Phytase Enzyme

Preparation from *Trichoderma reesei* Expressing a Gene encoding a Variant of Consensus Bacterial Phytase

is Generally Recognized As Safe

for Use as an Animal Food Ingredient

for Swine and Poultry

Prepared by Danisco US Inc.
(operating as DuPont Nutrition and Biosciences)

December 17, 2020

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1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

In accordance with 21 C.F.R. §570.225, Danisco US Inc. (operating as DuPont Nutrition and Biosciences, hereafter referred to as 'DuPont') submits the GRAS notice for phytase produced by submerged fermentation of *Trichoderma reesei* expressing a gene encoding a variant of consensus bacterial phytase. The enzyme is intended for use as a swine and poultry food additive to catalyze the reaction myo-inositol hexakisphosphate + H2O = 1D-myo-inositol 1,2,3,5,6-pentakisphosphate + phosphate, and, therefore, this phytase acts by increasing the nutritional availability of phosphate in the diet for swine and poultry.

The IUBMB nomenclature is 6-phytase. Other names used are phytase from *T. reesei* expressing a (gene encoding a) variant of consensus bacterial phytase, phytase, PhyG, and variant of consensus phytase as described in Section 2.2.1 of this submission. For consistency and transparency, the enzyme will be designated as "variant of consensus phytase" throughout the notice. The CAS number of this enzyme is 9001-89-2. The enzyme hydrolyzes *myo*-inositol hexakisphosphate + H_2O = 1D-*myo*-inositol 1,2,3,5,6-pentakisphosphate + phosphate.

The information provided in the following parts is the basis of our determination of GRAS status of this phytase enzyme preparation from *T. reesei* expressing a variant of consensus bacterial phytase.

(b) (4)

The safety of the production organism is considered to be the prime consideration in assessing the safety of an enzyme preparation intended for animal and human food use (Pariza & Foster, 1983; Pariza & Johnson, 2001; Pariza & Cook, 2010). The safety of the production organism (*T. reesei*) is discussed in Part 2 and 6 of this submission. The other essential aspect of the safety evaluation of enzymes derived from genetically engineered microorganisms is the identification and characterization of the inserted genetic material (Berkowitz & Maryanski, 1989; IFBC, 1990; OECD, 1993; Pariza & Johnson, 2001; Pariza & Cook, 2010; FDA/CFSAN, 2010; EFSA Panel on Genetically Modified Organisms (GMO), 2011). The genetic modifications used to construct this production organism are well defined and described in Part 2. The safety and exposure evaluation described in Part 3 and 6 shows no evidence to indicate that any of the inserted DNA sequences and incorporated code for or express a harmful toxic substance.

In addition to the information enabling the Pariza and Cook (2010) decision tree analysis, this GRAS Notice includes published studies outlining identity and biochemical characterization (Christensen *et al*., 2020), toxicology (Ladics *et al.,* 2020) and utility (Dersjant-Li, 2020a,b) of the phytase that corroborate DuPont's safety assessment.

1.1 § 570.225 (c)(1) GRAS Notice Submission

In accordance with 21 C.F.R. §570. 225, Danisco US Inc. (operating as DuPont Nutrition and Biosciences, hereafter referred to as 'DuPont') submits the GRAS Notice for phytase from *T. reesei* expressing a gene encoding a variant of the consensus bacterial phytase (Christensen *et al*., 2020).

1.2 § 570.225 (c)(2) Name and Address of Notifier

Danisco US Inc.
(operating as DuPont Nutrition and Biosciences, a division of 'DuPont')
925 Page Mill Road
Palo Alto, CA 94304

1.3 § 570.225 (c)(3) Common or Usual Name of Substance

Phytase from *T. reesei* expressing a variant of consensus bacterial phytase

1.4 § 570.225 (c)(4) Applicable Conditions of Use

The consensus phytase variant is intended to be used in swine and poultry feeds at levels up to a maximum specific activity of (b) (4) which equates to (b) (4) mg TOS/kg feed as a feed additive.

1.5 § 570.225 (c)(5) Basis for GRAS Determination

This GRAS determination is based upon scientific procedures in accordance with 21 C.F.R. §570.30 (a) and (b).

1.6 §570.225 (c)(6) Exemption from Pre-market Approval

Pursuant to the regulatory and scientific procedures established in 21 C.F.R. §570.225, DuPont has determined that its phytase from *T. reesei* expressing a variant of consensus bacterial phytase is a Generally Recognized As Safe ("GRAS") substance for the intended applications and is, therefore, exempt from the requirement for premarket approval.

1.7 §570.225 (c)(7) Availability of Information for FDA Review

A notification package providing a summary of the information that supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary

exposure. The complete data and information that are the basis for this GRAS determination are available for review and copying at 925 Page Mill Road, Palo Alto, CA 94304 during normal business hours or can be sent to the Food and Drug Administration upon request.

1.8 §570.225 (c)(8) and (c)(9) Disclosure and Certification

Several appendices of this GRAS Notice (notably Appendix 1 – 4, 7, 8 and 10) are marked as "CBI" to indicate that they contain confidential business information that is deemed exempt from disclosure under the Freedom of Information Act (FOIA; 5 U.S.C. §552).

We confirm that the data and information in this GRAS notice satisfactorily addresses Part 2-7 of a GRAS notice per 21 C.F.R. §570.230 to 570.255 as copied below.

> §570.230 Part 2 of a GRAS Notice: Identity, method of manufacture, specifications, and physical or technical effect.
> §570.235 Part 3 of a GRAS notice: Target animal and human exposure.
> §570.240 Part 4 of a GRAS notice: Self-limiting levels of use.
> §570.245 Part 5 of a GRAS notice: Experience based on common use in food before 1958.
> §570.250 Part 6 of a GRAS notice: Narrative.
> §570.255 Part 7 of a GRAS notice: List of supporting data and information in your GRAS Notice.

DuPont certifies that to the best of our knowledge this GRAS notice is a complete, representative, and balanced submission that includes unfavorable and favorable information known to us as well as relevant to the evaluation of the safety and GRAS status of the use of the notified substance.

(b)(6)

December 17, 2020

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Date

2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATION AND PHYSICAL OR TECHNICAL EFFECT

2.1 PRODUCTION ORGANISM

2.1.1 Production Strain

The production strain is *T. reesei* expressing a gene encoding a variant of the consensus bacterial phytase (Christensen *et al*., 2020). This strain is derived from *Trichoderma reesei* strain RL-P37, which has been genetically engineered resulting in the production strain. This production strain produces a variant of consensus bacterial phytase. Consensus bacterial phytase was described in Christensen *et al* (2020). Code names used to describe the variant of consensus phytase throughout this dossier and supporting appendixes include CRC2836-13855, Eclipse and PhyG.

The taxonomy of the genus *Trichoderma* has undergone revision. As a result, some strains that are known in literature as *T. longibrachiatum* are actually *T. reesei.* Therefore, in the remainder of this document and appendices, reference may be made to *T. longibrachiatum (reesei)* to indicate this taxonomic revision.

T. reesei is a non-pathogenic fungus. A review of the literature on the organism uncovered no reports that implicate *T. reesei* with a disease situation, intoxication, or allergenicity among healthy adult humans and animals (Nevalainen *et al*., 1994; Blumenthal, 2004; Paloheimo *et al*., 2016). It is classified as a Biosafety Level 1 (BSL1) microorganism by major culture collections worldwide[1]. BSL1 microorganisms are not known to cause diseases in healthy adult humans. In the US, *T. reesei* is not listed as a Class 2 or higher containment agent under the National Institute of Health (NIH) Guidelines for Recombinant or synthetic nucleic acid molecules (NIH, 2016). The US Environmental Protection Agency (EPA) completed a risk assessment on *T. reesei* in 2011 resulting in a Proposed Rule in 2012 and Final Rule in 2020, concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements,[2] if this fungus was to be used in submerged standard industrial fermentation for enzyme production.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed (Nevalainen *et al*., 1994; Blumenthal, 2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins

[1] ATCC 13631 *T. reesei* classified as BSL1 https://www.atcc.org/products/all/13631.aspx

[2] reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce.

or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme products used as processing aids in international food and feed industries. It is listed as a safe production organism for cellulases (Pariza & Johnson, 2001; Olempska-Beer *et al*., 2006), and various strains of *T. reesei* have been approved for the manufacture of commercial enzyme products internationally including Canada (Canada, 2017), the United States (FDA, 2015b), France, Australia/New Zealand, China, and Japan. In the US, numerous enzyme preparations for food use from *T. reesei* have been submitted in Generally Recognized as Safe (GRAS) notices to the Food and Drug Administration (FDA) for human consumption (for example, FDA, 2000; FDA, 2015a; FDA, 2015b). In animal feed, *T. reesei* is a common production organism for various enzymes including Cellulase, beta-Glucanase, Hemicellulase, beta-Mannanase, Xylanase, and Phytase as listed in American Association of Feed Control Officials Official Publication Table 30.1 (AAFCO, 2020). This presents a significant history of use that the enzymes produced from *T. reesei* as a production strain are generally nontoxic and safe for feed use. This includes the DuPont lineage of production organisms, which is used to produce, amongst others, variant *Buttiauxella* sp. phytase. The safety and utility of that phytase was previously reviewed by FDA/CVM prior to its recommendation to list the enzyme in the enzyme table (Table 30.1) of the AAFCO Official publication.

Similar to the construction of the *T. reesei* strain expressing an altered *Buttiauxella* sp. phytase gene, initial steps to construct the *T. reesei* production strain for the variant of consensus phytase involved inactivation of the endogenous cellobiohydrolase genes (*cbh1* and *cbh2*) and endoglucanase genes (*egl1* and *egl2*) by deletion or disruption using molecular genetic techniques. These steps are the same as those which occurred during the construction of the *T. reesei* strain expressing an altered *Buttiauxella* sp. phytase gene, which has previously been reviewed by CVM and subsequently listed in the AAFCO Official Publication. This resulted in an intermediate expression host M1-1.1. Subsequently, additional genes were inactivated, to create a highly productive recipient strain with reduced viscosity phenotype. The gene encoding a variant of consensus bacterial phytase was then introduced. These modifications were conducted to produce an exogenous, secreted phytase from a known safe production organism. The full construction outline of the production strain can be found in Appendix 1, which is marked "CBI", indicating that its entire content is business confidential.

2.1.2 Recipient Organism

The host strain is derived from *Trichoderma reesei* strain RL-P37, derivatives of which have a long history of safe use by DuPont Nutrition and Biosciences in the manufacture of commercial cellulase, xylanase, and phytase preparations for use in animal food, as well as multiple enzymes for use in human food. The derivation and characterization of strain RL-P37 has been published (Sheir-Neiss & Montenecourt, 1984). Strain RL-P37 is a cellulase over-producing strain that was obtained through several classical mutagenesis steps from the wild-type *T. reesei* strain (QM6a). Strain QM6a is present in several public culture collections, e.g. in the American Type Culture Collection as ATCC 13631.

RL-P37 is the same host organism reviewed by FDA to produce the AAFCO (AAFCO, 2020) listed phytase from *T. reesei* expressing an altered phytase gene from *Buttiauxella* sp.

2.1.3 Donor Organism

Consensus bacterial phytase was constructed using a synthetic phytase gene. As such it is not derived from a traditional singular donor organism. The consensus phytase amino acid sequence is based on protein engineering aimed at improved activity at low pH and increased thermostability, combining multiple microbially sourced phytase sequences including a *Buttiauxella* species variant. Therefore, a single "gene donor" was not used to generate the consensus phytase, rather a plurality of input phytase sequences. The final variant as expressed in the *T. reesei* production organism was further protein-engineered for increased thermostability and has high homology with consensus bacterial 6-phytase (Christensen, 2020). It also shares homology with a *Buttiauxella* sp. phytase. See Appendix 2 for additional information. A modified *Buttiauxella* sp. phytase has previously been reviewed by FDA for use in swine and poultry feed as listed in AAFCO Enzyme Table 30.1 (source organism from *T. reesei* expressing an altered phytase gene from *Buttiauxella* sp.) (AAFCO, 2020).

As the donor gene was synthesized (versus isolation from a donor organism), any risk for inadvertent transfer of cloning remnants of donor DNA of concern is eliminated.

2.1.4 Phytase Expression Plasmid

The production organism expresses a variant of consensus bacterial phytase gene, which was synthesized *in vitro* and inserted into the host as described in Part 2, Production Organism, and Appendix 1 under the control of the native *T. reesei cbh1* (cellobiohydrolase) promoter and terminator as summarized in Table 1 below.

Table 1: DNA sequences contained in production organism

Promoter	*cbh1* promoter (*T. reesei*)
Phytase	consensus bacterial phytase variant (synthesized)
Terminator	*cbh1* terminator (*T. reesei*)
Selective marker	*pyr2* (*T. reesei*)
Other introduced genetic material	*pyrG* remnant, *Aspergillus nidulans* (438 bp) used for cloning and expression of the synthetic phytase gene

The *Aspergillus nidulans pyrG* gene is a remnant from the strain construction. The entire *pyrG* gene is 1734 bases and contains a *KpnI* restriction site which cuts the plasmid. This results in 443 bases from the original *Aspergillus nidulans pyrG* gene left in the final expression cassette. This

remnant gene is non-functional and is not translated; it does not contain an open reading frame and is not expressed in the final cassette.

No antibiotic resistance markers were inserted into the new microorganism during construction. Rather, p*yr2* as a bidirectional metabolic marker was used. This gene encodes an orotate phosphoribosyl transferase. This allows for selection of transformants using uridine and 5-FOA plates (Schmoll, Dattenbock, 2016).

All these modifications were performed in such a way that no complete bacterial vectors were used in the construction. The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the intended chromosomal modifications were confirmed by Southern Blot (Appendix 3), PCR analyses (Appendix 4) and genome sequencing. Appendix 1 contains the full description (marked "CBI") of the scheme for the construction of the production strain for the variant of consensus bacterial phytase.

In addition, (b) (4) software was employed to perform an open reading frame (ORF) analysis (minimum size 90 bp, start codons (b)(6)). 23 novel ORFs were identified. All potential new ORFs that were created were analyzed for toxin homology using the UniProt annotated Protein Knowledge databasehttp://www.uniprot.org), release 2020_01 of 26 Feb 2020. This database contains 561,911 reviewed proteins, of which 6,229 sequences are manually annotated as animal toxins and 6,715 as venom proteins (http://www.uniprot.org/biocuration_project/Toxins/statistics). These toxin and venom sequences are grouped in the animal toxin database subset (http://www.uniprot.org/program/Toxins). A specific BLAST search for homology of the potential new ORF sequence against the UniProt animal toxin subset database was performed, with a threshold E-value of 0.1. This yielded no matches. As such, insertion of the variant phytase gene did not cause any shifts in ORFs that might lead to the expression of new genes of concern or repression of essential endogenous genes. A detailed ORF analysis report is available for FDA's viewing upon request.

2.1.5 Stability of the Introduced Genetic Sequences

The production strain was constructed in such a way that only purified restriction enzyme or PCR fragments remain the genome of the recipient organism as described in Appendix 1. The stability of the inserted genetic variant was confirmed through comparative genome sequence analysis at the beginning and end of fermentation confirming no rearrangement of the expression cassette. In addition, the production strain proved to be completely stable after at least 30 generations of fermentation (data available on file).

2.1.6 Antibiotic Resistance Gene

No antibiotic resistance genes were introduced in the construction of the production microorganism.

2.1.7 Absence of Live Production Microorganism in Product

Live production microorganisms will be removed at the end of fermentation to meet the commercial enzyme product specification (<1 CFU/g). As such, live production organism is not anticipated to be in the finished animal food and, therefore, the first step in the safety assessment as described by the International Food Biotechnology Council (IFBC)[1] is satisfactorily addressed.

2.2 ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

2.2.1 Enzyme Identity

Classification (name):	Phytase
IUBMB Nomenclature:	6-phytase
IUBMB Number:	3.1.3.26
CAS Number:	9001-89-2
Reaction catalyzed:	*myo*-inositol hexakisphosphate + H_2O = 1D-*myo*-inositol 1,2,3,5,6-pentakisphosphate + phosphate.

2.2.2 Amino Acid Sequence

The amino acid sequence of the consensus bacterial phytase was constructed from the combination of microbial phytase sequences including a *Buttiauxiella* species variant. Therefore, a single "gene donor" was not used. The consensus bacterial phytase as well as the variant that is the subject of this GRAS Notice are described in a biochemical characterization paper by Christensen *et al*. (2020) (included in Appendix 5 for reference), and the patent reference therein (Babe *et al*., 2020). For ease of reference, both amino acid sequences are also listed in Appendix 2.

The amino acid sequences of consensus bacterial phytase and its variant are 98.3% similar (see Appendix 2). A BLAST search for homology of the variant of consensus bacterial phytase variant against the complete Uniprot database was performed, with a threshold E-value of 0.1. The vast majority of matches were phosphoanhydride phosphorylases (phytases). The sequence of the variant of consensus bacterial phytase protein is similar to other phytases isolated from commercially relevant bacteria with an amino acid similarity range of 70 – 80% to such species including *Buttiauxella sp.* In addition, a BLAST search for homology to known toxins was also

[1] https://ac.els-cdn.com/S0273230005800807/1-s2.0-S0273230005800807-main.pdf?_tid=c89f62ce-5402-4e18-a3be-68ddbf116b10&acdnat=1530898844_165c4c45e811723d34f8db3e1878c745

conducted. None of the top 1,000 database matches were annotated as either a toxin or venom. The results are discussed in Part 6 of this dossier.

The protein engineering effort and subsequent amino acid variation from consensus bacterial phytase do not impact the integrity, functionality or safety of the expressed protein. The biochemical functionality of the expressed enzyme as a phytase and other properties were confirmed in enzymatic assays as described in Christensen *et al* (2020) contained in Appendix 5. Given the high structural similarity of phytase molecules from various sources, significant differences in toxicological properties between these homologous enzymes are not expected.

2.3 MANUFACTURING PROCESS

The manufacturing process for phytase preparations follows standard industry fermentation practice (Kroschwits, 1994; Aunstrup *et al*., 1979; Aunstrup, 1979). For a diagram of the respective manufacturing process, see Appendix 6. A written description to accompany the manufacturing process is presented in the subsequent sections. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. §110 and with FDA's requirements under the Food Safety Modernization Act.

2.3.1 Raw Materials

The raw materials used in the fermentation of the production strain for this phytase concentrate are standard ingredients used in the enzyme industry (Kroschwits, 1994; Aunstrup, 1979 and Aunstrup *et al*., 1979). All the raw materials used have appropriate specifications given intended use in animal feed. Many conform to the specifications of the Food Chemicals Codex, 12th edition, 2020 ("FCC"). DuPont uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt. Any defoamer used during fermentation is used in accordance with GMPs, per the September 11, 2003 FDA correspondence to ETA acknowledging the listed antifoams and flocculants (see Appendix 7 for list of raw materials used and Appendix 16 for FDA correspondence to ETA acknowledging the listed antifoams and flocculants).

2.3.2 Fermentation Process

The phytase enzyme is manufactured by submerged fermentation of a pure culture of the genetically engineered strain of *T. reesei* as described in Part 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. (b) (4)

(b) (4)

(b) (4)
(b)(6)

2.3.3 Recovery and Formulation

The enzyme is recovered from the culture broth through the following steps:

(b)(6)

This recovery- and formulation process is performed through the following series of operations:

Recovery and Formulation

(b)(6)

(b)(6) . All formulations contain the same 'clarified' enzyme preparation, (b) (4)) from which all solids including the production organism are removed by centrifugation and/or microfiltration as described above.

The final phytase products are analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the JEFCA in 2006 and FCC, 12th edition, 2020, as well as taking into account requirements for the final animal feed use. These specifications are set forth in Section 2.4 along with additional details on final composition.

2.4 COMPOSITION AND SPECIFICATIONS

2.4.1 Quantitative Composition

The phytase enzyme is composed of phytase enzyme protein in accordance with the specifications as described in Section 2.4.2. All food additives used in the manufacture and formulation of products are done so in accordance with ingredient feed regulatory status and use allowances under strict quality control standards. The products are then tested to confirm and demonstrate specifications as described in Section 2.4.2 are met.

2.4.2 Specifications

As discussed in previous sections, variant consensus phytase preparation meet the purity specifications for enzyme preparations set forth in Food Chemicals Codex 12th edition (USP, 202018). In addition, they also conform to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by JECFA (2006).

The results of analytical testing of a representative 3 lots of product are given in Appendix 8 verifying conformance with USP (2020) and JECFA (2006) specifications for food enzyme preparations.

2.5 APPLICATION

2.5.1 Mode of Action

The phytase functions in the catalysis of the reaction myo-inositol hexakisphosphate + H2O = 1D-myo-inositol 1,2,3,5,6-pentakisphosphate + phosphate. It acts by increasing the nutritional availability of phosphate in the diet. This phytase will be used on its own and in combination with other feed ingredients in accordance with their regulatory status to be added to animal feeds for poultry and swine. Phytase will increase the availability of phosphorus from phytate from various sources including corn, wheat, soybean, sunflower seed, hominy, milo, tapioca, barley, rice and potatoes and other cereals used in animal feeds.

2.5.2 Use Levels

This phytase will be used in swine and poultry feeds at levels up to a maximum specific activity of (b) (4) feed as a feed additive. One phytase unit (FTU) was defined as the amount of enzyme that released 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C. Per FDA guidance for food enzymes (FDA/CFSAN Division of Biotechnology and GRAS Notice Review, 2010), exposure is calculated as Total Organic Solids (TOS). The maximum recommended use levels in mg TOS/kg feed for the applications where the phytase may be used is (b) (4) mg TOS/kg feed, equivalent to (b) (4).

2.5.3 Enzyme in Final Animal Foods

Like all proteins, the phytase used in swine and poultry feed is expected to be broken down by the animal's digestive system into small peptides and amino acids, with the latter being absorbed and metabolized which is not expected to pose any animal health risk. The resulting amino acids are incorporated into animal products are universally present in meat, milk, or eggs and do not pose any human health risk.

3. DIETARY EXPOSURE

Target animals will be exposed to phytase from *T. reesei* expressing a variant of consensus bacterial phytase via its use as a direct feed additive in swine and poultry diets. Phytase will be used in swine and poultry feed up to a maximum level of (b) (4) TOS/kg feed, equivalent to (b) (4) for swine and poultry. To estimate the livestock exposure from this source in feed the following conservative assumptions are used:

a) Broilers Feed Consumption: 79 g DM/kg bird bw/day
b) Piglets Feed Consumption: 44 g DM/kg pig bw/day
c) Pigs for fattening Feed Consumption 37 g DM/kg pig bw/day

Using the maximum inclusion level in feed of (b) (4) TOS/kg feed consumption the levels of enzyme consumed for each species is presented in Table 2.

Table 2: Maximum total enzyme consumption when used in swine and poultry feed directly in feed

Species	**Poultry**	**Piglets**	**Pigs for Fattening**
Enzyme inclusion rate in feed (mg TOS/kg feed)	(b) (4)		
Feed Consumption (g DM/kg bw/day)	79	45	37
Average feed dry matter content	88%	88%	88%
Total Enzyme consumption (mg TOS/kg-bw/day)	**0.62**	**0.35**	**0.29**

Human exposure to phytase from *T. reesei* expressing a variant of consensus bacterial phytase is negligible, as, upon ingestion by the animal, the enzyme protein is not absorbed from the gastrointestinal tract. Hence there is negligible potential for the enzyme itself to accumulate in edible animal tissues or products. The largest peptide fragment that can be absorbed is only a few amino acids, which also eliminates any concern with regarding to potential allergenicity.

In addition to the enzyme protein, other metabolites resulting from *T. reesei* fermentation in the enzyme manufacture process are of little concern given that *T. reesei* is well-established as a safe host for food and feed enzymes.

4. SELF-LIMITING LEVELS OF USE

The self-limiting levels of use are primarily economical as customers are unlikely use more enzyme than is needed to achieve the technical effects in order to minimize production costs. As such, self-limiting levels of use for this enzyme are not applicable.

5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Information regarding this enzyme's common use in food and feed before 1958 is not provided as the statutory conclusion of our GRAS status, which is based on scientific procedures rather than common use before 1958.

6. SAFETY EVALUATION

6.1 SAFETY OF THE PRODUCTION STRAIN

The safety of the production organism is recognized as the prime consideration in assessing the safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). For microbially produced enzymes, the same principles of safety assessment as used in human food can be used in animal food (Pariza and Cook, 2010). If the production organism is non-toxigenic and non-pathogenic, then it is assumed that common foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC 1990). Pariza and Foster (1983) define a non-toxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a non-pathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances." *Trichoderma reesei* strains used in enzyme manufacture meet these criteria for non-toxigenicity and non-pathogenicity.

6.1.1 Safety of the host

T. reesei is a known safe host for enzyme production and is widely used by enzyme manufacturers around the world for production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. *T. reesei* is considered a benign organism that does not possess traits that cause disease. A review of the literature on the organism uncovered no reports that implicate *T. reesei* in any way with a disease situation, intoxication, or allergenicity among healthy adult human and animals (Nevalainen *et al*., 1994; Blumenthal, 2004; Paloheimo *et al*., 2016). This also applies to the DuPont *T. reesei* host strain, which has been demonstrated to be non-pathogenic, non-toxigenic and not cytotoxic.

Published data indicates *T. reesei* is non-toxigenic and non-pathogenic with toxins not found in the culture broth, the tox batch or the commercial enzyme preparation (Hjortkjaer *et al*., 1986). The potential pathogenicity of *T. reesei* in immunosuppressed animals was investigated in mice, guinea pigs and rabbits by the subcutaneous administration of a large dose of cortisone acetate (5 mg) 2 days before the administration of 10^7 or 10^5 viable or 10^7 killed spore suspensions. All animals were observed for 40 days then sacrificed (Modeweg-Hansen, 1978). The authors reported that spores were microscopically and microbiologically identified in different organs 40 days after administration of viable *T. reesei* by intravenous injection. No effects were seen after intraperitoneal administration of 10^5 viable spores. These investigations suggested that under special conditions (i.e., a large inoculum given to immunosuppressed animals), the microorganism could be pathogenic. However, this finding is not unexpected due to the nature of the immunological condition of the animals prior to test substance administration. Nevalainen and colleagues provided an overview of *T. reesei* and its safety as a production organism for cellulase enzymes (Nevalainen *et al*., 1994). The organism is considered non-pathogenic for humans and

does not produce fungal toxins or antibiotics under conditions used for enzyme production. In conclusion, there is a body of information to assert the lack of pathogenicity and toxicity of *T. reesei*. *T. reesei* is listed as a safe production organism for cellulases (Pariza & Johnson, 2001; Olempska-Beer *et al*., 2006) and various strains have been approved for the manufacture of commercial enzyme products internationally, for example, in Canada (Canada, 2017), France, Australia/New Zealand, China, and Japan.

In the US, numerous enzyme preparations from *T. reesei* have been submitted in Generally Recognized as Safe (GRAS) Notices to the Food and Drug Administration (FDA) for human consumption, a few, but not all include, GRAS 32, 566, and 567 (FDA, 2000; FDA, 2015a; FDA, 2015b). In addition to food use, the Environmental Protection Agency (EPA) completed a risk assessment on *T. reesei* in 2011 (https://www.regulations.gov/document?D=EPA-HQ-OPPT-2011-0740-0007, see Appendix 9) resulting in a Proposed Rule in 2012 (77 FR 54499) and Final Rule in 2020 (85 FR 13760), concluding that it is appropriate to consider *T. reesei* as a recipient microorganism eligible for exemptions from full reporting requirements,[1] if this fungus is used in submerged standard industrial fermentation for enzyme production. Additionally, *T. reesei* is listed in the Association of American Feed Control Official's Official Publication (AAFCO, 2020) Table 30.1 as a suitable species for production of numerous enzymes for use in animal food. One such enzyme produced by *T. reesei* is a modified *Buttiauxella* sp. phytase that was previously been reviewed by FDA/CVM for use in swine and poultry feed. This phytase has been listed in Table 30.1 since 2013.

A review of all toxicology studies conducted by DuPont with enzyme preparations produced by different strains of a *T. reesei* strain lineage indicate that, regardless of the production organism strain, all enzyme preparations are not irritating to the skin and eyes, are not skin sensitizers, are not mutagenic, clastogenic or aneugenic in genotoxicity assays and do not adversely affect any specific target organ. Due to the consistency of the findings from enzyme preparations derived from different *T. reesei* strains of the *T. reesei* strain lineage, it is expected that any new common enzyme produced with such a *T. reesei* strain would be similar from a toxicological standpoint. In addition, the safety of this production strain lineage and toxicology summary for 21 strains is provided in Appendix 15 and 17. Additional discussion on the safety of the enzyme preparation itself can be found in Section 6.3.

6.1.2 Safety of the 'gene donor'

This phytase was not derived from a single taxonomic gene donor. It is a variant of a synthetic phytase gene that was constructed using protein engineering starting from a plurality of microbial

[1] reporting procedures in place under the Toxic Substances Control Act (TSCA) for new micro-organisms that are being manufactured for introduction into the commerce.

phytase sequences (such as *Buttiauxiella* species*)* to achieve a phytase protein with high activity under commercial feed applications, which was subsequently termed consensus bacterial phytase (Christensen *et al.*, 2020). Similar methods to develop synthetic genes have been used to generate production organisms that result in more effective enzymes (Chang, 2013; Gumulya, 2018; Verma, 2019; Wilding, 2017). This technique was used previously to generate a consensus fungal phytase sequence, which was tested in livestock as discussed in Gentile *et al.* (2003). In the construction of the synthetic variant phytase gene, no pathogenic, toxigenic or virulence factors were included. This is further supported through sequence comparison to toxin and virulence factors databases (See Section 6.3.1 for more information).

The only genetic information expressed in the production host is a synthetic consensus bacterial phytase variant gene, no actual organism-derived DNA was transferred. This consensus bacterial phytase variant shares ~75% identity with the *Buttiauxella sp.* phytase amino acid sequence listed in Table 30.1 Enzyme/Source Organisms Acceptable for use in Animal Feeds since 2013.

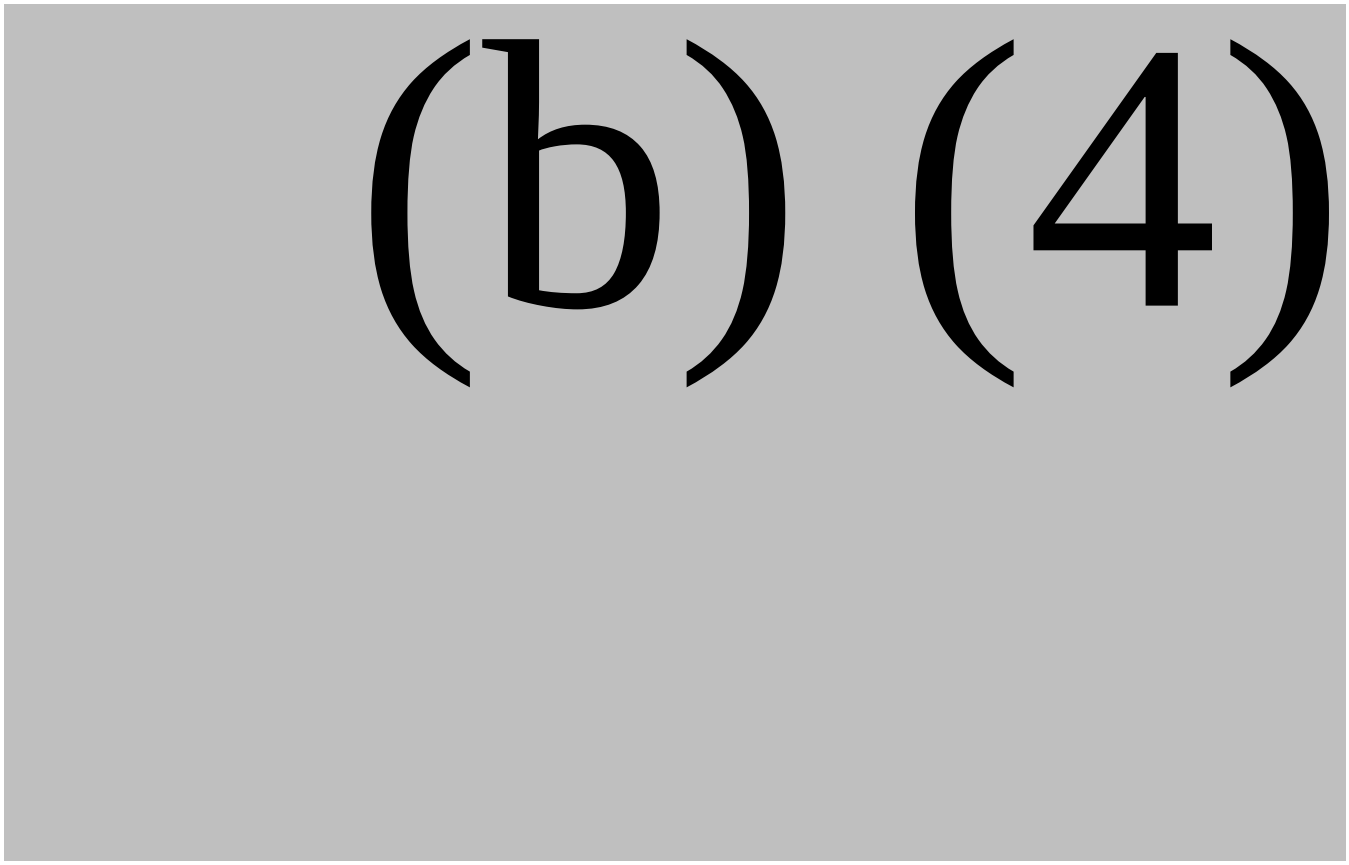
6.1.3 Safety of the production strain

The genetic modification of the *T. reesei* host involved recombinant DNA techniques to introduce a gene encoding an optimized phytase variant synthesized *in vitro* into the safe *T. reesei* host. The variant of consensus bacterial phytase gene was synthesized and was placed under the expression signals of the endogenous *T. reesei cbh1* promoter gene and terminator, using the *T. reesei pyr2* gene (orotate phosphoribosyl transferase) as a selectable marker. Southern blot analysis showed that no bacterial vector DNA present in the genome. Since there are no vector sequences present in the final strain, there is no increase in the transfer frequency of the integrated expression cassettes relative to any other (b) (4) sequence, which means that the insertion is genetically stable. The result is a production strain in which only the phytase encoding gene, the native *cbh1* promoter and terminator, and a *pyrG* remnant were introduced into the host strain. No antibiotic resistance markers were inserted into the new microorganism.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information, and the intended chromosomal modifications were confirmed by PCR analyses (Appendix 4) as well as Open Reading Frame analysis.

6.2 SAFETY OF THE MANUFACTURING PROCESS

The manufacturing process to produce this consensus bacterial phytase variant is conducted in a manner like other food and feed enzyme production processes. (b) (4)



The entire process is conducted in accordance with the current food good manufacturing practice (cGMP) as set forth in 21 C.F.R. §110 and in compliance with the Food Safety Modernization Act. The resultant products meet the purity specifications for enzyme preparations of the Food Chemicals Codex, 12th Edition (US Pharmacopeia, 2020) and the general specifications for enzyme preparations used in food processing proposed by FAO/WHO (JECFA, 2006).

6.3 SAFETY OF PHYTASE FROM *T. REESEI* EXPRESSING A CONSENSUS BACTERIAL PHYTASE VARIANT GENE

6.3.1 Toxin Homology

The UniProt annotated Protein Knowledge database,[1] release 2019_11 of 11 Dec. 2019, contains 561, 568 reviewed proteins, of which 6,220 sequences are manually annotated as toxins and 6,711 as venom proteins.[2] These toxin and venom sequences are grouped in the animal toxin database subset.[3] A BLAST search for homology of the consensus bacterial phytase variant sequence against the complete Uniprot database was performed, with a threshold E-value of 0.1. The

[1] The Uniprot Consortium (2019) Uniprot: The universal protein knowledgebase. Nucleic Acids Res. 45: D158-D169. http://www.uniprot.org
[2] http://www.uniprot.org/biocuration_project/Toxins/statistics)
[3] http://www.uniprot.org/program/Toxins

majority of matches were phosphoanhydride phosphorylases (phytases), with none of the top 1000 database matches being annotated as either toxin or venom. In addition, a specific BLAST search for homology of the mature phytase variant sequence was performed against the Uniprot animal toxin database. This yielded no matches. Therefore, the consensus bacterial phytase variant sequence does not share homology with a known toxin or venom sequence. The full report can be found in Appendix 10.

6.3.2 Safety of Use in Animal Food

The Enzyme Marketing Coordination document in the American Association of Feed Control Officials (AAFCO) Official Publication, which was compiled as a collaboration between FDA/CVM and the Enzyme Technical Association, states that any safety concerns for feed enzymes can be addressed either by answering all questions in the Pariza and Foster (1983) decision tree **or** by tolerance trials in the most sensitive target species. The Pariza and Foster (1983) decision tree was updated by Pariza and Johnson (2001) for enzymes produced with modern biotechnology and this update was documented by Pariza and Cook (2010) to be suitable for animal feed enzymes. DuPont has supported the safety of the consensus bacterial phytase variant in question via the decision tree route outlined in Pariza and Cook (2010) as detailed below. In addition, studies have been conducted in broilers and piglets to assess the functionality of the phytase product as it relates to phosphorus availability in the diet.

As noted in the safety Section 6.1, *T. reesei* and enzyme preparations derived there from are well recognized by qualified experts as being safe for use in human food processing. Published literature, government laws and regulations, reviews by expert panels such as JECFA, as well as DuPont's own unpublished safety studies, support such a conclusion that *T. reesei* is a safe host for enzyme production. In addition, *T. reesei* is listed in the AAFCO Official Publication (OP) Section 30.1 Enzyme Table as a production organism for numerous feed enzymes, including phytase.

A review of all internal and external toxicology and safety studies conducted with enzyme preparations produced by different strains of a *T. reesei* strain lineage indicate that, regardless of the production organism strain, all enzyme preparations are not irritating to the skin and eyes, are not skin sensitizers, are not mutagenic, clastogenic or aneugenic in genotoxicity assays, and do not adversely affect any specific target organ (Nevalainen *et al.*, 1994; Blumenthal, 2004; Paloheimo *et al.*, 2016). Due to the consistency of the findings from enzyme preparations derived from different *T. reesei* strains, it is expected that any new enzyme preparation produced from such a *T. reesei* strain would behave similarly from a toxicological standpoint.

To confirm and further support safety, a 90-day repeated dose rat toxicity study was conducted on phytase from *T. reesei* expressing a consensus bacterial phytase variant gene (Ladics *et al.*, 2020). The results of this safety study are discussed in Section 6.3.3 below and included in Appendix 11.

6.3.3 Safety Studies

Phytase from *T. reesei* expressing a consensus bacterial variant phytase gene is an enzyme preparation that can be used as a food additive for use in swine and poultry .

Notwithstanding the availability of existing toxicology data for enzymes produced in DuPont's *T. reesei* lineage, including the *Butiauxella* variant phytase listed in the AAFCO OP, new toxicology testing was conducted for this specific phytase enzyme. The results were evaluated, interpreted and assessed by Ladics *et al.* (2020), and the open access publication[1] is included in Appendix 11 for FDA's convenience.

All toxicology studies summarized here were conducted in compliance with current Good Laboratory Practice standards and Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals. The test materials used in all toxicology investigations were the clarified (b) (4) enzyme preparation collected from fermentation with the following characteristics (Table 5):

Table 5. Characteristics of Test Materials Used in Toxicology Testing

Test Material Characteristics	(b) (4) **(Ultra-filtered Concentrate)**
Physical	Fermentation liquid, Brown
Enzyme Activity (Phytase units, One FTU is the activity of phytase required to liberate 1 µmol of inorganic phosphorus per minute at pH 5.5 from an excess of 15 M sodium phytate at 37°C)	97,885 FTU/g
Total Protein	206.47 mg/g
Total Organic Solids (TOS)	21.75%
Specifications for contaminants	Compliant with JECFA (2006) and FCC (2018)

The 90-day repeated dose toxicity study was conducted to establish a No Adverse Effect Level (NOAEL) for the Margin of Safety calculation. The genotoxicity studies reported here were not deemed essential to support GRAS status as enzymes produced by *T. reesei* are not known to cause

[1] https://www.sciencedirect.com/science/article/pii/S2214750020303498

genotoxicity, based on a plethora of pre-existing studies (e.g., Pariza and Johnson, 2001; Ladics and Sewalt, 2018), but rather to satisfy regulatory requirements outside the USA. However, all studies are reported here as part of a balanced review of all available safety information. The studies demonstrated the consensus bacterial phytase variant is safe for its intended use and that the toxigenic potential of the production organism was no different from that of the non-toxigenic host organism.

90-Day Repeated Dose Toxicity Study
(Ladics *et al*., 2020)

This 90-day study was conducted in accordance with OECD guideline No. 408 (June 2018). This study is reported here in summary format, with much more detail in the peer-reviewed open-access publication (Ladics *et al*., 2020).

The objective of this study was to investigate the potential toxicity of Phytase from *T. reesei* expressing a gene encoding a variant of consensus bacterial phytase after 90-day oral gavage administration in Sprague-Dawley rats. The phytase was administered once daily to Sprague-Dawley rats (ten animals/sex/group) by oral gavage at the following doses:0 (vehicle control), 250, 500 and 1000 mg TOS/kg bw/day.

The rats were weighed on the first day of treatment and weekly thereafter during the study. Food consumption was measured weekly. Cage-side evaluations were conducted twice daily, except on the days the detailed clinical observations were evaluated. Detailed clinical observations were conducted weekly. Ophthalmological evaluations were performed on all animals once prior to randomization and again during the last week of treatment. Abbreviated neurobehavioral evaluations were performed on all animals once prior to dosing and again during the last week of treatment. Blood samples for hormone analyses were collected from all animals on the scheduled sacrifice day. Blood and urine samples for clinical pathological evaluations were collected at the termination of the study. All animals were fasted prior to collecting the clinical pathological samples. At the termination of the study, all animals were euthanized and examined macroscopically. Selected organs were weighed, and selected tissues were evaluated microscopically.

During the study, one male at 250 mg TOS/kg bw/day was found dead on Day 84. There were no test item-related changes in macroscopic and microscopic examinations. The cause of death was not determined but it was considered incidental and not test item-related. No test item-related clinical signs or ophthalmology findings were observed in any animals. No test item-related changes in body weight, body weight gain, food consumption, abbreviated neurobehavioral evaluations, hematology, coagulation, clinical chemistry, urinalysis, gross findings, organ weights or histopathology were observed during the study.

Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for Phytase from *T. reesei* expressing a variant of consensus bacterial phytase in male and female rats is 1000 mg TOS/kg bw/day, the highest concentration tested. The NOAEL is based on the lack of any adverse or test item-related effects on in life, clinical or anatomic pathology parameters in the study.

Bacterial Reverse Mutation Test (Ames test)
(Ladics *et al*., 2020)

This assay was conducted in accordance with OECD guideline No. 471 (1997).

Phytase from *T. reesei* expressing a gene encoding a variant of consensus bacterial phytase was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 uvrA in the presence and absence of Aroclor-induced rat liver S9. The dose levels tested were (b) (4) µg per plate. No positive mutagenic response was observed with any of the tester strains. Neither precipitate nor toxicity was observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, no positive mutagenic responses were observed. The dose levels tested were (b) (4) and (b) (4) µg per plate. Neither precipitate nor toxicity was observed.

The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, the phytase did not exhibit any mutagenic responses in either the presence or absence of Aroclor-induced rat liver S9. Therefore, it was concluded to be negative in this assay. This is consistent with other Ames test assay results for enzymes produced in *Trichoderma reesei* and other microorganisms reported in the literature (Ladics and Sewalt, 2018).

in vitro Mammalian Chromosomal Aberration Assay
(Ladics *et al*., 2020)

This assay was conducted in accordance with OECD guideline No. 473 (2016).

Phytase from *T. reesei* expressing a gene encoding a variant of consensus bacterial phytase was tested in the *in vitro* Mammalian Chromosomal Aberration Assay in human peripheral blood lymphocytes (HPBL) in both the absence and presence of an Aroclor-induced S9 activation system. The chromosomal aberration assay was used to evaluate the genotoxic potential of the phytase.

Based upon preliminary results, the doses chosen for the definitive chromosomal aberration assay ranged from (b) (4) to (b) (4) µg/mL for all three exposure groups. In the definitive chromosomal aberration assay, visible precipitate was not observed at any dose at the beginning and end of the treatment period. Cytotoxicity (≥ 50% reduction in mitotic index relative to the vehicle control) was not observed at any dose in any of the three exposure groups. The doses selected for evaluation of chromosomal aberrations were 1250, 2500, and 5000 µg/mL for all three exposure groups.

No significant or dose-dependent increases in structural or numerical (polyploid or endoreduplicated cells) aberrations were observed at any dose in treatment groups with or without S9 ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests). All positive and vehicle control values were within acceptable ranges, and all criteria for a valid assay were met.

These results indicate the phytase was negative in the in vitro Mammalian Chromosomal Aberration Assay in human peripheral blood lymphocytes (HPBL) under the conditions, and according to the criteria of the test protocol. These results are, again, consistent with chromosomal aberration assay results for other microbial enzymes reported in the literature (Ladics and Sewalt, 2018).

6.3.4 Utility of Phytase (Efficacy as Relates to Safety)

The variant of consensus bacterial phytase functions in the catalysis of the reaction myo-inositol hexakisphosphate + H2O = 1D-myo-inositol 1,2,3,5,6-pentakisphosphate + phosphate. This phytase will be used on its own and in combination with other feed ingredients in accordance with their regulatory status added to animal feeds for poultry and swine. Phytase will increase the availability of phosphorus from phytate from various sources including corn, wheat, soybean meal, sunflower seed, hominy, milo, tapioca, barley, rice and potatoes and other cereals used in animal feeds.

As per the Enzyme Marketing Coordination Document (AFFCO, 2020), the functionality of the enzyme may be documented either *in vivo* or *in vitro.* To assess the functionality of the consensus bacterial variant phytase in enhancing phosphorus availability to swine and poultry, two *in vivo* studies were conducted, one in broilers and one in piglets. The results were evaluated, interpreted and assessed by Dersjant-Li *et al.* (2020a), Dersjant-Li *et al.* (2020b), and the open access publications are included here for FDA's convenience as Appendix 12 and 13. Both studies demonstrate at dose of 250-1000 FTU/kg consensus phytase is highly effective in the tested dietary setting in both swine and poultry.

<u>Functionality of variant of consensus bacterial 6-phytase in pigs</u>
Dersjant-Li *et al.* (2020b)

The utility of a variant of consensus bacterial 6-phytase in restoring bone ash, bone phosphorus (P) content and performance in piglets depleted in P was evaluated. A negative control (NC) diet was formulated without inorganic P (1.1 g digestible P/kg) and reduced in calcium (Ca; 5.0 g/kg). This NC diet was supplemented with increasing levels of test phytase (PhyG, 250, 500 and 1,000 FTU/kg) and a commercial *Buttiauxella sp*. phytase (PhyB; 500 and 1,000 FTU/kg); and with mono-calcium phosphate (MCP) to obtain additional 0.7, 1.4 and 1.8 g digestible P/kg, with added limestone to maintain Ca: P ratio between 1.2 to 1.3. The latter, with 1.8 g digestible P/kg supplementation, was the positive control (PC) diet.

A total of 216 crossed Pietrain (large White x Landrace) 21-d-old piglets (50% males and 50% females) were fed adaptation diets until 42-d-old and then assigned to pens with 2 pigs/pen and 9 pens/treatment in a completely randomized block design. Piglets were fed mash diets based on corn and soybean meal *ad libitum* for 28 d. At the end of the study 1 piglet/pen was euthanized and the right feet collected for determination of bone strength, bone ash and mineral content.

Compared with the PC, the NC group had reduced average daily gain (ADG) and increased feed conversion ratio (FCR) during all growth phases and overall. At d 28 (70-d-old) NC pigs had bones with reduced ash, Ca and P content ($P < 0.05$). The PhyG variant of consensus bacterial phytase at 250 FTU/kg improved bone ash vs. NC. Increasing PhyG dose linearly/quadratically improved bone ash and other parameters ($P < 0.05$). At $\geq$ 500 FTU/kg, both PhyG and PhyB maintained ADG and FCR equivalent to PC. Linear regression analysis was done to compare the measured response parameters to increasing digestible P from MCP. Based on this analysis, it was shown that PhyG and PhyB at 1,000 FTU/kg could replace 1.83 g/kg and 1.66 g/kg digestible P from MCP in the diet, respectively. In addition, ileal IP6 digestibility and IP ester concentrations were determined from ileal contents of birds in this study and included in the biochemical characterization publication (Christensen *et al.*, 2020). PhyG at 1,000 FTU/kg achieved an ileal digestibility of phytic acid of 89.3%. These findings demonstrate utility of the consensus phytase variant in improving P availability from corn and soy, restoring bone ash in pigs with reduced overall dietary P.

<u>Functionality of variant of consensus bacterial 6-phytase in broilers</u>
Dersjant-Li *et al.* (2020a)

The effects of the variant of consensus bacterial 6-phytase on growth performance, tibia ash and P digestibility in broilers was evaluated. Treatments included a nutritionally adequate positive control (PC) diet, a negative control (NC) diet formulated with reductions in Ca and available P (avP) of 2.0 g/kg and 1.9 g/kg (starter phase) and 2.0 g/kg and 1.8 g/kg (finisher phase), respectively, and three further diets comprising the NC supplemented with three levels of the consensus bacterial phytase variant (PhyG, at 250, 500 or 1,000 FTU/kg).

Diets were fed in mash form to day-old Cobb 500 broilers housed in pens (24 birds/pen; 9 pens for NC and 10 pens for all other treatments), in two phases (starter days 1 to 21, finisher days 22 to 42). Tibias were collected from 4 birds on day 21 and from 6 birds on day 42 for determination of defatted tibia ash and ileal digesta collected on day 21 for determination of apparent ileal digestibility (AID) of nutrients.

Compared to PC, the NC exhibited reduced tibia ash at day 21 and 42, resulting in reduced average daily gain (ADG) and average daily feed intake (ADFI) during starter, finisher phases and overall, and increased feed conversion ratio (FCR) during finisher phase and overall ($P < 0.05$). Phytase at any dose-level during both phases improved tibia ash vs. NC ($P < 0.05$) and maintained feed intake and growth parameters equivalent to the PC. At a dose-level of 500 FTU/kg or above, phytase supplementation increased AID P vs NC ($P < 0.05$) and at 1,000 FTU/kg, phytase improved the AID of P compared with PC ($P < 0.05$). For all measures, response values were numerically highest with 1,000 FTU/kg and increased linearly or quadratically with increasing phytase dose ($P < 0.05$). On a grams per kilogram diet basis, phytase at 1,000 FTU/kg improved ileal digestible P by 1.76 g/kg above NC (at day 21). The monocalcium phosphate (MCP-P) replacement of the consensus bacterial phytase was estimated as 2.07 g/kg diet based on digestible P improvement.

The results suggest the novel variant of consensus bacterial phytase has high utility in restoring tibia ash in broilers in the tested reduced P dietary setting.

CONCLUSION

The safety of phytase from *T. reesei* expressing a gene encoding a variant of consensus bacterial phytase was assessed in a battery of toxicology studies investigating its genotoxic and systemic toxicity potential. Under the conditions of the mutagenicity assays, the phytase is not a mutagen or clastogen. Daily administration of the phytase by gavage for 90 continuous days did not result in observable signs of systemic toxicity. A NOAEL for the phytase is established at 1000 mg TOS/kg bw/day, equivalent to 450,450 FTU/kg bw/day (Ladics *et al.*, 2020). In addition, the variant of consensus bacterial phytase used at inclusion rates as low as 250 FTU/kg feed was efficacious in improving P availability to pigs and poultry fed reduced inorganic P diets, supporting that its use will not result in a safety concern from reduced phosphorus content in the diet as demonstrated by Dersjant-Li *et al.* (2020a) and Dersjant-Li *et al.* (2020b).

6.4 OVERALL SAFETY ASSESSMENT

6.4.1 Identification of the NOAEL

In the 90-day oral (gavage) studies in Sprague-Dawley rats, a NOAEL was established at 1000 mg TOS/kg bw/day (450,000FTU/kg bw/day) for the variant of consensus bacterial phytase. The studies were conducted in compliance with both the US and OECD Good Laboratory Practice

Regulations and were designed based on OECD guideline No. 408. Since animal exposure to consensus bacterial variant phytase is through oral ingestion, selection of this NOAEL is thus appropriate.

NOAEL: 1000 mg TOS/kg bw/day

6.4.2 Conclusion

Determination of the Margin of Exposure

The margin of exposure is calculated by dividing the NOAEL obtained from the 90-day oral (gavage) study in rats by the exposure (worst-case scenario) assessed in Part 3. If the margin of exposure is greater than 100, it suggests that the available toxicology data support the proposed uses and application rates. The Margin of Exposure is listed in Table 6.

$$\text{Margin of Exposure} = \frac{\text{No Observed Adverse Effect Level (NOAEL)}}{\text{Maximum Daily Exposure}}$$

Table 6: Margin of Exposure Calculation for phytase from *T. reesei* expressing a variant of consensus bacterial variant phytase

	Poultry	**Piglets**	**Swine**
Maximum intake of enzyme TOS (mg/kg BW) (see Table 2)	0.62	0.35	0.29
NOAEL (mg TOS/kg bw/day)	1000	1000	1000
Margin of safety	**1613**	**2857**	**3448**

The rodent data is suitable for pigs and poultry, with appropriate safety margin to accommodate minor species-to-species variation, as both poultry and pigs are monogastric and neither is expected to be significantly more sensitive to common enzymes.

6.5 Safety to Humans consuming animal products (eggs, meat)

Any enzyme protein ingested by animals will be readily digested into amino acids and not absorbed intact by the animal for deposit/transfer into edible tissues/animal products for human consumption. Furthermore, phytase enzymes do not require a metal co-factor for optimal functioning, so it is unlikely that ingestion of the phytase by the target production animals will result in significant metal accumulation in their edible products. In addition, appropriate limits for contaminants (heavy metals and microbial contaminants, per current FCC and JECFA specifications for food enzymes and accounting for intended use in livestock feed) minimize animal exposure to contaminants to such a high degree that any potential transfer to edible tissues, eggs or milk (and thus, exposure to humans) will be negligible as well. As per the Enzyme

Marketing Coordination Document (AAFCO, 2020) enzymes used in animal feed that pass the safety assessment proposed by Pariza and Foster should raise no human safety concerns. The Pariza and Foster (1983) decision tree was updated by Pariza and Johnson (2001) for enzymes produced with modern biotechnology and this update was documented by Pariza and Cook (2010) to be suitable for animal feed enzymes.

The safety of this variant of consensus bacterial phytase has also been established using the Pariza and Cook (2010) decision tree (herein incorporated by reference).

1. **Is the production strain[1] genetically modified[2,3]?** Yes, go to 2.

2. **Is the production strain modified using rDNA techniques?** Yes, go to 3a.

3a. **Does the expressed enzyme product which is encoded by the introduced DNA[4,5] have a history of safe use in food or feed[6]?** Yes, phytase (IUBMB No. 3.1.3.26) has been used for years in animal feed. Although this variant of consensus bacterial phytase is a new sequence, the variant phytase expressed in *T. reesei* is still a phytase with the same IUBMB designation and the same general phytase properties. Given that protein engineered enzymes have the same basic properties and toxicological profile as their non-engineered counterparts based on many studies (see review by Pariza and Cook, 2010) and given the high sequence similarities of this variant of consensus bacterial phytase to phytase molecules from various other sources along with demonstrated phytase activity, it is considered substantially equivalent to these phytases, all of which have an extensive history of safe use. Go to 3c.

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[1] Production strain refers to the microbial strain that will be used in enzyme manufacture. It is assumed that the production strain is nonpathogenic, nontoxigenic, and thoroughly characterized; steps 6–11 are intended to ensure this.

[2] The term "genetically modified" refers to any modification of the strain's DNA, including the use of traditional methods (*e.g.*, UV or chemically-induced mutagenesis) or rDNA technologies.

[3] If the answer to this or any other question in the decision tree is unknown, or not determined, the answer is then considered to be NO.

[4] Introduced DNA refers to all DNA sequences introduced into the production organism, including vector and other sequences incorporated during genetic construction, DNA encoding any antibiotic resistance gene, and DNA encoding the desired enzyme product. The vector and other sequences may include selectable marker genes other than antibiotic resistance, noncoding regulatory sequences for the controlled expression of the desired enzyme product, restriction enzyme sites and/or linker sequences, intermediate host sequences, and sequences required for vector maintenance, integration, replication, and/or manipulation. These sequences may be derived wholly from naturally occurring organisms or incorporate specific nucleotide changes introduced by *in vitro* techniques, or they may be entirely synthetic.

[5] If the genetic modification served only to delete host DNA, and if no heterologous DNA remains within the organism, then proceed to step 5.

[6] Enzymes from bioengineered organisms are considered *not* to have a history of safe use in food, unless they are derived from a safe lineage of previously tested engineered enzymes expressed in the same host using the same modification system.

3c. Is the test article free of transferable antibiotic resistance gene DNA[1]? No transferable antibiotic resistance gene DNA is present in the enzyme preparation, as no antibiotic resistance marker was used. Go to 3e.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce feed-grade products? Yes, inserted DNA is well characterized and free of unsafe attributes. For example, a specific BLAST search for homology of the consensus bacterial variant phytase sequence was performed against the Uniprot animal toxin database. The consensus bacterial variant phytase sequence does not share homology with any of the known toxin or venom sequences. As such, the production strain is not known to produce oral toxins. Go to 4.

4. Is the introduced DNA randomly integrated into the chromosome? Yes, the introduced DNA is randomly integrated into the chromosome. If yes, go to 5.

5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? Yes, the production strain proved to be 100% stable after at least 30 generations of fermentation. Comparative genomic analysis studies of the strain at the beginning and end of fermentation confirm that there is no rearrangement of the expression cassette into another locus in the genome. No other new genetic material is expressed with the exception of the partial *pyrG* gene marker from *Aspergillus nidulans*, and the consensus bacterial variant phytase gene. The non-toxic and non-pathogenic status of *T. reesei* is well known and as such no differences between the modified strain and its non-modified parent are expected. If yes, go to 6.

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure[2]? Yes, the production strain is derived from a safe strain lineage that is supported by repeated analysis in this decision tree, including toxicological studies. Nevertheless, this exact variant phytase enzyme was subjected to additional toxicological studies per regulatory requirements in various jurisdictions, which confirm the absence of any safety concerns. If yes, go to 11.

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[1] Antibiotic resistance genes are commonly used in the genetic construction of enzyme production strains to identify, select, and stabilize cells carrying introduced DNA. Principles for the safe use of antibiotic resistance genes in the manufacture of food and feed products have been developed (IFBC, 1990; "FDA Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants (https://www.gpo.gov/fdsys/pkg/FR-1998-09-08/pdf/98-24072.pdf)

[2] In determining safe strain lineage, one should consider the host organism, all of the introduced DNA, and the methods used to genetically modify the host. In some instances, the procedures described by Pariza and Foster (1983) and IFBC (1990) may be considered comparable to this evaluation procedure in establishing a safe strain lineage

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety? Yes. In a 90-day repeated gavage study, a NOAEL for phytase variant was established in male and female rats at 1000 mg TOS/kg/day. This provides a margin of safety that greatly exceeds 100 with a margin of 1613, 2857, and 3448 over the estimated exposure via feed poultry, piglets and swine respectively.

Conclusion: The test article is ACCEPTED.

6.6 BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the safety sections above, *T. reesei* and enzyme preparations produced by this organism, including phytase from *T. reesei* expressing a variant of consensus bacterial phytase, are well recognized by qualified experts as being safe for their intended uses. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO's JECFA, as well as DuPont's published and unpublished safety studies, support such a conclusion.

T. reesei is widely used by enzyme manufacturers around the world for production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is generally recognized as a safe host for enzyme production. In addition, the *T. reesei* lineage used by DuPont has been demonstrated to be safe based on extensive toxicity studies and history of safe use.

The exposure of phytase from *T. reesei* expressing a variant of consensus bacterial phytase as a food additive in poultry and swine applications is assessed based on a battery of toxicology studies as well as feeding studies in swine and poultry.

Results from published safety assessment including a 90-day toxicology study (Ladics *et al*., 2020) investigating the systemic toxicity potential of this phytase preparation, demonstrated the phytase preparation doesn't induce systemic toxicity when administered by gavage to rats for 90 continuous days. The consistent toxicology results allowed for establishment of the NOAEL at 1000 mg TOS/kg bw.

This enzyme preparation is manufactured using appropriate raw materials of suitable purity for the intended use per Good Manufacturing Practices, *i.e.* minimum amounts needed to achieve desired effect (Sewalt *et al*., 2016). In addition, once the manufacturing process is completed, an evaluation for microbial and chemical contaminants is performed (per the Food Chemical Codex (US Pharmacopeia, 2020) and FAO/WHO's Joint Evaluation Committee for Food Additives (JECFA, 2006), and only then is the final enzyme preparation product released. Furthermore, for both Quality and Intellectual Property (IP) protection reasons, enzyme manufacturers further evaluate the final enzyme product to make sure that they are free of the active production organism.

The phytase enzyme preparation from *T. reesei* expressing a variant of consensus bacterial phytase is intended for use in poultry and swine feed directly. Using the NOAEL derived from the 90-day oral toxicity test with phytase, it was calculated that exposure to animals is of no safety concern as the safety margin is far greater than 100, in fact even greater than 1000 (see Table 6). The proposed uses of phytase as a swine and poultry feed additive at a rate of (b) (4) is not a human health concern and are supported by existing toxicological data.

We conclude Phytase from *T. reesei* expressing a variant of consensus bacterial phytase is safe for use as a feed additive in swine and poultry feed, without health concern to animals consuming the feed, and without health concern to humans consuming animal products of animals fed the above feed additives as substantiated by existing toxicology data and corroborated by new published toxicology studies.

Based on the publicly available scientific data from the literature and additional supporting data published by DuPont (Ladics *et al*., 2020; Dersjant-Li *et al*, 2020a; 2020b), and the decision tree analysis using generally recognized evaluation methodology (Pariza and Johnson, 2001; Pariza and Cook, 2010, Sewalt *et al*., 2016), experts in toxicology, food and feed safety, and animal nutrition at DuPont have concluded that the Phytase from *T. reesei* expressing a variant of consensus bacterial phytase with properties as described in Christensen (2020) is safe and suitable for use as a feed additive in poultry and swine feed. Collectively, the use of published information and evaluation methods provide a strong common knowledge element, based upon which this phytase can be considered Generally Recognized as Safe (GRAS) for its intended uses. In addition, the safety determination, including construction of the production organism, the production process and materials, and safety of the product, were reviewed by an external expert in the field of microbial enzyme safety, Dr. Michael Pariza, who concurred with the company's conclusion that the product is GRAS (see Appendix 14).

7. SUPPORTING DATA AND INFORMATION

7.1 LIST OF THE APPENDIXES (CBI – Confidential Business Information)

1. Scheme and Detailed Description of Production Strain Construction – CBI
2. Amino Acid Sequence of Consensus Variant CRC2836-13885 Bacterial Phytase and its Alignment to Wild Type Phytases and Consensus Phytase - CBI
3. Southern Blot Analysis of Phytase Expressing Strains
4. PCR Amplification for detection of successful cassette integration.
5. "*In vitro* Characterization of a Novel Consensus Bacterial 6-Phytase and one of its Variants" by Christensen *et al*. (2020).
6. Manufacturing Flow Chart
7. Raw Materials Used During Manufacture - CBI
8. Certificate of Analysis (3 Lots)
9. EPA Final Rule: Microorganisms; General Exemptions from Reporting Requirements; Revisions to Recipient Organisms Eligible for Tier I and Tier II Exemptions. (85 FR 13760) [FRL-9991-60] 40 CFR Part 725
10. Toxin Homology Database Search – CRC2836-13885 - CBI
11. Ladics *et al.* (2020): Safety evaluation of a novel variant of consensus bacterial phytase. Toxicol Rep. 2020; 7: 844–851. Published online July 2020 (https://doi:10.1016/j.toxrep.2020.07.004)
12. Dersjant-Li *et. al* (2020a) Publication: Functionality of a next generation biosynthetic bacterial 6-phytase in enhancing phosphorus availability to broilers fed a corn-soybean meal-based diet. Animal Feed Science and Technology 264: 114481. Published online June 2020 (https://doi.org/10.1016/j.anifeedsci.2020.114481)
13. Dersjant-Li *et. al* (2020b) Publication: Functionality of a next generation biosynthetic bacterial 6-phytase in enhancing phosphorus availability to weaned piglets fed a corn-soybean meal-based diet without added inorganic phosphate. Animal Nutrition 6(1): 24-30. Published online November 2019 (https://doi.org/10.1016/j.aninu.2019.11.003)
14. GRAS opinion on the intended use of DuPont's variant of consensus bacterial phytase produced by a non-pathogenic, non-toxigenic strain of *Trichoderma reesei* by Dr. Pariza
15. *T. reesei* Safe Strain Lineage CBI
16. ETA 2003 Correspondence Regarding Defoaming and Flocculating Agents
17. *T. reesei* Toxicology Study Summary

7.2 REFERENCES

AAFCO. (2020). Association of American Feed Control Officials. *The Official Publication of the Association of American Feed Control Officials*.

Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K. (1979) Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Aunstrup, K. (1979) Production, isolation, and economics of extracellular enzymes. In *Applied Biochemistry and Bioengineering* (pp. 27-69).

Babe, L.M., Christensen, T. Haaning, S., Kim, H-S., Mejldal, R., Nikolaev, I., Prasad, J.C., Pricelius, S., Sorensen, J.F., and Sorg, R.A. (2020) Engineered Robust High Tm-Phytase Clade Polypeptides and Fragments Thereof. Patent Application Number WO/2020/106796 (https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2020106796, accessed 8/2/2020).

Blumenthal C.Z. (2004) Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. *Regulatory Toxicology and Pharmacology* **39**: 214-228.

Canada (2017) Food and Drug Regulations (Division 16, Table 5), Government of Canada. available at: http://laws-lois.justice.gc.ca/eng/regulations/C.R.C.,_c._870/page-72.html#h-108.

Chang, R., Andrews, K., Kim, D., Li, Z., Godzik, A., Palsson, B. (2013) Structural Systems Biology Evaluation of Metabolic Thermotolerance in *E. coli*. *Science* **340:** 1220-1223.

Christensen, T., Dersjant-Li, Y., Sewalt, V., Mejldal, R., Haaning, S., Pricelius, S., Nikolaev, I, Sorg, R.A., and de Kreij, A. (2020) *In vitro* Characterization of a Novel Consensus Bacterial 6-Phytase and one of its Variants. *Current Biochemical Engineering* **6**: 156-171.

Dersjant-Li, Y., Archer, G., Stiewert, A. Brown, A., Sobotik, E., Jasek, A., Marchal, L., Bello, A., Sorg, R., Christensen, T., Kim, H., Mejldal, R, Nikolaev, I., Pricelius, S., Haaning, S., Sørensen, J., de Kreije, A., Sewalt, V., (2020a) Functionality of a next generation biosynthetic bacterial 6-phytase in enhancing phosphorus availability to broilers fed a corn-soybean meal-based diet. Animal Feed Science and Technology 264: 114481. https://doi.org/10.1016/j.anifeedsci.2020.114481.

Dersjant-Li Y., Villca, B., Sewalt, V., de Kreij, A., Marchal, L., Velayudhan, D.E., Sorg, R.A., Christensen, T., Mejldal, R., Nikolaev, I., Pricelius, S., Kim, H-S., Haaning, S., Sørensen, J.F., Lizardo, R. (2020b) Functionality of a next generation biosynthetic bacterial 6-phytase in enhancing phosphorus availability to weaned piglets fed a corn-soybean meal-based diet without added inorganic phosphate. Animal Nutrition **6**: 24-34. https://doi.org/10.1016/j.aninu.2019.11.003.

FAO/WHO (Food and Agriculture Organization of the United States/World Health Organization) (2006) General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting. *FAO JECFA Monographs*, *3*, 63-67.

FDA US (2000) GRN No 32. Pectin lyase derived from *Trichoderma reesei* carrying a gene encoding pectin lyase from *Aspergillus niger*, available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=32.

FDA US (2015a) GRN No 566. Mannanase from *Trichoderma reesei*, available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=566&sort=GRN_No&order=ASC&startrow=1&type=basic&search=Trichoderma.

FDA US (2015b) GRN No 567. Xylanase from *Trichoderma reesei*, available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=567&sort=GRN_No&order=ASC&startrow=1&type=basic&search=Trichoderma.

FDA/CFSAN Division of Biotechnology and GRAS Notice Review (2010). Guidance for Industry – Enzyme Preparations: Recommendations for Submission of Chemical and Technological data for Food Additive Petitions and GRAS Notices. Available at: https://www.fda.gov/downloads/Food/GuidanceRegulation/UCM217735.pdf (last accessed March 2, 2019).

Gentile, J., Roneker, K., Crowe, S., Pond, W., and Lei, X. (2003) Effectiveness of an experimental consensus phytase in improving dietary phytate-phosphorus utilization by weanling pigs. *Journal of Animal Science.* **11**: 2751-7.

Gumulya, Y., Baek, J., Wun, S. *et al.* (2018) Engineering highly functional thermostable proteins using ancestral sequence reconstruction. *Nature Catalysts* **1:** 878–888.

Hjortkjaer RK, Bille-Hansen V, Hazelden KP, McConville M, McGregor DB, Cuthbert JA, Greenough RJ, Chapman E, Gardner JR & Ashby R (1986) Safety evaluation of Celluclast®, an acid cellulase derived from *Trichoderma reesei*. *Food and Chemical Toxicology* **24**: 55-63.

IFBC (International Food Biotechnology Council) (1990) Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. *Regulatory Toxicology and Pharmacology* **12**: S1-S196.

Kroschwitz, J.I. (1994) Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition. Volume 9, pp. 567-620.

Ladics, G.S., K-H. Han, M.S. Jang, H. Park, V. Marshall, Y.Dersjant-Li, and V.J. Sewalt (2020) Safety evaluation of a novel variant of consensus bacterial phytase. *Toxicology Reports* **7**: 844–851. https://doi.org/10.1016/j.toxrep.2020.07.004.

Ladics, G.S. and V. Sewalt. 2018. Industrial microbial enzyme safety: What does the weight-of-evidence indicate? Regul. Toxicol. Pharmacol. 98: 151-154. https://doi.org/10.1016/j.yrtph.2018.07.016.

EPA, 2020. Microorganisms; General Exemptions from Reporting Requirements; Revisions to Recipient Organisms Eligible for Tier I and Tier II Exemptions. (85 FR 13760) [FRL-9991-60] 40 CFR Part 725 https://beta.regulations.gov/document/EPA-HQ-OPPT-2011-0740-0018

Modeweg-Hansen L (1978) Acute oral toxicity of cellulase SP122 to rats, mice, and dogs, Novo Reports.

Nakashima, B., McAllister, T., Sharma, R., and Selinger, L. (2007) Diversity of phytases in the Rumen. *Microbial Ecology* **53:** 82-88

Nevalainen H, Suominen P & Taimisto K (1994) On the safety of *Trichoderma reesei*. *Journal of Biotechnology* **37**: 193-200.

NIH (2016) Guidelines for research involving recombinant of synthetic nucleic acid molecules, National Institutes of Health. available at: https://osp.od.nih.gov/biotechnology/nih-guidelines/

Olempska-Beer ZS, Merker RI, Ditto MD & DiNovi MJ (2006) Food-processing enzymes from recombinant microorganisms - a review. *Regulatory Toxicology and Pharmacology* **45**: 144-158.

Paloheimo M, Haarmann T, Mäkinen S & Vehmaanperä J (2016) Production of Industrial Enzymes in Trichoderma reesei, *Gene Expression Systems in Fungi: Advancements and Applications,* Eds. Schmoll M & Dattenböck C, Cham, Springer International Publishing: 23-57.

Pariza MW & Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regulatory Toxicology and Pharmacology* **33**: 173-186. https://doi.org/10.1006/rtph.2001.1466

Pariza, M.W. and Cook, M. (2010) Determining the safety of enzymes used in animal feed. *Regulatory Toxicology and Pharmacology* **56**: 332-342. https://doi.org/10.1016/j.yrtph.2009.10.005.

Pariza, M.W. and Foster, E.M. (1983) Determining the safety of enzymes used in food processing. *Journal of Food Protection* **46**: 453-468. https://doi.org/10.4315/0362-028X-46.5.453.

Schmoll, M. and Dattenbock, C. (Ed.) (2016) *Gene Expression Systems in Fungi: Advancements and Applications.*

Sheir-Neiss, G. and Montenecourt, B.S. (1984) Characterization of the secreted cellulases of *Trichoderma reesei* wild type and mutants during controlled fermentations. *Applied Microbiology and Biotechnology* **20**: 46-53.

U.S. Pharmacopeia (2020) Monograph: Enzyme Preparations. In: Food Chemicals Codex, *12th Edition*. Washington, D.C.

Wilding, M., Peat, T., Kalyaanamoorth, S., Newman, J, Scott, C., and Jermiin, L. (2017) Reverse engineering: transaminase biocatalyst development using ancestral sequence reconstruction. *Green Chemistry* **19:** 5375-5380.

Appendix 1 - Scheme and Detailed Description of Production Strain Construction

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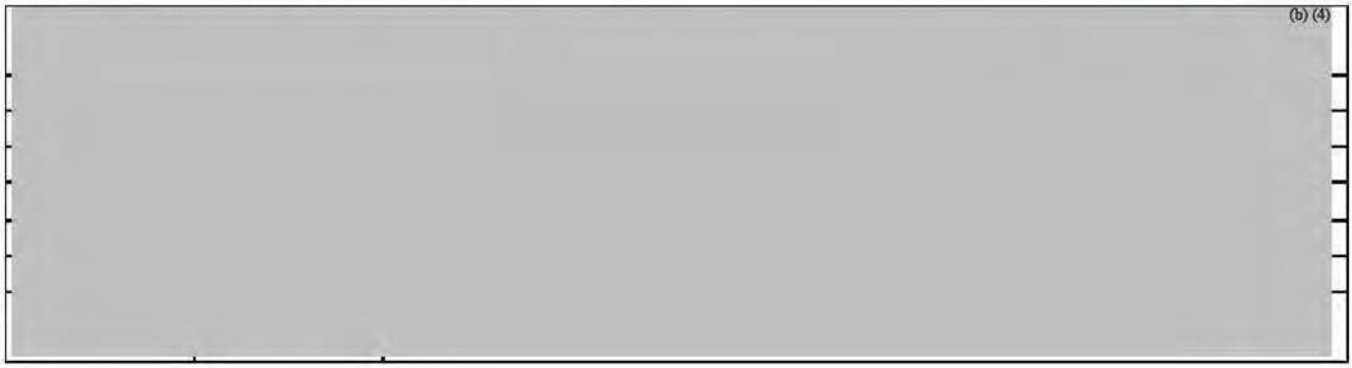

The new production strain *Trichoderma reesei* was deposited in the DuPont Industrial Biosciences culture collection as (b) (4)

Figure legends

Figure 1: (b) (4)

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Figure 30A and B: (b) (4)

Figure 31: (b) (4)

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Figure 43. (b) (4)

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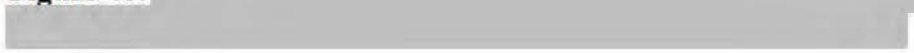

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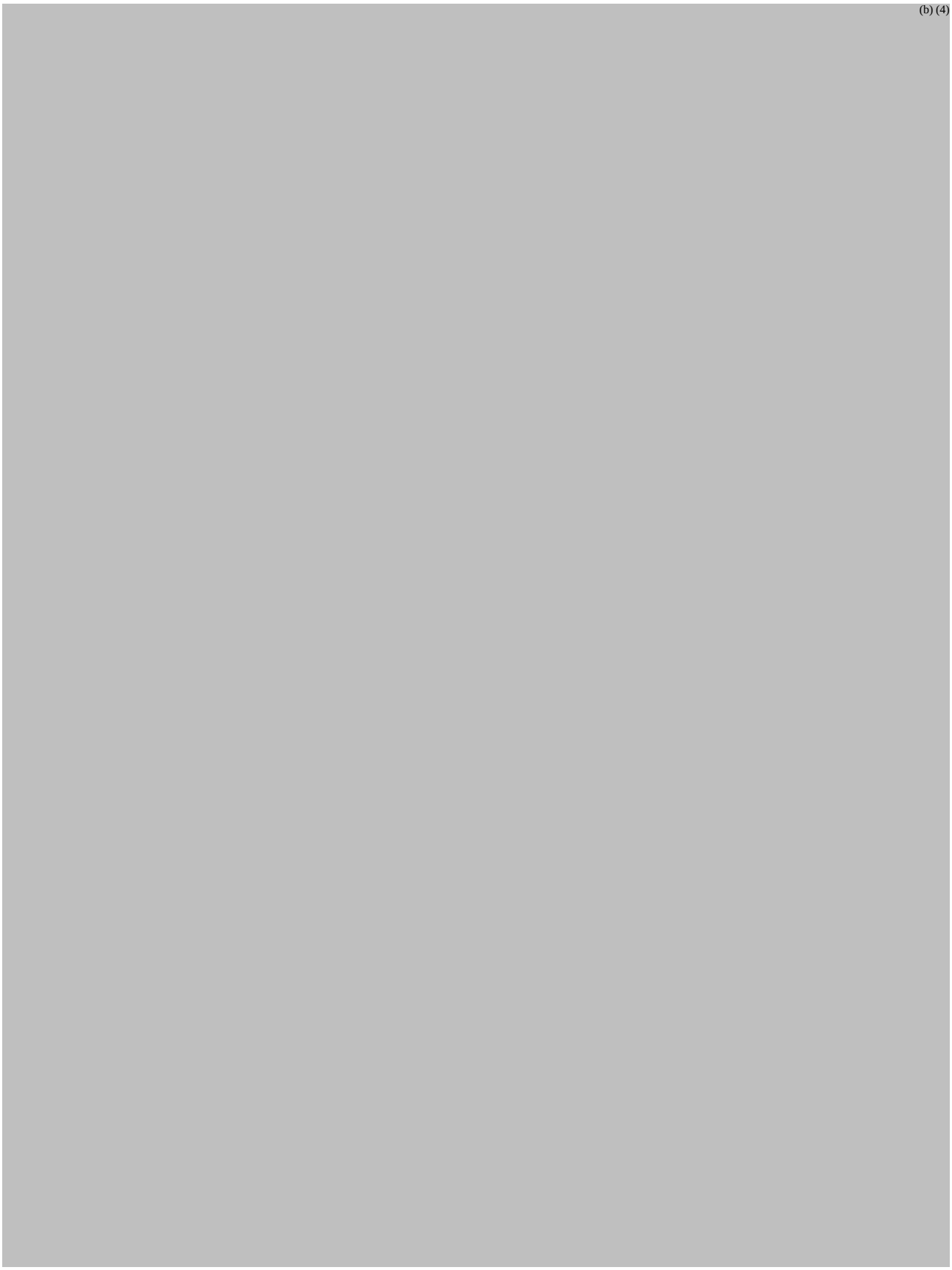

Figure 2

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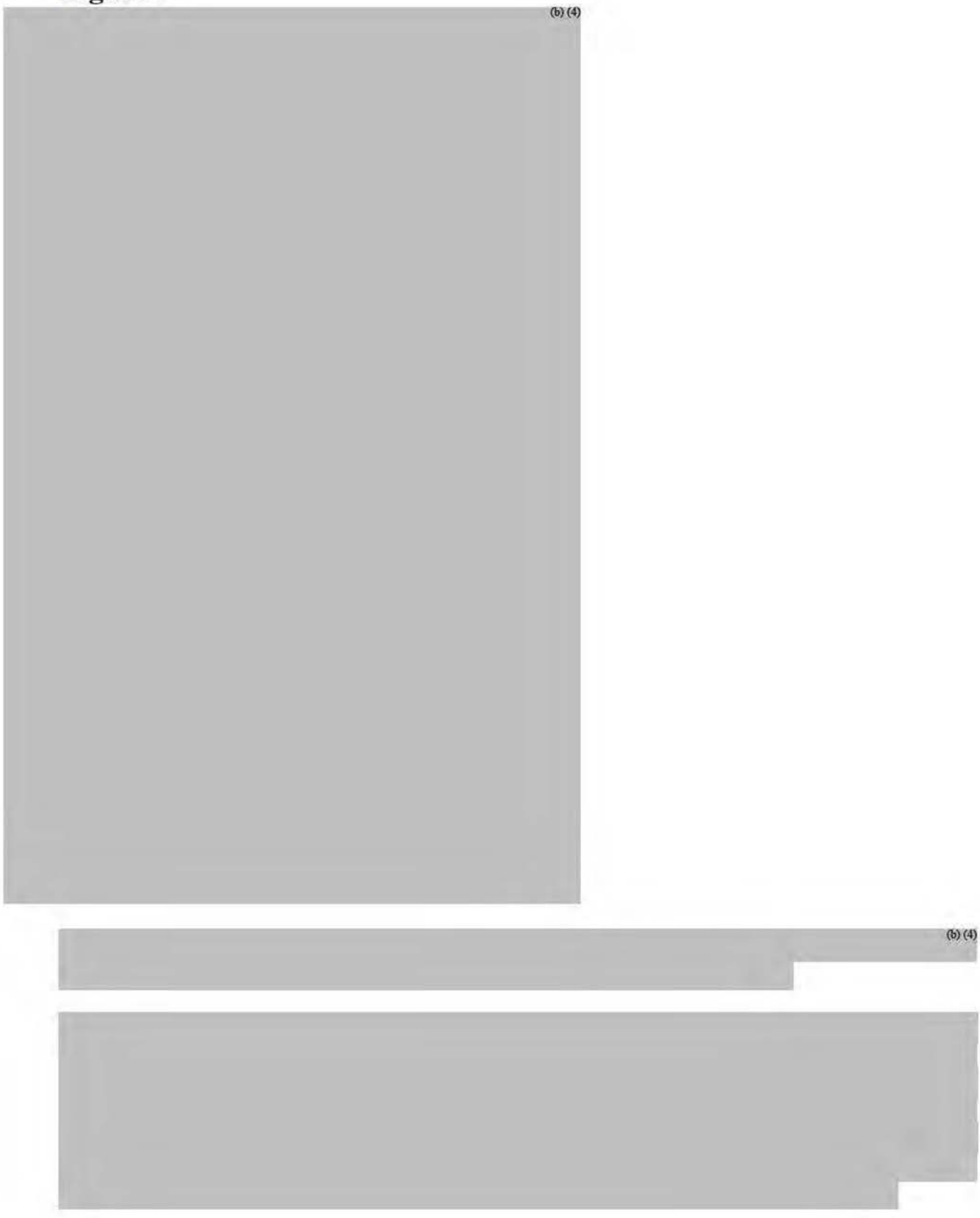

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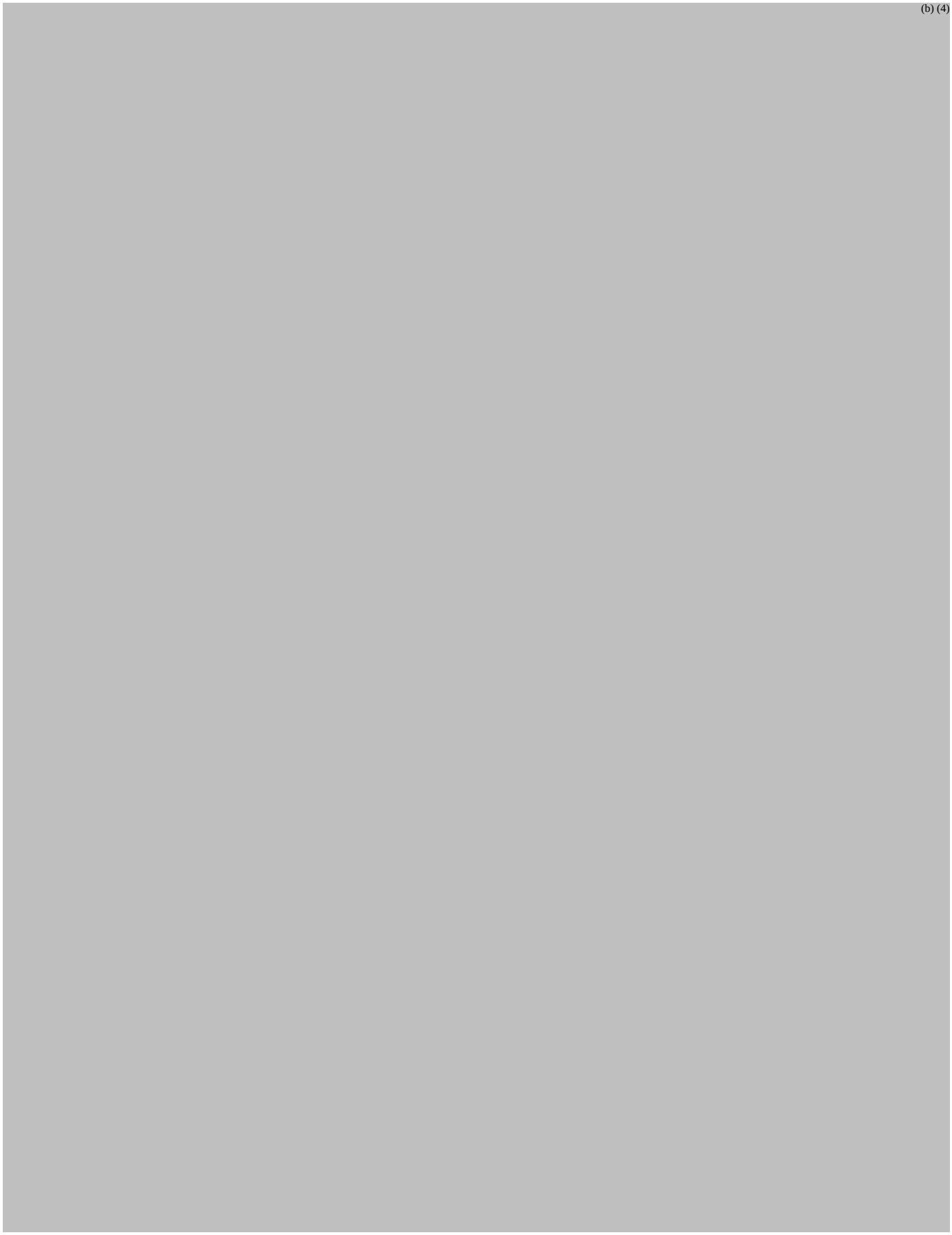
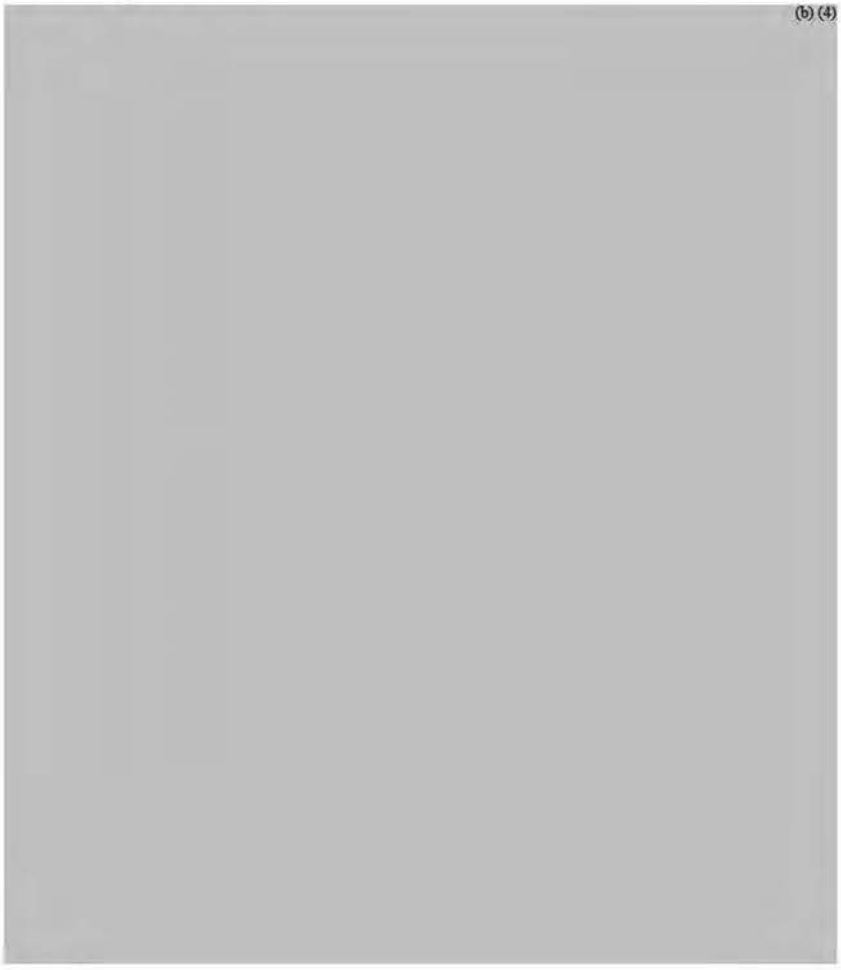


Figure 5

Figure 6



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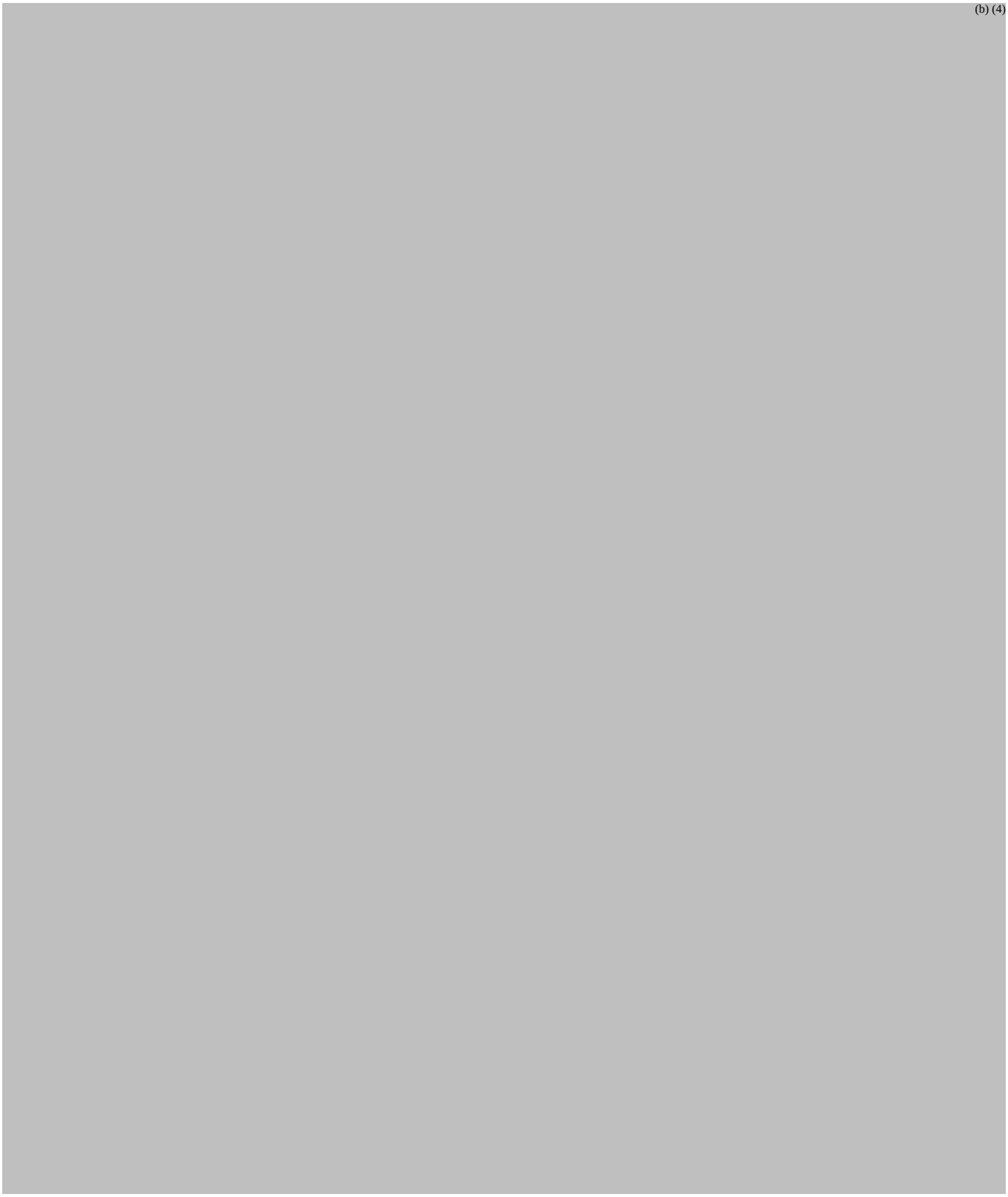


Figure 8

Figure 9

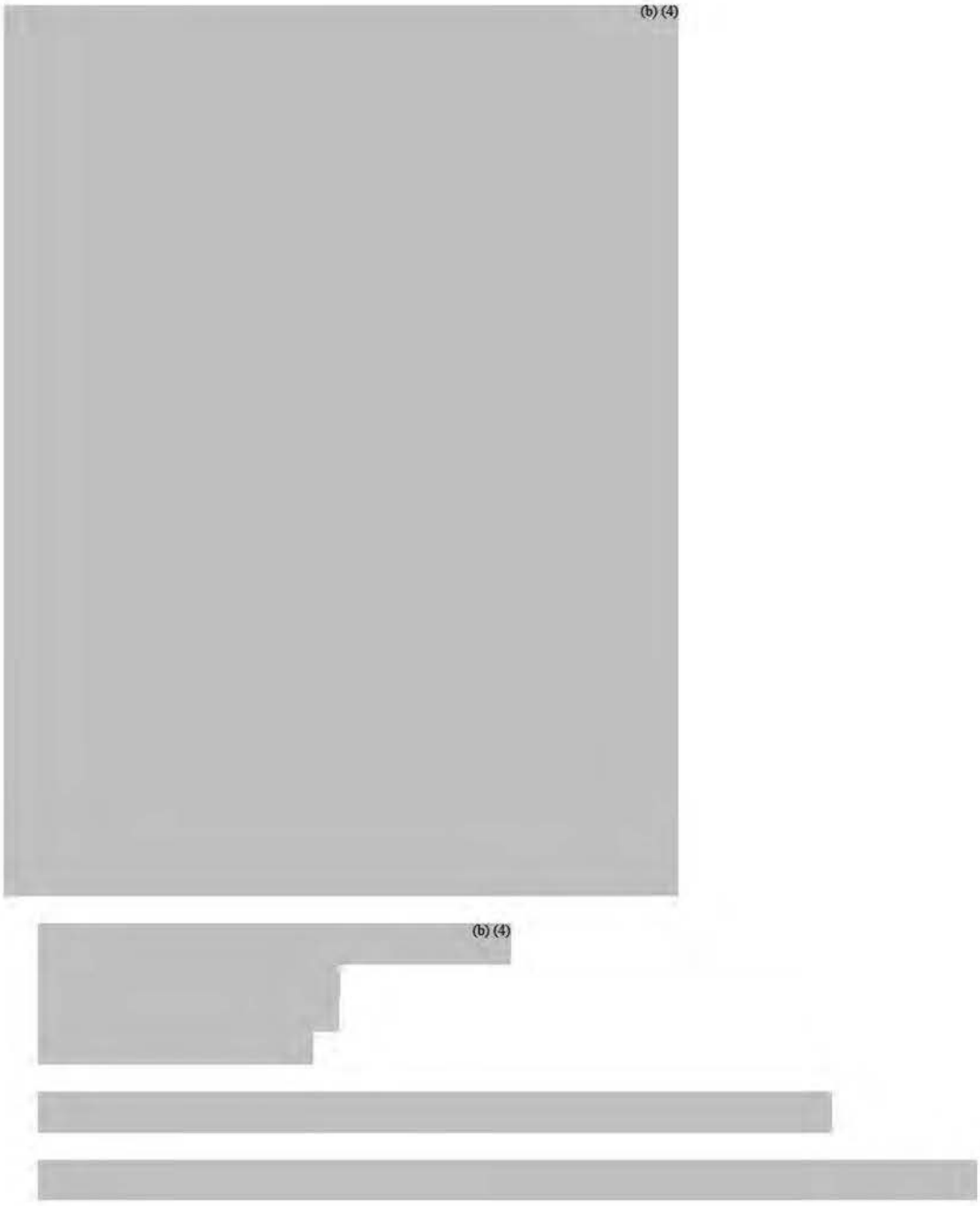


Figure 10

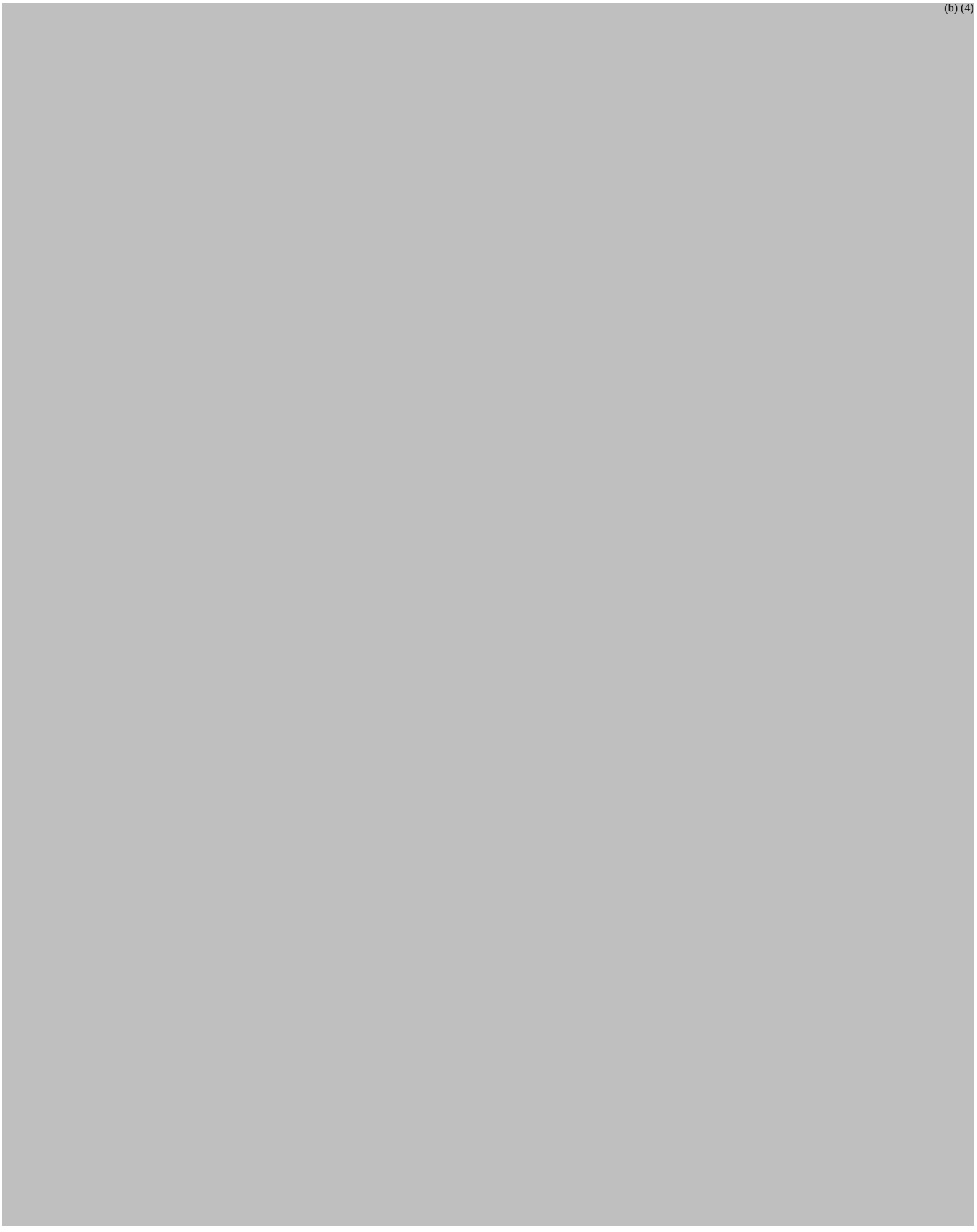


Figure 11

Figure 12

Figure 13

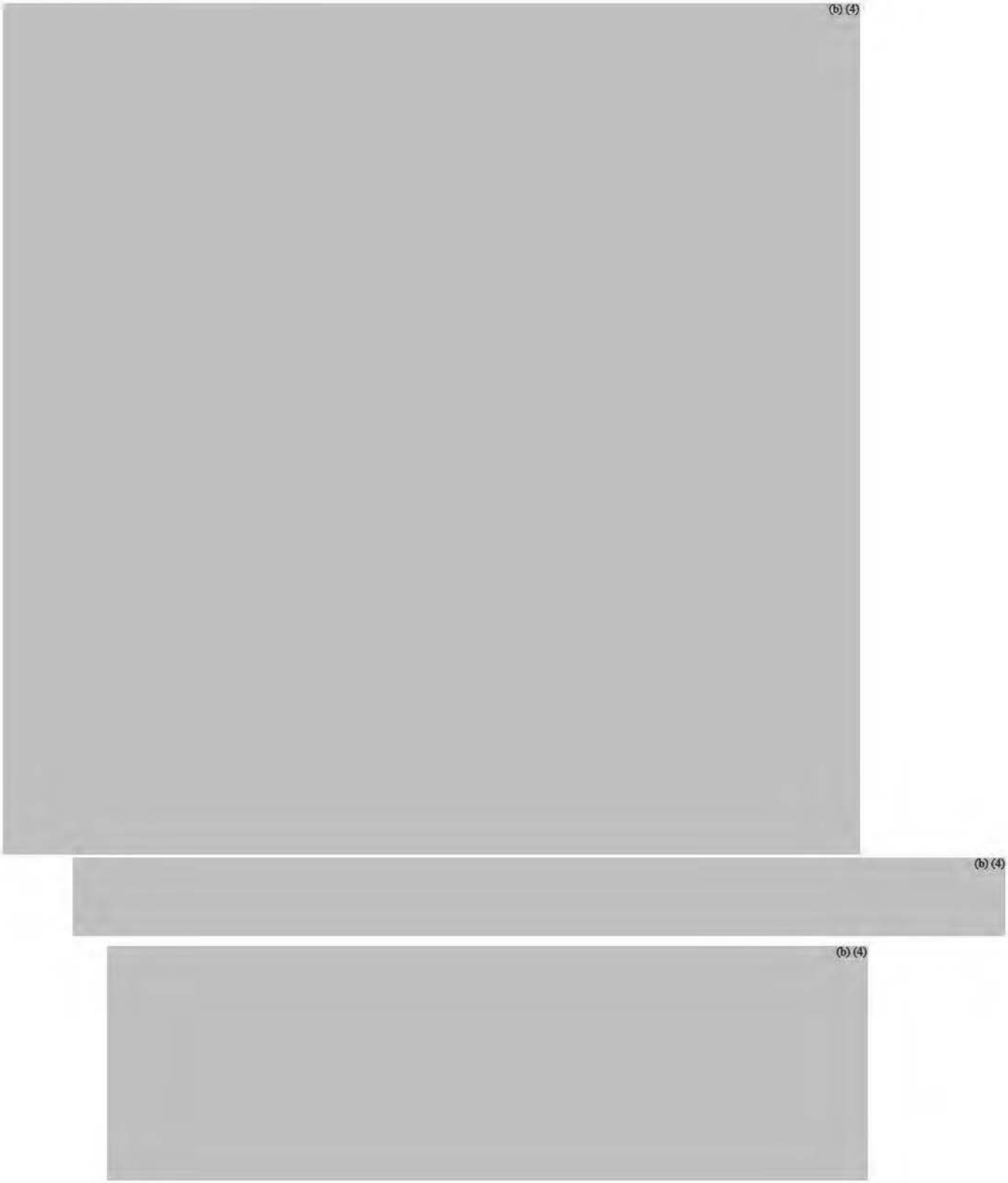
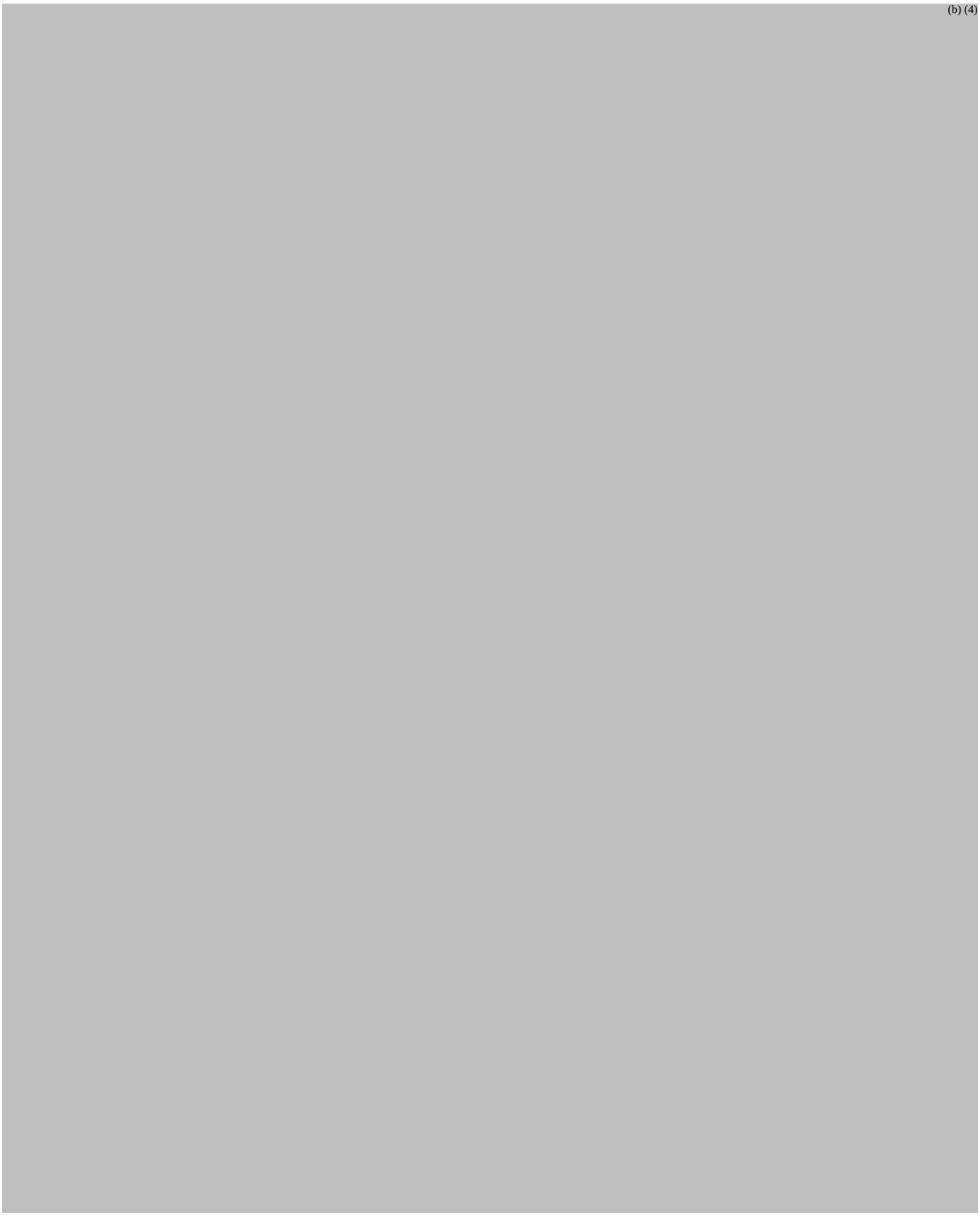


Figure 14



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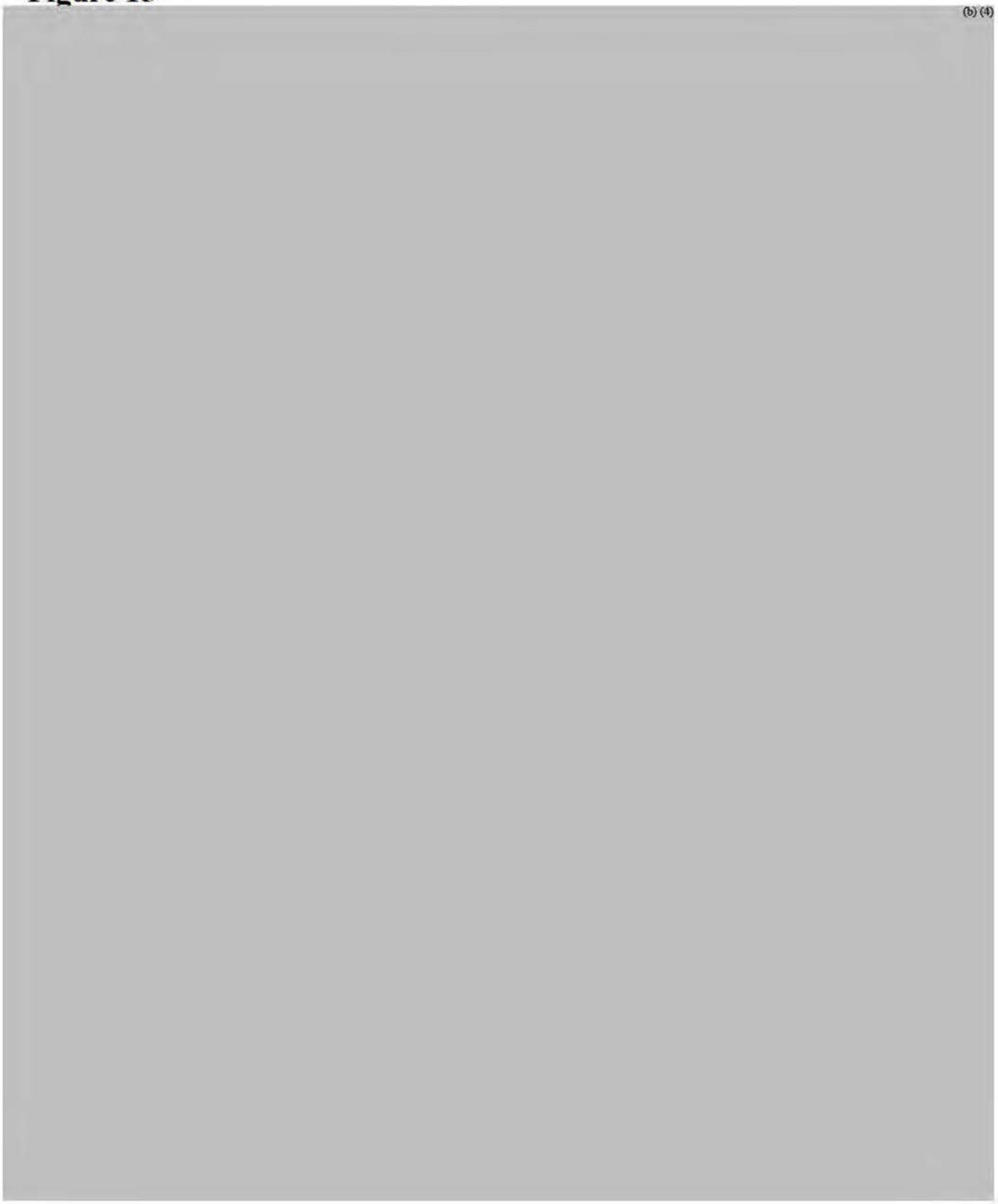

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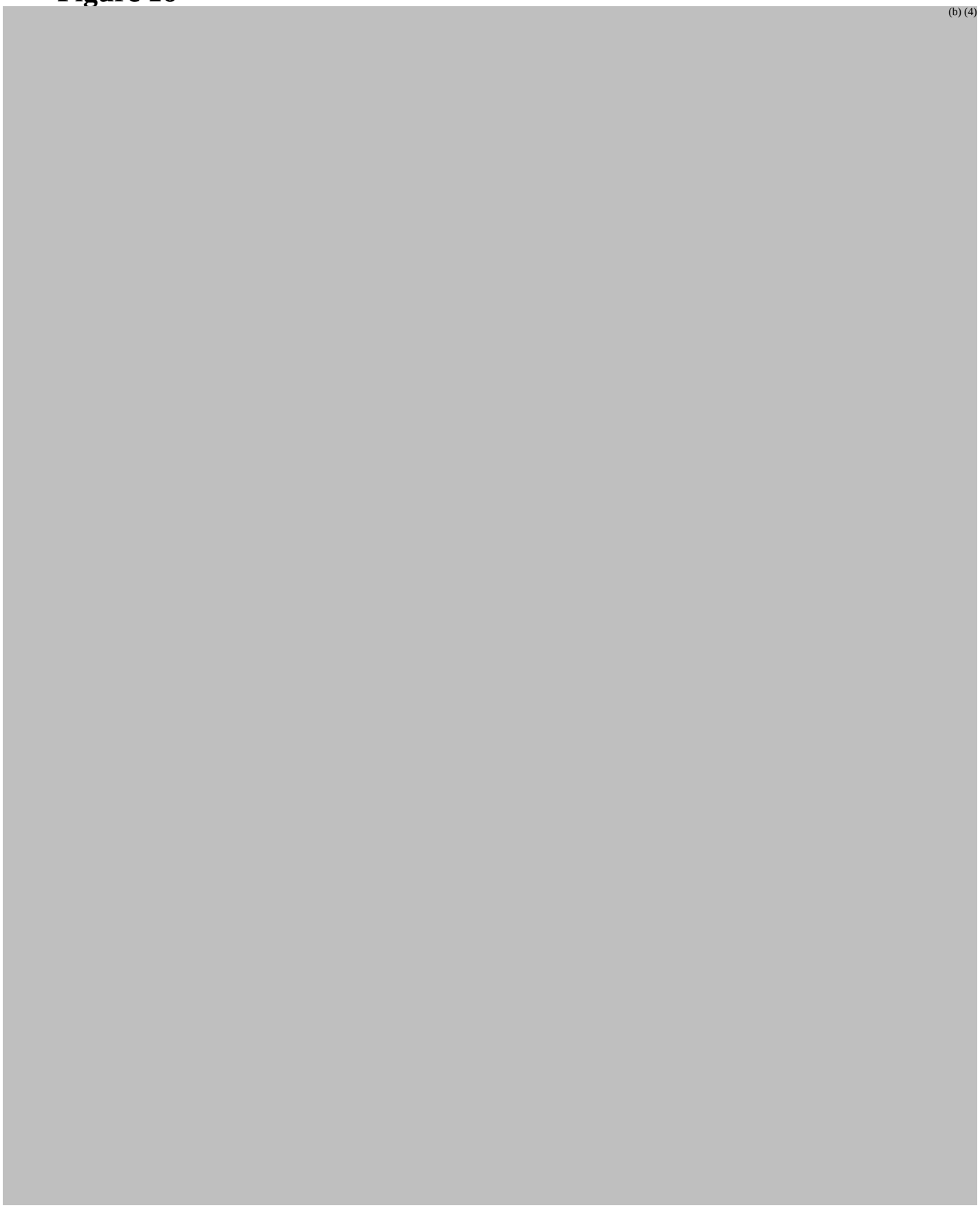

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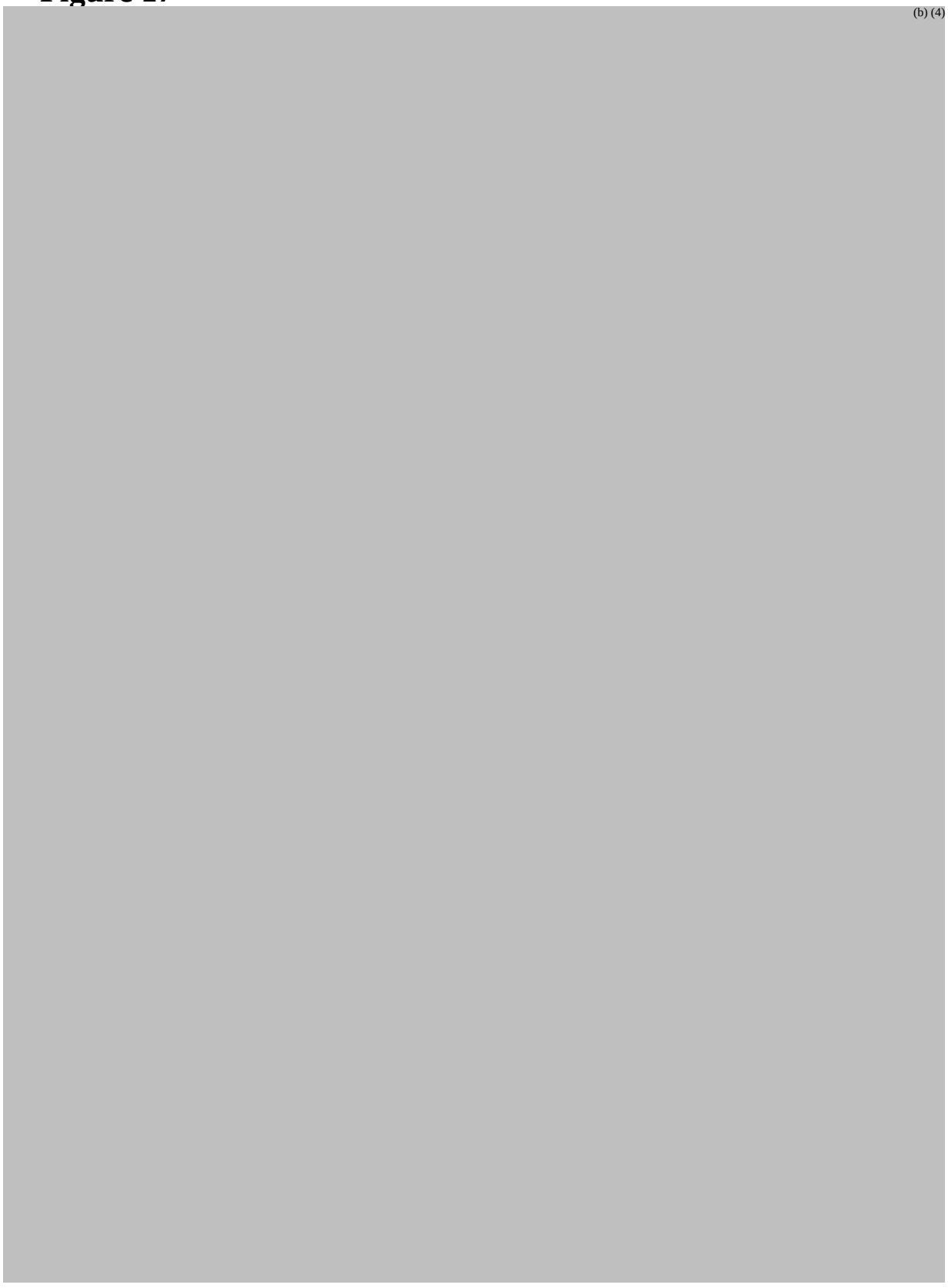
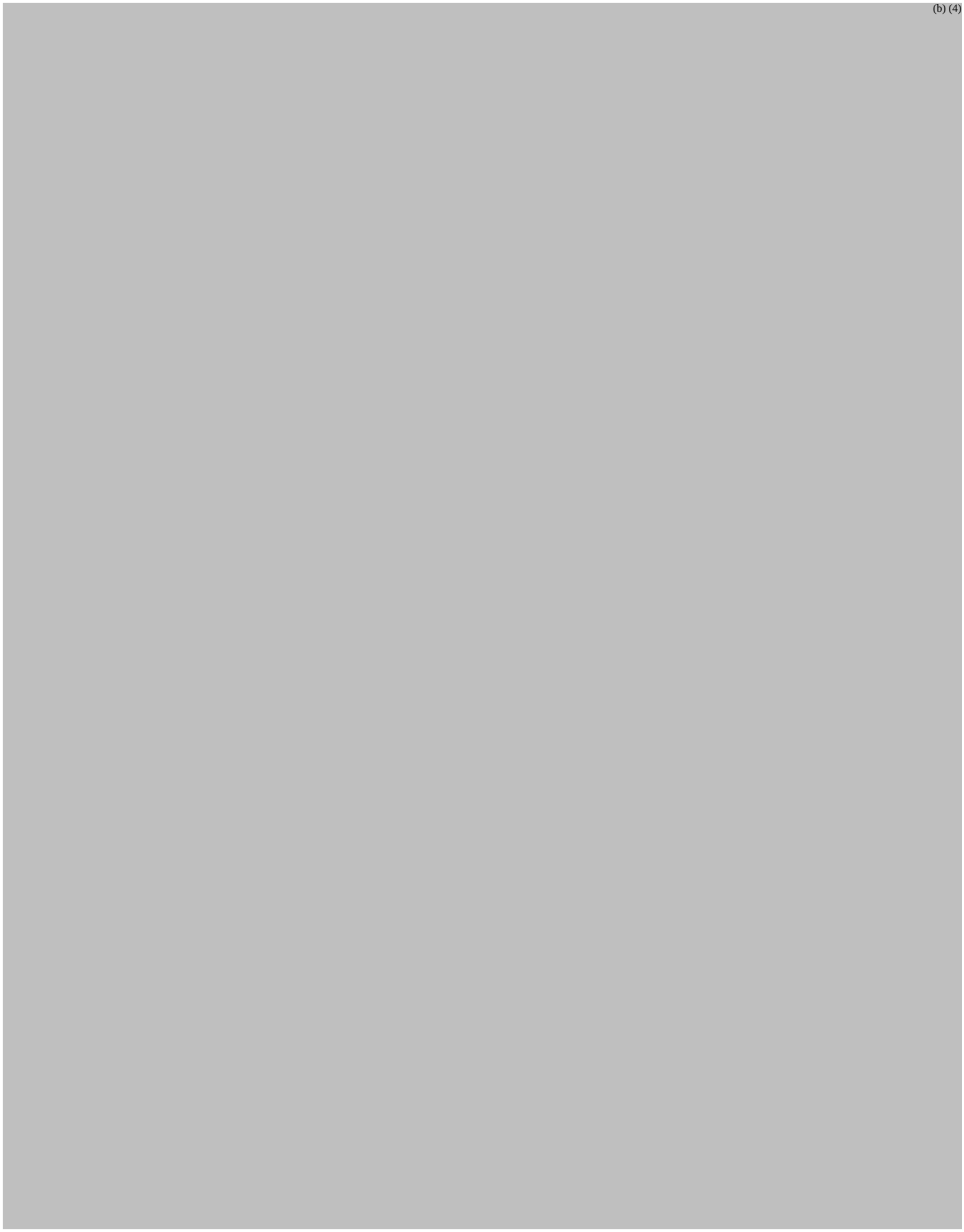


Figure 18

Figure 19

(b) (4)



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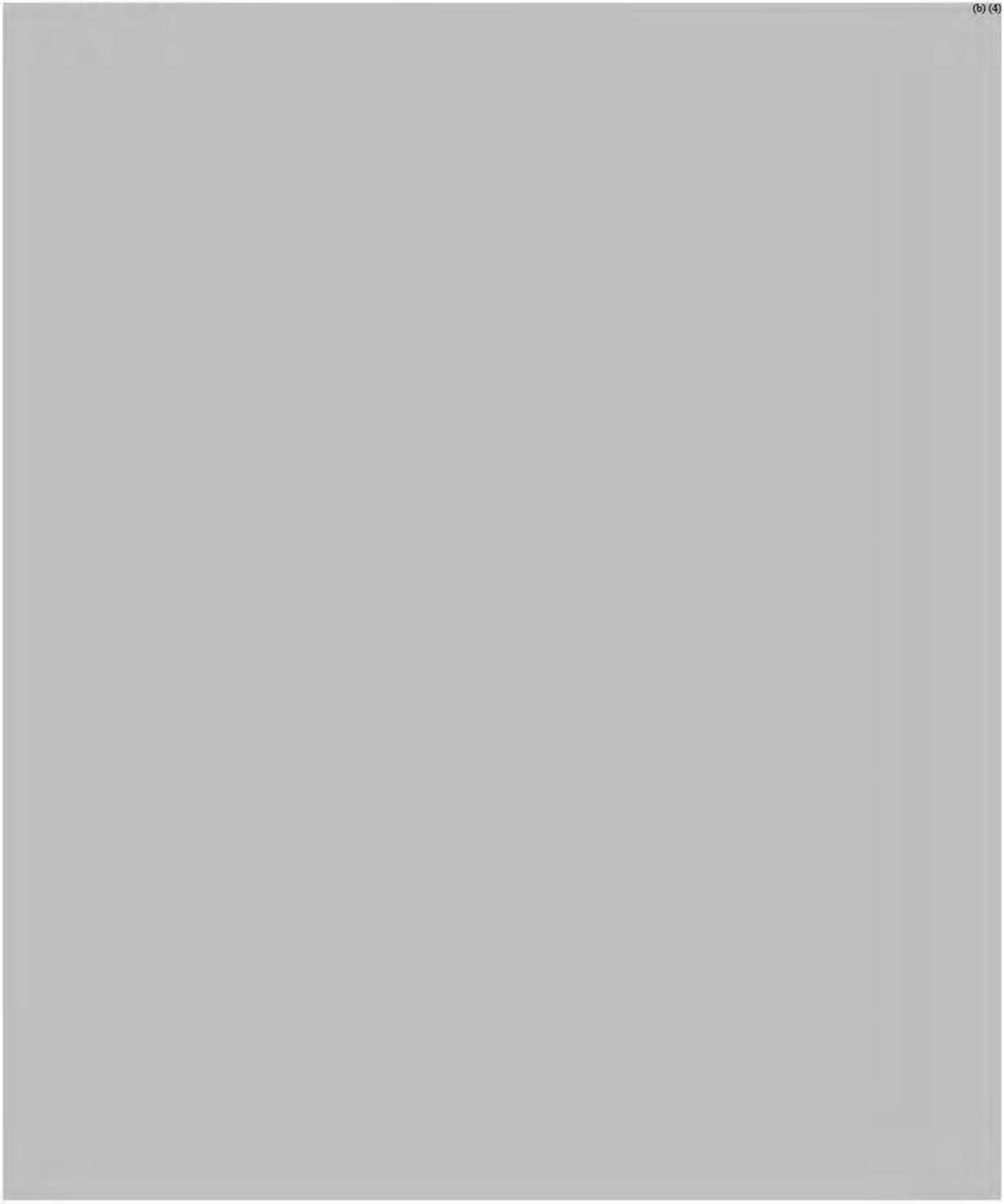

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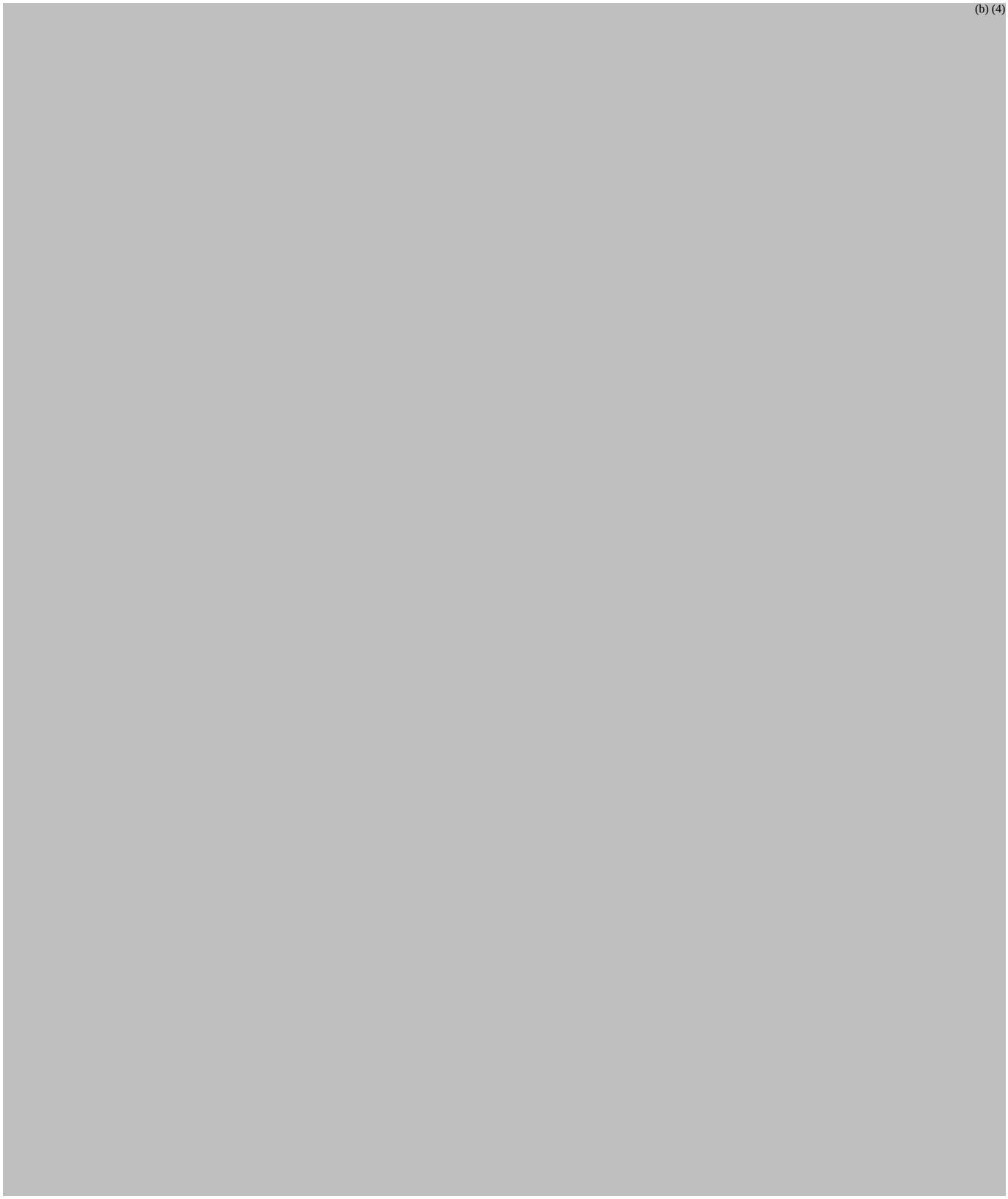

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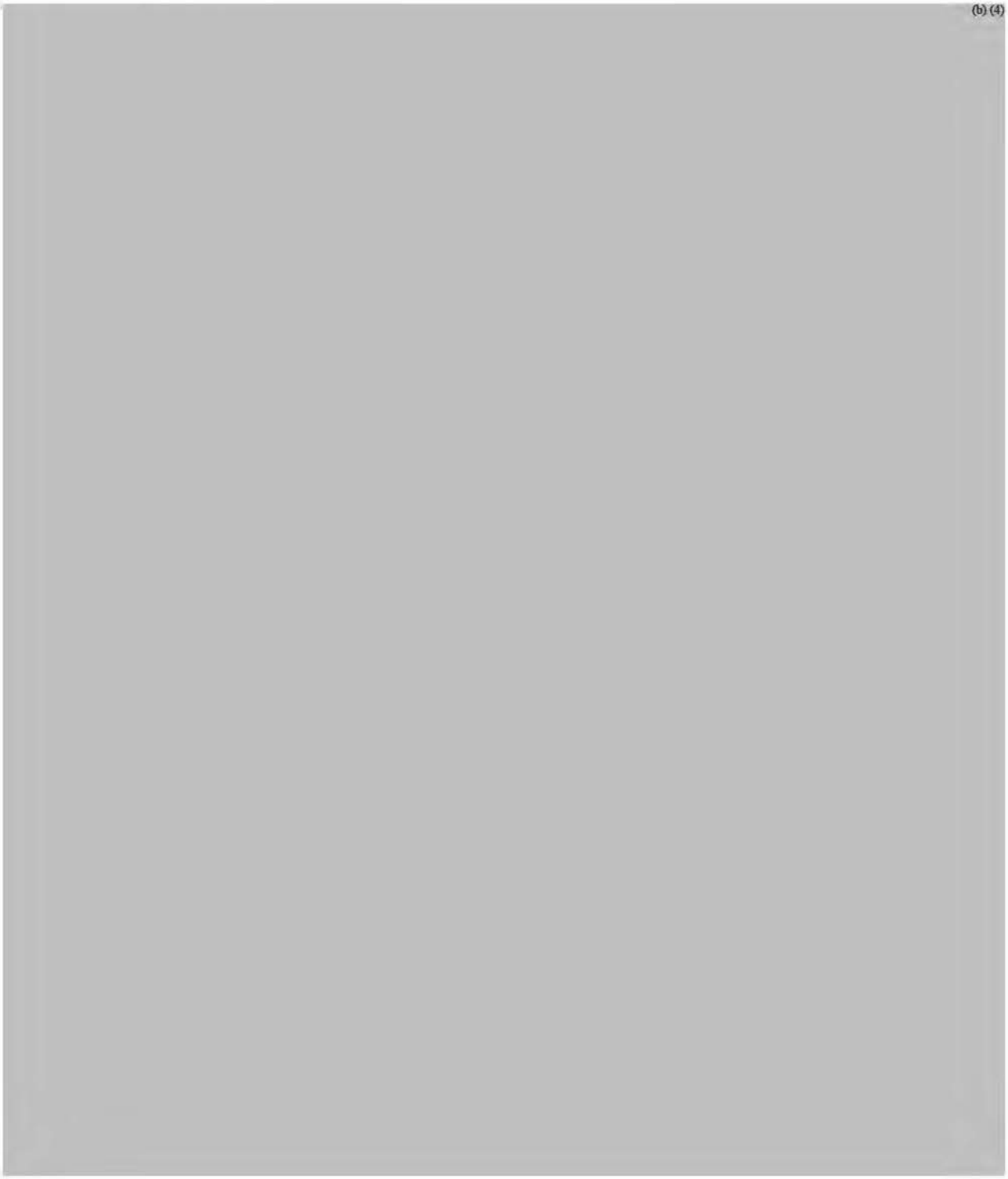

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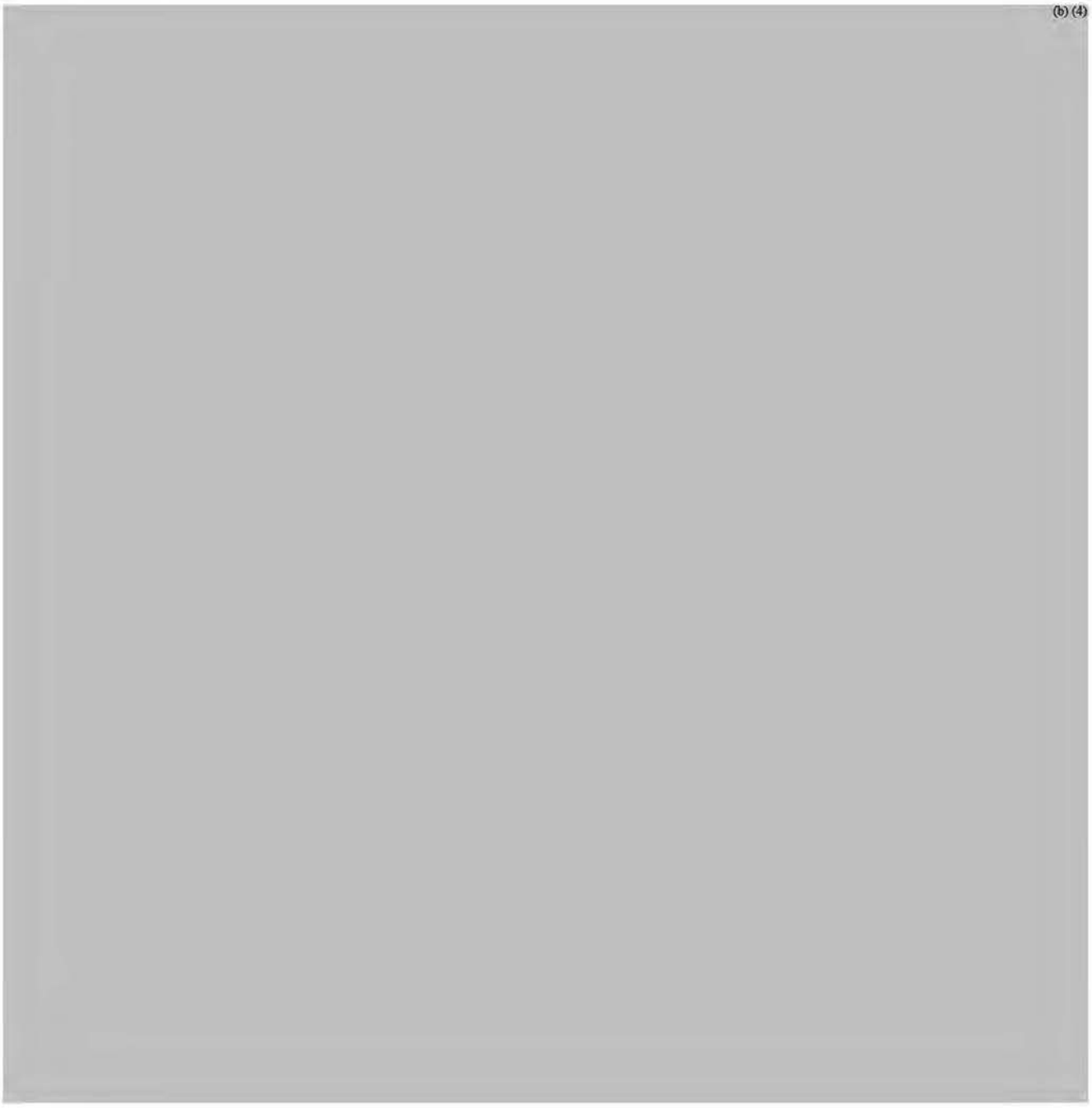

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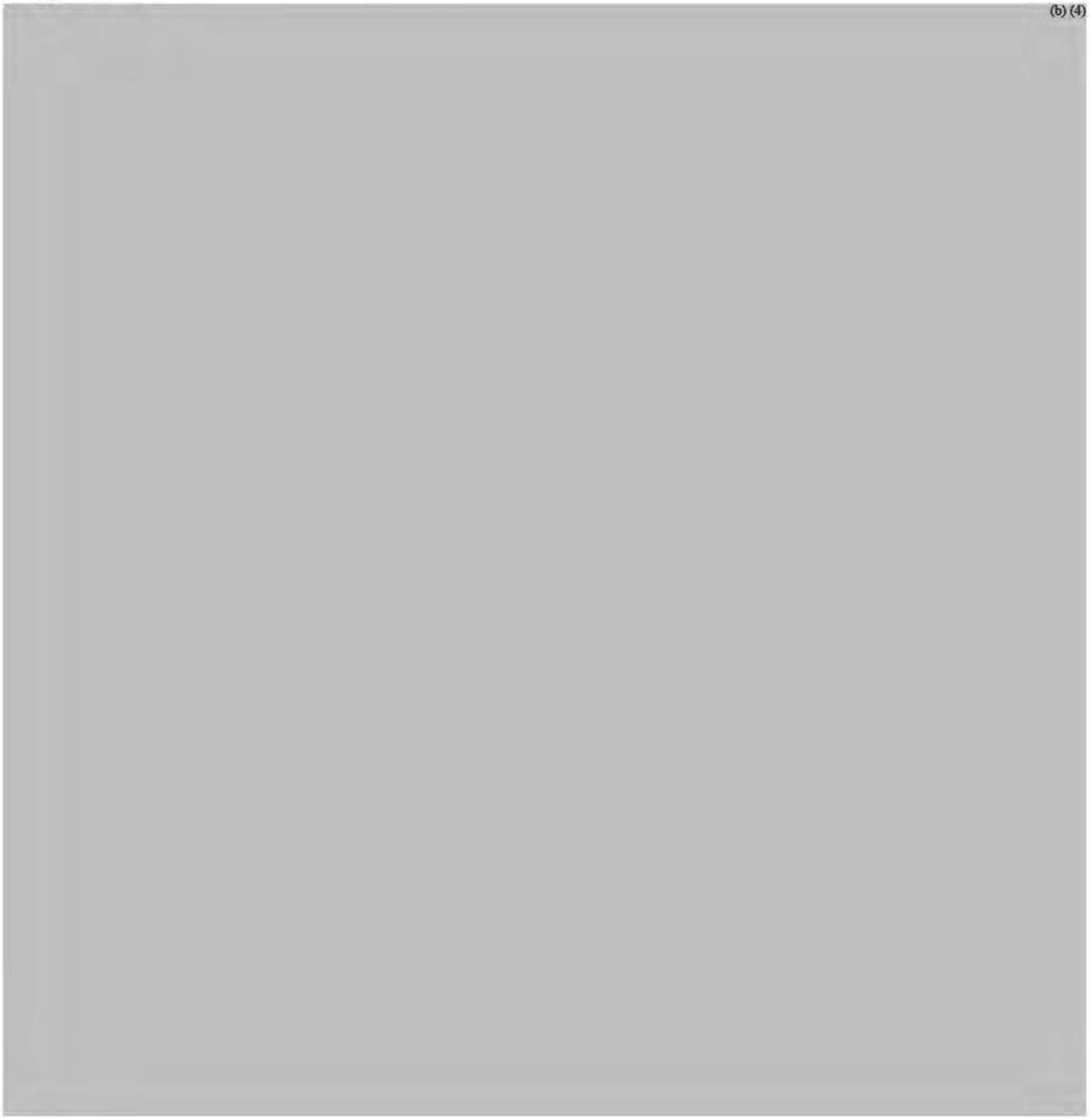
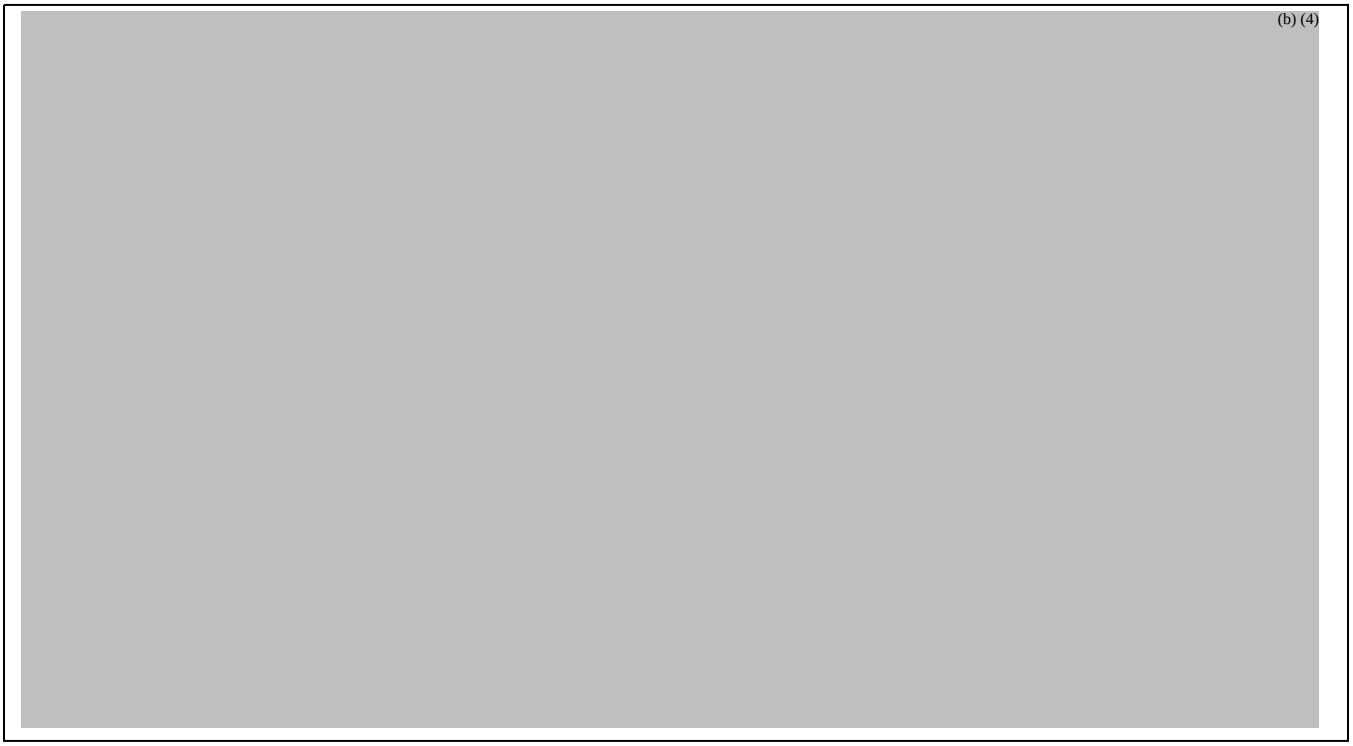


Figure 25. Nucleotide sequence



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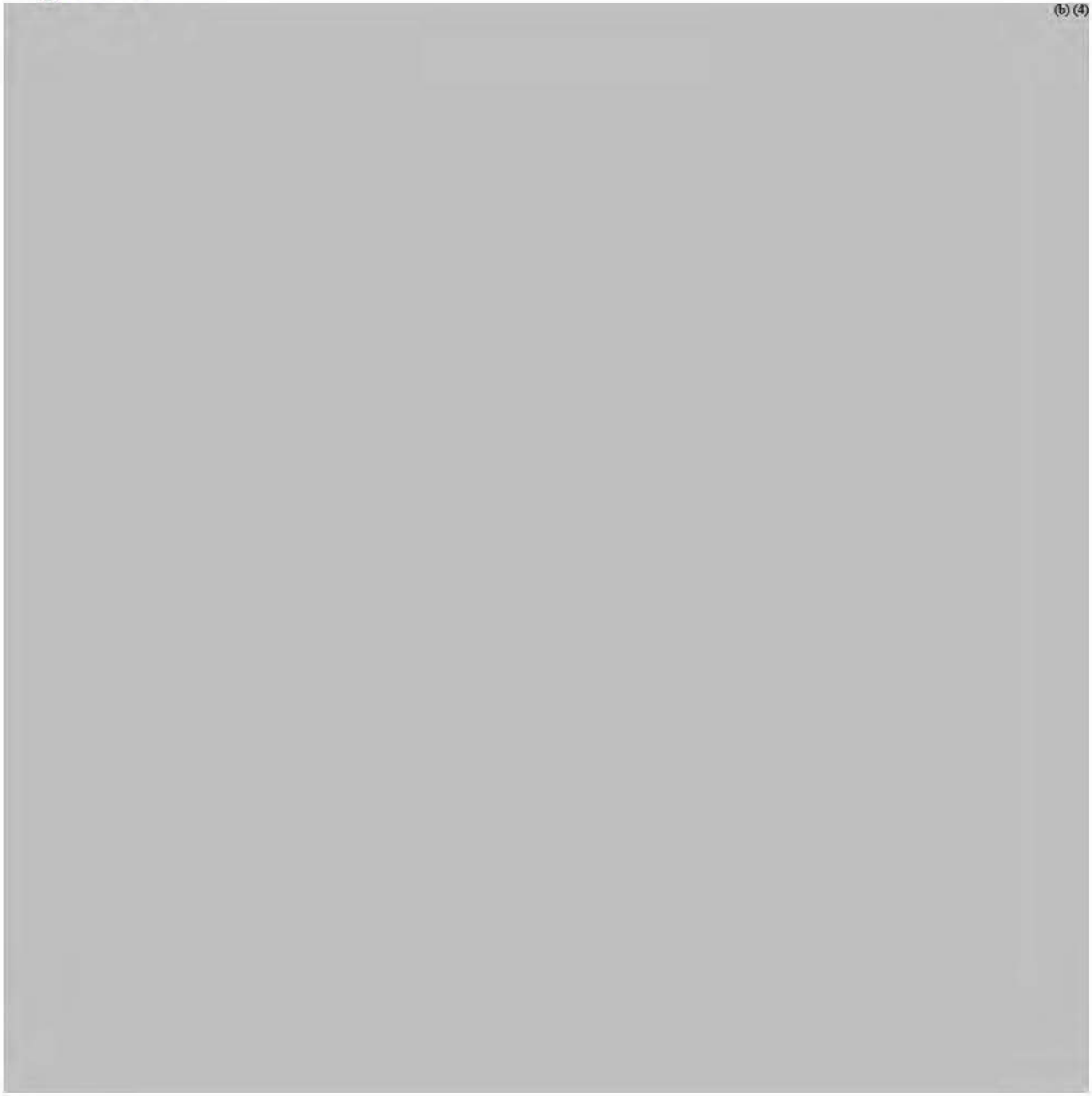
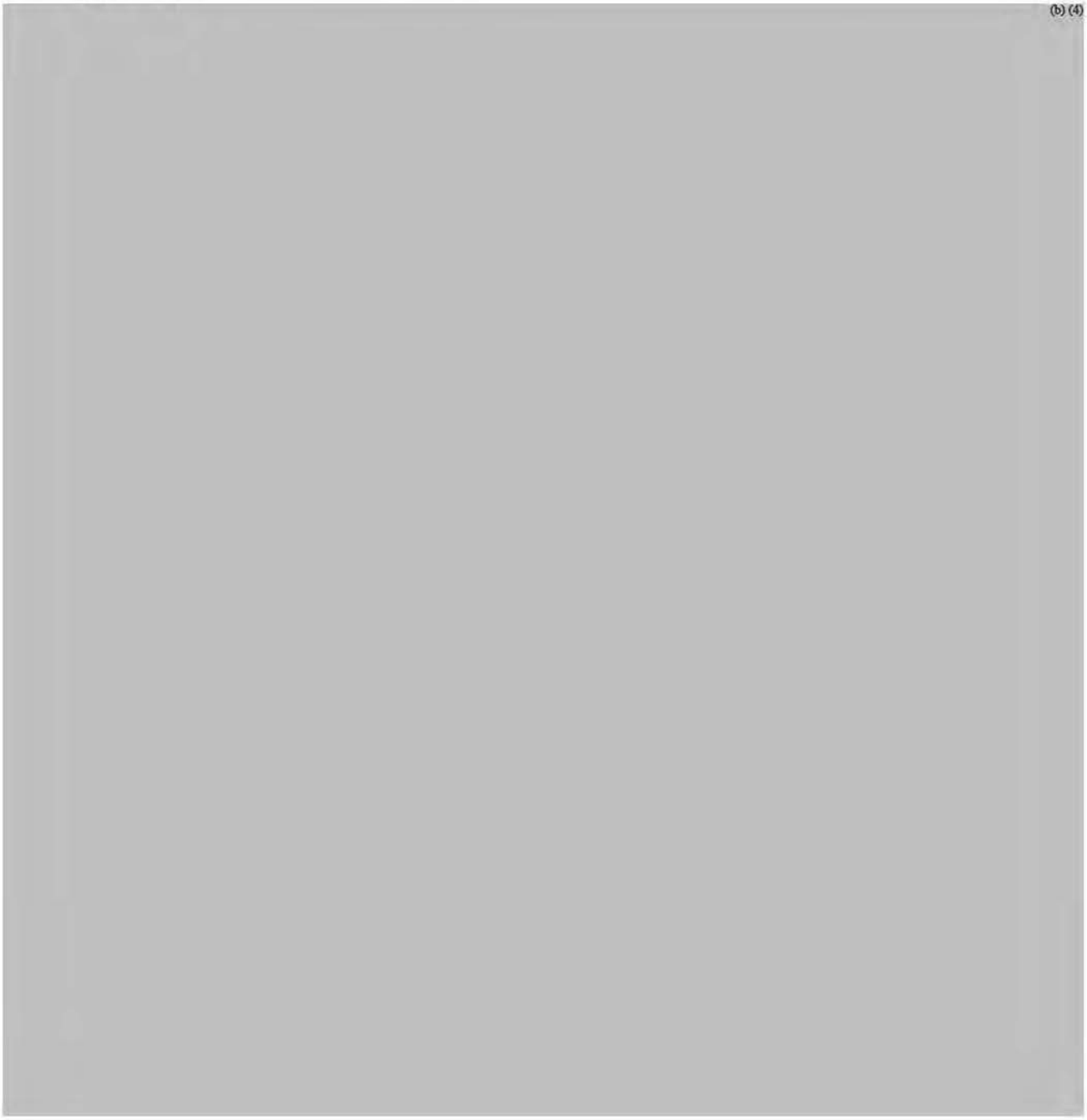


Figure 27

Figure 28



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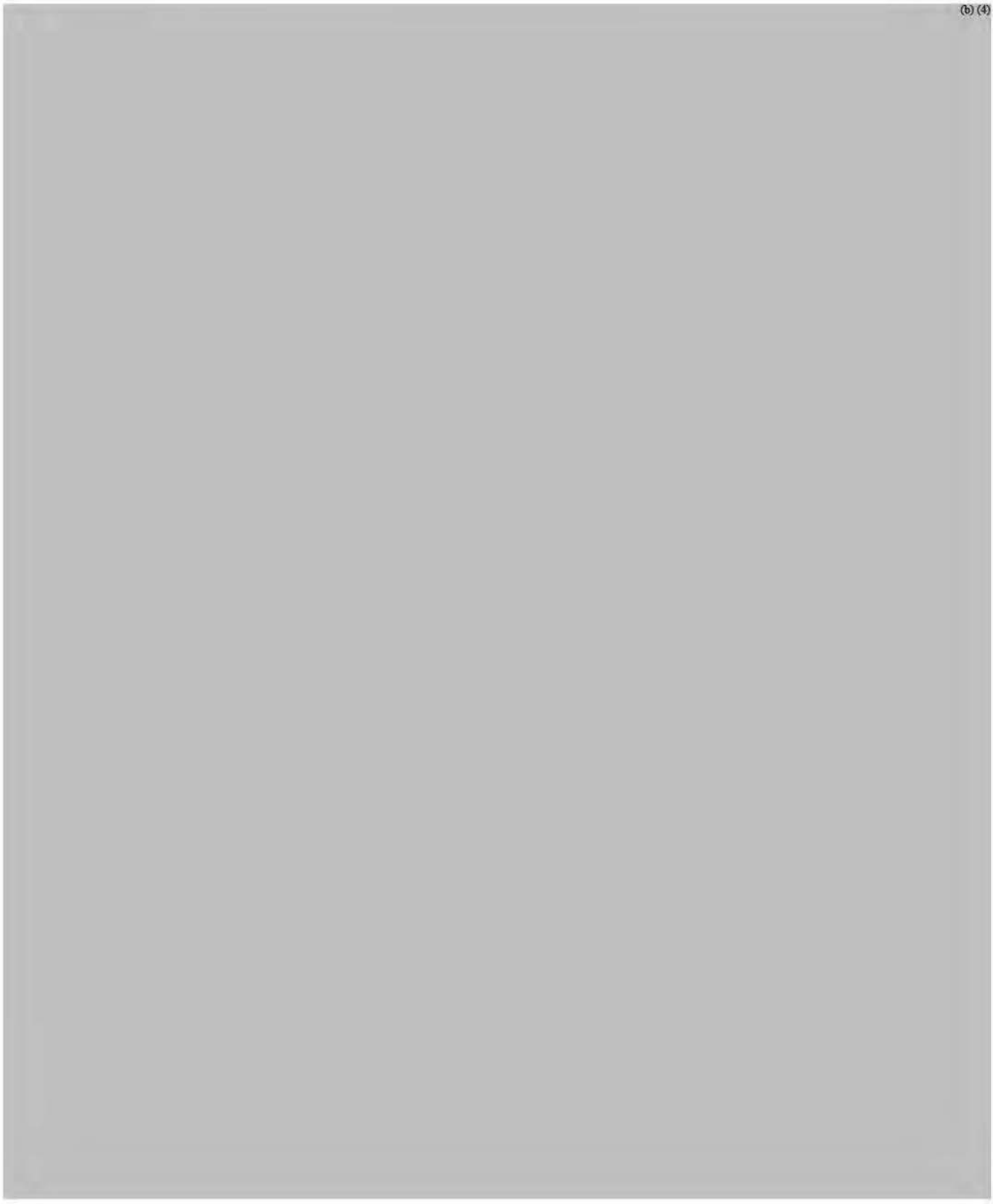


Figure 30A

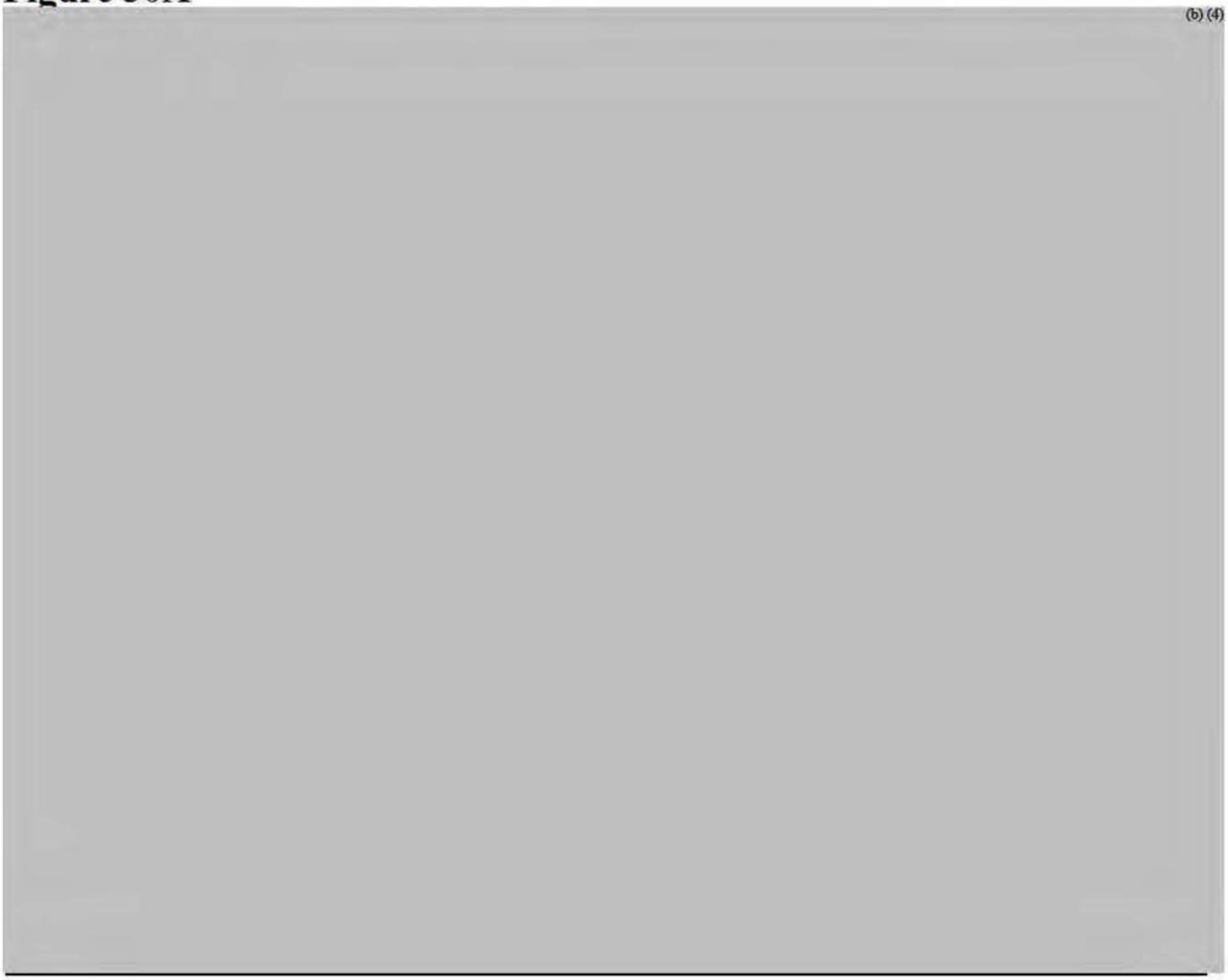
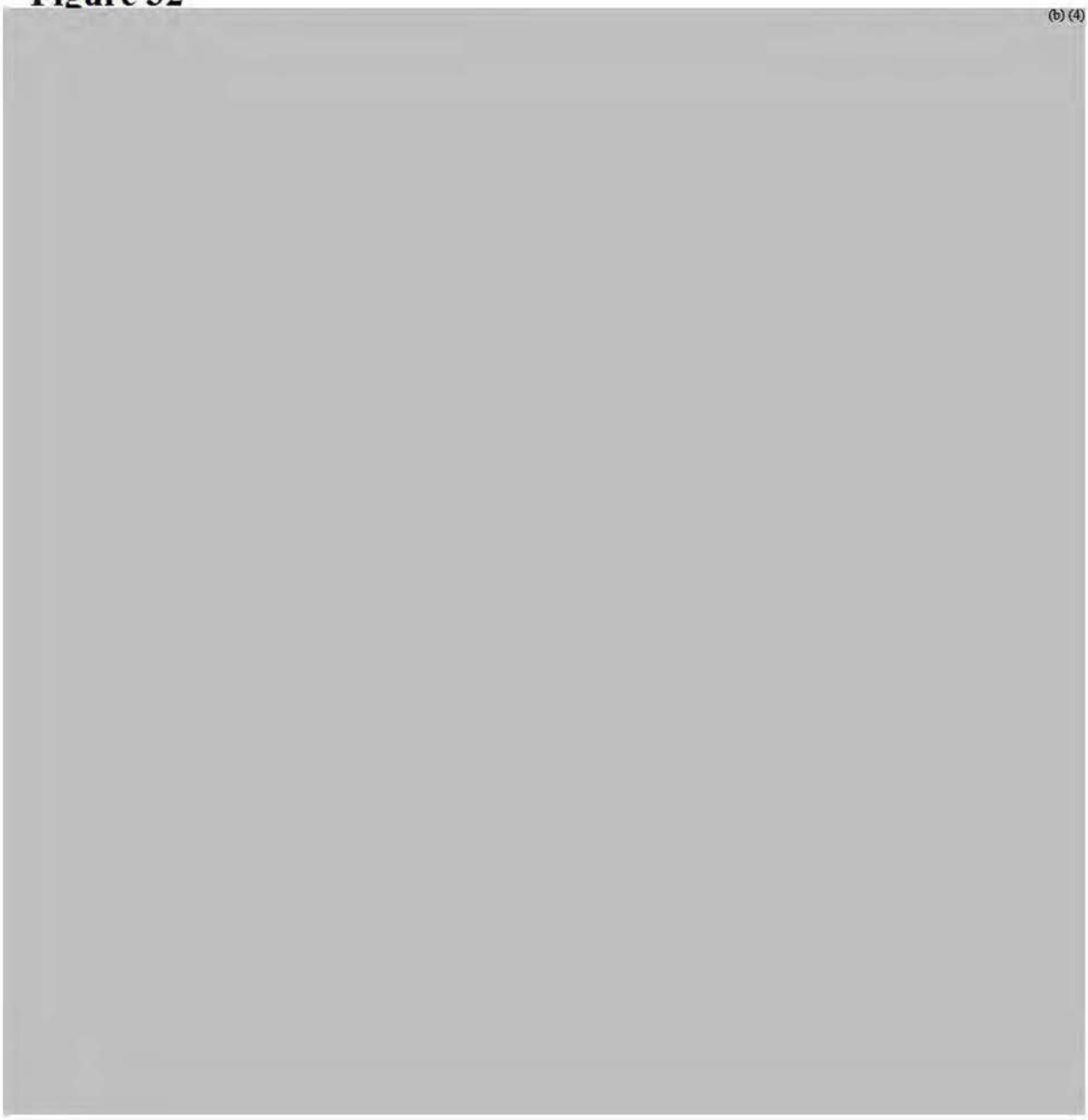


Figure 30B

Figure 31

Figure 32



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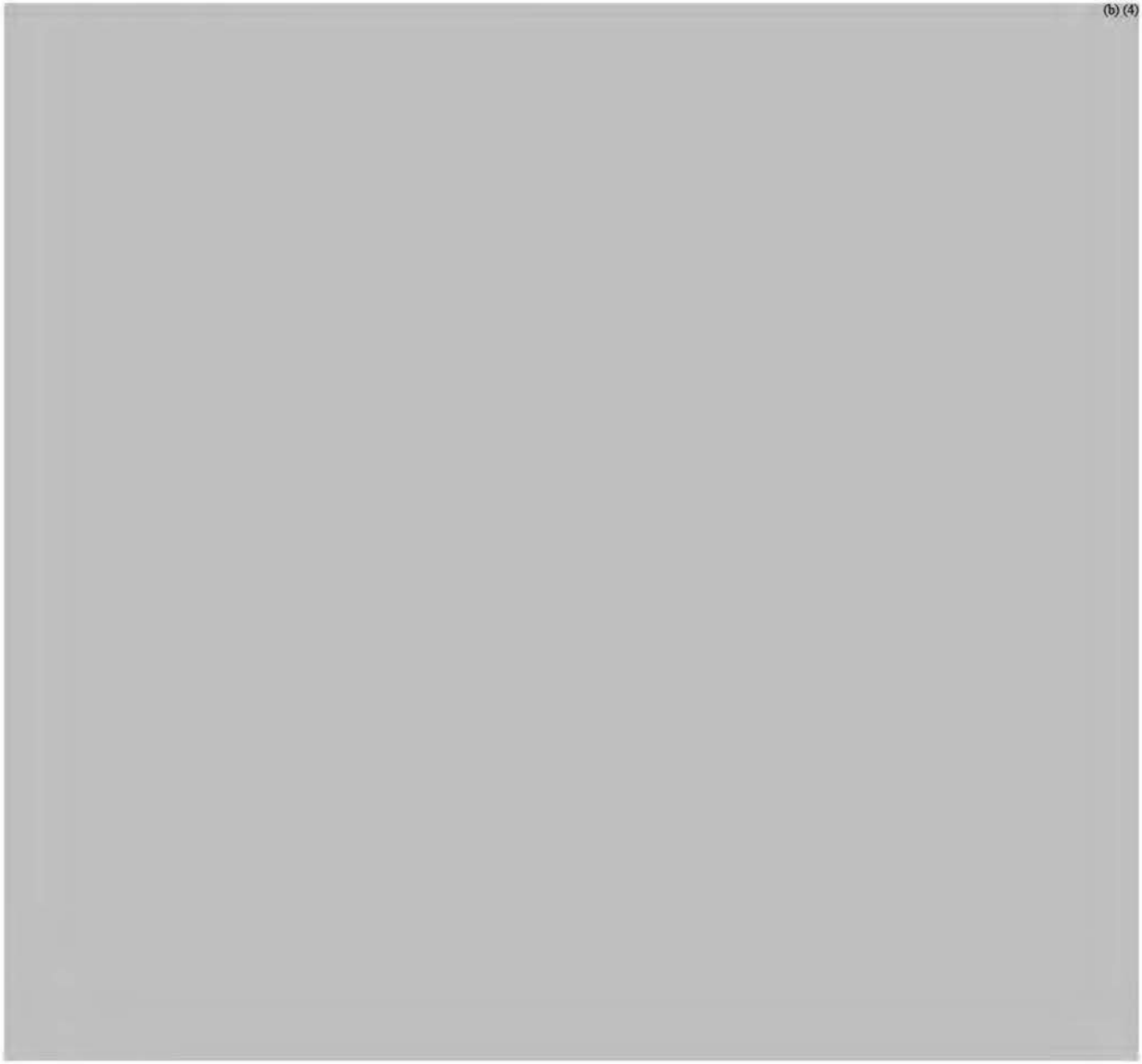

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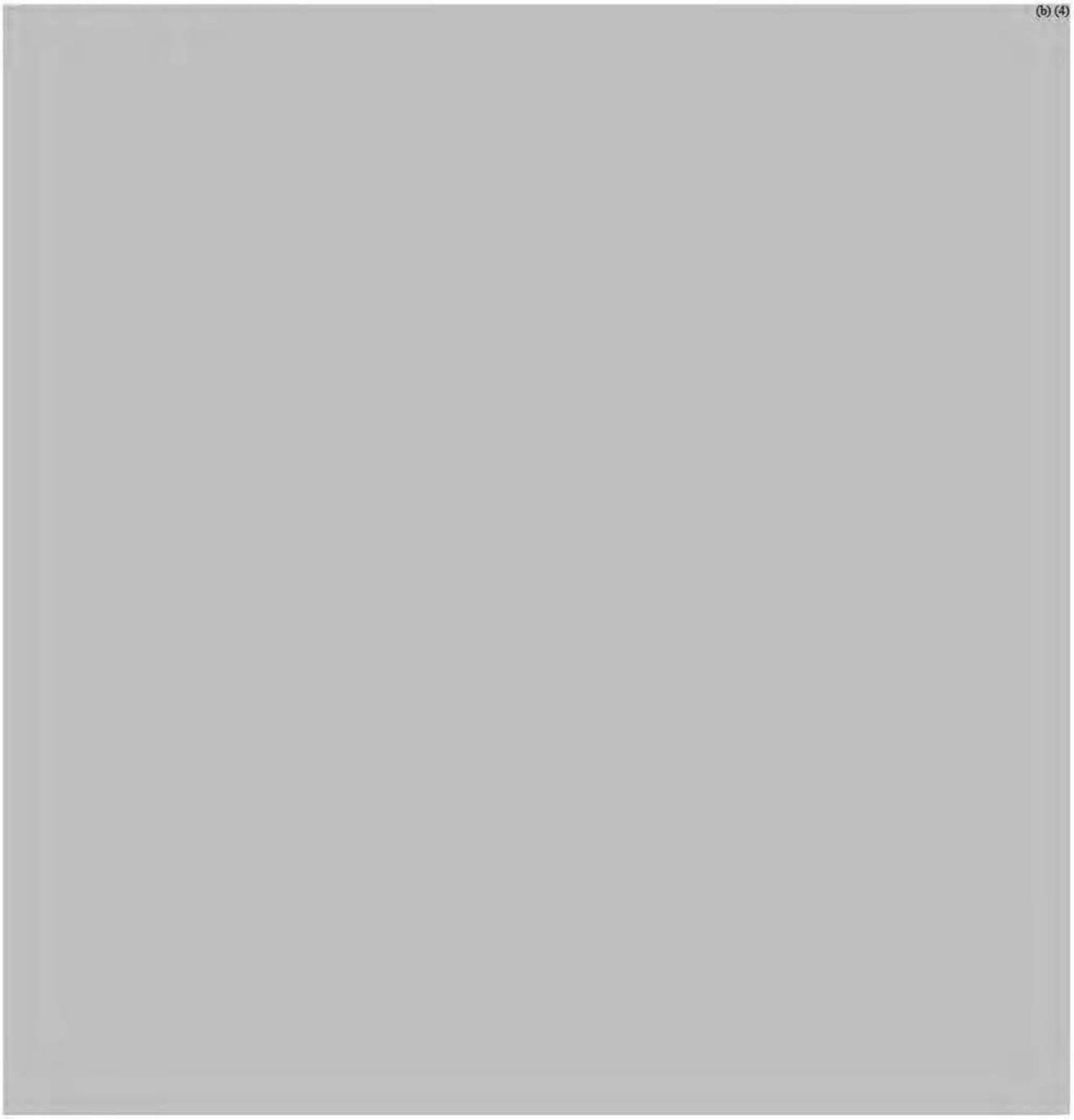

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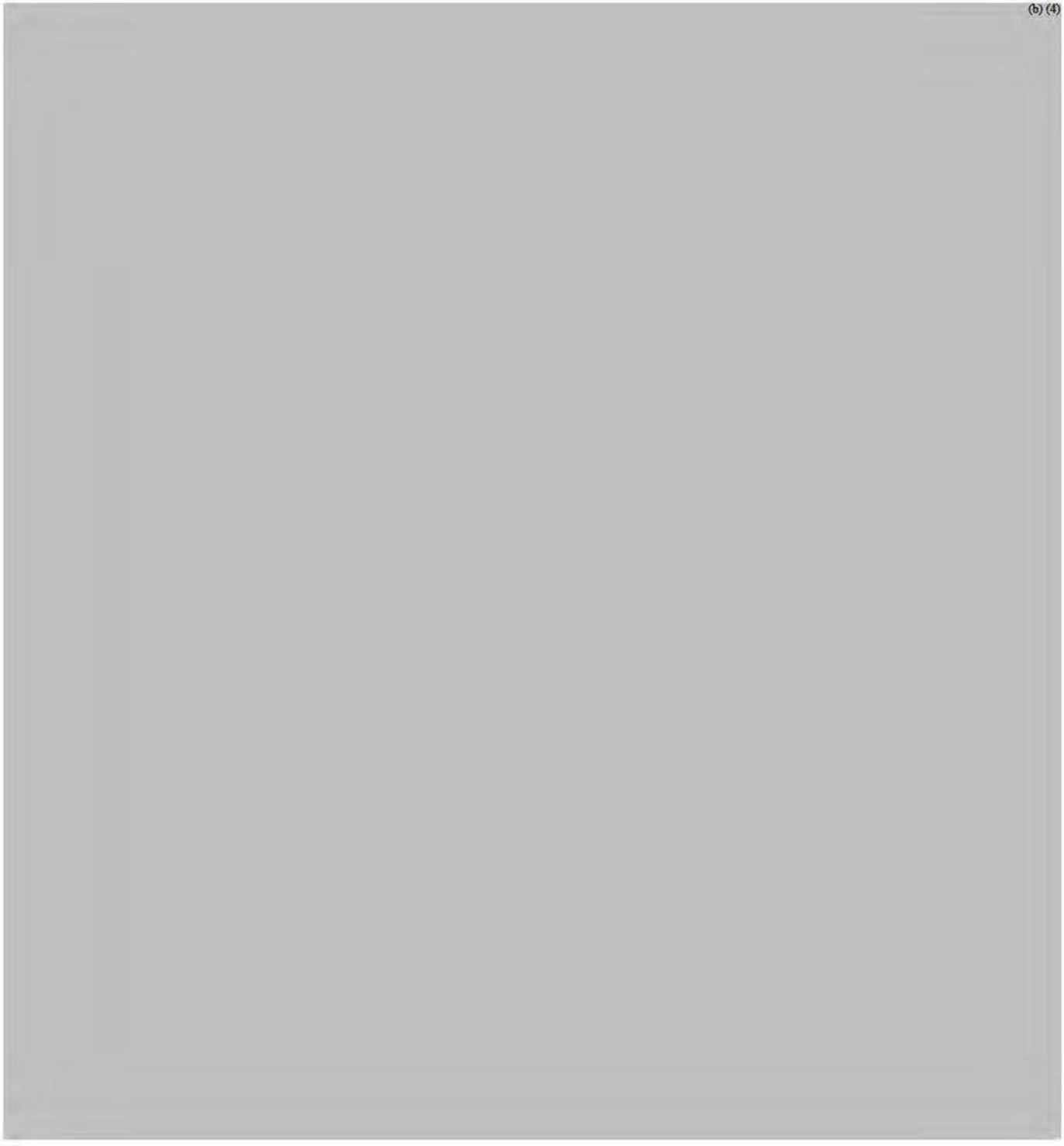

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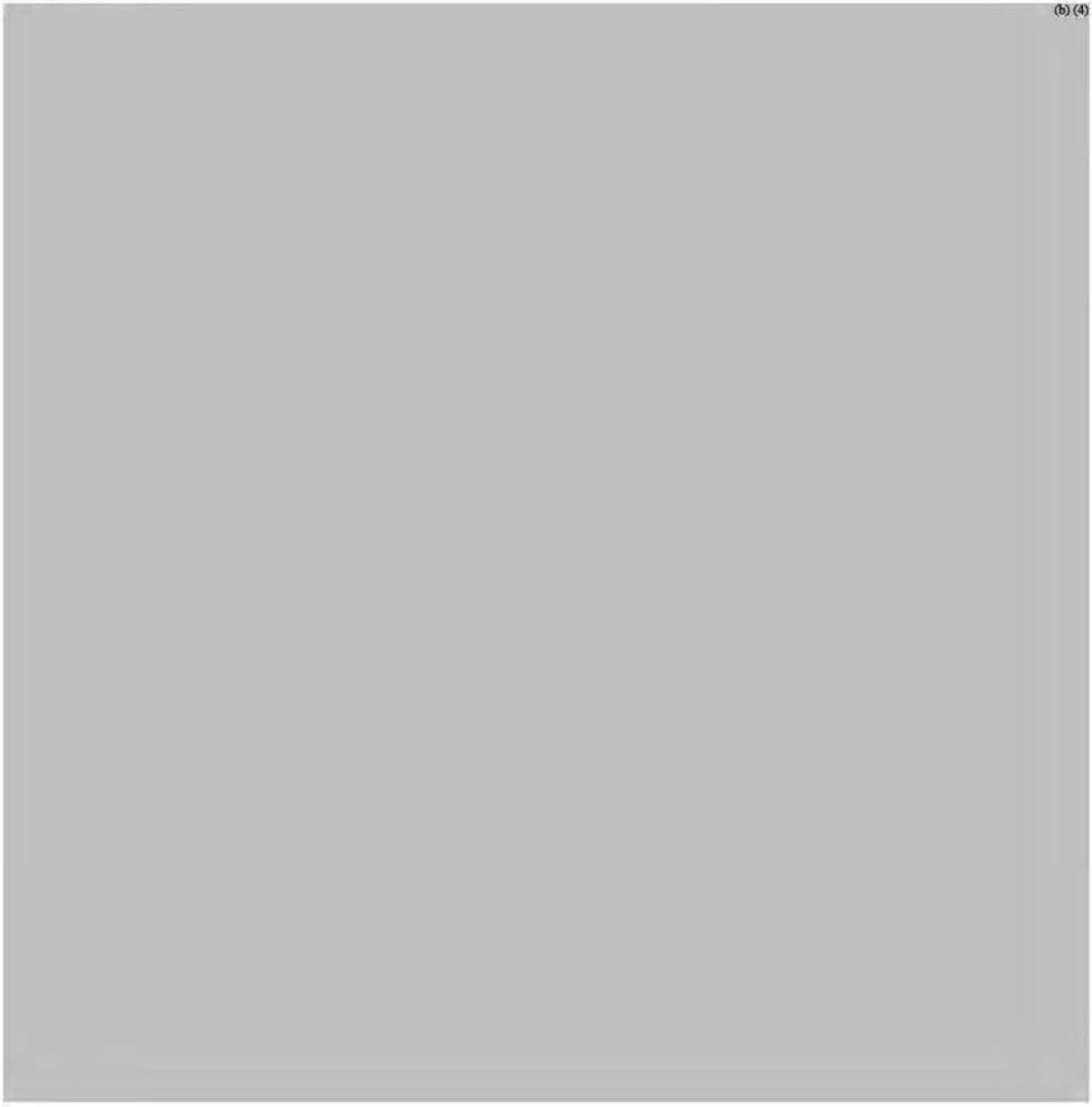

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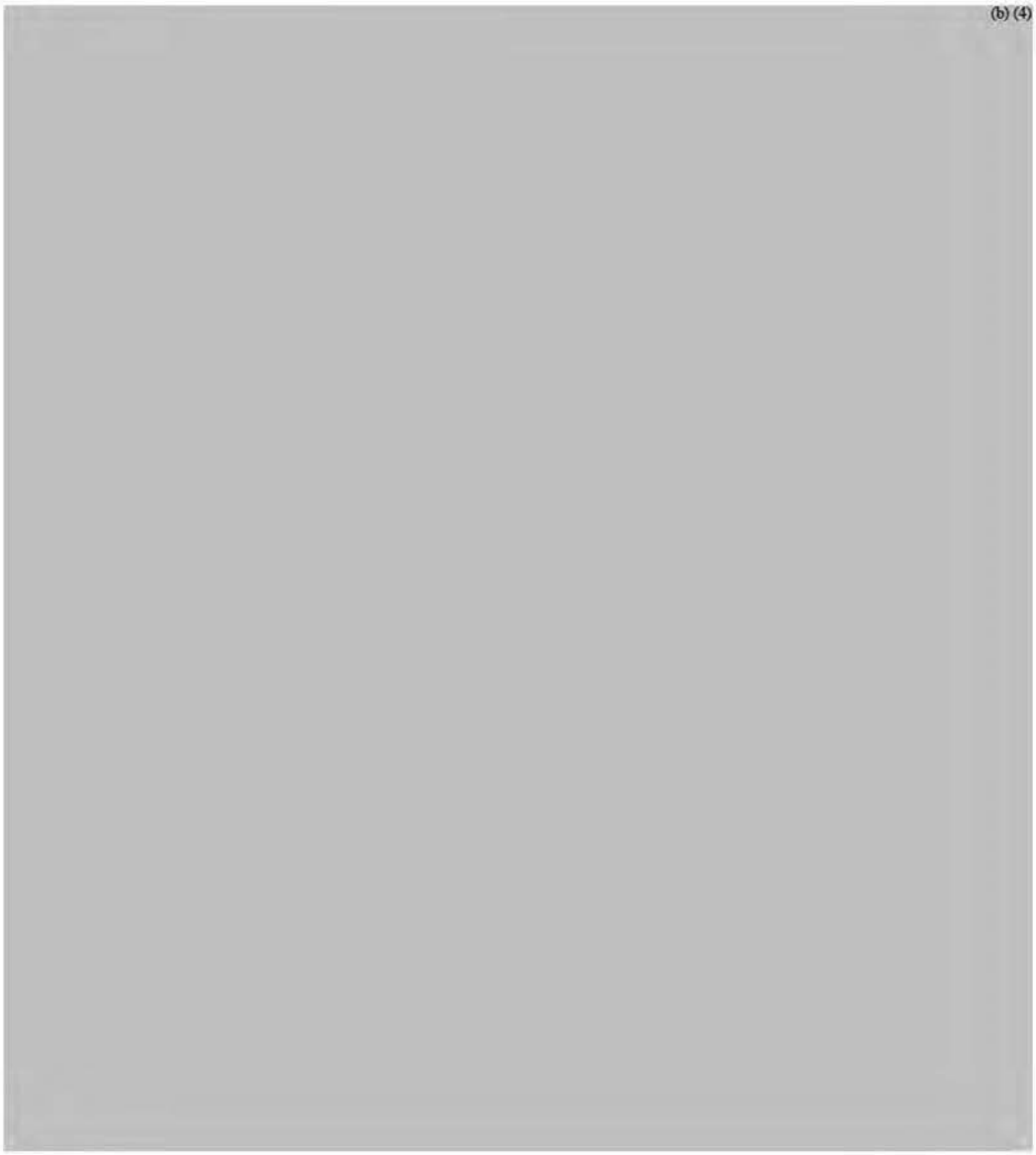

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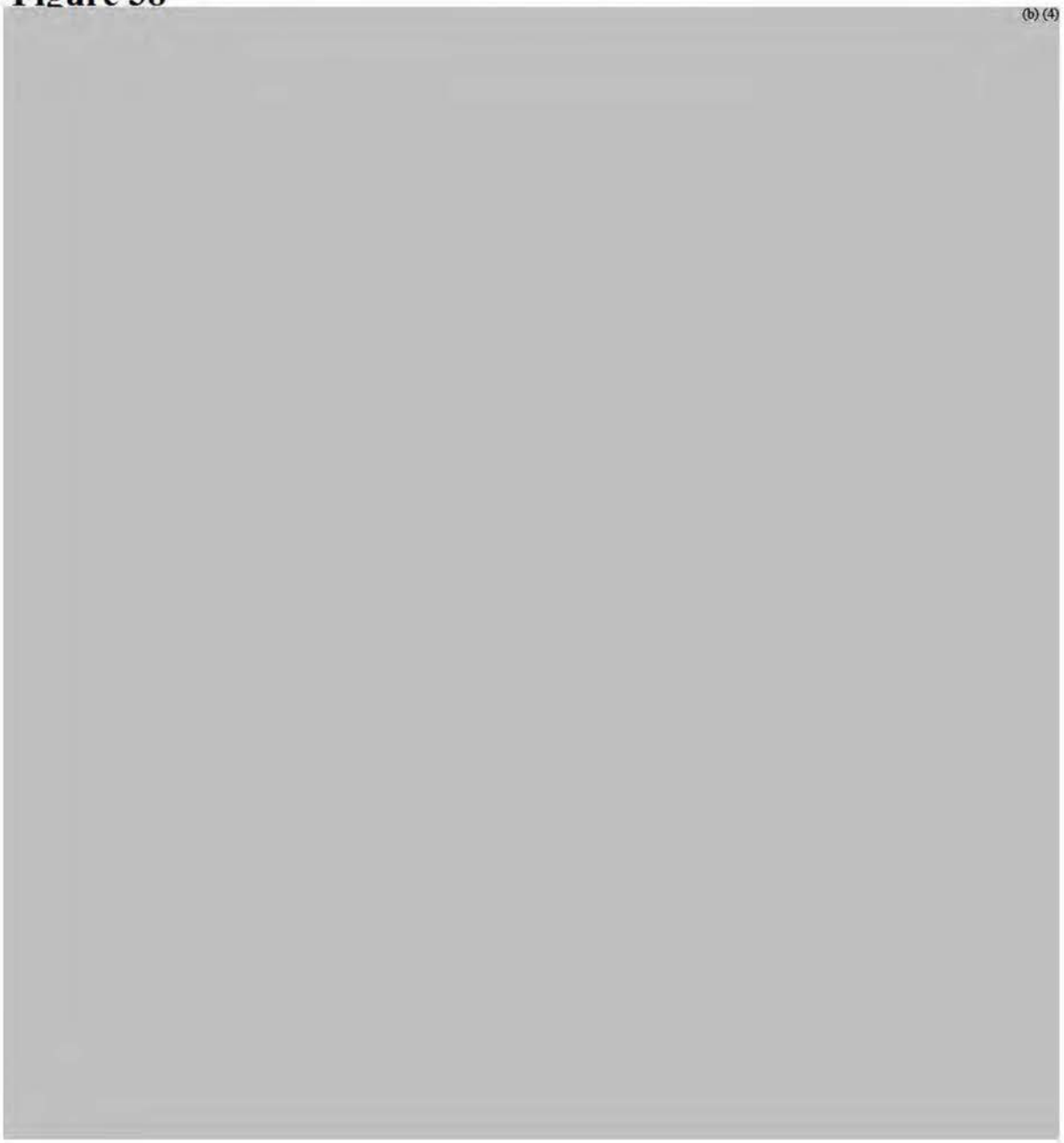

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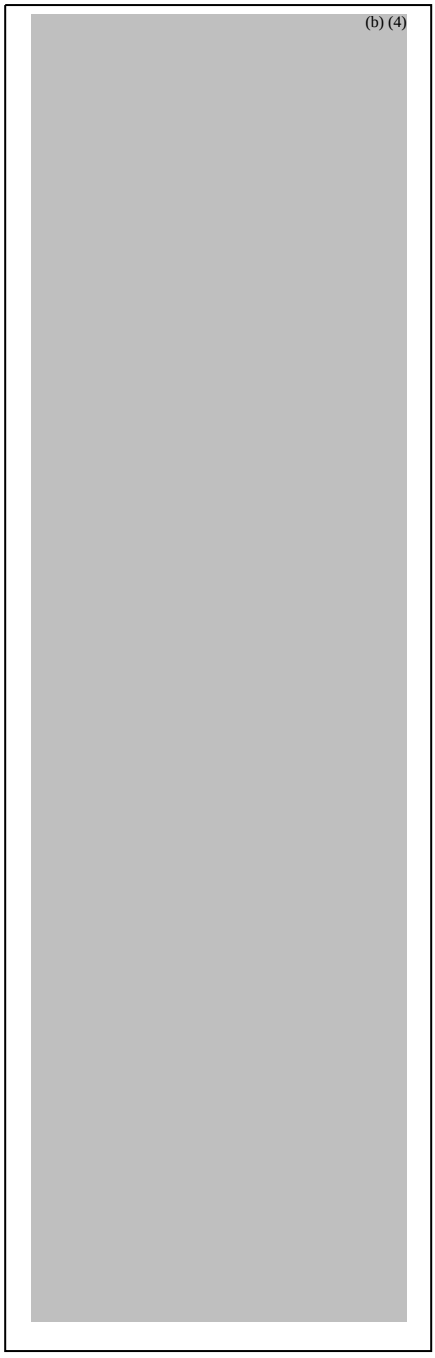
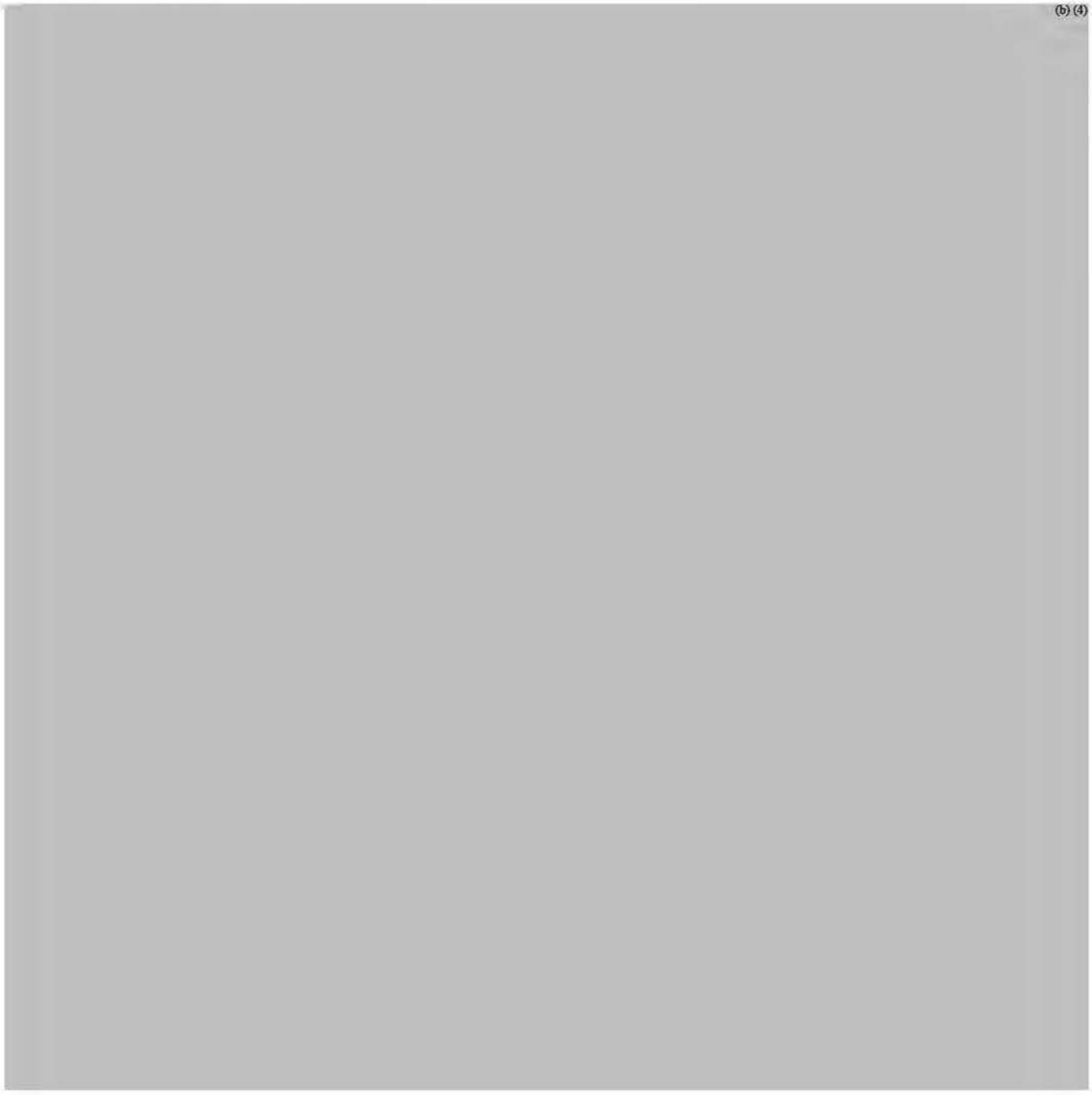


Figure 40

Figure 41

Figure 42

Figure 43



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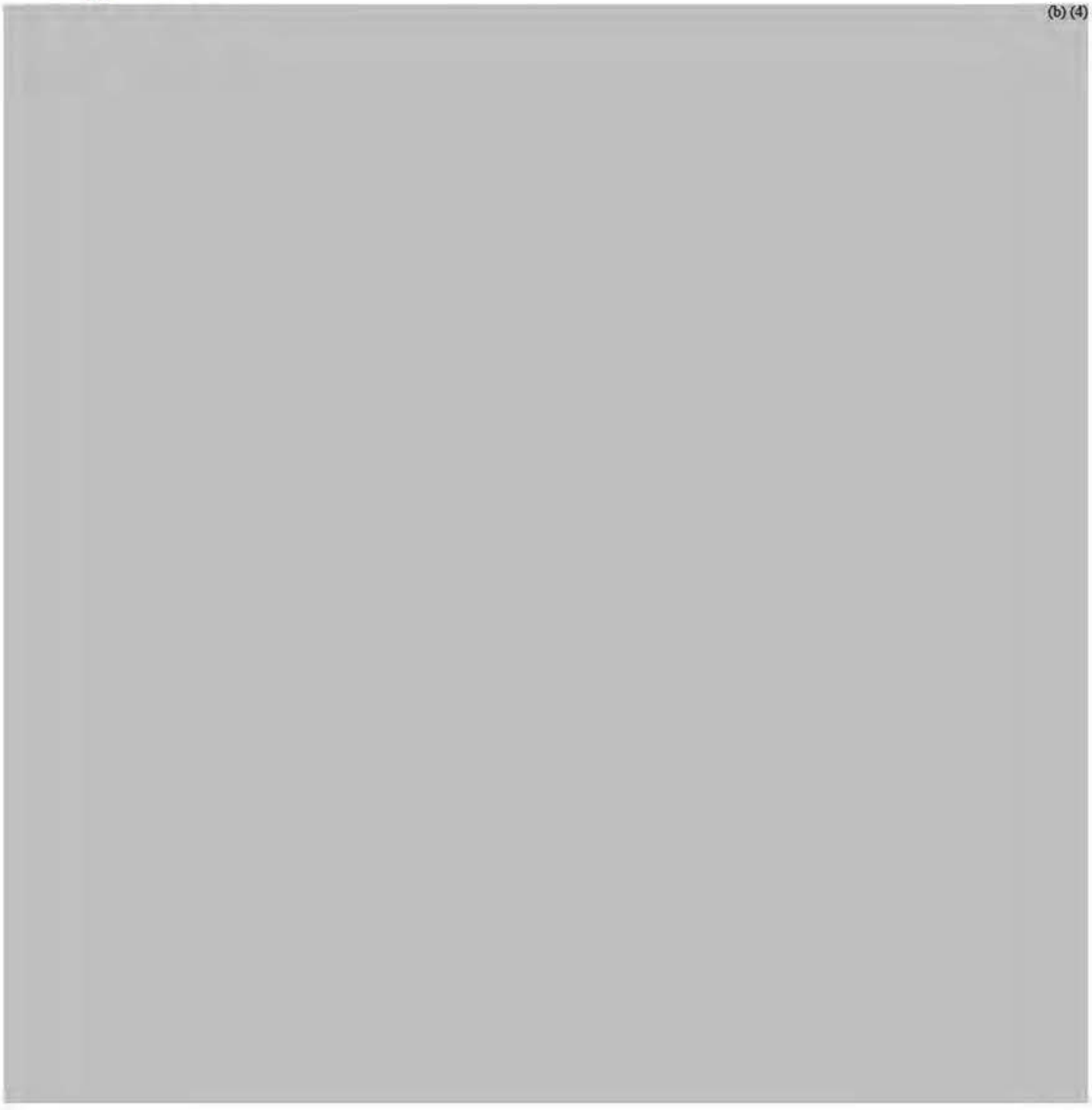


Figure 45.

Figure 46.

Figure 47.

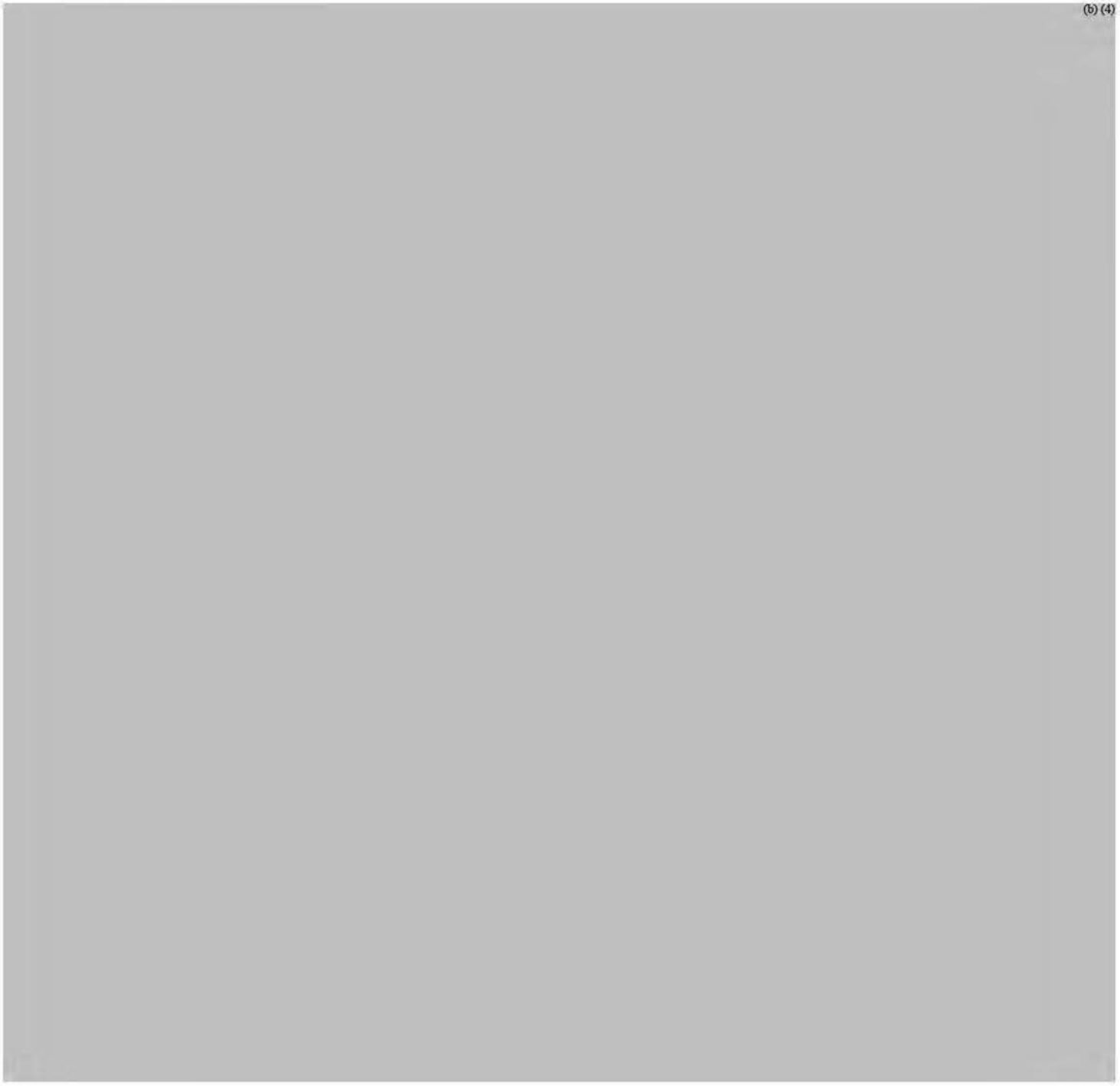
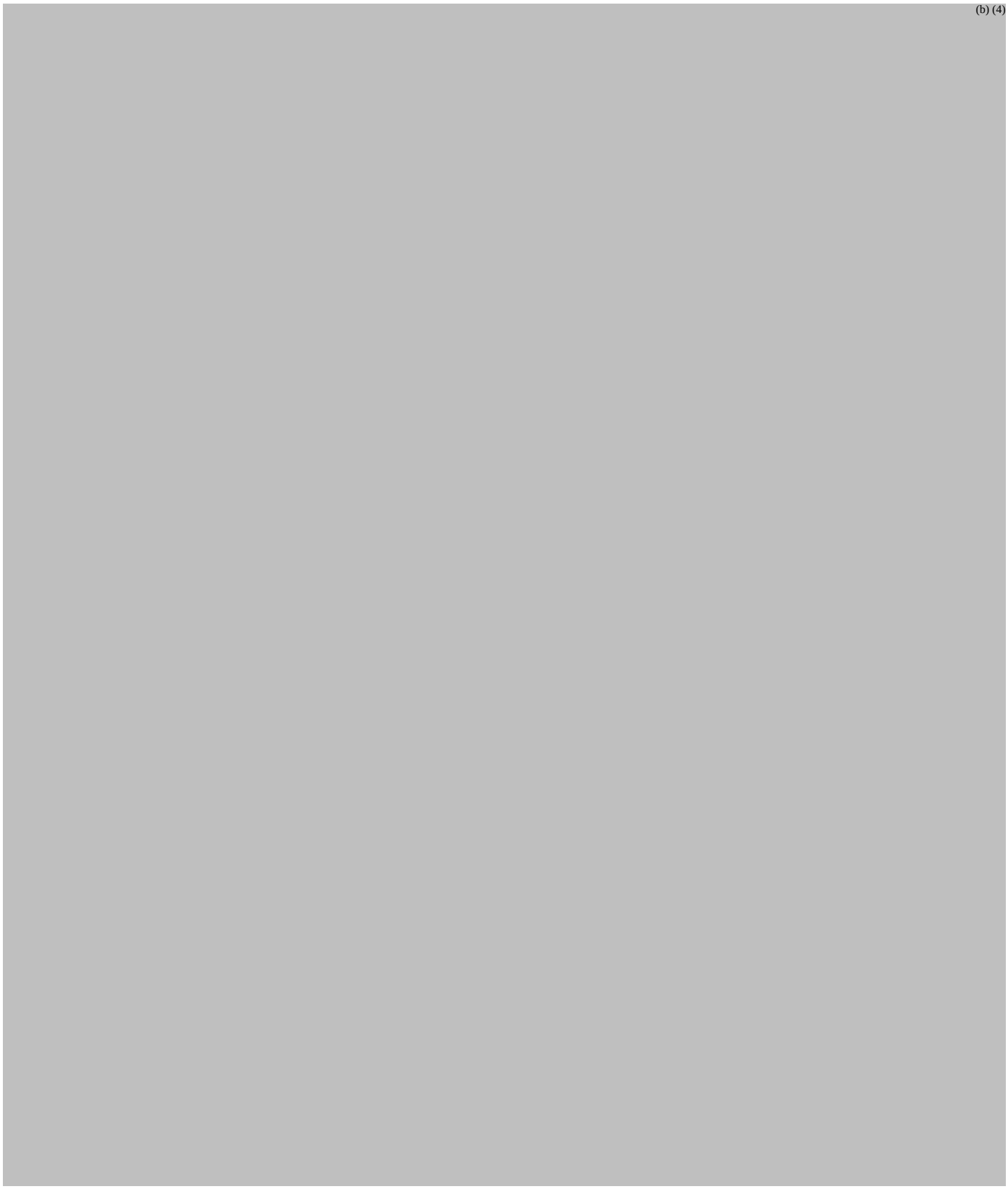


Figure 48.

Figure 49.

(b) (4)



(b) (4)

(b) (4)

Figure 50.

Figure 51.

Figure 52.

Figure 53.

Appendix 2: Amino Acid Sequence Variant (b) (4) of Consensus Bacterial Phytase and its Alignment to Wild Type Phytases and Consensus Phytase

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(b) (4)

(b) (4)

(b) (4)

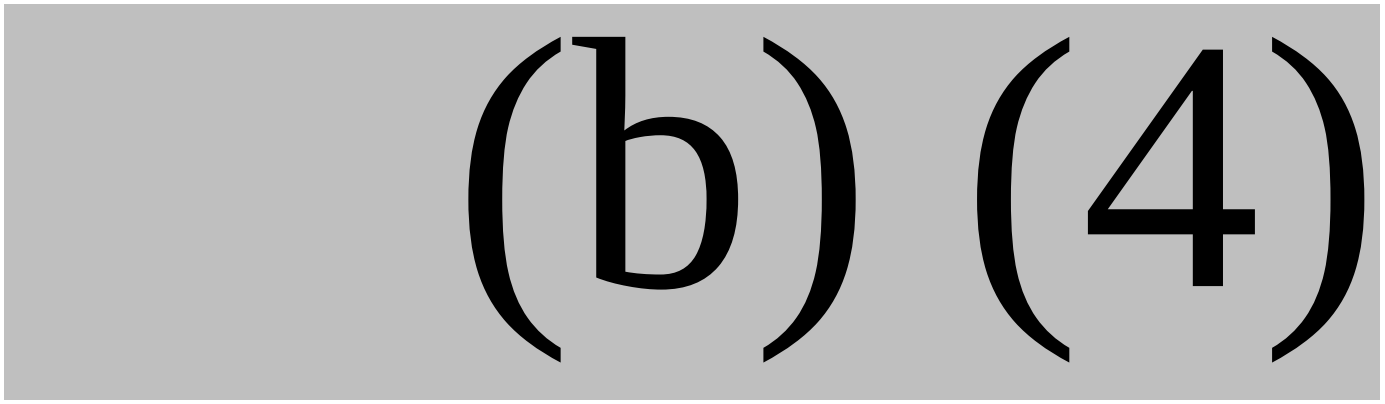
(b) (4)

(b) (4)

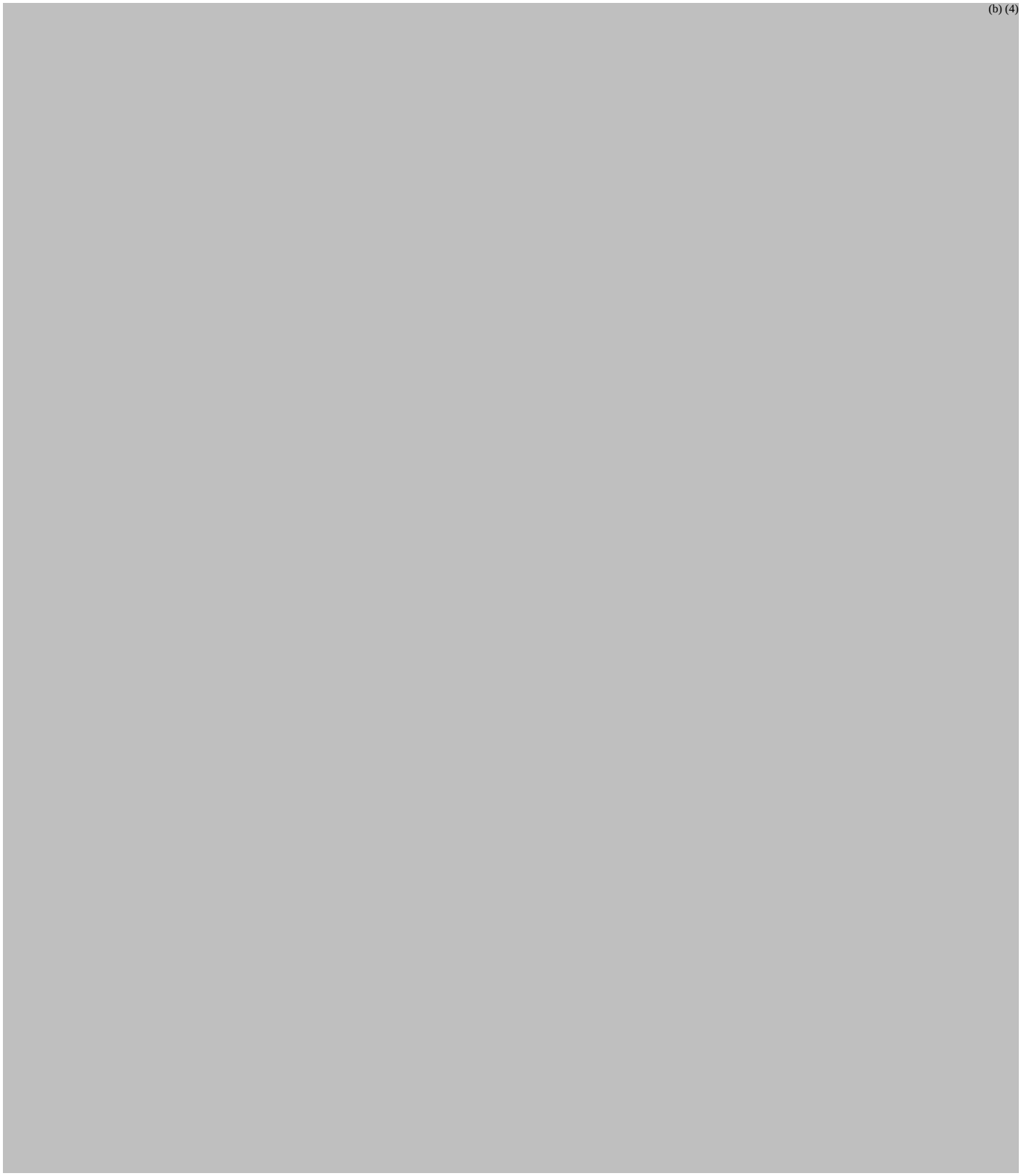
(b) (4)

Alignment to Wild Type Phytases

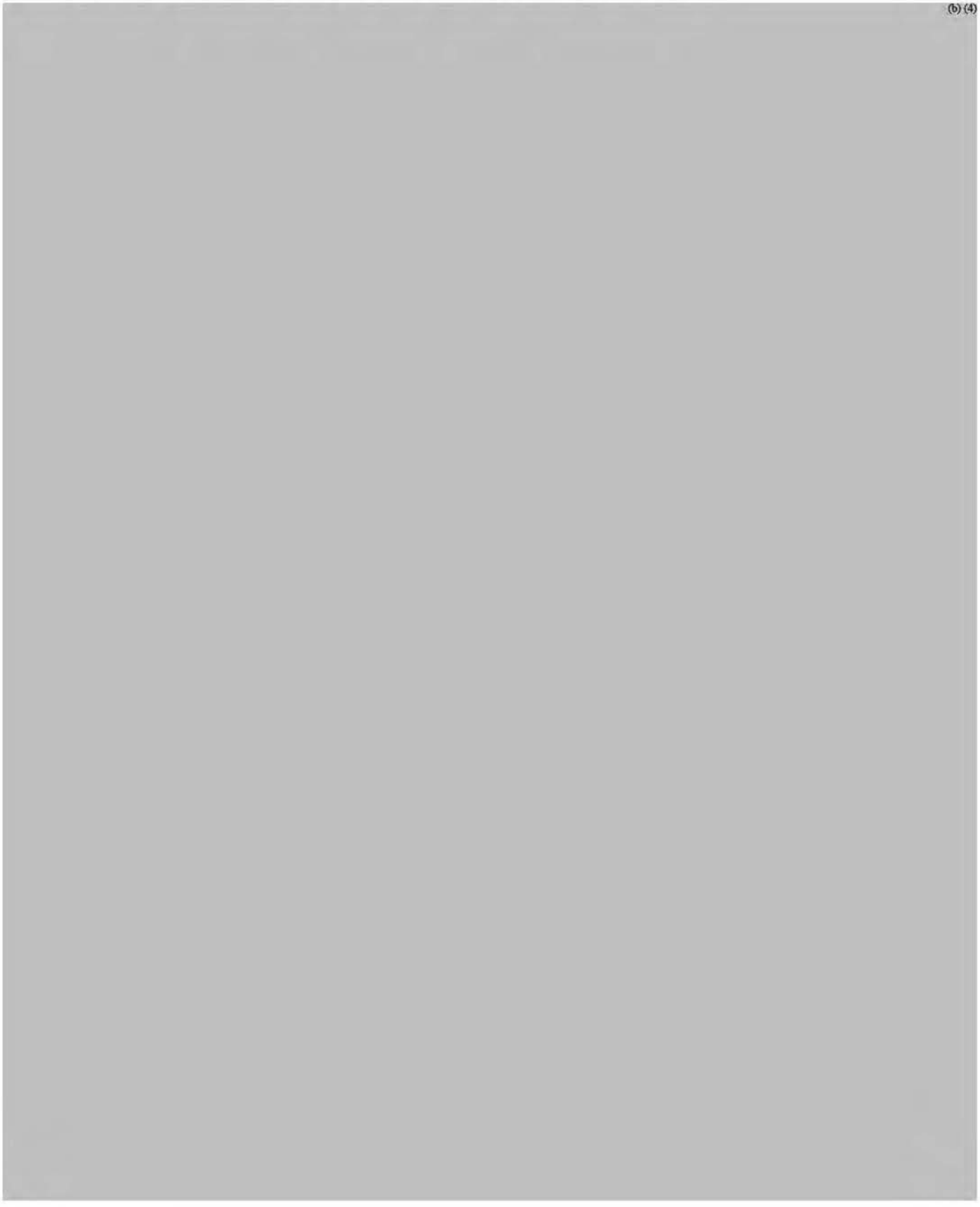
(b) (4)



(b) (4)

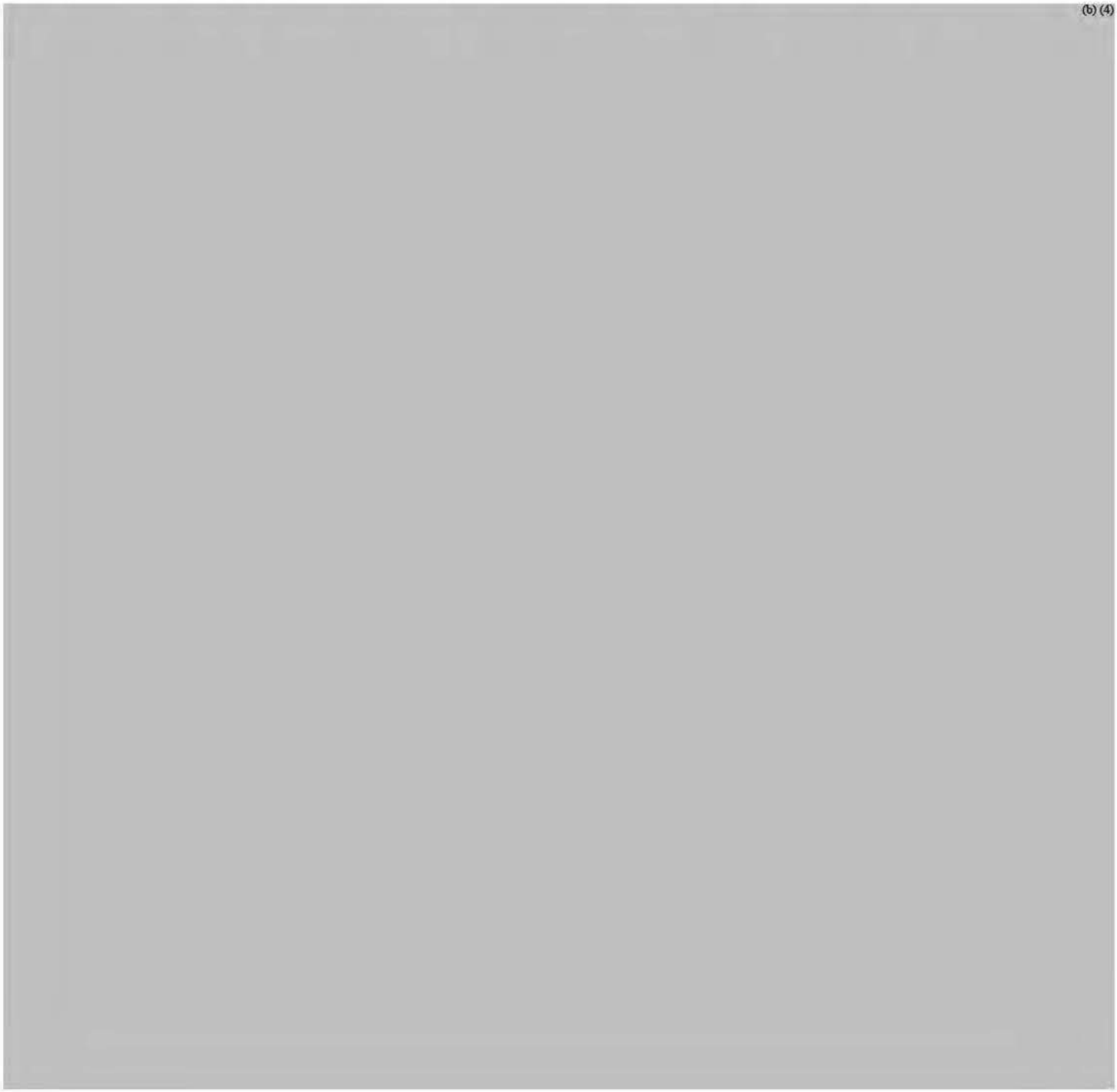


Appendix 3: Southern Blot Analysis of Phytase Expressing Strains



(b) (4)

Appendix 4: PCR Amplification for Detection of Successful Cassette Integration.



Appendix 5

Appendix 6: Manufacturing Flow Chart

Manufacturing Process

Appendix 7: Raw Materials Used During Manufacture

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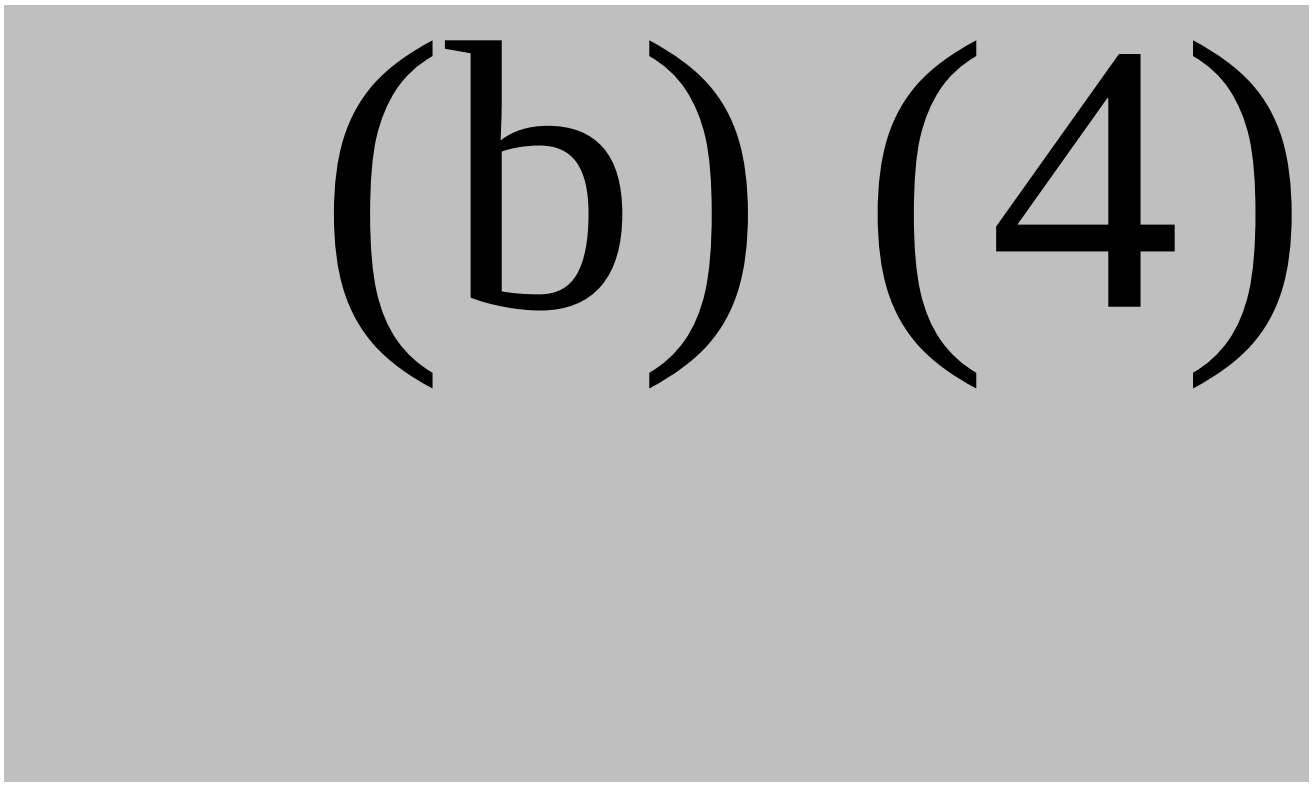
Representative media and ingredients which may be used in the manufacture are listed below. Different combinations are used at various manufacturing sites.

Production Tank, Recovery, and Formulation Ingredients	CAS#	Regulatory Status
(b) (4)		


(b) (4)

Appendix 8

DUPONT

CERTIFICATE OF ANALYSIS

PRODUCT: (b) (4)
LOT NUMBER:

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Phytase	U/g		
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
Antibacterial activity	/g		
PHYSICAL PROPERTIES			
Bulk Density			
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		

(b) (4)

This product is manufactured in accordance with FAO/WHO JECFA and FCC recommendations

23-Nov-2020
Date

(b)(6)
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

DUPONT

CERTIFICATE OF ANALYSIS

PRODUCT:
LOT NUMBER: (b) (4)

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			
Phytase	U/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
Antibacterial activity	/g		
PHYSICAL PROPERTIES			
Bulk Density			
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		

This product is manufactured in accordance with FAO/WHO JECFA and FCC recommendations

23-Nov-2020
Date

(b)(6)
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

DUPONT

CERTIFICATE OF ANALYSIS

PRODUCT: (b) (4)
LOT NUMBER:

ASSAY	UNIT	SPECIFICATION	FOUND
ENZYME ACTIVITY			(b) (4)
Phytase	U/g		
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
Antibacterial activity	/g		
PHYSICAL PROPERTIES			
Bulk Density			
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		

This product is manufactured in accordance with FAO/WHO JECFA and FCC recommendations

23-Nov-2020
Date

(b)(6)
QA/QC Department

This certificate of analysis was electronically generated and therefore has not been signed.

Appendix 9

Risk Assessment of *Trichoderma reesei* for Consideration of Addition to the List of Eligible Microorganisms for the Tiered 5(h)4 Exemptions from MCAN Reporting Requirements

I. INTRODUCTION

Trichoderma reesei is an ascomycetous wood-rot fungus that has been used for decades for the production of enzymes for agricultural, food, pharmaceutical, and industrial uses. It is the anamorph (asexual reproduction through mitotic division of conidia) of the teleomorph (sexual reproduction through the formation of ascospores) known as *Hypocrea jecorina*. QM6a is the designation given to the original strain of *T. reesei* isolated in 1944 from decaying tent fabric on the Solomon Islands in the South Pacific during World War II. According to Nevalainen et al. (1994) almost all, if not all, industrial strains of *T. reesei* are derivatives of QM6a. Recent taxonomic studies have shown that actually the species *T. reesei* consists only of this single isolate and its derivatives. Many other strains of this fungus isolated from other places, which have sometimes been referred to in the literature as *T. reesei,* have now been proposed as belonging to the newly named species, *T. parareesei* (Druzhinina et al., 2010). These other isolates differ from *T. reesei* in habitat, sporulation, and metabolic versatility. *T. reesei* has been shown to belong to a single species now referred to as *H. jecorina/T. reesei* (QM6a) which reflects its relationship to its teleomorph *H. jecorina* (Atanasova et al., 2010). The only anamorphic strains within the species *H. jecorina/T. reesei* are those of QM6a and its derivatives. Thus, given these recent taxonomic publications, all strains of *T. reesei* are, by definition, QM6a or a derivative (Druzhinina et al., 2010; Atanasova et al., 2010).

T. reesei is not pathogenic to humans, other animals, or plants. However, *T. reesei* can produce a secondary metabolite called paracelsin which is a compound known as a peptaibol. Peptaibols are small linear peptides characterized by a high content of the non-proteinogenic amino acid α-aminoisobutyric acid, with an acetylated N terminus, and a C-terminus that is linked to an amino alcohol. Paracelsin, like other peptaibols, modifies cell membranes of various organisms through the formation of ion channels which may result in loss of osmotic balance and in leakage of cell contents. Paracelsin is inhibitory to the growth of Gram-positive bacteria and to fungi, but it also has been shown to exhibit cytotoxicity to mammalian cells and to aquatic indicator species. However, paracelsin is apparently not produced by the fungus under the conditions of submerged standard industrial fermentation used for enzyme production. There is a long history of safe use of *T. reesei* for production of enzymes for food and animal feed. Data submitted in Generally Recognized as Safe (GRAS) petitions to the Food and Drug Administration (FDA) for numerous enzyme preparations from *T. reesei* for human and animal consumption demonstrate that the enzymes are nontoxic. Given the long history of safe use of *T. reesei* for production of enzymes, it is appropriate to

consider this microorganism eligible as a recipient microorganism for the 5(h)4 Tiered Exemptions from Microbial Commercial Activity Notice (MCAN) reporting requirements if this fungus was to be used in submerged standard industrial fermentation for enzyme production. However, scenarios have been proposed for use of *T. reesei* in cellulosic biofuel facilities under conditions not typical of submerged standard industrial fermentation. Publications have suggested on-site cellulase production at biofuel facilities by the growth of *T.* reesei directly on the pre-treated cellulosic hydrolysate (i.e., delignified plant material) for degradation of the plant material into sugars that are subsequently fermented into ethanol (Wooley et al., 1999; Kazi et al., 2010). The growth of *T. reesei* directly on plant material in biofuel facilities poses concern because paracelsin may be produced under these conditions. Historically, the conditions used by researchers to optimize peptaibol production by *Trichoderma* species, including *T. reesei,* have typically involved the addition of insoluble sources of carbon such as cellulose to the fermentation broth, the presence of solid surfaces in the fermentation broth, or the growth of the fungus on a solid surface. Presently there is no information on the quantities of paracelsin that could be formed if *T. reesei* was grown on pre-treated plant material in the saccharification tanks. There is no information on the stability of paracelsin, if formed, during the entire process stream of ethanol production in cellulosic biofuel facilities. There is no information available on its persistence or degradation during wastewater treatment of spent fermentation broth, nor its persistence and resulting potential effects if it made its way into the environment. Therefore, given paracelsin's demonstrated biological activity to bacteria, fungi, mammalian cells, and aquatic indicator species, *T. reesei* cannot be recommended as a recipient microorganism for the 5(h)4 Tiered Exemption if used under conditions other than those typical of submerged standard industrial fermentation for enzyme production.

History of Commercial Use and Products Subject to TSCA Jurisdiction

There is a long history of safe use of enzymes produced by various species of *Trichoderma* in agricultural, food, pharmaceutical, and industrial applications. According to the Enzyme Technical Association (ETA), *Trichoderma* enzymes are currently used in brewing processes (β-glucanses), in potable alcohol manufacture (glucoamylases), for maceration in fruit juice production (pectinases, cellulases, hemicellulases), as an additive to livestock feed (xylanases), and for pet food processing (ETA, 2010). In addition to their use for enzyme production, some species of *Trichoderma* are used as biocontrol agents of fungal plant pathogens, predominately strains of *T. harzianum* and *T. viride* (Verma et al., 2007).

The species *T. reesei* has been used extensively for enzyme production in both foods and in industrial applications, mainly for production of cellulases. Cellulases have been manufactured commercially in Japan since the 1960's (Toyama, 1969, as cited by Nevalainen et al., 1994). Cellulases are used in the food industry for baking, malting, brewing, and in grain alcohol production (Nevalainen et al., 1994; Olempska-Beer et al., 2006). Several cellulases produced by *T. reesei* have been approved for food use by

FDA in GRAS petitions (21 CFR §184.1250). GRAS notices for enzymes produced from *T. reesei* used in food include cellulase, hemicellulase, transglucosidase, pectin lyase, and acid fungal protease (http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm). Cellulases produced by *T. reesei* have also been used in the pharmaceutical industry as digestive aids, and in the animal feed industry to increase the digestibility of feed (Malmos, 1977, as cited by Nevalainen et al., 1994).

T. reesei produces a number of different enzymes that have TSCA uses, especially cellulases. Historically, *T. reesei* cellulases have been used in the wood processing and textile industries (Nevalainen et al., 1994). More recently, however, there has been an enormous interest in producing *T. reesei* cellulases for use in the saccharification step of plant material in cellulosic biofuel production facilities. *T. reesei* also produces numerous extracellular proteases (Kredics et al., 2005). *T. reesei* may also be used for production of hydrophobins, which are proteins with characteristics for potential use as biosurfactants (Askolin et al., 2001; Wösten, 2001). To date, the Agency has reviewed numerous Microbial Commercial Activity Notices of *T. reesei* for production of enzymes with TSCA applications such as α-amylase, β-glucosidase, acid protease, glucoamylase, phytase, laccase, and a number of cellulose-degrading preparations.

II. IDENTIFICATION AND TAXONOMY

A. Characterization of the Genus *Trichoderma*

The genus *Trichoderma,* which was first named by Persoon in 1794, is a member of the *Hypocreales* (fungi, Ascomycota). It is an anamorphic fungus that reproduces solely asexually through mitotic division of conidia. *Hypocrea* is the genus name of the teleomorph that reproduces sexually through the formation of ascospores. Members of the genus *Trichoderma* are ubiquitous in nature. They are geographically widely distributed and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. Most *Trichoderma* are aerobic mesophilic wood rot fungi found in soils and decaying wood and vegetative materials (Gams and Bissett, 1998). *Trichoderma* spp. are very common in agricultural, prairie, forest, and desert soils and in salt marshes with a variety of climates (Klein and Eveleigh, 1998). They are saprophytic fungi that produce copious amounts of degradative enzymes including cellulases, xylanases, and chitinases which enables them to live on a wide variety of organic materials and transform many xenobiotic compounds (Klein and Eveleigh, 1998). The production of cellulases and xylanases by various species, particularly *T. reesei,* has made them important industrial microorganisms. Members of the genus *Trichoderma* produce a vast array of secondary metabolites such as peptaibols and peptide antibiotics active against fungi and bacteria. Mycotoxins such as trichodermin, trichotoxin A, and trichothecenes are produced by some species of the genus. Some

Trichoderma strains produce chitinases and possess antagonistic properties to other fungi, and are thus, used as biocontrol agents (Jaklitsch, 2009). Species used as fungal biocontrol agents are predominately strains of *T. harzianum* and *T. viride, but also include T. lignorum, T. virens, T. hamatum, T. koningii, T. pseudokoningii, T. aureoviride, T. longibrachiatum,* and *T. asperellum* (Verma et al., 2007). The antagonistic properties to fungal plant pathogens by some *Trichoderma* strains are due to multiple mechanisms such as competition for nutrients and space, modification of environmental conditions, promotion of plant growth and plant defense mechanisms, and by mycoparasitism (Haran et al., 1996; Benitez et al., 2004; Harman et al., 2004; Woo et al., 2006; Verma et al., 2007). Some endophytic species confer plant protection, most likely through the production of secondary metabolites (Degenkolb et al., 2006). There are also *Trichoderma* biocontrol agents against bacteria, insects and nematodes, and even plants (weeds) (Verma et al., 2007; Bokari, 2009).

Other species of *Trichoderma*, such as *T. aggressivum, T. pleuroticola, T. pleurotom, T. pleuotrophilum,* and *T. fulvidum* are pathogenic to cultivated mushrooms such as *Agaricus bisporus* and *Pleurotus ostreatus* (Samuels et al., 2002; Komoń-Zelazowska et al., 2007a; Kubicek et al., 2007).

There is one species in the genus that is considered an opportunistic human pathogen, *T. longibrachiatum.* It has been shown to cause fungal mycosis in immunocompromised individuals (Pfaller and Diekema, 2010; Druzhinina et al., 2005).

B. Taxonomy of the genus *Trichoderma*

Currently, there are more than one hundred different *Trichoderma* species that have been molecularly characterized. However, the taxonomy of *Trichoderma* has been problematic and assignment of an accurate species name to an isolate may be challenging. Before the discovery and development of molecular biological identification methods, fungi were classified based solely on morphological characteristics, often incorrectly so. According to Druzhinina et al. (2005), approximately 50% of the *Trichoderma* isolates currently in culture collections under names based solely on morphological analysis are wrong. With the advent of molecular techniques for identification, the internal transcribed spacer (ITS) 1 and 2 fragments were first used for assignment of an isolate into a species. However, Druzhinina et al. (2005) stated that the ITS1 and ITS2 sequences do not provide sufficient phylogenetic resolution even though these sequences are still commonly used by some researchers. Proper identification methods are available however. The currently accepted mode of identification for *Trichoderma* and *Hypocrea* is a more comprehensive approach employing criteria of the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept, which often demands the use of genealogies of three or four genes, not just the spacer regions (Taylor et al., 2000). The GCPSR was meant to provide a common approach to classifying new strains of *Trichoderma*. Druzhinina et al. (2005) proposed an online identification tool using an oligonucleotide barcode, i.e., a diagnostic

combination of several oligonucleotides specifically allocated within the ITS1 and ITS2 sequences of the rDNA repeat which they called (b) (4). The latest version of this tool is available via the International Subcommission on Trichoderma and Hypocrea Taxonomy (ISTH) website at http://www.isth.info/index.php. More recently, Druzhinina et al. (2010) indicated that additional markers besides those in the GCPSR protocol are essential for differentiating among *H. jecorina* and its close relatives. In any case, these methods appear to be best suited for research laboratories doing *Trichoderma* systematics rather than routine identification methods needed by commercial entities or others. Without these protocols, it appears that differentiation of these closely related species will be difficult. Even though identification of new species of *Trichoderma* may be challenging, the lineage of all commercial strains of *T. reesei* from the original QM6a isolate has been established.

C. The Species *Trichoderma reesei,* strain QM6a and its Derivatives

1. The species *Trichoderma reesei*

The history of the well-known *T. reesei* QM6a strain is presented in Kubicek and Harman (1998). During World War II, because of the military's interest in determining what was causing the rapid rotting of canvas tent material and fabric in the South Pacific tropics, research on members of the genus *Trichoderma* accelerated greatly. A very cellulolytic fungal strain was isolated from an island, reported variously as either Bougainville or Guadalcanal, in the chain known as the Solomon Islands (now politically part of Papua New Guinea). This isolate was designated as *Trichoderma viride* QM6a. At that time there was only one species of *Trichoderma* which was *T. viride* (Druzhinina et al., 2005). Approximately 20 years later, QM6a was re-named by Simmons as *Trichoderma reesei* after Elwyn T. Reese who conducted the majority of investigations on this fungal strain (Kubicek and Harman, 1988). The American Type Culture Collection designation for this original strain of *T. reesei* QM6a (Simmons 1977) is ATCC13631.

As previously stated, *Trichoderma reesei* is the anamorphic form (asexual reproduction) of the fungus which also has a teleomorphic form (sexual reproduction) known as *Hypocrea jecorina* (Eveleigh, 1985). Kuhls et al. (1996) presented molecular evidence that *T. reesei* is indistinguishable from *H. jecorina* based on sequences of internal transcribed spacer (ITS) regions. In addition, failed attempts to cross *T. reesei* with wild type *H. jecorina* strains led Kuhls et al. (1996) to believe that *T. reesei* was a clonal derivative of *H. jecorina*.

Later Lieckfeldt et al. (2000) were successful in isolating anamorphic *H. jecorina* from South American soils and vegetation, rather than just the usual teleomorphic *H. jecorina* from ascospores. They demonstrated that *H. jecorina* was a heterothallic ascomycete having two mating types, with ascospores that exhibited bipolar segregation within the ascus, the sac containing the sexual spores during sexual

reproduction. Using ITS regions, identical sequences between these new anamorphic *H. jecorina* isolates and collection-based cultures of *H. jecorina* and *T. reesei* QM6a were observed. Thus, it appeared that *H. jecorina* and *T. reesei* had a teleomorph/anamorph relationship, sharing habitats and gene sequences. However, Lieckfeldt et al. (2000) noted differences in metabolic phenotype between strain QM6a and other putative *H. jecorina* (*T. reesei*) in that all of the new isolates utilized sucrose, nitrate, and nitrite, but QM6a did not.

In the years that followed there were several other reports in the literature on the isolation of *T. reesei* from geographical regions other than the Solomon Islands. Kar et al. (2006) reported the isolation of *T. reesei* from soil in West Bengal, India. Another paper reported the isolation of *T. reesei* from sea mud in the tideland in Lianyungang, Jiangsu Province, China (Sun et al., 2006). However, the identification of the fungal isolate as *T. reesei* was questionable as the method of identification used was not specified. There is one other article reporting the isolation of *T. reesei* from an area south of the Caspian Sea in Iran (Roodsari et al., 2007). Although robust molecular identification methods appear to have been used in the identification of the 22 species of *Trichoderma* found on wood or in soils from Iran, this report exists only as an abstract from a presentation at a meeting, and the data have not been published. It could also be that in these papers the authors used the better known species name of *T. reesei* rather than *H. jecorina.*

Later, mating experiments between *T. reesei* and *H. jecorina* conducted by Seidl et al. (2009) led to the discovery that *T. reesei* (strain QM6a) and *H. jecorina* were not identical in mating types. *T. reesei* has a single mating type known as *MAT1-2,* whereas *H. jecorina* possesses either a *MAT1-1* or a *MAT1-2* mating type. Thus, in experiments *T. reesei* was a successful male donor in mating with opposite type *H. jecorina,* but was unable to form female structures when undergoing mating with *H. jecorina,* or with an artificially modified *T. reesei* having opposite mating type. These results led Seidl et al. (2009) to a conclusion different from that of Kuhls et al. (1996) which was that *T. reesei* is not a clonal derivative of *H. jecorina*, but rather a cryptic species. Cryptic species are morphologically similar, or even virtually identical, but have been reproductively isolated. In this case Seidl et al. (2009) speculated that the reproductive isolation may be related to non-functional or absent genes required for female fruiting bodies. Therefore, Seidl et al. (2009) stated that *T. reesei* is a true anamorph of *H. jecorina,* i.e., a true member of the same species.

In the literature the names *T. reesei* and *H. jecorina* have been used synonymously as some researchers consider them to be identical fungi (except for mating type). Others think that the anamorph and teleomorph should be referred to as separate species. However, a recent taxonomic publication has now proposed a split of *H. jecorina* into as many as four species (Druzhinina et al., 2010). They proposed the name *T. parareesei* for those fungal strains of *H. jecorina* that were isolated as anamorphs from soils, and showed that the majority of these anamorphic strains were

genetically isolated from *H. jecorina/T. reesei.* They also suggested that the remaining strains that do not belong to *H. jecorina/T. reesei* probably belonged to at least one other species, but the number of isolates studied was so limited they did not propose species names for those few isolates. Later that year, building upon the work of Druzhinina et al. (2010), Atanasova et al. (2010) indicated that there were actually three species equivalents within *H. jecorina sensu lato* (*H. jecorina* in the broad sense). They provided data further justifying the proposal for the new species *T. parareesei* based upon differences in habitat, sporulation, and metabolic capabilities of these strains. *H. jecorina/T. reesei* strains were differentiated from *T. parareesei* by being more sensitive to light, having a narrower substrate range, growing less robustly at higher temperatures on many carbon substrates, and being less competitive with other fungi. Although more light tolerant, *T. parareesei* was viewed as primarily a soil fungus while *H. jecorina/T. reesei* seemed to be better adapted to plant surfaces. These papers therefore proposed that the species *T. parareesei* would consist of those strains formerly referred to as *T. reesei* from soils in Latin America, Africa, and Southeast Asia. These included fungal isolates from soils of subtropical and tropical areas in the South American countries of Brazil, Argentina, and Columbia, the Central American country of Mexico, from Ghana and Ethiopia in Africa, and from India (Druzhinina et al., 2010). Atanasova et al. (2010) also presented data showing that strains within the species *T. parareesei* had lost the ability to reproduce sexually. They stated that although *T. parareesei* strains contain both the *MAT1-1* and *MAT1-2* mating types, the *MAT1-2* locus in *T. parareesei* was altered, and was thus defective compared to that found in *H. jecorina.*

These recent taxonomic papers also showed that *T. reesei sensu stricto* (QM6a) was in the same clade as almost all teleomorphic strains of *H. jecorina* (Atanasova et al., 2010; Druzhinina et al., 2010). *T. reesei* QM6a was the only anamorphic strain within that clade, termed *H. jecorina sensu stricto.* Atanasova et al. (2010) stated that the strain QM6a (plus derivatives) and teleomorphic strains of *H. jecorina* are a single species now referred to as *H. jecorina/T. reesei* (QM6a). This, of course, implies that the species *T. reesei* consists solely of the strain QM6a and its derivatives.

In summary, recently Druzhinina et al. (2010) proposed splitting up some *T. reesei/H. jecorina* isolates into several new species, one of which was called *T. parareesei*. Atanosova et al. (2010) continued with that work and characterized the species *T. parareesei,* which would consist of those *H. jecorina* strains isolated from soils around the globe as an anamorph which differ from the original QM6a strain in sporulation, photosensitivity, substrate utilization, and habitat. Since this new species, *T. parareesei,* was formally described by Atanasova et al. (2010), it now awaits acceptance from the scientific community. Upon acceptance, the species *H. jecorina* will consist of the teleomorphic strains remaining after those called *T. parareesei* and some as yet to be named other isolates are removed, plus the single anamorphic isolate called *T. reesei* which encompasses strain QM6a and its derivatives. Only the latter has a stable taxonomic status at this time and can be unequivocally identified due to well-established culture collection provenance. Therefore, this exemption is being

considered only for *T. reesei* strain QM6a and its derivatives, and not for other potential or existing species of *Trichoderma* or *Hypocrea*.

2. Derivatives of QM6a

At the U.S. Army Natick Laboratories in Natick, Massachusetts, researchers created mutants of QM6a with increased cellulase production. Conidia of the strain *T. reesei* QM6a (called *T. viride* QM6a at the time) were suspended in distilled water at 20°C and were irradiated with high energy electrons from a 24-million electron volt, 18-kw linear accelerator (Mandels et al., 1971). A dose of 0.05 megarads killed more than 95% of the conidia, and all were killed with radiation above 0.2 megarads. Survivors (0.05 - 0.2 megarads) formed small colonies on deoxycholate agar even after extended incubation periods, but then sporulated and were transferred to potato dextrose agar where normal growth resumed. One isolate, designated QM9123 (dose, 0.05 megarads), was found to produce twice as much cellulase as the wild type QM6a strain. According to Morawetz et al. (1992), this *T. reesei* mutant strain QM9123 was apparently subjected to another radiation step to produce the mutant strain *T. reesei* QM9414. However, the literature is not clear in exactly what mutagenesis was conducted to arrive at strain QM9414. Nevalainen et al. (1994) says that this mutagenesis also took place at the Natick Laboratory and references the paper by Mandels et al. (1971) for this mutagenesis. The Mandels et al. (1971) paper, however, does not mention strain QM9414, but only QM9123 and another mutant obtained that was deficient in cellulase production designated QM9136. Montenecourt and Eveleigh (1977) stated that QM9414 was derived by a 2-step mutational process from QM6a, but again, there are no specifics regarding the second mutation. Brückner et al. (1984) stated that QM9414 was obtained from a second irradiation of the conidia of QM6a, but these authors reference the Mandels et al. (1971) paper and Montenecourt and Eveleigh (1977), neither of which specifically report exactly what the second mutational step consisted of. Morawetz et al. (1992) further stated that “the nature of the mutations leading from the original isolate QM6a (Simmons 1977) to QM9414 have never been elucidated”. It is apparently only in an abstract of a meeting where Simmons (1977) described QM9414, but this abstract is not available. The American Type Culture Collection (ATCC) gives a history of QM9414 as a mutant of QM9123, and says that it was originally Mandels’ strain 3019. The strain QM9123 from which QM9414 was derived was described by Mandels et al. (1971) as a mutant that appeared to only have an increased quantity of cellulase production and that other carbohydrases were not affected. Mandels et al. (1971) further reported that QM9123’s increased cellulase activity was stable for more than two years in subcultures and through a lyophilized state. Obviously the mutations of QM9123 to arrive at QM9414 resulted in a further increase in cellulase production. ATCC says that QM9414 produces 1.5-2.0 times more cellulase on cellulose medium than strain QM9123.

In an article on the safety of *T. reesei* in enzyme fermentation, Nevalainen et al. (1994) say that most, if not all, of the *T. reesei* strains used industrially are derived from

the *T. reesei* QM6a originally described by Mandels and Reese (1957). The authors present a figure showing the genealogy of the different high cellulase-producing *T. reesei* strains in use throughout the world. All of the following strains were apparently derived from the mutant QM9414: MCG77 (Natick Laboratory), the MG Series (Gulbenkian Institute of Science), the VTT-D Series (Technical Research Center, Finland), the L Series (Cetus Corporation, USA), the MHC Series (Slovak Academy for Sciences, Slovakia), the D1 Series (Indian Institute of Technology, India), the Kyowa Series (Kyowa Hakko Kogyo Co. Ltd., Japan), and the CL Series (Societe Cayla, France). The only series of *T. reesei* that was not derived from this QM9414 mutant is the Rut Series (Rutgers University, USA) which is a derivative directly from the wild type QM6a strain. The additional mutations associated with these various high cellulase-producing *T. reesei* series were not specified.

D. Related Species of Concern

There are a few species within the genus *Trichoderma* that present human health or ecological concerns. One of these is *Trichoderma longibrachiatum,* which has been referred to as a serious opportunistic pathogen that can cause human fungal infections (Pfaller and Diekema, 2010). Druzhinina et al. (2008) stated that *T. longibrachiatum* is the species most commonly associated with *Trichoderma* mycoses, which often has unfavorable outcomes. In addition to this species, *T. citrinoviride* is thought of as a potentially pathogenic species (Kuhls et al., 1999). Earlier reports on the isolation of *T. koningii* and *T. pseudokoningii* from clinical samples were found to be incorrect when the sequence analysis of the internal transcribed spacer (ITS) region was examined according to Antal et al. (2006). Invasive potential within the genus is apparently restricted to *T. longibrachiatum* and *T. citrinoviride* based on recent molecular taxonomic analyses (Pfaller and Diekema, 2010). Infection by these species has been associated with immunocompromised individuals, and those undergoing peritoneal dialysis (as summarized by Pfaller and Diekema, 2010). The species *T. longibrachiatum* is apparently closely related to *Hypocrea orientalis,* which is a member of the *H. schweinitzii* complex, even though it is not the anamorph of *H. orientalis* (Samuels et al., 1998, as cited by Antal et al., 2006).

In addition to potential infections from some strains of *Trichoderma*, other strains present human health concerns because of the production of mycotoxins or other secondary metabolites, some of which are highly toxic (Lübeck et al., 2000). The mycotoxin trichodermin is a strong inhibitor of protein synthesis, and thus, is highly toxic to mammalian cells (Wei et al., 1974). *T. viride* has been shown to produce a mycotoxin trichotoxin A (Hou et al., 1972) with a median LD_{50} (lethal dose required to kill 50% of the population) of 4.36 mg/kg in mice. Degenkolb et al. (2008b) stated that *T. harzianum* and *T. viride*, two biocontrol strains that are known to produce trichothecene toxins, have been re-identified as the closely related species *T. brevicompactum* and *T. arundinaceum.* Both of these species belong to the *T. brevicompactum* complex, a complex with members that have been proven to produce trichothecene-like mycotoxins

such as trichodermin and harzianum A (Degenkolb et al., 2008a). Some *Trichoderma* species and their toxins are associated with "sick building syndrome" from water damaged buildings (Lübeck et al., 2000). *T. longibrachiatum, T. citrinoviride, T. harzianum, T. viride, T. hamatum,* and *T. atroviride* were all isolated from water-damaged building materials in various Danish buildings (Lübeck et al., 2000).

In addition to human health concerns, species producing mycotoxins may present concerns for animals other than humans, including aquatic species, and also pose concerns for plants. For instance, trichodermin is a potent inhibitor of protein synthesis, so it is toxic to all eukaryotes, both animals and plants.

Some members of the genus *Trichoderma* are pathogens of commercial mushrooms. Earlier reports in the literature stated that the species *T. harzianum* caused disease in commercial mushrooms (Gams and Bissett, 1998). Later, new names were proposed for the species of *Trichoderma* that causes green mold of the commercial white button mushroom, *Agaricus bisporus* (Samuels et al., 2002). The new names proposed were *T. aggressivum* var. *aggressivum*, and *T. aggressivum* var. *europeaeum* for the fungi responsible for disease of commercial mushrooms in North American and Europe, respectively. The former designations were *T. harzianum* biotypes Th2 and Th4, respectively (Samuels et al., 2002). More recently, studies have reported that a number of genetically closely related, but phenotypically divergent species of *Trichoderma* are responsible for disease on the oyster mushroom, *Pleurotus ostreatus,* and a number of new species have been described (as summarized by Komoń-Zelazowska et al., 2007a). The ability of these strains to attack mushrooms is in part due to the production of chitinase that can degrade chitin, an important component of fungal cell walls, and mushrooms are fungi. Also, the ability of these particular species to cause disease in mushrooms is also due to the production of antifungal compounds. *T. aggressivum, T. pleuotrophilum,* and *T. fulvidum* are responsible for significant loss of the mushroom crops of *Agaricus bisporus* and *Pleurotus ostreatus* (Kubicek et al., 2007).

III. HAZARD ASSESSMENT

A. Human Health Hazards

1. Colonization and Pathogenicity

Trichoderma reesei is not a human pathogen. There is only one report in the literature associating *T. reesei* with an infection in an immunocompromised human. *T. reesei* is not the species thought to be of increasing importance with infection of immunocompromised individuals, which is *T. longibrachiatum.* The other species of the genus thought to have invasive potential is *T. citrinoviride* (Pfaller and Diekema, 2010). *T. reesei* has a long history of safe use for production of enzymes with food, agricultural,

pharmaceutical, and industrial uses with no incidences associated with human disease. Because of its long history of enzyme production, and due to the use of some of these enzymes in food and pharmaceuticals, the potential for *T. reesei* to cause disease and the potential toxicity of its enzyme products has been tested a number of times.

An elaborate series of studies designed to elucidate both consumer and occupational health effects were conducted over the period of 1978 - 1982 on a cellulase produced by Novo Industries used as a food processing aid (Hjortkjaer et al., 1986). The *T. reesei* microorganism used to produce an enzyme product known as Celluclast was tested for its pathogenic potential and for the production of toxins and antibiotics. Mice, guinea pigs, and rabbits were used for investigating the potential pathogenicity of the *T. reesei* strain. However, the animals were immunosuppressed first with a high dose of cortisone prior to inoculation with either viable or killed spores to facilitate survival and multiplication of the fungus in the animals. Nonpathogenic microorganisms are typically eliminated readily from a healthy animal by its immune system defenses. Preliminary studies for determining the lethality of the fungus in mice revealed that the median lethal dose (LD_{50}) was greater than 10^7 viable spores with interperitoneal (ip) injection in mice. In the main study, the immunosuppressed animals were injected either intravenously (iv) or ip with 10^5 to 10^7 viable spores, or with 10^7 killed spores. No effects were seen with ip injection of 10^5 viable spores, but microorganisms were found in different organs 40 days after iv injection of 10^5 or 10^7 viable spores or ip injection of 10^7 viable spores. In all animals, granulomatous inflammation was observed with histological examination. Additional studies on mice conducted to further elucidate the chronic nature of the lesions led the authors to conclude that *T. reesei* was pathogenic to cortisone-treated mice. However, interpretation of these data by Nevalainen et al. (1994) was that *T. reesei* should be regarded as non-pathogenic because it was only under the circumstances of using high doses of spores in immunocompromised animals that *T. reesei* behaved as a pathogen.

A pathogenicity study on a strain of *T. reesei* was conducted by (b) (4) that was described in a GRAS notice for an enzyme submitted to the FDA (GRN No. 333). With an ip dose of 2.2 x 10^7 viable spores of *T. reesei* strain A83 in Sprague-Dawley (SD) rats, *T. reesei* was judged to be non-pathogenic and non-toxic as no animals died, no adverse clinical symptoms were observed, and clearance of the microorganism from the rat spleen was observed. A different study in the same GRAS notice using an ip dose of 5 x 10^6 colony-forming units (cfus) in (b) (4) Sprague-Dawley (CD) rats also demonstrated a lack of pathogenicity and clearance of the fungus from the rats.

Data on the potential pathogenicity of *T. reesei* are available in another GRAS petition (GRN No. 32) for the enzyme pectin lyase submitted by Röhm Enzyme GmbH. A genetically modified *T. reesei* strain, ALKO 2224, and its parental strain were iv injected into Naval Medical Research Institute (NMRI) rats at a dosage of *5* x 10^6 viable spores. Even with the injection of cortisone acetate to one group of animals two days prior to inoculation to immunosuppress them, no pathogenicity of the *T. reesei* strains

was observed in any of the rats.

In GRAS notice number 315 from (b) (4) for the enzyme transglucosidase, data from another pathogenicity study were reported for *T. reesei* strain EG1-EP9 (GRN No. 315). CD rats were ip injected with 5.6 x 10^6 cfu viable microorganisms or with heat-killed cells. No deaths occurred and no adverse clinical observations were reported. Initial necroscopy on days 7 and 21 revealed enlarged spleens and decreased liver weight, however, the animals completely recovered thereafter.

There is no information available on the presence of any virulence factors in *T. reesei* that would enable it to be pathogenic. Potential fungal virulence factors include adherence factors, penetration factors, necrotic factors (enzymes), melanin pigments, toxins, and the ability to grow at elevated temperatures. *T. reesei* is a mesophilic fungus that has generally been cultured *in vitro* in the range of 25-30°C, and been grown between 25 and 28°C for optimal enzyme production. There is one report in the literature stating that *T. reesei* can grow near human body temperature at 36°C, however, the body of evidence suggests that *T. reesei* does not pose concerns regarding human pathogenicity.

2. Mycotoxin Production by *T. reesei*

Many fungi produce mycotoxins, and more produce other secondary metabolites that are inhibitory to various organisms but are typically less toxic. Mycotoxins are highly toxic secondary metabolites that are thought to provide the fungus with a competitive edge against other microorganisms, including other fungi. There is one report in the literature that *T.* reesei produces the mycotoxin trichodermin (Watts et al., 1988). Trichodermin is a member of a group of 12,13-epoxytrichothecenes that are potent inhibitors of protein synthesis in eukaryotes, and is therefore highly toxic to animals, including humans, and to plants (Wei et al., 1974). According to Blumenthal (2004) trichodermin is a member of this family of potent food contamination toxins but it is much less toxic than some mycotoxins.

Watts et al. (1988) reported that trichodermin and another secondary metabolite with antifungal activity designated as Tx were isolated from a mycelial extract of *T. reesei* strain P-12. They described the strain P-12 as a mutant of *T. reesei* strain QM9414 although the nature of the mutagenesis that resulted in their strain was not specified. As stated above, QM9414 is a radiation mutant of strain QM9123 (Morawetz et al., 1992) which is a radiation mutant of *T. reesei* QM6a (Mandels et al., 1971). In her paper on mycotoxin testing in food grade enzyme preparations produced by *A. niger, A. oryzae,* and *T. reesei,* Blumenthal (2004) questioned the identity of the strain used by Watts et al. (1988). She suggested that it was more likely a strain of *T. harzianum* (based upon a personal communication). However, Watts et al. (1988) specifically stated that they obtained the *T. reesei* strain QM9414 directly from the Army

Laboratories in Natick, Massachusetts where the QM9414 strain was originally created. Thus, it would appear that there should be no question as to the proper identity of the strain as *T. reesei*. Rather than the strain used by Watts et al. (1988) being incorrectly identified, it may be possible that the unspecified mutagenesis that QM9414 was subjected to produce the mutant strain P-12 resulted in trichodermin production even though trichodermin is not typically produced by *T. reesei*. There are no other reports in the literature suggesting that *T. reesei* is capable of producing trichodermin. On the contrary, more recent papers have demonstrated that mycotoxin production is restricted to only certain species of the *Trichoderma* genus that are not closely related to *T. reesei*.

In a review of peptaibiotics and mycotoxins from species in the genera *Trichoderma* and *Hypocrea,* Degenkolb et al. (2008b) stated that *T. harzianum* and *T. viride*, two biocontrol strains that are known to produce trichothecene toxins, have been re-identified as the closely related species *T. brevicompactum* and *T. arundinaceum.* Both of these species belong to the *T. brevicompactum* complex, a complex with members that have been proven to produce trichothecene-like mycotoxins such as trichodermin and harzianum A (Degenkolb et al., 2008a). Degenkolb et al. (2008b) specifically stated "only *T. brevicompactum* and three closely related species within the *T. brevicompactum* complex have been proven to produce trichothecenes-type mycotoxins (trichodermin, harzianum A)". They further stated that two studies have shown that trichothecenes were not produced by any species outside the *T. brevicompactum* complex. These more current papers suggest that *T. reesei* is not capable of producing the mycotoxin trichodermin.

According to Nevalanein et al. (1994) industrial strains of *T. reesei* are routinely checked by enzyme producers to confirm the absence of antibiotic activity and toxins including aflatoxin B, ochratoxin A, sterigmatocystin, T-2 toxin, and zearalenone according to the recommendations of the Joint FAO/WHO Expert Committee on Food Additives (JEFCA, 1981). Given that *T. reesei* has a long history of safe use in the production of food enzymes where there is a need to routinely check for the absence of toxins and harmful metabolites, *T. reesei* strains utilized for production of enzymes used in industrial applications likely also do not produce these compounds under the growth conditions used in submerged standard industrial enzyme fermentation.

3. Production of Secondary Metabolites by *T. reesei*

Secondary metabolites, like mycotoxins, may also provide the fungus with competitive mechanisms against other microorganisms. Martinez et al. (2008) stated that *T. reesei* has numerous genes that code for biosynthetic pathways of secondary metabolites, and the authors speculated that these may help it survive in its natural soil habitat. *T. reesei* has been shown to produce a secondary metabolite known as paracelsin which is a compound known as a peptaibol (Brückner and Graf, 1983). Peptaibols are small (up to 20 amino acids) linear peptides of 1000-2000 Daltons

characterized by a high content of the non-proteinogenic amino acid α-aminoisobutyric acid (Aib), with an N-terminus that is typically acetylated, and a C-terminus that is linked to an amino alcohol which is usually phenylalaninol, or sometimes valinol, leucinol, isoleucinol, or tryptophanol (Szekeres et al., 2005). The term peptaibol was formed from the names of its three characteristic components, peptide, Aib, and amino alcohol. Peptaibiotics (peptide antibiotics) are similar secondary metabolites but the N-terminus is rarely acetylated (Szekeres et al., 2005). Peptaibols are associated with a wide variety of biological activities as they modify membranes and cause pore formation, resulting in leakage from the cell. They can have antifungal, antibacterial, sometimes antiviral, antiparasitic, and neurotoxic activity, and many fungal strains producing peptaibols are used as biological control agents against plant pathogens (Szekeres et al., 2005). Part of their role as biocontrol agents is that they induce plant defense mechanisms. They are also being investigated as therapeutic agents because of their anti-cancer activity (Mukherjee et al., 2010).

It is known that peptaibols are synthesized by large non-ribosomal peptide synthetases by a gene referred to as *Tex1*, and two other genes that code for different size peptaibols bearing high homology to *Tex1,* referred to as *Tex2* and *Tex3* (Kubicek et al., 2007; Mukherjee et al., 2010). However, peptaibol formation by *Trichoderma* is still apparently poorly understood. According to Kubicek et al. (2007) in a review article on peptaibols in *Trichoderma*, there are numerous data gaps regarding how peptaibols are actually produced. Kubicek et al. (2007) point out that in most studies investigating peptaibol formation, surface cultures were used, but with the same fungus under submerged conditions, peptaibols were not formed. There were a few exceptions to this, however, as peptaibol production in submerged culture was observed when an insoluble component, for instance, a carbon source such as cellulose, was added to the culture medium (Brewer et al., 1987). *T. viride* was shown to produce the peptaibol alamethicin in culture when the growth medium was supplemented with one of several insoluble components such as dextrin, pharmamedia, or fish meal. Yields of the peptaibol were greatly reduced in the absence of these solids (Brewer et al., 1987). These authors reported that a number of insoluble polysaccharides added to the culture medium increased the production of the peptaibol alamethicin, and that methylcellulose resulted in the largest increase. The production of the compound paracelsin by *T. reesei* as originally discovered by Brückner and Graf (1983) was accomplished by the solid phase fermentation of the fungus on bagasse and other cellulose-containing substrates. Schirmböck et al. (1994) also got peptaibol production with addition of a solid component to a *T. harzianum* culture. However, in this study, the solid surface was cells walls of the fungus *Botyrtis cinerea.* Further investigation revealed it was the presence of the cell wall material from the other fungus that triggered both peptaibol production and production of hydrolytic enzymes including chitinase, glucanase, and protease to degrade the fungal cell walls. Song et al. (2007) used solid-state fermentation (SSF) to optimize production of the peptaibols known as trichokonins from *T. koningii.*

Kubicek et al.'s (2007) summary of the literature also reported that peptaibol production in *Trichoderma* occurred in older cultures, usually more than 15 days old. They speculated that with older cultures sporulation was likely occurring. However, they stated that there was no definitive link between conidiation and peptaibol production. Later that year, researchers in Kubicek's laboratory published a paper that demonstrated that the formation of the peptaibol atroviridin by *Hypocrea atroviridis* was conidiation-associated, and was regulated by both blue light and by a protein known as GNA3 (Komoń-Zelazowska et al., 2007b). It was shown that light triggered peptaibol formation, but it was only because the light triggered conidiation. No atroviridin was produced during vegetative growth of the fungus.

Stress conditions have also been shown to result in peptaibol production by *Trichoderma.* An earlier study by Vizcaino et al. (2006) showed that the *T. harzianum* peptaibol synthetase transcript could only be detected under nitrogen starvation. To date more than 900 peptaibol sequences have been published (http://www. cryst.bbk.ac.uk/ peptaibol/sequence.htm). However, Mukherjee et al. (2010) stated that despite the knowledge of the sequences, the genetics of peptaibol formation are not well-understood.

Peptaibols have been shown to be produced in very high amounts in cultures of *Trichoderma* strains in some studies. Ooka et al. (1966) showed that *T. viride* produced a peptaibol in the extremely high concentration of 20 g/L over the course of 70 - 90 hours (< 3 - < 4 days) with shake-flask culture of a broth supplemented with 4% soluble starch and 1% Polypepton at 30°C. Meyer and Reusser (1967) used submerged culture supplemented with molasses, dextrin, fish meal, and Pharmamedia to get production of 8 - 10 mg/L of a peptaibol from a strain of *T. viride* grown at 25°C for 4 - 5 days. Song et al. (2007) obtained the maximum production of trichokonin VI of 4.07 mg/g dry substrate under optimized conditions in solid-state fermentation.

Several different paracelsins have been discovered since the term was first used by Brückner and Graf (1983) for the toxic secondary metabolite produced by *T. reesei*. Paracelsins have 20 amino acids (Brückner and Graf, 1983), and are characterized by the presence of phenylalaninol as the C-terminal amino alcohol, and by the specific amino acid content and sequence (Ritieni et al., 1995). The structures of paracelsin A, B, C, and D (Brückner et al., 1984), which were isolated from both the fungal mycelium and the culture broth of *T. reesei* QM9414, were elucidated by Fast Atom Bombardment Mass Spectroscopy (fabms) by Przybylski et al. (1984). These four paracelsins, A, B, C, and D, were individual components in a microheterogeneous paracelsin mixture. Przybylski et al. (1984) stated that a characteristic of peptaibols is their microheterogeneity which is a result of their non-ribosomal biosysthesis mechanism. Paracelsin E was later isolated from *T. saturnisporum* which also produced paracelsin A, B, C, and D (Ritieni et al., 1995).

Brückner and Graf (1983) demonstrated that paracelsin extracted from *T. reesei*

QM9414 had antibacterial action against *Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus, Streptococcus faecalis, Streptococcus lactis,* and *Streptococcus thermophilus.* It was not inhibitory to *Candida albicans, Escherichia coli, Porteus vulgaris, Serratia marcescens, Salmonella typhimurium, Pseudomonas aeruginosa,* or *Enterobacter aerogenes.* Paracelsin was shown to have membrane-modifying properties and antibiotic activity, particularly against Gram-positive bacteria.

In a study on peptaibols produced by 12 different species of *Trichoderma*, most of which had potential as biocontrol agents, a strain of *T. reesei* was shown to produce paracelsin (Solfrizzo et al., 1994). Paracelsin production was common among the *Trichoderma* species studied as it was also observed in *T. citrinoviride,* in one of two strains of *T. hamatum,* in *T. inhamatum, T. koningii, T. longibrachiatum, T. parceramosum, T. polysporum,* and *T. pseudokoningii,* in both strains of *T. saturnisporum,* and in *T. viride*. The only species in which paracelsin production was not observed was in the two strains of *T. harzianum.*

Paracelsin has been shown to have toxicity toward mammalian cells. Brückner et al. (1984) reported that paracelsin exhibited hemolytic activity on human erythrocytes. They stated that erythrocyte hemolysis is a common characteristic of other peptaibols such as alamethicin, suzukacillin, and trichotoxin. The *in vitro* hemolytic activity of paracelsin was reported as $C_{50} = 3.7 \times 10^{-5}$ mol/liter (where C_{50} is the concentration that causes 50% hemolysis). Paracelsin exhibited lesser hemolytic activity than trichotoxin ($C_{50} = 2.1 \times 10^{-5}$ mol/liter) and alamethicin F-50 ($C_{50} = 1.6 \times 10^{-5}$ mol/liter). Brückner et al. (1984), when discussing the hemolytic activity of paracelsin, also stated that "*in vivo* hemolysis is also suspected to be the reason why the lethal dose is about 5 mg/kg mice when paracelsin is administered intraperitoneally". However, the authors do not present any data for determination of this lethal dose, nor do they cite a reference for this study. Therefore, it is unknown whether this value is from unpublished data from the authors or whether these are data from another study that cannot be located in the literature.

Abu Raya et al. (1993) reported that paracelsin showed toxicity to PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) with a CC_{50} (cytotoxicity concentration of 50%) of 21.8 µM. Grigoriev et al. (2003) showed that paracelsin A was toxic to mice at an intraperitoneal dosage of 20 mg/kg body weight. In this study, all of the highly membrane active peptaibols which included alamethicin F-30, paracelsin A, chrysospermin C, and the lipopeptaibol texenomycin A were toxic to mice. Long lasting hypothermia in mice, which was an indicator of neuroleptic activity (affecting the brain, especially by reducing the intensity of nerve function, e.g., tranquilizing) was not observed in this study because the mice died shortly after administration of the compounds (Grigoriev et al., 2003).

The toxicity of paracelsin reported in the two studies above seems quite high, particularly in comparison to the reported toxicity of trichodermin, an actual mycotoxin with high toxicity to both animals and plants (Wei et al., 1974). Blumenthal (2004)

stated that trichodermin had an LD_{50} of 500 mg/kg of body weight with intraperitoneal or subcutaneous administration in mice according to the Registry of Toxic Effects of Chemical Substances. This Registry has other toxicity values for trichodermin in addition to this one reported value. The LD_{50}'s of trichodermin were 500 and 354 mg/kg in mice with intraperitoneal injection, and 500 mg/kg in mice with subcutaneous injection. LD_{50} values were approximately two orders of magnitude lower with oral administration of trichodermin. The Registry of Toxic Effects of Chemical Substances reported an LD_{50} of 6.2 mg/kg with oral administration of trichodermin to mice, and an LD_{50} of 7.5 mg/kg with oral administration of trichodermin to rats.

In addition to paracelsin, other secondary metabolites have been reported from *T. reesei*. Trichodermatides, which are polyketide compounds that exhibit weak cytotoxicity against the A 375-S2 melanoma cell line, were reported by Sun et al. (2006) and Sun et al. (2008) as being from the marine fungus *T. reesei*. As stated previously, these two papers do not specify how the particular strain of *Trichoderma* was identified. *T. reesei* is not known as a marine fungus, so it is unknown whether the fungus studied in these two papers was actually *T. reesei.* There apparently are, however, marine strains of *T. longibrachiatum* (Mohamed-Benkada et al., 2006; Ruiz et al., 2007) and *T. koningii* (Landreau et al., 2002), the former of which was isolated from blue mussel and the latter from a shellfish (cockle) farm.

4. Test Data on Toxin Production

A number of different studies have been conducted assessing the potential for *T. reesei* to produce toxins during submerged fermentation for production of enzymes for food, pharmaceutical, or industrial uses. In addition to the pathogenicity testing of the *T. reesei* strain used to produce the enzyme Cellulast as discussed above, the enzyme product itself has also been tested for toxicity (Hjortkjaer et al., 1986). The test material, referred to as the "tox-batch" was tested for general oral toxicity, effects on reproduction, mutagenic potential, inhalation toxicity, irritation to skin and to eyes, and for skin sensitization. Acute oral toxicity studies were conducted in mice, rats, and dogs. No toxicity was observed in any species and the acute oral toxicity was > 8 g/kg in rats, >16 g/kg in mice, and >5 g/kg in dogs (the highest dose tested in each species). Subchronic toxicity studies, which consisted of feeding the tox batch for 91 days at 0, 1, 2, or 5%, were conducted in rats and dogs. There was no evidence of systemic effects in dogs up to 3.0 g/kg of bodyweight (bw) per day, the highest dose tested. In the 91-day subchronic toxicity study in rats, the highest dietary level of the Celluclast enzyme product (tox-batch) of 5% depressed body weights in male rats but not female rats. Rats tolerated the tox-batch at the 1% or 2% dietary level (~1000 mg/kg/bw).

Additional toxicity studies have been conducted on other enzymes produced by *T. reesei.* In GRAS petition No. 315, a 91-day subchronic feeding study with a cellulase was conducted in CD rats to assess the potential toxicity of the enzyme product. No mortalities were recorded throughout the entire investigation. There were no treatment-

related adverse effects on any of the parameters measured (systemic toxicity, food consumption, opthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology). It was concluded that the cellulase from *T. reesei* did not result in toxicity up to and including a dose level of 5% w/w in the diet. A No Observable Effect Level (NOEL) was established at 5% in the diet corresponding to 3.35 and 4.05 g cellulase/kg bw/day for male and female rats, respectively (GRN No. 315).

Data from a number of other studies are also presented in this GRAS petition (GRN No. 315) for different enzymes from the same *T. reesei* strain including a high pI xylanase, a low pI xylanase, endoglucanase III, endoglucanase I, and protease. A 91-day subchronic feeding study in CD rats at concentrations up to 50,000 mg/kg of a high pI xylanase reported two deaths, but they were not attributed to the treatment. No other treatment-related adverse effects were observed on any of the parameters measured (organ weight, urine analysis, hematology, necropsy, and histopathology) with the high pI xylanase indicating a lack of toxicity of the enzyme. Likewise, a 91-day subchronic feeding study was conducted with a low pI xylanase produced by *T. reesei* with feeding concentrations up to 3000 mg/kg/day. There were no deaths and no adverse treatment-related effects on any of the same parameters measured as listed previously. The NOEL was established at 3000 mg/kg for the low pI xylanase.

Also presented in GRAS Notice 315 is data from a 28-day oral toxicity study in rats for a *T. reesei* endoglucanse III administered at concentrations up to 1000 mg/kg/day for four weeks. No mortalities were observed in the (b) (4) rats during the study. There were no treatment-related adverse effects either, which established a NOEL of 1000 mg/kg/day (GRN No. 315). For another *T. reesei* enzyme, endoglucanase I, there was a 14-day oral feeding study and a 91-day subchronic feeding study conducted. There were no adverse effects reported for any parameter monitored in the 14-day oral study in (b) (4) BR rats dosed with up to 1000 mg/kg/day by oral gavage using a dosage volume of 10 ml/kg bw. Likewise, there was no toxicity observed in the 91-day subchronic feeding test. There were no mortalities, and no adverse treatment-related effects were observed on any of the parameters measured (organ weight, urine analysis, hematology, necropsy, and histopathology). At study termination, there were no differences in the behavioral test (open field) and functional tests (gripping reflex, startle reflex) between the control and treatment groups. A NOEL was established for this enzyme of 1000 mg/kg/day.

A protease produced by *T. reesei* was also tested for toxicity with a 91-day subchronic feeding test (GRN No. 315). There were no treatment-related deaths or adverse effects observed. A NOEL of 31.25 mg total protein/kg bw/day was established (the highest dosage studied).

Data from an acute toxicity test for another *T. reesei* enzyme, glucoamylase, are also presented in GRAS petition 315. Under the conditions of this study where the rats

received a single oral dose followed by a 14-day period of observation, the oral LD_{50} was > 1996 mg total protein/kg bw which was the highest possible dose volume of 10 ml/kg. This same enzyme was also tested in a 91-day subchronic feeding test in SD rats. There were no overt signs of systemic toxicity or adverse clinical observations attributable to treatment (GRN No. 315).

5. Allergic Reactions/Sensitization to *T. reesei* and its Enzymes

Although fungi in general can be allergens, there are no reports in the literature specifically suggesting that *T. reesei* causes allergenicity in humans but there are reports for other *Trichoderma* species. There are two reported cases of hypersensitivity pneumonitis, also known as extrinsic allergic alveolitis, caused by *T. koningii* and by *T. viride* (Halpin et al., 1994; Enriquez-Matas et al., 2009). In addition, there is one report of *T. longibrachiatum* causing allergic fungal sinusitis (Tang et al., 2003).

Enzymes themselves are known allergens. However, there are no allergenicity issues specifically attributable to enzymes produced by *T. reesei* vs. enzymes produced by other fungi.

6. Human Health Hazard Conclusions

There is only one potential concern for human health hazards associated with *T. reesei* and that is paracelsin production. There is no concern for potential pathogenicity of *T. reesei.* Test data on various industrial strains typically do not show adverse effects, except in one case where pathogenicity to mice was demonstrated, but only under the conditions where the mice were intentionally immunosuppressed and then subjected to high inoculation rates. Toxicity testing on a number of enzymes produced by *T. reesei* indicates that the fungus does not produce toxins under the conditions used in submerged standard industrial fermentation for enzyme production. There is, however, concern for the production of the secondary metabolite paracelsin if *T. reesei* was to be grown under certain other conditions. Paracelsin has been shown to be capable of hemolysis of human erthyrocytes, and has been reported to be toxic to mice at a concentration of 20 mg/kg (Grigoriev et al., 2003) which is a lower amount than that of an actual mycotoxin known as trichodermin that is produced by some *Trichoderma* spp.

Paracelsin production is not expected to occur under the conditions of submerged standard industrial fermentation for enzyme production. However, according to information available in the literature, extended fermentations, or fermentations that contain insoluble carbon sources such as cellulose may result in paracelsin production. Therefore, the potential for paracelsin production during fermentation of plant material in a cellulosic biofuel facility, and the potential environmental introduction of paracelsin resulting from the use of *T. reesei* in cellulosic material degradation warrants further investigation. Even before interest in using *T.*

reesei for biofuels, Brückner et al. (1984) came to a similar conclusion years ago with other uses of *Trichoderma,* and it was Brückner and Graf (1983) that first isolated paracelsin. Brückner et al. (1984) stated "With regard to the most intensive efforts for a biotechnological application of *Trichoderma* – both in submerged and solid-substrate fermentation – for the enzymatic conversion of cellulosic materials used for animal feed or human food and, moreover, the contribution of these microfungi and their relatives to the moldiness, spoilage and deterioration of organic matter, the excretion of paracelsin and related metabolites should be seriously taken into account. These mycotoxins exhibit a great variety of biological activity and are sometimes produced in extraordinarily high amounts".

B. Environmental Hazards

1. Hazards to Animals

T. reesei is not pathogenic to animals, and there are no reports in the literature suggesting it can cause infection or disease in animals. In a summary of the literature Klein and Eveleigh (1998) stated that some of the secondary metabolites produced by some *Trichoderma* spp. such as glioviridin (a diketopiperazine), sesquiterpenoids, trichothecenes, cyclic peptides, and isocyanide-containing metabolites (trichoviridin) may be inhibitory to organisms other than microorganisms. Some of these compounds may result in ill-thrift of sheep due to their inhibitory action on cellulolytic rumen microflora (Brewer et al., 1982). It is known that paracelsin, the peptaibol produced by *T. reesei*, is inhibitory to Gram-positive bacteria such as *Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus, Staphylococcus faecalis, Streptococcus lactis,* and *Streptococcus thermophilus* (Brückner and Graf, 1983). Brückner et al. (1984), in a discussion of the potential effects of paracelsin, stated "the increase of the digestion of starch and cellulose in rumen liquor by the addition of low concentrations of paracelsin should be considered in the discussion of the association of metabolites from *Trichoderma* with the ill-thrift of pasture fed ruminants, and may be explained by the suppression of various feed rivals of the cellulolytic activ(e) microorganisms".

Paracelsin has been shown to exhibit toxicity to mammalian cells. Data from mice and rat studies were previously presented in the human health effects section. The toxicity of paracelsin to aquatic species has also been directly tested. Solfrizzo et al. (1994) studied the toxicity of paracelsin and other compounds extracted from six *Trichoderma* species to *Artemia salina* (brine shrimp) larvae. Paracelsin was shown to have an LC_{50} (concentration that is lethal to 50% of the organisms) of 2.2 µM which was equivalent to approximately 4 µg/ml (Solfrizzo et al., 1994). The toxicity of the extracts of five of the six *Trichoderma* strains to *A. salina* could be attributed to the production of paracelsin by these strains, whereas in *T. harzianum,* toxicity was attributed to an unidentified peptaibol(s).

Favilla et al. (2006) tested the toxicity of various secondary metabolites of

biocontrol fungi to both *Artemia salina* and *Daphnia magna* (water flea). The three peptaibols tested included alamethicin, paracelsin, and antiamoebin, the first two of which are produced by some *Trichoderma* species. The paracelsin used in these studies was a mixture of paracelsin homologs (purity 88.4%) purchased from a chemical supply company. With 24 hr. exposure to *A. salina*, the LC_{50} of paracelsin was 21.26 µM (equivalent to 40.84 µg/ml). The LC_{50} decreased with 36 hr. exposure to 9.66 µM (equivalent to 18.56 µg/ml). One of the most toxic fungal compounds was alamethicin, which is produced by some *Trichoderma* species including all the members of the *Trichoderma brevicompactum* clade that previously included strains of *T. viride* (Degenkolb et al., 2008a). For comparison, the LC_{50} of alamethicin was 5.32 µM (10.43 µg/ml) and 1.96 µM (3.84 µg/ml) for 24 and 36 hr. exposure, respectively, so it is approximately four to five times more toxic to brine shrimp than paracelsin. However, paracelsin was about twice as toxic to brine shrimp as gliotoxin, an actual mycotoxin produced by several fungi such as *Aspergillus, Penicillium,* and *Trichoderma.* In experiments with *D. magna,* the LC_{50} of paracelsin was 7.70 µM (equivalent to 14.79 µg/ml) with a 24 hr. exposure, and 5.60 µM (equivalent to 10.76 µg/ml) with a 36 hr. exposure (Favilla et al., 2006). These LC_{50} values for *D. magna* are considered moderately toxic. Favilla et al. (2006) stated that the biological activity of peptaibols is a result of their ability to form pores in lipid membranes by forming gated ion channels. The pores lead to the loss of osmotic balance from leakage.

Poirier et al. (2007a) reported that peptaibols and peptaibol-producing *Trichoderma* were detected in marine sediments that exhibited a high toxicity to mussel larvae from a shellfish farming area in France. Peptaibols were also detected in the marine bivalves. Poirier et al. (2007b) conducted bioassays with oyster (*Crassostrea gigas*) embryos with alamethicin and different groups of peptaibols that were produced by a strain of *T. longibrachiatum* that was isolated from the mussels. They found that peptaibols of both long and short sequence disturbed embryogenesis of oysters at nanomolar concentrations. Toxicity of the peptaibols was between 1.3 and 8 times greater than the toxicity induced by copper which was used for reference, and the longer peptaibols were found to be more toxic than the shorter ones. The toxicity was suggested to be associated with the membrane-modifying properties of peptaibols wherein they interact with biological membranes and modify their permeability by forming voltage-dependent membrane ion channels through which cytoplasmic contents can leak out (Poirier et al., 2007b).

In an earlier study, Sallenave et al. (1999) examined the experimental bioaccumulation in filter-feeding mollusks of fungal “mycotoxins”, which were later referred to as peptaibols rather than mycotoxins by Poirier et al. (2007b). Sallenave et al. (1999) exposed mussels to culture filtrates of *Trichoderma koningii* that was previously isolated from cockles (*Cerastiderma edule*). The toxic metabolites bioaccumulated in the mussels, and the digestive gland of the mussels had the greatest toxicity indicating that contamination was from filtration. There are no reports in the literature of bioaccumulation of peptaibols by aquatic species in the environment.

However, it was not until 2007 when peptaibols were actually detected in the environment in marine sediments and in shellfish (Poirier et al., 2007a).

In a review of peptaibiotics and mycotoxins in the genera *Trichoderma* and *Hypocrea,* Degenkolb et al. (2008b) commented that the high toxicity reported for the peptaibols by Poirier et al. (2007b) (alamethicin in oysters) and Favilla et al. (2006) (alamethicin, paracelsin, and others in *A. salina* and *D. magna*) in aquatic invertebrate studies may be a result of contamination of the alamethicin used in these experiments. Degenkolb et al. (2008b) suggested that the alamethicin standard used in these studies (Sigma-Alrich A-4665) may have been contaminated with the trichothecene mycotoxin harzianum A which was shown to be the case in some of their own studies. Harzianum A has been shown to be highly toxic to *A. salina*. However, this possible explanation for the toxicity of alamethicin in these two studies has no relevance to the toxicity values of paracelsin to brine shrimp or *D. magna* presented by Favilla et al. (2006), nor on the toxicity values of paracelsin to brine shrimp reported by Solfrizzo et al. (1994).

Landreau et al. (2002) speculated that the neurotoxicity observed with peptaibols from *T. koningii* was related to the amphiphilic nature of the molecules that form pores in phospholipid membranes. In addition to pore formation, peptaibols have been shown to uncouple oxidative phosphorylation of mitochondria, stimulate catechol secretions of adrenal cells, activate membrane-bound enzymes, and impart cytotoxicity, neurotoxicity, and neuroleptic effects (as summarized in Poirier et al., 2007b; Szekeres et al., 2005).

2. Hazards to Plants

Trichoderma reesei is not a pathogen of plants. Although it is capable of degrading cellulose and hemicellulose due to the copious quantities of these enzymes it can produce, it is not a primary colonizer on plant tissue. Genetic studies have shown that it does not contain any genes for ligninases that are required for initial breakdown of plant material (Maheshwari, 2008). *T. reesei* is known as a wood rot fungus, but it apparently attacks decaying plant material, not live plants. This species does not appear to pose a hazard to plants.

3. Effects on Other Organisms

As previously mentioned, peptaibols, including paracelsin, are toxic to Gram-positive bacteria and to some fungi. Peptaibols are apparently not inhibitory to Gram-negative bacteria, and it assumed that this is a result of the differences in the cell walls of Gram-positive and Gram-negative bacteria. Paracelsin has also been shown to be inhibitory to the fungus *Phoma destructiva* (Grigoriev et al., 2003). They measured differences in membrane pore formation by a number of peptaibols produced by a number of different fungi including paracelsin isolated from an unspecified *Trichoderma* sp. The 19-20mer peptaibols such as alamethicin F-30, paracelsin A, and chrysospermin C all had very high membrane activity, and the larger peptaibols

exhibited higher activity by several orders of magnitude compared with the smaller compounds, the 11-17mer peptaibols. Watts et al. (1988) reported that two metabolites produced by *T. reesei,* trichodermin, and another compound designated as Tx, were inhibitory to species of *Aspergillus, Cladosporium, Fusarium, Rhizoctonia,* and *Pythium*. However, as mentioned previously, the identity of that fungus as *T. reesei* has been questioned. Other studies have investigated the biocontrol potential of *T. reesei.* Harjono and Widyastuti (2001) found that high concentrations of an endochitinase produced by *T. reesei* lysed the hyphae of the plant pathogenic fungus *Ganoderma philipii*. Seidl et al. (2004) reported that the teleomorph of *T. reesei, H. jecorina,* was antagonistic to the plant pathogen *Pythium ultimatum* in plate confrontation tests. Apparently *T. reesei* can be antagonistic to other fungi, but it has not been developed commercially as a biocontrol agent because its antifungal activity is inferior to that of other strains of the genus.

As previously mentioned, some species of *Trichoderma,* specifically *T. aggressivum, T. pleuotrophilum,* and *T. fulvidum* are pathogens of mushrooms, which of course, are fungi. However, *T. reesei* is not a pathogen of mushrooms.

4. Stability of Peptaibols in the Environment

There is no information in the literature on the stability of the paracelsin molecule in the environment. There is little literature on the stability of other peptaibols either, but it is known that peptaibols form helical structures in phospholipid membranes. In a paper on the structure-functional relationship of model α-aminoisobutyric acid peptides, Higashimoto et al. (1999) stated that " the presence of α-aminoisobutyric acid, because of its preference for certain dihedral angles most suitable for 3_{10} – or α-helical structures, gives a particular stability to their conformations in membranes in a variety of environments".

Poirier et al. (2007a) were the first to detect the presence of peptaibols produced by *Trichoderma* in the environment, in marine sediments. Their isolation of peptaibols from the bivalve mussels cultivated in those sediments led them to state that theirs was "the first observation of contamination of the marine human-food chain by fungal metabolites". The fact that they could still detect peptaibols in sediments samples that had been frozen for years led Poirier et al. (2007a) to suggest that peptaibols could be considered as stable markers for fungal presence in the marine environment.

Song et al. (2006) described the stability of secondary metabolites produced by *T. koningii* called trichokonins, which they referred to as peptaibol-like antimicrobial compounds. These trichokonins were referred to as peptaibols, rather than just peptaibol-like compounds, in a review article by Samuels et al. (2006). They are also considered to be peptaibols by others as they are included in the peptaibol database (http://www.cryst.bbk.ac.uk/peptaibol/home.shtml). Trichokonins also contains the non-proteinogenic α-aminoisobutyric acid as do peptaibols. Song et al. (2006) reported that

trichokonins were inhibitory to Gram-positive bacteria and a number of plant pathogenic fungi. To evaluate their stability to temperature, a solution of the antimicrobial compounds dissolved in buffer was heated for 30 minutes at 50, 70, 90, or 100°C, and was also subjected to autoclaving. In addition, the solution was stored at 4°C for 10 days, at -20°C for 30 days, and the frozen solution was then lyophilized. The trichokonins were found to be extremely heat resistant, with no loss of activity at any temperature tested, including autoclaving at 121°C and 15 psi for 15 minutes. Cold storage and lyophilization did not affect their activity either. Their biological activity was retained over a pH range of 3.0 to 10.0 as well. The sensitivity of trichokonins to proteolytic enzymes such as trypsin, neutral protease, basic protease, and multiplex protease was also tested by Song et al. (2006). The trichokonins were insensitive to treatment with proteolytic enzymes, and the majority of their antimicrobial activity was retained.

5. Environmental Hazard Conclusions

The issues for environmental hazards are similar to those for human health hazards. The primary hazard concern for *T. reesei* is for the production of paracelsin, the moderately toxic secondary metabolite. Under usual conditions of submerged culture for standard enzyme production, well-established commercial strains of this species do not seem to produce paracelsin. However, fungal secondary metabolites can be produced after extended fermentation, particularly when sporulation occurs after depletion of nutrients. It has also been demonstrated that these peptaibol compounds may be produced in the presence of an insoluble carbon source such cellulose. Therefore, its growth on plant material as proposed for on-site production of enzymes in cellulosic biofuel facilities by NREL (Wooley et al., 2009; Kazi et al., 2010) may potentially result in the production of significant quantities of paracelsin in the fermentation broth. Large quantities of paracelsin could be possible with extended fermentations and/or the absence of exponential growth (i.e., the stationary growth phase) in the presence of a surplus of carbon with a shortage of nitrogen, conditions that are known to stimulate the formation of secondary metabolites by fungi.

There are two issues warranting consideration with the use of *T. reesei* cells in cellulosic biofuel plants. One is the potential for cells of *T. reesei* to escape containment, and *T. reesei* is expected to survive in the environment if inadvertently released. However, the releases expected from closed system use are expected to be low. It is unknown whether *T. reesei* would produce any quantity of paracelsin in the environment that would be of any concern. However, there needs to be a consideration of the concentrations of paracelsin that could be released in wastewater from a cellulosic biofuel facility. It is unknown whether paracelsin, if it was formed, would be denatured, with a resulting loss of toxicity, at some point in the process stream, such as with high temperatures used for ethanol distillation. It is also not known whether paracelsin would be rapidly degraded during wastewater treatment, or whether it could

possibly remain intact in the stable α-helical form known to occur in membranes. There are no data in the literature on the degradation of peptaibols in the environment, except for the one paper of Poirier et al. (2007a) that reported the persistence of peptaibols in marine sediments and stability of the compounds for years when in frozen sediments. Extraordinary stability was observed for the peptaibols known as trichokonins, as extremes in temperature, including autoclaving, and freezing followed by lyophilization, did not denature the compounds. The compounds also functioned over a wide pH range. In addition, they also were fairly resistant to attack by trypsin and proteases as judged by the retention of their antimicrobial activity. It is unknown if paracelsin would be similarly resistant to temperature extremes, pH levels, or to enzymatic degradation as are the trichokonin peptaibols.

IV. EXPOSURE ASSESSMENT

Estimates of occupational exposure and releases of the microorganism from an enzyme fermentation facility with the resulting exposures to the environment and to the general human population are given below. Note that these numbers have been revised since the worker exposure and environmental release and exposure estimates used in the risk assessments of the original ten eligible recipient microorganisms listed at §725.420 in the Final Microbial Biotechnology Rule promulgated in 1997. Information gleaned from Microbial Commercial Activity Notices received over the years since the rule was published has allowed for better estimates of exposures using a generic scenario (Chemical Engineering Branch, 2011). For this generic scenario, it is assumed that a large-scale closed system enzyme fermentation plant operates 24 hours per day for 350 days/yr and that there are 100 batches/yr.

A. Worker Exposure

No data were available for assessing the releases and exposures specifically for enzyme fermentation facilities using *T. reesei.* Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated using information available from a number of submissions to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms. These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing, and decontamination procedures. A typical fermentation facility has a total of 36 workers with four workers per shift involving fermentation, four workers per shift for recovery operations, three workers per shift for laboratory procedures, one supervisory worker, all with three shifts per day.

The National Institute for Occupational Safety and Health (NIOSH) has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Typically, area monitoring data would not be used to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. However, personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous microbial biotechnology submissions. Applying these NIOSH data to the workers in the various plant areas (laboratory, fermentation, recovery, control room) yields a range of potential inhalation exposures of 81 to 9800 cfu/day. The uncertainty associated with this estimated range of exposures is not known.

The potential dermal exposure for workers in the various plant areas ranges from 6.0×10^6 to 1.8×10^7 cfu/day. However, the typical personal protection equipment (PPE) used in these types of facilities (i.e., gloves and lab coats) is expected to be adequate to protect against dermal exposure to the microorganism. In the unlikely event that dermal exposure was to occur, unbroken skin is considered to be a protective barrier to entry of the microorganism into the body. Even in the event of dermal contact with broken skin, there is little risk of infection because this microorganism is not pathogenic to humans.

B. Releases and Environmental and General Population Exposure

Estimates of the number of *T. reesei* cells released during typical enzyme production are tabulated in Table 1. The uncontrolled/untreated scenario assumes no control features for the fermentor off-gases, and no inactivation of the fermentation broth for the liquid and solid waste releases. The containment criteria required for the full exemption scenario assume the use of features or equipment that minimizes the number of viable cells in the fermentor off-gases. They also assume inactivation procedures resulting in a validated 6-log reduction of the number of viable microorganisms in the liquid and solid wastes relative to the maximum cell density of the fermentation broth.

Table 1. Estimated Numbers of Viable *T. reesei* Cells Released During Production

Release Media	Uncontrolled/ Untreated (cfu/day)	Full Exemption (cfu/day)	Release (days/yr)
AIR			
Fermentor exhaust	2×10^5	$< 2 \times 10^5$	350
Rotary Drum Filter	7×10^5	$< 7 \times 10^5$	100
WATER - to WWT			
Fermentor cleaning	1.4×10^{13}	1.4×10^7	100
LAND - to landfill			
Filter cakes	7×10^{14}	7×10^8	100

These are "worst-case" estimates for releases from the facility that assume a maximum cell density in the fermentation broth of 10^7 cfu/ml for fungi, a fermentor size of 70,000 liters, and a separation efficiency for the rotary drum filter of 99 percent. For the Full Exemption there is also a release to wastewater treatment (WWT) 100 days/yr of 6.9×10^5 cfu/day each from treatment of fermentor and rotary drum exhausts.

1. Releases to Air

Specific data which indicate the survivability of *T. reesei* in the atmosphere after release are currently unavailable. Survival of vegetative cells during aerosolization is typically limited due to stresses such as shear forces, desiccation, temperature, and UV light exposure. Human exposure may occur via inhalation if the organisms are dispersed in the atmosphere attached to dust particles, or lofted through mechanical or air disturbance.

Air releases from fermentor off-gas and rotary drum filtration could potentially result in nonoccupational inhalation exposures due to point source releases. To estimate exposures from these sources, the sector averaging form of the Gaussian algorithm described in Turner (1970) was used. For purposes of this assessment, a release height of 3 meters and downward contact at a distance of 100 meters were assumed. Assuming that there is no removal of organisms by controls/equipment for aerosol releases, the potential human inhalation dose rates are estimated to be < 1 cfu/yr from both the fermentor off-gas and from the rotary drum filtration exhaust, and less than that for systems meeting the full exemption criteria. It should be noted that

these estimates represent hypothetical exposures under reasonable worst case conditions.

2. Releases to Water

The concentrations of *T. reesei* in surface water were estimated using stream flow values for water bodies receiving process wastewater discharges from facilities within SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals). The surface water release values (cfu/day) tabulated in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/l). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Dischargers (IFD) database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers facilities that have a NPDES permit to discharge to surface water). Tenth percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10-year period. The use of this methodology to estimate concentrations of *T. reesei* in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by any component of the treatment process. Estimated concentrations of *T. reesei* in surface water for the uncontrolled/untreated and the full exemption scenarios are tabulated in Table 2.

TABLE 2. *T. reesei* Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	7Q10	Mean	7Q10
Uncontrolled/Untreated				
10th Percentile	156	5.60	8.97×10^{4}	2.50×10^{6}
50th Percentile	768	68.13	1.82×10^{4}	2.05×10^{5}
Full Exemption				
10th Percentile	156	5.60	8.97×10^{-2}	2.50
50th Percentile	768	68.13	1.82×10^{-2}	2.05×10^{-1}

*MLD = million liters per day

C. Release and Exposure Summary

Although direct monitoring data are unavailable, worst-case estimates do not suggest high levels of exposure of *T. reesei* to either workers or the public resulting from typical enzyme fermentation operations.

D. Survival of the Organism in the Environment

The species *T. reesei* is known only from the single original isolate QM6a from the Solomon Islands in the 1940s. Therefore, there is little information on its prevalence or behavior in the environment. However, microcosm studies conducted on *T. reesei* suggests that it would survive in the environment. Providenti et al. (2004) studied the survival of a derivative of QM6a marked with a recombinant hygromycin-B (HygB) resistance gene, designated as QM6a#4, added to laboratory contained intact soil-core microcosms containing three different textured soils. In one of those soils, survival was also tested with or without a plant rhizosphere (bush lima beans, *Phaseolus limensis*). Soil cores were incubated at 22℃ and were sampled periodically over a 4-month period. Then the soil cores were subjected to a simulated winter treatment (approximately 2 weeks at 4℃, 3 weeks at -20℃, and 2 weeks at 4℃), and then returned to 22℃ for another 2 weeks. The levels and viability of *T. reesei* were determined by quantitative polymerase chain reaction (PCR) of total soil DNA extracts and by dilution-plating of soil on a semiselective growth medium, respectively. QM6a#4 was added at a rate of 2.0 ml of inocula (consisting of 0.35 g of wet fungal mycelia per ml that had been processed at high speed in a Waring blender) onto the top 1 cm of the soil core. In Soil 1 (sandy loam), QM6a#4 numbers decreased over the first 2 - 3 months (40- to 170-fold for the soil with or without a plant rhizosphere, respectively), after which a steady state was reached. In Soil 2, (medium texture, soil only), the decrease in QM6a#4 was observed within the first month of incubation (80-fold) after which a steady state was reached. In Soil 3 (clay loam, soil only), no clear trend was apparent, but there was an approximate 4-fold decrease observed by day 114.

After the cores were subjected to the simulated overwinter treatment, QM6a#4 levels did not change in any of the three soils without the plant rhizosphere. In the one soil with the bean rhizosphere (Soil 1), there was a statistically significant approximately 3-fold increase in QM6a#4 levels, reflecting a “bloom” associated most likely with an increase in nutrients from the decaying dead plant roots after thawing of the cores. In all three soils, QM6a#4 remained viable and culturable, even after the simulated winter treatment.

From this data given in Providenti et al. (2004) above, it appears that even though *T. reesei* was originally isolated from a tropical climatic region, it is able to persist in soils for extended periods of time. *T. reesei* survived in soil even after colder temperatures of a simulated overwintering treatment of 4℃ for 2 weeks, -20℃ for 3 weeks, followed by 4℃ for 2 weeks. In addition to mere persistence, fungal growth

actually occurred after the winter treatment in the soil with the bean rhizosphere, presumably due to the nutrient release from the dead roots.

V. INTEGRATION OF RISK

The fungus *T. reesei* has a long history of safe use in submerged standard industrial fermentation for enzyme production with food, feed, pharmaceuticals and industrial uses. It is not pathogenic to humans, animals, or to plants. The fungus has been shown to be nonpathogenic to healthy rats in a number of acute and chronic pathogenicity tests. The enzyme fermentation industry routinely tests for toxin production in their enzyme products, and *T. reesei* has not been associated with mycotoxin production during typical enzyme fermentation conditions. Acute and chronic toxicity tests on a number of enzymes for which GRAS petitions were made to the FDA have demonstrated the lack of toxicity of *T. reesei* enzymes such as cellulase, xylanase, endoglucanase, glucanase, protease, and pectin lyase. The use of *T. reesei* in submerged standard industrial fermentation poses low risk. *T. reesei* has a long history of safe use in enzyme production and is a good candidate for the list of eligible recipient microorganisms in the Tiered Exemptions if used for production of enzymes under the conditions of submerged standard industrial fermentation.

However, *T. reesei* is not an appropriate recipient microorganism for the 5(h)4 Tiered Exemptions for any other closed system use or under conditions unlike those used in standard submerged enzyme fermentation. *T. reesei* is known to produce a secondary metabolite known as paracelsin in submerged fermentation in the presence of insoluble substrates such as cellulose. Cellulose is, of course, the main component of plant material, and in some form or other, the substrate of choice for biofuel production. Cellulosic biofuel facilities pre-treat various types of plant material such as sugarcane, switchgrass, corn stover, wood chips, etc. with acid or other chemicals to initiate breakdown of the lignin in the plant material. Then, the fungus might actually be grown on the plant hydrolysate in the seed fermentor, which would then be transferred to a cellulase fermentor, for on-site enzyme production. One approach would be to subsequently transfer both the broth containing high levels of cellulases and live cells to the saccharification tank wherein the cellulases would breakdown the cellulose in the pretreated plant material into sugars which could then be fermented into ethanol.

Given this knowledge that an insoluble substrate such as cellulose could stimulate peptaibol synthesis, there are a number of different scenarios to consider. The first of which is whether paracelsin would actually be produced in a cellulosic biofuel facility in the plant biomass breakdown step, and if so, at what levels. It is possible that paracelsin would not be produced in submerged fermentation even in the presence of plant material, particularly if other conditions known to be conducive to peptaibol formation such as the use of older cultures, exposure to light, exposure to stress such as nitrogen limitation, and sporulation are all avoided. It is known that, in

general, that production of secondary metabolites in fungal liquid culture usually occurs late in the growth stage, i.e., the stationary phase, after exponential growth has ceased and when there is a nitrogen limitation in the presence of excess carbon substrates. These conditions are known to cause fungal metabolism to shift into production of secondary metabolites.

If in fact paracelsin was produced in the plant biomass breakdown tank, the ethanolic fermentation step following degradation of the plant material into sugars could be inhibited because paracelsin is inhibitory to the growth of other fungi, and a yeast (a fungus) is typically used for the ethanolic conversion step. However, a particular yeast, *Candida*, studied by Brückner and Graf (1983) was not inhibited by paracelsin, so it is not known whether the particular yeasts used for ethanol production would be inhibited. If paracelsin was produced at high concentrations with the growth of *T. reesei* on the plant material rather than using purified enzymes void of the fungus, the manufacturer may find that efficient ethanol fermentation was not possible. However, microorganisms other than yeast, such as the Gram-negative bacteria *Escherichia coli* or *Zymonomas mobilis,* could alternatively be used as the ethanologen. Gram-negative microorganisms would not be inhibited by paracelsin, so paracelsin production could possibly go undetected.

Another scenario for consideration is that the production of paracelsin in the fermentation tanks used for plant material breakdown may be inconsequential if at some other point in the ethanol production process the compound is denatured. It is possible that the high temperatures (78°C) used for ethanol distillation could break down the peptide. However, other peptaibols have been shown to retain their biological activity after high temperature treatment. It is unknown if paracelsin would be denatured or whether it would retain its conformation and biological activity. Another possibility is that if paracelsin is not degraded during the latter stages of the ethanol production process, it could be broken down during wastewater treatment of the spent fermentation broth, and thus, would not enter the environment. However, there is no information available in the literature that addresses the potential breakdown of paracelsin in a wastewater treatment system, nor is there information on the breakdown or persistence of paracelsin in the environment. There is some indication that other peptaibols appear to be relatively stable due to formation of α-helical structures resulting from the presence of the α-aminoisobutyric acid which favors that structure. There is one article in the literature that detected peptaibols in marine sediments that were causing toxicity to shellfish. The peptaibols were apparently rather persistent in the environment, and the authors suggested that peptaibols could be used as stable markers for the presence of *Trichoderma* fungi in the marine environment (Poirier et al., 2007a). There is also the one article on the stability of peptaibol-like compounds produced by *Trichoderma koningii* called trichokonins that also contain high proportions of α-aminoisobutyric acid and have antimicrobial properties like other peptaibols. These trichokonins, which now are recognized as peptaibols, were found to be stable at high temperatures, up to 100°C, and even after autoclaving. They retained their biological activity after low

temperature treatment as well, including freezing followed by lyophilization, and over a pH range of 3.0 to 10.0. They were also found to be resistant to enzymes such as trypsin and other proteases that normally break down proteinaceous materials (Song et al., 2006).

In the absence of any data or literature specifically on paracelsin and its degradation or persistence in the environment, the consequences of paracelsin exposure to environmental receptors must be considered. At the far extreme, it is possible that large quantities of paracelsin could be produced with the growth of *T. reesei* on plant material, and that paracelsin would not be denatured/inactivated at any other point in the ethanolic fermentation process stream. Under this scenario, paracelsin could adversely affect the functioning of wastewater treatment by inhibition of growth of Gram-positive bacteria in the POTW or on-site treatment facility with disposal of liquid wastes. There is no information available in the literature on whether paracelsin would be rapidly broken down in the wastewater treatment system, or whether the molecule is resistant to proteases or other enzymes present in wastewater. If the paracelsin remains intact through wastewater treatment, then the potential consequences of exposure of paracelsin to the environment must be considered.

Recommendations

T. reesei (QM6a and its derivatives) is recommended as an eligible recipient microorganism for the 5(h)4 Tiered Exemption when used with submerged standard industrial fermentation conditions for enzyme production where no solid material is present in the fermentation broth. However, in the absence of data on potential production of paracelsin, *T. reesei* is not recommended for an exemption from review when used in closed systems under conditions unlike those encountered in submerged standard enzyme fermentation, such as the growth directly on plant material.

VI. REFERENCES

Abu-Raya., S., V. Trembovier, E. Shohami, and P. Lazarovici. 1993. Cytolysins increase intracellular calcium and induce eicosanoids release by pheochromocytoma PC12 cell cultures. Natural Toxins 1:263-270.

Antal, Z., J. Varfa, L. Kredics, A. Szekeres, L. Hatvani, L. Manczinger, C. Vágvölgyi, and E. Nagy. 2006. Intraspecific mitochondrial DNA polymorphism within the emerging filamentous fungal pathogen *Trichoderma longibrachiatum.* J. Medical Microbiol. 55:31-35.

Askolin, S., T. Nakari-Setala, and M. Tenkanen. 2001. Overproduction, purification and characterization of the *Trichoderma reesei* hydrophobin HFBI. Appl. Microbiol. Biotechnol. 57:124-130.

Atanasova, L., W.M. Jaklitsch, M. Komoń-Zelazowska, C.P. Kubicek, and I.S. Druzhinina. 2010. Clonal species *T. parareesei* sp. nov. likely resembles the ancestor of the cellulase producer *Hypocrea jecorina/T. reesei*. Appl. Environ. Microbiol. 76:7259-7267.

Avcin, J. 2011. Engineering Assessment for *Trichoderma reesei* for the 5(h)4 Tier Exemptions. Unpublished. U. S. Environmental Protection Agency, Washington, DC.

Benitez, T., A.M. Rincon, M.C. Limon, and A.C. Codon. 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. 7(4):249-260.

Blumenthal, C. 2004. Production of toxic metabolites of *Aspergillis niger, Aspergillis oryzae,* and *Trichoderma reesei*: Justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Reg. Tox. Pharmacol. 39:214-228.

Bokari, F. 2009. Efficacy of some *Trichoderma* species in the control of *Rotylenchulus reniformis* and *Meloidogyne javanica*. Archives Phytopathology and Plant Protection 42:361-369.

Brewer, D., A. Feicht, A. Taylor, J. W. Keeping, A. A. Taha, and V. Thaller. 1982. Ovine ill-thrift in Nova Scotia. 9. Production of experimental quantities of isocyanide metabolites of *Trichoderma hamatum*. Can. J. Microbiol. 28:1252-1260.

Brewer, D, F.G. Mason, and A. Taylor. 1987. The production of alamethicins by *Trichoderma* sp. Can. J. Microbiol. 33:619-625.

Brückner, H. and H. Graf. 1983. Paracelsin, a peptide antibiotic containing α-aminoisobutyric acid, isolated from *Trichoderma reesei* Simmons. Experientia 39:528-530.

Brückner, H., H. Graf, and M. Bokel. 1984. Paracelsin; characterisation by NMR spectroscopy and circular dichroism, and hemolytic properties of a peptaibol antibiotic from the cellulolytical active mold *Trichoderma reesei* . Part B. Experientia 40:1189-96.

Chemical Engineering Branch. 2011. Generic scenario for fungal releases from an enzyme fermentation facility. Unpublished. U. S. Environmental Protection Agency, Washington, DC.

Degenkolb, T., T. Grafenhan, A. Berg, H.I. Nirenbrg, W. Gams, and H. Brückner. 2006. Peptabiomics: Screening for polypeptide antibiotics (peptaibiotics) from plant-protective *Trichoderma* species. Chemistry & Biodiversity 3:593-610.

Degenkolb, T., R. Dieckmann, K.F. Nielsen, T. Grafenhan, C. Thies, D. Zafari, P. Chaverri, A. Ismaiel, H. Brückner, H. von Dorhe, U. Thrane, O. Petrini, and G.J. Samuels. 2008a. The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibols, and mycotoxins. Mycol. Progress 7:177-219.

Degenkolb, T., H. von Dorhen, K.F. Nielsen, G.J. Samuels, and H. Brückner. 2008b. Recent advances and future prospects in peptaibiotics, hydrophobin, and mycotoxin research, and their importance for chemotaxonomy of *Trichoderma* and *Hypocrea.* Chem. Biodiversity 5:671-680.

Druzhinina I., M. Komoń-Zelazowska, L. Atanasova, V. Seidl, C. Kubicek. 2010. Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (anamorph *Trichoderma reesei*) and a new sympatric agamospecies related to it. PLoS ONE 5(2):e9191

Druzhinina, I.S., M. Komoń-Zelazowska, L. Kredics, L. Hatvani, Z. Antal, T. Belayneh, and C.P. Kubicek. 2008. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. Microbiol. 154:3447-59.

Druzhinina I. S., A.G. Kopchinskiy, M. Komoń, J. Bissett, G. Szakacs, and C. P. Kubicek. 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet. Biol. 42: 813–828.

Enriquez-Matas, A., S. Quirce, N. Cubero, J. Sastre, and R. Melchor. 2009. Hypersensitivity pneumonitis caused by *Trichoderma viride*. Archivos de Bronconeumologia 45:304-305.

ETA. 2010. Letter from the Enzyme Technical Association to EPA, Re: Response to request for additional information regarding the application to list *Trichoderma reesei* as

a recipient microorganism eligible for exemption under 40 CFR Part 725, Subpart G; relating to the potential production of paracelsin by the organism during enzyme manufacture. Letter from Anthony T. Pavel to Greg Schweer, August 24, 2010.

Eveleigh, D. E. 1985. *Trichoderma*. *In*: A.L. Demain and N.A. Solomon (eds.). Biology of Industrial Microorganisms. Chapter 16, pp. 487-509. Benjamin/Cummings Publishing Co., Menlo Park, CA.

Favilla, M., L. Macchia, A. Gallo, and C. Altomare. 2006. Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina* and *Daphnia magna*) invertebrate assays. Food Chem. Toxicol. 44:1922-31.

Gams, W. and J. Bissett. 1998. Morphology and identification of *Trichoderma*. *In*: C.P. Kubicek and G.E. Harman (eds.). *Trichoderma* and *Gliocladium*. Volume 1. Basic Biology, Taxonomy, and Genetics. Taylor and Frances Inc. Bristol, PA, pp. 3-34.

Grigoriev, P.A., B. Schlegel, M. Kronen, A. Berg, A. Harti, and U. Grafe. 2003. Differences in membrane pore formation by peptaibols. J. Peptide Sci. 9(11-12):763-68. Published online 5 Nov 2003, Wiley Interscience.

GRN No. 32. Röhm Enzyme GmbH Kirschenallee 45; D-64293 Darmstadt, GERMANY. Pectin lyase derived from *Trichoderma reesei* carrying a gene encoding pectin lyase from *Aspergillus niger*. Sept, 29, 1999.

GRN No. 315. Genencor, a Danisco Division, Palo Alto, CA. 2009. Transglucosidase enzyme preparation derived from *Trichoderma reesei* expressing the transglucosidase gene from *Aspergillus niger.* Dec. 4, 2009.

GRN No. 333. Genencor, a Danisco Division, Palo Alto, CA. 2010. Acid fungal protease enzyme preparation from *Trichoderma reesei* expressing the gene encoding acid fungal protease from *T. reesei*. March 26, 2010.

Halpin, D.M., B.J. Graneek, M. Turner-Warwick, and A.J. Taylor Newman. 1994. Extrinsic allergic alveolitis and asthma in a sawmill worker: case report and review of the literature. Occup. Environ. Med. 51:160-164.

Haran, S., H. Schickler, and I. Chet. 1996. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum.* Microbiol. 142:2321-31.

Harjono and S.M. Widyastuti. 2001. Antifungal activity of purified endochitinase produced by biocontrol agent *Trichoderma reesei* against *Ganoderma philippii.* Pakistan J. Biological Sciences 4:1232-34.

Harman, G.E., C.R. Howell, A. Viterbo, I. Chet, and M. Lorito. 2004. *Trichoderma*

species -- opportunistic, avirulent plant symbionts. Microbiol. 2:43-56.

Higashimoto, V., H. Kodama, M. Jelokhani-Niaraki, F. Kato, and M. Kondo. 1999. Structure-function relationship of model Aib-containing Peptides as ion transfer intermembrane templates. J. Biochem. 125:705-712.

Hjortkjaer, R.K., V. Billie-Hansen, K.P. Hazelden, M. McConville, D.B. McGregor, E. Chapman, J.R. Gardner, and R. Ashby. 1986. Safety evaluation of Celluclast®, an acid cellulase derived from *Trichoderma reesei*. Fd. Chem. Toxic. 24:55-63.

Hou, C.T., A. Ciegler, and C.W. Hesseltine. 1972. New mycotoxin, trichotoxin A, from *Trichoderma viride* isolated from southern leaf blight-infected corn. Appl. Microbiol. 23:183-185.

Jaklitsch, W. 2009. European species of *Hypocrea* Part I. The green-spored species. Studies in Mycology 63:1-91 (http:www.studiesinmycology.org) doi:10.3114/ sim.2009.63.01.

JEFCA. 1981. Joint FAO/WHO Expert Committee on Food Additives. Specifications for identity and purity of carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavoring agents, food colours, sweetening agents and other food additives, FAO Food and Nutrition Paper 19, p. 214.

Jones-Brown, Y. 2011. TSCA uses and the history of safe use of *Trichoderma reesei* for the 5(h)4 Tier Exemptions. Unpublished. U. S. Environmental Protection Agency, Washington, DC.

Kar, S. A. Mandal, P.K. das Mohapatra, K.C. Mondal, and B.R. Pati. 2006. Production of cellulase-free xylanase by *Trichoderma reesei* SAF3. Brazilian J. Microbiol. 37:462-464.

Kazi, F.K., J. Fortman, R. Anex, G. Kothandaraman, D. Hsu, A. Aden, and A. Dutta. 2010. Techno-Economic Analysis of Biochemical Scenarios for Production of Cellulosic Ethanol. Technical Report NREL/TP-6A2-46588, June 2010.

Klein, D. and D.E. Eveleigh. 1998. Ecology of *Trichoderma*. In: C.P. Kubicek and G.E. Harman (eds.). *Trichoderma* and *Gliocladium*. Volume 1. Basic Biology, Taxonomy, and Genetics. Taylor and Frances Inc. Bristol, PA, pp. 57-74.

Komoń-Zelazowska, M., J. Bissett, D. Zafari, L. Hatvani, L. Manczinger, S. Woo, M. Lorito, L. Kredics, C.P. Kubicek, and I.S. Druzhinina. 2007a. Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide. Appl. Environ. Microbiol. 73:7415-26.

Komoń-Zelazowska, M., T. Neuhof, R. Dieckmann, H. von Döhren, A. Herrera-Estrella, C.P. Kubicek, and I.S. Druzhinina. 2007b. Formation of atroviridin by *Hypocrea atroviridis* is conidiation associated and positively regulated by blue light and the G protein GNA3. Eukaryotic Cell 6:2332-2342.

Kredics, L., Z. Antal, A. Szekeres, L. Hatvani, L. Manczinger, C. Vágvölgyi, and E. Nagy. 2005. Extracellular proteases of *Trichoderma* species. A Review. Acta Microbiol. Immunol. Hung. 52:169-84.

Kubicek, C.P. and G.E. Harman. 1988. Preface. *In*: C.P. Kubicek and G.E. Harman (eds.). *Trichoderma* and *Gliocladium*. Volume 1. Basic Biology, Taxonomy, and Genetics. Taylor and Frances Inc. Bristol, PA. pp. ix.

Kubicek, C.P., M. Komoń-Zelazowska, E. Sándor, and I.S. Druzhinina. 2007. Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*. Chemistry & Biodiversity – Vol. 4, pp.1068-1082.

Kuhls, K. E. Lieckfeldt, G.J, Samuels, W. Kovacs, W. Meyers, O. Petrini, W. Gams, T. Borner, and C.P. Kubicek. 1996. Molecular evidence that the asexual fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc. Natl. Acad. Sci. 93:7755-7760.

Kuhls**,** K., E. Lieckfeldt, T. Börner, and E. Guého. 1999. Molecular re-identification of human pathogenic *Trichoderma* isolates as *Trichoderma longibrachiatum* and *Trichoderma citrinoviride.* Medical Mycology 37:25-33.

Landreau, A., Y.F. Pouchus, C. Sallenave-Namont, J.-F. Biard, M.-C. Boumard, T.R. du Pont, F. Mondeguer, C. Goulard, and J.-F. Verbist. 2002. Combined use of LC/MS and a biological test for rapid identification of marine mycotoxins produced by *Trichoderma koningii*. J. Microbiol. Methods 48:181-194.

Lieckfeldt, E., C. Kullnig, G.J. Samuels, and C.P. Kubicek. 2000. Sexually competent, sucrose- and nitrate-assimilating strains of *Hypocrea jecorina* (*Trichoderma reesei*) from South American soils. Mycologia 92:374-380.

Lübeck, M., S.K. Poulsen, P.S. Lübeck, D.F. Jensk, and U. Thrane. 2000. Identification of *Trichoderma* strains from building materials by ITS1 ribotyping, UP-PCR fingerprinting and UP-PCR cross hybridization. FEMS Microbiology Letters 185:129-134.

Lynch, D. 2011. Exposure assessment of *Trichoderma reesei* for the 5(h)4 Tier Exemptions. Unpublished. U. S. Environmental Protection Agency, Washington, DC.

Maheshwari, R. 2008. Genome sequence of a fungus for the biofuel industry. Current Sci. 95(2):161-162.

Malmos, H. 1977. Enzyme applications in food, pharmaceutical, and other industries: Industrial applications of cellulase, American Institute Chem. Engineers Symposium Series 74:93-99.

Mandels, M. and E.T. Reese. 1957. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J. Bacteriol. 73:269-278.

Mandels, M. J. Weber, and R. Parizek. 1971. Enhanced cellulase production by a mutant of *Trichoderma reesei*. Appl. Microbiol. 21:152-154.

Martinez, D., R.M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S.E. Baker, J. Chapman, O. Chertkov, P.M. Coutinho, D. Cullen, E.G.J. Danchin, I.V. Grigoriev, P. Harris, M. Jackson, C.P. Kubicek, C.S. Han, I. Ho, and L.F. Larrondo, A.L. Leon, J.K. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robbertse, A.A.Salamov, M. Schmoll, A. Terry, N. Thayer, A. Westerholm-Parvinen, C.L.Schoch, J. Yao, R. Barabote, M.A. Nelson, C.Detter, D. Bruce, C.R. Kuske, G. Xie, P. Richardson, D.S. Rokhsar, S.M. Lucas, E.M. Rubin, N. Dunn-Coleman, M. Ward, T.S. Brettin. 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nature Biotechnol. 26:553-560.

McClung, G. 2011. Ecological hazard assessment of *Trichoderma reesei* for the 5(h)4 Tier Exemptions. Unpublished. U. S. Environmental Protection Agency, Washington, DC.

Meyer, C.E. and F. Reusser. 1967. A polypeptide antibacterial agent isolated from *Trichoderma viride.* Specialia 15:85-86.

Mohamed-Benkada, M., M. Montaqu, J.F. Baird, F. Mondequer, P. Verite, M. Dalgalarrondo, J. Bissett, and Y.F. Pouchus. 2006. New short peptaibols from a marine *Trichoderma* strain. Rapid Commun. Mass Spectrom. 20(8);1176-1180.

Montenecourt, B.S. and D.E. Eveleigh. 1977. Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. Appl. Environ. Microbiol. 34:777-782.

Morawetz, R. F. Granz, R. Messner, and C.P. Kubicek. 1992. Presence, transcription and translation of cellobiohydrolase genes in several *Trichoderma* species. Current Genetics 21:31-6.

Mukherjee, P., A. West, N. Ruiz, A. Keighttley, M.E. Moran-Diez, K. McCluskey, Y.F. Pouchus, and C.M. Kenerley. 2010. Two classes of new peptaibols are synthesized by a single non-ribosomal peptide synthetase of *Trichoderma virens.* J. Biological

Chemistry 286:4544-4554. Published on-line Dec 1, 2010 (http://www.jbc.org/cgi/doi/10.1074/jbc.M110.159723).

Nevalainen, H., P. Suominen, and K. Taimisto. 1994. On the safety of *Trichoderma reesei*. J. Biotechnol. 37:193-200.

Olempska-Beer, Z., R.I. Merker, M.D. Ditto and M.J. DiNovi. 2006. Food-processing enzymes from recombinant microorganisms – a review. Regulatory Toxicology Pharmacology 45:144-158.

Ooka, T., Y. Shimojima, T. Akimoto, I. Takeda, S. Senoh, and J. Abe. 1966. A new antibacterial peptide "Suzukacillin". Agr. Biol. Chem. 30:700-702.

Pfaller, M. and D.J. Diekema. 2010. Epidemiology of invasive mycoses in North America. Critical Rev. Microbiol. 36:1-53.

Poirier, L., M. Montagu, A. Landreau, M. Mohamed-Benkada, O. Grovel, C. Sallenave-Namont, J.-F. Biard, C. Amiard-Triquet, J.-C. Amiard, and Y.F. Pouchus. 2007a. Peptaibols: Stable markers of fungal development in the marine environment. Chem. & Biodiversity 4:1116-1128.

Poirier, L., F. Quiniou, N. Ruiz, M. Montagu, J.-C. Amiard, and Y.F. Poucheus. 2007b. Toxicity assessment of peptaibols and contaminated sediments on *Crassostrea gigas* embryos. Aquatic Toxicology 83:254-62.

Providenti, M.A., S.I. Mautner, O. Chaudhry, M. Bombardier, R. Scroggins, E. Gregorich, and M.L. Smith. 2004. Determining the environmental fate of a filamentous fungus, *Trichoderma reesei,* in laboratory-contained intact soil-core microcosms using competitive PCR and viability plating. Can. J. Microbiol. 50:623-631.

Przybylski, M., I. Dietrich, I. Manz, and H. Brückner. 1984. Elucidation of structure microheterogeneity of the polypeptide antibiotics paracelsin and trichotoxin A-50 by fast atom bombardment mass spectrometry in combination with selective *in situ* hydrolysis. Biomed. Mass Spectrom. 11: 569–582.

Ritieni, A, V. Fogliano, D. Nanno, G. Randazzo, C. Altomare, G. Perrone, A. Bottalico, L. Maddau, and F. Marras. 1995. Paracelsin E, a new peptaibol from *Trichoderma saturnisporum*. J. Nat. Prod. 58(11):1745-8.

Roodsari, F.N., D. Zafari, S.A. Khodaparast, and H. Rouhani. 2007. Introducing some new species of *Trichoderma* for Iran. Rostaniha 8:14-18.

Ruiz, N. G. Wielgosz-Collin, L. Poirier, O. Grovel, K.E. Petit, M. Mohamed-Benkada, T.R. du Pont, J. Bissett, P. Verite, G. Barnathan, and Y.F. Pouchus. 2007. New

trichobrachins, 11-residue peptaibols from a marine strain of *Trichoderma longibrachiatum.* Peptides 28: 1351-1358.

Sallenave, C., Y.F. Pouchus, M. Bardouil, P, Lassus, M.-F. Roquebert, and J.-F. Verbist. 1999. Bioaccumulation of mycotoxins by shellfish: Contamination of mussels by metabolites of a *Trichoderma koningii* strain isolated in the marine environment. Toxicon 37:77-83.

Samuels, G., S.L. Dodd, W. Gams, L.A. Castlebury, and O. Petrini. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia 94:146-170.

Samuels, G., S.L. Dodd, B.-S. Lu, O. Petrini, H.-J. Schroers, and I.S. Druzhinina. 2006. The *Trichoderma koningii* aggregate species. Studies in Mycology 56:67-133.

Samuels, G.J., O. Petrini, K. Kuhls, E. Lieckfeldt, and C.P. Kubicek. 1998. The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *longibrachiatum.* Studies in Mycology 41:1-54.

Schirmböck, M. Lorito, Y.-L. Wang, C.K. Hayes, I. Arisan-Atac, F. Scala, G.E. Harman, and C.P. Kubicek. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against pathogenic fungi. Appl. Environ. Microbiol. 60:4364-4370.

Segal, M. 2011. Taxonomy of *Trichoderma reesei* for the 5(h)4 Tier Exemptions. Unpublished. U.S. Environmental Protection Agency, Washington, DC.

Seidl, V., M. Schmoll, B. Scherm, V. Balmas, B. Seiboth, Q. Migheli, and C.P. Kubicek. 2004. Use of *Hypocrea jecorina* (anamorph *Trichoderma reesei*) as a model system for *Trichoderma* biocontrol of *Pythium* blight identifies new targets for genetic strain improvement. J. Zhejiang University 30(4):404.

Seidl, V., C. Seibel, C. Kubicek, and M. Schmoll. 2009. Sexual development in the industrial workhorse *Trichoderma reesei.* Proc Natl. Acad. Sci. 106:13909-13914.

Simmons, E.G. 1977. Classification of some cellulase-producing *Trichoderma* species, p. 618. Abstract of the Second International Mycological Congress, Tampa, FL.

Solfrizzo, M., C. Altomare, A. Visconti, A. Bottalico, and G. Perrone. 1994. Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. Nat. Toxins 2(6):360-5.

Song, X.Y., Q.-T. Shen, S.-T. Xie, X.-L. Chen, C.-Y. Sun, and Y.-Z. Zhang. 2006.

Broad spectrum antimicrobial activity and high stability of trichokonins from *Trichoderma koningii* SMF2 against plant pathogens. FEMS Microbiol. Letters260:119-125.

Song, X.Y., S.-T. Xie, X.-L. Chen, C.-Y. Sun, and Y.-Z. Zhang. 2007. Solid-state fermentation for trichokonins production from *Trichoderma koningii* SMF2 and preparative purification of trichokonin VI by a simple protocol. J. Biotech. 131:209-215.

Sun,Y., L. Tian, Y.-F. Huang, Y. Sha, and Y.-H. Pei. 2006. A new cyclotetrapeptide from marine fungus *Trichoderma reesei.* Pharmazie 61:809-810.

Sun, Y., L. Tian, J. Huang, H.-Y. Ma, Z. Zheng, A.-L. Lv, K. Yasukawaw, and Y.-H. Pei. 2008. Trichodermatides A-D, novel polyketides from the marine-derived fungus *Trichoderma reesei*. Organic Letters 10:393-396.

Szekeres, A., B. Leitgeb, L. Kredics, A. Antal, L. Hatvani, L. Manczinger, and C. Vagvolgyi. 2005. Peptaibols and related peptaibiotics of *Trichoderma*. A review. Acta Microbiol. Immunol. Hung. 52(2):137-68.

Tang, P., S. mohan, L. Sigler, I. Witterick, R. Summerbell, I. Campbell, and T. Mazzulli. 2003. Allergic fungal sinusitis associated with *Trichoderma longibrachiatum.* J. Clin. Microbiol. 41:5333-36.

Taylor, J.W., D.J. Jacobson, S. Kroken, T. Kasuga, D.M. Geiser, D.S. Hibbett, and M.C. Fisher. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31:21-32.

Toyoma, N. 1969. Applications of cellulase in Japan. *In*: R.F. Gould (ed.). Cellulases and Their Application. Advances in Chemistry Series 95, American Chemical Society, Washington, DC, pp. 359-390.

Turner, D.B. 1970. Workbook of Atmospheric Dispersion Estimates. PHS Publication No. 999-AP-26. U.S. Department of Health, Education, and Welfare, National Air Pollution Control Administration, Cincinnati, OH.

Verma, M., S.K. Brar, R.D. Tyagi, R.Y. Surampalli, and J.R. Valero. 2007. Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. Biochem. Engineering J. 37:1-20.

Vizcaino, J.A., R.E. Cardoza, L. Dubost, B. Bodo, S. Gutierrez, and E. Monte. 2006. Detection of peptaibols and partial cloning of a putative peptaibol synthetases gene from *Trichoderma harzianum* CECT 2413. Folia Microbiol. 51:114-120.

Ward, R. 2011. Human health effects of *Trichoderma reesei* for the 5(h)4 Tier Exemptions. Unpublished. U.S. Environmental Protection Agency, Washington, DC.

Watts, R., J. Dahiya, K. Chaudhary, and P. Tauro. 1988. Isolation and characterization of a new fungal metabolite of *Trichoderma reesei*. Plant and Soil 107:81-84.

Wei, C.-M., B.S. Hansen, M.H. Vaughan Jr., and C.S. McLaughlin. 1974. Mechanisms of action of the mycotoxins trichodermin, a 12,13-epoxytrichothecene. Proc. Natl. Acad. Sci. 71:713-717.

Woo, S.L., F. Scal, M. Ruocco, and M. Lorito. 2006. The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. Phytopatholgy 96:181-5.

Wooley, R., M. Ruth, J. Sheehan, K. Ibsen, H. Majdeski, and A. Galvez. 1999. Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis Current and Future Scenarios. Report No. NREL/TP-580-26157. National Renewable Energy Laboratory, Golden, CO.

Wösten, H.A. 2001. Hydrophobins: Multipurpose proteins. Ann. Rev. Microbiol. 55: 625-646.

Appendix 10

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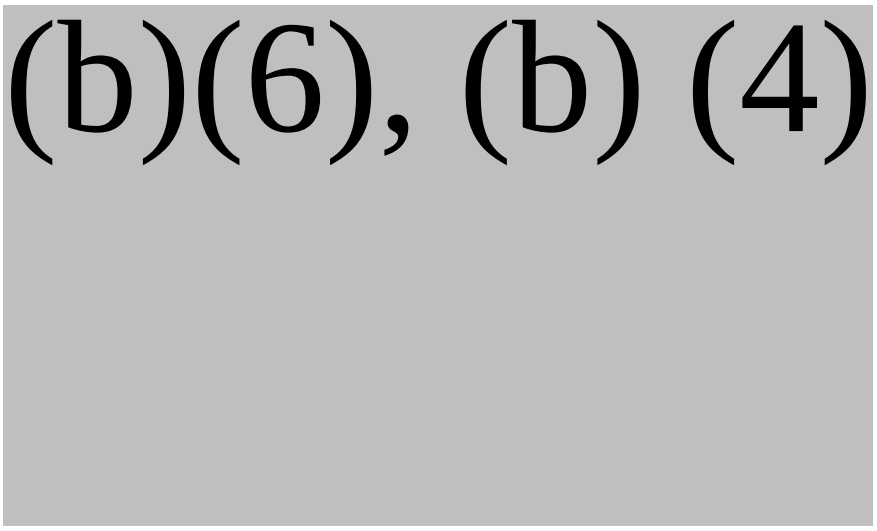
(b)(4)

CONFIDENTIAL

TOXIN HOMOLOGY DATABASE SEARCH

(b) (4)

(b) (4)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Appendix 11

Appendix 12

Appendix 13

Appendix 14

Michael W. Pariza (b)(4) LLC
(b)(6)
Michael W. Pariza, Member

September 21, 2020

Vincent Sewalt, Ph.D.
Head of Regulatory Science & Advocacy
Danisco US, Inc (Operating as DuPont Nutrition & Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

RE: GRAS opinion on the intended use of DuPont's *variant consensus bacterial phytase* produced by a non-pathogenic, non-toxigenic strain of *Trichoderma reesei*

Dear Dr. Sewalt,

I have critically reviewed and evaluated the information you provided on DuPont's *variant consensus bacterial phytase*, presented in a dossier prepared by Danisco US Inc. (operating as DuPont Nutrition and Biosciences), dated August 26, 2020, entitled, "Phytase Enzyme Preparation from *Trichoderma reesei* Expressing a Gene encoding a Variant of Consensus Bacterial Phytase is Generally Recognized As Safe for Use as an Animal Food Ingredient for Swine and Poultry".

The *variant consensus bacterial phytase* exhibits a high degree of similarity (83.3%) to a native *Buttiauxella* sp. phytase amino acid sequence. The *variant consensus bacterial phytase* also contains sequence motifs from other bacterial phytases, the purposes of which are to increase the enzyme's thermostability and improve its activity at low pH. *Trichoderma reesei* strain RL-P37, the strain from which the production strain for *variant consensus bacterial phytase* is derived, was also the host strain for the *Buttiaxella* sp variant phytase previously reviewed by CVM and currently listed by AAFCO as an approved source of phytase (AAFCO, 2020). The *variant consensus bacterial phytase* is intended for use as an additive for swine and poultry feed, to increase the bioavailability of dietary phosphate.

In evaluating the use of the *variant consensus bacterial phytase* as an additive for swine and poultry feed, I considered the biology of *T. reesei* and its history of safe use in food-grade enzyme manufacture; safety evaluation studies on the *variant consensus bacterial phytase* enzyme produced by its *T. reesei* production strain; safety evaluation studies on other food grade enzymes expressed by DuPont's safe lineage of *T. reesei* production strains; the information that you provided regarding the safe lineage of the production organism, protein engineering techniques, cloning methodology,

manufacturing materials and procedures, and product specifications; and information available in the peer-reviewed scientific literature.

Trichoderma reesei is used widely by enzyme manufacturers worldwide for the production of enzyme preparations that are, in turn, used in human food, animal feed, and numerous industrial enzyme applications. DuPont's lineage of safe *T. reesei* production strains, including the *T. reesei* production strain for the *variant consensus bacterial phytase*, was derived through a series of modifications from *T. reesei* QM6a, the original non-pathogenic and non-toxigenic wild-type parental strain used to produce this safe lineage of *T. reesei* enzyme production strains. Published literature, government laws and regulations, for example FR 64:28658-28362 (1999), reviews by expert panels such as FAO/WHO JECFA (1992), and DuPont's (legacy Genencor and Danisco) unpublished safety studies, all support the conclusion that the lineage to which these production strains belong is safe and suitable for use in the manufacture of food-grade and feed-grade enzymes.

Strains within this safe lineage are used to manufacture many food and feed enzymes including chymosin, transglucosidase, β-glucanase, glucoamylase, α-amylase, α-glucosidase, β-glucosidase/cellulase, other cellulases, acid fungal protease, lipase, xylanase and phytase. The enzyme products from 21 production strains within this safe lineage, and in two cases production strains themselves, have been subjected to toxicology testing and rigorous safety evaluation in accordance with the Pariza-Johnson and Pariza-Cook decision trees (MW Pariza and EA Johnson. *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology 33: 173-186, 2001; MW Pariza and M Cook. *Determining the safety of enzymes used in animal feed*, Regulatory Toxicology and Pharmacology 56: 332–342, 2010). Some of these enzymes are also the subjects of GRAS notification documents that are listed on the CFSAN GRAS Notice Inventory, specifically GRN 230, 315, 333, 372, 567, 703, 727, and 808, all of which carry the decision statement, "FDA has no questions."

The primary structure of the *variant consensus bacterial phytase* was analyzed for sequences associated with protein/peptide toxins using the UniProt Annotated Protein Knowledge Database (December 2019 version). The results of this analysis indicated that the *variant consensus bacterial phytase* does not share homology with any known toxin sequence.

The *variant consensus bacterial phytase* was assessed for subchronic toxicity and genotoxicity. A 90-day repeated dose study using Sprague-Dawley rats (ten animals/sex/group, treated by oral gavage at doses of 0, 250, 500 and 1000 mg TOS/kg bw/day) was conducted to establish a No Observed Adverse Effect Level (NOAEL) for the Margin of Safety calculations. (The genotoxicity studies were conducted to satisfy regulatory requirements outside the USA, not to support GRAS status because genotoxicity is not an issue for enzymes-- MW Pariza and EA Johnson. *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology 33: 173-186, 2001; MW Pariza and M Cook. *Determining the safety of enzymes used in animal feed*, Regulatory Toxicology and Pharmacology 56: 332–342, 2010). No dose-related adverse events were observed in any of these studies.

The NOAEL was established as the highest dose of the *variant consensus bacterial phytase* tested in the 90-day repeated dose study: 1000 mg TOS/kg bw/day, equivalent to 450,000 FTU/kg bw/day (TOS is the abbreviation for Total Organic Solids; FTU is the abbreviation for phytase unit, defined as the amount of enzyme that will release 1 µmol of inorganic orthophosphate from sodium phytate

per minute at pH 5.5 and 37 °C). For reference, the maximum recommended use level for the *variant consensus bacterial phytase* is (b) (4) TOS/kg feed, equivalent to (b) (4). From these considerations, together with estimates of maximum intake of the enzyme by target species, the Margins of Safety for poultry, piglets and swine, respectively, are 1613, 2857, and 3448, respectively.

The protein engineering methodologies used to construct the *variant consensus bacterial phytase* gene, and the cloning methodologies employed to create the *T. reesei* production strain that expresses this gene, are appropriate for use in the genetic modification of production strains for food and feed ingredient manufacture. (b) (4)

Based on the foregoing, I concur with the evaluation made by DuPont that the *Trichoderma reesei* production strain that expresses the gene encoding the *variant consensus bacterial phytase* is safe and appropriate, based on scientific procedures, to use for the manufacture of food-grade *variant consensus bacterial phytase*.

I also concur with the evaluation made by DuPont that *the variant consensus bacterial phytase* enzyme, manufactured in a manner that is consistent with *current Good Manufacturing Practice* (*cGMP*) and meeting appropriate food-grade specifications, is *GRAS* (*Generally Recognized As Safe*) based on scientific procedures for use as an additive for swine and poultry feed at levels up to (b) (4) mg TOS/kg feed (equivalent to (b) (4)

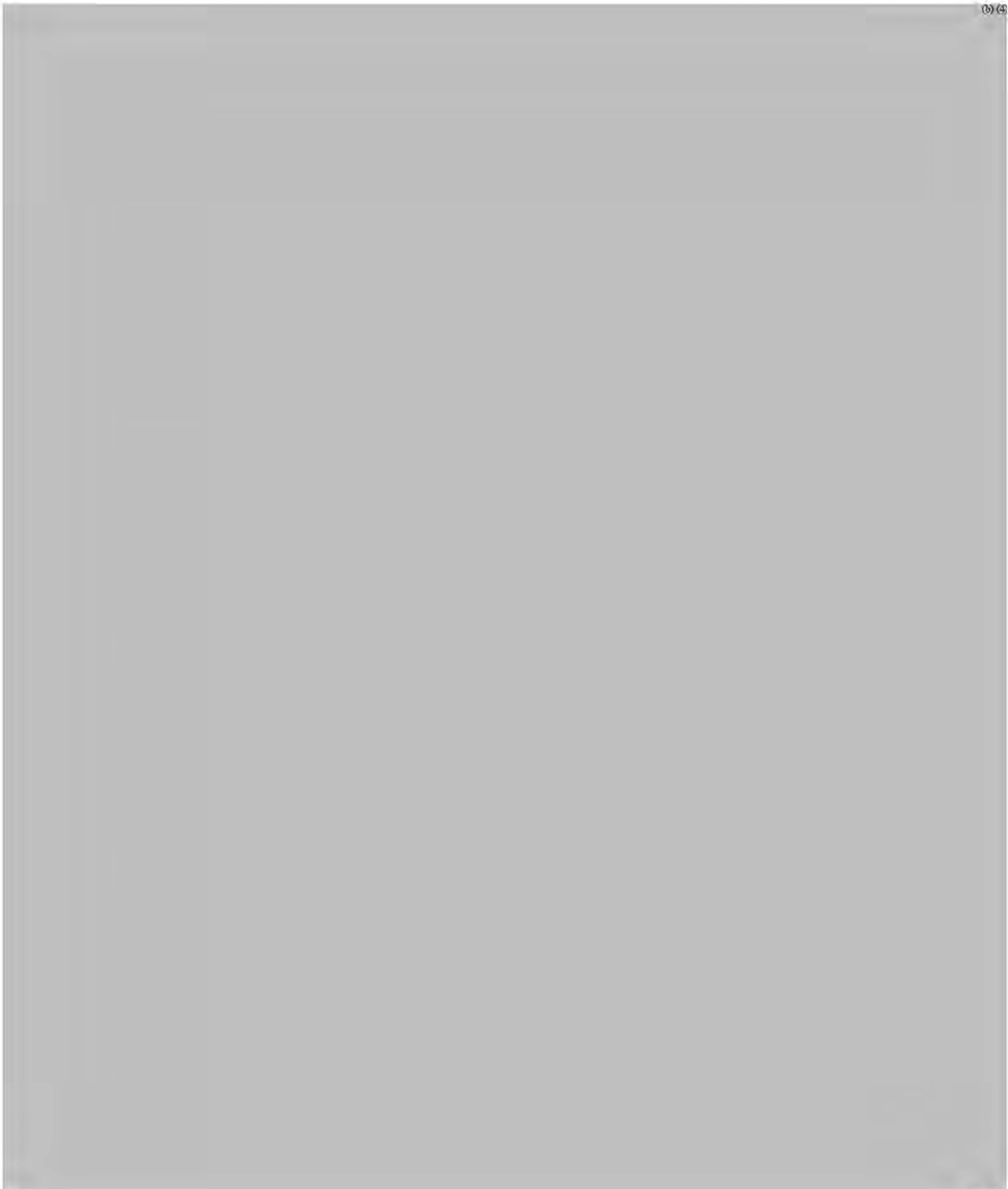
It is my professional opinion that other qualified experts would also concur in these conclusions.

Sincerely,

(b)(6)

Michael W. Pariza
Member, Michael W. Pariza Consulting, LLC
Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

Appendix 15



Appendix 16

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

(b) (4), (b)(6)

Dear (b)(6)

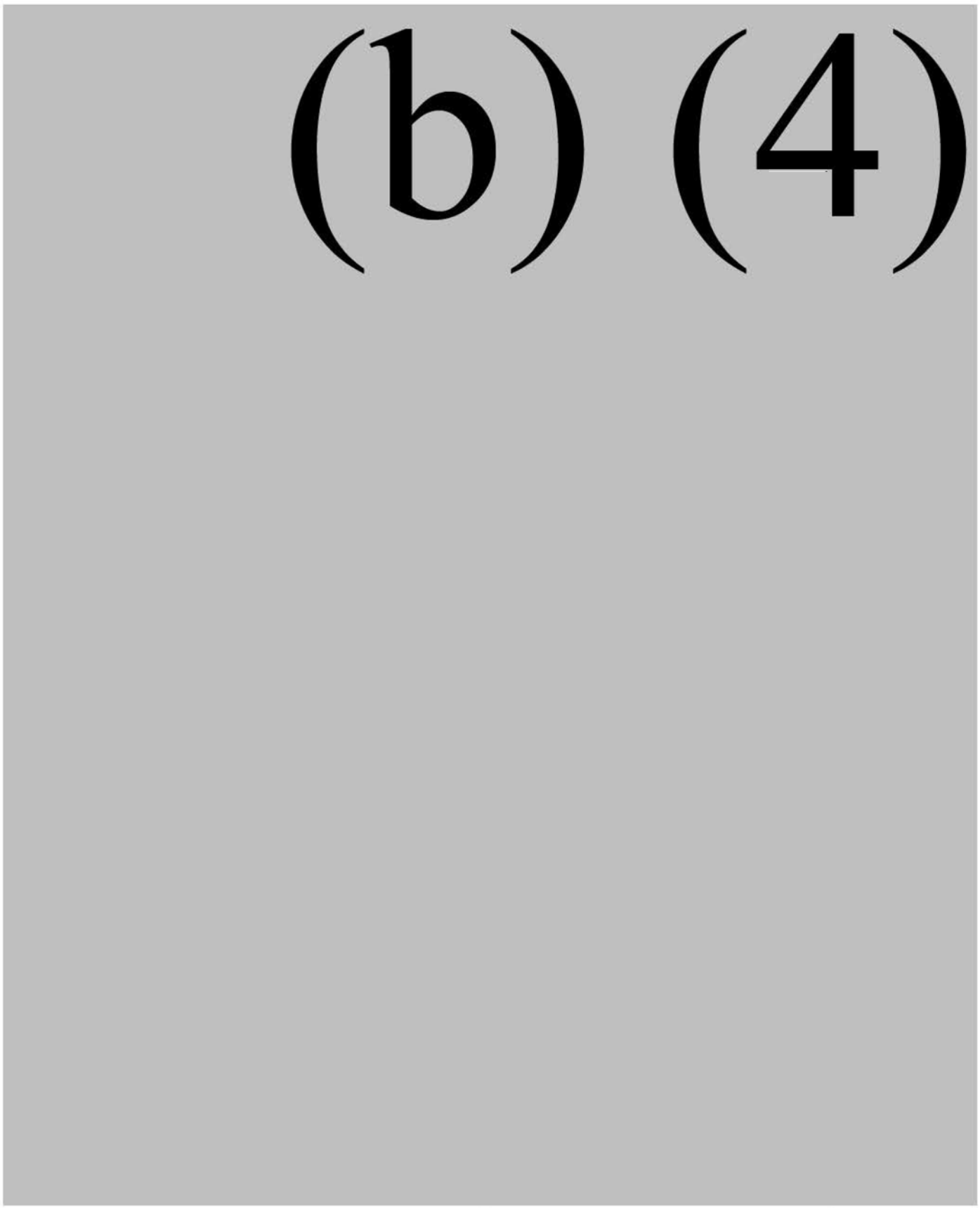
You requested, on behalf of the (b) (4) that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer), and 11-30-99 (to Dr. Zofia Olempska-Beer). You also arranged for a teleconference between (b) (4) members and OFAS representatives, facilitated telephone contacts with technical experts from (b) (4) member companies, and responded to numerous requests for clarification. We appreciate your and (b) (4) cooperation.

We reviewed the information on defoaming and flocculating agents that you submitted as well as the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. The enclosed attachment provides a brief overview of our evaluation and itemizes the evaluated defoamers (Table 1) and flocculants (Table 2). We conclude that these compounds are used by enzyme manufacturers in accordance with the principles of good manufacturing practice (GMP).

Sincerely yours,

(b)(6)

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition


(b) (4)

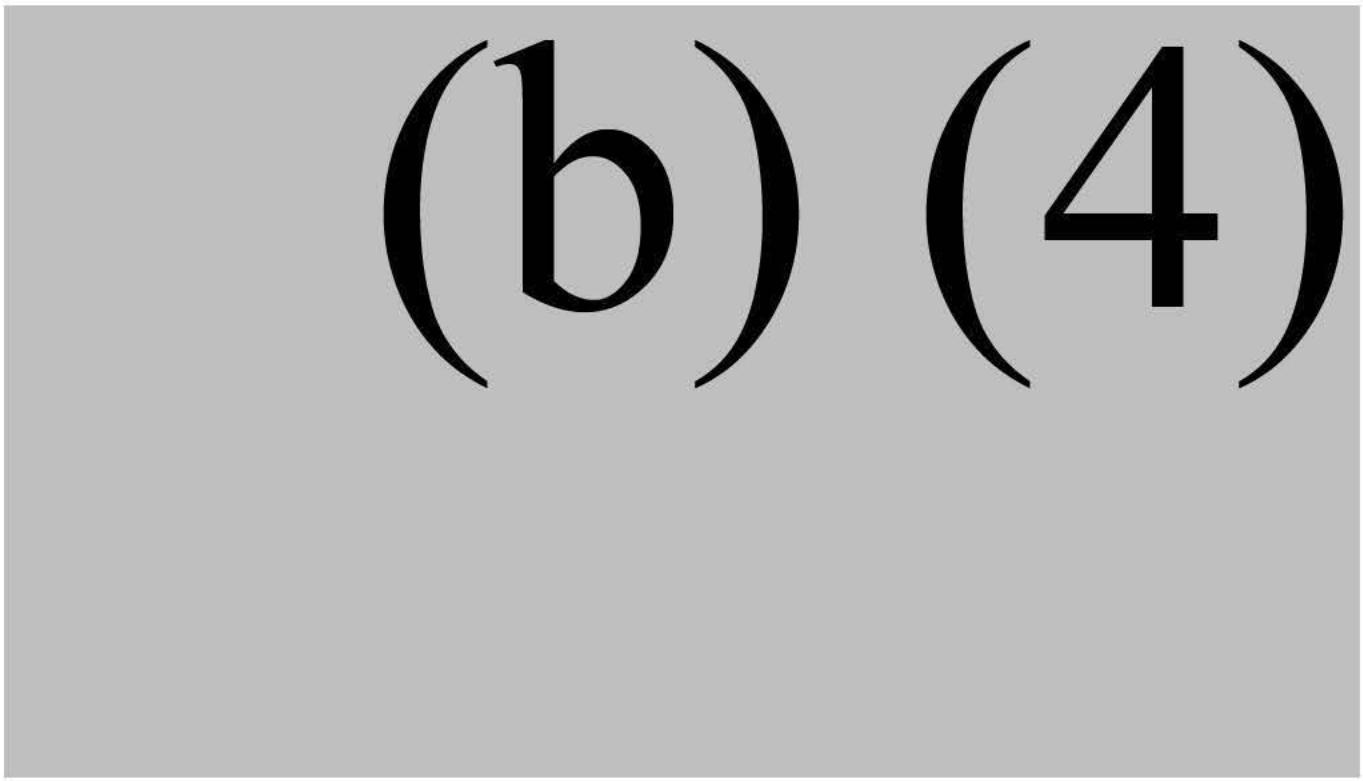

(b) (4)

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
(b) (4)		Average MW: 2000
		Average MW: 2000
		Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations

Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
(b) (4)		Cationic polyamine
		Cationic polyamine
		Cationic polyamine
		Cationic polyacrylamide
		Cationic polyacrylamide
		Anionic polyacrylamide

Appendix 17

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Toxicology Test Summaries

Summary of safety studies on Trichoderma reesei derived enzymes and Trichoderma reesei strains in support of DuPont's Safe Strain Lineage

The safety of 21 DuPont *Trichoderma reesei* strains and 25 enzyme preparations derived from recombinant production strains were assessed in a number of toxicology tests as shown in the table below. All strains tested were found to be non-cytotoxic/pathogenic and all enzyme preparations were found to be non-toxic, non-mutagenic and not clastogenic.

PRODUCTION ORGANISM	ENZYME	TOXICOLOGY TEST	RESULT
1) ***T. reesei*** (traditionally modified)	Cellulase	• 90-day subchronic study, rats	No adverse effects
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
2) ***T. reesei*** (traditionally modified)	Cellulase	• Pathogenicity test, rats	Non-toxigenic, non-pathogenic
		• 91-day subchronic oral toxicity study, rats	No adverse effects detected
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
3) ***T. reesei*** (heterol. rDNA)	Cellulase, Hemicellulase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
		• Local lymphe node assay in mice	No adverse effects
		• 90-day subchronic oral toxicity study rats	No adverse effects

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4) ***T. reesei*** (homol. rDNA)	Endo glucanase I	• Pathogenicity study, rats	Non pathogenic
		• 14-day oral feeding study, rats	No adverse effects
		• 91-day subchronic oral toxicity study rats	No adverse effects
		• *In vitro* chromosome assay, human lymphocytes	Not clastogenic
5) ***T. reesei*** (homol. rDNA)	Endo glucanase II	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
		• 90-day subchronic oral toxicity study rats	No adverse effects
6) ***T. reesei*** (heterol. rDNA)	Chymosin	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes.	Not clastogenic
		• 90-day subchronic oral toxicity study rats	No adverse effects detected
7) ***T. reesei*** (homol. rDNA)	High pI xylanase	• 91-day subchronic oral toxicity study rats	No adverse effects
		• Bacterial reverse mutation assay	Not mutagenic
		• *In vitro* chromosomal aberration assay with Chinese Hamster Ovary (CHO) cells	Not clastogenic
8) ***T. reesei*** (homol. rDNA)	Endo glucanase III	• 28-Day subacute oral toxicity study, rats	No adverse effects
		• Bacterial reverse mutation assay (Ames)	Not mutagenic

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9) ***T. reesei*** (homol. rDNA)	Low pI xylanase	• 91-day subchronic oral toxicity study rats	No adverse effects
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
10) ***T. reesei*** (homol. rDNA)	Xylanase Y5	• 91-day subchronic oral toxicity study rats	No adverse effects
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
11) ***T. reesei*** (heterol. rDNA)	Lipase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic
		• 91-day subchronic oral toxicity study rats	No adverse effects
12) T. reesei (heterol. rDNA)	Phytase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic
		• 91-day subchronic oral toxicity study rats	No adverse effects
13) ***T. reesei*** (heterol. rDNA)	Trans glucosidase / Alpha-glucosidase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes.	Not clastogenic
		• 18-week Oral (Gavage) Toxicity Study in Wistar Rats	No adverse effects

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14) *T. reesei* (homol. rDNA)	Acid Fungal Protease	• 91-day subchronic oral toxicity study rats	No adverse effects
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
15) *T. reesei* (heterol. rDNA)	Glucoamylase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic
		• 90-day subchronic oral toxicity study rats	No adverse effects
16) *T. reesei* (heterol. rDNA)	Catalase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
		• 90-day subchronic oral toxicity study rats	No adverse effects
17) *T. reesei* (homol. rDNA)	Trehalase	• 90-day subchronic oral toxicity study rats	No adverse effects
		• *In vitro* chromosomal aberration assay, human lymphocytes	Not clastogenic
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
18) T. reesei (heterol. rDNA)	Xylanase	• 90-day subchronic oral toxicity study rats	No adverse effects
		• *In vitro* chromosomal aberration assay, Human lymphocytes	Not clastogenic
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
19) *T. reesei* (heterol. rDNA)	Glucoamylase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• I*n vitro* chromosome assay, human lymphocytes	Not clastogenic
		• 90-day subchronic oral toxicity study rats	No adverse effects

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20) *T. reesei* (heterol. rDNA)	Xylanase	• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes	Not clastogenic
		• Repeated dose 90-day oral toxicity in rats	No adverse effects
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes	Not clastogenic
21) *T. reesei* (heterol. rDNA)	Alpha-amylase	• 90-day subchronic oral toxicity study rats	No adverse effects
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* chromosomal aberration assay, Human lymphocytes	Not clastogenic
22) *T. reesei* (heterol. rDNA)	Glucoamylase	• Subchronic toxicity 90-day gavage study in rats	No adverse effects
		• 90-day subchronic oral toxicity study rats	No adverse effects observed
		• Bacterial reverse mutation assay (Ames	Not mutagenic
23) *T. reesei* (heterol. rDNA)	Xylanase	• *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes	Not clastogenic

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24) *T. reesei* (heterol. rDNA)	Phytase	• 90-day subchronic oral toxicity study rats	No adverse effects observed
		• *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes	Not clastogenic
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* EpiOcular™ Eye Irritation Test (EIT)	not considered an eye irritant
		• Local Lymph Node Assay (LLNA)	not considered a contact dermal sensitizer
		• EpiDerm™ Skin Irritation Test (SIT)	considered a non-irritant
		• Acute Oral Toxicity Study (rats)	the acute oral LD50 of Phytase A was greater than 5000 mg/kg
25) *T. reesei* (heterol. rDNA)	Phytase	• 90-day subchronic oral toxicity study rats	No adverse effects observed
		• *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes	Not clastogenic
		• Bacterial reverse mutation assay (Ames)	Not mutagenic
		• *In vitro* EpiOcular™ Eye Irritation Test (EIT)	not considered an eye irritant
		• Local Lymph Node Assay (LLNA)	not considered a contact dermal sensitizer
		• EpiDerm™ Skin Irritation Test (SIT)	considered a non-irritant
		• Acute Oral Toxicity Study (rats)	the acute oral LD50 of Phytase A was greater than 5000 mg/kg

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1. *T. reesei* - cellulase

A. 90-day feeding study, rats (b) (4)

A 13-week dietary feeding study was conducted in rats with the unformulated cellulase concentrate. Three groups of male and female CD rats received cellulase preparation in the diet at 3 concentrations for 90 consecutive days. Body weights and body weight changes were recorded weekly during the study. Hematology, clinical chemistry, urine analysis, necropsy and pathological observation were recorded at study termination.

The only adverse effect noted in this study was a slight increase in urinal acidity and specific gravity noted in males. No evidence of toxicity was noted in this study relative to body weight, body weight changes, organ weights, clinical observation, food consumption, hematology, clinical chemistry, ophthalmology, necropsy and pathological observations up to and including the highest dose tested.

Under the conditions of this experiment, cellulase was non-toxic to rats and the NOEL (No observed effect level) was established at the highest dose.

B. Bacterial reverse mutation assay ((b) (4)).

In a bacterial reverse mutation assay (Ames assay), cellulase was tested at 8, 40, 200, 1000 or 5000 ug/plate for its ability to induce mutation in five *Salmonella* strains and one *Escherichia coli* strain. No positive mutagenic response was observed in both the presence and absence of metabolic activation up to and including the highest dose tested, 5000 ug/plate. Under the conditions of this investigation, cellulase from *Trichoderma reesei* RUT C30 was not mutagenic in the bacterial reverse mutation assay using *Salmonella* and *E. coli* Cellulase in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with Human lymphocytes ((b) (4)).

The clastogenic activity of cellulase from *T.reesei* RUT C30 was investigated in a human peripheral blood lymphocyte (HPBL) assay. Cellulase was tested at 0, 500, 1,667 or 5,000 ul/ml in the absence of metabolic activation and at 0, 1,000, 3,333, or 10,000 ug/ml in the presence of metabolic activation. Whole blood cultures from two donors were treated with cellulase for 3 hours in the presence of S9 and continuously for 24 hours in the absence of S9. No statistical significant increases in chromosome aberrations were noted. Under the conditions of this assay,

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cellulase was not clastogenic to HPBL in both the presence and absence of metabolic activation.

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2. *T. reesei* - Cellulase

A. Pathogenicity study in rats ([redacted]).

This study was conducted in compliance with the Good Laboratory Practice Standards as published by the OECD Guidelines, the US FDA and the US EPA. In this investigation, *Trichoderma reesei* strain A83 was administered as a single intraperitoneal dose of 2.2 x 10^7 cfu to 15 male and 15 female Sprague Dawley rats. Five additional animals per sex were used as the untreated control group. Groups of animals were killed on Days 4, 8, 15, 22 and 32 for microbial enumeration and necropsy observations. No deaths occurred and no adverse clinical observations were noted. No treatment related differences in body weights or body weight changes were observed at any time point, when compared to controls. No abnormal findings were noted at necropsy. By Day 15, *T. reesei* was detected in only the spleen and liver of both males and females and, by Day 22, in only the spleen of both male and female rats. Total clearance of the test microbe from the spleen was achieved by Day 32. Under the conditions of this investigation, *T. reesei* strain A83 was not found to be toxic or pathogenic in rats.

B. 91-day subchronic feeding study in rats ([redacted]).

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of 20 male and female CD rats were fed with 4 different concentrations of the test material in the diet for 13 consecutive weeks.

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* cellulase. Based upon these findings, it was concluded that the treatment of male and female rats with cellulase from *T. reesei* did not result in toxicity at the highest dosage in the diet.

A NOEL (No Observed Effect Level) was established at the highest dosage tested.

C. Bacterial reverse mutation assay (Ames assay) [redacted]).

The test material, a cellulase from *T. reesei* A83 was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation. The assay was conducted in accordance with OECD Guideline 471.

In the first assay, eight dose levels ranging from 2.5 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the

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exposure groups. In the confirmatory assay, dose levels ranging from 75 to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, *T. reesei* cellulase was not a mutagen.

D. *In vitro* chromosomal aberration assay with Human Peripheral Blood Lymphocytes (HPBL) [redacted](b) (4)).

The test material, a cellulase from *T. reesei* A83 was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation. The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels of 625, 1250, 2500 and 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, cellulase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

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3. *T. reesei* – (Hemi)Cellulase

A. Bacterial Reverse Mutation (Ames) Assay. (b) (4) .

The objective of this assay was to assess the potential of Cellulase, Beta-glucosidase, Hemicellulase to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and E.coli strain WP2 uvrA-. The test material was tested both in the presence and absence of a metabolic activation system (rat liver homogenate metabolizing system, S9). In order to select appropriate dose levels for the main test, a preliminary test was conducted. Dose levels from 0-5000 µg total protein/plate were used. The highest dose level tested (5000 µg TP/plate) is the maximum required by the OECD guideline. Subsequently, two main tests were performed. The first main test used 5 dose concentrations (50-5000 µg total protein/plate). Each dose level was assayed in triplicate for each bacterial strain and for each concentration with and without S9 mix using the direct plate incorporation method. The second main test used fresh cultures, test material and control solutions. Dose range was the same as in the first main test, but used the pre-incubation method followed by the direct plate incorporation method. This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the preliminary test, the material was not found to be toxic to the tester strains. In the two main tests, no test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9 mix. No significant increases in the frequency of revertant colonies were observed for any of the bacterial strains, at any dose level, either with or without S9 or exposure method. The positive controls used in the test yielded marked increases in the frequency of revertant colonies, confirming the efficacy of the S9 mix and the sensitivity of the bacterial strains. Under the conditions of this assay, Cellulase, Beta-glucosidase, Hemicellulase was found to be non-mutagenic in the Ames assay.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes. (b) (4)

The objective of this assay was to investigate the potential of Cellulase, Beta-glucosidase, Hemicellulase to induce numerical and/or structural changes in mammalian chromosomes of mammalian systems (human peripheral lymphocytes).Cellulase, Beta-glucosidase, Hemicellulase was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic

activation (rat liver induced with phenobarbitone and b-naphthoflavone; S-9 mix). This assay included a preliminary cytotoxicity test prior to the two main tests to select appropriate dose levels. In the preliminary assay, dose ranges from 19.53-5000 µg/ml were used. Based on the results of this assay, dose levels were then selected for the two main assays with the highest dose producing a 50% reduction in mitotic index (number of cells in mitosis/1000 cells examined). If no cytotoxicity is observed, the highest dose level would be 5000 µg/ml, as recommended by the OECD guideline. The lymphocytes were stimulated to divide by the addition of phytohemagglutinin, or PHA. All dose levels are expressed in terms of µg total protein/ml.

In the preliminary test, cultures were exposed for 4-hours with and without metabolic activation followed by a 20 hour recovery period, as well as continuously for 24 hours without metabolic activation. Dose levels ranged from 19.53-5000 µg/ml. In the main test, Experiment 1 involved a 4 hour exposure to the test material with and without S9-mix (2%) followed by a 20 hour culture in treatment-free media prior to cell harvest. In Experiment 2, cells were exposed to test material continuously for 24 hours in the absence of S9-mix, as well as for 4 hours in the presence of S9-mix (1%) followed by 20-hour culture in treatment free media. Dose range used for both experiments was 312.5-5000 µg/ml. To arrest mitosis, Colcemid at 0.1 µg/ml was added two hours before required harvest time. Cells were then centrifuged, supernatant discarded and cells resuspended in hypotonic KCL. After approximately 14 minutes (including centrifugation), most of the solution was drawn out and discarded. The cells were resuspended and then fixed on slides, stained and scored for chromosomal aberrations: Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1000 cells examined. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main tests.

Metaphase analysis was conducted on at least 100 metaphases per culture dose level. In the absence of S9-mix, mitomycin C (dissolved in minimal essential medium) was used at 0.4 and 0.2 µg/ml for Experiments 1 and 2, respectively. In the presence of S9-mix, cyclophosphamide (dissolved in dimethyl sulphoxide) was used at 5 µg/ml in both experiments. This assay was conducted in accordance with OECD guideline No. 473 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the preliminary assay, the dose range was 19.53 to 5000 µg/ml. A precipitate of test material was observed above 1250 µg/ml in the 24-hour exposure group only. Upon slide examination, exposure cultures showed metaphase cells were present up to 5000 µg/ml in the 4(20) -hour exposure group without S9-mix and in the 24 hour exposure group. An absence of cells and metaphases in the 4(20) hour exposure group with S9-mix at 5000 µg/ml was considered to be due to loss of cell

pellet during harvesting and not as a result of toxicity. Some evidence of toxicity was observed in the 4(20)-hour exposure group in the presence of S9-mix at 2500 µg/ml. The maximum dose level selected for both main experiments was 5000 µg/ml.

In Experiment 1, evaluation of the slides determined there was no apparent toxicity, and at the highest dose level there were metaphases suitable for scoring in both exposure groups. The absence of cells and metaphase at 5000 µg/ml observed in the preliminary assay was not duplicated in this experiment, confirming pellet loss in the preliminary assay. No precipitate was seen in the beginning or end of exposure in either group. The maximum dose level was selected for analysis. The test material did not induce any statistically significant increases in the frequency of cells with aberrations either with or without S9-mix, nor did it induce a statistically significant increase in the numbers of polyploidy cells at any dose level in either exposure group.

In Experiment 2, assessment of the slides determined metaphases suitable for scoring were present at the highest dose level (5000 µg/ml) in both exposure groups (with and without S9-mix). Therefore, the maximum dose level was selected for analysis. No precipitate was seen at the beginning or end of exposure in either exposure group. The test material did not induce any statistically significant increases in the frequency of cells with aberrations either with or without S9-mix, nor did it induce a statistically significant increase in the numbers of polyploidy cells at any dose level in either exposure group. Under the conditions of this assay, Cellulase, Beta-glucosidase, Hemicellulase did not induce chromosomal aberrations (both structural and numerical) in this in-vitro test using human peripheral lymphocytes both in the presence and absence of metabolic activation up to the highest dose level (5000 µg/ml) as recommended by the guidelines. Cellulase, Beta-glucosidase, Hemicellulase is neither a clastogen nor an aneugen.

C. Local Lymph Node Assay in Mice. (b) (4)

The objective of this study was to investigate the potential of Cellulase, Beta-glucosidase, Hemicellulase to induce dermal sensitization in the mouse when administered topically using flow cytometry analysis. A preliminary dermal irritation screening was conducted with 3 groups of 2 mice. Increasing concentrations (25%, 50% and 100%) of Cellulase, Beta-glucosidase, Hemicellulase in DMSO were applied topically to each ear once daily for 3 consecutive days. Ear thickness was measured to determine irritation. No irritation was measured at any concentration; therefore the same doses were used in the main test. In the definitive study, 5 groups of 5 mice each were treated with increasing concentrations of Cellulase,

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Beta-glucosidase, Hemicellulase applied topically to the dorsum of each ear (25%, 50%, 100% Cellulase, Beta-glucosidase, Hemicellulase a positive control with 25% HCA and a negative control of DMSO) once daily for 3 consecutive days. Five days following the initial application and 5 hours prior to sacrifice, all mice received an intraperitoneal injection of BrdU. At sacrifice, auricular nodes were isolated, single cell suspensions of lymph node cells (LNC) were generated and then analyzed by flow cytometry for BrdU incorporation and total number of LNC. A stimulation index (SI) was calculated for each group and test articles that yielded a SI > 3.0 were characterized as sensitizing substances.

This study was conducted in accordance with the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

No deaths occurred during the study. Body weight changes were noted but were not considered significant. Ear thickness measurements and animal observations did not result in excessive local irritation. The group SI values were 1.8, 1.0 and 1.2 for the 25%, 50% and 100% dose concentrations, respectively. Under the conditions of this experiment, Cellulase, Beta-glucosidase, Hemicellulase was not a dermal sensitizer.

D. 90-day Oral Gavage Study in Rats. (b) (4)

The objective of this study was to investigate the potential of Cellulase, Beta-glucosidase, Hemicellulase to induce systemic toxicity after repeated daily oral administration by gavage to Wistar rats of both sexes for 90 consecutive days. Four treatments groups (10 males, 10 females in each group for a total of 80 rats) received a low, medium, high or control dose. Dose levels selected were as follows: control group received 0 mg total protein/kg with 5.0 ml of 0.9% saline/kg; low dose group received 4.5 mg total protein/kg with 4.9 ml of 0.9% saline/kg; medium dose group received 11.25 mg total protein/kg with 4.75 ml of 0.9% saline/kg; high dose group received 56.25 mg total protein/kg with 3.75 ml of 0.9% saline/kg. Corresponding TOS levels in each dose group were 0, 5.14, 12.85 and 64.25 mg/kg, respectively. Animals were housed in groups of 3 or 4 (by same sex) and were kept under controlled temperature, humidity and lightning conditions. Environmental enrichment was provided for and all animals had free access to food (pelleted diet) and water.

All animals were observed for clinical observations immediately prior to dosing, up to 30 minutes post-dosing and 1-5 hours post dosing during the work week (1 hour after dosing on weekends and holidays). Functional/behavioral observations were observed and recorded prior to the start of treatment and at weekly intervals

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thereafter. During Week 12, functional tests were performed on all animals to assess reactions to different stimuli. Water intake was observed daily, and food consumption and body weight was recorded weekly (body weight was also recorded on Day 1 of the study). Ophthalmoscopic examination was performed on high dose group and control animals pre-treatment and prior to termination of treatment (Week 12). At the end of the study (Day 90), hematological and blood chemical investigations were performed for all animals. They were then sacrificed and organs weighed and histopathology examined.

This study was conducted in accordance with OECD guideline No. 408 (September 1998), EU Annex B method B26 “Subchronic Oral Toxicity Test-Repeated Dose 90-Day Oral Toxicity Study in Rodents,” 21 August 2001, USEPA Health Effects Test Guidelines OPPTS 870.3100 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

There were no unscheduled deaths. No clinical signs of toxicity and no changes in behavior were observed. No functional performance changes were detected, and no treatment-related changes in sensory reactivity were observed. No adverse effects on body weights were recorded. No adverse changes in body weight, food and water consumption, ophthalmoscopy, hematology, bloodchemistry and organ weights were noted. No abnormalities in necropsy and histopathology were seen

Oral administration of Cellulase, Beta-glucosidase, Hemicellulase to rats at doses of 0, 4.5, 11.25 and 56.25 mg total protein/kg/d (corresponding to 0, 5.14, 12.85 and 64.25 mg TOS/kg/d, respectively) did not result in any toxicologically significant effects. The NOAEL (“No Observed Adverse Effect Level”) was determined to be 56.25 mg total protein/kg/d (highest dose tested).

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4. *T. reesei* - Endoglucanase I

A. Pathogenicity study in rats ([redacted]).

This study was conducted in accordance with the US. EPA Microbial Pesticide Test Guideline OPPTS 885.3200 and the US. EPA Good Laboratory Practice Standards (40CFR.160). In this investigation, the pathogenicity potential of *T. reesei* Endoglucanase I production strain was tested in male and female CD rats following an acute intraperitoneal injection of 5.6 x 10^6 colony forming units (cfu). Groups of animals of both sexes were sacrificed on Days 0, 7, 21, and 35 after injection of the test substance for microbial enumeration. The results were compared to those obtained from heat-killed test substance group, naïve control group and shelf control group. No deaths occurred and no adverse clinical observations were noted. The major necropsy findings were enlarged spleens noted in treated females on Days 7 and 21 and in treated males on Day 21. On Day 7, a significant decrease in relative liver weight was noted in treated males but was no longer observed on subsequent necropsy days. A statistical difference in body weight was noted in treated males between Days 0-7 but all animals showed complete recovery thereafter. By Day 21, *T. reesei* was detected in the peritoneal lavage fluid and masses of treated male rats and from the liver and peritoneal masses of treated female rats. By Day 35, the test substance was detected only from the peritoneal masses. Complete clearance was estimated to be 67 and 43 days for male and female rats, respectively. Under the conditions of this investigation, *T. reesei* Endoglucanase I production strain was not found to be toxic or pathogenic in rats.

B. 14-day oral feeding study in rats ([redacted]).

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase I was administered orally by gavage to groups of male and female [redacted] BR rats at a dosage volume of 10 ml/kg body weight for 14 days at 4 different concentrations for 14 consecutive days. There were no adverse effects on any parameter monitored in this study for rats treated with the enzyme. It was concluded that the treatment of male and female rats with the enzyme preparation did not produce evidence of systemic toxicity in any of the parameters that were monitored.

C. 91-day subchronic oral study in rats ([redacted]).

A 13-week oral gavage study with endoglucanase from *T. reesei* was conducted in accordance with OECD Guideline 408 in CD rats. Groups of ten male and females rats each were exposed to 3 different concentrations of the test material

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by gavage in a constant volume of 5 ml/kg/day. Control animals received deionized water following the same treatment regimen.

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with endoglucanase from *T. reesei*. There were no differences in behavioral test (open field) and functional tests (gripping reflex, startle reflex) conducted at study termination between the control and treated groups. Based upon these findings, it was concluded that the treatment of male and female rats with the endoglucanase from *T. reesei* did not result in toxicity up to and including the highest dosage tested. A NOEL (No Observed Effect Level) was established at the highest dosage tested.

D. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes, ((b) (4)).

Endoglucanase I from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation. The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml as well as solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels of 625, 1250, 2500 and 5000 µg/ml were selected for the chromosome aberration assay.
In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. In the non-activated 4 hour exposure group, mitotic inhibition was 10% relative to the solvent control at the 5,000 µg/ml dose level. A mitotic inhibition of 7% was also noted at the 5,000 µg/ml dose level in the activated assay. However, the percentage of HPBL cells with structural and numerical aberrations in both 5,000 µg/ml groups (with and without metabolic activation) was not significantly increased above that of the solvent control. Under the conditions of this investigation, endoglucanase I was negative for the induction of structural and numerical chromosome aberrations in both the presence and absence of metabolic activation.

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5. *T. reesei* - Endoglucanase II (EGII)

A. Bacterial Reverse Mutation Assay – Ames assay ((b) (4)).

The objective of this assay was to assess the potential of Endoglucanase II to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherichia coli* strain WP2 uvr in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008, and the US EPA (TSCA) OPPTS harmonized guidelines. The assay also complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the main test, five dose levels (50, 150, 500, 1500 and 5000 µg TP/plate) were tested. The plates incubated with the test material showed normal background growth up to 5000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item in either main test. There was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the assay and the efficacy of the metabolic activation mixture.

Under the conditions of this assay, Endoglucanase II from *T. reesei* has not shown any evidence of mutagenic activity in the Ames assay. The glucanase did not induce gene mutations by base pair changes or frame-shifts in the genome of the strains used.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ((b) (4)).

The objective of this assay was to investigate the potential of the endoglucanase II to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1977) and the Commission Regulation (EC) No. 440/2008 dated May 2008. The assay complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the preliminary test, all cultures (with or without S-9 mix) were treated for 4 hours. In the main test, cultures without S-9 mix were treated for 22 hours and those with S-9 mix for 4 hours. Three hours before harvesting, colcemid was added to all cultures at the concentration of 0.2 µg/ml to arrest all cells at the

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metaphase stage of mitosis. All cultures (with and without S-9 mix) were harvested by centrifugation 22 hours after the start of treatment. The supernatant was discarded and the cell pellets were re-suspended in a KCl hypotonic solution. The cell suspension was allowed to stand at 37°C for 25 minutes and then centrifuged. The hypotonic solution was removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations.

Under the conditions of this test, Endoglucanase II did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration recommended by guidelines.

C. 90-day repeated dose oral (gavage) toxicity Study in the rat ((b) (4) (b) (4)).

The objective of this study was to investigate the potential of Endoglucanase II to induce systemic toxicity after repeated daily oral (gavage) administration to Wistar rats of both sexes. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. “Subchronic Oral Toxicity”, 30 September 1996 and in compliance with the OECD Principles of Good Laboratory Practice [November 1997] and the US EPA Health Effects Test Guidelines, OPPTS 870.3100.

Daily administration of Endoglucanase II by oral gavage to Wistar rats at 4 different dosages for 13 consecutive weeks resulted in some effects in high dose males but was not considered as adverse. Indeed, centrilobular hepatocytes hypertrophy of the liver was noted in 3 out of 10 high dose males. However, hepatocyte enlargement is a common histopathologic finding in rodent liver and, in the absence of associated degenerative or inflammatory changes; this finding is generally considered to be an adaptive response. Both relative and absolute adrenal weights were increased in high dose males but this finding is a non-specific common stress response in rodents usually associated with increased steroidogenesis. In the absence of corresponding histopathologic changes in the adrenal glands, the toxicological significance of this finding is questionable. This reviewer agrees with the conclusion from the study director and pathologist that the effects noted in high dose males were not considered to represent an adverse effect to health.

Under the conditions of this assay, the NOAEL was established at the highest dose tested

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6. *T. reesei* - Chymosin

A. Bacterial Reverse Mutation Assay – Ames assay ((b) (4)).

The test material, chymosin from *T. reesei,* was tested in five strains of *Salmonella typhimurium* (TA 98, TA 100, TA 102, TA 1535 and TA 1537) in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

In the screening assay, chymosin was not toxic to the test bacteria except at the highest dose level (5000 µg/plate) in the presence of S-9 mix where the growth of the background lawn was slightly reduced. Therefore, 5000 µg/plate was selected as the highest dose level for the main tests. In the main tests, five dose levels (50, 160, 500, 1600 and 5000 µg/plate) were tested. Insoluble material was observed on most of the plates treated at all dose levels. Slight reductions in the growth of the background lawn were observed in strains TA 98 and TA 1537 in the presence of S-9 mix. A statistically significant reduction in the number of revertant colonies was observed in the second main test with TA 1537 in the absence of S-9 mix.

No biologically significant increases in the number of revertant colonies were observed in any dose level in either main test. A statistically significant increase in the number of revertant colonies were observed in the first main test with TA 1537 at the lowest dose tested, 50 µg/plate, in the absence of S-9 mix and at the 500 µg/plate in the presence of S-9 mix. These increases were not considered as treatment-related as they are not dose-related and are found only in the first main test. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture. Under the conditions of this assay, Chymosin has not shown any evidence of mutagenic activity in the Ames assay.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ((b) (4)).

The objective of this assay was to investigate the potential of Chymosin to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes).

This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

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In both the presence and absence of S-9 mix, no biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with chymosin. Significant increases in aberrant metaphases were demonstrated with the positive controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix. Under the conditions of this test, Chymosin did not induce chromosomal aberrations (both structural and numerical) in this *in* vitro cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

C. A 13-week Oral (Gavage) Toxicity Study in Rats ((b) (4)).

The objective of this study was to investigate the potential of Chymosin to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats ((b) (4), (b)(6) of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

One mid-dose female (# 51) was found dead on day 13 and necropsied. Since no clinical signs were observed in this animal prior to death, the cause of death was not related to treatment. No mortalities were recorded in other groups throughout the entire investigation. There were no overt signs of systemic toxicity or clinical observations that could be considered treatment-related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations. On day 90, the “Total Distance Parameter” in the open-field test was statistically significantly higher for high dose males. All other parameters of the Functional Observation Battery Test were not significantly different from controls. The biological significance of this finding was not clear since the significant value was still within the range of the historical control data collected at the testing facility.

Incidental macroscopic findings were noted but were not considered as treatment-related. Scattered histopathologic findings were noted, but in the absence of a dose-response relationship all findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals. Daily administration of Chymosin by gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL was established at the highest dose tested.

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7. *T. reesei* - High pI Xylanase

A. 91-day subchronic feeding study in rats ((b) (4)).

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of 10 male and female CD rats were fed with 4 different concentrations of the test material in the diet for 13 consecutive weeks. Two high dose rats (1 male and 1 female) died but the deaths were not attributed to be treatment related. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with *T. reesei* High pI xylanase. Based upon these findings, it was concluded that the treatment of male and female rats with High pI xylanase from *T. reesei* did not result in toxicity up to and including the highest dose tested in the diet. A NOEL was established at this dose.

B. Bacterial reverse mutation assay (Ames assay) ((b) (4) (b) (4)).

The test material, high pI xylanase from *T. reesei* was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation. The assay was conducted in accordance with OECD Guideline 471.

In the first assay, various dose levels ranging from 0 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels up to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, high pI xylanase from *T. reesei* is not a mutagen.

C. *In vitro* chromosomal aberration assay with Chinese Hamster Ovary (CHO) cells (b) (4)).

The objective of this *in vitro* assay was to evaluate the ability of High pI Xylanase to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation. For the dose range finding assays with or without metabolic activation, the stabilizer for the test article, was tested at various concentrations ranging from 0 to the maximum dose. The maximum concentration used was selected based on osmolality tests conducted. The elevations in osmolality at higher concentrations were deemed unacceptable, and the elevation

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observed at the highest concentration was used for testing. All dosing was done using the neat stabilizer and final concentrations achieved by altering the dosing volume. No evidence of cytotoxicity was observed in the cultures analyzed.
Based on data from the dose range finding assay, replicate cultures of CHO cells were incubated at various concentrations with and without metabolic activation. Cultures treated with these concentrations (including the max dose) were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations or in percent polyploidy was observed at the concentrations analyzed. The test article, high pI xylanase was considered negative for inducing chromosomal aberrations or polyploidy in CHO cells with or without metabolic activation. These results were verified in independently conducted confirmatory trials.

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8. *T. reesei* - Endoglucanase III (EGIII)

A. 28-day oral study in rats ((b) (4)).

This study was conducted in accordance with OECD Guideline 408 in rats. Endoglucanase III was given by gavage to groups of male and female (b) (4) rats at 4 different concentrations for 28 consecutive days ((b)(6), (b) (4)).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with the enzyme. Based upon these findings, it was concluded that the treatment of male and female rats with EG III did not result in toxicity and a NOEL was established at the highest dosage tested.

B. Bacterial reverse mutation assay (Ames assay) ((b) (4)).

The test article was examined for mutagenic activity in 5 strains of *Salmonella typhimurium* and *Escherichia coli* strain WP2*uvr*A in both the presence and absence of metabolic activation (b) (4)). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, various dose levels ranging from 2.5 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels of 0, 333, 667, 1,000, 3,330, or 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this investigation, the enzyme was not mutagenic in the bacterial reverse mutation assay using *Salmonella* and *E. coli*.

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9. *T. reesei* - Low pI Xylanase

A. 91-day subchronic oral study in rats ((b) (4), 1997).

This study was conducted in accordance with OECD Guideline 408 in CD rats. Groups of male and female rats were treated orally with 4 concentrations of the test material for 13 consecutive weeks.

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with Low pI xylanase from *T. reesei*. Based upon these findings, it was concluded that the treatment of male and female rats with low pI xylanase from *T. reesei* did not result in toxicity up to and including the highest dose tested. A NOEL was established at the highest dose rate.

B. Bacterial reverse mutation assay (Ames assay) ((b) (4) (b) (4)).

The test material, low pI xylanase from *T. reesei,* was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation ((b) (4)). The assay was conducted in accordance with OECD Guideline 471.

In the first assay, eight dose levels ranging from 0 to 5,000 µg/plate were used and no positive mutagenic response was observed at any dose level in any of the exposure groups. In the confirmatory assay, dose levels up to 5,000 µg/plate were used. No positive mutagenic response was observed in both the presence and absence of metabolic activation. Under the conditions of this assay, low pI xylanase from *T. reesei* was not a mutagen up to the maximum recommended dose level of 5,000 µg/plate in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes (b) (4)).

The test material, low pI xylanase from *T. reesei* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation. The assay was conducted in accordance with OECD Guideline 473.

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In a preliminary toxicity assay, HPBL cells were exposed to 9 concentrations of the test material ranging from 0.5 to 5000 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 5000 µg/ml were selected for the chromosome aberration assay. In the main chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted in the test material treated cells. Under the conditions of this investigation, low pI xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

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10. *T. reesei* - Xylanase

A. 91-day subchronic oral study in rats [redacted](b) (4)).

A 13-week oral gavage study with xylanase from *T. reesei* was conducted in accordance with OECD Guideline 408 in CD rats. Groups of ten male and females rats each were exposed to 3 different concentrations of the test material by gavage in a constant volume of 5 ml/kg/day. Control animals received deionized water following the same treatment regimen ([redacted](b) (4)).

No mortalities were recorded throughout the entire investigation. There were no treatment-related adverse effects on any of the parameters (systemic toxicity, food consumption, ophthalmology, clinical chemistry, body weight, organ weight, urine analysis, hematology, necropsy, and histopathology) monitored in this study for rats treated with xylanase from *T. reesei*. There were no differences in behavioral test (open field) and functional tests (gripping reflex, startle reflex) conducted at study termination between the control and treated groups. Based upon these findings, it was concluded that the treatment of male and female rats with *T. reesei* xylanase did not result in toxicity up to and including the highest dosage tested. A NOAEL was established at this concentration.

B. Bacterial reverse mutation assay (Ames assay) ([redacted](b) (4)).

The test material, xylanase *T. reesei* (homologous rDNA) was tested in four strains of *Salmonella typhimurium* (TA98, TA100, TA 1535 and TA1537) and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of metabolic activation. The assay was conducted in accordance with OECD Guideline 471 using the plate incorporation method (first assay) and "treat and plate" method (second assay).

In the first assay, dose levels ranging from 52 to 5,000 µg/plate were used and no treatment-related positive mutagenic response was observed at any dose level in any of the exposure groups. In the second assay, dose levels ranging from 492 to 5,000 µg/plate were used. No precipitate or cytotoxicity was noted. Random changes in the number of revertants were noted in TA 98, TA 100 and TA 1535. However, in the absence of a dose response relationship, these changes were considered as spontaneous variations. Under the conditions of this assay, xylanase from *T. reesei* was not a mutagen in both the presence and absence of metabolic activation.

C. *In vitro* chromosomal aberration assay with human peripheral blood lymphocytes, ([redacted](b) (4)).

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The test material, a xylanase from *T. reesei,* was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of metabolic activation ((b) (4)). The assay was conducted in accordance with OECD Guideline 473.

In a preliminary toxicity assay, HPBL cells were exposed to various concentrations of the test material ranging from 0.0 to 3600 µg/ml and solvent controls in both the presence and absence of metabolic activation. Cytotoxicity was not observed at any dose level in any of the exposure groups. Based on these findings, dose levels up to 3600 µg/ml were selected for the chromosome aberration assay. In the chromosome aberration assay, HPBL cells were incubated with the test material for 4 and 20 hours in the non-activated test system and for 4 hours in the activated test system. All cells were harvested 20 hours after treatment. Two hours prior to harvest, Demecolcine (0.1 µg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. Mitomycin C and cyclophosphamide served as positive controls. No statistically or biologically significant increases in the number of cells with aberrations (structural or numerical) were noted for the test material treated cells. Under the conditions of this investigation, xylanase from *T. reesei* did not induce any clastogenic or aneugenic effects in cultured human lymphocytes, either with or without metabolic activation.

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11. *T. reesei* - Lipase

A. Bacterial Reverse Mutation Assay – Ames assay (b) (4)).

The objective of this assay was to assess the potential of lipase from *T. reesei* to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherichia coli* strain WP2 uvrA in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 (July 21, 1997), EPA OPPTS 870.5100 (August 1998) and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

The plates incubated with the test material showed normal background growth up to 5000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item. There was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture. Under the conditions of this assay, the test article has not shown any evidence of mutagenic activity in the Ames assay; it did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes (b) (4)).

The objective of this assay was to investigate the potential lipase from *T. reesei* to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test; February 1998) and complied with Commission Regulation (EC) No. 440/2008 B.10: “Mutagenicity – *In Vitro* Mammalian Chromosome Aberration Test” dated May 30, 2008. The study was performed in compliance with the Chemicals Act of the Federal Republic of Germany (July 25, 1994; revised June 27, 2002) and the OECD Principles of Good Laboratory Practice (1997).

In both the preliminary and main assays, no increase in polyploidy metaphases was noticed. Under the conditions of this test, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration recommended by guidelines.

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C. 13-week Oral (Gavage) Toxicity Study in Wistar Rats ([(b) (4)], [(b) (4)]).

The objective of this study was to investigate the potential of lipase from *T. reesei* to induce systemic toxicity after repeated daily oral administration (gavage) to SPF-bred Wistar rats of both sexes. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. "Subchronic Oral Toxicity", 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice (May 18, 2005) and the OECD Principles of Good Laboratory Practice (1997).

Daily administration of the test article for 13 consecutive weeks by oral gavage to Wistar rats daily at 4 different concentrations resulted in no treatment-related deaths, clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength and locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested.

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12. *T. reesei* - Phytase

A. Bacterial Reverse Mutation Assay – Ames assay (b) (4)).

The objective of this assay was to assess the potential of phosphatase (phytase) to induce point mutations (frame-shift and base-pair) in five strains of *Salmonella typhimurium* (TA 98, TA 100, TA 102, TA 1535 and TA 1537) both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents. Under the conditions of this assay, phosphatase (phytase) from *T. reesei* has not shown any evidence of mutagenic activity in the Ames assay.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ((b) (4)).

The test material, a phosphatase (phytase) from *T. reesei,* was tested *in vitro* in the chromosome of mammalian systems (i.e., human peripheral lymphocytes) both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

In the presence of S-9 mix, no biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with Phosphatase (phytase).

In the absence of S-9 mix, statistically significant increases were observed at three test points, but none of these increases are considered to be biologically significant. Significant increases in aberrant metaphases were demonstrated with the positive controls. Under the conditions of this test, Phosphatase (phytase) did not induce chromosomal aberrations (both structural and numerical) in this *in* vitro cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

C. A 13-week Oral (Gavage) Toxicity Study in Rats ((b) (4)).

The objective of this study was to investigate the potential of phytase to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (b) (4) (b)(6)) of both sexes for 90 consecutive days. This study

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was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

No mortalities were recorded throughout the entire investigation. There were no overt signs of systemic toxicity or clinical observations that could be considered treatment-related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations.

Daily administration of phytase by gavage for 90 consecutive days did not result in adverse systemic toxicity or adverse effects on clinical chemistry, hematology, functional observation tests and macroscopic and histopathologic examinations. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested.

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13. *T. reesei* - Transglucosidase / alpha-glucosidase

A. Bacterial Reverse Mutation Assay – Ames assay ([redacted](b) (4)).

The objective of this assay was to assess the potential of transglucosidase from *T. reesei* to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherichia coli* strain WP2 uvrA in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

In the screening assay, the transglucosidase was not toxic to the test bacteria up to and including the highest dose level (5000 µg TP/plate) in both absence and presence of S-9 mix. Therefore, 5000 µg TP/plate was selected as the highest dose level for the main test. In the main test, five dose levels (33, 100, 333, 1000, 2500 and 5000 µg TP/plate) were tested. The plates incubated with the test material showed normal background growth up to 5000 µg/plate with and without metabolic activation. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item in either main test. There was also no tendency of higher mutation rates with increasing concentrations of the test material. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

Under the conditions of this assay, transglucosidase from *T. reesei* has not shown any evidence of mutagenic activity in the Ames assay. The test material did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ([redacted](b) (4)).

The objective of this assay was to investigate the potential of transglucosidase from *T. reesei* to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1977), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

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Preliminary assay (Experiment I): Ten dose levels were used. Exposure period was 4 hours for both cultures with and without S9 mix. No clear cytotoxicity was observed up to highest concentration tested 5000 µg TP/ml. No visible precipitation of the test material in the culture medium was observed. No biologically relevant increases in cells with chromosomal aberrations were noted in the three highest dose levels selected for analysis. Since the cultures fulfilled the requirements for cytogenicity evaluation, this preliminary assay was designated as Experiment I.

In experiment II: Exposure period was 4 hours for cultures with S9 mix and 22 hours for cultures without S9 mix. No visible precipitation of the test material in the culture medium was observed. In the absence of S9 mix (22-hour cultures); mitotic index was reduced to 53.85 of control after treatment with 5000 µg/ml. In the presence of S9 mix (4-hour cultures), no clear cytotoxicity was observed up to the highest concentration tested, 5000 µg/ml. No biologically relevant increases in cells with chromosomal aberrations were noted in the three highest dose levels selected for analysis. (1632.7, 2857.1 and 5000 µg TP/ml).

Under the conditions of this test, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration recommended by guidelines.

C.18-week Oral (Gavage) Toxicity Study in Wistar Rats (b) (4), (b) (4)).

The objective of this study was to investigate the potential of transglucosidase from *T. reesei* to induce systemic toxicity after repeated daily oral administration to SPF-bred Wistar rats of both sexes. This study lasted 18 weeks and consisted of 2 phases. During the first phase (5-week duration), groups of 10 animals per sex were treated by oral gavage daily with 4 different concentrations. Due to the absence of overt signs of toxicity in phase 1, both study director and study sponsor agreed to increase the dose levels. The higher dose levels were administered by oral gavage to the animals for an additional 13-week period (phase 2). All animals were sacrificed at termination of phase 2 (week 18 of study).

This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. “Subchronic Oral Toxicity”, 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

Daily administration of transglucosidase by oral gavage to Wistar rats at lower doses for a 5-week period and at higher doses for an additional 13-week period

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resulted in no treatment-related deaths, clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength and locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested.

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14. *T. reesei* - AFP

A. A 13-week Oral (Gavage) Toxicity Study in Rats ((b) (4)).

The objective of this study was to investigate the potential of protease from *T. reesei* to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats ((b)(6), (b) (4)) of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

There were no treatment-related deaths in this study. No clinical signs were seen that could be considered to be treatment related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, body weights, body weight gains, hematology, clinical chemistry, and ophthalmologic examinations. At study termination, in the males, the absolute and relative liver weights were statistically significantly increased and the relative testes weight was statistically significantly decreased compared to the concurrent control group. However, there were no treatment-related macroscopic and histopathologic changes. In the functional observation battery testing, there were no statistically significant changes noted in treated groups.

Although statistically significant variations in liver and testes weights were noted in high dose males, these weight variations were still within the 95% confidence interval of historical control data collected at (b) (4). Further, in the absence of corresponding histopathologic changes, these weight variations were considered to be of no toxicological significance. Under the conditions of this assay, the NOAEL is established at the highest dose tested.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ((b) (4)).

The objective of this assay is to investigate the potential of protease from *T. reesei* to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with AFP concentrate both in the presence and absence of metabolic activation. Significant

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increases in aberrant metaphases were demonstrated with the positive controls. Under the conditions of this test, the enzyme concentrate did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation.

C. Bacterial Reverse Mutation Assay – Ames assay ((b) (4)).

The objective of this assay is to assess the potential of protease from *T. reesei* to induce point mutation (frame-shift and base-pair) in five strains of *Salmonella typhimurium* (TA 98, TA 100, TA 102, TA 1535 and TA 1537) in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

Although a statistically significant reduction in the number of revertant colonies was noted in TA 1535 with S-9 mix, this is not considered to be biologically significant in the absence of a dose-related effect. Some variations in revertant colonies were noted but the variations were not reproducible between the three replicate plates and none of these variations meet the positive criteria recognized by regulatory agencies worldwide. Under the conditions of this assay, the test article has not shown any evidence of mutagenic activity in the Ames assay.

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15. *T. reesei* Glucoamylase

A. Bacterial Reverse Mutation Assay – Ames assay ((b) (4)).

The objective of this assay was to assess the potential of glucoamylase from *T. reesei* to induce point mutations (frame-shift and base-pair) in five strains of *Salmonella typhimurium* (TA 98, TA 100, TA 102, TA 1535 and TA 1537) in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix. This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

In the screening assay, Glucoamylase was not toxic to the test bacteria up to and including the highest dose level (5000 µg/plate) in both absence and presence of S-9 mix. In the main tests, five dose levels (50, 160, 500, 1600 and 5000 µg/plate) were tested. No marked reductions in the number of revertant colonies or growth of the background lawn of non-revertant bacteria were observed. No biologically significant increases in the number of revertant colonies were observed at any dose level of the test item in either main test. Small, statistically significant increases in the number of revertant colonies were observed at several test points, but none are considered to be biologically significant (i.e., at least a 2-fold higher than the corresponding negative control value) since the increases were not clearly dose-related and were not reproducible. Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture. Under the conditions of this assay, Glucoamylase has not shown any evidence of mutagenic activity in the Ames assay.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ((b) (4)).

The objective of this assay was to investigate the potential of Glucoamylase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes).This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

In both the presence and absence of S-9 mix, no biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with Glucoamylase in either test. Significant increases in aberrant metaphases were demonstrated with the positive

controls demonstrating the sensitivity of the tests and the efficacy of the S-9 mix. Under the conditions of this test, Glucoamylase did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation.

C. 90-day oral (gavage) study in Rats (b) (4)).

The objective of this study was to investigate the potential of glucoamylase to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats ((b)(6), (b) (4)) of both sexes for 90 consecutive days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations. There were no differences in the functional observation battery and stimuli assays between treated and control animals. At study termination, the mean cell hemoglobin in high dose females was statistically lower than control values and the mean cell hemoglobin concentration in mid dose females was statistically significantly higher than control values, but these findings were considered incidental in the absence of a dose-response relationship and the observation of finding in only one gender. Changes in creatinine levels were also observed in treated females but all values were within the historical control range and were not considered as treatment-related.

At necropsy, there were no treatment related findings on organ weights, macroscopic findings and histopathologic examinations. In males, focal tubular basophilia/dilatation including hyaline casts in the kidneys and minimal testicular atrophy were noted in both control and high dose groups. In light of these findings, microscopic examination was extended to the kidneys and testes of males in the low and mid dose groups. Upon examination of all groups, the findings in the kidneys were considered incidental in the absence of a dose response relationship (the most severe findings were noted in mid dose group) and no statistical differences were detected among groups. Further, the incidences of focal tubular basophilia/dilatation with hyaline casts in this study were within the range of historical control data for this species and strain. Testicular atrophy (minimal severity) was noted in all groups, including the control, and was considered as incidental and of no toxicological significance.

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All other microscopic findings were considered to be within the background incidence of findings reported in this age and strain of laboratory animals.
Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested.

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16. *T. reesei* - Catalase

A. Bacterial Reverse Mutation Assay – Ames assay ((b) (4)).

The objective of this assay was to assess the potential of Catalase to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherichia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system. The assay was performed in two phases using the plate incorporation methodology for the positive control, 2-aminoanthracene, with *E. coli* and the treat and plate methodology for the all remaining strains and assays. This assay was conducted in accordance with OECD guideline No. 471 (1997). Under the conditions of this assay Catalase has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ((b) (4)).

The objective of this assay was to investigate the potential of Catalase to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the addition of a mitogen. This assay was conducted in accordance with OECD guideline No. 473 (1977).
Under the conditions of this test, Catalase did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration recommended by guidelines. All of the vehicle control cultures had frequencies of cells with chromosomal aberrations within the expected range. The positive control items inducted statistically significant increases in the frequency of cells with aberrations.

C. Subchronic toxicity 90-day gavage in rats ((b) (4)).

The objective of this study was to investigate the potential of Catalase to induce systemic toxicity after repeated daily oral administration to (b) (4) CD rats of both sexes for 90 continuous days. This study was conducted in accordance with OECD guideline No. 408 (September 1998). Daily administration of Catalase by oral gavage to CD at various doses for 90 consecutive days did not result in treatment-related effects on clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength or locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested.

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17. *T. reesei* - Trehalase

A. Repeated Dose 90-day Oral toxicity in rats (DuPont Haskell Global Centers for Health & Environmental Sciences, 2017).

The objective of this study was to investigate the potential toxicity of *T. reesei* Trehalase (Test article, H-32153) to induce systemic toxicity after repeated daily oral administration to (b) (4) CD rats of both sexes for 90 continuous days. This study was conducted in accordance with OECD guideline No. 408 (September 1998).

No test article-related effects were reported among clinical observations, ophthalmic observations, body weight measurements, food consumption or food efficiency values, functional observation battery tests, locomotor activity evaluations, hematology, coagulation, clinical chemistry, or urinalysis parameters, or organ weight, macroscopic or microscopic pathology findings. Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) was established at the high dose.

B. *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL) (DuPont Haskell Global Centers for Health & Environmental Sciences, 2017).

The purpose of this study was to evaluate the potential of Trehalase and/or its metabolites to induce structural chromosomal aberrations in Human Peripheral Blood Lymphocytes (HPBL) in the presence and absence of an exogenous metabolic activation system. A preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test. This assay was conducted in accordance with OECD guideline No. 473 (2016). Under the conditions of the assay described in this test, Trehalase was concluded to be negative for the induction of structural and numerical chromosome aberrations in both the non-activated and S9-activated test systems. Trehalase was negative in the *In Vitro* Mammalian Chromosome Aberration Assay in HPBL.

C. Bacterial reverse mutation assay (Ames assay) (DuPont Haskell Global Centers for Health & Environmental Sciences, 2017).

The test article, Trehalase was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 uvrA in the presence and absence of Aroclor-induced rat liver S9. This assay was conducted in accordance with OECD

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guideline No. 471 (1997). All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Trehalase did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

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18. *T. reesei* - Xylanase

A. Repeated Dose 90-day Oral toxicity in rats ((b) (4)).

The objective of this study was to investigate the potential toxicity of *T. reesei* Xylanase (Test article, H-31435) to induce systemic toxicity after repeated daily oral administration to (b) (4) CD rats of both sexes for 90 continuous days. This study was conducted in accordance with OECD guideline No. 408 (September 1998).

No test article-related effects were reported among clinical observations, ophthalmic observations, body weight measurements, food consumption or food efficiency values, functional observation battery tests, locomotor activity evaluations, hematology, coagulation, clinical chemistry, or urinalysis parameters, or organ weight, macroscopic or microscopic pathology findings. Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) was established at the high dose.

B. *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL) ((b) (4)).

The purpose of this study was to evaluate the potential of a test article and/or its metabolites to induce structural chromosomal aberrations in Human Peripheral Blood Lymphocytes (HPBL) in the presence and absence of an exogenous metabolic activation system. A preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test. This assay was conducted in accordance with OECD guideline No. 473 (1977). Under the conditions of the assay described in this test, Xylanase was concluded to be negative for the induction of structural and numerical chromosome aberrations in both the non-activated and S9-activated test systems. Xylanase was considered to be negative in the *In Vitro* Mammalian Chromosome Aberration Assay in HPBL.

C. Bacterial reverse mutation assay (Ames assay) (b) (4)).

The test article, Xylanase was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 uvrA in the presence and absence of Aroclor-induced rat liver S9. All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Xylanase did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-

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induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

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19. *T. reesei* - Glucoamylase

A. Bacterial Reverse Mutation Assay – Ames assay [redacted]).

The objective of this assay was to assess the potential of glucoamylase (GA) to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, A 1535 and TA 1537) and *Escherichia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). A screening (dose range) test was performed with strains TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA. Subsequently, two independent main tests were performed with all 5 strains in both the presence and absence of S-9 mix. Triplicate plates were used at each test point. All dose levels were expressed in terms of total protein (TP). Negative control plates were treated by the addition of sterile deionized water. This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

Under the conditions of this assay, GA has not shown any evidence of mutagenic activity in the Ames assay. GA did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ([redacted]).

The objective of this assay was to investigate the potential of GA to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the addition of a mitogen (e.g., phytohemagglutinin, PHA).

GA was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main assays. Ten concentrations of GA were used in the preliminary assay and at least 4 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). In the absence of cytotoxicity, the highest dose was selected, and which is recommended by the OECD guideline. All dose levels were expressed in terms of total protein.

In the preliminary assay was conducted in accordance with OECD guideline No. 473 (1977), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

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Under the conditions of this test, GA did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration recommended by guidelines. All of the vehicle control cultures had frequencies of cells with chromosomal aberrations within the expected range. The positive control items inducted statistically significant increases in the frequency of cells with aberrations.

C. 90-day Oral (Gavage) Toxicity Study in Wistar Rats (b) (4)**,** (b) (4)

The objective of this study was to investigate the potential of GA to induce systemic toxicity after repeated daily oral administration to SPF-bred Wistar rats of both sexes for 90 continuous days. This study was conducted in accordance with OECD guideline No. 408 (September 1998) and Directive 96/54/EC, B.26. “Subchronic Oral Toxicity”, 30 September 1996 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

Daily administration of GA by oral gavage to Wistar rats at various doses for 90 consecutive days did not result in treatment-related effects on clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength or locomotor activities. No macroscopic or microscopic changes could be attributed to treatment. The increase in hepatocellular glycogen noted in the high dose animals was not accompanied by any other indicators of liver injury or liver function and thus is considered to be of metabolic nature and not a treatment related effect. Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested.

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20. *T. reesei* - Xylanase

A. Bacterial Reverse Mutation Assay – Ames assay ([redacted]).

In a bacterial reverse mutation assay, no biologically significant increases in the number of revertant colonies were observed at any dose level in the presence and absence of metabolic activation up to the maximum recommended dose. Under the conditions of this assay, Xylanase is not a mutagen.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes, [redacted].

In an *in vitro* assay with human peripheral lymphocytes, Xylanase did not induce chromosomal aberrations (both structural and numerical) in this cytogenetic test in both the presence and absence of metabolic activation up to the maximum recommended dose. Under the conditions of this assay, *T. reesei* Xylanase is not a clastogen.

C. Subchronic toxicity 90-day Oral Gavage Study in Rats [redacted]).

In a 90-day oral (gavage) study, groups of 10 rats/sex each were gavaged daily at various doses depending on body weight at a constant volume. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, water consumption, body weights, body weight gains, hematology, and ophthalmologic examinations. There were no differences in the functional observation battery and stimuli assays between treated and control animals. At necropsy, there were no treatment related findings on organ weights and macroscopic findings. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested.

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21. *T. reesei* - Alpha-amylase

A. Bacterial Reverse Mutation (Ames) Assay (b) (4)).

The objective of this assay was to investigate the mutagenic potential of alpha-amylase from *T. reesei* using *Salmonella typhimurium* strains TA 102, TA 100, TA 98, TA 1537, and TA 1535. This assay was conducted in accordance with OECD guideline No. 471 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

The test item was not toxic to any tester strain at any dose level with or without metabolic activation. No biologically significant increases were seen in any tester strain at any dose level with or without metabolic activation. The positive controls used in the test yielded marked increases in the frequency of revertant colonies, confirming the efficacy of the S9 mix and the sensitivity of the bacterial strains. Under the conditions of this assay, the test article was found to be non-mutagenic in the Ames test.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes ((b) (4)).

The objective of this procedure was to investigate the potential of alpha-amylase from *T. reesei* to induce numerical and/or structural changes in mammalian chromosomes (human peripheral lymphocytes). This assay was conducted in accordance with OECD guideline No. 473 (1997), the Commission Regulation (EC) No. 440/2008 dated May 2008 and complied with OECD Principles of GLP (1997) and all subsequent OECD consensus documents.

The test item did not cause marked toxicity at any concentration tested in the presence or absence of S-9 mix in any test. In the initial test 1, exposure to the alpha-amylase with metabolic activation at 5000 µg total protein/ml produced a small but statistically significant increase in the mean frequency of aberrant metaphases. This result was not reproducible in the replicate culture so it was further investigated in the repeated test 1. In the initial test 2, three of the four positive control treatments did not produce adequate increases in the frequency of aberrant metaphases. Exposure to 1250 µg total protein/ml without S-9 mix yielded a statistically significant increase in the mean frequency of aberrant metaphases. However, this result was not reproducible between the replicate cultures and it was further investigated in the repeated test 2. No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with the alpha-amylase in the presence or absence of S-9 mix in the repeated test 1. The repeated test 2 was considered valid since all

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controls performed as expected. No biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with the alpha-amylase in the presence or absence of S-9 mix in the repeated test 2. The increases in frequency of aberrant metaphases in both initial tests 1 and 2 are not considered to be biologically significant since they were not reproduced in the replicate cultures or in the repeated tests. Under the conditions of this assay, the test article did not induce chromosomal aberrations (both structural and numerical) in this *in-vitro* test using human peripheral lymphocytes both in the presence and absence of metabolic activation up to the highest dose level (5000 µg total protein/ml) as recommended by the guidelines. The alpha-amylase is neither a clastogen nor an aneugen.

C. 90-day Oral Gavage Study in Rats ((b) (4)).

The objective of this study was to investigate the potential of alpha-amylase from *T. reesei* to induce systemic toxicity after repeated daily oral administration by gavage to Sprague Dawley rats of both sexes for 13 consecutive weeks. This study was conducted in accordance with OECD guideline No. 408 (September 1998), EU Annex B method B26 “Subchronic Oral Toxicity Test- Repeated Dose 90-Day Oral Toxicity Study in Rodents,” 21 August 2001, USEPA Health Effects Test Guidelines OPPTS 870.3100 and in compliance with the Swiss Ordinance relating to Good Laboratory Practice [May 18, 2005] and the OECD Principles of Good Laboratory Practice [November 1997].

No adverse clinical signs were observed that could be related to the test item. No observations were noted with regards to the weekly assessment. No adverse effects attributed to the test item were observed during the functional battery before termination of treatment. All animals gained weight during the study, and no effects on food or water consumption were observed. No abnormalities were noted upon ophthalmoscopic examination. No effects in hematology, clinical chemistry and organ weights were noted that were considered toxicologically significant. No abnormalities in necropsy and microscopic examinations were seen.

Oral administration of the alpha-amylase to rats at 4 different doses did not result in any toxicologically significant effects. The NOAEL (“No Observed Adverse Effect Level”) was established at the highest dosage tested.

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22. *T. reesei* - Glucoamylase

A. Bacterial Reverse Mutation Assay – Ames assay ([redacted]).

The objective of this assay was to assess the potential of Glucoamylase (GA) to induce point mutations (frame-shift and base-pair) in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and *Escherichia coli* strain WP2 uvrA. The test material was tested both in the presence and absence of a metabolic activation system. This assay was conducted in accordance with OECD guideline No. 471 (1997). Under the conditions of this assay, GA has not shown any evidence of mutagenic activity in the Ames assay in both presence and absence of metabolic activation.

B. *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes [redacted]).

The objective of this assay was to investigate the potential of GA to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). In this assay, human lymphocytes were stimulated to divide by the addition of a mitogen. This assay was conducted in accordance with OECD guideline No. 473 (1977). Under the conditions of this test, GA did not induce chromosomal aberrations (both structural and numerical) in this *in vitro* cytogenetic test using cultured human lymphocytes cells both in the presence and absence of metabolic activation up to the highest concentration recommended by guidelines. All of the vehicle control cultures had frequencies of cells with chromosomal aberrations within the expected range. The positive control items inducted statistically significant increases in the frequency of cells with aberrations.

C. Subchronic toxicity 90-day gavage study in rats ([redacted]).

The objective of this study was to investigate the potential of GA to induce systemic toxicity after repeated daily oral administration to [redacted] CD rats of both sexes for 90 continuous days. This study was conducted in accordance with OECD guideline No. 408 (September 1998).

Daily administration of GA by oral gavage to CD rats at various doses for 90 consecutive days did not result in treatment-related effects on clinical observations, feed consumption, body weight changes, hematology, clinical chemistry, urinalysis, organ weights, functional observation, grip strength or locomotor activities. No macroscopic or microscopic changes could be attributed

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to treatment. Under the conditions of this assay, the NOAEL (no observed adverse effect level) was established at the highest dose tested.

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23. *T. reesei* - Xylanase

A. 90-Day Gavage (oral) Study in Rats. DuPont Haskell Global Centers for Health & Environmental Sciences (2016)

The objective of this study was to evaluate the potential subchronic toxicity of Fungal Xylanase when administered by oral gavage to male and female rats for approximately 90 days. Four groups of young adult male and female Crl:CD(SD) rats (10/sex/dose) were dosed by oral gavage for 91 days (males) or 92 days (females) with the test substance, diluted with deionized water, at doses of 250 (low dose), 500 (mid dose), or 1000 (high dose) mg total protein/kg body weight (bw)/day. Controls were dosed with deionized water. All rats received evaluations of body weight and nutritional parameters, clinical observations, ophthalmology and neurobehavioral parameters (pretest and end of study). Clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), and anatomic pathology parameters (organ weights and gross and microscopic pathology) were evaluated at the end of the study. The dosing formulations were demonstrated to be at the targeted concentrations, with the exception of the low dose at week 11 which was 36% below nominal. Concentration verification for all other dosing formations at weeks 1, 6, and 11 were within 10% of nominal. The test substance was demonstrated to be stable for up to 5 hours at room temperature (neat, ½, and ¼ dilution), up to 27 days refrigerated (neat material), and up to 16 months frozen (neat material).

All animals survived to scheduled necropsy except for one high-dose female that died due to gavage error. No test substance-related effects were observed on body weight and nutritional parameters, clinical or ophthalmology observations, neurobehavioral parameters, clinical pathology parameters, or anatomic pathology parameters. Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for H-31334 was 1000 mg total protein/kg bw/day for male and female rats, based on a lack of test substance-related effects at the highest dose tested. This NOAEL is equivalent to 1214.4 mg total organic solids (TOS)/kg body weight/day in male and female rats.

B. Bacterial reverse mutation assay (Ames assay) (b) (4)).

The test article was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and Escherichia coli tester strain WP2 uvrA in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases using the treat and plate modification of the pre-incubation method except as noted below. The plate incorporation methodology was used only for the positive control, 2-aminoanthracene (2AA), with E. coli in the presence of S9 activation. The first

phase, the initial toxicity-mutation assay, was used to establish the dose range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test article. Dosing formulations were adjusted for total protein content based on the concentration as supplied at 86.34 mg/mL.

In the treat and plate method, the volumes of S9 mix, Sham mix, bacteria and test article, vehicle or positive control were increased by a factor of 2.5 or 3.5 to ensure sufficient volume of resuspended bacteria to plate the desired number of replicates. Water was selected as the solvent of choice based on information provided by the Sponsor and compatibility with the target cells.

In the initial toxicity-mutation assay, the maximum dose tested was 5000 µg per plate; this dose was achieved by diluting the test article at the Sponsor-provided concentration of 86.34 mg/mL to a concentration of 50 mg/mL and using a 100 µL plating aliquot. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. The test article formed clear solutions in sterile water for injection-quality, cell culture grade water (hereafter referred to as sterile water) from 0.015 to 50 mg/mL. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Increases in revertant counts (1.5- to 2.5-fold maximum increases) were observed with a few test conditions. However, these increases were not considered indicative of mutagenic activity because (1) the increases were non-dose responsive and (2) the maximum revertant counts were still within the historical vehicle control ranges for each tester strain. No precipitate was observed. Toxicity was observed at 5000 µg per plate with tester strain TA100 in the absence of S9 activation. This test condition was retested to clarify the toxicity profile observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the retest and confirmatory mutagenicity assays was 5000 µg per plate.

In the retest of the initial toxicity-mutation assay, no positive mutagenic responses were observed with tester strain TA100 in the absence of S9 activation. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed.

In the confirmatory mutagenicity assay, no positive mutagenic responses were observed with tester strains TA100, TA1535, TA1537 or WP2 uvrA in either the presence or absence of S9 activation. Increases in revertant counts (1.6- and 2.8-fold maximum increases) were observed with several test conditions. However, these increases were not considered indicative of mutagenic activity because:

(1) The increases were non-dose responsive and (2) the maximum revertant counts were still within the historical vehicle control ranges for each tester strain. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. Due to confluent bacterial growth, tester strain TA98 was not evaluated for mutagenicity but was retested. In the retest of the confirmatory mutagenicity assay, no positive mutagenic responses were observed with tester strain TA98 in either the presence or absence of S9 activation. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, the test article did not exhibit any mutagenic responses in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

C. *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL). ((b) (4)).

The test article was tested in the chromosome aberration assay using human peripheral blood lymphocytes (HPBL) in both the absence and presence of an Aroclor-induced rat liver S9 metabolic activation system. A preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test. The chromosome aberration assay was used to evaluate the clastogenic potential of the test article. In both assays, HPBL cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9-activated test system. All cells were harvested 20 hours after treatment initiation. Dosing formulations were adjusted for total protein content based on the concentration as supplied at 86.34 mg/mL. Water was used as the vehicle based on information provided by the Sponsor and compatibility with the target cells. The test article was supplied in water at a concentration of 86.34 mg/mL of total protein.

In the preliminary toxicity assay, the doses tested ranged from 0.5 to 5000 µg/mL. The test article formed workable suspensions in water at concentrations ≥ 15 mg/mL, while concentrations ≤ 5 mg/mL were soluble in water. The test article was soluble in treatment medium at all dose levels tested at the beginning and conclusion of the treatment period. The osmolality in treatment medium of the highest dose level tested, 5000 µg/mL, was 254 mmol/kg. The osmolality of the vehicle (water«12») in the treatment medium was 243 mmol/kg. The osmolality of the test article dose level in treatment medium is acceptable because it did not exceed the osmolality of the vehicle by more than 20%. The pH of the highest dose level of test article in treatment medium was 7.0. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was not observed at any dose level in any of the treatment conditions. Based on these findings, the doses

chosen for the chromosome aberration assay ranged from 1000 to 5000 µg/mL for all three treatment conditions.

In the chromosome aberration assay, the test article formed workable suspensions in water at all concentrations tested. The test article was soluble in treatment medium at all dose levels tested at the beginning and conclusion of the treatment period. The pH of the highest dose level of test article in treatment medium was 7.0. Substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was not observed at any dose level in any of the treatment conditions. Based on these findings and upon consultation with the Sponsor, the doses chosen for microscopic analysis were 1000, 2500, and 5000 µg/mL for all three treatment conditions.

The percentage of cells with structural or numerical aberrations in the test article-treated groups was not significantly increased relative to the vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). Under the conditions of the assay described in this report, H-31334«2» was concluded to be negative for the induction of structural and numerical chromosome aberrations in both the non-activated and S9-activated test systems. H-31334 was considered to be negative in the In Vitro Mammalian Chromosome Aberration Assay in HPBL.

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24. *T. reesei* - Phytase

A. Repeated Dose 90-day Oral toxicity in rats (b) (4), (b) (4)).

The objective of this study was to investigate the potential toxicity of *T. reesei* Phytase A to induce systemic toxicity after repeated daily oral administration to (b) (4) CD rats of both sexes for 90 continuous days. Three groups of young adult male and female rats (10/sex/group) were dosed by oral gavage with Phytase A, diluted in deionized water, at doses of 250, 500, or 1000 mg total organic solids (TOS)/kg body weight/day. The control group was dosed with deionized water. This study was conducted in accordance with OECD guideline No. 408 (June 2018).

No test article-related effects were reported among clinical observations, ophthalmic observations, body weight measurements, food consumption or food efficiency values, functional observation battery tests, locomotor activity evaluations, hematology, coagulation, clinical chemistry, or urinalysis parameters, or organ weight, macroscopic or microscopic pathology findings. Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) was established at the high dose of 1000 mg TOS/kg bw/day.

B. *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL) ((b) (4)).

The purpose of this study was to evaluate the potential of Phytase A and/or its metabolites to induce structural chromosomal aberrations in Human Peripheral Blood Lymphocytes (HPBL) in the presence and absence of an exogenous metabolic activation system. A preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test. This assay was conducted in accordance with OECD guideline No. 473 (July 2016). In the preliminary toxicity assay, the highest concentration tested was the OECD recommended limit dose of 5000 µg/ml. The cells were exposed to 9 concentrations of the test substance ranging from 0.5 to 5000 µg/ml, as well as to a vehicle control. Test substance precipitation was not observed and substantial toxicity (i.e., ≥ 50% mitotic reduction in relation to the vehicle control) was not observed at any concentration in any test condition. The concentrations chosen for the assay ranged from 300 to 5000 µg/ml for all test conditions. Neither test substance precipitation nor substantial toxicity was observed at any concentration in any test condition. Under the conditions of the assay described in this test, Phytase A was concluded to be negative for the induction of structural and numerical chromosome aberrations in both the non-activated and S9-activated test systems. Phytase A was negative in the *In Vitro* Mammalian Chromosome Aberration Assay in HPBL.

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C. Bacterial reverse mutation assay (Ames assay) ((b) (4)).

The test article, Phytase A was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 uvrA in the presence and absence of Aroclor-induced rat liver S9. This assay was conducted in accordance with OECD guideline No. 471 (1997). In the toxicity-mutation test, the maximum dose evaluated was 5000 µg/plate for the tester strains. All tester strains and test conditions were evaluated at 8 dose levels along with the negative (vehicle) and positive controls. The dose levels used in the test were (b) (4) µg per plate for all tester strains. No positive mutagenic responses were observed at any dose level or with any tester strain in either the absence or presence of S9 metabolic activation. No appreciable toxicity was observed at any dose level with any tester strain in either the absence or presence of S9. Based on the toxicity-mutation test, the maximum dose evaluated in the mutagenicity test was 5000 µg/plate for each of the tester strains in the absence and presence of S9 metabolic activation. All tester strains and test conditions were evaluated at 6 dose levels along with the negative (vehicle) and positive controls. The dose levels used were 15.0, 50.0, 150, 500, 1500 and 5000 µg/plate for all tester strains. No positive mutagenic responses, appreciable toxicity, or precipitation was observed at any dose level or with any tester strain in either the absence or presence of S9 metabolic activation. All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Phytase A did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

D. In Vitro (b) (4) Eye Irritation Test (EIT) ((b) (4))

The in vitro (b) (4) eye irritation test (FIT) was performed using the (b) (4) tissue construct, which models the cornea epithelium with progressively stratified, but non-cornified cells. The assay was conducted according to OECD Guideline Test No. 492 (2016). The assay measures destruction of the ocular tissue, one component predictive of ocular irritation. The reduction of the viability of tissues exposed to chemicals in comparison to negative control treated tissue was used to predict the ocular irritation potential. Viable tissue constructs were randomly allocated to three groups of two matrices each; one test substance group, a negative control group, and a positive control group. Methyl acetate and deionized water were used as the positive and negative controls, respectively. The test substance and negative control were tested in duplicate.The (b) (4) tissue was conditioned to the assay media

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prior to exposure to test or control substances. Each substance was applied directly to the surface of duplicate tissue matrices. Treated tissues were exposed to the test or control substances for 30 minutes and rinsed thoroughly with DPBS. Rinsed tissues were incubated for approximately 2 hours, followed by MTT exposure for 3 hours. The tissues were then extracted with isopropanol in a refrigerator overnight for approximately 18 hours. The resulting extraction solution from each tissue was sampled and optical density (CD) measured using a spectrophotometer. The relative viability of each treated tissue was calculated based on the mean CD compared to that of the negative control treated tissue. Under the conditions of this study, Phytase A is not considered an eye irritant. Based on the results of this study, Phytase A meets the requirement for UN GHS Category: No Category.

E. Local Lymph Node Assay (LLNA) in Mice (b) (4))

The assay was conducted according to OECD Guideline Test No. 429 (2010). A dermal sensitization test was conducted with mice to determine the potential for Phytase A to produce sensitization after repeated topical applications. Two concentrations (25% and 50%) of the test substance in 1% (b) (4) Surfactant w/w in distilled water (1% (b) (4) and the neat test substance were topically applied to fifteen healthy test mice (5 mice/group) for three consecutive days. Three days after the last application, the mice were given an IV injection containing 20 µCi of ^{3}H-methyl thymidine. Approximately five hours later, all animals were euthanized via an overdose of inhaled Isoflurane and the draining (auricular) lymph nodes were harvested and prepared for analysis in a scintillation counter. The results are presented in disintegrations per minute per mouse (dpm/mouse). Each animal's ears were also evaluated for erythema and edema prior to each application and again on Day 6, prior to the IV injection. A vehicle control group (five animals) and a positive control group (five animals) were maintained under the same environmental conditions and treated in the same manner as the test animals. The vehicle control animals were treated with 1% (b) (4) and the positive control group animals were treated with a 25% w/w mixture of HCA. in 1% (b) (4). To reduce the total number of animals used, this study was run concurrently with another study to utilize a common positive control and common vehicle control group. Based on the results of this study, the test substance is not considered to be a contact dermal sensitizer in the LLNA. Proper conduct of the LLNA was confirmed via a positive response with 25% RCA, a moderate contact sensitizer.

F. EpiDerm™ Skin Irritation Test (SIT) (b) (4))

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The assay was conducted according to OECD Guideline for Test No. 439 (2015). The in vitro (b) (4) skin irritation test (SIT) was performed using three-dimensional reconstructed human epithelium tissue obtained from (b) (4) The assay measures the destruction of skin cells in the tissue, one component predictive of skin irritation. The decrease in viability of tissue exposed to test substance(s) in comparison to those treated with a negative control is measured using a colorimetric assay involving the reduction of (3-4,5-dimethyhhiazol-2-yl)-2,5-diphenyltetrazolium bromide (MIT) to formazan to predict their skin irritation potential. Nine viable tissue constructs were randomly allocated to three groups containing three matrices each; one test substance treated group, one vehicle control treated group and one positive control treated group. Dulbecco's phosphate buffered saline (DPBS) and 5% Sodium dodecyl sulfate (5% SDS) were used as the negative and positive controls, respectively. The (b) (4) Tissue inserts were conditioned to the assay media prior to exposure with lest and control substances. Each substance was applied directly to the surface of the tissue matrices in triplicate. Treated tissues were exposed to the test or control substances under standard CO_2 incubation conditions ['-37°C (5% CO_2)] then moved to ambient temperature for a total exposure time of 60 minutes. The tissue inserts were rinsed thoroughly with DPBS. placed in fresh assay medium and further incubated for approximately 45 hours with a change of media after 26 hours. Following post-exposure incubation, the tissue inserts were transferred to a 24-well plate with wells containing 300 ul of MET medium and incubated for 3 hours at —37°C (5% CO_2). The tissues were then rinsed with DPBS and extracted with isopropanol for 2 hours at room temperature while agitating. The resulting extraction solution from each tissue was sampled and evaluated for optical density (OD) using a spectrophotometer. The relative viability of each treated tissue was calculated based on the mean OD compared to that of the negative control treated tissue. The positive control group demonstrated a decrease in viability which was acceptable for the assay. Under the conditions of this study, Phytase A is considered a non-irritant. Based on the results of this study, Phytase A meets the requirement for UN GI-IS Category: No Category.

G. Acute Oral Toxicity Study ((b) (4))

The study was conducted according to OECD Guideline 423 (2001). An acute oral toxicity study (Acute Toxic Class Method) was conducted with rats to determine the potential for Phytase A to produce toxicity from a single dose via the oral route. Under the conditions of this study, the acute oral LD50 of Phytase A was greater than 5000 mg/kg of body weight in female rats.

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25. *T. reesei* - Phytase

A. Repeated Dose 90-day Oral toxicity in rats ((b) (4), (b) (4)).

The objective of this study was to investigate the potential toxicity of *T. reesei* Phytase B to induce systemic toxicity after repeated daily oral administration to (b) (4) CD rats of both sexes for 90 continuous days. Three groups of young adult male and female rats (10/sex/group) were dosed by oral gavage with Phytase B, diluted in deionized water, at doses of 250, 500, or 1000 mg total organic solids (TOS)/kg body weight/day. The control group was dosed with deionized water. This study was conducted in accordance with OECD guideline No. 408 (June 2018).

No test article-related effects were reported among clinical observations, ophthalmic observations, body weight measurements, food consumption or food efficiency values, functional observation battery tests, locomotor activity evaluations, hematology, coagulation, clinical chemistry, hormone analysis, urinalysis parameters, or organ weight, macroscopic or microscopic pathology findings. Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) was established at the high dose of 1000 mg TOS/kg bw/day.

B. *In vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL) (b) (4)).

The purpose of this study was to evaluate the potential of Phytase B and/or its metabolites to induce structural chromosomal aberrations in Human Peripheral Blood Lymphocytes (HPBL) in the presence and absence of an exogenous metabolic activation system. A preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test. This assay was conducted in accordance with OECD guideline No. 473 (July 2016). In the preliminary toxicity assay, the highest concentration tested was the OECD recommended limit dose of 5000 µg/ml. The cells were exposed to 9 concentrations of the test substance ranging from 0.5 to 5000 µg/ml, as well as to a vehicle control. Test substance precipitation was not observed and substantial toxicity (i.e., ≥ 50% mitotic reduction in relation to the vehicle control) was not observed at any concentration in any test condition. The concentrations chosen for the assay ranged from 625 to 5000 µg/ml for all test conditions. Neither test substance precipitation nor substantial toxicity was observed at any concentration in any test condition. Under the conditions of the assay described in this test, Phytase B was concluded to be negative for the induction of structural and numerical chromosome aberrations in both the non-

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activated and S9-activated test systems. Phytase B was negative in the *In Vitro* Mammalian Chromosome Aberration Assay in HPBL.

C. Bacterial reverse mutation assay (Ames assay) (b) (4)).

The test article, Phytase B was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 uvrA in the presence and absence of Aroclor-induced rat liver S9. This assay was conducted in accordance with OECD guideline No. 471 (1997). In the toxicity-mutation test, the maximum dose evaluated was 5000 µg/plate for the tester strains. All tester strains and test conditions were evaluated at 8 dose levels along with the negative (vehicle) and positive controls. The dose levels used in the test were (b)(6) µg per plate for all tester strains. No positive mutagenic responses were observed at any dose level or with any tester strain in either the absence or presence of S9 metabolic activation. No appreciable toxicity was observed at any dose level with any tester strain in either the absence or presence of S9. Based on the toxicity-mutation test, the maximum dose evaluated in the mutagenicity test was 5000 µg/plate for each of the tester strains in the absence and presence of S9 metabolic activation. All tester strains and test conditions were evaluated at 6 dose levels along with the negative (vehicle) and positive controls. The dose levels used were (b)(6) µg/plate for all tester strains. No positive mutagenic responses, appreciable toxicity, or precipitation was observed at any dose level or with any tester strain in either the absence or presence of S9 metabolic activation. All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Phytase B did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test article was concluded to be negative in this assay.

D. In Vitro EpiOcular™ Eye Irritation Test (EIT) (b) (4))

The in vitro (b) (4) eye irritation test (FIT) was performed using the (b) (4) tissue construct, which models the cornea epithelium with progressively stratified, but non-cornified cells. The assay was conducted according to OECD Guideline Test No. 492 (2016). The assay measures destruction of the ocular tissue, one component predictive of ocular irritation. The reduction of the viability of tissues exposed to chemicals in comparison to negative control treated tissue was used to predict the ocular irritation potential. Viable tissue constructs were randomly allocated to three groups of two matrices each; one test substance group, a negative control group, and a positive control group. Methyl acetate and deionized water were used as the positive and

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negative controls, respectively. The test substance and negative control were tested in duplicate. The (b) (4) tissue was conditioned to the assay media prior to exposure to test or control substances. Each substance was applied directly to the surface of duplicate tissue matrices. Treated tissues were exposed to the test or control substances for 30 minutes and rinsed thoroughly with DPBS. Rinsed tissues were incubated for approximately 2 hours, followed by MTT exposure for 3 hours. The tissues were then extracted with isopropanol in a refrigerator overnight for approximately 18 hours. The resulting extraction solution from each tissue was sampled and optical density (CD) measured using a spectrophotometer. The relative viability of each treated tissue was calculated based on the mean CD compared to that of the negative control treated tissue. Under the conditions of this study, Phytase B is not considered an eye irritant. Based on the results of this study, Phytase B meets the requirement for UN GHS Category: No Category.

E. Local Lymph Node Assay (LLNA) in Mice (b) (4))

The assay was conducted according to OECD Guideline Test No. 429 (2010). A dermal sensitization test was conducted with mice to determine the potential for Phytase B to produce sensitization after repeated topical applications. Two concentrations (25% and 50%) of the test substance in 1% (b) (4) Surfactant w/w in distilled water (1% (b) (4)) and the neat test substance were topically applied to fifteen healthy test mice (5 mice/group) for three consecutive days. Three days after the last application, the mice were given an IV injection containing 20 µCi of ^{3}H-methyl thymidine. Approximately five hours later, all animals were euthanized via an overdose of inhaled Isoflurane and the draining (auricular) lymph nodes were harvested and prepared for analysis in a scintillation counter. The results are presented in disintegrations per minute per mouse (dpm/mouse). Each animal's ears were also evaluated for erythema and edema prior to each application and again on Day 6, prior to the IV injection. A vehicle control group (five animals) and a positive control group (five animals) were maintained under the same environmental conditions and treated in the same manner as the test animals. The vehicle control animals were treated with 1% (b) (4) and the positive control group animals were treated with a 25% w/w mixture of HCA. in 1% (b) (4). To reduce the total number of animals used, this study was run concurrently with another study to utilize a common positive control and common vehicle control group. Based on the results of this study, the test substance is not considered to be a contact dermal sensitizer in the LLNA. Proper conduct of the LLNA was confirmed via a positive response with 25% RCA, a moderate contact sensitizer.

Genencor International B.V.
Archimedesweg 30
2333 CN Leiden
The Netherlands

F. EpiDerm™ Skin Irritation Test (SIT) ([redacted])

The assay was conducted according to OECD Guideline for Test No. 439 (2015). The in vitro [redacted] skin irritation test (SIT) was performed using three-dimensional reconstructed human epithelium tissue obtained from [redacted]. The assay measures the destruction of skin cells in the tissue, one component predictive of skin irritation. The decrease in viability of tissue exposed to test substance(s) in comparison to those treated with a negative control is measured using a colorimetric assay involving the reduction of (3-4,5-dimethyhhiazol-2-yl)-2,5-diphenyltetrazolium bromide (MIT) to formazan to predict their skin irritation potential. Nine viable tissue constructs were randomly allocated to three groups containing three matrices each; one test substance treated group, one vehicle control treated group and one positive control treated group. Dulbecco's phosphate buffered saline (DPBS) and 5% Sodium dodecyl sulfate (5% SDS) were used as the negative and positive controls, respectively. The [redacted] Tissue inserts were conditioned to the assay media prior to exposure with lest and control substances. Each substance was applied directly to the surface of the tissue matrices in triplicate. Treated tissues were exposed to the test or control substances under standard CO_2 incubation conditions ['-37°C (5% CO_2)] then moved to ambient temperature for a total exposure time of 60 minutes. The tissue inserts were rinsed thoroughly with DPBS. placed in fresh assay medium and further incubated for approximately 45 hours with a change of media after 26 hours. Following post-exposure incubation, the tissue inserts were transferred to a 24-well plate with wells containing 300 ul of MET medium and incubated for 3 hours at —37°C (5% CO_2). The tissues were then rinsed with DPBS and extracted with isopropanol for 2 hours at room temperature while agitating. The resulting extraction solution from each tissue was sampled and evaluated for optical density (OD) using a spectrophotometer. The relative viability of each treated tissue was calculated based on the mean OD compared to that of the negative control treated tissue. The positive control group demonstrated a decrease in viability which was acceptable for the assay. Under the conditions of this study, Phytase B is considered a non-irritant. Based on the results of this study, Phytase B meets the requirement for UN GI-IS Category: No Category.

G. Acute Oral Toxicity Study ([redacted])

The study was conducted according to OECD Guideline 423 (2001). An acute oral toxicity study (Acute Toxic Class Method) was conducted with rats to determine the potential for Phytase B to produce toxicity from a single dose via the oral route. Under the conditions of this study, the acute oral LD50 of Phytase B was greater than 5000 mg/kg of body weight in female rats.

September 2, 2021

Megan Hall, M.S.
U.S. Food and Drug Administration
Center for Veterinary Medicine
Division of Animal Feeds, HFV-220
12225 Wilkins Avenue
Rockville, Maryland 20852

RE: Amendment to GRAS Notice No. AGRN 43 REF# GRAS M-000101-Z0004

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Dear Ms. Megan Hall,

Thank you for the comments and additional questions via the August 6, 2021 teleconference call between Center for Veterinary Medicine and Danisco US Inc. to discuss GRAS Notice AGRN 43. Please find our response below addressing the questions highlighted on the August 6th call and later provided in the meeting minutes on August 12, 2021 via email. We have included your questions below in *italics* along with our response for convenience. Note, in this document additional appendices are provided and referenced throughout. In the case where an appendix in the AGRN 43 already provided is referenced, this will be specified.

Several appendices have been marked "CBI" at the end of this document to indicate they are confidential business information that is deemed exempt from disclosure under the Freedom of Information Act (FOIA; 5 U.S.C.§552). In some of these appendices the files themselves may not be marked confidential. IFF would like to emphasis however the CBI consideration should include all those appendices marked as "CBI" at the end of this letter. In addition, specific sections of this response have been highlighted and in red font to indicate CBI sections.

Chemistry, Manufacturing and Controls (CMC)

1. *CVM stated that the notifier should provide concentration of the substrate in the definition of one phytase unit (FTU) of its enzyme. The notifier equated specific activity of* (b) (4) *the notifier should provide information on how they determined TOS along with relevant formula/equation and calculation they used for TOS. The notifier should provide information on how they determined TOS for test article(s) and other articles, as applicable, along with calculation.*

Health & Biosciences
Animal Nutrition –
Danisco US Inc
925 Page Mill Rd/
Palo Alto, CA
94304
T 650-799-0871
iff.com

The notified substance, phytase from *T. reesei* expressing a gene encoding a variant of the consensus bacterial phytase, is to be used at (b)(6). (b) (4). (b) (9) feed to increase the digestibility of phytin-bound phosphorus in poultry and swine diets.

One phytase unit (FTU) in this notice has been defined as the amount of enzyme that released 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C.

Per FDA guidance for food enzymes (FDA/CFSAN Division of Biotechnology and GRAS Notice Review, 2010), exposure should be calculated as Total Organic Solids (TOS). The calculation provided in the Guidance document and included below was used to convert the intended maximum use rate of (b) (4) to TOS for this phytase.

TOS is the sum of all organic compounds present in the enzyme preparation derived from the enzyme source and manufacturing process. TOS is calculated as follows:

TOS (%) = 100 - A - W - D

Where:
A = % ash
W = % water
D = % diluents and other formulation ingredients

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The 90-day toxicity study referenced in Ladics, 2020 included in appendix 11 of the AGRN 43 Notification utilized the attached (appendix 1) certificate of analysis (COA) of the (b) (4) of this notified phytase to characterize the test article in the study. This COA is attached in appendix 1 for your convenience.

The (b) (4)) was analyzed by Silliker Laboratories with the following results. The ash method used by Siliker is based on the AOAC Method No. 935.42, and a copy titled "Ash of Cheese" is included in appendix 2. (b) (4) is utilized as the concentrated enzyme ingredient, which then is blended into the various formats for commercial sale.

(b) (4)

In addition to the TOS value described above the (b) (4) test article contained (b) (4) per gram of test material. The phytase activity was measured using the same method utilized in the specification tables in response 2.

With this information, (b) (4)

This TOS conversion was utilized in the GRAS notice to conduct the exposure assessment in TOS.

For example, (b) (4) mg TOS/kg feed.

The maximum recommended use levels in mg TOS/kg feed for the applications where the phytase may be used is (b) (4) TOS/kg feed, equivalent to (b) (4). For purposes of feeding directions and label guarantees FTU/kg will be used.

Specifications

2. *The notifier provided certificates of analysis (COAs) for specifications for Axtra PHY GOLD 65G. The notifier should clarify the identity of Axtra PHY GOLD 65G relative to the notified substance. The notifier should provide a specification table for each of the market formulations, listing the specification parameters along with the specification limits, units, average result obtained, as applicable, and the methods used for analysis of each parameter. If the notifier used an official method, then it should provide the method number/name. If in-house/third party methods were used, then the notifier should indicate that accordingly. The notifier should also provide validation information as appropriate for in-house/third party methods.*

Axtra PHY GOLD 65G is a commercial product formulation that contains the notified substance as the source of phytase. A certificate of composition for this product is provided in appendix 3, which includes the full formula of the product, of which the notified substance is an ingredient. Axtra PHY GOLD 65G is a granular market formulation. In addition, a certificate of composition has been provided for Axtra PHY GOLD 30T (appendix 4) and Axtra PHY GOLD 30L (appendix 5), which are additional market

formulations of the thermostable granular and liquid forms, respectively containing the notified substance as an ingredient.

Specification tables for each of the market formulations (Axtra PHY GOLD 65G, Axtra PHY GOLD 30T, and Axtra PHY GOLD 30L) are provided below. In addition, three CoAs for each formulation are provided in appendix 6 confirming that each market formulation meets the designated specifications provided in the tables. The methods of analysis used for each parameter are indicated in the specification tables below. All methods, with the exception of phytase activity and absence of production strain, are published methods. The in-house method for phytase activity is provided in appendix 7 and the validation for this method is provided in appendix 8. For absence of production strain, see our response to CVM's question 5.

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PRODUCT SPECIFICATION TABLE

Product Name: Axtra® PHY GOLD 30 L

Material code: A14171

ASSAY	UNIT	SPECIFICATION	Reference test Method / Method Description
ENZYME ACTIVITY 6-Phytase	FTU/g	≥30,000	In-House method: Animal feed additives – determination of phytase activity – Colorimetric method (F_029/02)
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g	0-500000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Coliforms	CFU/g	0-30	ISO 4832 and FDA Bacteriological Analytical Manual; 8th edition; AOAC International
E. coli	/25g	Negative by test	Method 1 – ISO 7521 Method 2 - FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Salmonella	/25g	Negative by test	Method 1 – Nordic Committee on food Analysis; Salmonella Bacteria Detection in Foods No 71; 4th Edition 1991; Method 2 - FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Production Strain	/g	Negative by test	In house method; R-DOC-GM-2006
PHYSICAL PROPERTIES			
Specific gravity	g/ml	1.0 – 1.25	Determined at 20°C using oscillating U-tube densitometer
OTHER ASSAYS			
Lead	mg/kg	0-5	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Arsenic	mg/kg	0-2	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Cadmium	mg/kg	0-0.5	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Mercury	mg/kg	0-0.1	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)

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PRODUCT SPECIFICATION TABLE

Product Name: Axtra® PHY GOLD 30 T

Material code: A14168

ASSAY	UNIT	SPECIFICATION	Reference test Method / Method Description
ENZYME ACTIVITY 6-Phytase	FTU/g	≥30,000	In-House method: Animal feed additives – determination of phytase activity – Colorimetric method (F_029/02)
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g	0-500000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Coliforms	CFU/g	0-30	ISO 4832 and FDA Bacteriological Analytical Manual; 8th edition; AOAC International
E. coli	/25g	Negative by test	Method 1 – ISO 7521 Method 2 - FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Salmonella	/25g	Negative by test	Method 1 – Nordic Committee on food Analysis; Salmonella Bacteria Detection in Foods No 71; 4th Edition 1991; Method 2 - FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Production Strain	/g	Negative by test	In house method; R-DOC-GM-2006
PHYSICAL PROPERTIES			
Bulk Density	Kg/l	1.0 – 1.5	Determination of weight of granulated sample per volume (ASTM D7481)
OTHER ASSAYS			
Lead	mg/kg	0-5	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Arsenic	mg/kg	0-2	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Cadmium	mg/kg	0-0.5	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Mercury	mg/kg	0-0.1	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)

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PRODUCT SPECIFICATION TABLE

Product Name: Axtra® PHY GOLD 65 G

Material code: A14165

ASSAY	UNIT	SPECIFICATION	Reference test Method / Method Description
ENZYME ACTIVITY 6-Phytase	FTU/g	≥65,000	In-House method: Animal feed additives – determination of phytase activity – Colorimetric method (F_029/02)
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g	0-500000	ISO 4833 and FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Coliforms	CFU/g	0-30	ISO 4832 and FDA Bacteriological Analytical Manual; 8th edition; AOAC International
E. coli	/25g	Negative by test	Method 1 – ISO 7521 Method 2 - FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Salmonella	/25g	Negative by test	Method 1 – Nordic Committee on food Analysis; Salmonella Bacteria Detection in Foods No 71; 4th Edition 1991; Method 2 - FDA Bacteriological Analytical Manual; 8th edition; AOAC International
Production Strain	/g	Negative by test	In house method; R-DOC-GM-2006
PHYSICAL PROPERTIES			
Bulk Density	Kg/l	0.8 – 1.2	Determination of weight of granulated sample per volume (ASTM D7481)
OTHER ASSAYS			
Lead	mg/kg	0-5	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Arsenic	mg/kg	0-2	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Cadmium	mg/kg	0-0.5	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)
Mercury	mg/kg	0-0.1	Determination of heavy metals and minerals in food and feed matrices by means of Inductively coupled Plasma Mass Spectrometry (ICPMS) after microwave digestion (AS-CC-012)

3. *The notifier should provide 3 representative COAs, including enzyme activity, each for each of the 3 market formulations. The notifier should clarify what U/g stands for in the provided COAs and if U/g is equivalent to FTU/g.*

The U/g listed on the CoAs provided previously for Axtra PHY GOLD 65G is equivalent to FTU/g. One phytase unit (FTU) was defined as the amount of enzyme that released 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C.

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Three representative COAs, including enzyme activity, each for each of the 3 market formulations has been provided in appendix 6. Note, the FTU/g expressed on each CoA has the same definition as in the previous paragraph.

4. *The proposed specification limits are not supported by the 3 batch analyses provided for some of the parameters, for example, total viable count specification is listed as 0-500000 CFU/g even though <1000 CFU/g was reported for each of the 3 batches of COAs provided and similar high specification limits was noted for other parameters such as the heavy metals (lead, arsenic, cadmium, and mercury) where the 3 batch analyses did not support the proposed specification limit. The notifier should adjust the specification limit to reflect the values obtained from multiple analyses or justify the high value in the specification limit of these parameters.*

As per the Enzyme Marketing Coordination document in the 2021 AAFCO Official Publication (OP), our enzyme preparation complies with the chemical and microbiological purity standards established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), European Commission Directive on undesirable substances in animal food, and Food Chemical Codex (FCC). The reference standard and reference specification for each relevant is included in the table below. The FCC Enzyme Monograph is included in appendix 9, JECFA Compendium of Food Additive Specifications in appendix 10 for reference. The JECFA guidance document stipulates: "Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food." Our total viable count (TVC) specification is based on this JECFA guidance, taking into account that certain batches of solid raw materials may have higher and more variable TVC counts inherent to those materials while still being acceptable for use in food. Production strain absence specifications are primarily internally driven to ensure that the strains, and Intellectual Property (IP) associated with the strains are not released.

Assay	Unit	Specification	Standard
Total Viable Count	CFU/g	0-500000	JECFA (2006): Enzyme preparations should cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.
Coliforms	CFU/g	0-30	FCC Enzyme Monograph (2021)
E. coli	/25g	Negative by test	JECFA (2006)
Salmonella	/25g	Negative by test	FCC Enzyme Monograph (2021)
Production Strain	/g	Negative by test	
Lead	mg/kg	0-5	FCC Enzyme Monograph (2021)
Arsenic	mg/kg	0-2	DIRECTIVE 2002/32/EC on undesirable substances in animal feed
Cadmium	mg/kg	0-0.5	DIRECTIVE 2002/32/EC on undesirable substances in animal feed
Mercury	mg/kg	0-0.1	DIRECTIVE 2002/32/EC on undesirable substances in animal feed

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5. *The notifier should provide information on how it determined the absence of live production organisms, including detection method and related information, for example, how LOD was determined. The notifier should address, as appropriate, any contaminants, toxins of concern etc. that may be present and provide the appropriate validated methods, as needed.*

To confirm absence of the live production organism in the enzyme ingredient a (b) (4) method is used. (b) (4)

Three CoAs to support this on the phytase ingredient are included in appendix 11. The test substance for these CoAs is the phytase (b) (4) which is utilized in all product formulations. The in-house method utilized is R-DOC-GM-2006 which has been included in appendix 12.

The limit of detection for this method is determined based on the (b) (4)

With respect to any potential contaminants or toxins of concern which may originate from the production strain please refer to the response to question 20.

Manufacturing Process

6. *The notifier should provide a narrative of the manufacturing process clearly addressing the process conditions for sterilization conditions, scale of fermentation, growth medium description, composition of media, pH, temperature, duration, purification, recovery, etc., and control points.*

The notified substance, phytase from *T. reesei* expressing a gene encoding a variant of the consensus bacterial phytase, is manufactured by fermentation of a genetically engineered *Trichoderma reesei*, expressed as a variant of consensus bacterial phytase.

The enzyme feed additive manufacturing process is controlled by a number of documented procedures, which comply with the ISO 9001 quality standard. The enzyme preparation is also manufactured in accordance with FDA's current Good Manufacturing Practices ("cGMP") and with FDA's requirements under the Food Safety Modernization Act. Quality control of raw materials, intermediates and finished goods is carried out according to the management system requirements. Documented procedures and records are used and maintained to control the processes. All unit operations in the process are monitored and kept within set limits. Several quality controls are made during the production as well as every batch produced is subjected to quality control analyses prior to its release to sale. The production process uses standard methods employed in the enzyme industry for fermentation, recovery, concentration and standardization of enzyme preparations. The manufacturing process is a two-part process consisting of submerged fermentation and recovery.

Raw Materials

The raw materials used in the fermentation of the production strain for this phytase concentrate are standard ingredients used in the enzyme industry (Kirk et al., 2005; Aunstrup, 1979 and Aunstrup et al., 1979). All the raw materials used have appropriate specifications given intended use in animal feed. IFF uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Note, while all materials and inputs are not called out specifically in the description below, they are all provided in the response to question 7 separated by manufacturing step as requested.

Fermentation

The phytase enzyme is manufactured by submerged fermentation of a pure culture of the genetically engineered strain of *T. reesei* as described in Part 2 of AGRN 43. The fermentation process consists of (b) (4)

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All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. Prior to inoculation with the production strain the fermentation media is sterilized (described below) through (b) (4) During the various stages of fermentation, the raw material feeds are also sterilized using the same approach.

Sterilization of the fermentation broth and feed source uses (b) (4) . Cell death occurs at (b) (4) . Steam is utilized in this process as opposed to dry sterilization. In dry sterilization air acts as an insulator reducing the heat transfer coefficient of the exchange between the spores environment and the spore itself resulting in a slower cell kill. The use of steam allows for faster sterilization as well as lowering the partial pressure of steam in the system, reducing the required temperature.

Cell death within the sterilizers is measured by the Fo value. Fo is an equivalent exposure time at 121.1°C of the actual exposure time (τ, or the residence time in minutes) at the actual temperature (T, in °C), with a temperature coefficient of destruction (Z value) of 10 (typical for the model organism). A Fo value of 1 would be a one minute residence time at 121.1°C, and is a 90% reduction in viable organisms.

$$F_o = \tau 10^{\frac{\tau - 121.1}{Z}}$$

Minimum Fo value used in the sterilization of the fermentation media is (b) (4) with typical being greater than (b) (4) A Fo value of (b) (4) means that a spore would have a (b) (4) chance of survival given an initial background of one million ($1x10^6$) spores. As the Fo value increases the chance of cell survival decreases.

A new lyophilized stock culture vial of the *T. reesei* production organism as described in Part 2 of AGRN 43 is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use. During the main fermentation in the controlled environment on specific growth medium growth characteristics are observed microscopically. Fermentation is performed at a pH range from (b) (4) and a temperature from (b) (4) for a duration of approximately (b) (4). Samples are taken from each fermentation stage before inoculation, at regular intervals during growth and before harvest or transfer to monitor various process parameters, including enzyme activity. The procedure and controls used to obtain samples from the fermenter are equipped and designed to prevent microbial contamination of the fermentation, and to minimize the environmental release and exposure of the producing organism to employees.

Recovery (cell separation and concentration)

Once the fermentation is completed, the fermentation broth enters the recovery process. The primary purpose of the recovery process is to separate the biomass and to concentrate and stabilize the desired enzyme activity level.

In the cell separation step, the production microorganisms are separated from the enzyme containing liquid. The fermentation broth is held at temperature (b) (4) and pH (b) (4) controlled conditions, waiting for cell separation to take place. The post-fermentation broth hold time is optimized and as a result any potential cell lysis is limited. The typical hold time is (b) (4) The broth then goes through a filtration step such as (b) (4) Certain process chemicals and flocculating agents are added at various concentrations to maximize biomass separation from the enzyme containing filtrate. Producing a relatively solids-free stream which contains the notified enzyme (filtrate) is the purpose of this filtration.

All waste biomass from the separation process is inactivated using combinations of heat, pH or other chemical inactivation steps to ensure the killing of all living production microorganisms. The enzyme containing filtrate passes through (b) (4) moving to the concentration stage.

The enzyme containing filtrate after cell separation is then concentrated via (b) (4) The concentration occurs simultaneously with (b) (4) The filtrate containing the notified

[1] (b) (4)

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substance then (b)(6) . The notified substance is stabilized with various chemicals such as (b)(6) . The pH is controlled to (b)(6) o produce the phytase intermediate called (b) (4) at target specifications.

No antimicrobial substances, such as antibiotics, are used in fermentation and recovery steps of the manufacturing process.

All commercial product formulations contain the same (b) (4) enzyme preparation from which all solids including the production organism are removed as described above.

Liquid Formulation (Axtra PHY GOLD 30L)

(b) (4) from the above recovery step is pH adjusted to (b) (4) and held at temperature for (b) (4) . Formulation ingredients are added to the (b) (4) to target concentrations. (b) (4). The formulated (b) (4) then undergoes (b) (4) . In this (b) (4) . Enzyme activity is tested (b) (4) Water is added to the formulated (b) (4) after (b) (4) to achieve the desired final activity and formulation chemical specifications. The final formulated material is mixed (b) (4), and a final sample is pulled for QA/QC inspection and release.

Solid Formulation and Packaging (Granulation of Axtra PHY GOLD 30T, 65G)

Using the (b) (4) from the above recovery step, the notified enzyme is mixed with (b) (4) The final product is sieved to the desired particle size and the product is QA/QC tested and released upon passing.

7. *The notifier should provide a table listing the raw ingredients categorized by each manufacturing process stage, like production tank, recovery, formulation, etc. and describe which ingredient is used for which part of the manufacturing process such that we can understand at which stage of the manufacturing process these ingredients are added, for example, in market formulation or fermenter. CVM notes that the notifier referenced FDA correspondence regarding defoamers/flocculants; the notifier should specify which defoamers/flocculants are used in the manufacturing process. The notifier should provide information on each market formulation, including clearly stating each ingredient and its quantity used in each of these formulations.*

Raw materials used in the manufacture are listed below categorized by each manufacturing process stage as described in response 6. All information provided in the table below is considered CBI.

Each market formulation with ingredient and quantity used in the formulations is provided in appendix 3-5.

Entire Table is Confidential Business Information (CBI)

(b)(6), (b) (4)

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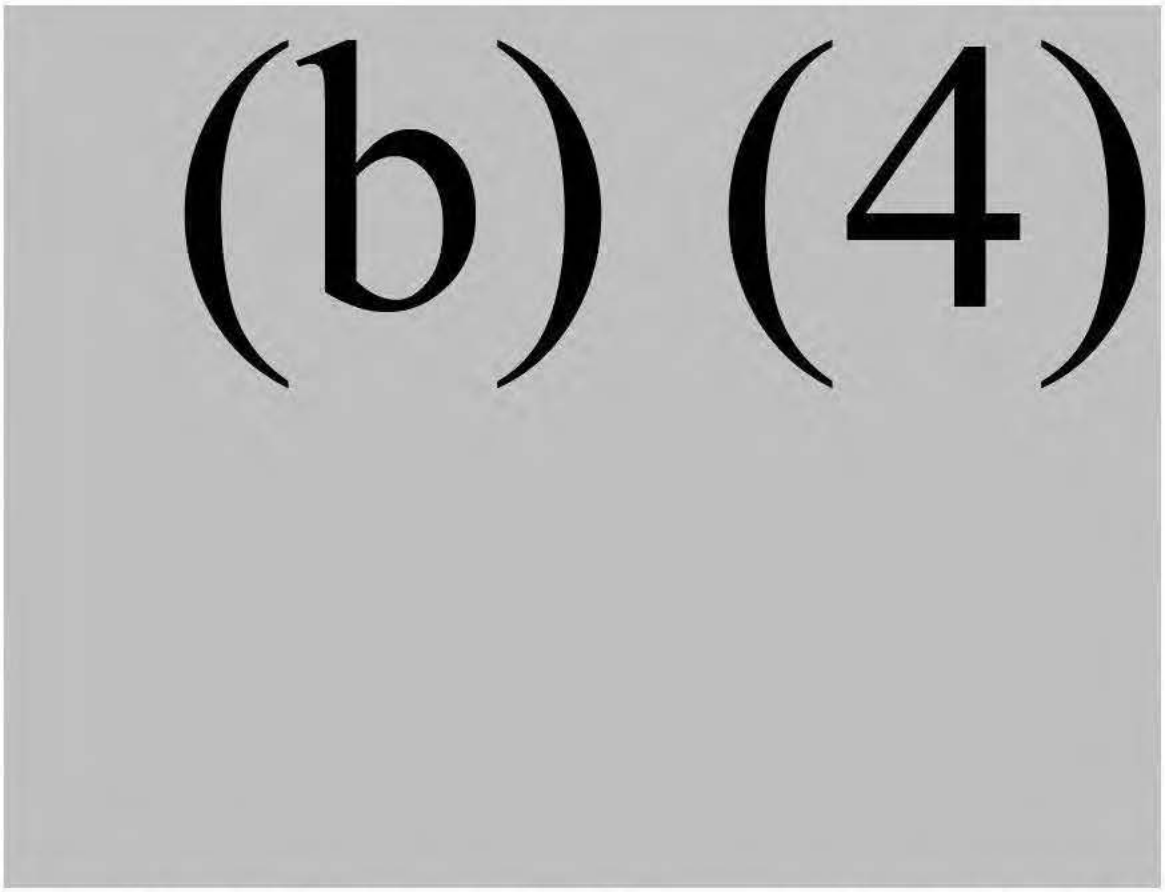
(b) (4)



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8. *The notifier should provide description of the packaging used for the substance. CVM recommended that the notifier refer to GFI #221 and ICCF #04 for further guidance.*

Final enzyme will be formulated with suitable carriers to achieve desired enzyme concentration in commercial product as described in the response to question 7. The commercial product will be packaged into 25kg Paper/ high density polyethylene bags, 200kg high density polyethylene plastic drums, or 1000kg high density polyethylene totes depending on the needed size and formulation with the Liquid products being in totes or drums. These are standard packaging materials suitable for the type of product. Once packaged the commercial label will be applied to the product meeting AAFCO OP 2021 Enzyme Marketing Coordination requirements as well as FDA 21 CFR 501. Example label for each product type is included in appendix 13.

Stability and Homogeneity

9. *CVM stated that the notifier should provide stability information for the notified substance. The notifier should provide stability for three batches each of each formulation of its enzyme and three batches each of each applicable formulation in premix, in feed, and in pelleted feed with suitable time points and conditions unless the notifier can provide suitable justification on why some of these may not be needed. Typically, the stability of premix is for at least six months and for feed at least three months. The notifier should ensure that the conditions used for the studies are representative of the conditions in the United States.*

Stability studies have been provided on three commercial formulations of the phytase product in each format, Axtra PHY GOLD 65G (appendix 3 composition) is a granular market formulation, Axtra PHY GOLD 30T (appendix 4 composition) and Axtra PHY GOLD 30L (appendix 5 composition) which are market formulations of the thermostable granular and liquid market formulation respectively. All three formulations contain the same phytase ingredient subject of this notification.

Stability of the phytase activity in the marketed product, animal feed (mash and pellet as appropriate), premix, and processing as applicable is included in the appendices and discussed below. In addition,

in-house phytase activity methods of analysis used in each study and their associated validation has been included. Refer to the table below for a listing of the provided appendixes.

Product	Study Type and Appendix	Method Used and Appendix	Method Validation and Appendix
Axtra PHY GOLD 30T	12 month stability in product Appendix 14	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_029/02) Appendix 7	Validation - Determination of phytase activity in animal feed additives - Colorimetric method (F_029/02) Appendix 8
Axtra PHY GOLD 30L	12 month stability in product Appendix 15	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_029/02) Appendix 7	Validation - Determination of phytase activity in animal feed additives - Colorimetric method (F_029/02) Appendix 8
Axtra PHY GOLD 65G	12 month stability in product Appendix 16	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_029/02) Appendix 7	Validation - Determination of phytase activity in animal feed additives - Colorimetric method (F_029/02) Appendix 8
Axtra PHY GOLD 30T	6 month stability in premix Appendix 17	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_030/03) Appendix 18	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 65G	6 month stability in premix Appendix 20	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_030/03) Appendix 18	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 30T	3 month stability in pellet feed - swine Appendix 21	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 30T	3 month stability in pellet feed - poultry Appendix 23	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 30L	3 month stability in pellet feed - swine	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08)	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08)

	Appendix 24	Appendix 22	Appendix 19
Axtra PHY GOLD 30L	3 month stability in pellet feed – poultry Appendix 25	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 65G	3 month stability in pellet feed - swine Appendix 26	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 65G	3 month stability in pellet feed – poultry Appendix 27	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 30T	3 month stability in mash feed - swine Appendix 28	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 30T	3 month stability in mash feed - poultry Appendix 29	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 65G	3 month stability in pellet feed - swine Appendix 30	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 65G	3 month stability in pellet feed – poultry Appendix 31	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 30L	Processing stability in feed Appendix 32	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19

Axtra PHY GOLD 30T	Processing stability in feed Appendix 33	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19
Axtra PHY GOLD 30G	Processing stability in feed Appendix 34	Animal feed additives – Determination of phytase activity – Colorimetric method" (F_031/08) Appendix 22	Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08) Appendix 19

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Enzyme Additive Stability in Product

The stability of the notified substance was determined by measuring the phytase activity of three batches of Axtra® PHY GOLD 30L, Axtra® PHY GOLD 30T and Axtra® PHY GOLD 65G , stored at (b) (4) in packaging that is representative of the commercial conditions for 12 months. Samples were taken at time points 0, 3, 6, and 12 months and the stability, as defined by phytase activity, was determined. The mean percent recovery of phytase activity at both (b) (4) is provided in the table below, in support of 12 month product stability at (b) (4)

Product	Percent recovery mean at (b) (4) at 12 months
Axtra® PHY GOLD 30 L	(b) (4)
Axtra® PHY GOLD 30T	
Axtra® PHY GOLD 65G	

Enzyme Stability in Pre-mix

Three batches of Axtra® PHY GOLD 30 T and Axtra® PHY GOLD 65 G were mixed with a commercial ready-to-use vitamin/mineral premixture for poultry. The 6-phytase inclusion level was (b) (4) premixture (average of three batches) for Axtra® PHY GOLD 30 T and 65 G, respectively. After storage at (b) (5) for 6 months, the average loss in phytase activity was (b) (4) for Axtra® PHY GOLD 30 T and Axtra® PHY GOLD 65 G, respectively. The results demonstrate that Axtra® PHY GOLD 30 T and 65 G when mixed into a vitamin/mineral premixture are stable up to 6 months at (b) (4)

Axtra® PHY GOLD 30 L was not included in the stability testing for premix as it is not practical for use in premix and will be labeled as such.

Enzyme stability in pelleted feed

Axtra® PHY GOLD 30 L

Axtra® PHY GOLD 30 L Batch 2019L001 was used for storage stability in pelleted feeds for piglets, sows, laying hens and broilers. After 3 months storage at (b) (4), the phytase activity in the pelleted feed diets were measured with an average activity loss of (b) (4) was found. These studies demonstrate the stability of Axtra® PHY GOLD 30 L in pelleted feeds when stored for 3 months at (b) (4)

Axtra® PHY GOLD 30 T

Axtra® PHY GOLD 30 T Batch 3099275159 was used for storage stability in pelleted feeds for piglets, sows, laying hens and broilers. After 3 months storage at (b) (4) the phytase activity in the

pelleted feed diets were measured and average activity loss of (b) (4) was found. These studies demonstrate the stability of Axtra® PHY GOLD 30 T in pelleted feeds when stored for 3 months at (b) (4)

Axtra® PHY GOLD 65 G

Axtra® PHY GOLD 65 G Batch 3099281163 was used for storage stability in pelleted feeds for piglets, sows, laying hens and broilers. After 3 months storage at (b) (4), the phytase activity in the pelleted feed diets were measured and average activity loss of (b) (4) was found. These studies demonstrate the stability of Axtra® PHY GOLD 65 G in pelleted feeds when stored for 3 months at (b) (4)

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Enzyme Stability in Mash feed

One batch for each formulation of Axtra® PHY GOLD (30T and 65G) was tested for storage stability in four animal feeds (piglets, sows, laying hens and broilers). Stability was determined by measuring the loss in phytase activity over time.

Note, this assessment was not done for Axtra® PHY GOLD 30 L as only the dry formulations will be used in this application.

Axtra® PHY GOLD 30 T

Axtra® PHY GOLD 30 T Batch 3099275159 was used for storage stability in feeds for piglets, sows, laying hens and broilers. After 3 months storage at (b) (4)), the phytase activity in the mash feed diets were measured and average activity loss of (b) (4) was found. Thus, these studies support the stability of Axtra® PHY GOLD in mash feed when stored at (b) (4) during a time period of 3 months.

Axtra® PHY GOLD 65 G

Axtra® PHY GOLD 65 G Batch 3099281163 was used for storage stability in feeds for piglets, sows, laying hens and broilers. After 3 months storage at (b) (4)), the phytase activity in the mash feed diets were measured and average activity loss of (b) (4) was found. Thus, these studies support the stability of Axtra® PHY GOLD in mash feed when stored at (b) (4) during a time period of 3 months.

Stability of the enzyme during processing (pelletizing)

Axtra® PHY GOLD 30 L

Axtra® PHY GOLD 30 L Batch 2019L001 was tested for demonstrating the stability of the additive in feed pelleting at [redacted]. Axtra® PHY GOLD 30 L was mixed into feed for piglets, sows, laying hens and broilers to achieve a total phytase activity of approximately 400 - 830 FTU/kg feed, and then pelleted at (b) (4). The pelleting stability in feeds pelleted at (b) (4) was then evaluated by comparing phytase activity from the pelleted data to that of the feed for the initial time point. The results are shown in the table below.

Pelleted feeds had recoveries for the 6-phytase between (b) (4) hence demonstrating stability to pelleting at (b) (4).

Pelleting stability of Axtra® PHY GOLD 30 L in feeds (pelleting at (b) (4)

Phytase Activity (FTU/kg feed)				
Batch 2019L008341	Feed for Broilers	Feed for Laying Hens	Feed for Sows	Feed for Piglets
Initial	(b) (4)			
Pelleted (b) (4)				
Recovery %				

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Axtra® PHY GOLD 30 T

Axtra® PHY GOLD 30 T Batch 3099275159 was tested for demonstrating the stability of the additive in feed pelleting at (b) (4) Axtra® PHY GOLD 30 T was mixed into feed for piglets, sows, laying hens and broilers to achieve a total phytase activity of approximately 400-820 FTU/kg and then pelleted at (b) (4). The pelleting stability in feeds pelleted at (b) (4) was then evaluated by comparing phytase activity from the pelleted data to that of the feed for the initial time point. The results are shown in the Table below.

Recoveries for the 6-phytase were between (b) (4) hence demonstrating stability to pelleting at (b) (4).

Pelleting stability of Axtra® PHY GOLD 30 T in feeds (pelleting at (b) (4)

Phytase Activity (FTU/kg feed)				
Batch 3099275159	Feed for Broilers	Feed for Laying Hens	Feed for Sows	Feed for Piglets
Initial	(b) (4)			
Pelleted (b) (4)				
Recovery %				

Axtra® PHY GOLD 65 G

Axtra® PHY GOLD 65 G Batch 3099281163 was tested for demonstrating the stability of the additive in feed pelleting at (b) (4). Axtra® PHY GOLD 65 G was mixed into feed for piglets, sows, laying hens and broilers to achieve a total phytase activity of approximately 500-1000 FTU/kg, and then pelleted at (b) (4). The pelleting stability in feeds pelleted at (b) (4) was then evaluated by comparing phytase activity from the pelleted data to that of the feed at the initial time point. The results are shown in the table below.

Recoveries for the 6-phytase were between (b) (4) hence demonstrating stability to pelleting at (b) (4)

Pelleting stability of Axtra® PHY GOLD 65 G in feeds (pelleting at (b) (4)

Phytase Activity (FTU/kg feed)				
Batch 3099281163	Feed for Broilers	Feed for Laying Hens	Feed for Sows	Feed for Piglets
Initial	(b) (4)			
Pelleted (b) (4)				
Recovery %				

10. *CVM stated that the notifier should provide inclusion rate of each of the three market formulations in the finished feed. The notifier may have to provide homogeneity of the substance in premix and/or feed depending on the level of inclusion. CVM recommended that the notifier may refer to GFI #221, ICCF #01, and ICCF #03 for further guidance.*

Axtra® PHY GOLD 65 G

Contains a minimum of 65,000 FTU/g phytase. The product should be used at a rate of 0.008-0.12 lbs/short ton (US) or 0.004-0.061 kg/tonne (0.0004-0.0061%) of finished feed, included either directly or via a premix.

This inclusion rate results in 260 – 3965 FTU/kg feed.

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Axtra® PHY GOLD 30 T

Contains a minimum of 30,000 FTU/g phytase. Use at a rate of 0.018-0.26 lbs/short ton (US) or 0.009-0.133 kg/tonne (0.009-0.0133%) of finished feed, included either directly or via a premix.

This inclusion rate results in 270 – 3990 FTU/kg feed.

Axtra® PHY GOLD 30 L

Contains a minimum of 30,000 FTU/g phytase. Use at a rate of 0.016-0.266 lbs/short ton (US) or 0.008-0.133 kg/tonne (0.0008-0.0133%) of finished feed.

This inclusion rate results in 270 – 3990 FTU/kg feed.

Homogeneity

The homogeneity of Axtra® PHY GOLD 30T and 65G when mixed into mash feed for swine and poultry was studied in feed by analyzing sets of ten sub-samples of the feed supplemented at approximately 500 -1000 FTU/kg feed. The coefficients of variation ranged between (b) (4) hence demonstrating homogeneous mixtures of Axtra® PHY GOLD 30 T and 65 G were obtained when Axtra® PHY GOLD 30 T and 65 G was included in mash feed. The results are provided in appendixes 35-38.

The method of analysis for phytase activity in the feed is provided in Appendix 22.

Analytical Method

11. *CVM stated that the notifier should provide a validated analytical method used to determine enzyme activity. The notifier should ensure that the method description clearly addresses the conditions of assay, including proper description of the substrate. The notifier should indicate the method used for other studies such as stability. The notifier should provide other analytical methods as appropriate. The notifier should clearly state if any of the analytical methods used are official methods and provide the method name/number. If the notifier used an in-house/third party method, then the method validation should be provided. CVM stated that to support utility the notifier should provide validation of the plate assay method from Yu et. al. 2012 that was referenced in the paper for poultry and was used to analyze "Assay of Phytase from Feed Samples" and provide a copy of the J. AOAC Int 1994 referred to in the paper for utility in swine. The notifier provided different methods and enzyme definitions for enzyme activity in published articles that relate to pivotal papers for swine and poultry. The notifier should address the suitability of the methods and provide appropriate validation as applicable. CVM recommended that the notifier refer to GFI #64 for further guidance regarding method validation information for any in-house/third party method that can be provided in the amendment.*

Refer to question 9 response for a list and associated appendices of the in-house methods used and validation as it relates to stability.

The CoAs provided typically used official methods for each assay. The method names and numbers are clearly provided in each specification table included in response 2. Two in-house methods have been utilized in the CoAs, phytase activity and detection of production strain. These methods have been provided in Appendix 7 and Appendix 12 respectively.

Method used in functionality studies

In both the Dersjant-Li (2020a,b) papers demonstrating functionality in swine and poultry the method used to analyze phytase activity in the feed is the in-house method provided in Appendix 22 with validation in Appendix 19.

In the paper Dersjant-Li (2020a) discussing the broiler study, analysis of exogenous phytase activity in feeds was described using the method in Yu et al. (2012). This description of the method used in Dersjant-Li (2020) broiler study was incomplete. Two methods were described in Yu et al. (2012) instead of one. One method is the semi-quantitative plate assay CVM refers to. The second method is the quantitative method described in detail in the Chemicals and Materials section of Yu et al. is based on the 2001 AOAC method (Engelen et al., 2001). This AOAC based method in Yu et al. (2012) is the method referenced by the Dersjant-Li (2020a) broiler study and is the in house method (Appendix 22).

A similar situation is also applicable to the Dersjant-Li (2020b) piglet study. In this paper we cited Engelen et al. (1994) which was a prior version of the AOAC method. This unfortunately is a mis quotation of the method as we used the same in-house method provided in Appendix 22 as published by Yu et al (2012), which was derived from the AOAC method.

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With respect to the definition of enzyme activity and the unit of one phytase unit (FTU) there is a typo in the two efficacy papers. In the papers it was listed as:

> One phytase unit (FTU) was defined as the amount of phytase that liberated 1 mmol of inorganic phosphate per minute from 0.0051 mol/L of sodium phytate at a standard pH of 5.5 and temperature of 37C.

However, as the methods used throughout to measure phytase activity are the same the correct FTU definition is provided below:

> One phytase unit (FTU) is defined as the amount of enzyme that released 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C.

12. *As a general note, the notifier should ensure that all analytical methods are suitable for the purpose. The notifier should be consistent throughout the narrative in the notice/amendment in the description of the enzyme, activity, units, etc., and in the method(s) used.*

This has been noted, future filings will be sure to maintain consistent description of the enzyme, activity, units, etc., and in the method(s) used in the narrative.

Intended Use and Use Rate

13. *The intended use of the notified substance and target animal species are inconsistently addressed in the notice. In section 1.4 of the notice, the intended use is stated as "to be used in swine and poultry diets" but elsewhere in section 1, use of the enzyme is stated to "increase the nutritional availability of phosphate in swine and poultry diets". In section 2.5.1 and 6.3.4, your firm clarifies that the phytase enzyme increases the availability of phosphorus from phytate from various plant-based ingredients used in animal feeds. In any amendment the firm should clearly state the intended use, including use rate and the purpose for which the notified substance will be used, presumably to increase the availability of phytate-bound phosphorus, and the specific target animal species.*

The notified substance, phytase from *T. reesei* expressing a gene encoding a variant of the consensus bacterial phytase, is to be used at (b) (4) feed to increase the digestibility of phytin-bound phosphorus in poultry and swine diets.

14. *A use rate in terms of the minimum amount of enzymatic activity required to achieve the intended use of the notified enzyme is not clearly discussed in the notice (sections 1.4 and 2.5.2). However, in section 6, the firm concludes that the inclusion of the notified substance at a level as low as 250 phytase units (FTU)/kg feed improves phytate phosphorus availability in swine and poultry diets. Phytase functionality is based on the minimum amount of enzymatic activity necessary for the enzyme to accomplish the intended use. The minimum amount of enzymatic activity/amount of feed required to achieve the intended use should be clearly stated in the notice and the units for this enzymatic activity defined. This information should also be incorporated into section 1.4.*

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The minimum amount of enzymatic activity/amount of feed required to achieve the intended use for the notified substance is 250 FTU/kg feed in both swine and poultry diets.

One phytase unit (FTU) is defined as the amount of enzyme that released 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C.

15. *The notice includes two published articles to support functionality of the notified phytase in poultry and swine. In the amendment, the firm should explain how the measures of enzymatic activity as defined in these publications and in various sections of the notice differ from each other and state which unit is used by the firm to measure the enzymatic activity present in the notified substance. In addition, the narrative of the notice should address how these differences in the measurement of enzymatic activity impact the intended use rate(s) of the notified substance.*

The measures of phytase activity in the published articles to support functionality of phytase in poultry and swine diets are the same as those used throughout the notice. One phytase unit (FTU) is defined as the amount of enzyme that released 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C in the product activity, stability studies, and functionality of the phytase in poultry and swine studies. The methods used to assess the phytase activity in product and applicable studies are the same as provided in response 9. As such, the use rates provided from the functionality studies can be used to in a one to one ratio to support the intended use rate of the notified substance in this GRAS notice of (b) (4) feed to increase the digestibility of phytin-bound phosphorus in poultry and swine diets.

16. *The phytase activity in the experimental diets utilized in the broiler study reported in Dersjant-Li, et al., 2020a, was determined using the methods described in Yu et al. 2012. CVM notes that Yu et al. 2012 describes this method as semi-quantitative yet based on the results reported in Dersjant-Li, et al., 2020a, it appears to be quantitative. This discrepancy should be addressed in the narrative of the amended notice.*

This discrepancy originates from incomplete description in the published articles as described in response 11. We did not used the semi quantitative plate assay method from Yu et al. but rather the quantitative AOAC method also described in Yu et al. The in-house method used in both publications (and provided in Appendix 22) is a quantitative method.

Functionality

17. *The analyzed phytase activities of diets supplemented with the notified substance in the swine study, reported in the publication Dersjant-Li, et al., 2020b, were 20% less and 27% more than the formulated values of 250 and 500 FTU/kg, respectively. The firm should address the discrepancy between the formulated and analyzed values and the relevance of this discrepancy when establishing a minimum use rate for the proposed notified substance.*

The analytical method used to measure activity in the feed has as acceptable reproducibility coefficient of variation of (b) (4) associated with it. In addition to assay reproducibility, there are a few other factors that may have contributed to the differences between analyzed phytase activity and

formulated values, such as incomplete homogeneity (ranging from (b) (4) in our homogeneity studies) and potential loss of enzyme activity during sample shipping and storage prior to analysis. Every effort is made to minimize these possible errors and use pooled representative samples however all errors cannot be removed.

Regardless of the measured and formulated value differences noted in the study as the analyzed value at the formulated 250 FTU is lower than the formulated and the results showed at this dose level the phytase improved bone ash vs NC, indicating this minimum effective dose of 250 FTU can be confirmed.

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18. *The bone ash percentage and ultimate force to break the metacarpi bones in pigs fed diets with 250 FTU/kg of the notified phytase were significantly higher than in negative control group. However, a significant increase in metacarpi bone phosphorus content compared to the negative control was observed only with inclusion of 500 FTU/kg notified phytase. Thus, there is a difference in the amount of enzyme needed to achieve a significant increase in bone ash vs. bone phosphorus content. The firm should provide a potential explanation for this unanticipated response.*

As clarified with CVM on the August 6th call, bone ash is the pivotal parameter in confirming functionality in vivo of phytase, and observations on bone P are, therefore, corroborative. In other words, bone P results need to be consistent with the bone ash findings, but they cannot and don't need to support functionality by themselves.

Bone ash is commonly used as the parameter for the phosphorus (P) release of phytase in typical phytase efficacy studies in swine and poultry. One of the reasons for this is that bone ash is a more sensitive parameter than specific mineral content in bone such as P content in bone and thus gives the best indication on the mineralization and P status in animals. Variation in bone P content is higher than that for bone ash. Regardless, the significant increase in bone ash with phytase addition is accompanied by meaningful numerical increases in both P and Ca between the Negative control and 250 FTU phytase treatment, so this is not inconsistent. Phytase is also known to make other minerals more bioavailable (Zeng, 2015), which may further elevate the sensitivity of bone ash as a marker for utility of phytase.

The numerical difference between negative control and the lowest dose (250 FTU) exceeded 3 x SEM (the variation for bone P across all treatment groups), which confirms a strong trend toward significance, consistent with the significant difference for bone ash, the pivotal parameter in establishing phytase functionality. In addition, there was no significant difference in bone P between the 250 FTE and 500 FTE treatments (even though only for the 500 FTE treatment the difference with NC was significant), which is an indicator that much of the improvement in bone P seen with 500 FTU is already achieved with 250 FTE, consistent, again, with the findings for bone ash.

Target Animal Safety

19. *The firm provided information of the in vitro characterization of the notified enzyme by citing the study by Christensen et al., 2020. The authors report the notified enzyme is not degraded by pepsin, and in the notice, the notifier states as part of the safety narrative, that the metabolic fate of the notified enzyme is break down into its constituent amino acids. The firm should address this discrepancy in the amended notice.*

Like all enzymes, the notified substance is a protein. As with typical protein digestion, proteolysis begins in the stomach where the primary proteolytic enzyme is pepsin. Protein digestion also occurs in the lumen of the intestine via a variety of proteolytic enzymes, which are secreted by the pancreas. These enzymes have a wide range of specificity thus degrading their substrates, such as the notified substance, info free amino acids as well as di- and tripeptides. In addition to the pancreas-secreted proteases, there is further digestion by proteases at the plasma membrane of the intestinal cells (Berg,

2002). In general, most proteases are relatively nonspecific for substrates with some targeting multiple substrates (Lopez-Otin, 2008).

Along with the endogenous digestive enzymes in the animals, throughout the intestinal system there will also be bacterial fermentation by the naturally occurring microbiome of the animal. This fermentation will also likely further break down the phytase into amino acids and smaller peptides.

The resulting single amino acids as well as the di- and tripeptides resulting from the digestion of the notified substance are transported into the intestinal cells from the lumen and released into the blood, as the degradation products from any other protein would be (Berg, 2002).

While it is true that the notified substance is largely resistant to pepsin in a 1-hour incubation at low pH resembling stomach conditions as reported in Christensen et al., 2020, the residual activity for the phytase was comparable to other commercial phytases (from *E. coli* and *Buttiauxella* sp.) under similar conditions, as discussed in the publication. As such, this is common for most phytases that rely on hydrolysis of phytic acid in feed for longer time than the short residence time in the stomach and under more neutral conditions.

Finally, any phytase protein that were to remain active and undigested in the lower intestinal tract will highly unlikely be absorbed by the target animals, as under normal circumstances, absorption of most proteinaceous material is largely limited to amino acids, dipeptides, and maybe tripeptides. The submitter is aware that proteases can be absorbed intact (e.g., Lorkowski, 2012) and that excessive proteolysis (resulting from dysregulated protease/antiprotease balance) can lead to gut epithelial damage and increased permeability (Van Spaendonk et al., 2017) associated with diseases such as inflammatory bowel disease, but the relevance of these conditions to commercial pig and poultry production is unclear. Furthermore, exposure to high concentration of free protease from the addition of protease enzymes to commercial poultry and pig feed is unlikely, as these enzymes are not added in high doses and are largely bound to the feed matrix to which they are added, therefore they are not expected to be present as free protease in high concentrations.

20. *The host organism may have the potential to produce trichodermin and peptaibol depending on fermentation conditions. The certificate of analysis included in appendix eight of the notice is unclear about whether the presence of these two substances would be assessed for the market formulation.*

These two substances are not routinely assessed in market formulations as, based on the manufacturing process and production strain characteristics, it has been determined to not be necessary. This is further discussed below with respect to these two substances.

Trichodermin:
Trichodermin is a member of the trichothecene toxin family—a group of sesquiterpenes. While some toxins in this family, such as T-2 toxin, are potent toxins, trichodermin is much less toxic although it is similar to T-2 toxin in structure and biochemical activity. Trichodermin has an established LD50 of 500mg/kg BW administered IP or SC in mouse (de Benito et al., 2017) and as such, is actually not highly toxic. Besides LD50, no toxicological studies have been conducted with trichodermin.

Trichodermin was reported to be produced by a strain of *T. reesei* (Watts et al., 1988) but this has never been confirmed since that original report even though the finding has been perpetuated in reviews (e.g., Blumenthal, 2004), which also resulted in its inclusion in the recent review by EFSA (de Benito et al., 2017).

The trichothecene trichodermin was one of the first secondary metabolites for which the chemical structure was elucidated (Godtfredsen & Vangedal, 1964). Trichothecene biosynthesis potential has been associated to the whole genus up until 2005 when it was shown that this class of compounds was restricted to *T. brevicompactum* and its close relatives (Nielsen et al., 2005; Degenkolb et al., 2008b; Degenkolb et al., 2008a).

The subsequent review by Frisvad et al. (2018) lists only two mycotoxins that are reported in literature to be produced by *T. reesei*, gliotoxin and trichodermin, but both were dismissed because the genes needed to synthesize trichodermin are not present in the *T. reesei* genome, and because gliotoxin has never been detected in any culture of *T. reesei*. The fact that trichodermin has never been reported since the original report by Watts (1998) is consistent with the conclusions by Nielsen et al. (2005), Degenkolb (2008a,b) and Frisvad (2018).

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Due to its lack of prevalence in original research reports, absence of genes needed for its biosynthesis, and relative low toxicity, trichodermin has raised little concern in relation to *T. reesei* used in industrial fermentation to produce enzymes (see also Olempska-Beer et al., 2006).

Peptaibols, notably paracelsin:

The only secondary metabolites of potential concern that appear to be naturally produced by *T. reesei* are the paracelsin peptaibols (Brückner & Graf, 1983; Brückner et al., 1984; Przybylski et al., 1984; Pócsfalvi et al., 1997). These peptaibols show antimicrobial activity, and have been shown to be toxic to mammalian cells, i.e. it exerts hemolytic activity towards human erythrocytes, and it is acutely toxic to mice when administered intraperitoneally (Brückner et al., 1984; Solfrizzo et al., 1994). The *T. reesei* genome contains genes for two peptaibol synthases (Kubicek et al., 2011). However, the bulk of the literature investigating the capability of *T. reesei* to produce peptaibols is based on fermentation conditions designed either to mimic natural (and poor) growth conditions or attempt to optimize the conditions for secondary metabolite production. Efficient production of peptaibols occurs in solid cultivation (surface growth) and correlates with conidiation (Kubicek et al., 2007; Tisch & Schmoll, 2010). These cultivation methods are not representative of the conditions used in controlled industrial fermentation practices including the one used to produce the notified substance. As a result, *T. reesei* has been described as not producing mycotoxins or antibiotics under conditions used for enzyme production.

In the 2020 Federal Register EPA-HQ-OPPT-2011-0740 FRL-9991-60[2] publication on the EPA assessment of *T. reesei* it is stated that "Paracelsin has not been detected in the use of *T. reesei* QM6a under the submerged standard industrial fermentation operations used for enzyme production, and numerous toxicity studies on enzyme products of *T. reesei* QM6a have demonstrated a lack of toxicity to laboratory animals. EPA therefore expects that paracelsin production would be of insignificant concern, provided the microorganisms are produced with submerged standard industrial fermentation operations used for enzyme production as described at 40 CFR725.3."

In addition, data from a 90-day oral toxicity study was included in Appendix 11 of the original AGRN43 notification (Ladics, 2020) which reported a NOAEL for the test article of 1000 mg TOS/kg bw/day. This is additional evidence to support no oral toxin concerns for potential secondary metabolites such as trichodermin or paracelsin.

21. *The firm should address the levels of* (b) (4) *in the notified phytase, and the potential exposure in the target animals.*

(b)(6)

. Three batches of Axtra® PHY GOLD 30 L, Axtra® PHY GOLD 30 T and Axtra® PHY GOLD 65 G were analyzed for boron level. All batches were found to be < 1 mg/kg

[2] https://www.federalregister.gov/documents/2020/03/10/2020-04746/microorganisms-general-exemptions-from-reporting-requirements-revisions-to-recipient-organisms

market product (Appendix 6) hence demonstrating there is no expected significant exposure of (b) (4) to target animals from the notified substance.

Molecular Biology

22. *The notifier states* (b) (4) *of the phytase gene were inserted into the T. reesei source organism. However, other than a single statement there does not appear to be any information about these* (b) (4) *elsewhere in the notice. Thus, further clarification is needed on the molecular characterization and bioinformatics analyses of* (b) (4) *The firm should amend its notice to include detailed information addressing changes in the genome of the source strain T. reesei CRC2836-13885* (b) (4) *This information includes, but is not limited to, whether the phytase expression cassette was inserted using homologous recombination or random insertion; if homologous recombination was used, which parts of the sequence from the expression cassette served as the homology arms; what method was used to determine copy numbers of the phytase insertion(s); information about the exact sequences from the phytase expression cassette integrated at each site; information about the genetic locations of the insertions and flanking genes at each site in the T. reesei genome.*

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Genomic DNA of notified substance production strain was sequenced by PacBio (one time) and Illumina (five times). A hybrid assembly of the highest coverage Illumina and PacBio data was generated using Canu software (Koren et al., 2017). The data assembly was corrected using PacBio data with Arrow and subsequently error corrected using Illumina data and three rounds of Pilon (Walker et al., 2014). Read coverage was determined by aligning the reads onto the parent genome.

The copy number of the (b) (4) based on Illumina read coverage against the parent genome and the transformation cassette with (b) (4). The *de novo* assembly indicated that the transforming cassette (b) (4) relative to the parent reference genome of strain *Trichoderma reesei* QM6a sequenced by the JGI (Martinez et al., 2008). *De novo* assembly was unable to completely resolve the assembly of the insertion cassette, however, (b) (4) confirming the copy number found by read coverage. Due to the (b) (4). Appendix 39 included in this response provides additional information on the expression cassette insertion.

23. *The notifier states in the notice that an ORF analysis was performed at the phytase* (b) (4) *, but it is unclear whether the ORF analysis addressed a single or multiple insertion site(s) of the phytase gene cassette and whether it included junction sequences. The notifier should provide detailed information including whether the ORF analysis was carried out at all the phytase* (b) (4) *sites and how many potential new ORFs were identified at each site, as well as a narrative addressing whether any of the putative proteins share homology to known toxins listed in the pertinent databases. Information about the database(s) used for the search should also be included. In addition,* (b) (4) *The notifier should provide information about the ORF analyses carried out at these sites, as well as a narrative addressing whether any of any putative proteins at these sites share homology to known toxins listed in the pertinent databases.*

The updated ORF analysis and report has been included in Appendix 40. Note, this report includes additional detail as requested by CVM above. This report in Appendix 40 evaluates potential (b) (4) resulting from the engineered deletions/disruptions introduced in this strain, as described in Appendix 1 of original filling of AGRN 43. This report should be used alongside the original report provided in

Appendix 39 and referenced in Appendix 40. In Appendix 39 the ORF analysis was done to assess potential novel ORF formation resulting from the integration of the phytase cassette.

All novel open reading frames ≥30 amino acids in length were analyzed for toxin and allergen homology. Any corresponding theoretical peptide sequences were compared to (b) (4) (Appendix 39) or COMPARE (Appendix 40) database and UniProKB annotated Protein Knowledge database version 2021_02, to identify significant similarities to known food allergens or toxin. Homologies in the databases having an E-value < 0.1 were assessed for significance, using pairwise identity > 80% combined with query coverage > 60% as cut-off.

(b) (4)

These ORFs are 'hypothetical' and unlikely to be expressed, as they lack the minimally required transcription signals (promoter and terminator) and translation signals (ribosome binding site and start codon). Further, no matches of concern between the 'hypothetical' ORFs and known toxins or food allergens were found.

In addition, data from a 90-day oral study was included in Appendix 11 of the original AGRN43 notification (Ladics, 2020) which reported a NOAEL of 1000 mg TOS/kg bw/day. This is additional evidence to support no oral toxin concerns regarding these novel translated stop codon-bracketed frames in the optimized Eclipse phytase *Trichoderma reesei* strain. Taken together, these data indicate that the newly created 'hypothetical' ORFs are unlikely to pose a risk of food allergenicity or protein toxicity.

24 *The notifier states the stability of the inserted genetic variant was confirmed by comparative genome sequence analysis at the beginning and end of fermentation. The notifier further indicates there is no rearrangement of the expression cassette, and the source organism was proved to be stable after at least 30 generations. However, as addressed in the previous questions it is unclear whether there is a single or multiple insertion site(s) of the phytase expression cassette. Thus, the notifier should clarify* (b) (4) .

Five independent fungal culture samples of *Trichoderma reesei* production strain for the notified substance were sequenced. One of these samples originated via flask growth as a representative of the starting genomic sequence, prior to fermenter cultivation. The other four samples were taken at end-of-fermentation from independent fermenter cultivations.

Insertion of the phytase cassette into the *T. reesei* genome was by (b) (4). As described in the response to question 22, (b) (4) of the expression cassette were (b) (4)

Next-Generation Sequencing (NGS) analysis was used to characterize the production strain for the insertion site prior to and at the end of a (b) (4) fermentation protocol. Any rearrangement of the inserted phytase expression cassette DNA in the strain would result in a change of flanking DNA sequence in the analysis. No change in flanking DNA sequence was observed between the genomic DNA samples extracted from the shake flask culture, which represented the genome prior to fermentation, and samples taken at the end of four independent fermentations. This indicates that there had been no insertion of the expression cassette at new sites in the *T. reesei* genome during these fermentations.

In conclusion, the notified substance production strain maintains the inserted DNA stably during fermentations that last for the intended period of commercial-scale production.

The following documents are included in this response:

1. Cover letter response
2. Appendix 1: CoA used to determine TOS (CBI)
3. Appendix 2: Method of Analysis for Ash (CBI)
4. Appendix 3: Certificate of Composition for Axtra PHY GOLD 65G (CBI)
5. Appendix 4: Certificate of Composition for Axtra PHY GOLD 30T (CBI)
6. Appendix 5: Certificate of Composition for Axtra PHY GOLD 30L (CBI)

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7. Appendix 6: Certificate of Analysis (CBI)
8. Appendix 7: Method of Analysis for Phytase Activity in Enzyme Products (CBI)
9. Appendix 8: Method of Analysis Validation for Phytase Activity in Enzyme Products (CBI)
10. Appendix 9: FCC Enzyme Monograph
11. Appendix 10: JECFA Food Enzyme Specifications
12. Appendix 11: Certificate of Analysis for Absence of Production Strain (CBI)
13. Appendix 12: Method of Analysis for Absence of Production Strain (CBI)
14. Appendix 13: Example US Product Labels
15. Appendix 14: 12 month stability of Axtra PHY GOLD 30T product (CBI)
16. Appendix 15: 12 month stability of Axtra PHY GOLD 30L product (CBI)
17. Appendix 16: 12 month stability of Axtra PHY GOLD 65G product (CBI)
18. Appendix 17: 6 month stability in premix of Axtra PHY GOLD 30T (CBI)
19. Appendix 18: Method of Analysis for Phytase Activity in Premix (CBI)
20. Appendix 19: Method of Analysis Validation for Phytase Activity in Premix and Feed (CBI)
21. Appendix 20: 6 month stability in premix of Axtra PHY GOLD 65G (CBI)
22. Appendix 21: 3 month stability in pelleted feed for swine of Axtra PHY GOLD 30T (CBI)
23. Appendix 22: Method of Analysis for Phytase Activity in Feed (CBI)
24. Appendix 23: 3 month stability in pelleted feed for poultry of Axtra PHY GOLD 30T (CBI)
25. Appendix 24: 3 month stability in pelleted feed for swine of Axtra PHY GOLD 30L (CBI)
26. Appendix 25: 3 month stability in pelleted feed for poultry of Axtra PHY GOLD 30L (CBI)
27. Appendix 26: 3 month stability in pelleted feed for swine of Axtra PHY GOLD 65G (CBI)
28. Appendix 27: 3 month stability in pelleted feed for poultry of Axtra PHY GOLD 65G (CBI)
29. Appendix 28: 3 month stability in mash feed for swine of Axtra PHY GOLD 30T (CBI)
30. Appendix 29: 3 month stability in mash feed for poultry of Axtra PHY GOLD 30T (CBI)
31. Appendix 30: 3 month stability in mash feed for swine of Axtra PHY GOLD 65G (CBI)
32. Appendix 31: 3 month stability in mash feed for poultry of Axtra PHY GOLD 65G (CBI)
33. Appendix 32: Processing stability of Axtra PHY GOLD 30L (CBI)
34. Appendix 33: Processing stability of Axtra PHY GOLD 30T (CBI)
35. Appendix 34: Processing stability of Axtra PHY GOLD 65G (CBI)
36. Appendix 35: Homogeneity Stability in Mash Feed for Poultry for Axtra PHY GOLD 30T (CBI)
37. Appendix 36: Homogeneity Stability in Mash Feed for Swine for Axtra PHY GOLD 30T (CBI)
38. Appendix 37: Homogeneity Stability in Mash Feed for Poultry for Axtra PHY GOLD 65G (CBI)
39. Appendix 38: Homogeneity Stability in Mash Feed for Swine for Axtra PHY GOLD 65G (CBI)
40. Appendix 39: Open Reading Frame Analysis Original Report (CBI)
41. Appendix 40: Open Reading Frame Analysis Report Updated (CBI)
42. Copies of all references used in this response

We trust the provided information in this response addresses each of the questions and will allow CVM to complete the evaluation of the notice. Should you have any questions or require additional information for the processing of this application, please do not hesitate to contact me at 650-799-0871.

Sincerely,

(b)(6)

Vincent Sewalt, Ph.D.
Head, Regulatory Science & Advocacy
Global Regulatory Affairs
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IFF
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Palo Alto, CA 94304
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References

Aunstrup, K., Andersen, O., Falch, E.A., and Nielsen, T.K. (1979) Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Aunstrup, K. (1979) Production, isolation, and economics of extracellular enzymes. In *Applied Biochemistry and Bioengineering* (pp. 27-69).

Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Section 23.1, Proteins Are Degraded to Amino Acids. Available from: https://www.ncbi.nlm.nih.gov/books/NBK22600/

Blumenthal C.Z. (2004) Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology and Pharmacology 39: 214-228.

Brückner H & Graf H (1983) Paracelsin, a peptide antibiotic containing alphaaminoisobutyric acid, isolated from *Trichoderma reesei* Simmons. Part A. *Experientia* **39**: 528-530.

Brückner H, Graf H & Bokel M (1984) Paracelsin; characterization by NMR spectroscopy and circular dichroism, and hemolytic properties of a peptaibol antibiotic from the cellulolytically active mold *Trichoderma reesei*. Part B. *Experientia* **40**: 1189-1197.

Christensen, T., Dersjant-Li, Y., Sewalt, V., Mejldal, R., Haaning, S., Pricelius, S., Nikolaev, I, Sorg, R.A., and de Kreij, A. (2020) *In vitro* Characterization of a Novel Consensus Bacterial 6-Phytase and one of its Variants. *Current Biochemical Engineering* **6**: 156-171.

de Benito A, Ibáñez C, Moncho W, Martínez D, Vettorazzi A & de Cerain AL (2017) Database on the taxonomical characterisation and potential toxigenic capacities of microorganisms used for the industrial production of food enzymes and feed additives, which do not have a recommendation for Qualified Presumption of Safety. *EFSA Supporting Publications* **14**: 1274E.

Degenkolb T, von Döhren H, Nielsen KF, Samuels GJ & Brückner H (2008a) Recent advances and future prospects in peptaibiotics, hydrophobin, and mycotoxin research, and their importance for chemotaxonomy of *Trichoderma* and *Hypocrea*. *Chemistry & biodiversity* **5**: 671-680.

Degenkolb T, Dieckmann R, Nielsen KF, *et al.* (2008b) The *Trichoderma* brevicompactum clade: a separate lineage with new species, new peptaibiotics, and mycotoxins. *Mycological Progress* **7**: 177-219.

Dersjant-Li, Y., Archer, G., Stiewert, A. Brown, A., Sobotik, E., Jasek, A., Marchal, L., Bello, A., Sorg, R., Christensen, T., Kim, H., Mejldal, R, Nikolaev, I., Pricelius, S., Haaning, S., Sørensen, J., de Kreije, A., Sewalt, V., (2020a) Functionality of a next generation biosynthetic bacterial 6- phytase in enhancing phosphorus availability to broilers fed a corn-soybean meal-based diet. Animal Feed Science and Technology 264: 114481. https://doi.org/10.1016/j.anifeedsci.2020.114481.

Dersjant-Li Y., Villca, B., Sewalt, V., de Kreij, A., Marchal, L., Velayudhan, D.E., Sorg, R.A., Christensen, T., Mejldal, R., Nikolaev, I., Pricelius, S., Kim, H-S., Haaning, S., Sørensen, J.F., Lizardo, R. (2020b) Functionality of a next generation biosynthetic bacterial 6-phytase in enhancing phosphorus availability to weaned piglets fed a corn-soybean meal-based diet without added inorganic phosphate. Animal Nutrition 6: 24-34. https://doi.org/10.1016/j.aninu.2019.11.003

Engelen, A., Van der Heeft, F., Randsdorp, P., Somers, W., Schaefer, J., and van der Vat, B. (2001) "Determination of phytase activity in feed by a colorimetric enzymatic method: Collaborative interlaboratory study", J. AOAC Int., vol. 84, no. 3, pp. 629-633. http://dx.doi.org/10.1093/jaoac/84.3.629 PMID: 11417623

Engelen AJ, van der Heeft FC, Randsdorp PH, Smit EL. (1994) Simple and rapid determination of phytase activity. J AOAC Int;77:760 4.

FDA/CFSAN Division of Biotechnology and GRAS Notice Review (2010). Guidance for Industry – Enzyme Preparations: Recommendations for Submission of Chemical and Technological data for Food Additive Petitions and GRAS Notices. Available at: https://www.fda.gov/downloads/Food/GuidanceRegulation/UCM217735.pdf (last accessed August 23, 2021).

Frisvad JC, Moller LLH, Larsen TO, Kumar R & Arnau J (2018) Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of Aspergillus niger, Aspergillus oryzae, and *Trichoderma reesei*. *Appl Microbiol Biotechnol* **102**: 9481-9515.

Godtfredsen W & Vangedal S (1964) Trichodermin, a new antibiotic, related to trichothecin. *Proc Chem Soc* **6**: 188–189.

Kirk, O., Damhus, T., Borchert, T.V., Fuglsang, C.C., Olsen, H.S., Hansen, T.T., Lund, H., Schiff, H.E., and Kierstein-Nielsen. L. (2005). In: Kirk-Othmer (Ed.), Kirk-Othmer Encyclopedia of Chemical Technology, Volume 10, 5th edition. Wiley. (ISBN 978-0-471-48513-1), pp. 248-317.

Koren S, Walenz BP, Berlin K, Miller JR, Phillippy AM (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Research 27(5): 722–736.

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, *et al.* (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome Biol* **12**: R40.

Kubicek CP, Komoń-Zelazowska M, Sándor E & Druzhinina IS (2007) Facts and challenges in the understanding of the biosynthesis of peptaibols by *Trichoderma*. *Chemistry & biodiversity* **4**: 1068-1082.

López-Otín C, Bond JS. Proteases: multifunctional enzymes in life and disease. (2008) J Biol Chem; 283(45):30433-30437. doi:10.1074/jbc.R800035200

Lorkowski G. (2012) Gastrointestinal absorption and biological activities of serine and cysteine proteases of animal and plant origin: review on absorption of serine and cysteine proteases. Int J Physiol Pathophysiol Pharmacol. 4(1):10-27. Epub 2012 Feb 28. PMID: 22461953; PMCID: PMC3312459.

Martinez, D., R.M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S.E. Baker, J. Chapman, O. Chertkov, P.M. Coutinho, D. Cullen, E.G.J. Danchin, I.V. Grigoriev, P. Harris, M. Jackson, C.P. Kubicek, C.S. Han, I. Ho, and L.F. Larrondo, A.L. Leon, J.K. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robbertse, A.A.Salamov, M. Schmoll, A. Terry, N. Thayer, A. Westerholm-Parvinen, C.L.Schoch, J. Yao, R. Barabote, M.A. Nelson, C.Detter, D. Bruce, C.R. Kuske, G. Xie, P. Richardson, D.S. Rokhsar, S.M. Lucas, E.M. Rubin, N. Dunn-Coleman, M. Ward, T.S. Brettin. (2008). Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nature Biotechnol. 26:553-560.

Nielsen KF, Gräfenhan T, Zafari D & Thrane U (2005) Trichothecene production by *Trichoderma* brevicompactum. *Journal of agricultural and food chemistry* **53**: 8190- 8196.

Olempska-Beer ZS, Merker RI, Ditto MD & DiNovi MJ (2006) Food-processing enzymes from recombinant microorganisms - a review. Regulatory Toxicology and Pharmacology 45: 144-158.

Pócsfalvi G, Ritieni A, Ferranti P, Randazzo G, Vékey K & Malorni A (1997) Microheterogeneity characterization of a paracelsin mixture from *Trichoderma reesei* using high-energy collision-induced dissociation tandem mass spectrometry. *Rapid communications in mass spectrometry : RCM* **11**: 922-930.

Przybylski M, Dietrich I, Manz I & Brückner H (1984) Elucidation of structure and microheterogeneity of the polypeptide antibiotics paracelsin and trichotoxin A-50 by fast atom bombardment mass spectrometry in combination with selective in situ hydrolysis. *Biomedical Mass Spectrometry* **11**: 569-582.

Solfrizzo M, Altomare C, Visconti A, Bottalico A & Perrone G (1994) Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. *Natural toxins* **2**: 360- 365.

Tisch D & Schmoll M (2010) Light regulation of metabolic pathways in fungi. *Appl Microbiol Biotechnol* **85**: 1259-1277.

Van Spaendonk H, Ceuleers H, Witters L, et al. (2017) Regulation of intestinal permeability: The role of proteases. World J Gastroenterol. 23(12):2106-2123. doi:10.3748/wjg.v23.i12.2106

Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J & Young SK (2014) Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PloS one 9.

Watts, R., J. Dahiya, K. Chaudhary, and P. Tauro. (1988). Isolation and characterization of a new fungal metabolite of Trichoderma reesei. Plant and Soil 107:81-84.

Yu, S., Cowieson, A., Gilbert, C., Plumstead, P., and Dalsgaard, S. (2012) "Interactions of phytate and myo-inositol phosphate esters (IP1-5) including IP5 isomers with dietary protein and iron and inhibition of pepsin", J. Anim. Sci., vol. 90, no. 6, pp. 1824-1832. http://dx.doi.org/10.2527/jas.2011-3866 PMID: 22228039

Zeng, Z., Li, Q., Tian, Q. et al. (2015). Super High Dosing with a Novel *Buttiauxella* Phytase Continuously Improves Growth Performance, Nutrient Digestibility, and Mineral Status of Weaned Pigs. Biol Trace Elem Res 168, 103–109. https://doi.org/10.1007/s12011-015-0319-2

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CERTIFICATE OF ANALYSIS

925 Page Mill Road · Palo Alto, CA 94304-1013 USA · +1.650.846.7500 tel · www.dupont.com

Name of Test Article: Eclipse B

Production/Strain Name: *T. reesei,* [redacted]

Production Site: PALO ALTO, CA

Genencor International Culture Collection Number: GICC 03538

Designation of Lot Tested: 20188029

Description: Fermentation liquid, filtered

All of the analytical studies listed below were conducted in accordance with GLP regulations and/or ISO 9002 standards.

RESULTS:

1. Activity: [redacted]

2. Total and TCA Protein
The samples were measured for TCA and total protein by nitrogen analysis (with a conversion factor [redacted] – Assays completed by BAMQC per [redacted]

   Total Protein: [redacted]
   TCA Protein:
   % Total Organic Solids: [redacted]

3. Specific gravity: [redacted]

4. pH:

5. Microbial analysis: Conducted by Dupont Industrial Biosciences (Rochester, NY).

Analysis	Results
Total viable count	[redacted]
Coliform	
E. Coli	
Salmonella	
Production strain	
Antimicrobial activity	

6. Mycotoxin analysis: Completed by [redacted]

Total aflatoxin	(b) (4)
T-2	
Zearalenone	
Ochratoxin	
Fumonisin	
Vomitoxin	

7. Heavy metals analysis: Completed by (b) (4)

Analysis	Results
Heavy metals as Pb	(b) (4)
Arsenic	
Lead	
Mercury	
Cadmium	

8. Stability Data: All activity units are reported in FTU/g based on the method (b) (4)

Room Temperature

Sample ID	Dilution	T = 0	T = 5 hours	% of T = 0
20188029	(b) (4)			
20188029				
20188029				

Refrigerator (4°C): Undiluted Material

Sample ID	Dilution	T=0	T = 4 days	% of T = 0	T = 7 days	% of T = 0
20188029	(b) (4)					
20188029						
20188029						

Frozen (-20°C) : Undiluted Material

Sample ID	Dilution	T = 0	T = 30 days	% of T = 0	T = 63 days	% of T = 0	T = 90 days	% of T = 0	T=248 days	% of T = 0
20188029	(b) (4)									

(b) (6)

(b) (6) ..... Date: 7-11-2019

BAMQC Group
DuPont IB

(b) (6)

..... Date: 7-12-2019

Central Research & Development
DuPont

(b) (4)

CHEMISTRY METHOD

ASH BY IGNITION
(DRY ASHING)
Synopsis
QA-0225-2001.05

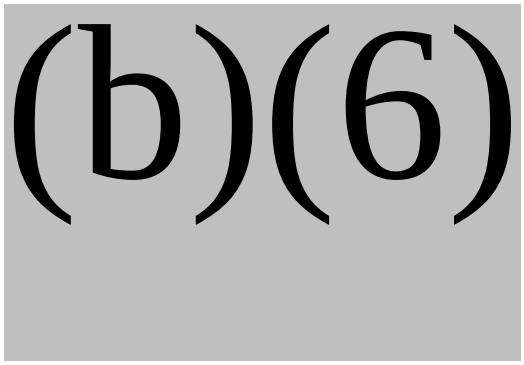
Effective: August 16, 1999

(b) (4)

1. OVERVIEW

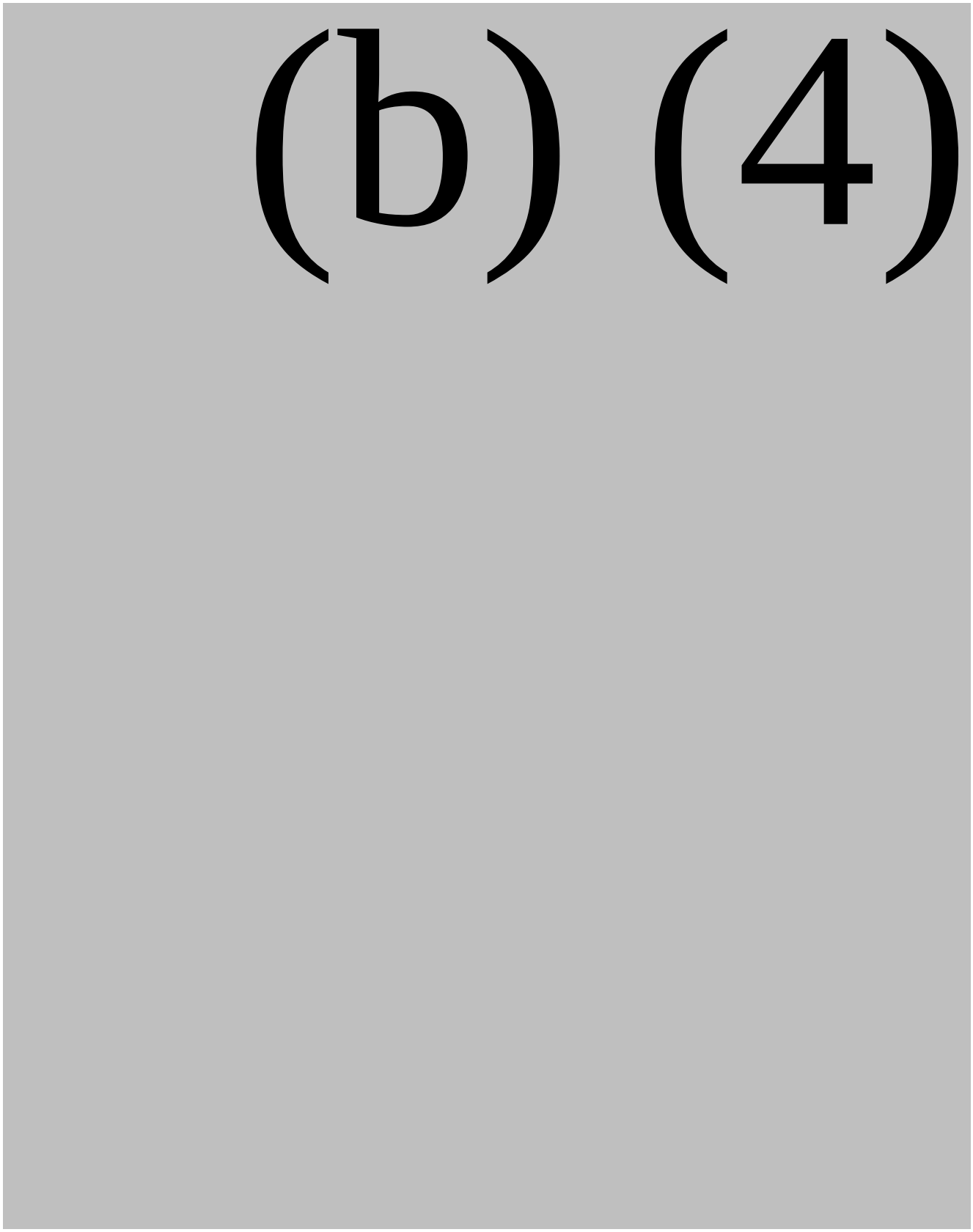
Purpose and Background

(b) (4)



(b)(

2. (b) (4)


(b) (4)

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CERTIFICATE OF COMPOSITION

Axtra® PHY GOLD 65 G

We hereby certify that this is a typical composition of the Danisco Animal Nutrition product Axtra® PHY GOLD 65 G

Ingredients	% w/w
(b) (4)	(b) (4)
	100% (Total)

Guaranteed minimum activity of Axtra® PHY GOLD 65 G

Active Ingredient	Guaranteed Minimum
6-phytase (EC 3.1.3.26)	(b) (4)

(b) (4)

Sincerely

(b) (6)

Emile Steijns
Regional QA manager

Date: August 18, 2021

Health & Biosciences
3490 Winton Place
Rochester, NY 14623
iff.com

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COMPANY CONFIDENTIAL

CERTIFICATE OF COMPOSITION

Axtra® PHY GOLD 30 T

We hereby certify that this is a typical composition of the Danisco Animal Nutrition product Axtra® PHY GOLD 30 T

Ingredients	% w/w
(b) (4)	(b) (4)
	100% (Total)

Guaranteed minimum activity of Axtra® PHY GOLD 30 T

(b) (4)

Health & Biosciences
3490 Winton Place
Rochester, NY 14623
iff.com

Sincerely (b) (6)

Emile Steijns
Regional QA manager

Date: August 18, 2021

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COMPANY CONFIDENTIAL

CERTIFICATE OF COMPOSITION

Axtra® PHY GOLD 30 L

We hereby certify that this is a typical composition of the Danisco Animal Nutrition product Axtra® PHY GOLD 30 L

Ingredients	% w/w
(b) (4)	(b) (4)
	100% (Total)

Guaranteed minimum activity of Axtra® PHY GOLD 30 L

(b) (4)

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Sincerely

Emile Steijns
Regional QA manager

Date: August 18, 2021

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 30 T

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577976**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

28-Sep-2020 Date	(b) (6) Regional QA manager

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Product Name: Axtra® PHY GOLD 30 T

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577978**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
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MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
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Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

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Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577980**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
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Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
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OTHER ASSAYS			
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Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

28-Sep-2020 Date	(b) (6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 30 L

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**20200513-001**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Specific gravity	g/ml		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

05-Oct-2020 Date	(b) (6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 30 L

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**202004001-002**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Specific gravity	g/ml		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

05-Oct-2020 Date	(b) (6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 30 L

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**4903674228**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Specific gravity	g/ml		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

02-Oct-2020 Date	(b) (6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 65 G

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577981**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

28-Sep-2020 Date	(b) (6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 65 G

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**3099294170**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

28-Sep-2020 Date	(b) (6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 65 G

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**3099294171**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron *	mg/kg		

(b) (4)

28-Sep-2020 Date	(b) (6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 30 T

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577976**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

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Product Name: Axtra® PHY GOLD 30 T

Material code (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577978**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

28-Sep-2020 Date	(b) (6), (b) (4) Regional QA manager

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Product Name: Axtra® PHY GOLD 30 T

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577980**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

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28-Sep-2020 Date	(b) (6), (b) (4) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 30 L

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**20200513-001**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Specific gravity	g/ml		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

05-Oct-2020 Date	(b)(6) Regional QA manager

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Product Name: Axtra® PHY GOLD 30 L

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**202004001-002**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Specific gravity	g/ml		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

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05-Oct-2020 Date	(b)(6) Regional QA manager

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Product Name: Axtra® PHY GOLD 30 L

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**4903674228**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Specific gravity	g/ml		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
Mercury	mg/kg		
Boron*	mg/kg		

(b) (4)

02-Oct-2020 Date	(b)(6) Regional QA manager

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Certificate of Analysis

Product Name: Axtra® PHY GOLD 65 G

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**1663577981**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
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PHYSICAL PROPERTIES Bulk Density	Kg/l		
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28-Sep-2020 Date	(b)(6) Regional QA manager

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Product Name: Axtra® PHY GOLD 65 G

Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**3099294170**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
E. coli	/25g		
Salmonella	/25g		
Production Strain	/g		
PHYSICAL PROPERTIES Bulk Density	Kg/l		
OTHER ASSAYS			
Lead	mg/kg		
Arsenic	mg/kg		
Cadmium	mg/kg		
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28-Sep-2020 Date	(b)(6) Regional QA manager

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Material code: (b) (4)

CERTIFICATE OF ANALYSIS

LOT NUMBER:	**3099294171**

ASSAY	**UNIT**	**SPECIFICATION**	**FOUND**
ENZYME ACTIVITY 6-Phytase	FTU/g	(b) (4)	
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	CFU/g		
Coliforms	CFU/g		
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28-Sep-2020 Date	(b)(6) Regional QA manager

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DANISCO
First you add knowledge...

Animal feed additives – Determination of phytase activity – Colorimetric method (b) (4)

Authorisation and Issue Control

I hereby declare that this method is an accurate reflection of the method conducted in our laboratory, and that the method was developed and written under my supervision and in accordance with standard operating procedures.

Senior Manager, Feed Technical Service
(b) (4)

Signature: (b) (4) Date 2/5 - 2012

This report is approved by Danisco Animal Nutrition Management:
Development and Technical Director, Danisco Animal Nutrition
(b) (4)

Signature (b) (4) Date 3/4/12

Contents

Foreword

Danisco Animal Nutrition has developed an in-house method of analysis in order to accurately analyse the phytase content of Danisco Animal Nutrition feed enzymes containing phytase derived from *Schizosaccharomyces pombe* or *Trichoderma reesei*.

(b) (4)

.

This method was developed according to the principles set out in ISO 9001 [2] and Good Laboratory Practice and has been written in accordance with the rules given in ISO 78-2:1999 [3].

Introduction

This in-house method of analysis has been developed to determine the activity of phytase in Danisco Animal Nutrition feed enzymes containing phytase derived from *Schizosaccharomyces pombe* or *Trichoderma reesei*. However, the method cannot be used to evaluate the in vivo efficacy of the phytase products.

DANISCO
First you add knowledge...

Animal feed additives – Determination of phytase activity – Colorimetric method (b) (4)

(b) (4)

(b) (4)

(b) (4)

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(b) (4)

[redacted]

(b) (4)

[redacted]

(b) (4)

DANISCO

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(b) (4)

(b) (4)

[redacted]

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(b) (4)

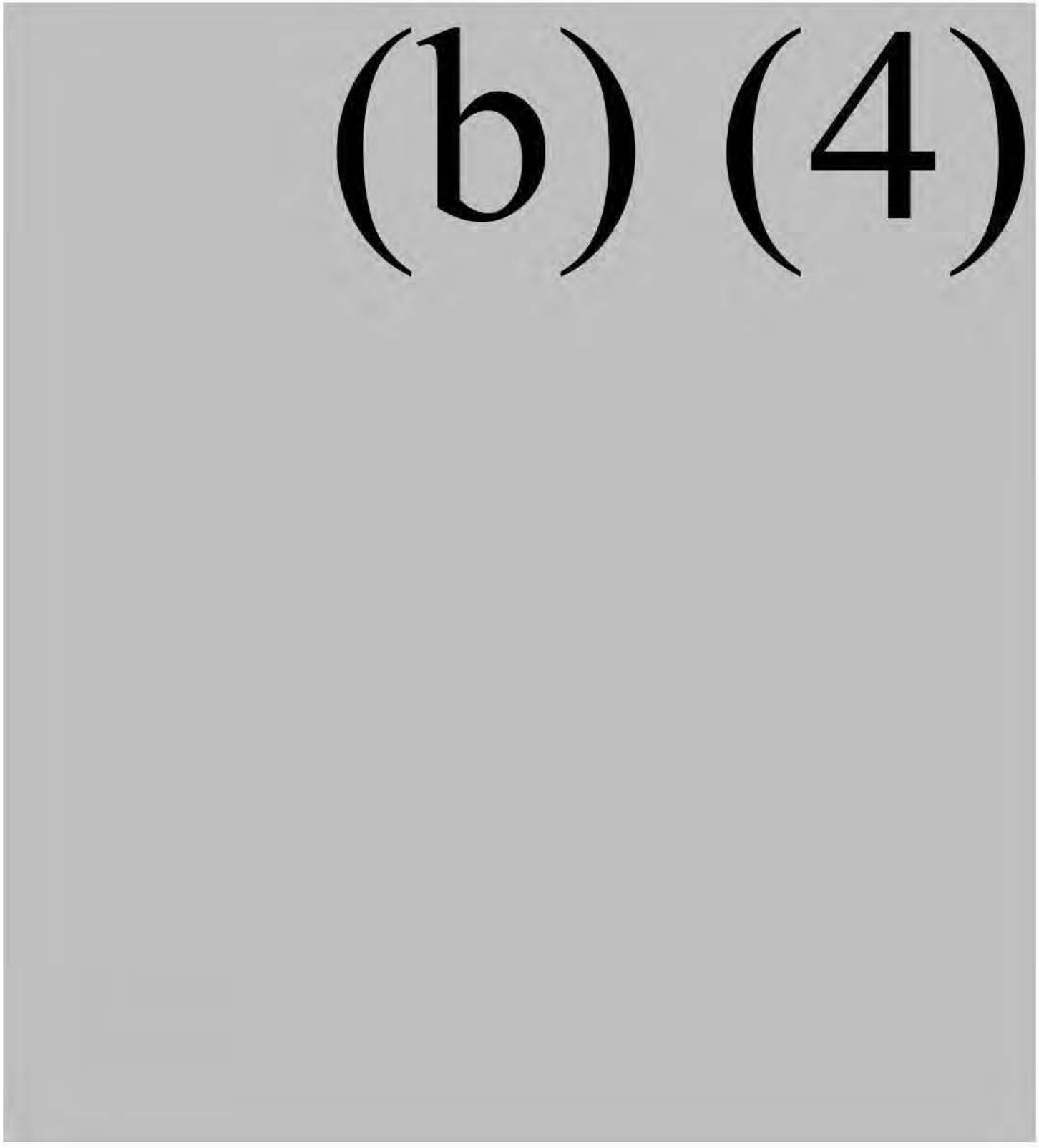
(b) (4)

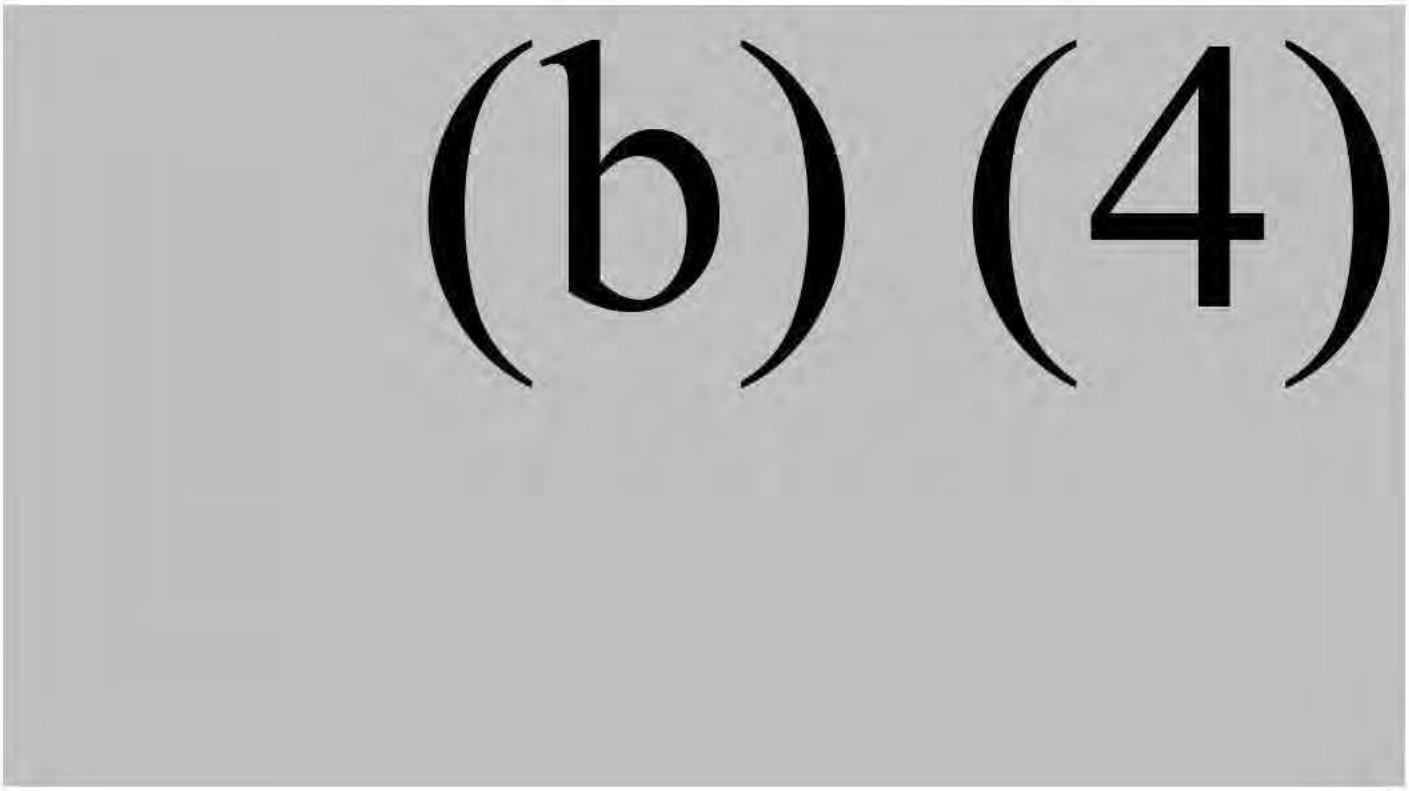
(b) (4)


(b) (4)

Annex 2

Safety (Danger classification)




(b) (4)

Bibliography

[1]

[2]

[3]

[4]

(b) (4)

DANISCO
First you add knowledge...

Validation - Determination of phytase activity in animal feed additives - Colorimetric method (b) (4)

Authorisation and Issue Control

I hereby declare that this in-house validation study is an accurate reflection of the study conducted in our laboratory, and that the study was developed and written under my supervision and in accordance with standard operating procedures and IUPAC harmonised guidelines for single laboratory validation of methods of analysis.

Senior Manager, Feed Technical Service
S. Dalsgaard

Signature: [redacted] (b) (6) Date 18 15-2012

This report is approved by Danisco Animal Nutrition Management:
Technical Services Director
Dr Gary Partridge

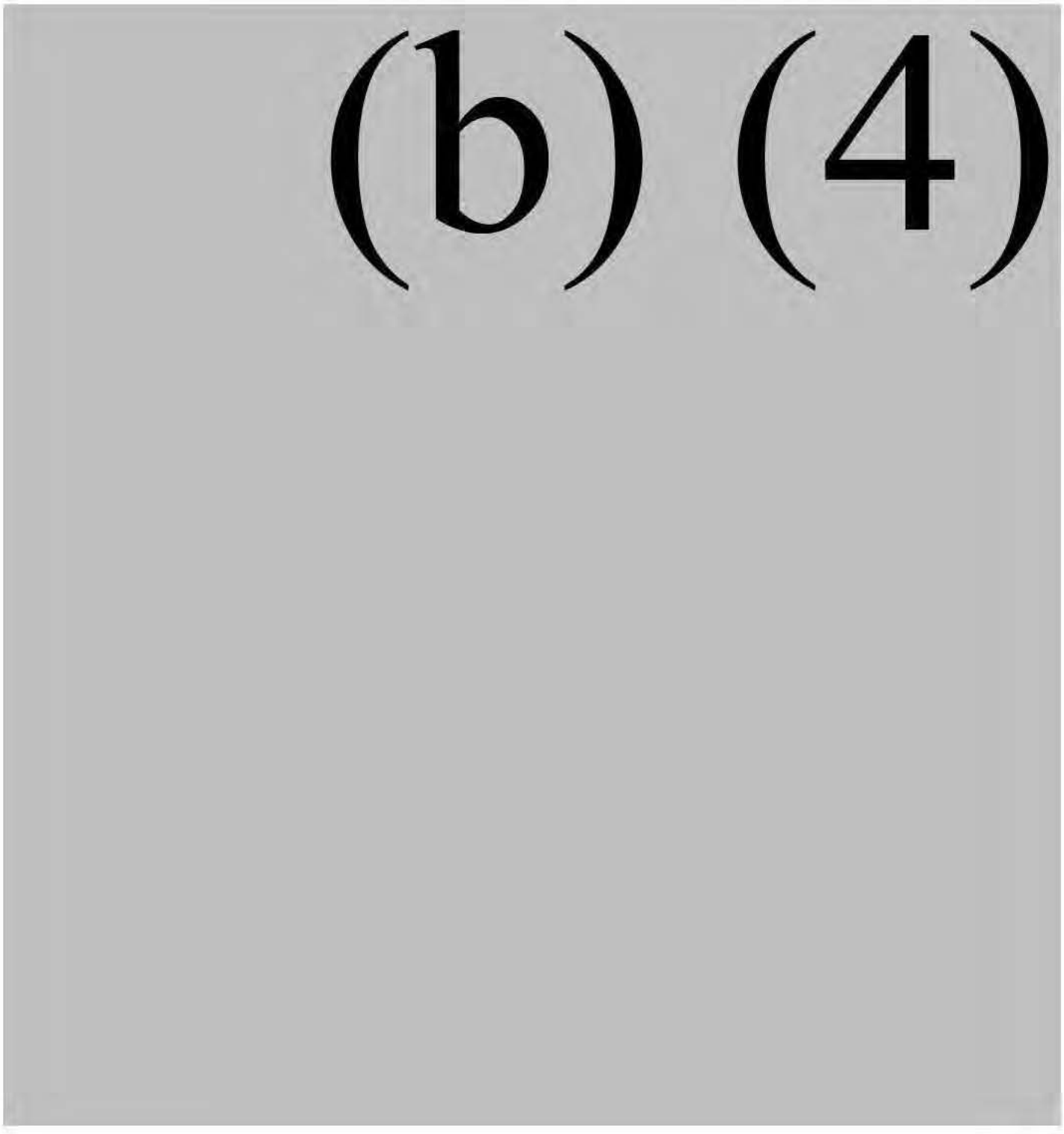
Signature: [redacted] (b) (6) Date 14/5/12

Contents

1. Introduction

(b) (4)

(b) (4)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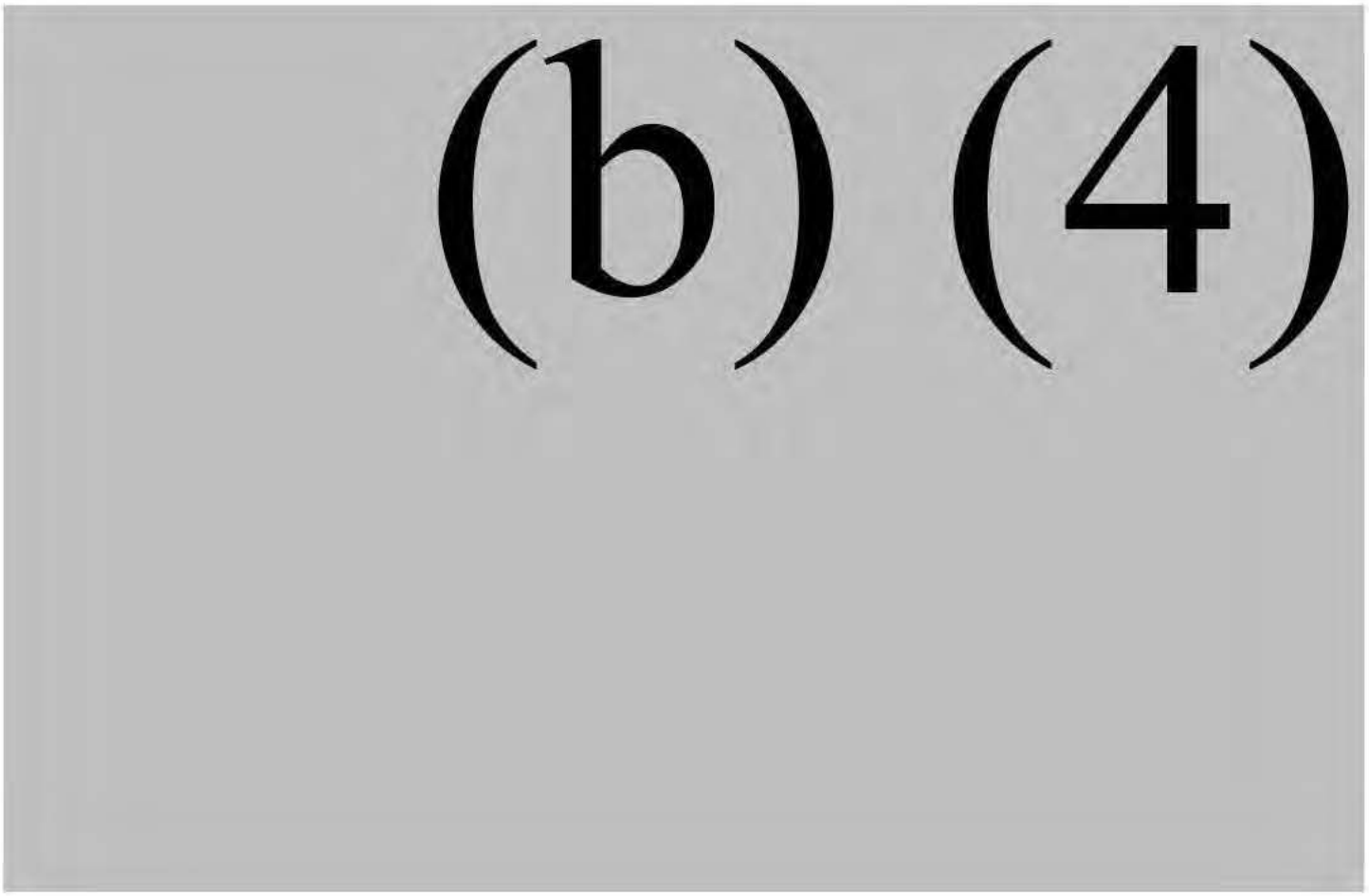
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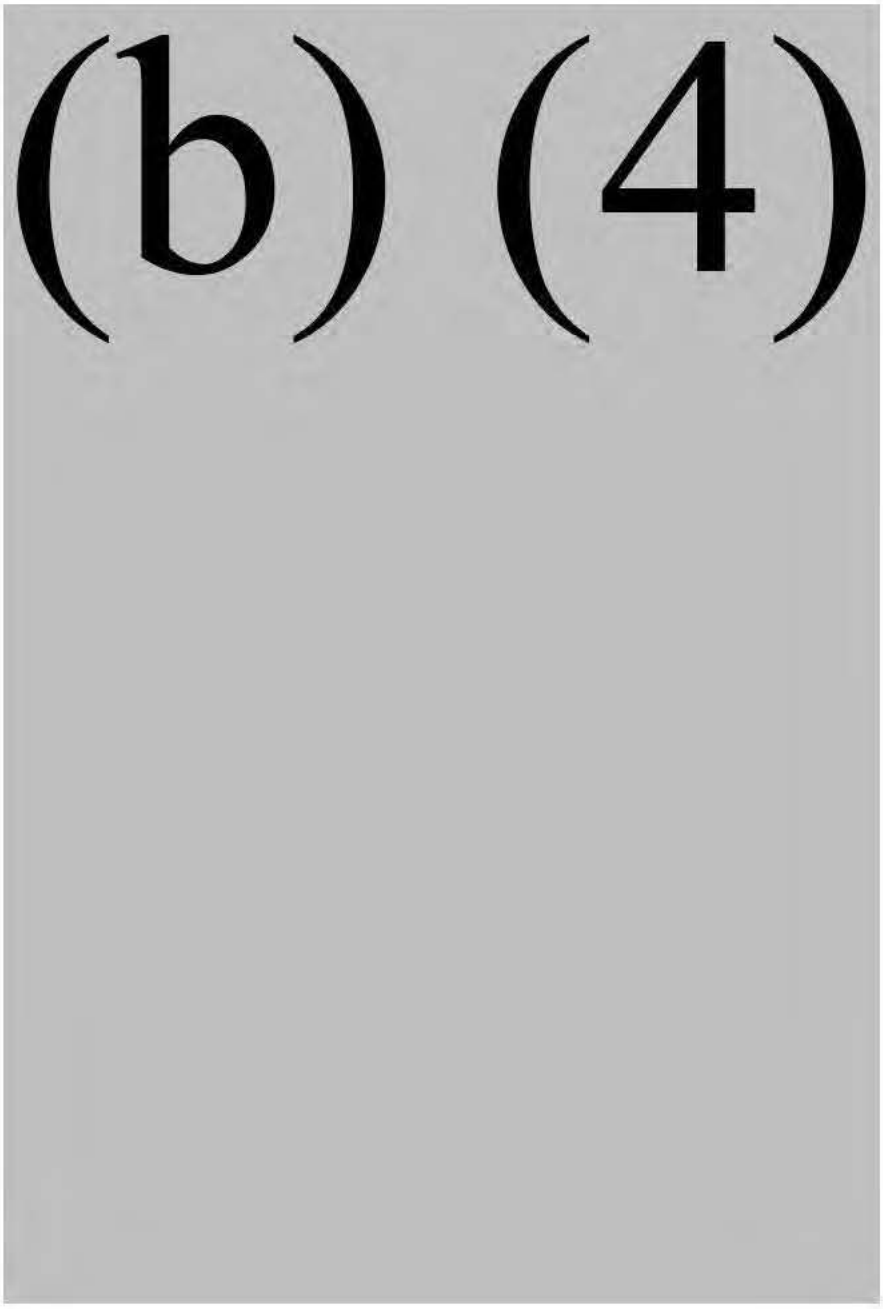
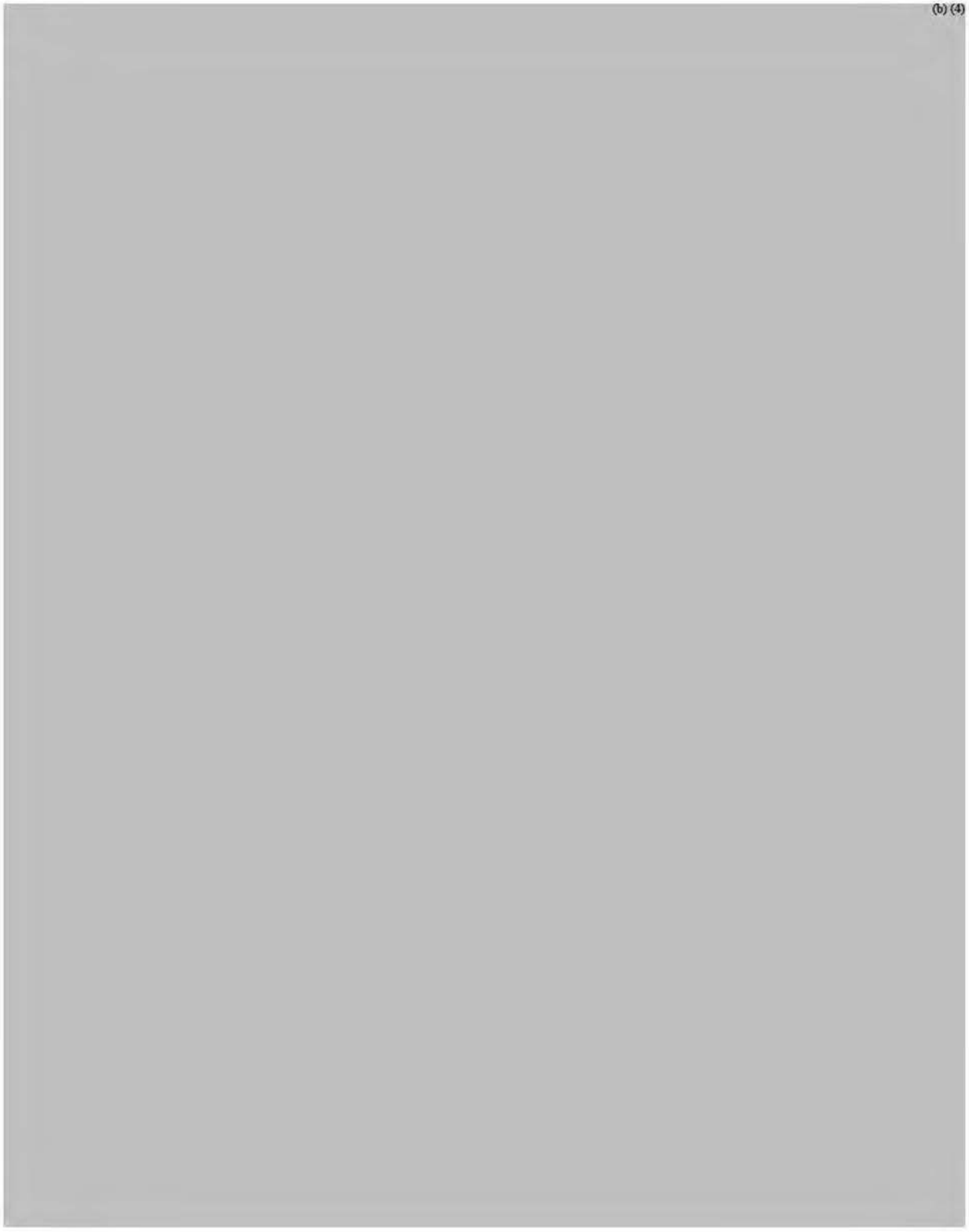
Appendix 1 – Limit of detection and limit of quantification data



Appendix 2 – Data for repeatability & reproducibility calculations

(b) (4)



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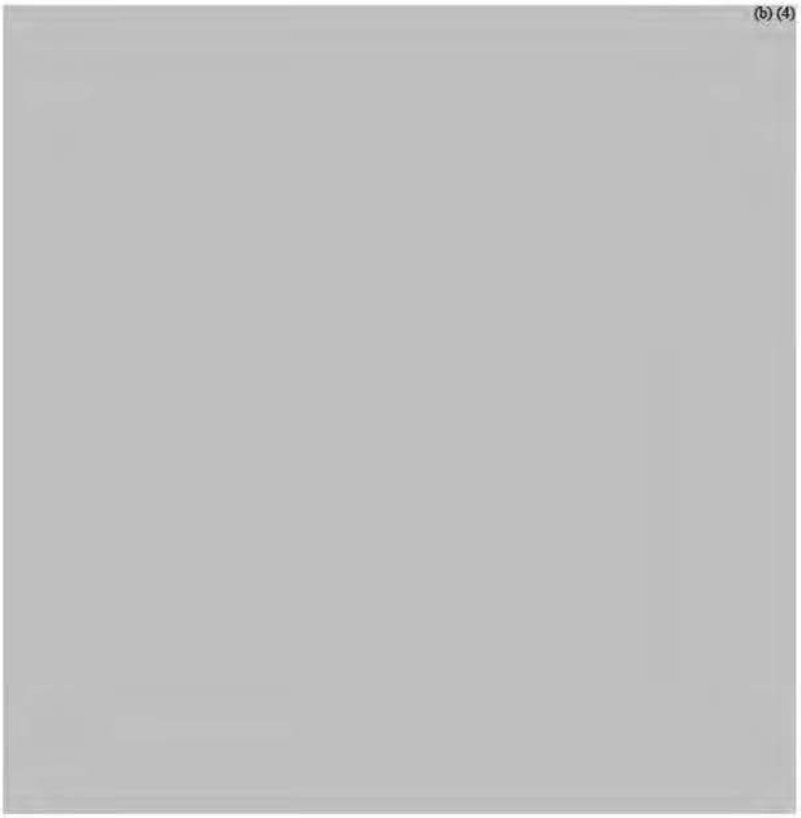
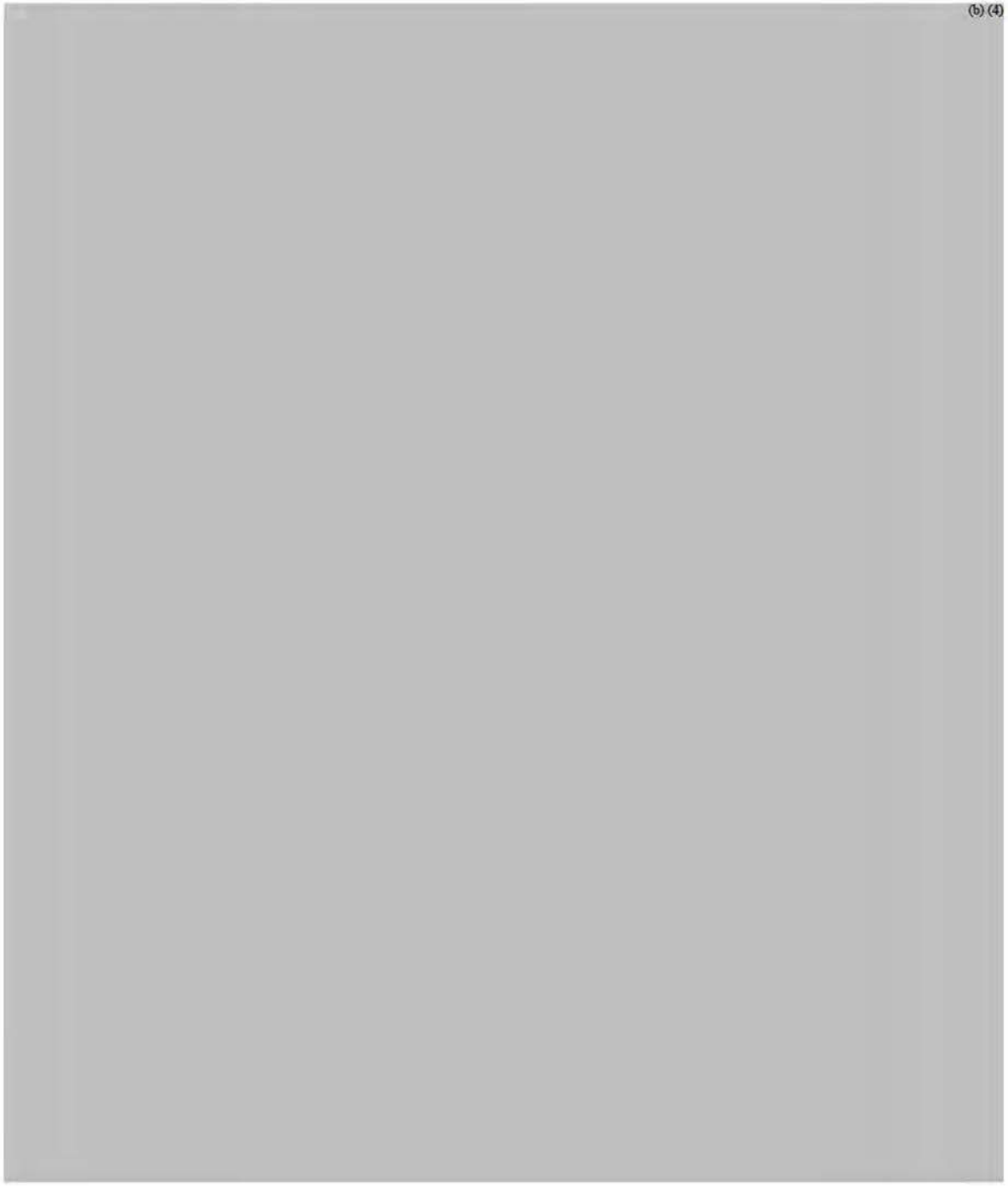
(b) (4)

Appendix 3 - Trueness data



Appendix 4 – Linearity data

Residual activity



Enzyme Preparations

DESCRIPTION

Enzyme Preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification*, below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one active component or, more commonly, a mixture of several, as well as food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practices. The individual preparations usually are named according to the substance to which they are applied, such as *Protease* or *Amylase*. Traditional names such as *Malt, Pepsin*, and *Rennet* also are used, however. The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand. The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food to achieve the desired effect. Additional information relating to the nomenclature and the sources from which the active components are derived is provided under *Appendix V: Enzyme Assays*

Function: Enzyme (see discussion under *Classification*, below)

Packaging and Storage: Store in well clo con ers in a cool, dry place.

IDENTIFICATION

Classification

- **ANIMAL-DERIVED PREPARAT NS**

Catalase, Bovine Liver: P uced partially purified liquid or powdered extract o bovine iver. Major active principle: *catalase*. Typ appli tion: used in the manufacture of certain cheeses.

Chymotrypsin: Obtained from pur ied extracts of bovine or porcine pancreatic tissue. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymotrypsin*. Typical application: used in the hydrolysis of protein.

Lipase, Animal: Obtained from the edible forestomach tissue of calves, kids, or lambs; and from animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts dispersible in water, but insoluble in alcohol. Major active principle: *lipase*. Typical applications: used in the manufacture of cheese and in the modification of lipids.

Lysozyme: Obtained from extracts of purified chicken egg whites. Generally prepared and used in the hydrochloride form as a white powder. Major active principle: *lysozyme*. Typical application: used as an antimicrobial in food processing.

Pancreatin: Obtained from porcine or bovine (ox) pancreatic tissue. Produced as a white to tan, water-soluble powder. Major active principles: (1) α-amylase; (2) protease; and (3) lipase. Typical applications: used in the preparation of precooked cereals, infant foods, and protein hydrolysates.

Pepsin: Obtained from the glandular layer of hog stomach. Produced as a white to light tan, water-soluble powder; amber paste; or clear, amber to brown, aqueous liquids. Major active principle: *pepsin*. Typical applications: used in the preparation of fishmeal and other protein hydrolysates and in the clotting of milk in the manufacture of cheese (in combination with rennet).

Phospholipase A_2: Obtained from porcine pancreatic tissue. Produced as a white to tan powder or pale to dark yellow liquid. Major active principle: *phospholipase* A_2. Typical application: used in the hydrolysis of lecithins.

Rennet, Bovine: Aqueous extracts made from the fourth stomach of bovines. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (pepsin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of sheep or goats.

Rennet, Calf: Aqueou racts made from the fourth stomach of calves. oduced as a clear, amber to dark brown liquid or a w ite to t powder. Major active principle: *prote se* (c mos). Typical application: used in the manufac re of cheese. Similar preparations may be made om th fourth stomach of lambs or kids.

Trypsin Obt d om purified extracts of porcine or bovin ancreas. oduced as white to tan, amorphous o ders luble in water, but practically insoluble in hol, in loroform, and in ether. Major active prin ple: *trypsin*. Typical applications: used in baking, in the te rizing of meat, and in the production of protein hydrolysates.

- **PL T-DERIVED PREPARATIONS**

A ylase: Obtained from extraction of ungerminated barley or extraction from grains of wheat. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: β-*amylase*. Typical applications: used in the production of alcoholic beverages and sugar syrups.

Bromelain: The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. (Fam. Bromeliaceae). Produced as a white to light tan, amorphous powder soluble in water (the solution is usually colorless to light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, in the production of protein hydrolysates, and in baking.

Ficin: The purified proteolytic substance derived from the latex of *Ficus* sp. (Fam. Moraceae), which includes a variety of tropical fig trees. Produced as a white to off-white powder completely soluble in water. (Liquid fig latex concentrates are light to dark brown.) Major active principle: *ficin*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, and in the conditioning of dough in baking.

Malt: The product of the controlled germination of barley. Produced as a clear amber to dark brown liquid preparation or as a white to tan powder. Major active principles: (1) α-*amylase* and (2) β-*amylase*. Typical applications: used in baking, in the manufacture of alcoholic beverages and of syrups.

Papain: The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as a white to light tan, amorphous powder or a liquid soluble in water (the solution is usually colorless or light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2)

chymopapain. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, and in the production of protein hydrolysates.

• **MICROBIALLY-DERIVED PREPARATIONS**

α-Acetolactatedecarboxylase: (*Bacillus subtilis* containing a *Bacillus brevis* gene) Produced as a brown liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually a light yellow to brown). Major active principle: *decarboxylase*. Typical application: used in the preparation of beer.

Aminopeptidase, Leucine: (*Aspergillus niger* var., *Aspergillus oryzae* var., and other microbial species) Produced as a light tan to brown powder or as a brown liquid by controlled fermentation using *Aspergillus niger* var., *Aspergillus oryzae* var., or other microbial species. The powder is soluble in water (the solution is usually light yellow to brown). Major active principles: (1) *aminopeptidase*, (2) *protease*, and (3) *carboxypeptidase* activities in varying amounts. Typical applications: used in the preparation of protein hydrolysates and in the development of flavors in processed foods.

Carbohydrase: (*Aspergillus niger* var., including *Aspergillus aculeatus*) Produced as an off-white to tan powder or a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. (including *Aspergillus aculeatus*). Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase* (a mixture of enzymes, including *pectin depolymerase, pectin methyl esterase, pectin lyase* and *pectate lyase*), (3) *cellulase*, (4) *glucoamylase* (amyloglucosidase), (5) *amylo-1,6-glucosi se*, (6 *hemicellulase* (a mixture of enzymes, incl ing *poly(galacturonate) hydrolase, arabino dase anno mannanase*, and *xylanase*), (7) *lacta* (8) β-*g anase*, (9) β-D-*glucosidase*, (10) *pentosanase*, an 1) α-*galactosidase*. Typical applica ons: use n the preparation of starch syrups nd dextrose, cohol, beer, ale, fruit juices, chocolate rups kery pro ucts, liquid coffee, wine, dairy produc ce als, and spice and flavor extracts.

Carbohydrase: (*Aspergillus oryz* a Produced as an off-white to tan, amorphous pow or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *glucoamylase* (amyloglucosidase), and (3) *lactase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, ale, bakery products, and dairy products.

Carbohydrase: (*Bacillus acidopullulyticus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Bacillus acidopullulyticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *pullulanase*. Typical applications: used in the hydrolysis of amylopectins and other branched polysaccharides.

Carbohydrase: (*Bacillus stearothermophilus*) Produced as an off-white to tan powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus stearothermophilus*. Soluble in water, but practically insoluble in alcohol, in ether, and in chloroform. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, and bakery products.

Carbohydrase: (*Candida pseudotropicalis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Candida pseudotropicalis*. Soluble in water (the solution is usually light yellow to dark brown) but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Kluyveromyces marxianus* var. *lactis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Kluyveromyces marxianus* var. *lactis*. Soluble in water (the solution is usually light yellow to dark brown), but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Mortie lla vinaceae* var. *raffinoseutilizer*) Produced as an off- hite tan powder or as pellets by controlled ferment on usin *Mortierella vinaceae* var. *raffinoseutilizer* Solu in w ter (pellets may be insoluble in water), but p ctica y soluble in alcohol, in chloroform and n ether. Major active principle: α-*g lactosi* T pical application: used in the produ on of sug from sugar beets.

rbohy se: (*Rhizopus niveus*) Produced as an off-white brown, orphous powder or a liquid by controlled fer ntation using *Rhizopus niveus*. Soluble in water (the soluti usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in e r. Major active principles: (1) α-*amylase* and (2) *g coamylase*. Typical application: used in the hydrolysis of starch.

Carbohydrase: (*Rhizopus oryzae* var.) Produced as a powder or a liquid by controlled fermentation using *Rhizopus oryzae* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase*, and (3) *glucoamylase* (amyloglucosidase). Typical applications: used in the preparation of starch syrups and fruit juices, vegetable purees, and juices and in the manufacture of cheese.

Carbohydrase: (*Saccharomyces* species) Produced as a white to tan, amorphous powder by controlled fermentation using a number of species of *Saccharomyces* traditionally used in the manufacture of food. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *invertase* and (2) *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: [(*Trichoderma longibrachiatum* var.) (formerly *reesei*)] Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Trichoderma longibrachiatum* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *cellulase*, (2) β-*glucanase*, (3) β-D-*glucosidase*, (4) *hemicellulase*, and (5) *pentosanase*. Typical applications: used in the preparation of fruit juices, wine, vegetable oils, beer, and baked goods.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus megaterium* α-*amylase* gene) Produced as an off-white to brown, amorphous powder or liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*amylase*. Typical

applications: used in the preparation of starch syrups, alcohol, beer, and dextrose.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus stearothermophilus* α-amylase gene) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: maltogenic *amylase*. Typical applications: used in the preparation of starch syrups, dextrose, alcohol, beer, and baked goods.

Carbohydrase and Protease, Mixed: (*Bacillus licheniformis* var.) Produced as an off-white to brown, amorphous powder or as a liquid by controlled fermentation using *Bacillus licheniformis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *protease*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, fishmeal, and protein hydrolysates.

Carbohydrase and Protease, Mixed: (*Bacillus subtilis* var. including *Bacillus amyloliquefaciens*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Bacillus subtilis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) β-*glucanase*, (3) *protease*, and (4) *pentosanase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, bakery products, and fishmeal in the tenderizing of meat, and in the preparation of protein hydrolysates.

Catalase: (*Aspergillus niger* var.) Produced as a off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical applications: used in the manufacture of cheese, egg products, and soft drinks.

Catalase: (*Micrococcus lysodeikticus*) Produced by controlled fermentation using *Micrococcus lysodeikticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical application: used in the manufacture of cheese, egg products, and soft drinks.

Chymosin: (*Aspergillus niger* var. *awamori, Escherichia coli K-12*, and *Kluyveromyces marxianus*, each microorganism containing a calf *prochymosin* gene) Produced as a white to tan, amorphous powder or as a light yellow to brown liquid by controlled fermentation using the above-named genetically modified microorganisms. The powder is soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymosin*. Typical application: used in the manufacture of cheese and in the preparation of milk-based desserts.

Glucose Isomerase: (*Actinoplanes missouriensis, Bacillus coagulans, Streptomyces olivaceus, Streptomyces olivochromogenes, Microbacterium arborescens, Streptomyces rubiginosus* var., or *Streptomyces murinus*) Produced as an off-white to tan, brown, or pink amorphous powder, granules, or liquid by controlled fermentation using any of the above-named organisms. The products may be soluble in water, but practically insoluble in alcohol, in chloroform, and in ether; or if immobilized, may be insoluble in water and partially soluble in alcohol, in chloroform, and in ether. Major active principle: *glucose* (or *xylose*) *isomerase*. Typical applications: used in the manufacture of high-fructose corn syrup and other fructose starch syrups.

Glucose Oxidase: (*Aspergillus niger* var.) Produced as a yellow to brown solution or as a yellow to tan or off-white powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2) *catalase*. Typical applications: used in the removal of sugar from liquid eggs and in the deoxygenation of citrus beverages.

Lipase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical application: used in the hydrolysis of lipids (e.g., fish oil concentrates and veal-derived lipids).

Lipase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids (e.g., fish oil concentrates) and in the manufacture of cheese and cheese flavors.

Lipase (*Candida rugosa;* formerly *Candida cylindracea*) Produced as an off-white to tan powder by controlled fermentation using *Candida rugosa*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of dairy products and confectionery goods, and in the development of flavor in processed foods.

Lipase: [*Rhizomucor (Mucor) miehei*] Produced as an off-white to tan powder or as a liquid by controlled fermentation using *Rhizomucor miehei*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of cheese, and in the removal of haze in fruit juices.

Phytase: (*Aspergillus niger* var.) Produced as an off-white to brown powder or as a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *3-phytase* and (2) *acid phosphatase*. Typical applications: used in the production of soy protein isolate and in the removal of phytic acid from plant materials.

Protease: (*Aspergillus niger* var.) Produced by controlled fermentation using *Aspergillus niger* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the production of protein hydrolysates.

Protease: (*Aspergillus oryzae* var.) Produced by controlled fermentation using *Aspergillus oryzae* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical applications: used in the chillproofing of beer, in the production of bakery products, in the tenderizing of meat, in the production of protein hydrolysates, and in the development of flavor in processed foods.

Rennet, Microbial: (nonpathogenic strain of *Bacillus cereus*) Produced as a white to tan, amorphous powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus cereus*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: (*Endothia parasitica*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using nonpathogenic strains of *Endothia parasitica*. The powder is soluble in water (the solution is usually tan to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: [*Rhizomucor (Mucor)* sp.] Produced as a white to tan, amorphous powder by controlled fermentation using *Rhizomucor miehei*, or *pusillus* var. Lindt. The powder is soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Transglutaminase: (*Streptoverticillium mobaraense* var.) Produced as an off-white to weak yellow-brown, amorphous powder by controlled fermentation using *Streptoverticillium mobaraense* var. Soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *transglutaminase*. Typical applications: used in the processing of meat, poultry, and seafood; production of yogurt, certain cheeses, and frozen desserts; and manufacture of pasta products and noodles, baked goods, meat analogs, ready-to-eat er , and other grain-based foods.

• Reactions Catalyzed

[NOTE—The reactions catalyzed by any given ive component are essentially the same, g rdles f the source from which that component is rived.]

α-Acetolactatedecarboxylas Decarboxy on of α-cetolactate to acetoin

Aminopeptidase, Leucine: yd ysis of *N*-terminal amino acid, which is preferably leu but m be other amino acids, from proteins and oligop tid , yielding free amino acids and oligopeptides o wer molecular weight

α-Amylase: Endohydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides

β-Amylase: Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding maltose and betalimit dextrins

Bromelain: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

Catalase: $2H_2O_2 \leftrightarrow O_2 + 2H_2O$

Cellulase: Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins

Chymosin (calf and fermentation derived): Cleaves a single bond in kappa casein

Ficin: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

α-Galactosidase: Hydrolysis of terminal nonreducing α-D-galactose residues in α-D-galactosides

β-Glucanase: Hydrolysis of β-1,3- and β-1,4-linkages in β-D-glucans, yielding oligosaccharides and glucose

Glucoamylase (amyloglucosidase): Hydrolysis of terminal α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose (dextrose)

Glucose Isomerase (xylose isomerase): Isomerization of glucose to fructose, and xylose to xylulose

Glucose Oxidase: β-D-glucose + $O_2 \leftrightarrow$D-glucono-δ-lactone + H_2O_2

β-D-Glucosidase: Hydrolysis of terminal, nonreducing β-D-glucose residues with the release of β-D-glucose

Hemicellulase: Hydrolysis of β-1,4-glucans, α-L-arabinosides, β-D-mannosides, 1,3-β-D-xylans, and other polysaccharides, yielding polysaccharides of lower molecular weight

Invertase (β-fructofuranosidase): Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar)

Lactase (β-galactosidase): Hydrolysis of lactose to a mixture of glucose and galactose

Lysozyme: Hydrolysis of cell-wall polysaccharides of various bacterial species leading to the breakdown of the cell wall most often in Gram-positive bacteria

Maltogenic Amylase: H drolysis of α-1,4-glucan bonds

Lipase: Hydrolysis o rigly erides of simple fatty acids, yielding mono- and dig yce es, glycerol, and free fatty acids

Pancreatin

α-Amyla Hyd lysis of α-1,4-glucan bonds

Protease: Hy o is of proteins and polypepticles

Lipase Hydrolysis of triglycerides of simple fatty acids

c nase
tate lya Hydrolysis of pectate to oligosaccharides

Pec depolymerase: Hydrolysis of 1,4 galacturonide bon

Pe tin lyase: Hydrolysis of oligosaccharides formed by ctate lyase

P ctinesterase: Demethylation of pectin

Pepsin: Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues, yielding peptides of lower molecular weight

Phospholipase A_2: Hydrolysis of lecithins and phosphatidylcholine, producing fatty acid anions

Phytase

3-Phytase: *myo*-Inositol hexakisphosphate + $H_2O \leftrightarrow$1,2,4,5,6-pentakisphosphate + orthophosphate

Acid Phosphatase: Orthophosphate monoester + $H_2O \leftrightarrow$an alcohol + orthophosphate

Protease (generic): Hydrolysis of polypeptides, yielding peptides of lower molecular weight

Pullulanase: Hydrolysis of 1,6-α-D-glycosidic bonds on amylopectin and glycogen and in α-and β-limit dextrins, yielding linear polysaccharides

Rennet (bovine and calf): Hydrolysis of polypeptides; specificity may be similar to pepsin

Transglutaminase: Binding of proteins

Trypsin: Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight

ASSAY

• Procedure

Analysis: The following procedures, which are included under *Appendix V: Enzyme Assays*, are provided for application as necessary in determining compliance with the declared representations for enzyme activity[1] : α-Acetolactatedecarboxylase Activity, Acid Phosphatase Activity, α-Amylase Activity (Nonbacterial); Bacterial α-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Chymotrypsin Activity; Diastase Activity (Diastatic

[1] Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, pectinase suppliers and users should develop their own assay procedures that would relate to the specific application under consideration.

Power); α-Galactosidase Activity, β-Glucanase Activity; Glucoamylase Activity (Amyloglucosidase Activity); Glucose Isomerase Activity; Glucose Oxidase Activity; β-D-Glucosidase Activity; Hemicellulase Activity; Invertase Activity; Lactase (Neutral) (β-Galactosidase) Activity; Lactase (Acid) (β-Galactosidase) Activity; Lipase Activity; Lipase/Esterase (Forestomach) Activity; Maltogenic Amylase Activity; Milk-Clotting Activity; Pancreatin Activity; Pepsin Activity; Phospholipase Activity; Phytase Activity; Plant Proteolytic Activity; Proteolytic Activity, Bacterial (PC); Proteolytic Activity, Fungal (HUT); Proteolytic Activity, Fungal (SAP); Pullulanase Activity; and Trypsin Activity.

Acceptance criteria: NLT 85.0% and NMT 115.0% of the declared units of enzyme activity

IMPURITIES

• **LEAD,** *Lead Limit Test, Appendix IIIB*
Control: 5 μg Pb (5 mL of *Diluted Standard Lead Solution*)
Acceptance criteria: NMT 5 mg/kg

SPECIFIC TESTS

• **MICROBIAL LIMITS**

[NOTE—Current methods for the following tests may be found in the Food and Drug Administration's Bacteriological Analytical Manual online at www.fda.gov/Food/default.htm.]

Acceptance criteria
Coliforms: NMT 30 CFU/g
Salmonella: Negative in 25 g

OTHER REQUIREMENTS

Enzyme preparations are produced in accordance with d manufacturing practices. Regardless of the s urce derivation, they should cause no increase the to l microbial count in the treated food over th el ac pted for the respective food.

Animal tissues used to produce enzyme ust com ly with the applicable U.S. meat inspect n re ments d must be handled in accordance with good hygie practices.

Plant material used to produc enzym or cul e media used to grow microorganis con t of components that leave no residues harmful to h in th nished food under normal conditions of use

Preparations derived from microbia rces shall be obtained using a pure culture fermentation of a non-pathogenic and non-toxigenic strain and are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of microorganisms that could be the source of toxic materials and other undesirable substances.

The carriers, diluents, and processing aids used to produce the enzyme preparations shall be substances that are acceptable for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.

DuPont Nutrition & Biosciences
BA&F Microbiology Group
1700 Lexington Avenue
Rochester, NY 14606

Production Strain QC Testing - Certificate of Analysis

Product Information:

Product Name:	Eclipse B Form Conc
Product Code:	C14101
Lot Number:	1683520613

Detection of Production Strain Method Sheet (R-DOC-GM-2006)

DuPont Industrial Biosciences uses a (b) (4) method to detect for the presence of production strain in end-product. To ensure the absence of production strain in end-product, DuPont utilizes (b) (4) This method is sensitive to detect <1 CFU/ml for liquid samples or <10 CFU/g for granular samples.

Summary of Test Method Parameters and Control Information

Product GICC Number	(b) (4)		
Media	(b) (4)		
Media Processing Aid w/ concentration	Not Applicable		
Dilution Tested	(b) (4)	g/ml dissolution	Not Applicable
Amount Plated	(b) (4)		
Number of Analyses / Sample	3		
Number of Replicate Plates / Analysis	2		
Incubation Temperature (°C)	(b) (4)		
Total Incubation Time (hrs)	(b) (4)		

Result Table

	Plate Type	Final Plate Count	End Result
Analysis 1	Plate 1 Replicate	0	(b) (4)
	Plate 2 Replicate	0	
Analysis 2	Plate 1 Replicate	0	
	Plate 2 Replicate	0	
Analysis 3	Plate 1 Replicate	0	
	Plate 2 Replicate	0	
Production Strain Control		(b) (4)	
Overall Production Strain Test Result		**Negative/mL**	

Prepared By:

Name:	(b) (4)
Position:	Microbiology Specialist
Date:	9/28/2020

DuPont Nutrition & Biosciences
BA&F Microbiology Group
1700 Lexington Avenue
Rochester, NY 14606

Production Strain QC Testing - Certificate of Analysis

Product Information:

Product Name:	(b)(6), (b) (4)
Product Code:	
Lot Number:	1663624495

Detection of Production Strain Method Sheet (R-DOC-GM-2006)

DuPont Industrial Biosciences uses a (b) (4) method to detect for the presence of production strain in end-product. To ensure the absence of production strain in end-product, DuPont utilizes (b) (4) This method is sensitive to detect <1 CFU/ml for liquid samples or <10 CFU/g for granular samples.

Summary of Test Method Parameters and Control Information

Product GICC Number	(b) (4)		
Media	(b) (4)		
Media Processing Aid w/ concentration	Not Applicable		
Dilution Tested	(b) (4)	g/ml dissolution	Not Applicable
Amount Plated	(b) (4)		
Number of Analyses / Sample	3		
Number of Replicate Plates / Analysis	2		
Incubation Temperature (°C)	(b) (4)		
Total Incubation Time (hrs)			

Result Table

	Plate Type	Final Plate Count	End Result
Analysis 1	Plate 1 Replicate	0	(b) (4)
	Plate 2 Replicate	0	
Analysis 2	Plate 1 Replicate	0	
	Plate 2 Replicate	0	
Analysis 3	Plate 1 Replicate	0	
	Plate 2 Replicate	0	
Production Strain Control			(b) (4)
Overall Production Strain Test Result			Negative/mL

Prepared By:

Name:	(b) (4)
Position:	Microbiology Specialist
Date:	7/17/2020

DuPont Nutrition & Biosciences
BA&F Microbiology Group
1700 Lexington Avenue
Rochester, NY 14606

Production Strain QC Testing - Certificate of Analysis

Product Information:

Product Name:	(b) (4)
Product Code:	
Lot Number:	1663723699

Detection of Production Strain Method Sheet (R-DOC-GM-2006)

DuPont Industrial Biosciences uses a (b) (4) method to detect for the presence of production strain in end-product. To ensure the absence of production strain in end-product, DuPont utilizes (b) (4). This method is sensitive to detect <1 CFU/ml for liquid samples or <10 CFU/g for granular samples.

Summary of Test Method Parameters and Control Information

Product GICC Number	(b) (4)		
Media	(b) (4)		
Media Processing Aid w/ concentration	Not Applicable		
Dilution Tested	(b) (4)	**g/ml dissolution**	Not Applicable
Amount Plated	(b) (4)		
Number of Analyses / Sample	3		
Number of Replicate Plates / Analysis	2		
Incubation Temperature (°C)	(b) (4)		
Total Incubation Time (hrs)			

Result Table

	Plate Type	Final Plate Count	End Result
Analysis 1	**Plate 1 Replicate**	0	(b) (4)
	Plate 2 Replicate	0	
Analysis 2	**Plate 1 Replicate**	0	
	Plate 2 Replicate	0	
Analysis 3	**Plate 1 Replicate**	0	
	Plate 2 Replicate	0	
Production Strain Control		(b) (4)	(b) (4).
Overall Production Strain Test Result		**Negative/mL**	

Prepared By:

Name:	(b) (4)
Position:	Microbiology Specialist
Date:	7/17/2020

IFF Health & Biosciences
Global Microbiology Group
1700 Lexington Avenue
Rochester, NY 14606

Confidential Information

Detection of Production Strain - Method Sheet

Purpose:

IFF Health & Biosciences uses a [redacted] to detect for the presence of production strain in end-product. To ensure the absence of production strain in end-product, IFF utilizes [redacted]

References:

[redacted] (Intellectual Property)

Materials	Equipment
General Nutrient Agar for Bacteria [redacted]	Incubator [redacted]
General Nutrient Agar for Yeast and Mold [redacted]	Water Bath [redacted]
[redacted] or Another Suitable Diluent	Biological Safety Cabinet
Processing Aid for the Selection of Production Strain (if applicable)	Microwave/Autoclave to Melt Agar
Production Strain Microorganism	Sterile Empty Petri Dishes
	Sterile Pipets
	Sterile Spreaders
	Plate Spinner

Method:

[redacted]

Results Interpretation:

If the production strain microorganism is not detected, report results as negative/mL (g). If the production strain microorganism is detected, report as positive/mL (g).

AXTRA®
PHY GOLD 30 L

Not for human consumption.

Liquid enzyme containing a source of phytase which increases the digestibility of phytin-bound phosphorus in poultry and swine diets.

Guaranteed analysis:

Phytase (from Trichoderma reesei) activity not less than:	852,000 units/oz (30,000 units/gram)

(b) (4)

Ingredients:
Water. Sorbitol. Liquid Trichoderma reesei fermentation product. Sodium chloride. Sodium citrate. Potassium sorbate. Sodium benzoate.

Directions for use:
Use at a rate of 0.016-0.266 lbs/short ton (US) or 0.008-0.133 kg/tonne (0.0008-0.0133%) of finished feed.
Where conditioning and pelleting temperatures exceed (b) (4) apply post pelleting.

(b) (4)

Country of origin: United States

Manufactured for: Danisco US Inc. 3490 Winton Place, Rochester, New York 14623 USA
+1 (585) 256-5200 +1 (800) 847-5311

Manufactured by: Danisco US Inc, Cedar Rapids Plant, 1000 41st Avenue Drive SW, Cedar Rapids, Iowa 52404.

(b) (4)

EAN No:

Net Weight: 441 lbs (200kg)

Batch No:

Best Before:

DANISCO

AXTRA®
PHY GOLD 30 T

Not for human consumption. Contains a source of phytase which increases the digestibility of phytin-bound phosphorus in poultry and swine diets.

Guaranteed analysis:
Phytase (from Trichoderma reesei) activity not less than: 852,000 units/oz (30,000 units/gram)

(b) (4)

Ingredients:
Sodium sulfate. Dried Trichoderma reesei fermentation product. Poly-vinyl alcohol (PVA). Talc.

Directions for use:
(b) (4)
.

(b) (4)

Country of origin: United States

Manufactured for: Danisco US Inc. 3490 Winton Place, Rochester, New York 14623 USA
+1 (585) 256-5200 +1 (800) 847-5311

Manufactured by: Danisco US Inc, Cedar Rapids Plant, 1000 41st Avenue Drive SW, Cedar Rapids, Iowa 52404.

(b) (4)

EAN No:

Net Weight: 2205 lbs (1000kg)

Batch No:

Best Before:

DANISCO

AXTRA®
PHY GOLD 65 G

Not for human consumption. Contains a source of phytase which increases the digestibility of phytin-bound phosphorus in poultry and swine diets.

Guaranteed analysis:

Phytase (from Trichoderma reesei) activity not less than:	1,846,000 units/oz (65,000 units/gram)

Definition of units :
One phytase unit of activity liberates 1 µmol of phosphate from phytate in one minute under the conditions of the assay at 99°F (37°C) and pH 5.5.

Ingredients:
Sodium sulfate. Dried Trichoderma reesei fermentation product. Poly-vinyl alcohol (PVA).

(b) (4)

Country of origin: United States

Manufactured for: Danisco US Inc. 3490 Winton Place, Rochester, New York 14623 USA
+1 (585) 256-5200 +1 (800) 847-5311

Manufactured by: Danisco US Inc. Cedar Rapids Plant, 1000 41st Avenue Drive SW, Cedar Rapids, Iowa 52404.

(b) (4)

EAN No:

Net Weight: 2205 lbs (1000kg)

Batch No:

Best Before:

DANISCO

REPORT

ID: 23345-02/2020

Determination of the stability of "Axtra PHY® GOLD 30T" after 12 months storage at (b) (4)

Author: (b)(6)

Study completed: November 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Study scientist:

(b)(6)

Signature: (b) (6) Date: 29/01/2021

This report is approved by:

Product Manager:

Arno de Kreij

Signature: (b) (6) Date: 02/02/2021

RESPONSIBLE PERSONNEL

Study Scientist(s) (b)(6)

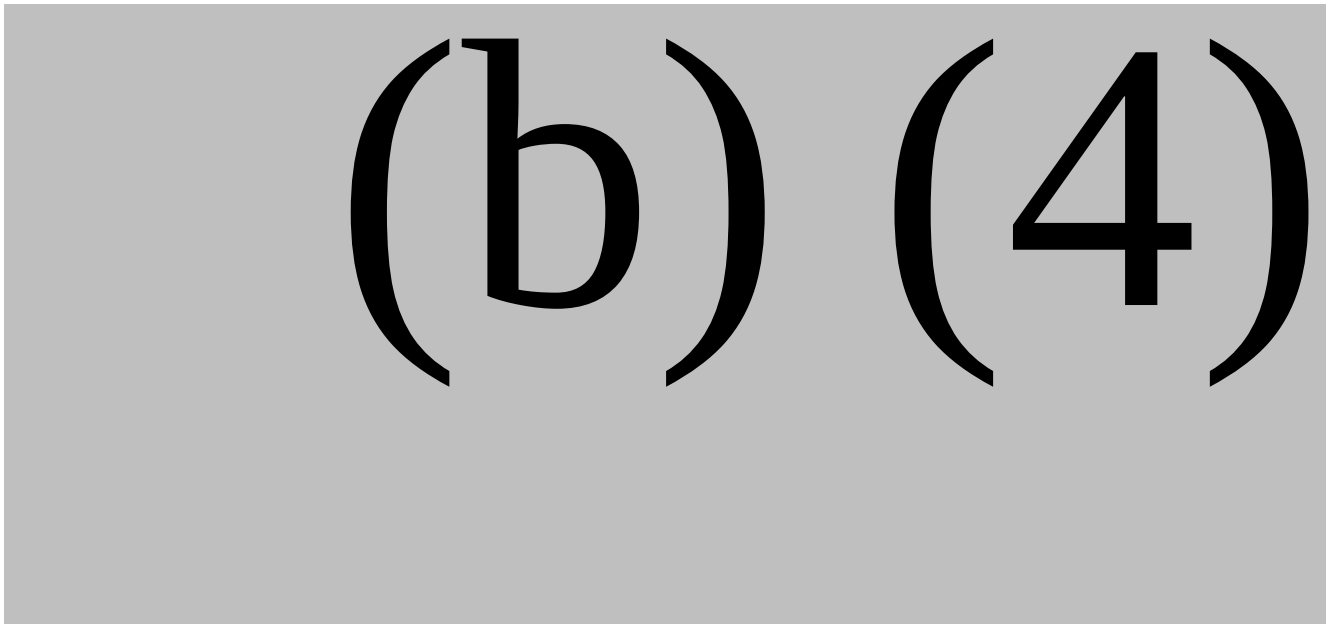
Product manager: Arno de Kreij

Regulatory manager: Lisa Merethe Jensen

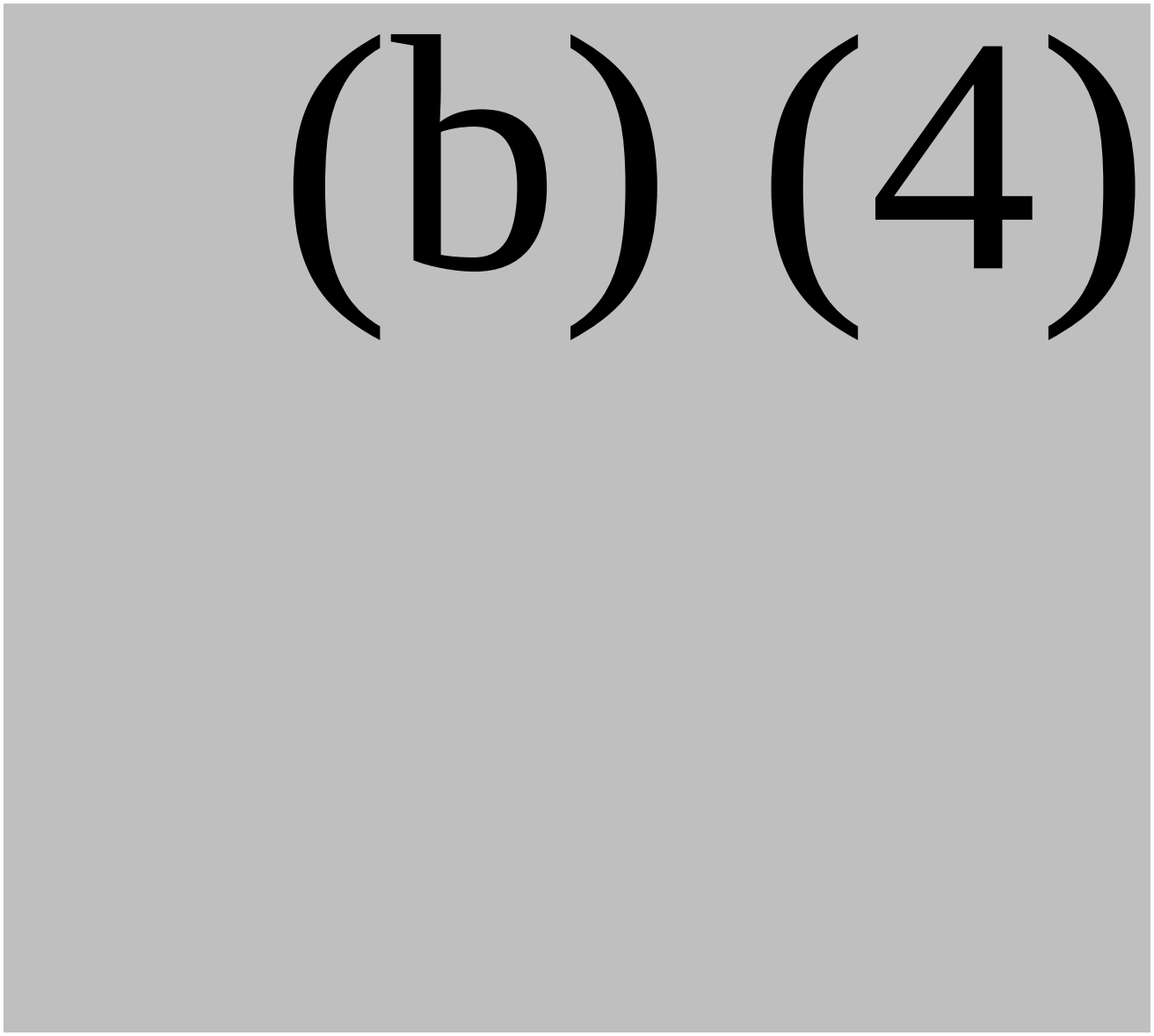
ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY



2. OBJECTIVE(S)



7. RESULTS

Phytase activity is reported in the tables below.

Table 1 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30T, when stored for 12 months at (b) (4)

	Time (months)			
FTU/g	0	3	6	12
3099269156	(b) (4)			
3099273157				
3099274158				
Mean	**53969**	**52308**	**52365**	**55463**
SD	(b) (4)			
%CV				
%t=0				

Notes: SD = Standard Deviation; %CV = Coefficient of variation; %t=0 = percentage recovery at each time point respect to t=0. (b) (4)

Table 2 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30T when stored for 12 months at (b) (4)

	Time (months)			
FTU/g	0	3	6	12
3099269156	(b) (4)			
3099273157				
3099274158				
Mean	**53969**	**46245**	**42186**	**37508**
SD	(b) (4)			
%CV				
%t=0				

Notes: SD = Standard Deviation; %CV = Coefficient of variation; %t=0 = percentage recovery at each time point respect to t=0. (b) (4)

Figure 1. Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30T, when stored for 12 months at (b) (4)

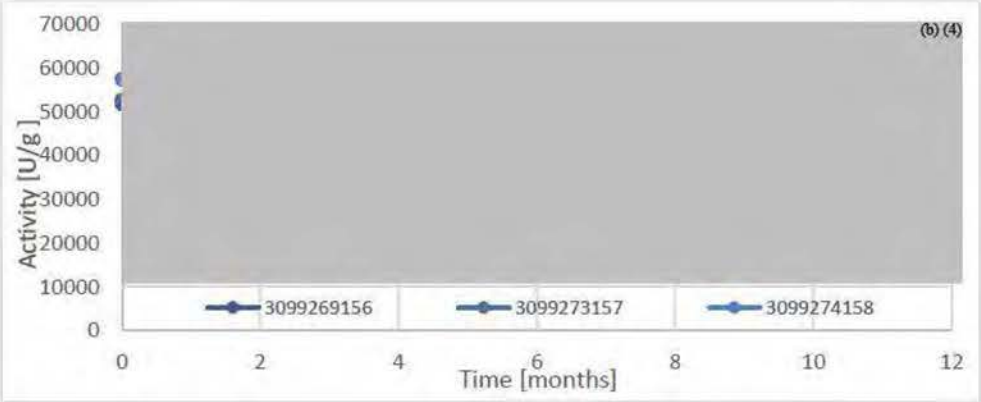
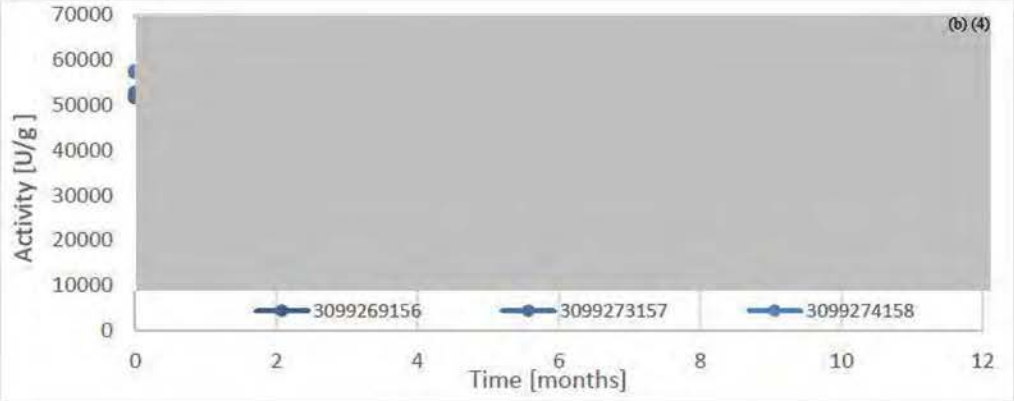


Figure 2. Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30T when stored for 12 months at (b) (4)



8. CONCLUSION

(b) (4)

(b) (4)

REPORT

ID: 23394-04/2020

Determination of Stability of "Axtra® PHY GOLD 30 L" after 12 months storage at (b) (4)

Authors: (b)(6)
Study completed: August/2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Study scientist:
(b)(6)

Signature: (b) (6) Date: 29/01/2021

This report is approved by:
Product Manager:
Arno de Kreij

Signature: (b) (6) Date: 02/02/2021

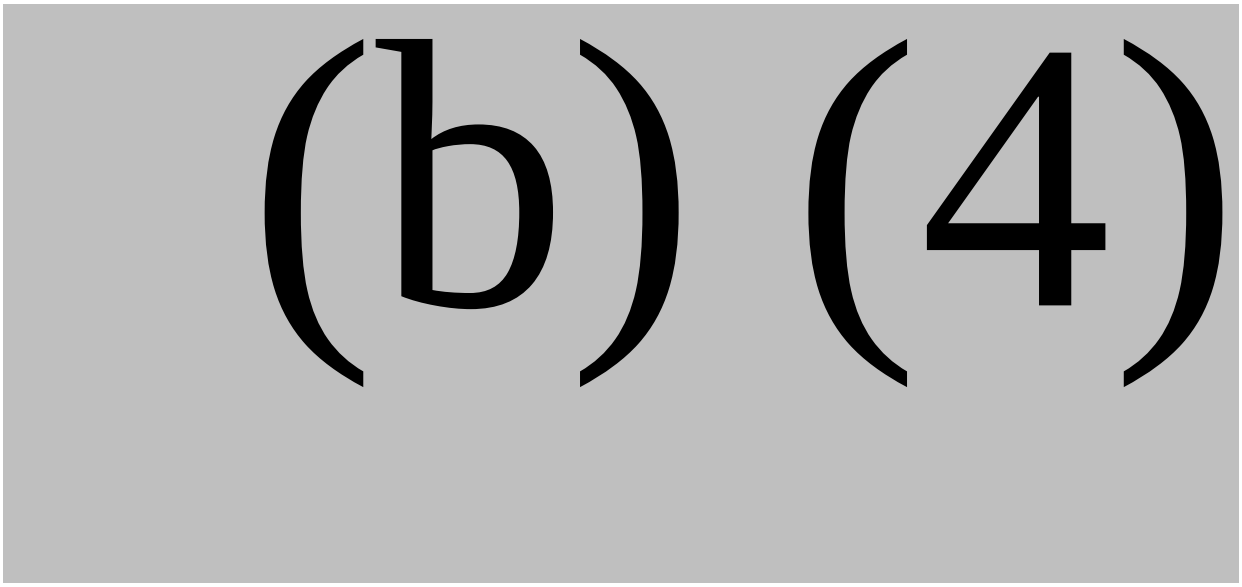
RESPONSIBLE PERSONNEL

Study Scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory specialist	:	Lisa Merethe Jensen

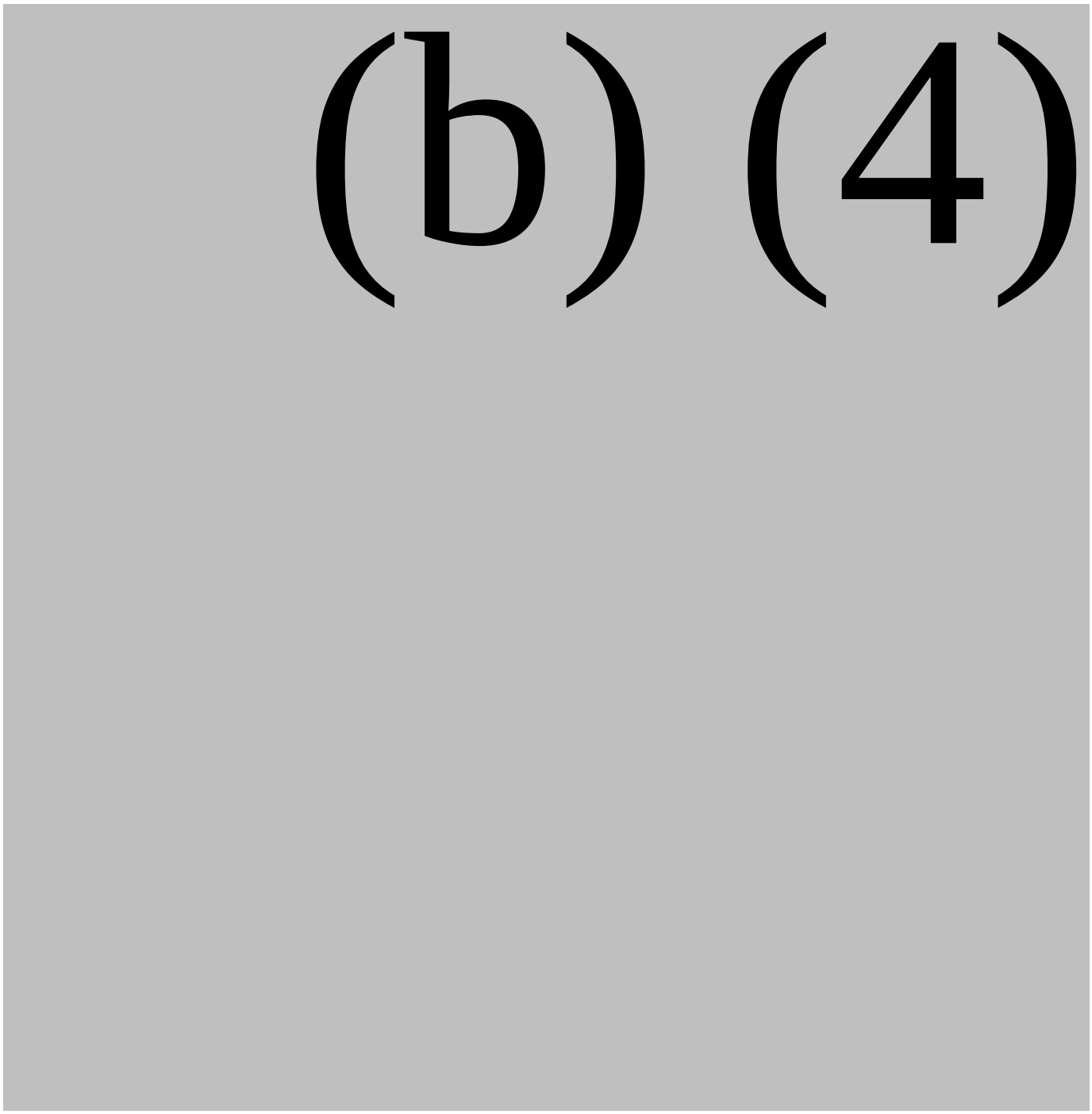
ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY



2. OBJECTIVE(S)



7. RESULTS

Data in the summary table below show the analysed results for phytase activity.

Table 1 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30 L, when stored for 12 months at (b) (4)

Phytase FTU/kg	Time (months)			
	0	3	6	12
2019L001	(b) (4)			
2019L005				
2019L009				
Mean	**52937**	**49787**	**47215**	**50033**
Std Dev	(b) (4)			
%CV				
%t=0				

Notes: SD = Standard Deviation; %CV = Coefficient of variation; %t=0 = percentage recovery at each time point respect to t=0. (b) (4)

Table 2 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30 L, when stored for 12 months at (b) (4)

Phytase FTU/kg	Time (months)			
	0	3	6	12
2019L001	(b) (4)			
2019L005				
2019L009				
Mean	**52937**	**46398**	**37328**	**29370**
Std Dev	(b) (4)			
%CV				
%t=0				

Notes: SD = Standard Deviation; %CV = Coefficient of variation; %t=0 = percentage recovery at each time point respect to t=0. (b) (4)

Figure 1. Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30 L, when stored for 12 months at (b) (4)

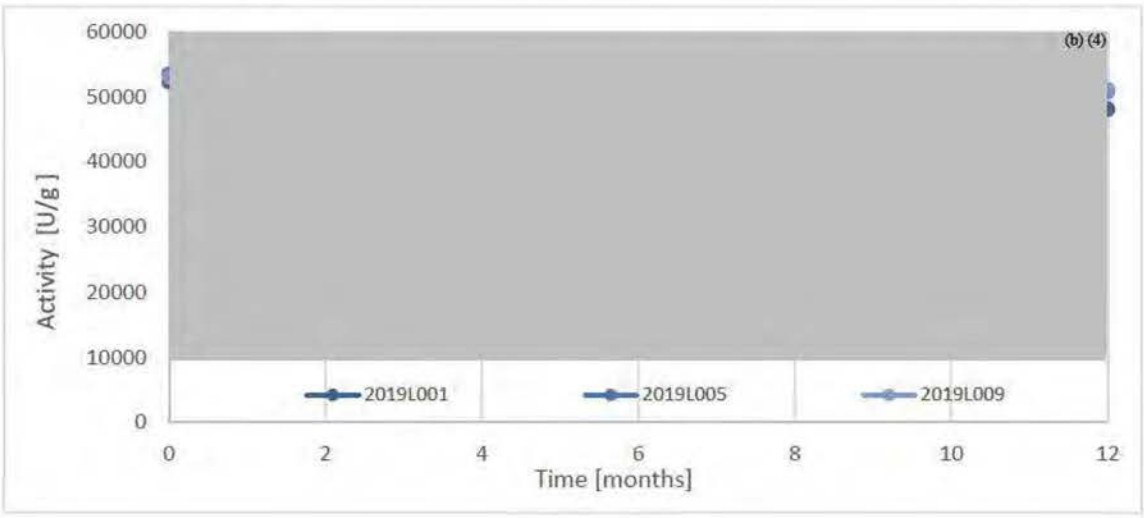
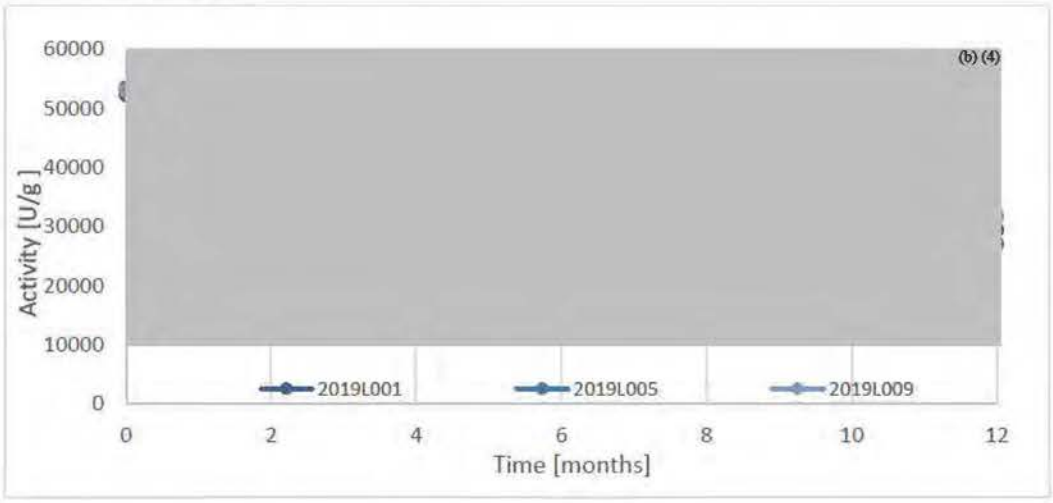


Figure 2. Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 30 L, when stored for 12 months at (b) (4)



8. CONCLUSION

(b) (4)

(b) (4)

REPORT

ID: 23413-02/2020

Determination of the stability of "Axtra PHY® GOLD 65G" after 12 months storage at (b) (4)

Author: (b)(6)

Study completed: November 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Study scientist:

(b)(6)

Signature: (b) (6) Date: 29/01/2021

This report is approved by:

Product Manager:

Arno de Kreij

Signature: … (b) (6) Date: 02/02/2021

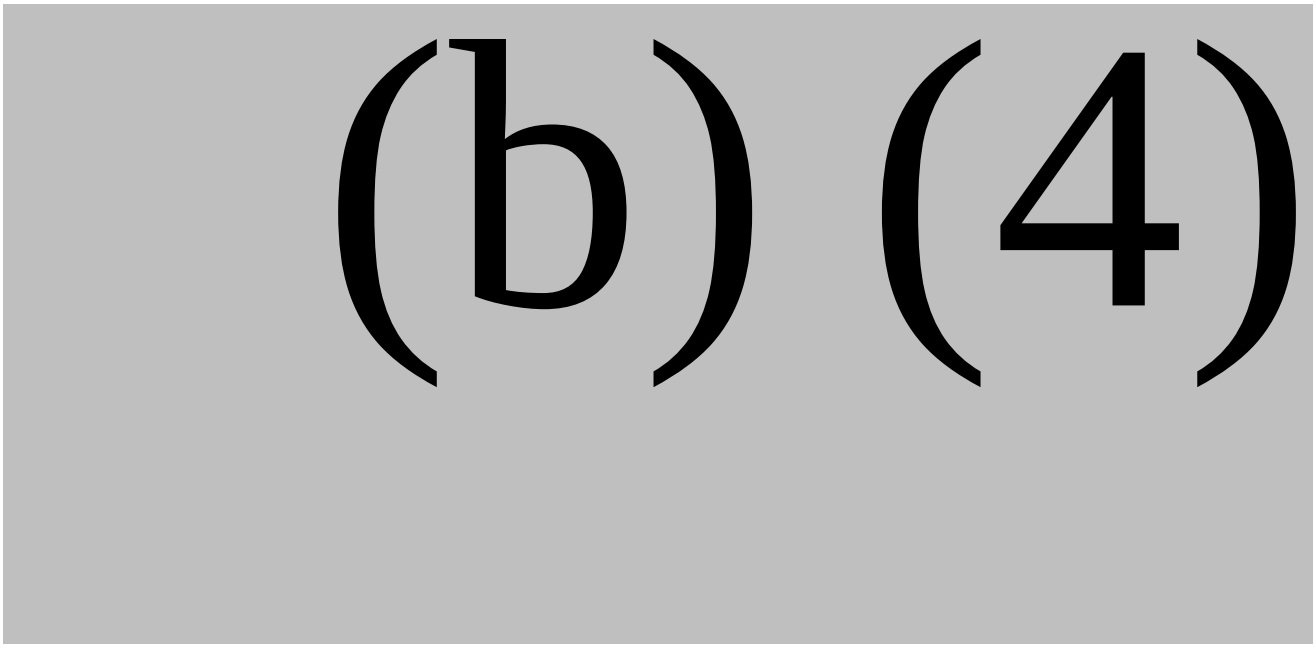
RESPONSIBLE PERSONNEL

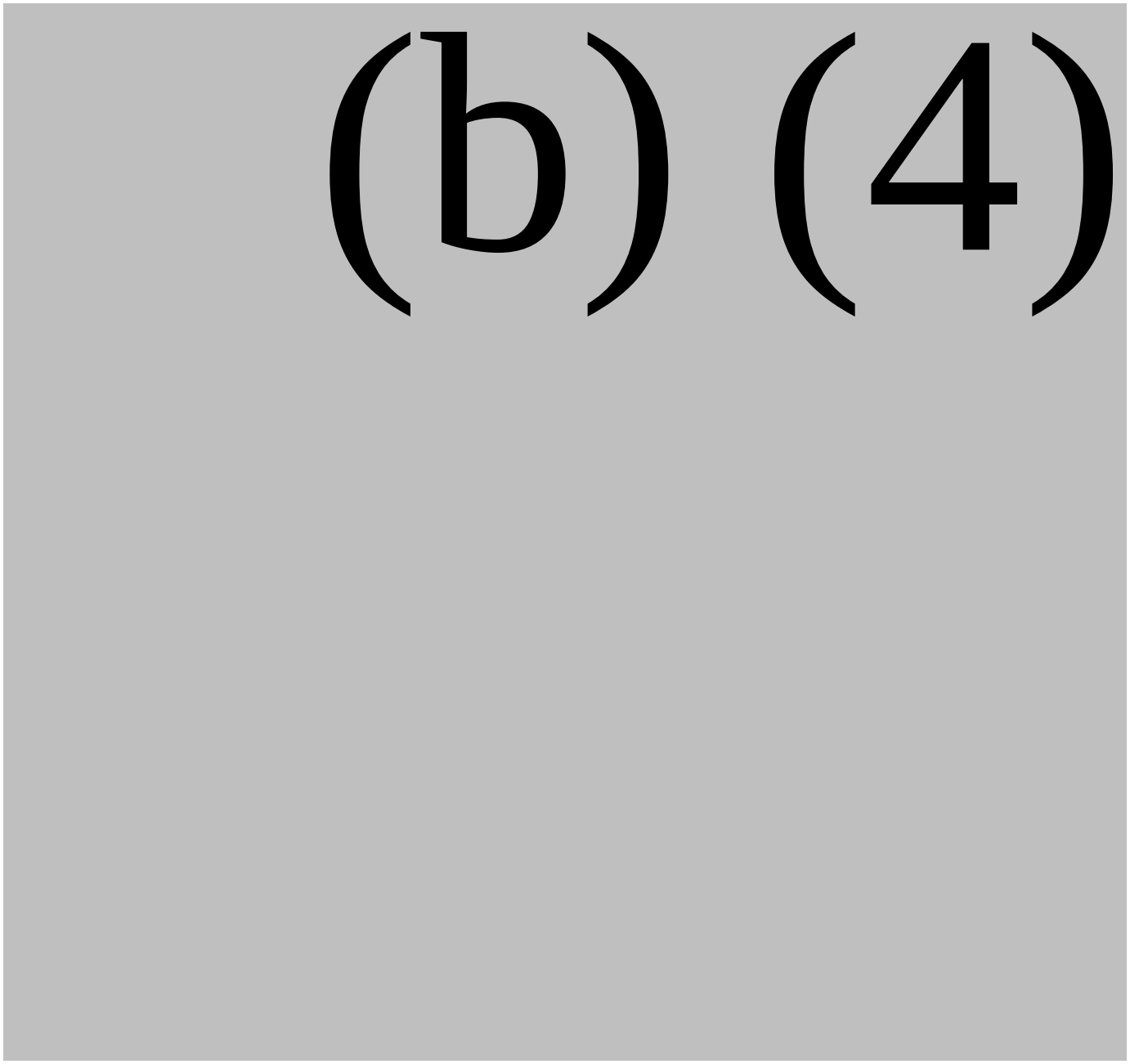
Study Scientist(s) : (b)(6)
Product manager : Arno de Kreij
Regulatory manager : Lisa Merethe Jensen

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY




(b) (4)

7. RESULTS

Phytase activity is reported in the tables below.

Table 1 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 65G, when stored for 12 months at (b) (4)

	Time (months)			
FTU/g	0	3	6	12
3099281163				(b) (4)
3099283165				
3099294170				
Mean	**117825**	**117986**	**119240**	**110004**
SD				(b) (4)
%CV				
%t=0				

Notes: SD = Standard Deviation; %CV = Coefficient of variation; %t=0 = percentage recovery at each time point respect to t=0. Guaranteed minimum: (b) (4)

Table 2 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 65G, when stored for 12 months at (b) (4)

	Time (months)			
FTU/g	0	3	6	12
3099281163				(b) (4)
3099283165				
3099294170				
Mean	**117825**	**112759**	**108919**	**83980**
SD				(b) (4)
%CV				
%t=0				

Notes: SD = Standard Deviation; %CV = Coefficient of variation; %t=0 = percentage recovery at each time point respect to t=0. Guaranteed minimum: (b) (4)

Figure 1 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 65G, when stored for 12 months at (b) (4)

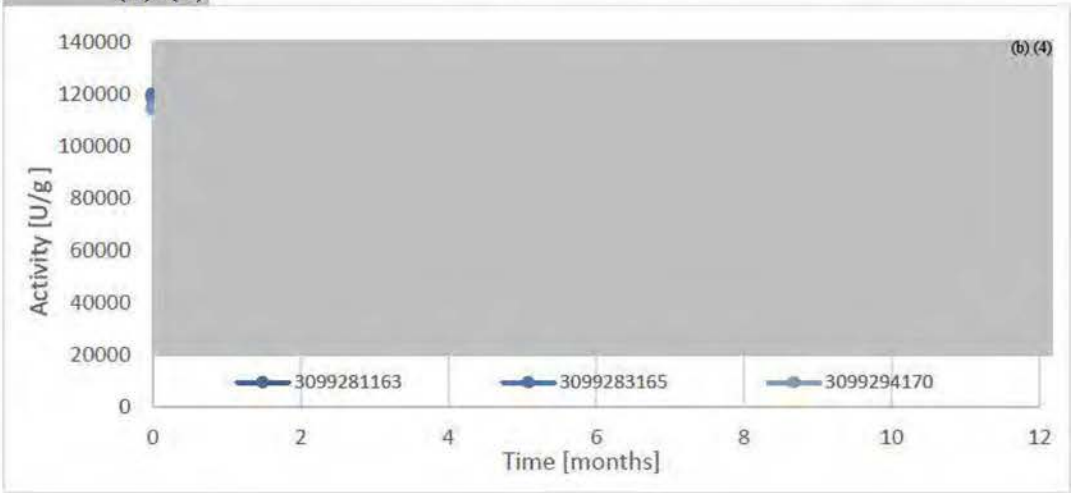
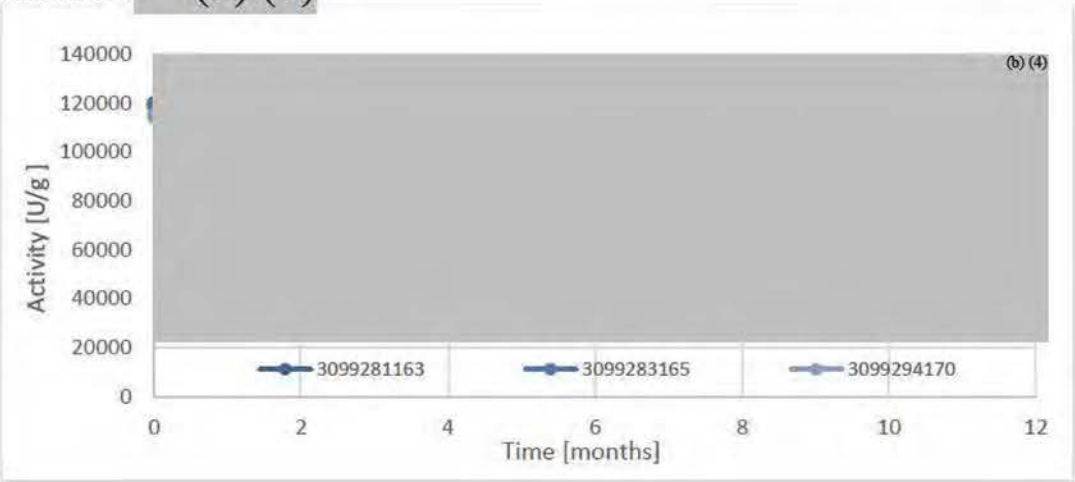


Figure 2 Stability of phytase activity (FTU/g) of Axtra PHY® GOLD 65G, when stored for 12 months at (b) (4)



8. CONCLUSION

(b) (4)

(b) (4)

REPORT

ID: 23496/2020

Stability of "Axtra® PHY GOLD 30 T" in vitamin-mineral premix when stored for 6 months at (b) (4)

Author: A. Tossoli

Study completed: Jul 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature (b)(6) ..... Date: 15/07/2020

Study Scientist:

(b)(6)

Signature:..... Date: 16/07/2020

This report is approved by:

Product Manager:

Arno de Kreij (b)(6)

Signature (b)(6) Date: 16/07/2020

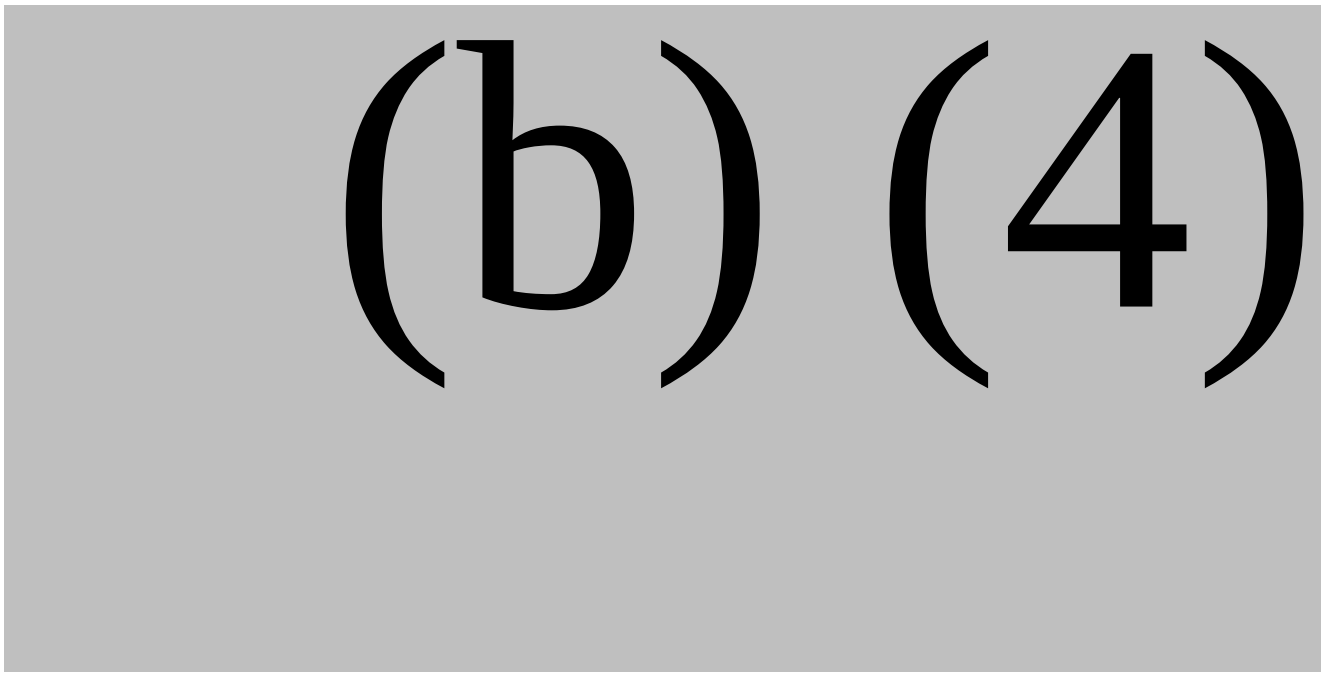
RESPONSIBLE PERSONNEL

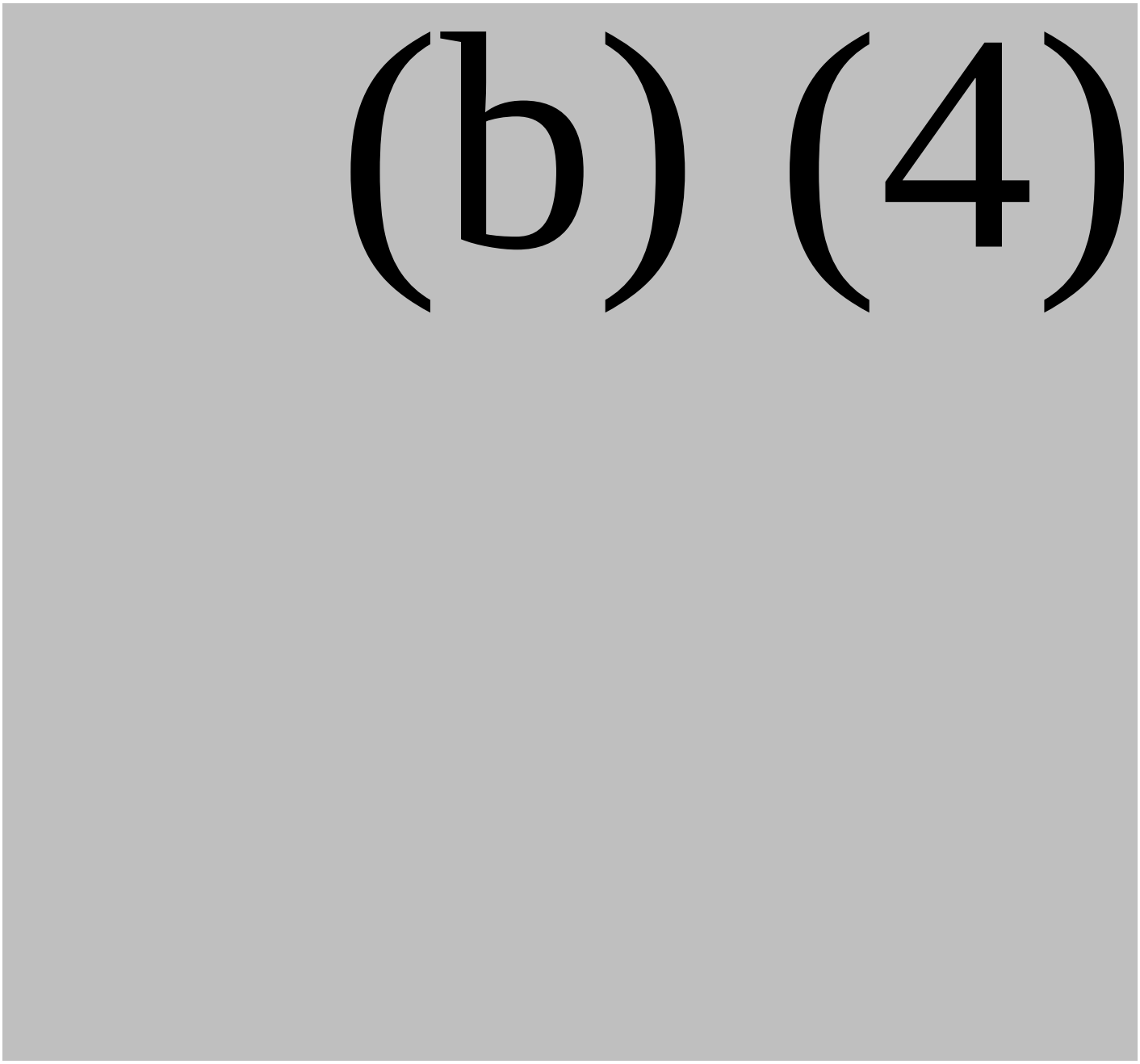
Study scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY

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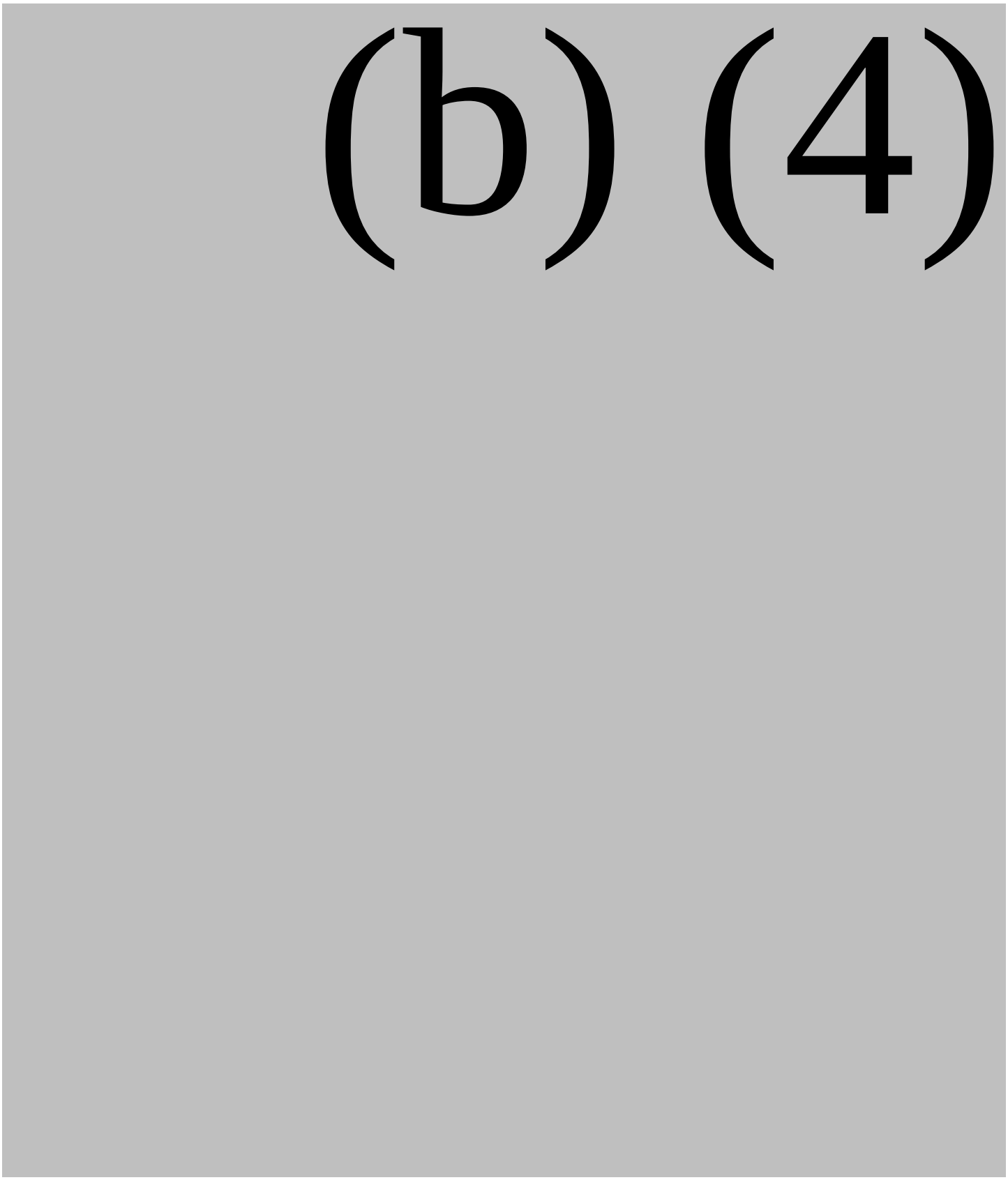
(b) (4)

(b) (4)

Animal feed premixtures
– Determination of phytase activity
– Colorimetric method
F_030/03)

Authorisation and Issue Control

I hereby declare that this method is an accurate reflection of the method conducted in our laboratory, and that the method was developed and written under my supervision and in accordance with standard operating procedures.

Scientist, Feed Biochemistry/Scientific Business support
(b)(6)

Signatur (b) (4) Date: 30.09.2020

This report is approved by Dupont Animal Nutrition Management:
Senior Principal Scientist, Feed Biochemistry/Scientific Business support
(b)(6)

Signatur (b)(6) Date: 30.09.2020

Contents

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Validation - Determination of phytase activity in animal feeding stuffs and premixtures – Colorimetric method (F_031/08)

Authorisation and Issue Control

I hereby declare that this in-house validation study is an accurate reflection of the study conducted in our laboratory, and that the study was developed and written under my supervision and in accordance with standard operating procedures and IUPAC harmonised guidelines for single laboratory validation of methods of analysis.

(b) (4), (b) (6)
Scientist
Animal Nutrition, Technology & Innovation

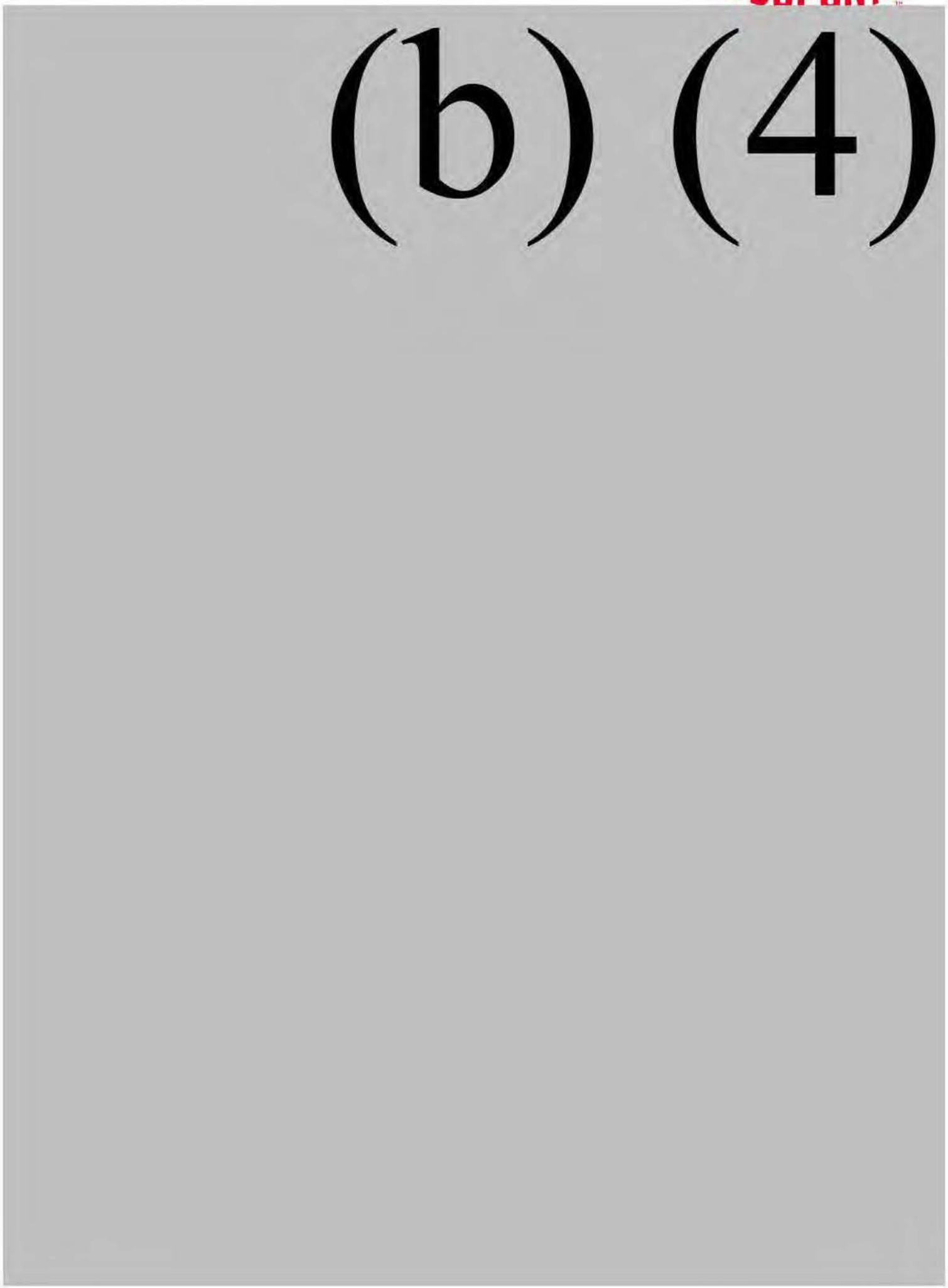
Signature (b) (4), (b) (6) Date: 2020-09-22

This report is approved by DuPont Animal Nutrition Management:

(b) (4), (b) (6)
(b)(6)
Animal Nutrition, Technology & Innovation

Signature (b) (4), (b) (6) Date: 2020-09-24

Contents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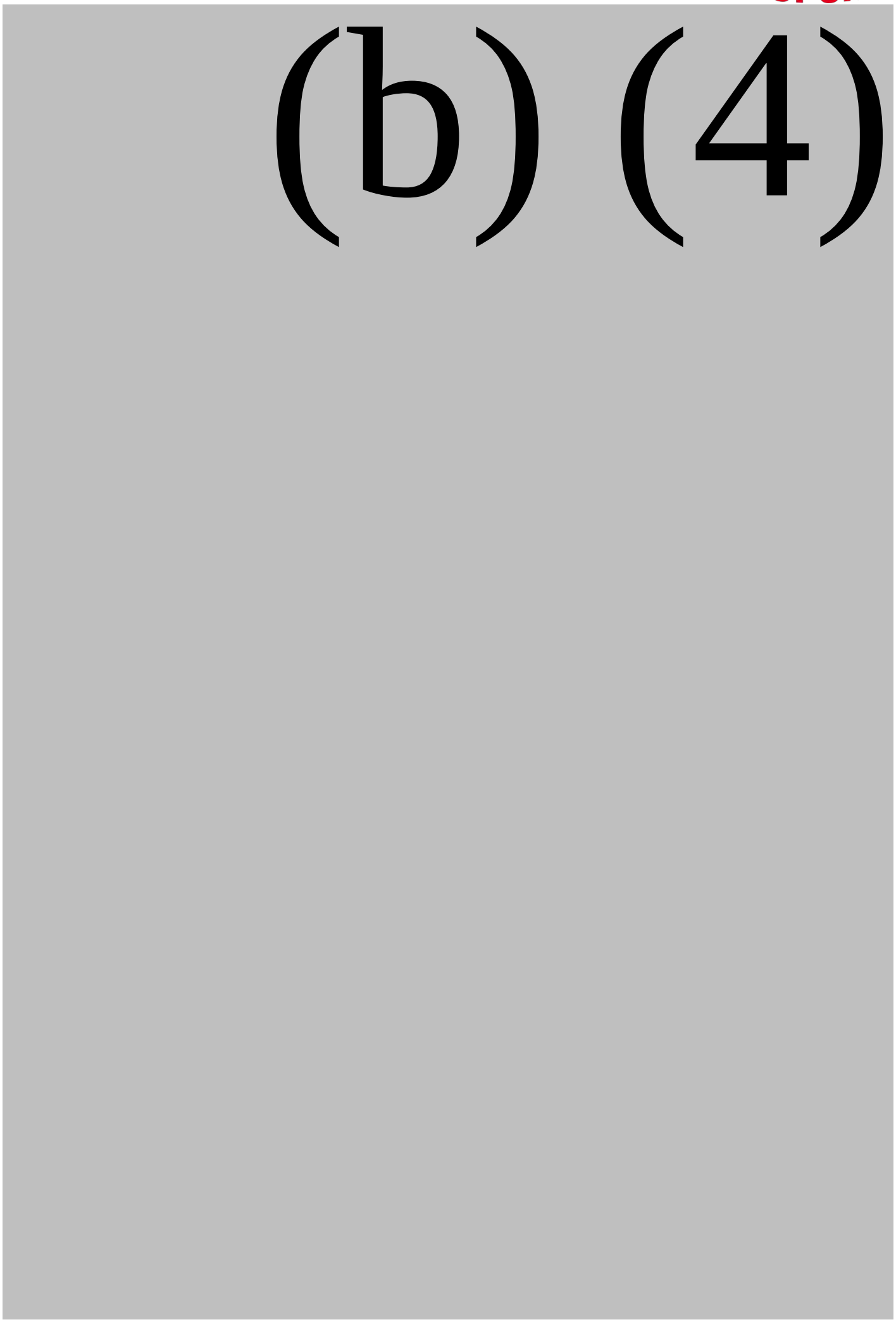
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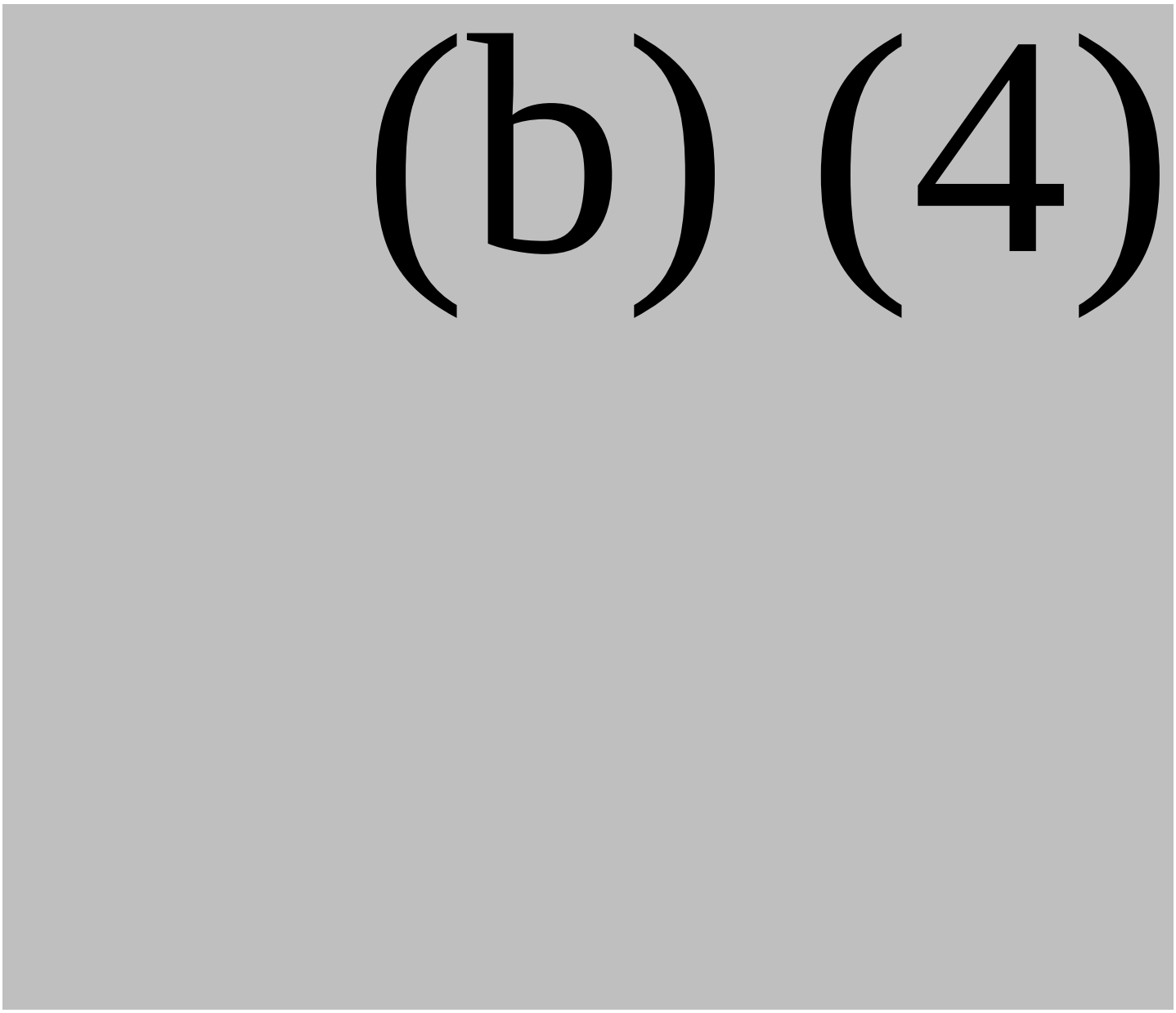
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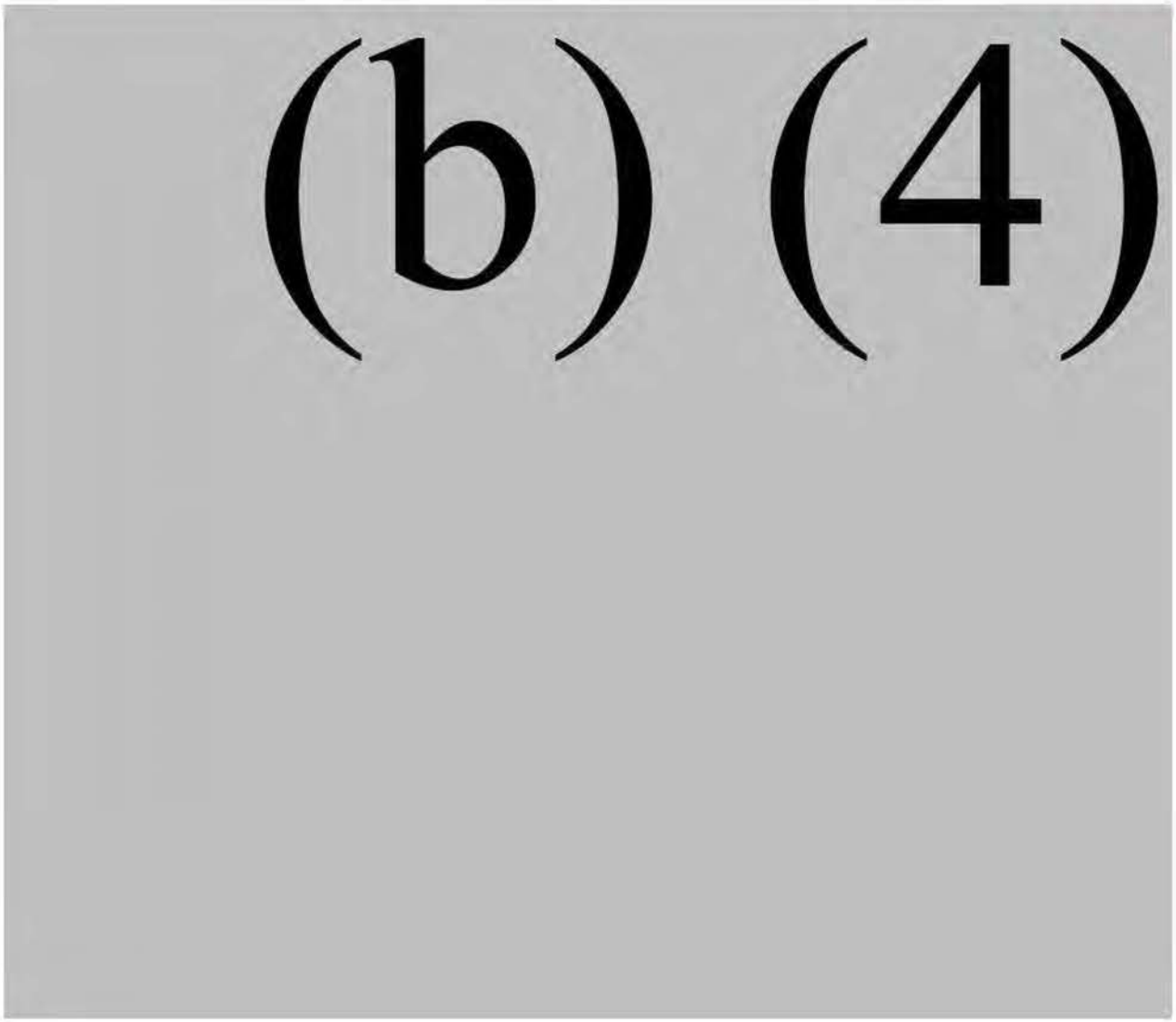
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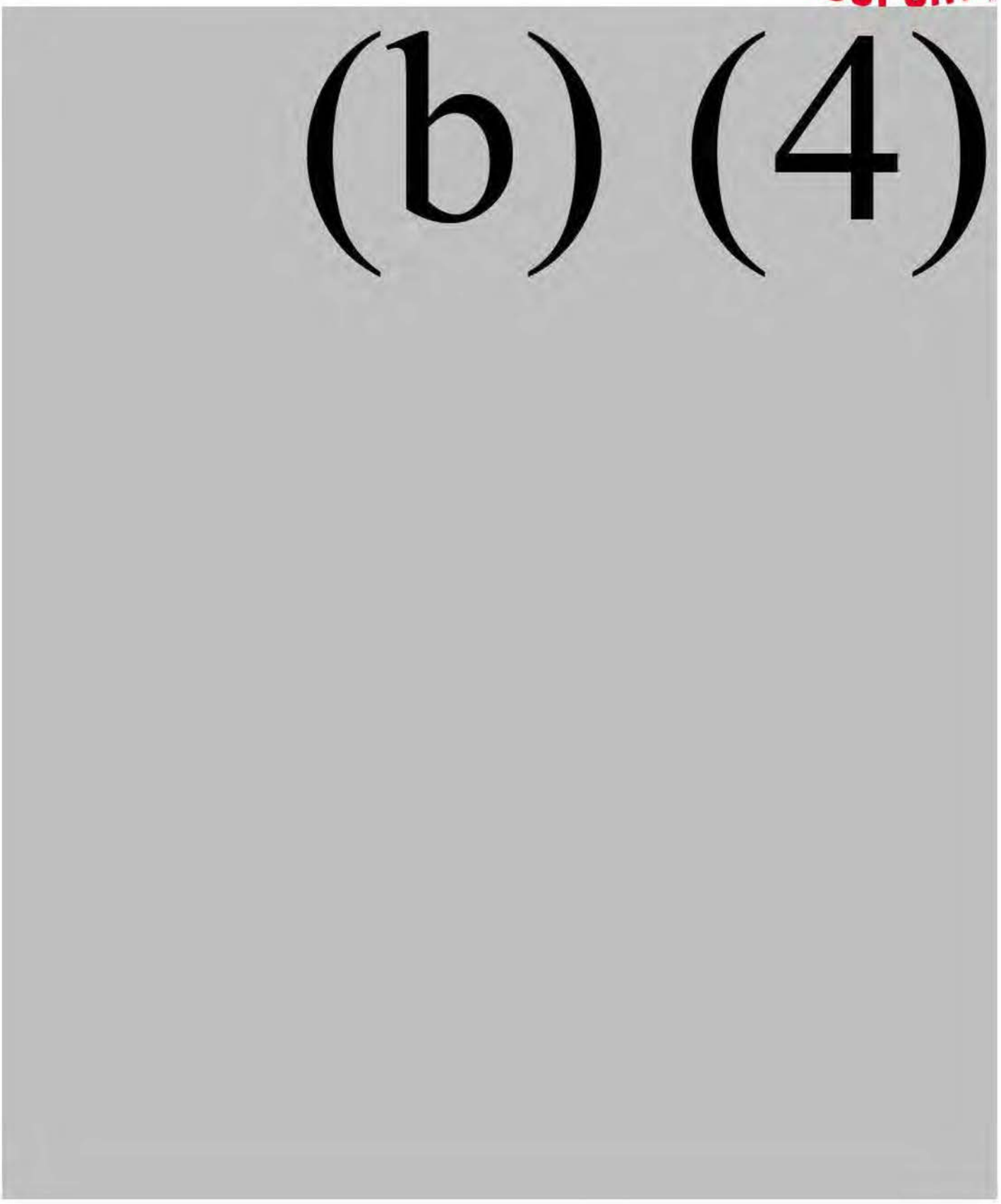
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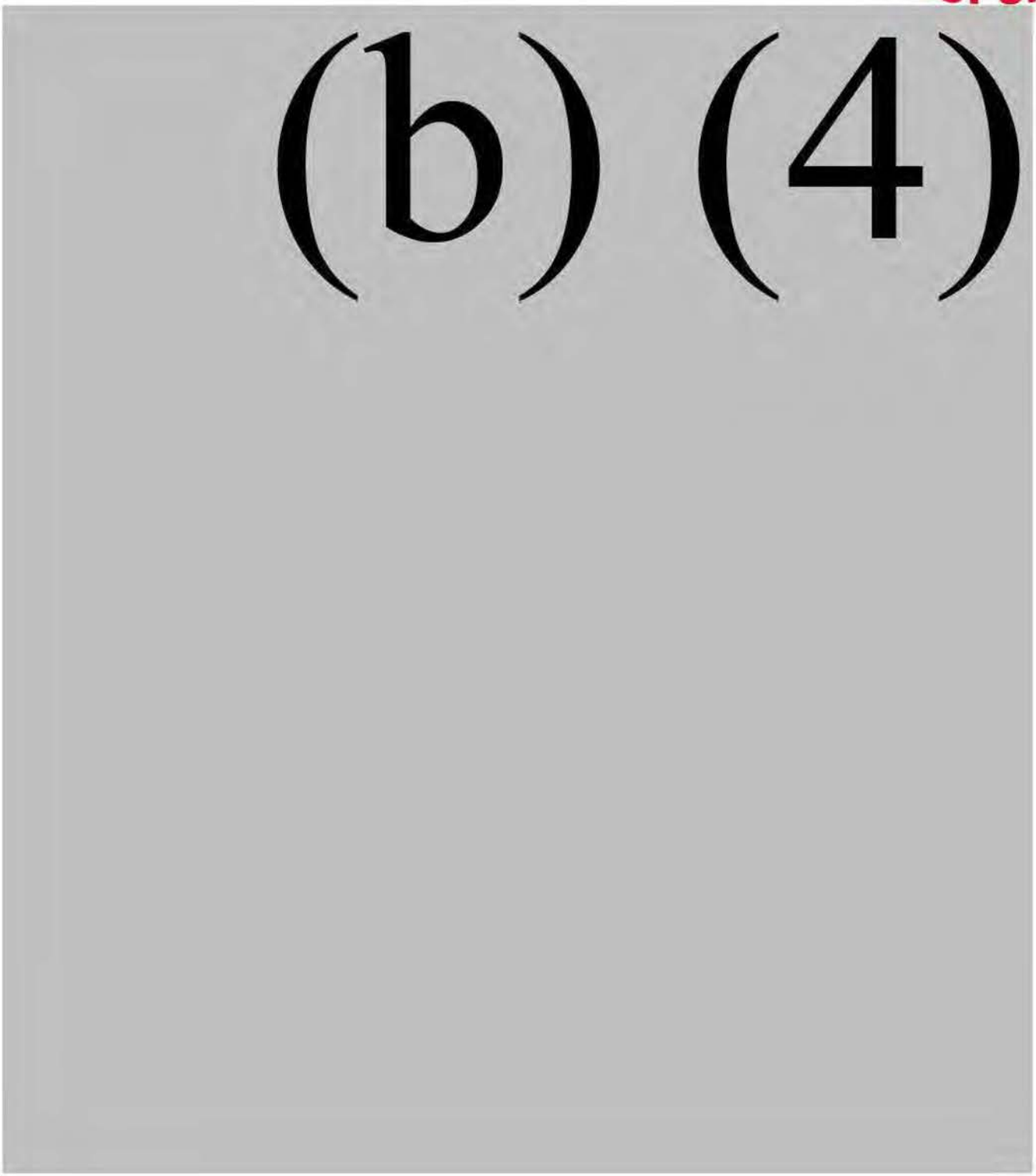
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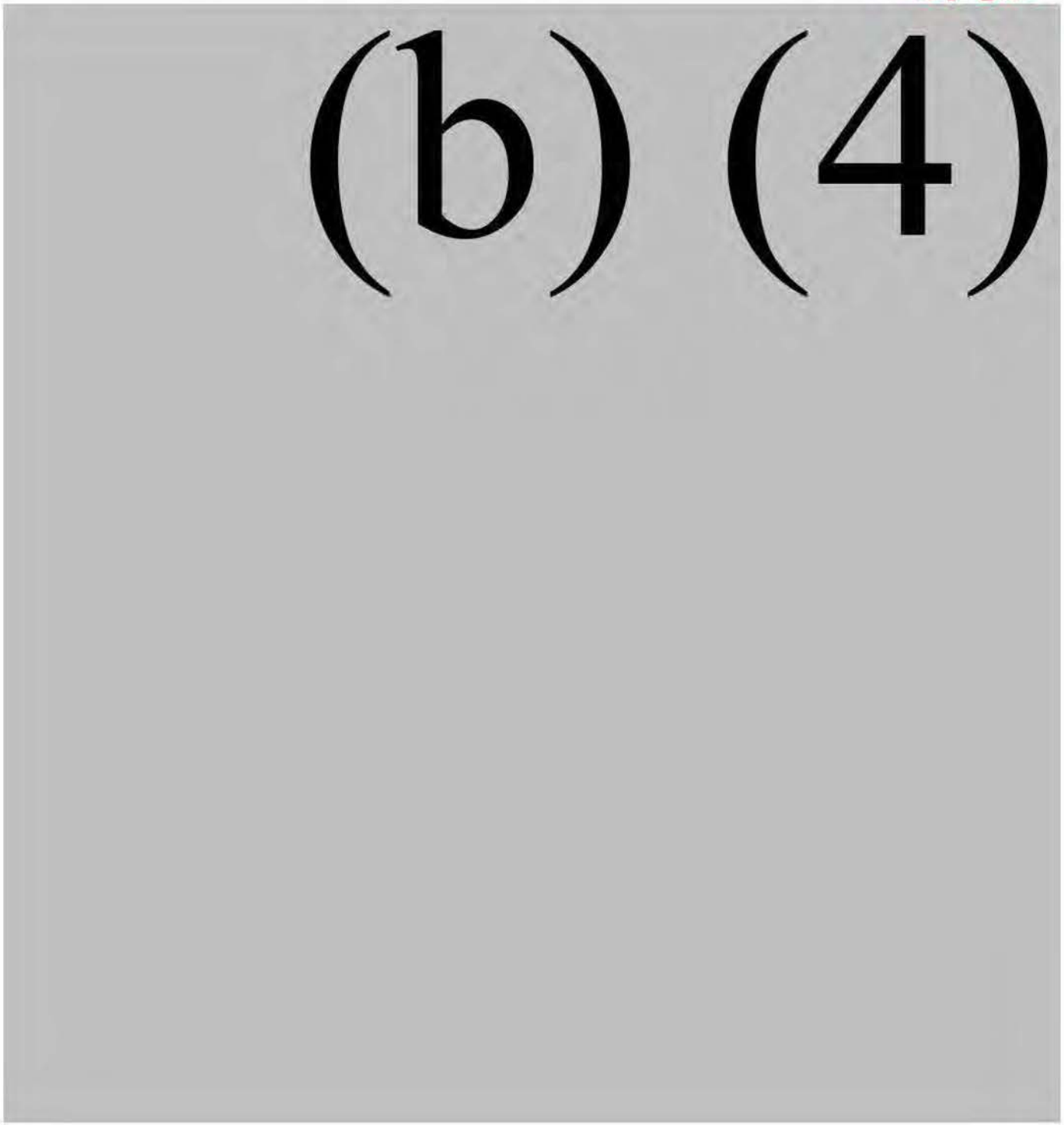
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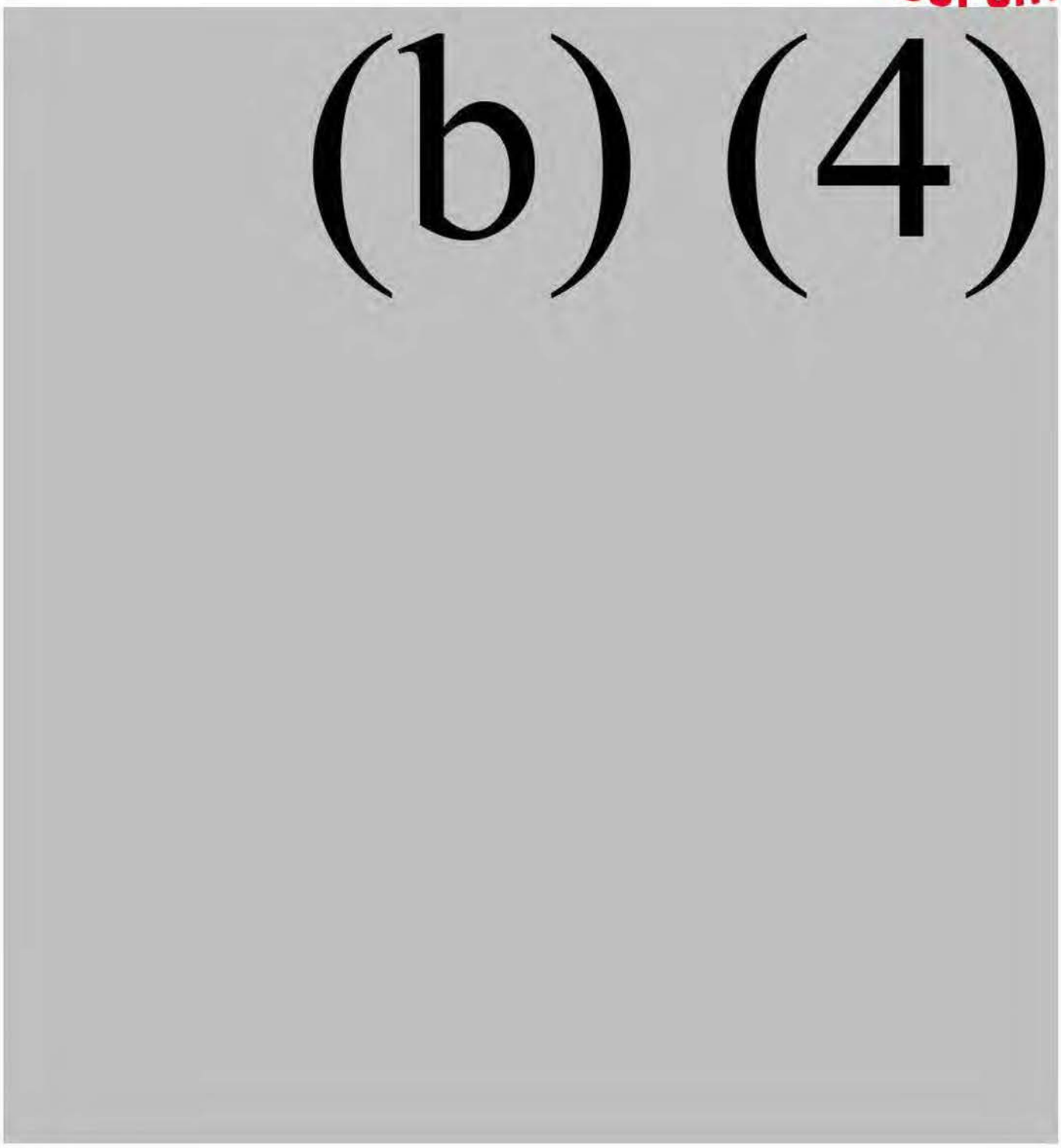
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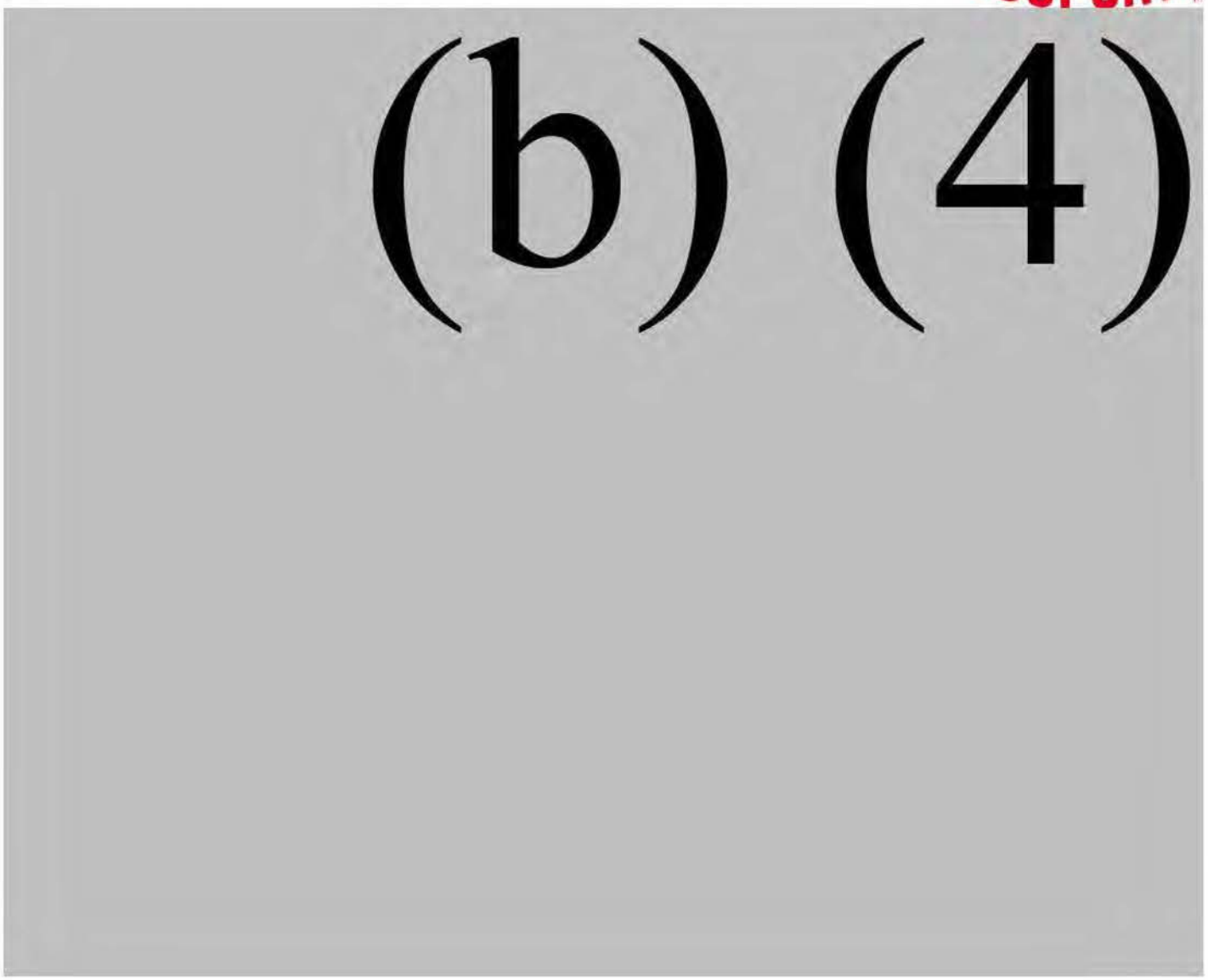
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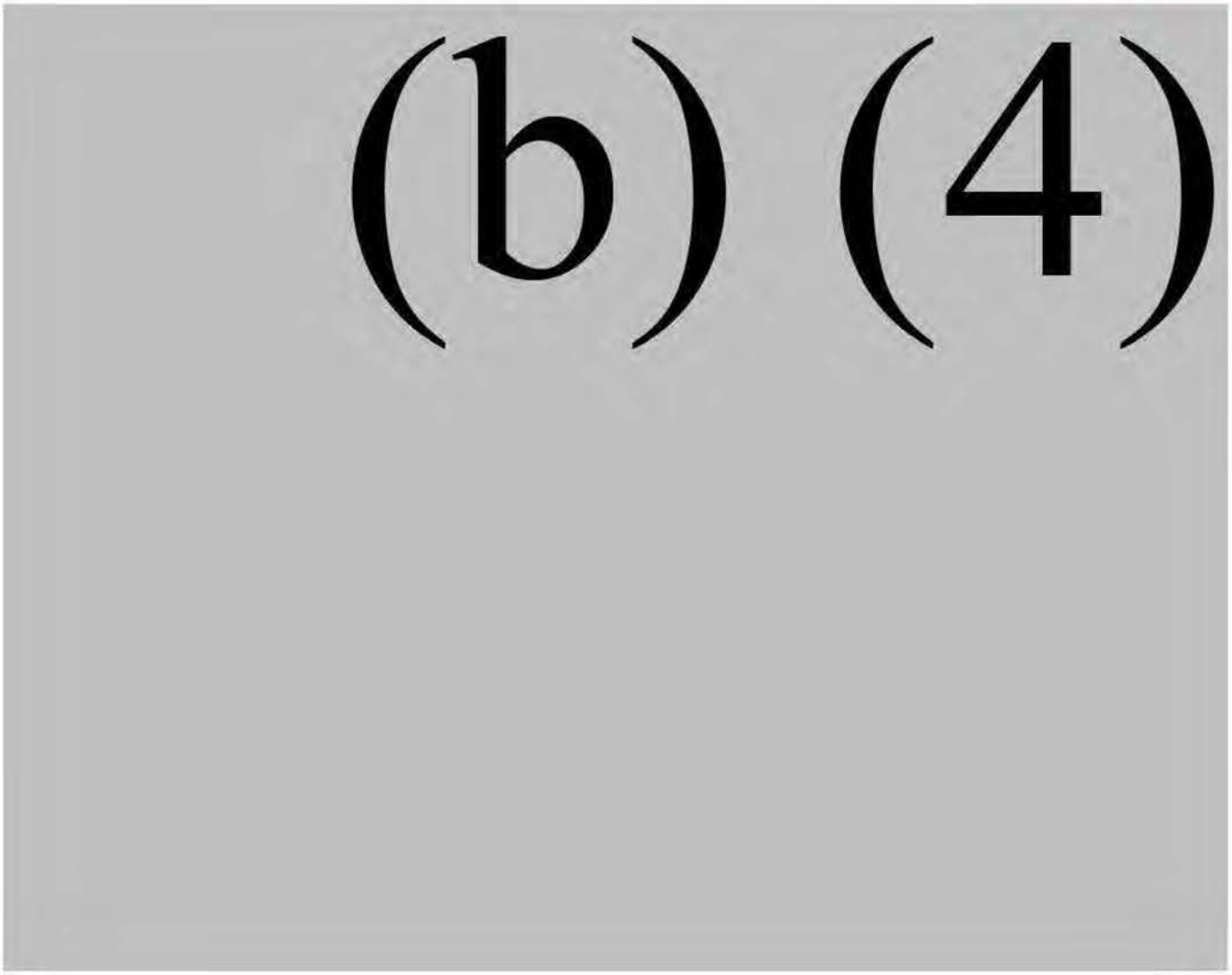
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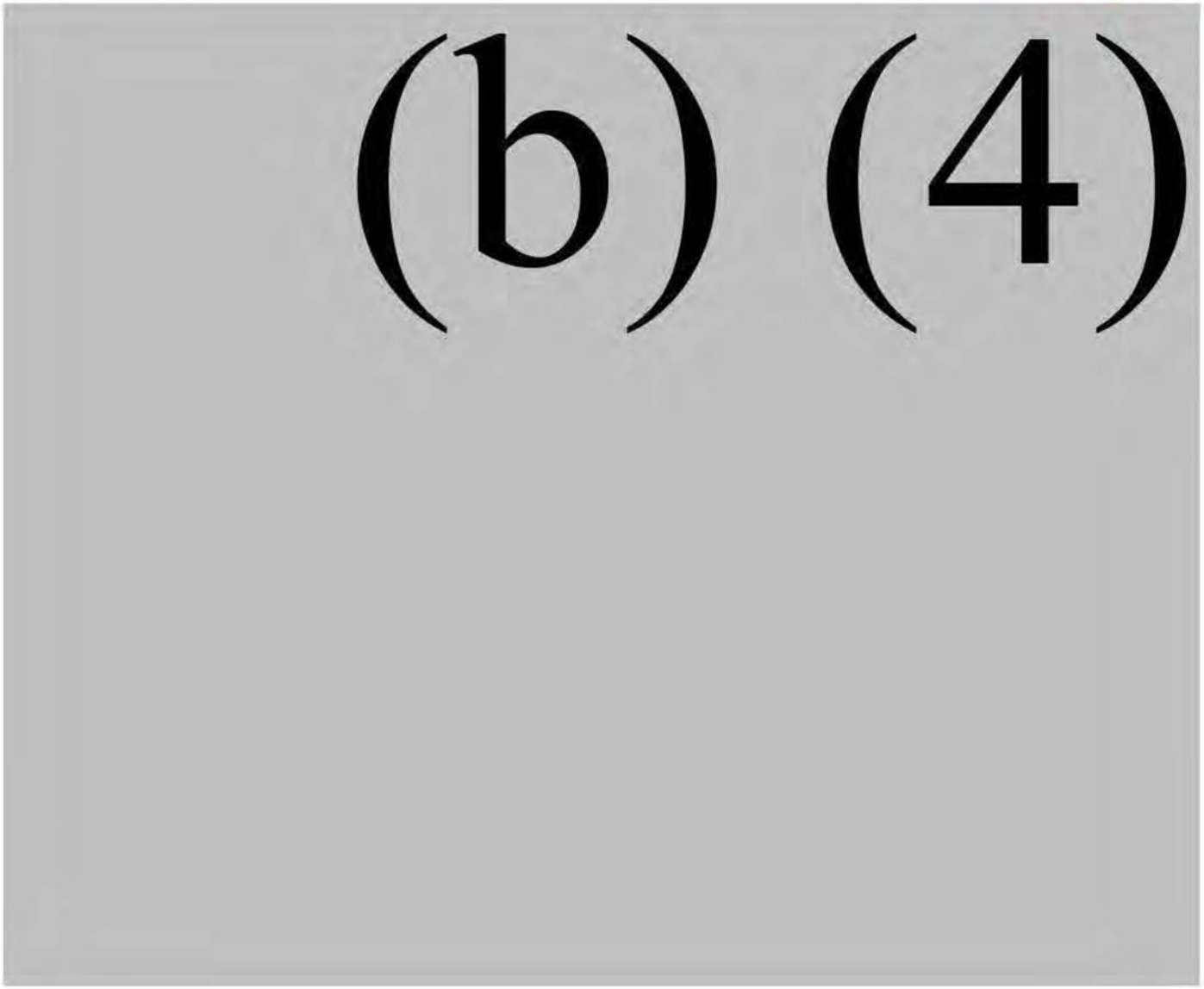
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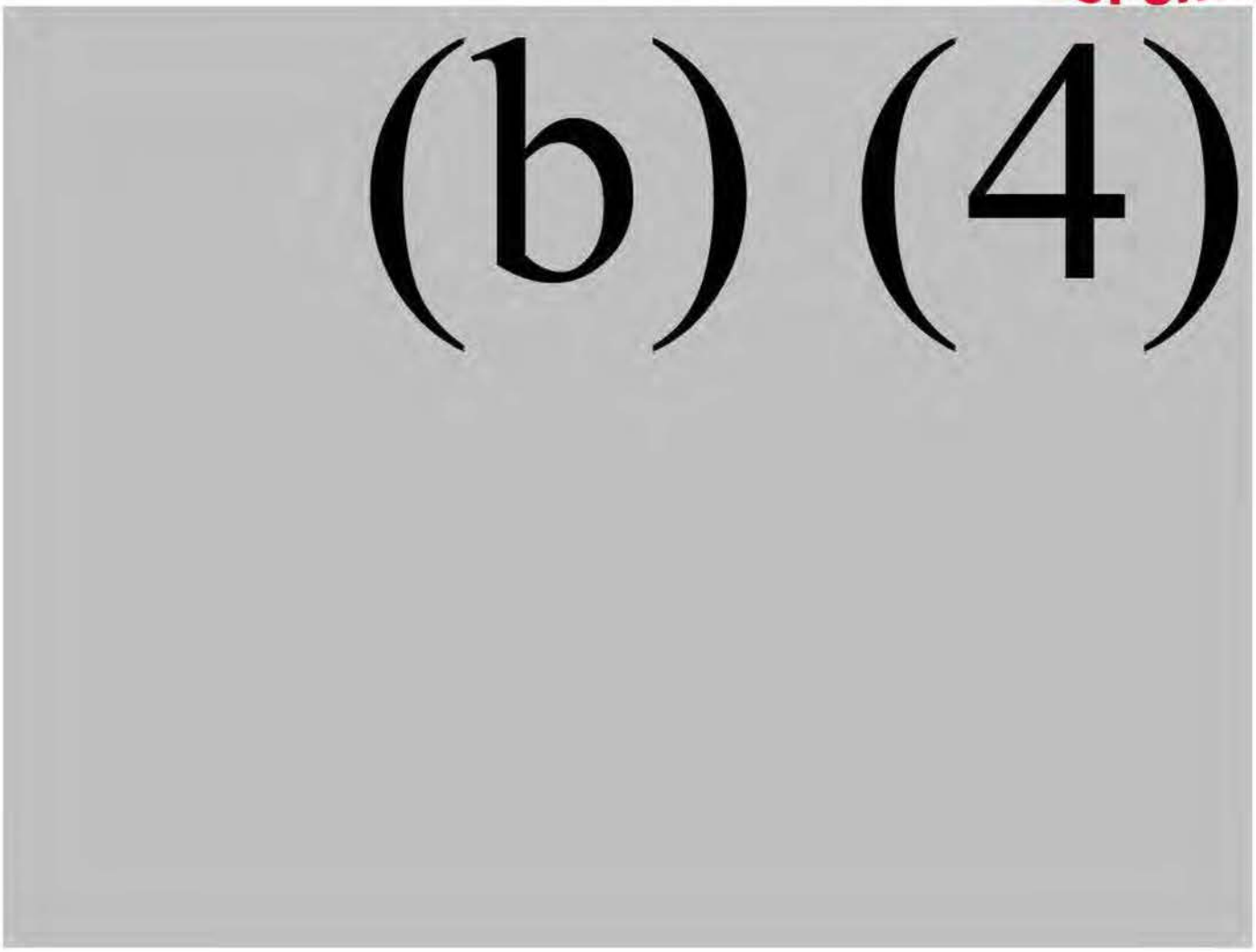
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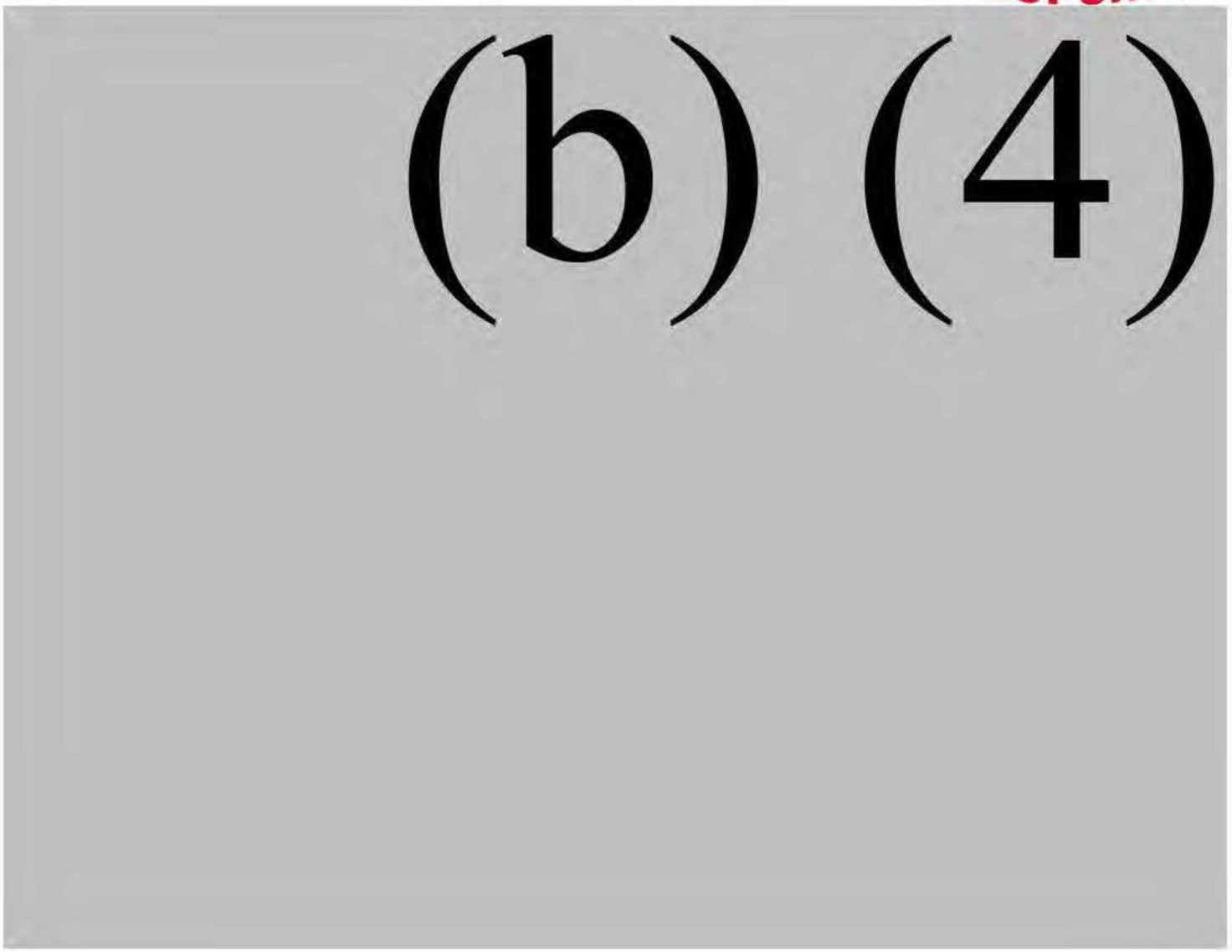
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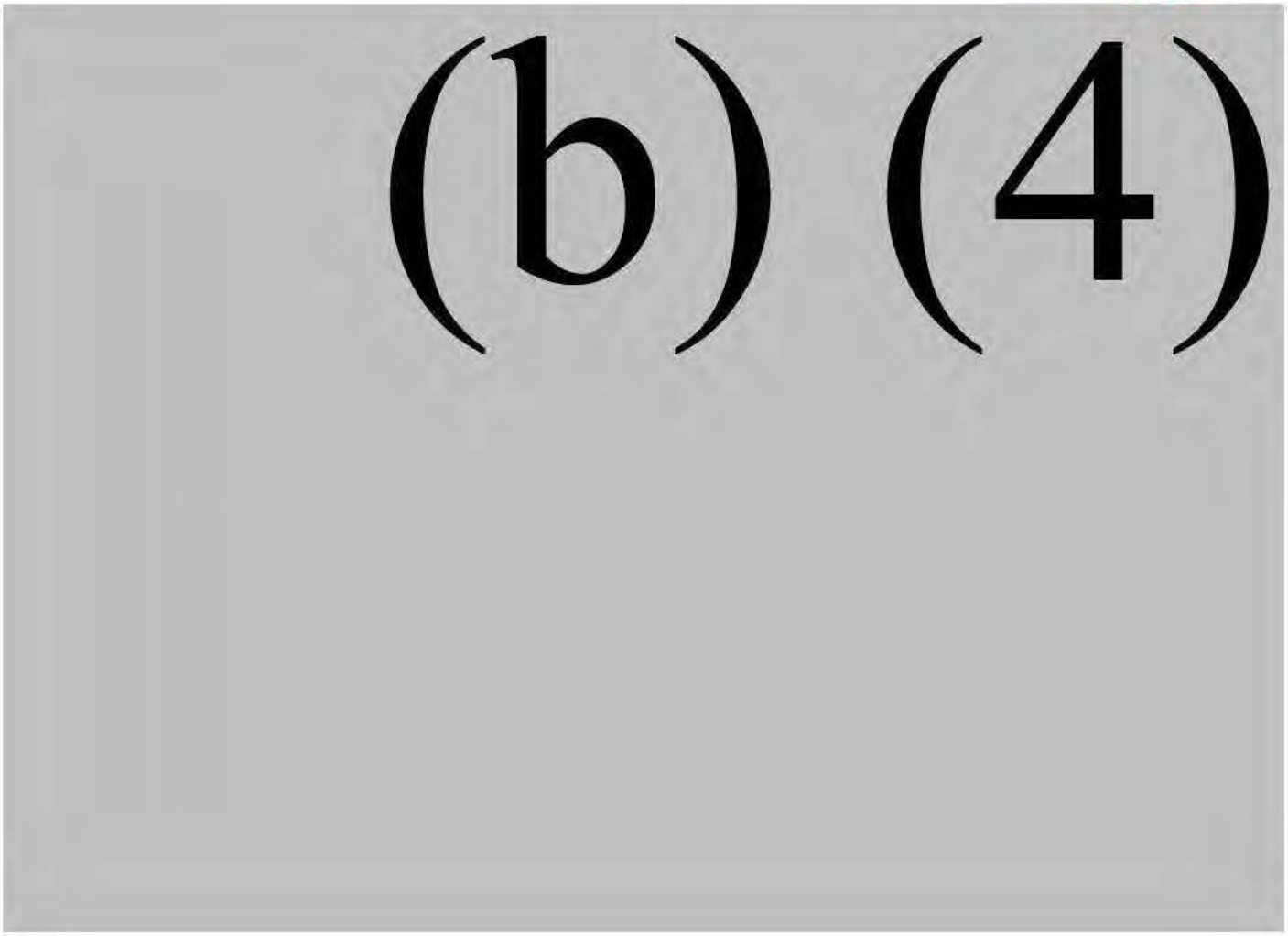
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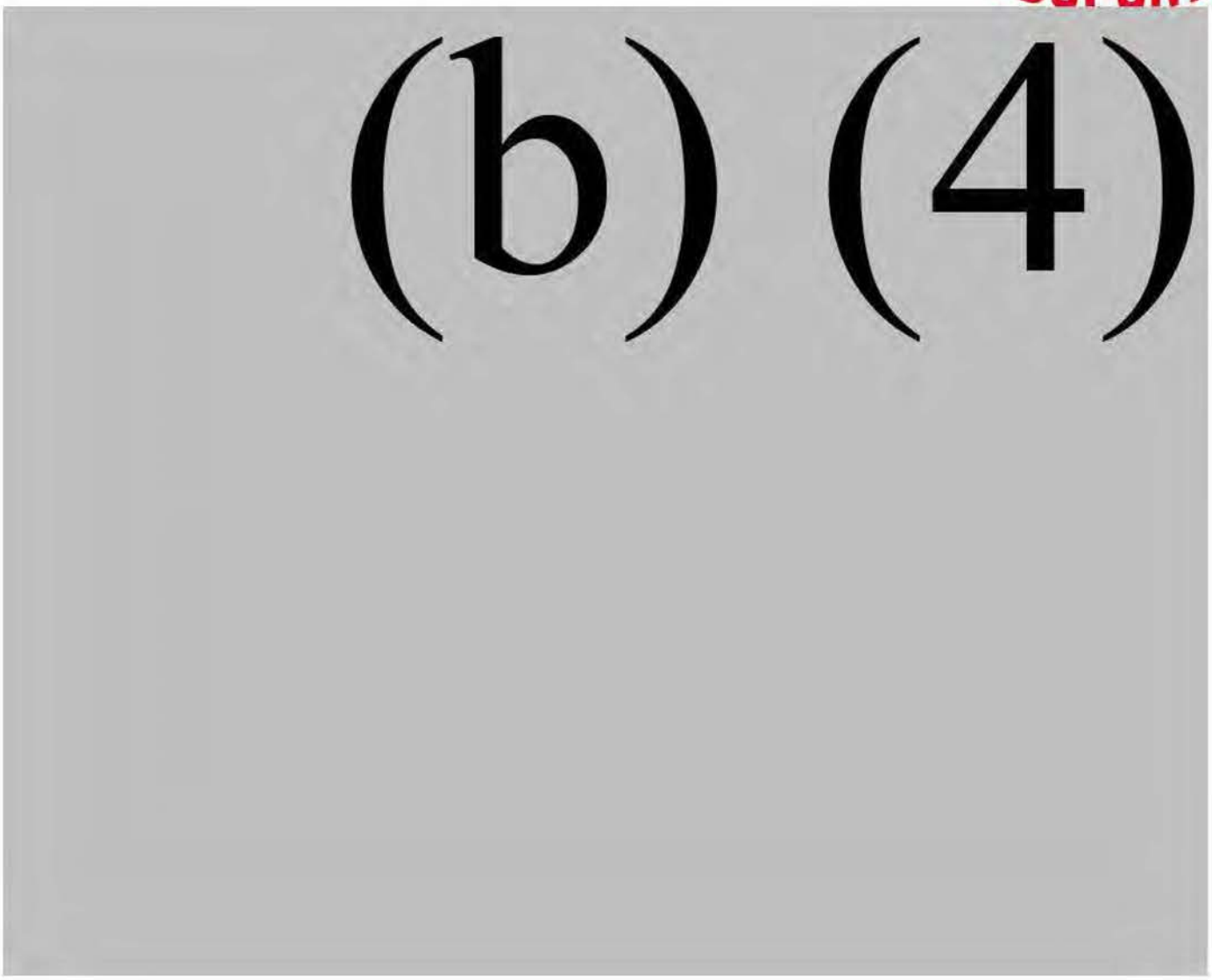
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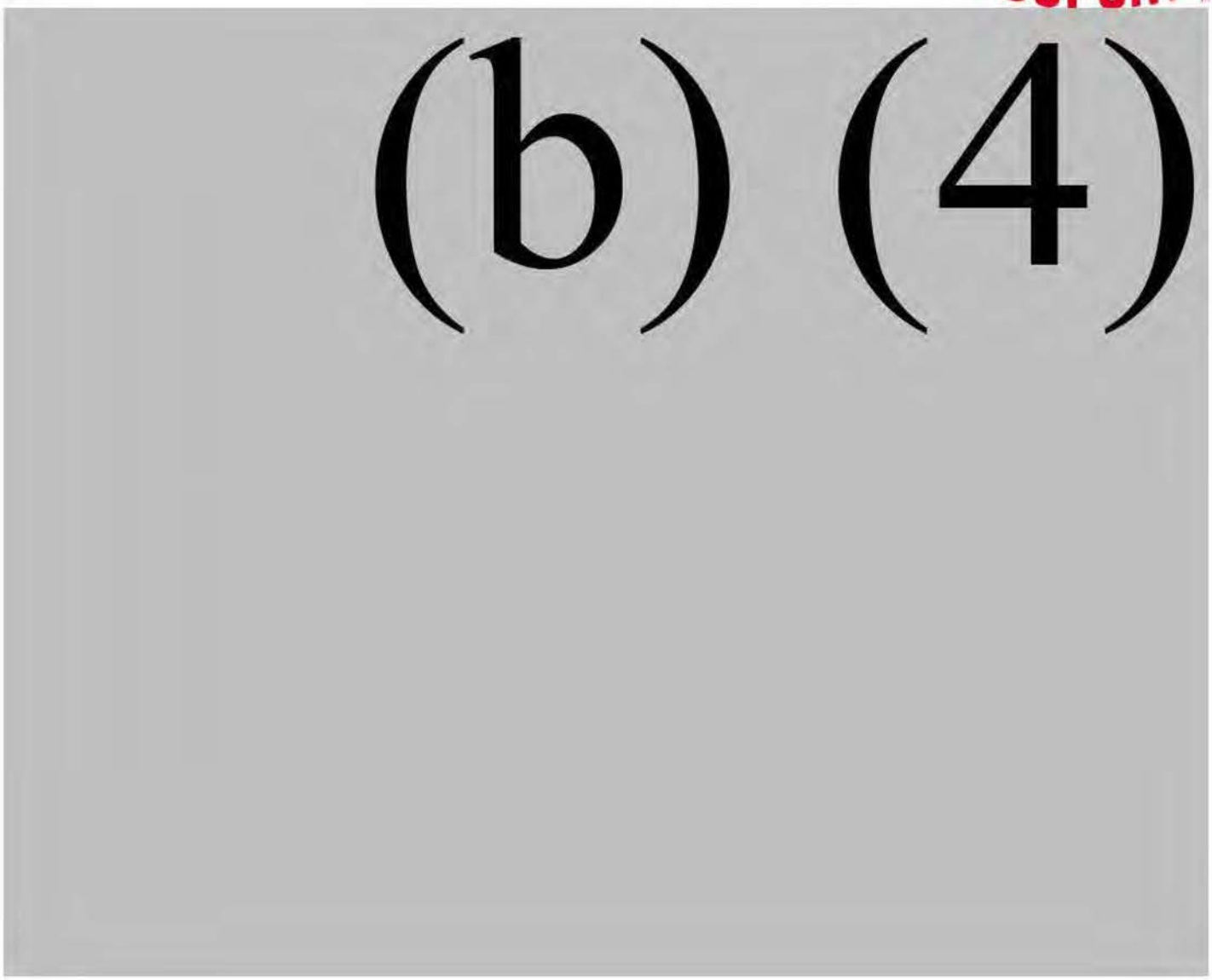
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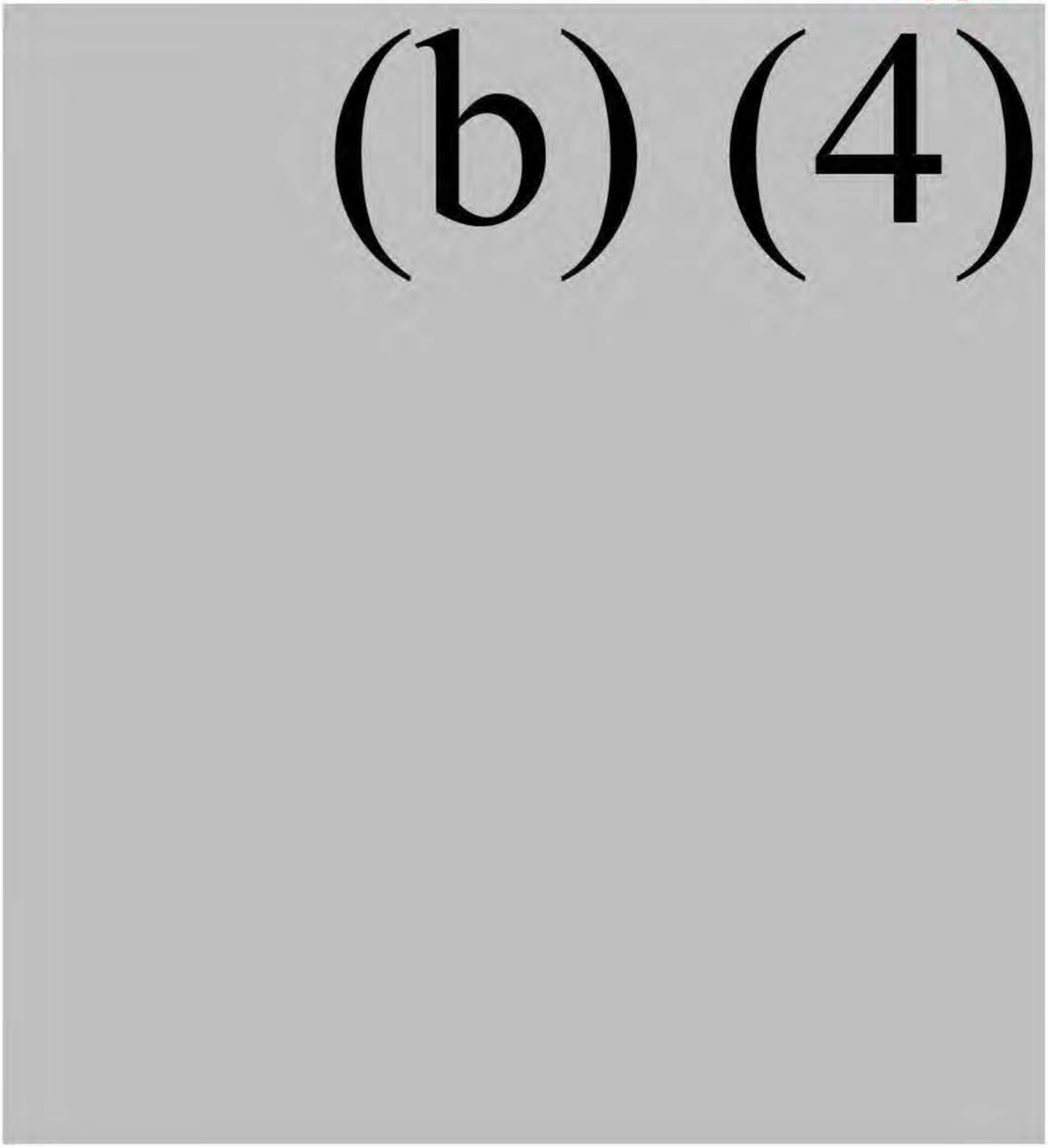
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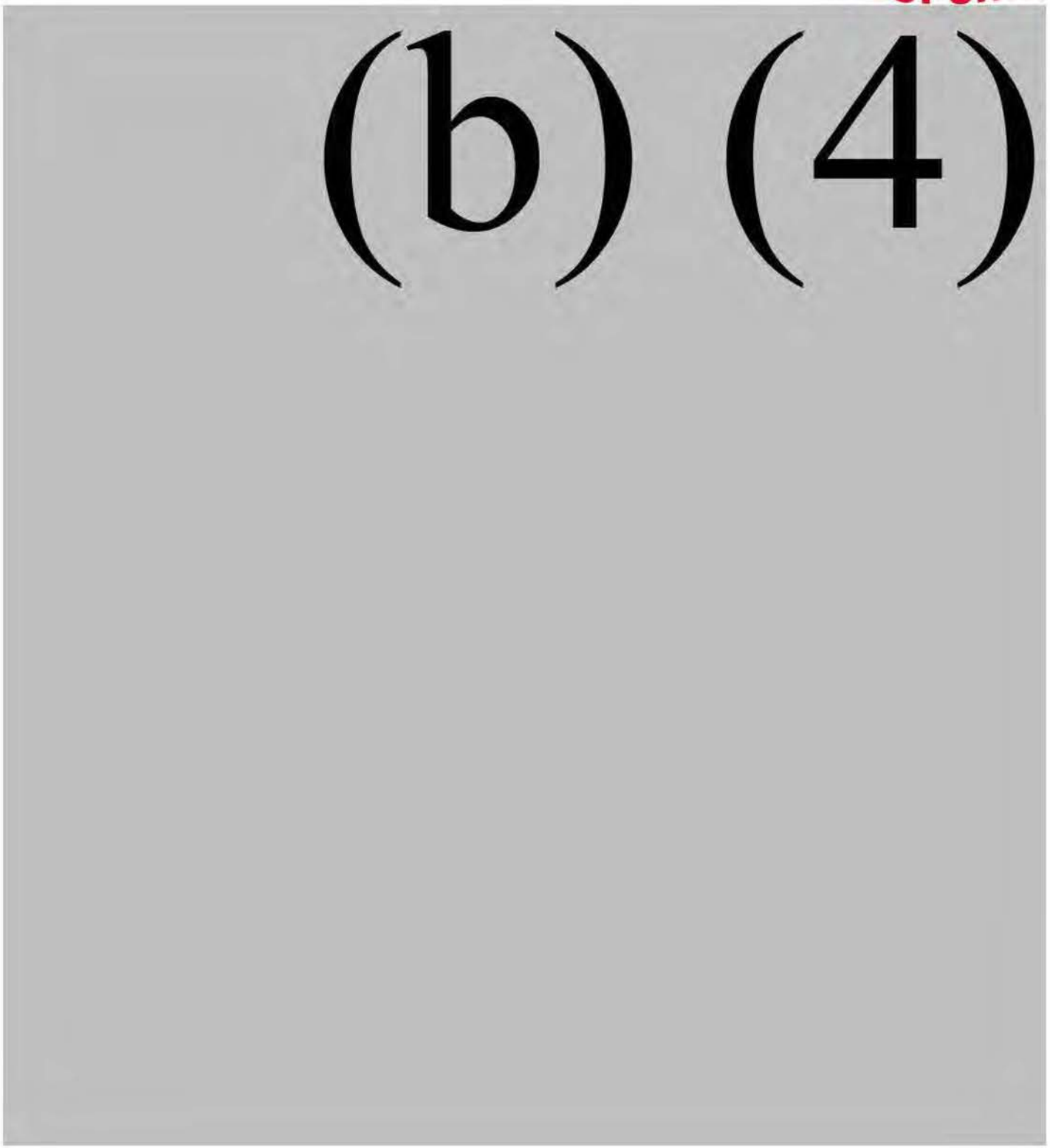
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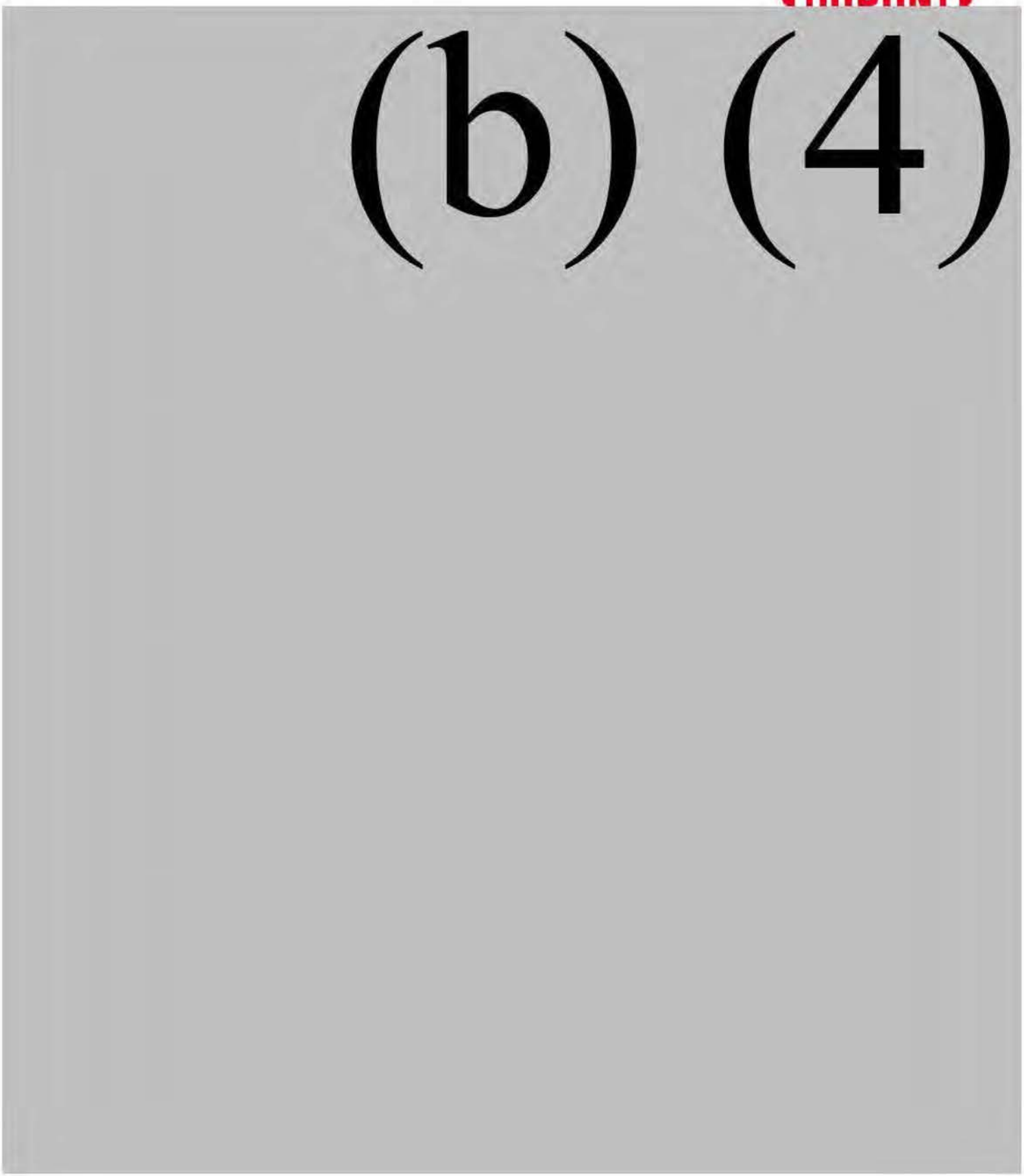
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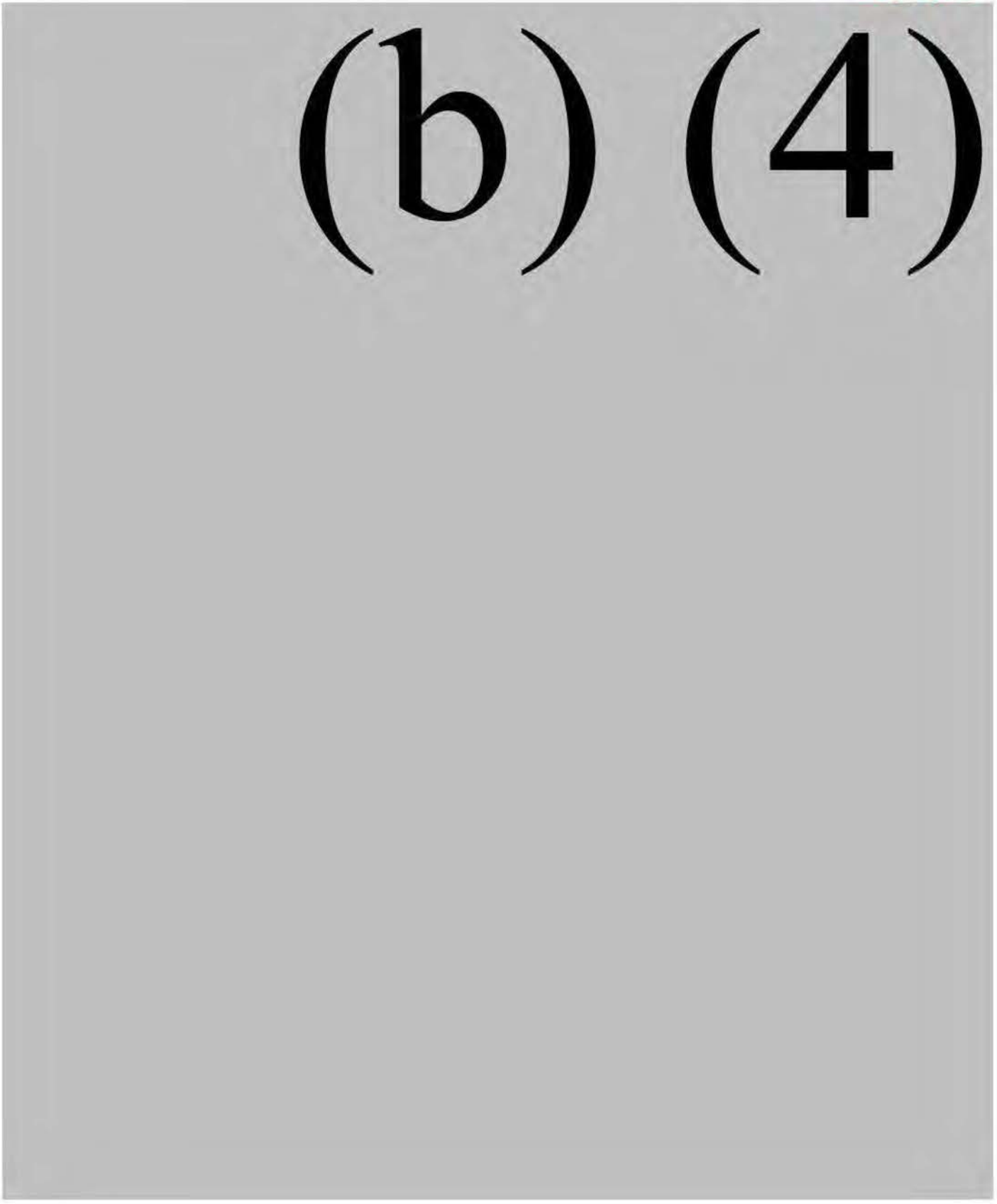
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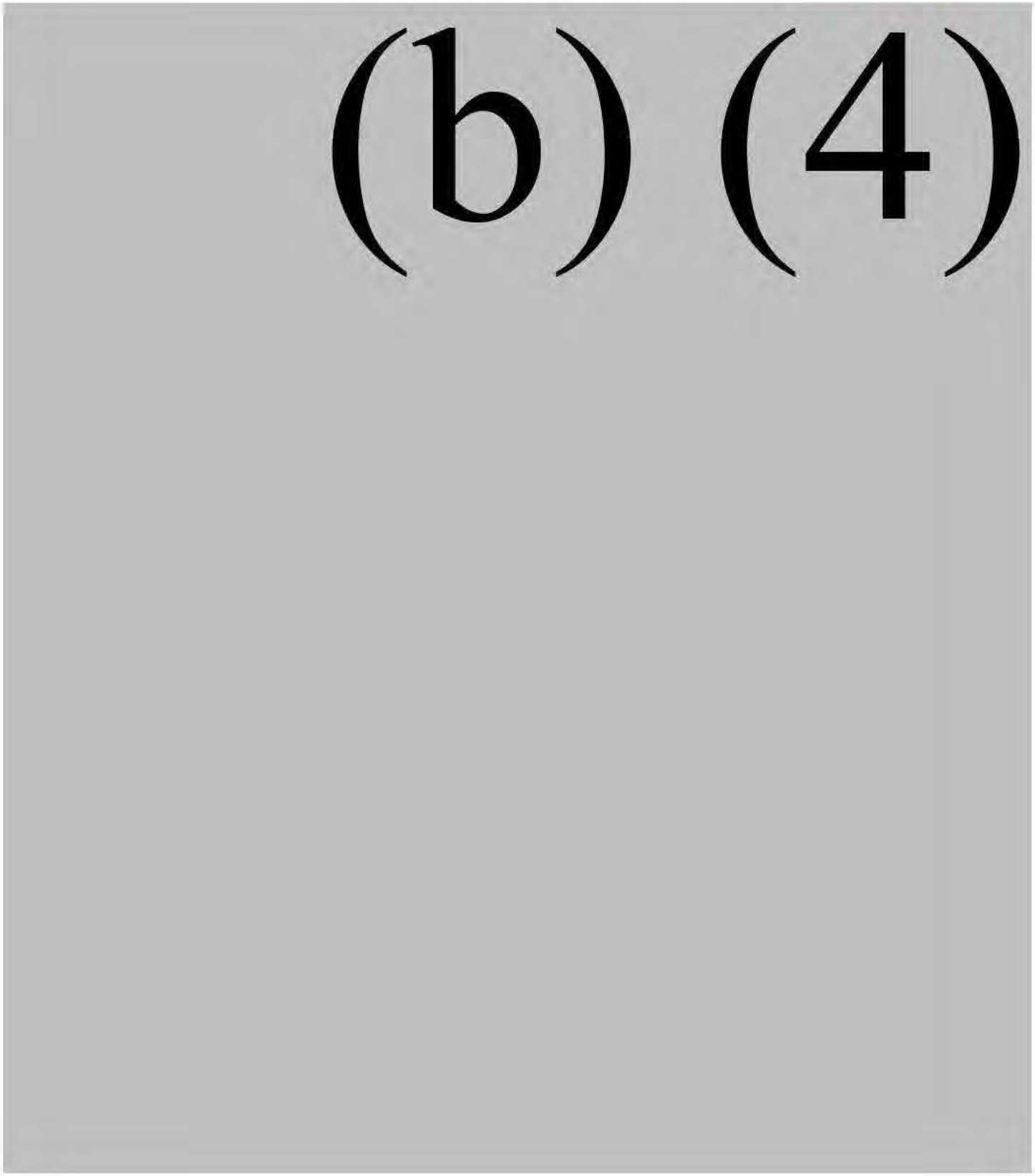
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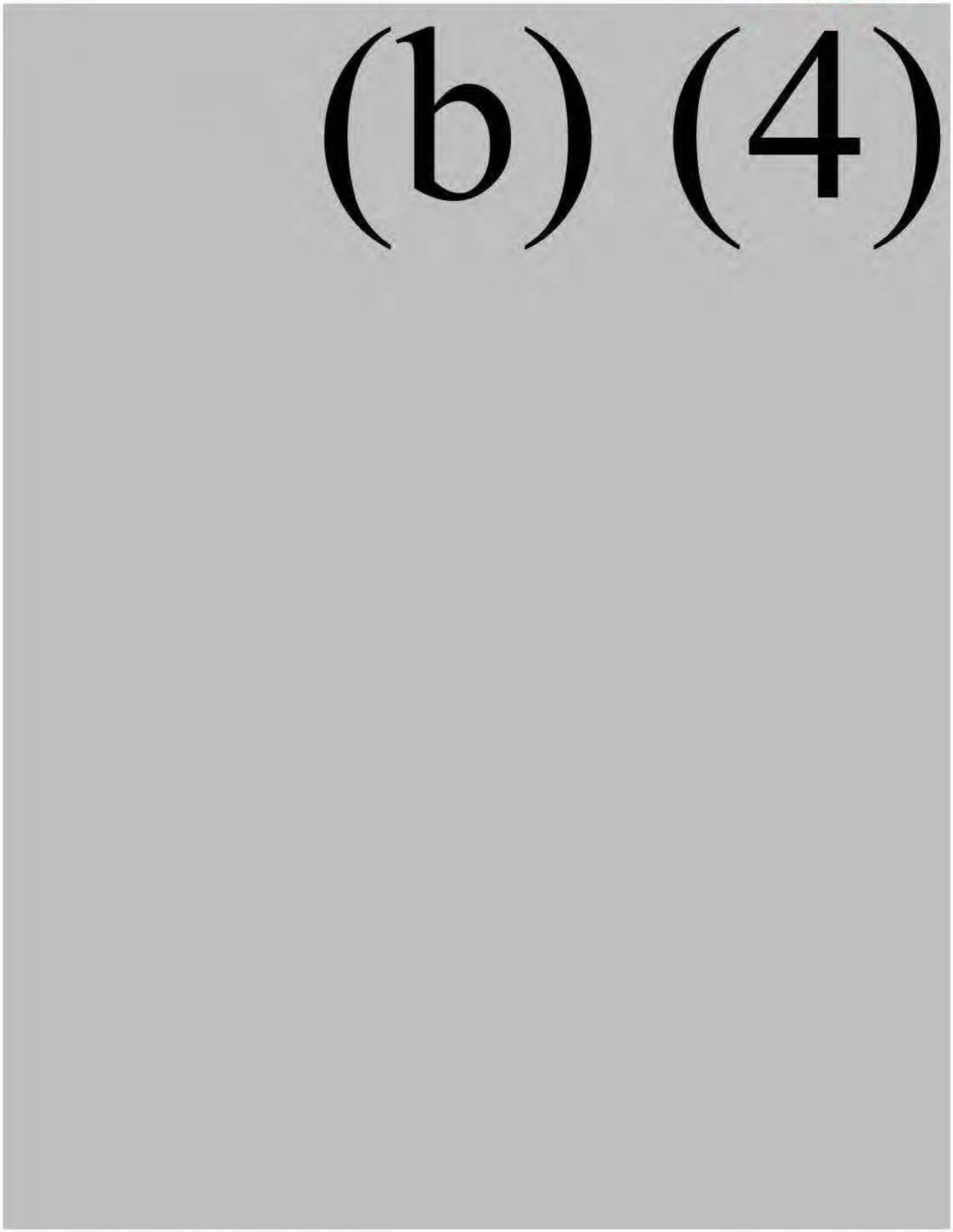
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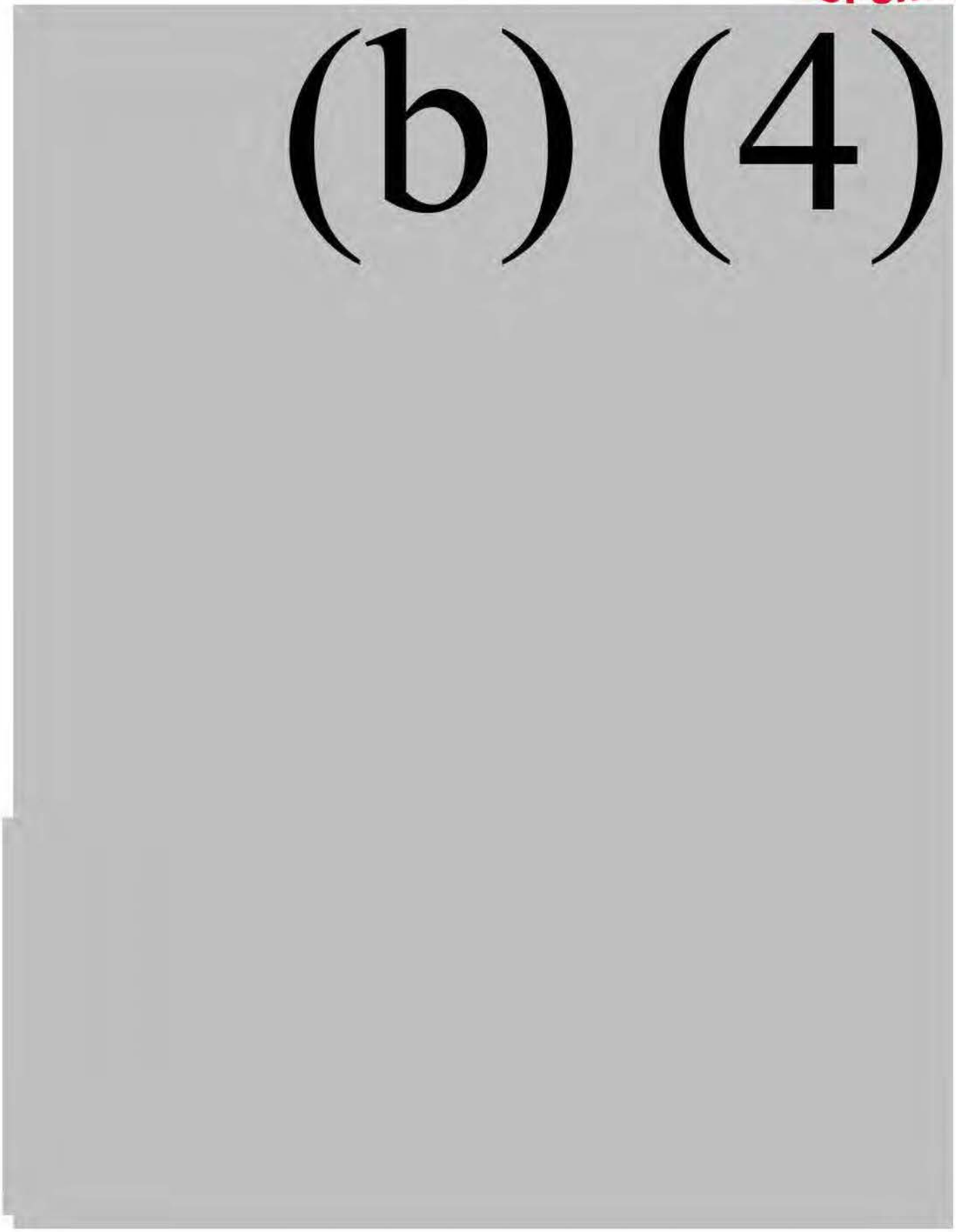
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REPORT

ID: 23497/2020

Stability of "Axtra® PHY GOLD 65 G" in vitamin-mineral premix when stored for 6 months at (b) (4)

Author: A. Tossoli

Study completed: Jul 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature: (b) (6) …… Date:….15/07/2020……..

Study Scientist:

(b)(6)

Signature:…… Date:16/07/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

Signature:… (b) (6) Date:16/07/2020……..

(b) (4)

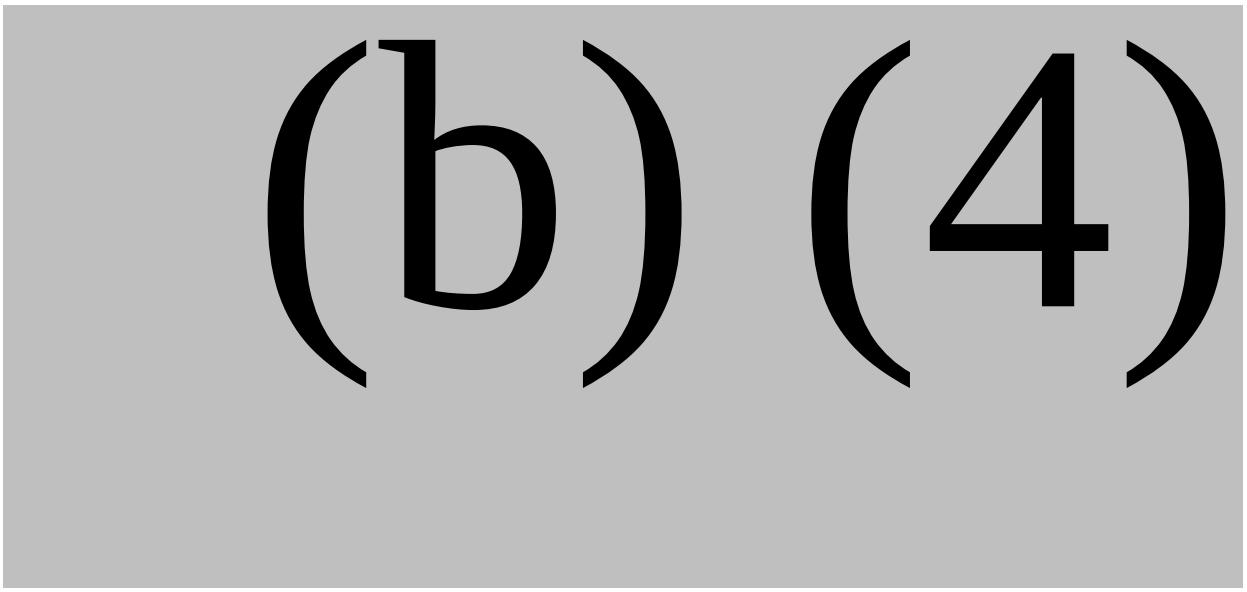
RESPONSIBLE PERSONNEL

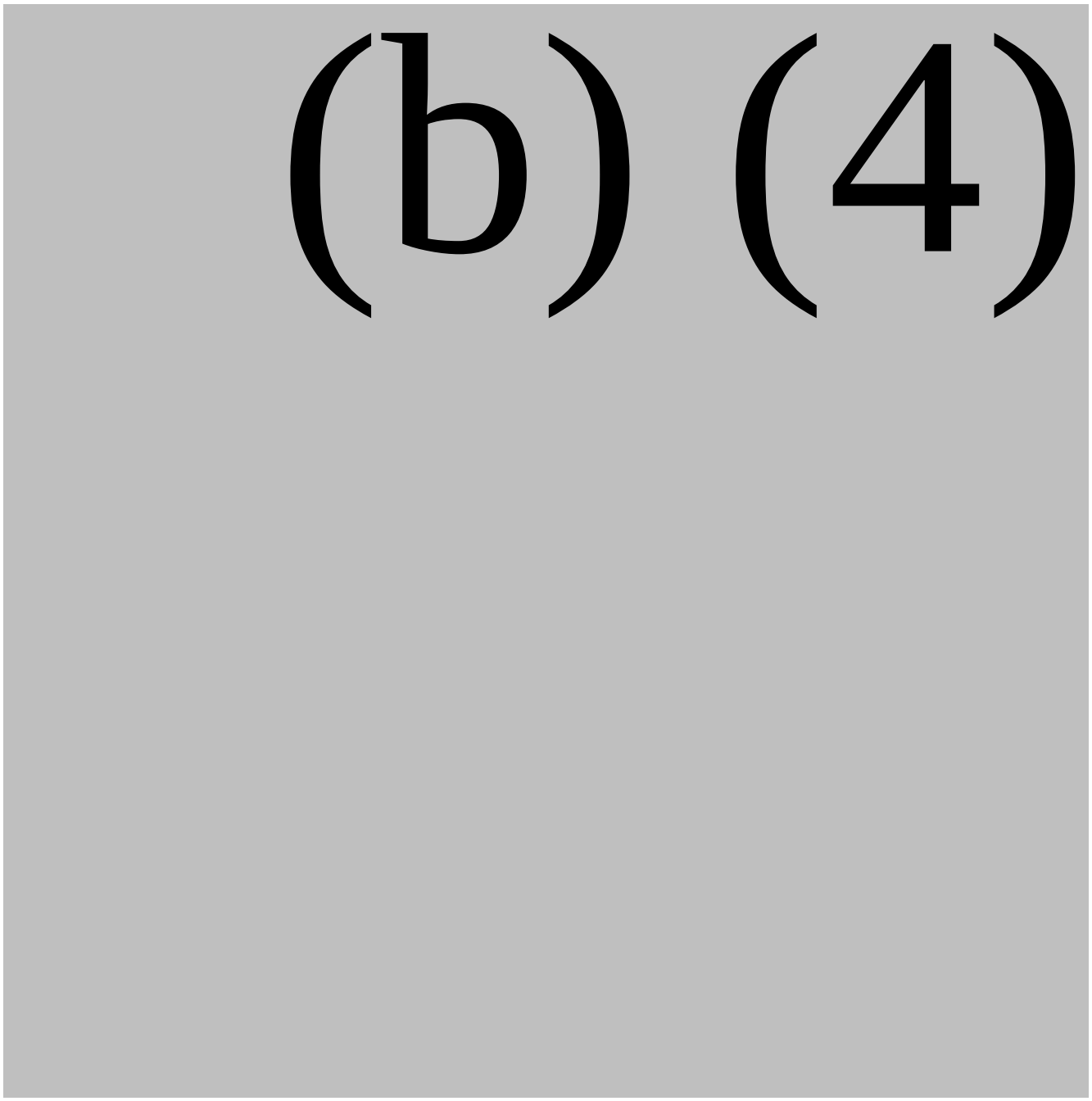
Study scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY





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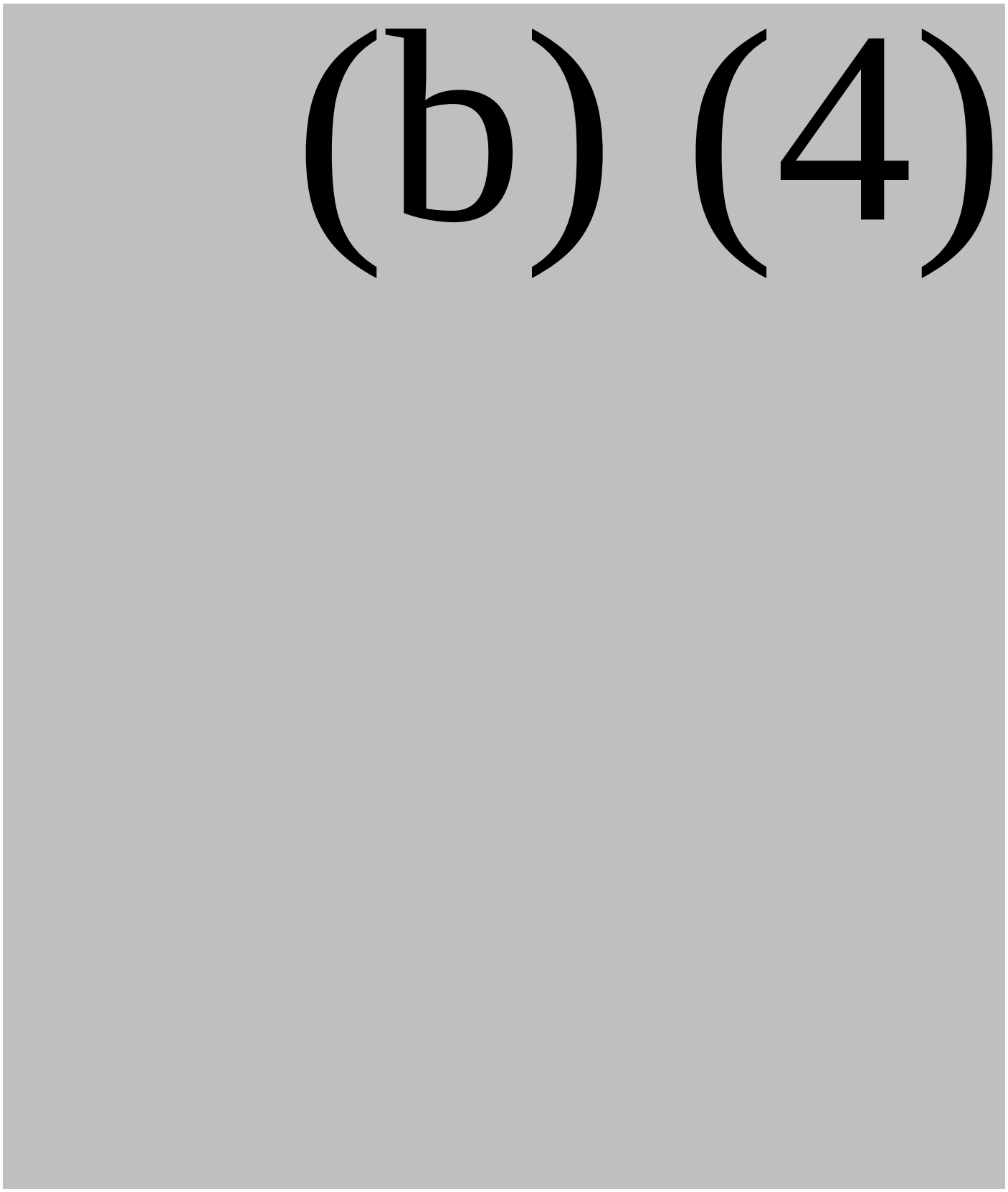

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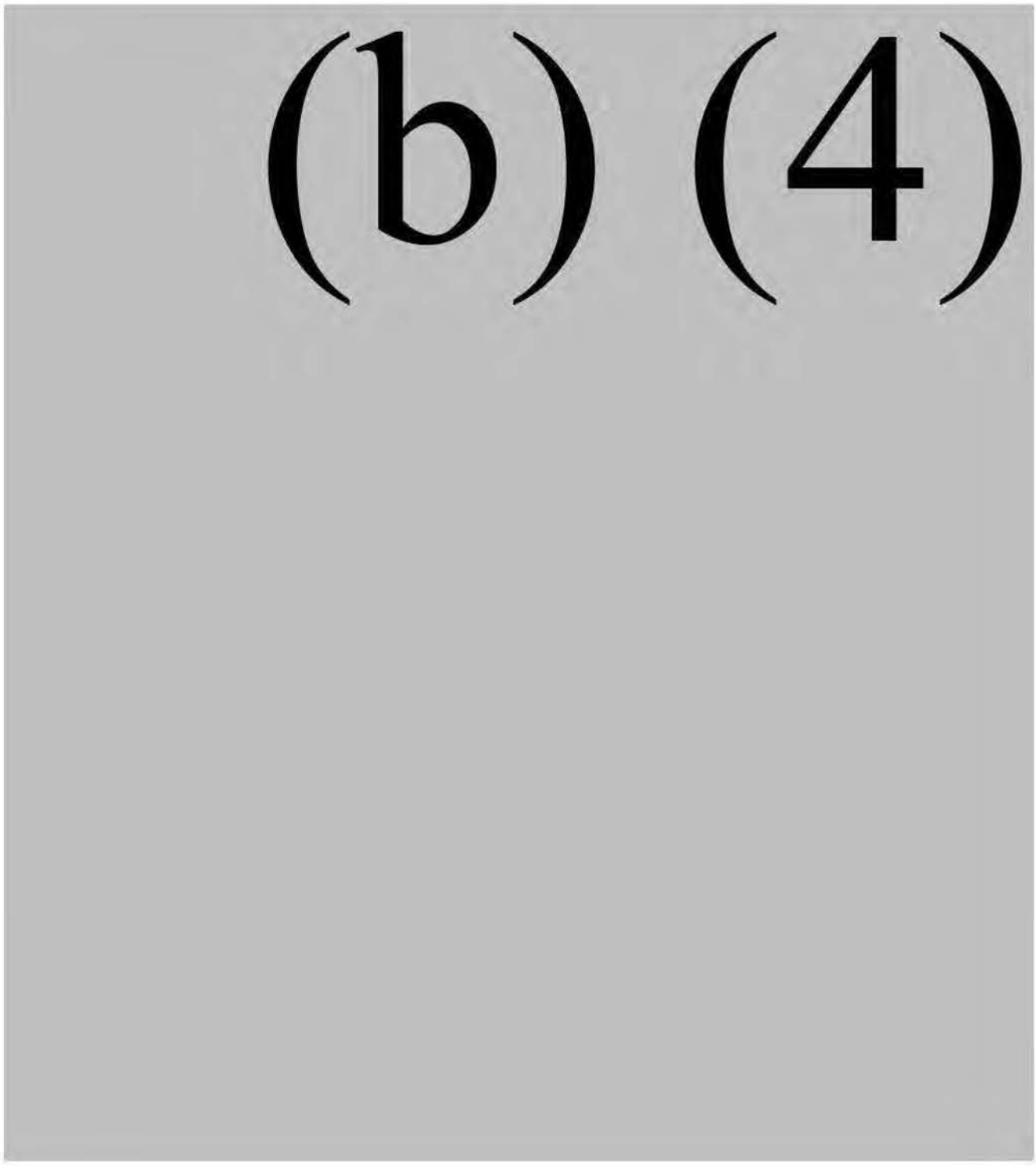

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REPORT

ID: 23566-1/2020

Stability of "Axtra® PHY GOLD 30 T" in pelleted feed for Swine, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra

Signature:… (b) (6) …… Date:29/09/2020……..

Study Scientist:

(b)(6)

Signature:… (b) (6) … Date:27/09/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

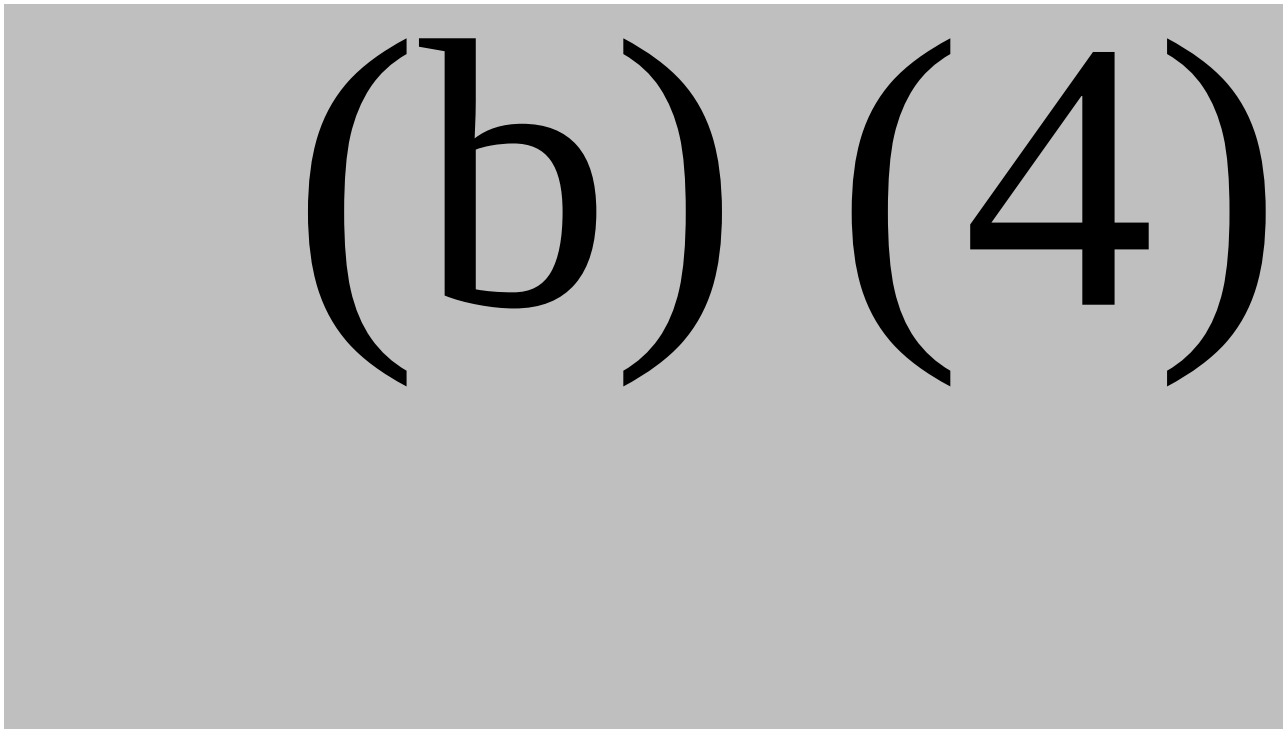
Signature: (b) (6) Date:28/09/2020……..

RESPONSIBLE PERSONNEL

Study scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.



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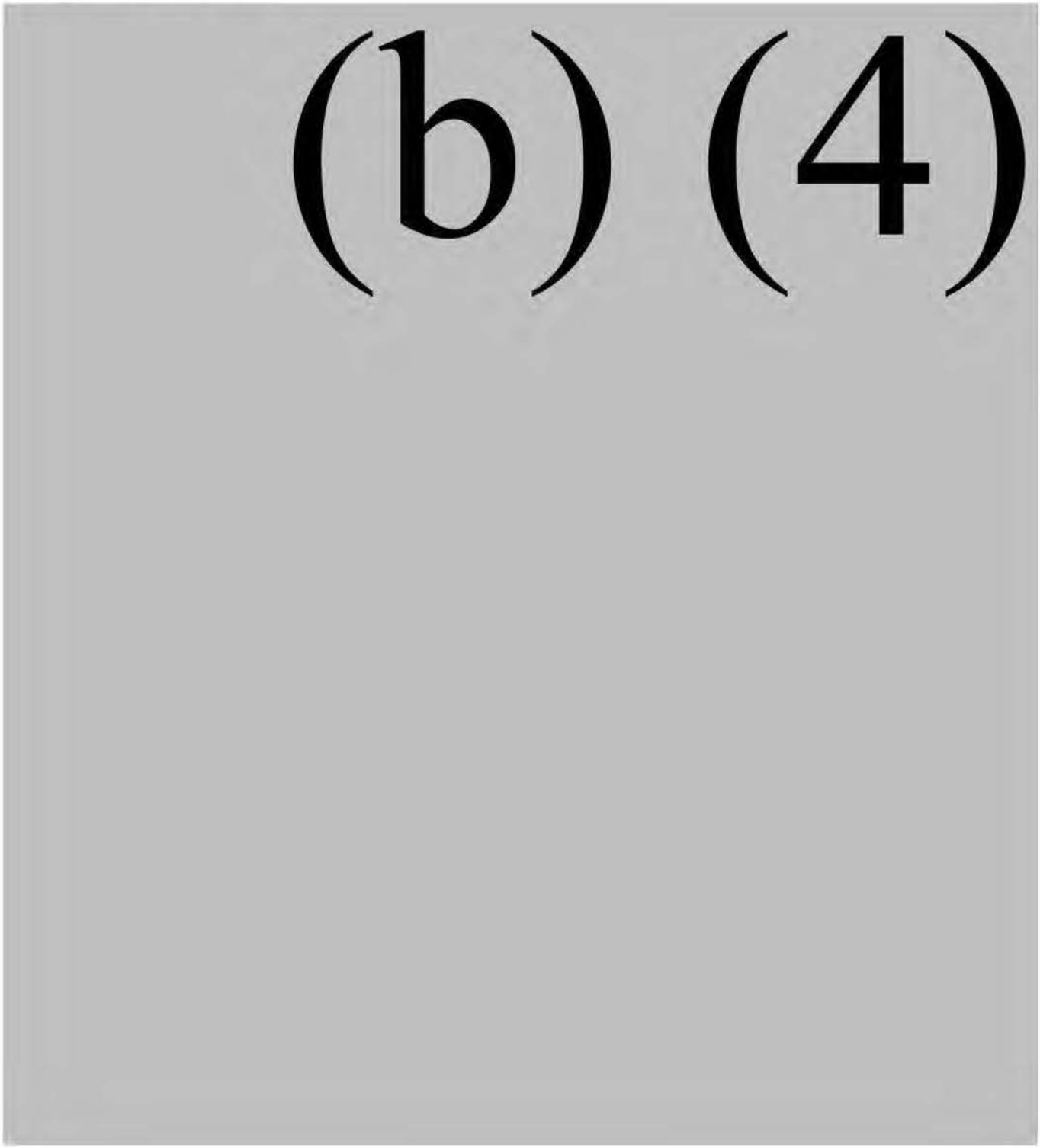

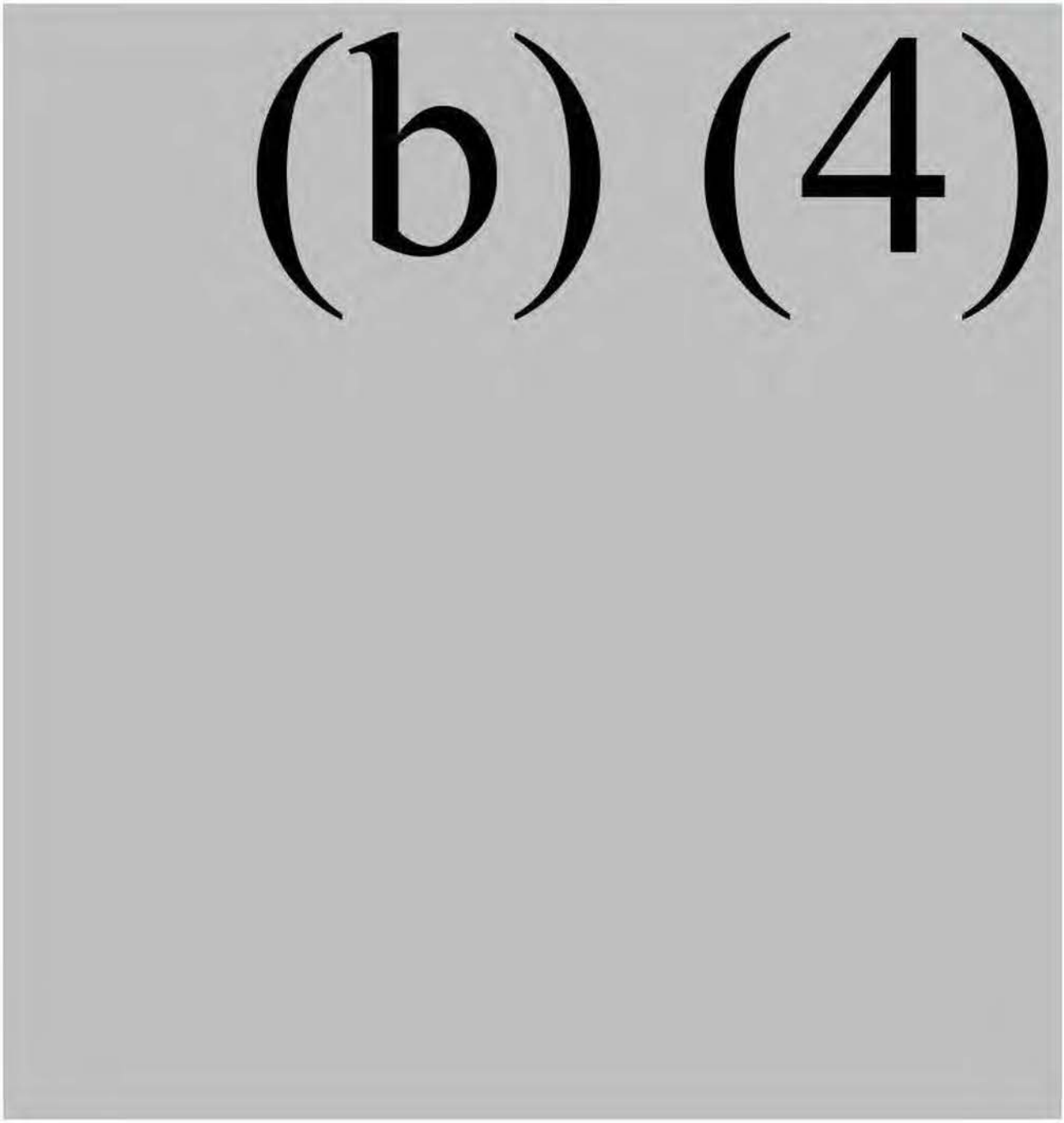

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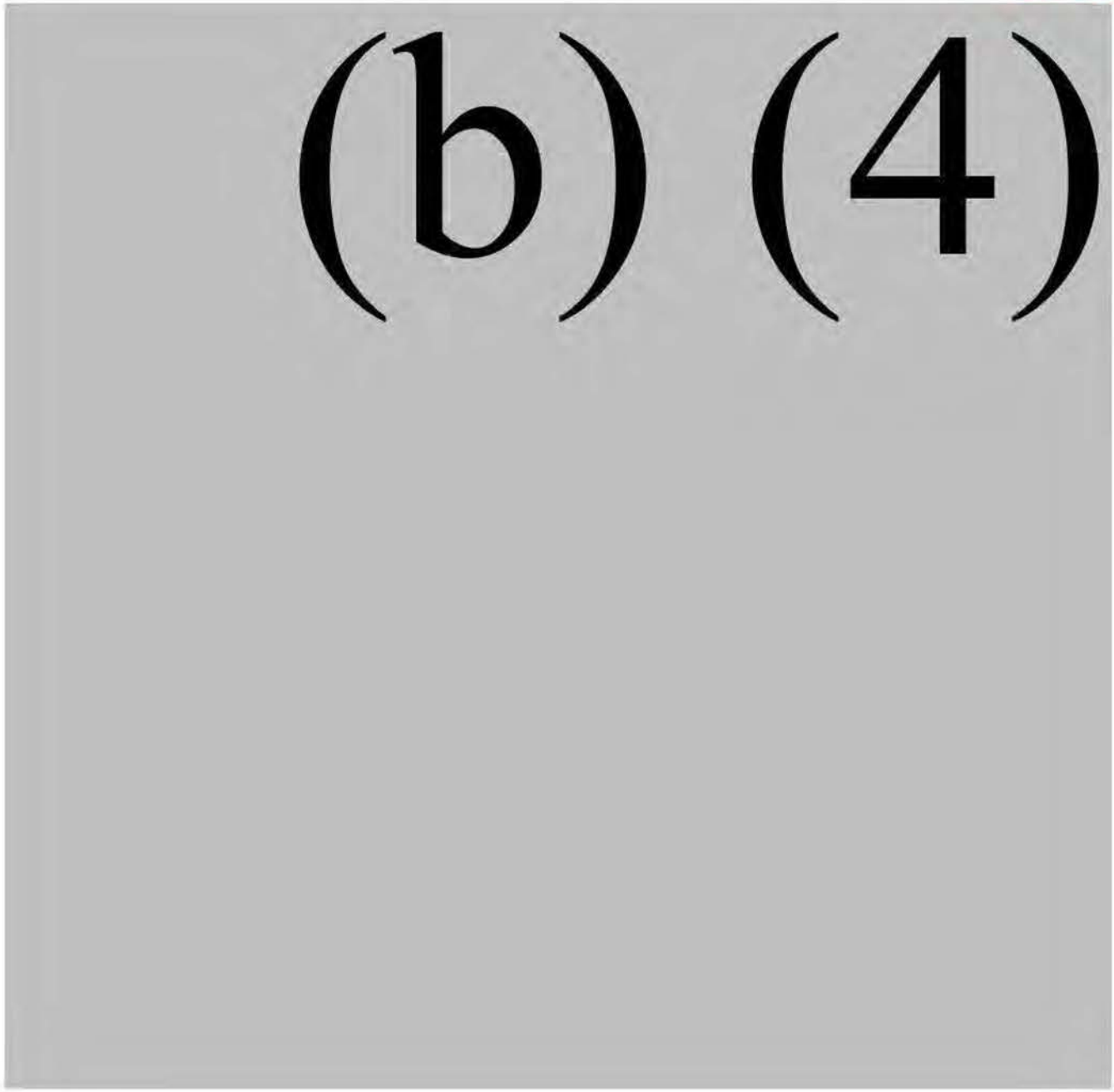

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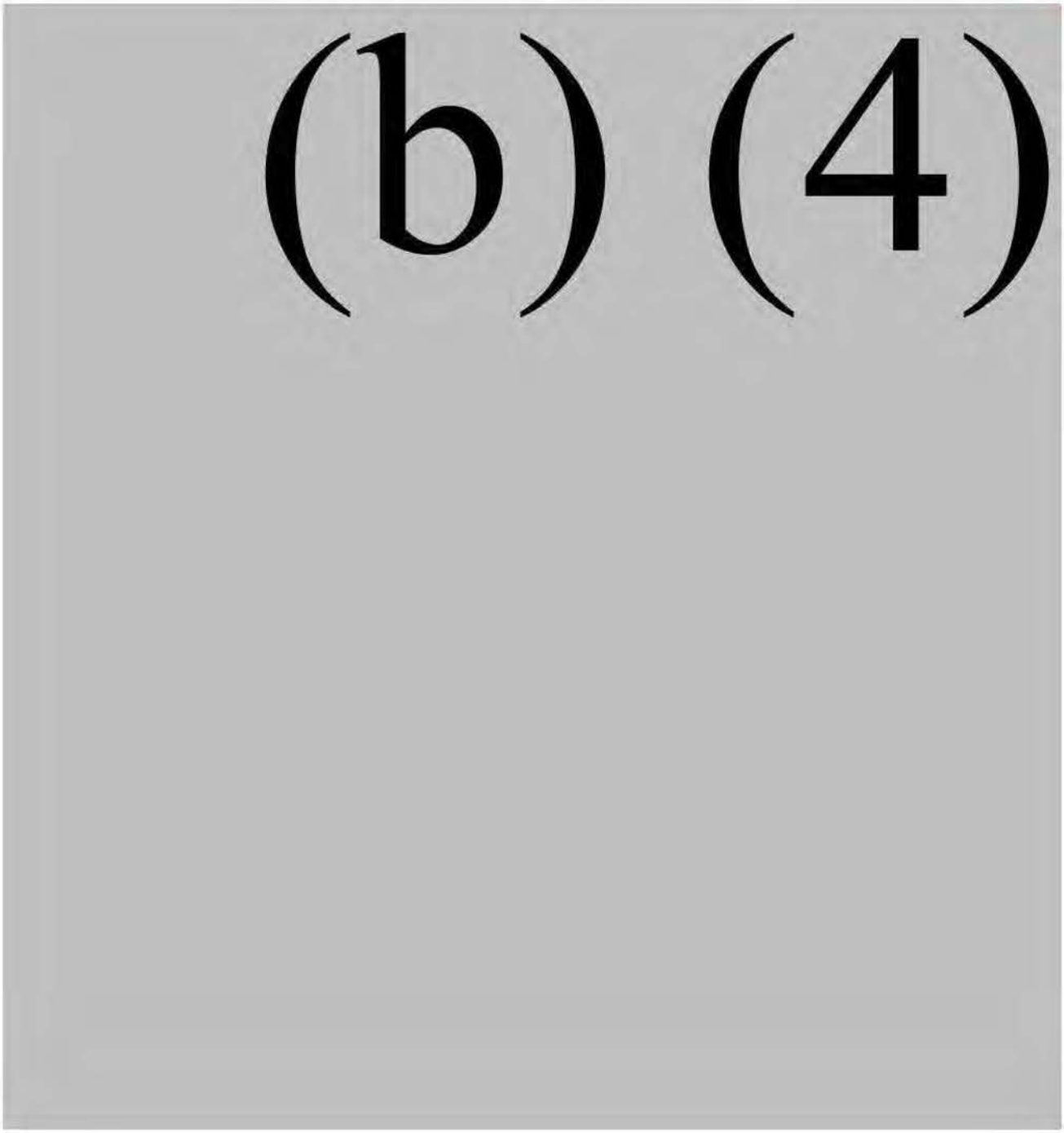

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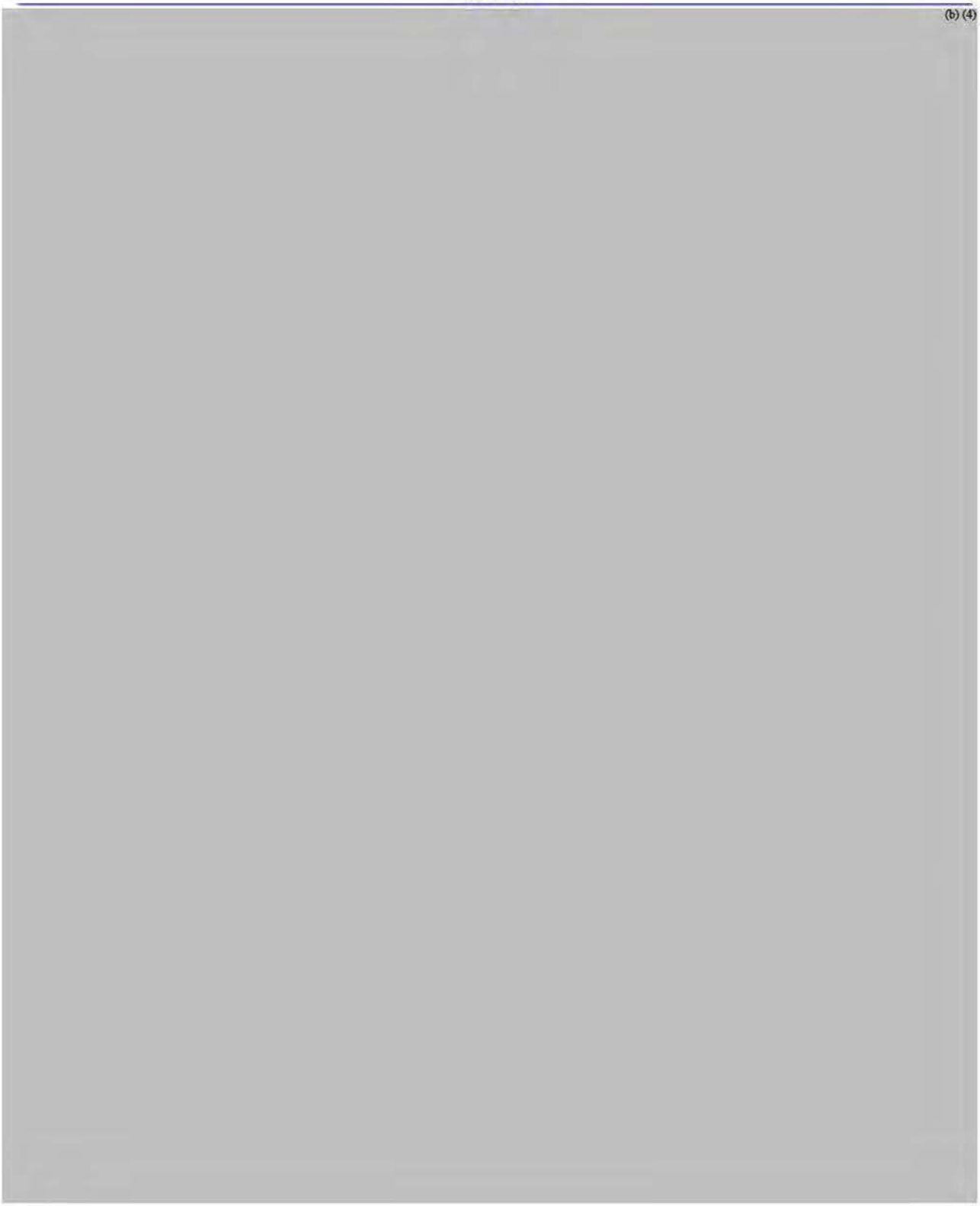

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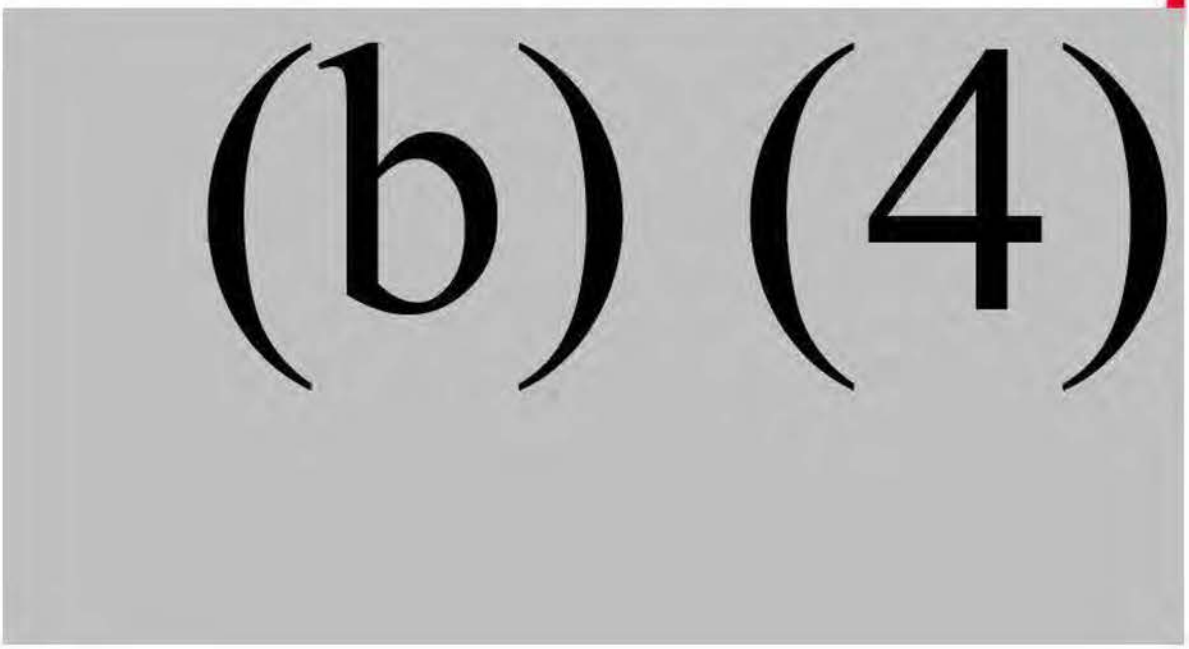

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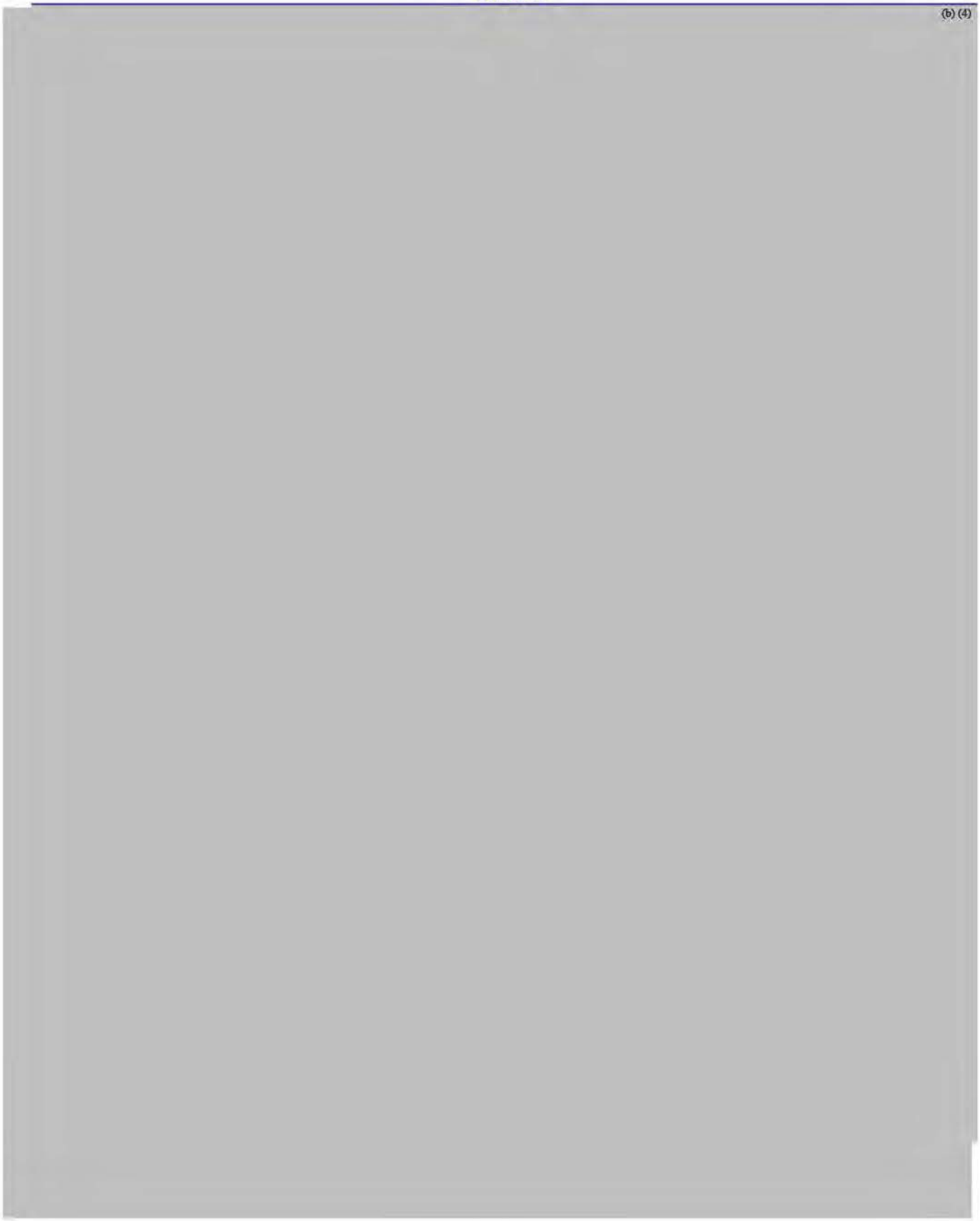

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Animal feeding stuffs
– Determination of phytase activity
– Colorimetric method

(b) (4)

Authorisation and Issue Control

I hereby declare that this method is an accurate reflection of the method conducted in our laboratory, and that the method was developed and written under my supervision and in accordance with standard operating procedures.

Scientist, Feed Biochemistry/Scientific Business support
(b)(6)

Signatu (b)(6) Date: 30.09.2020

This report is approved by Dupont Animal Nutrition Management:
Senior Principal Scientist, Feed Biochemistry/Scientific Business support
Karsten M. Kragh

Signatur (b)(6))ate: 30.09.2020

Contents


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Table 1 shows the dilutions steps needed to obtain the final phosphate concentrations of 0.0 to 4.0mM using (b) (4)

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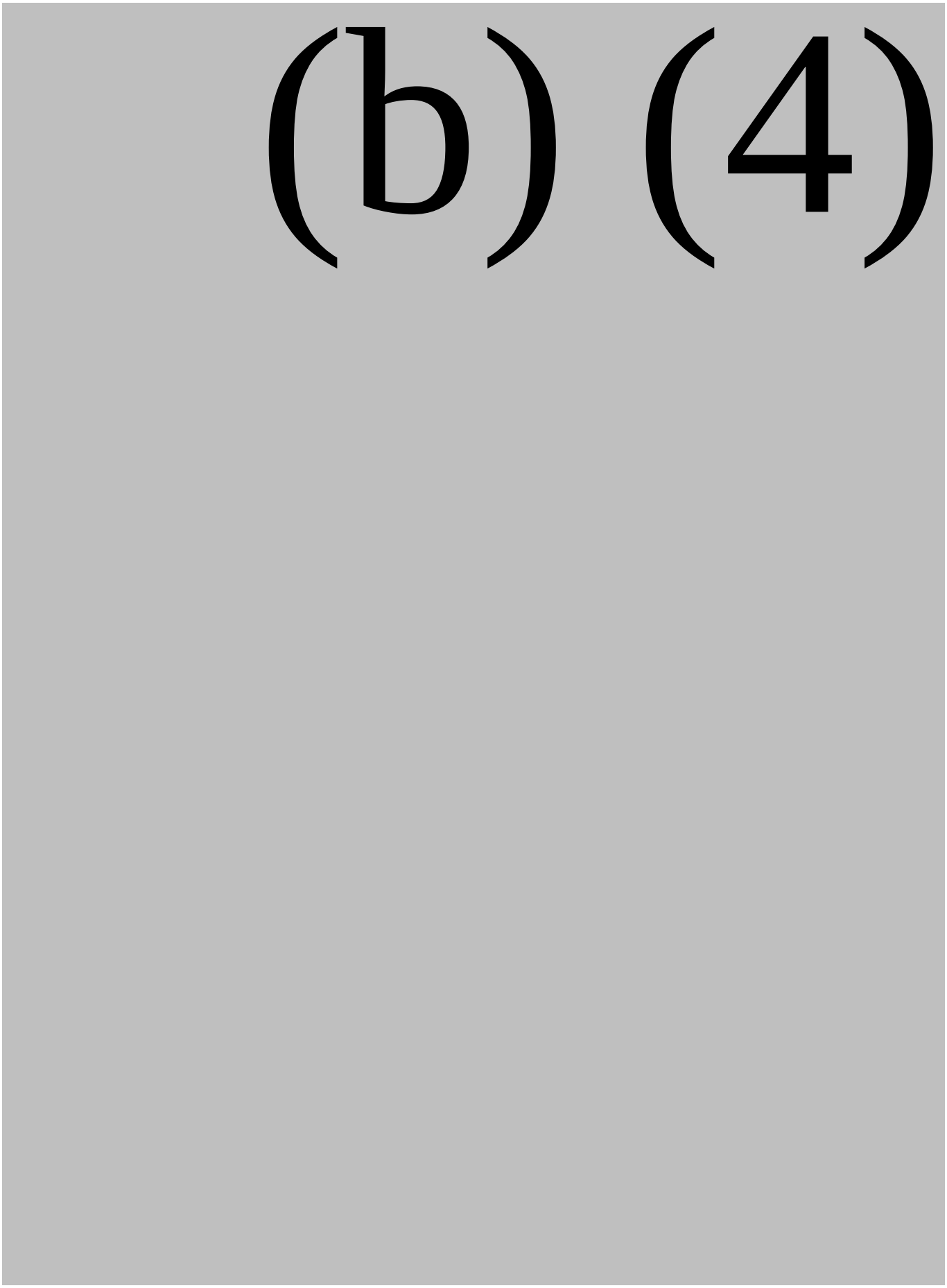
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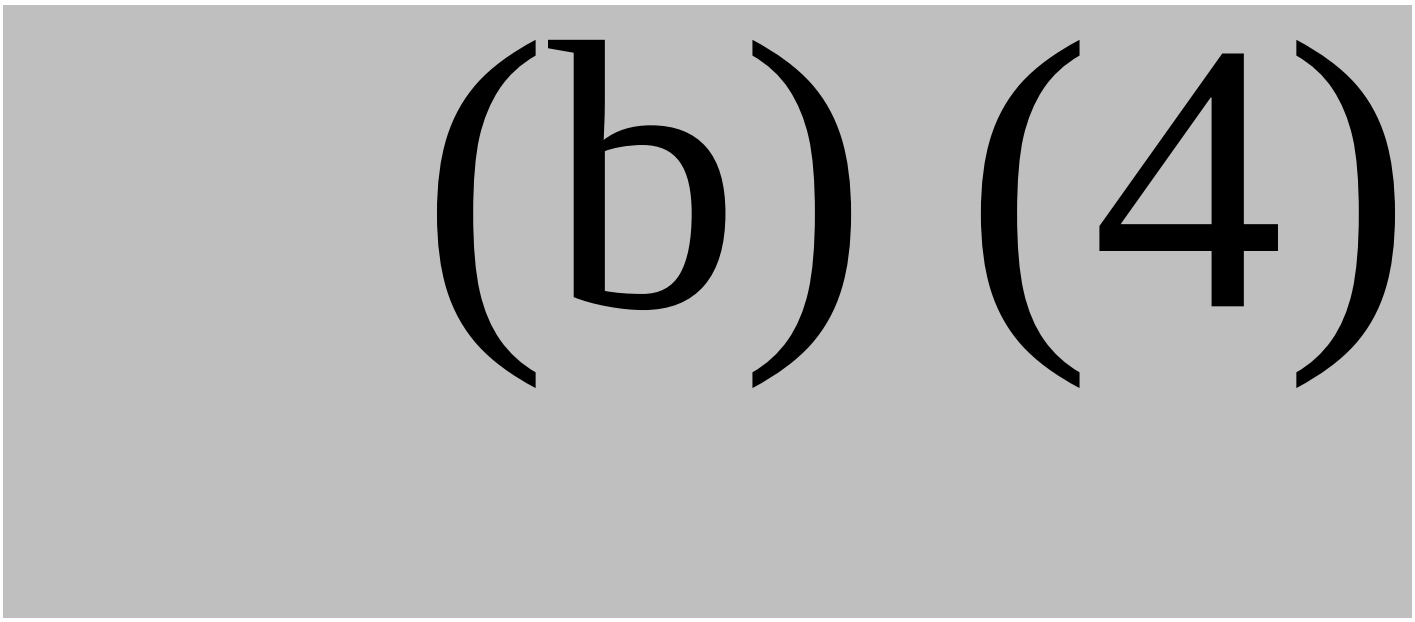
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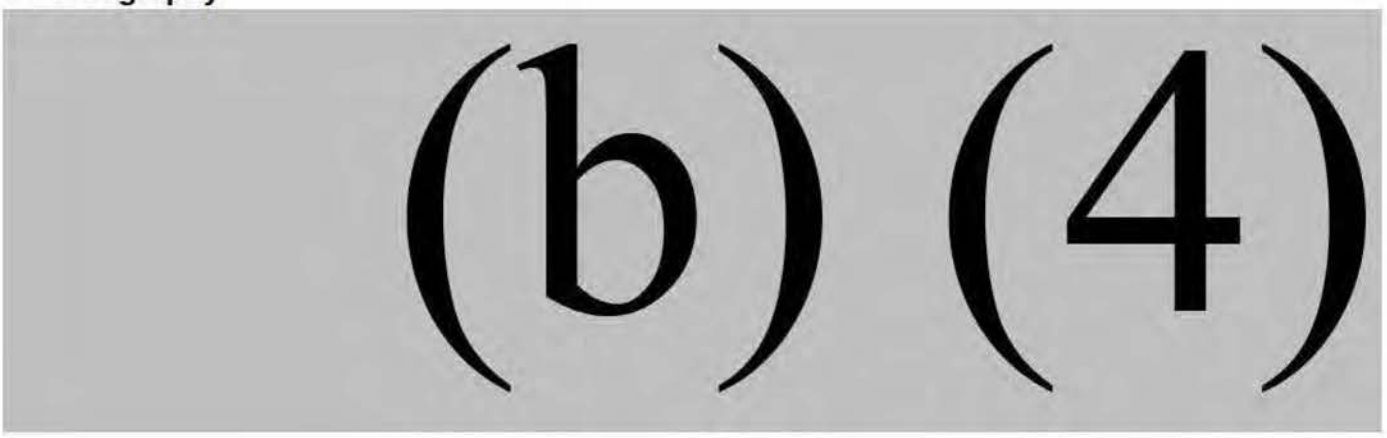

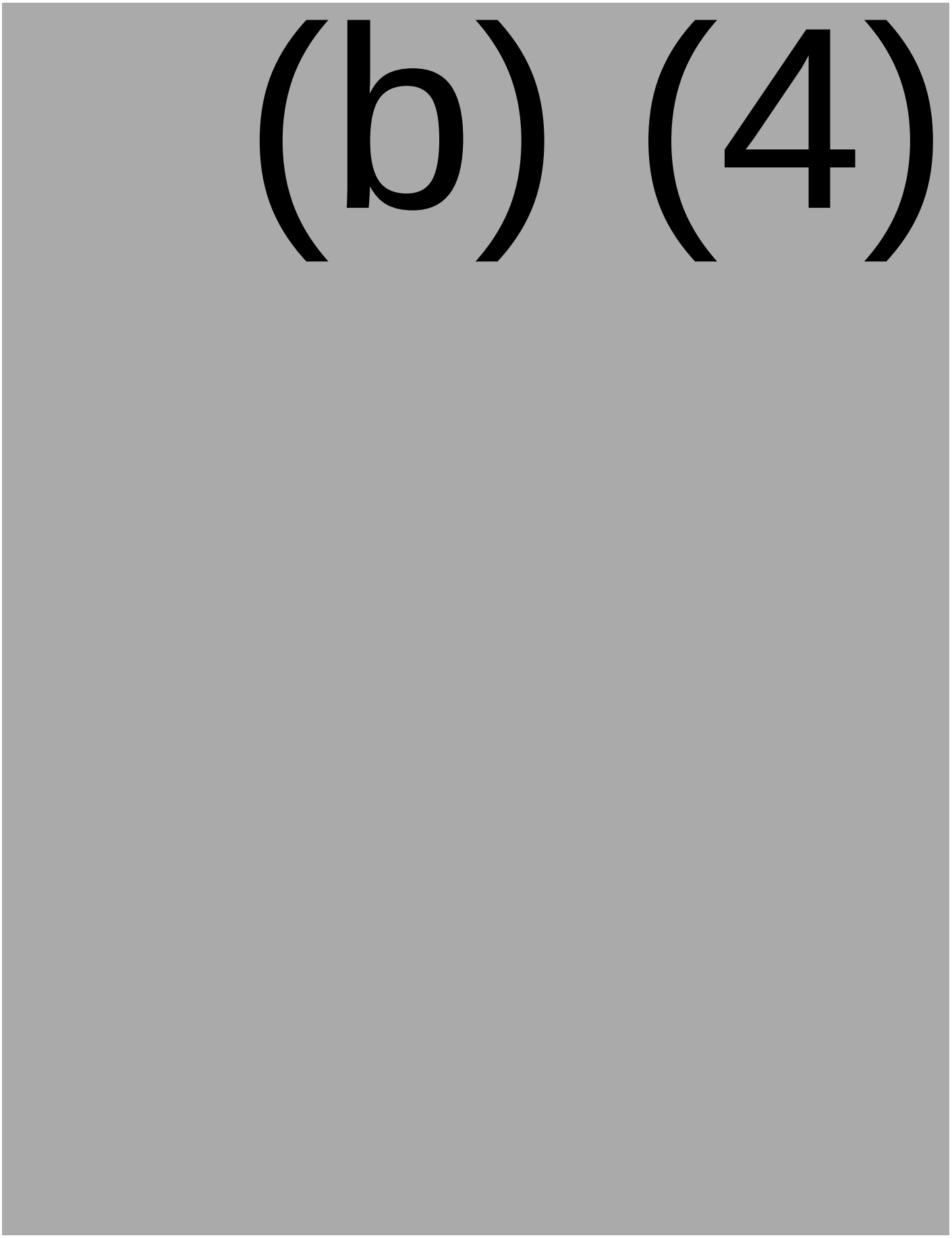
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REPORT

ID: 23566-3/2020

Stability of "Axtra® PHY GOLD 30 T" in pelleted feed for Poultry, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

(b) (4)

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signatur (b)(6) …… Date: 29/09/2020……..

Study Scientist:

(b)(6)

Signature:… (b)(6) Date: 27/09/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

Signature:… (b)(6) Date: 28/09/2020……..

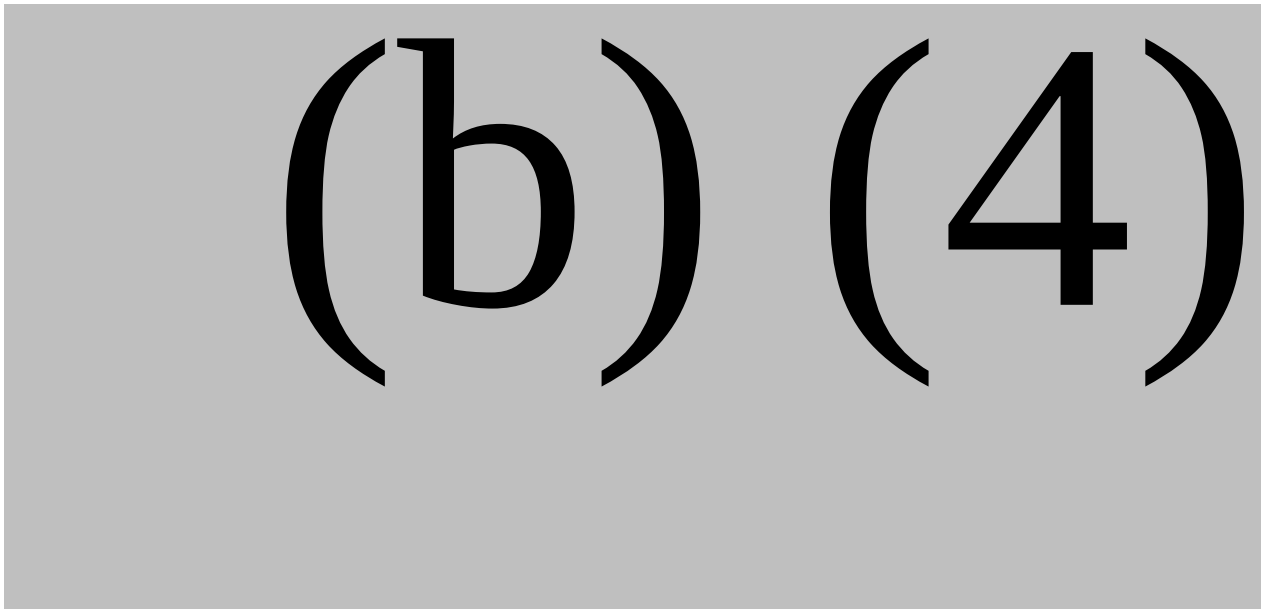
RESPONSIBLE PERSONNEL

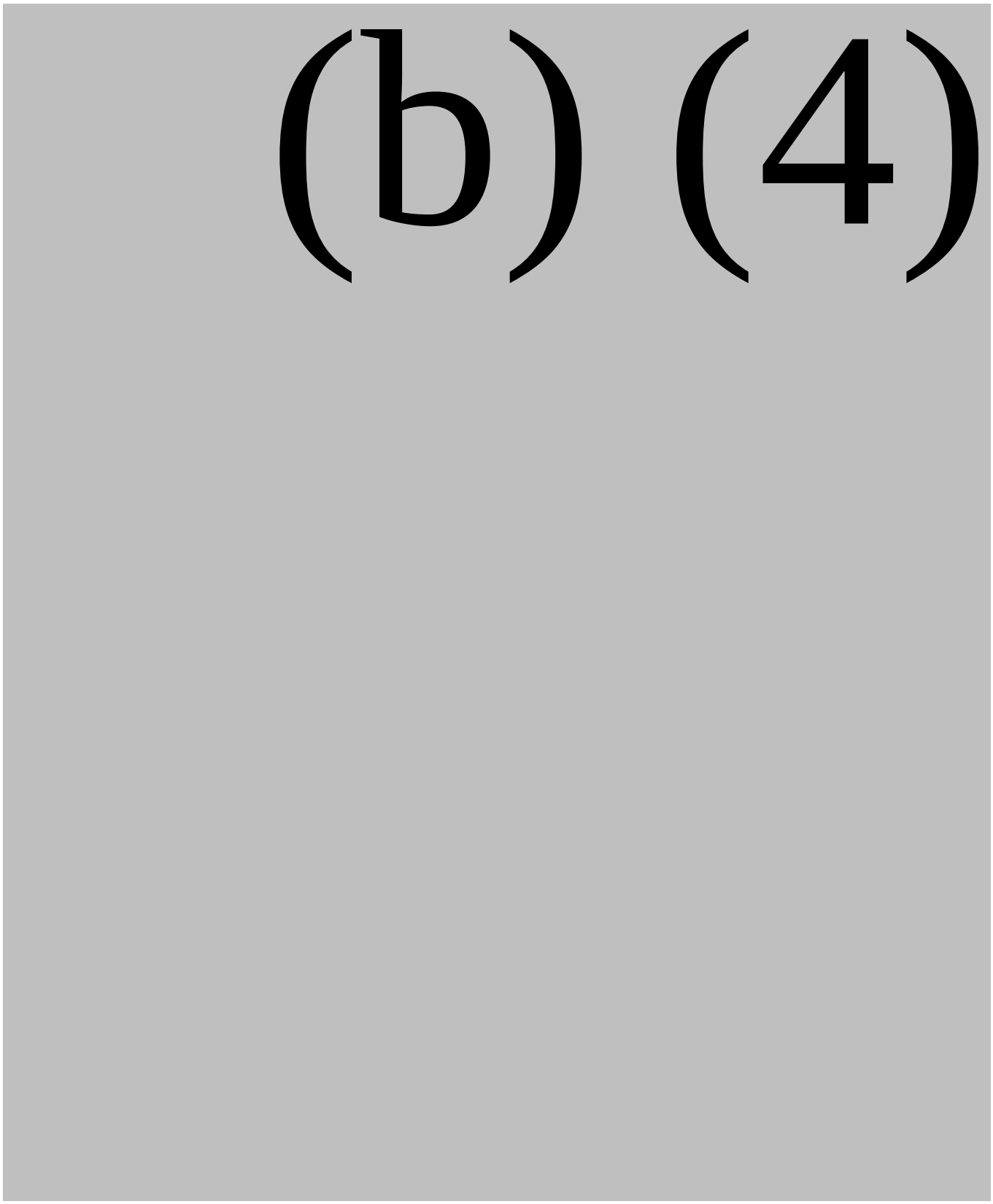
Study scientist(s)	:	[redacted]
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY

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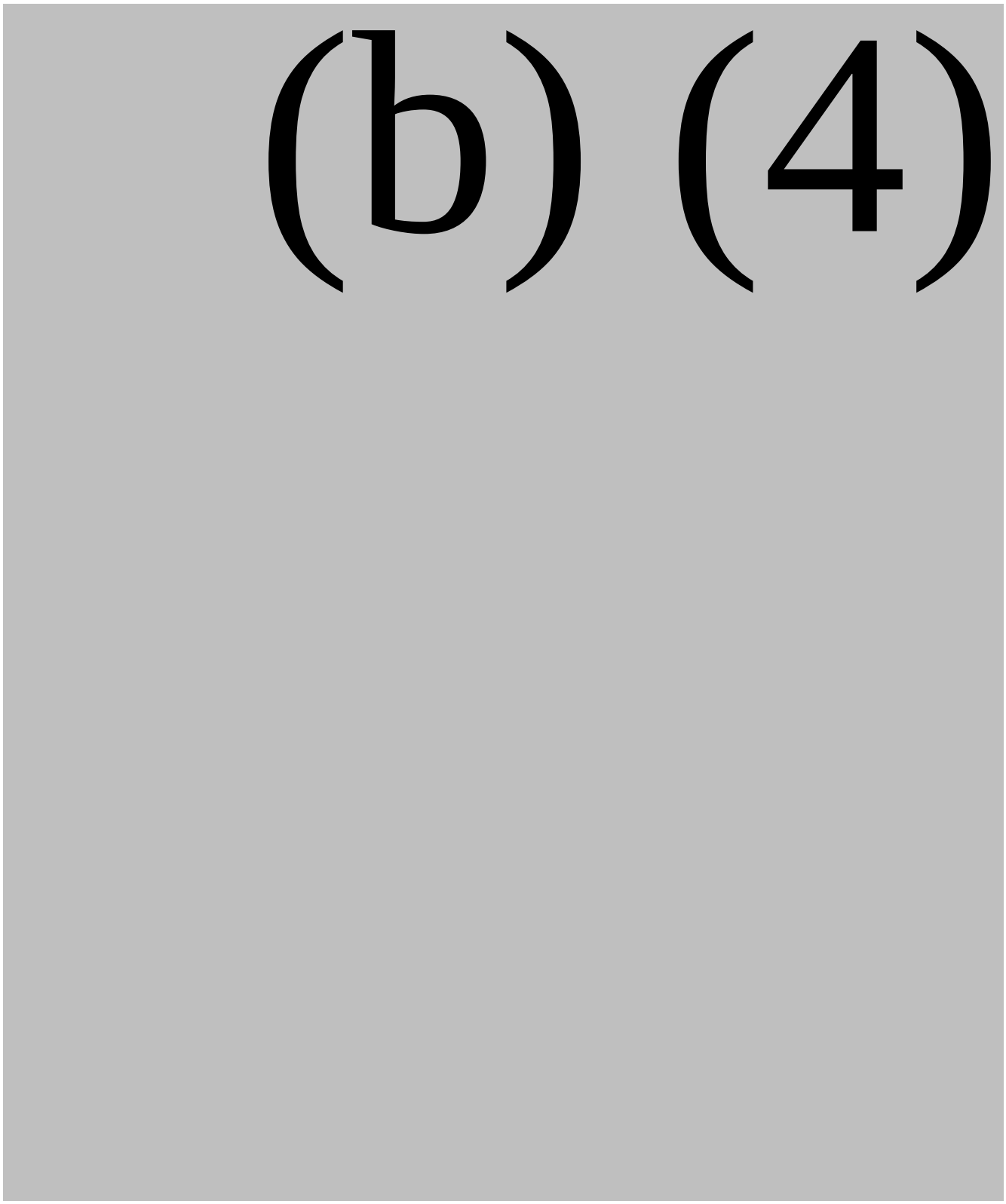
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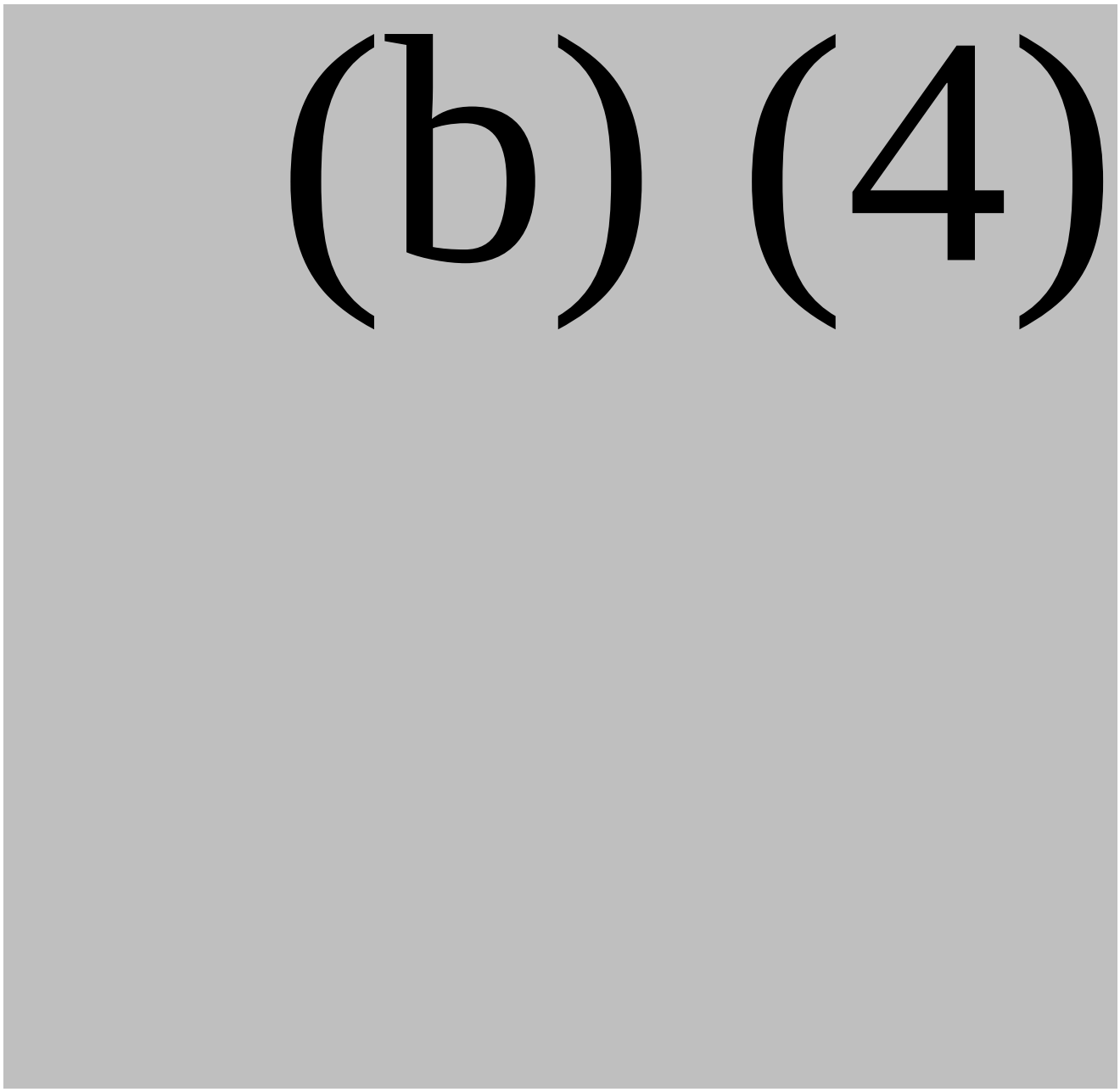

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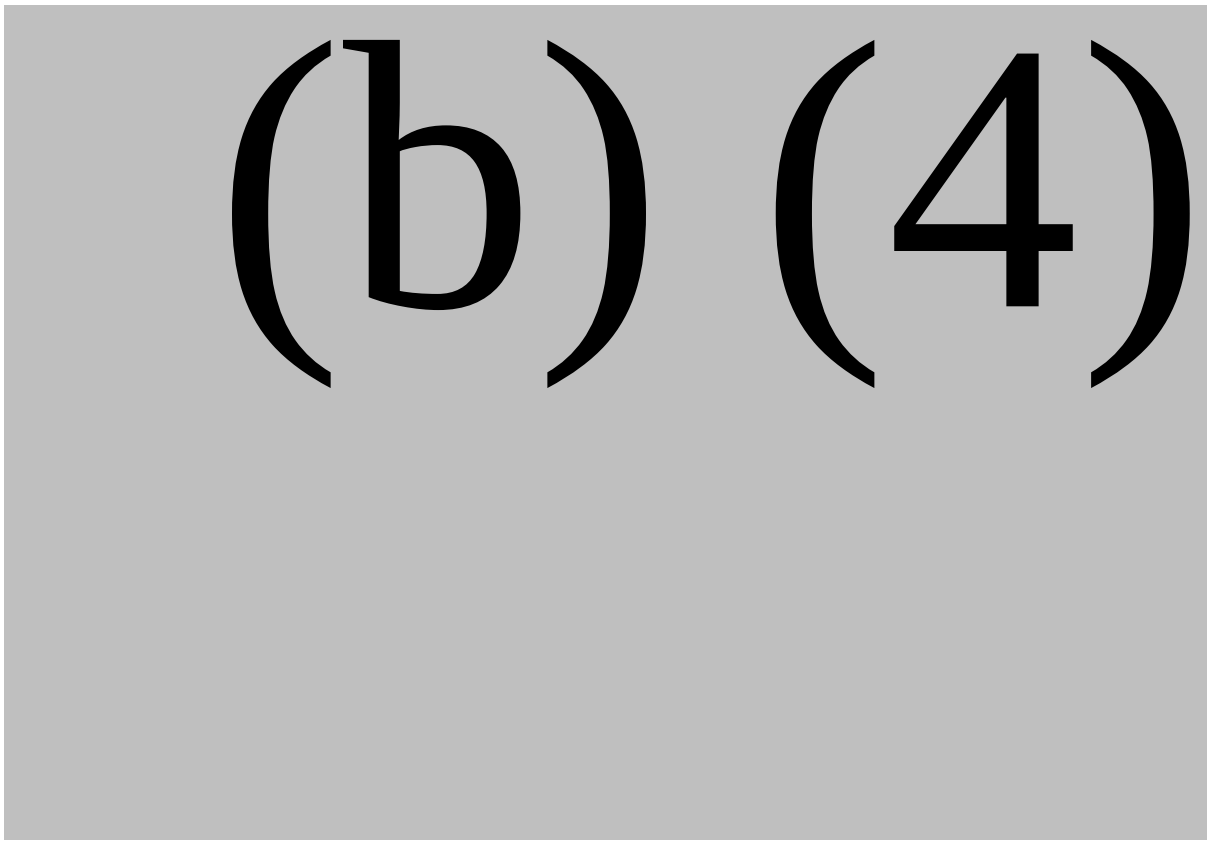
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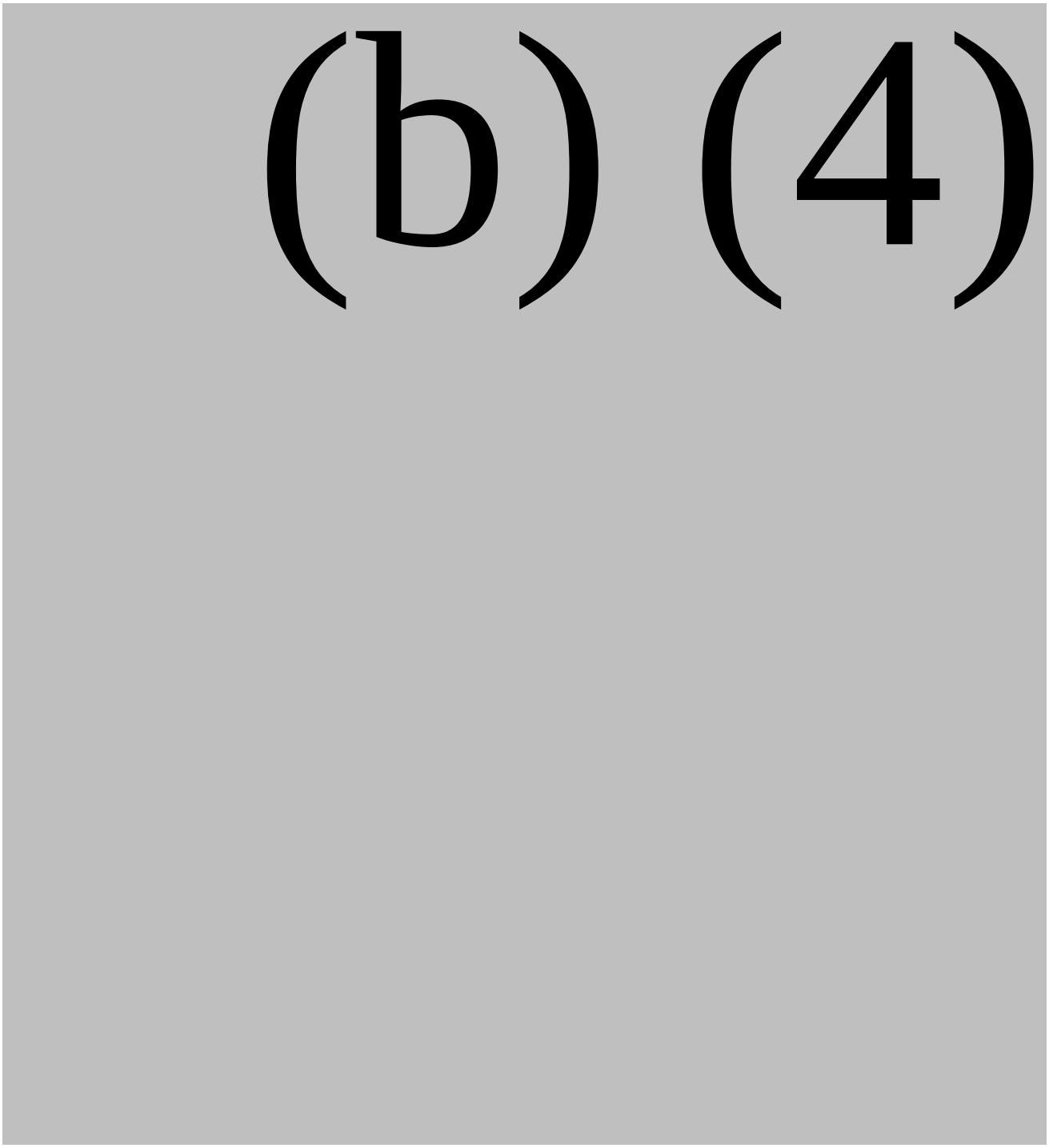

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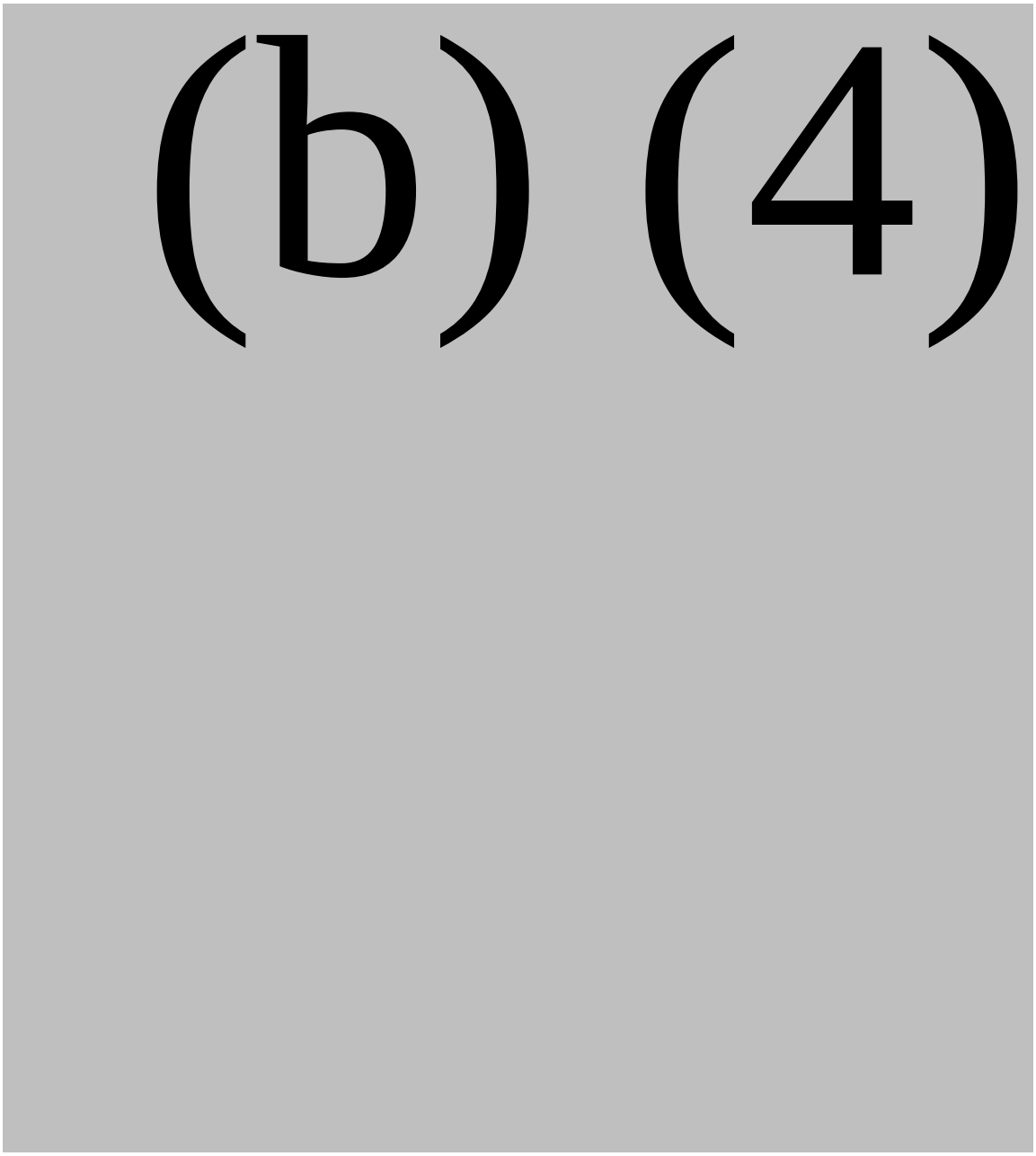

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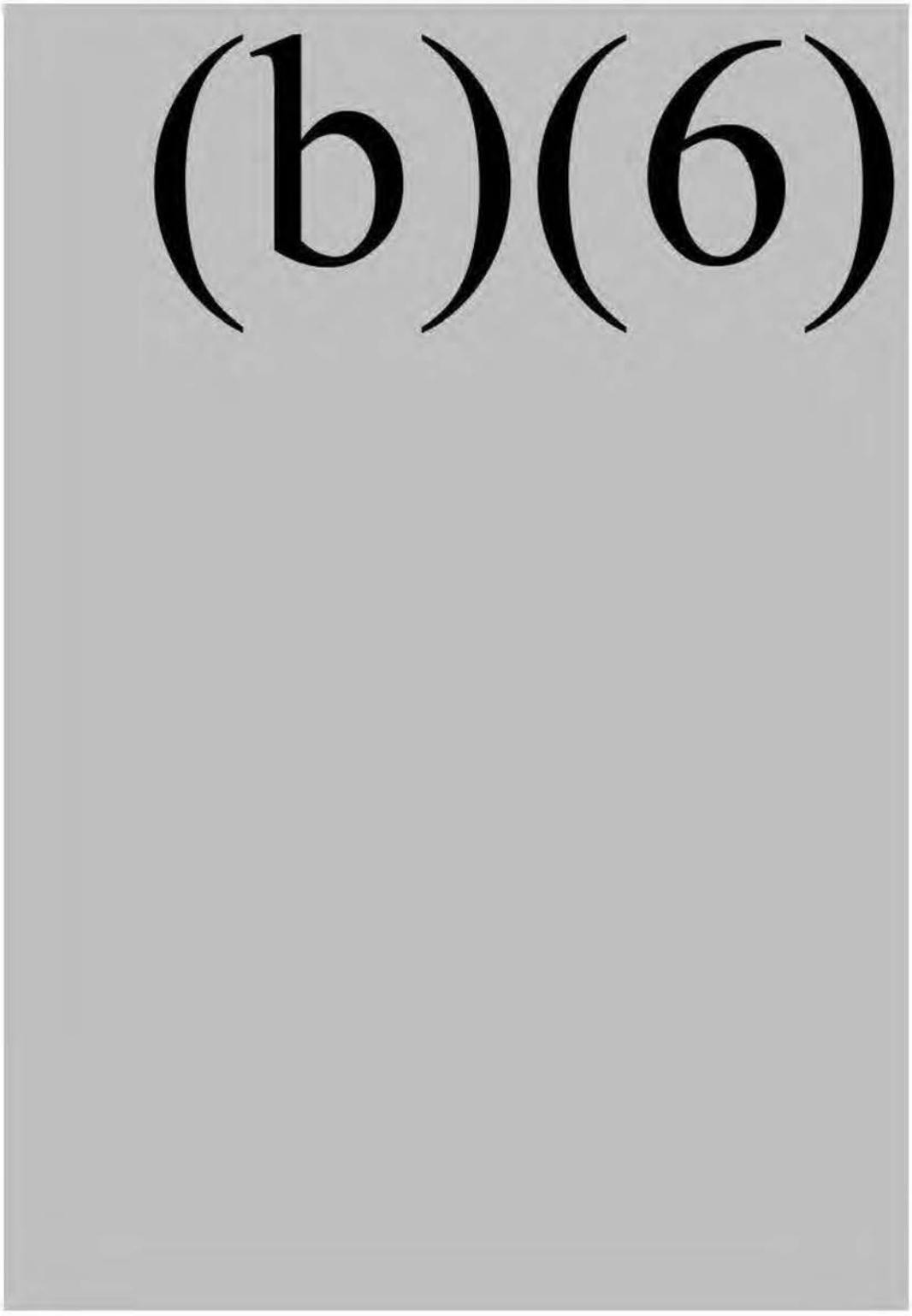
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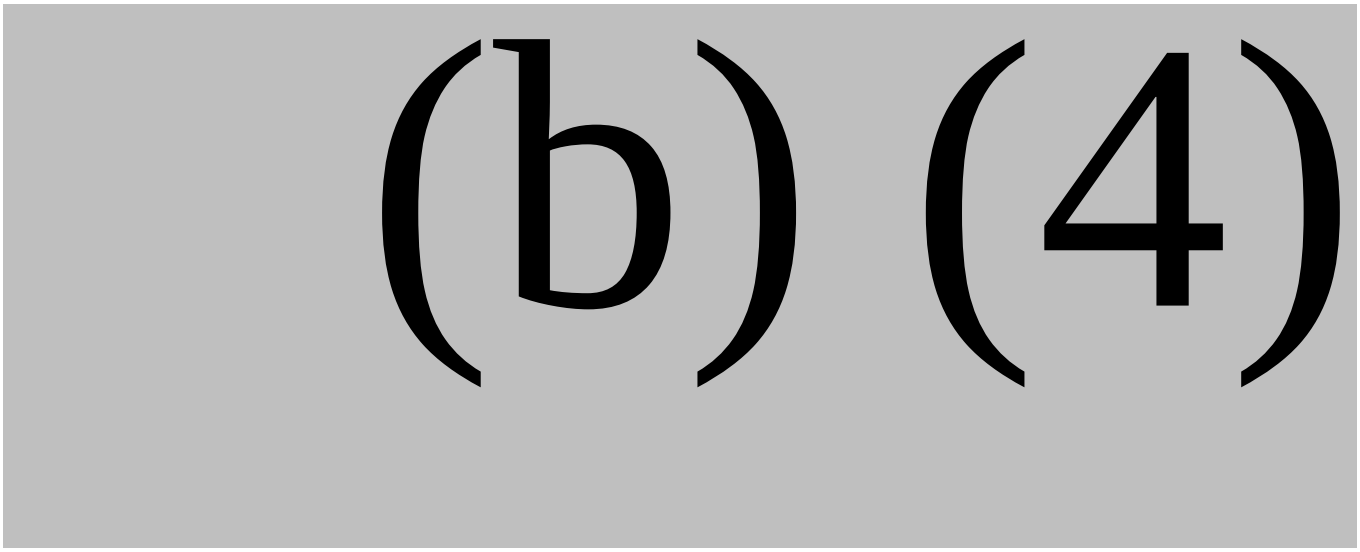


(b) (4)

REPORT

ID: 23612-1/2020

Stability of "Axtra® PHY GOLD 30 L" pelleted feed for Swine, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature:… (b) (6) …… Date:29/09/2020……..

Study Scientist:

(b)(6)

Signature:… (b) (6) …… Date:27/09/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

Signature: (b) (6) Date:28/09/2020……..

(b) (4)

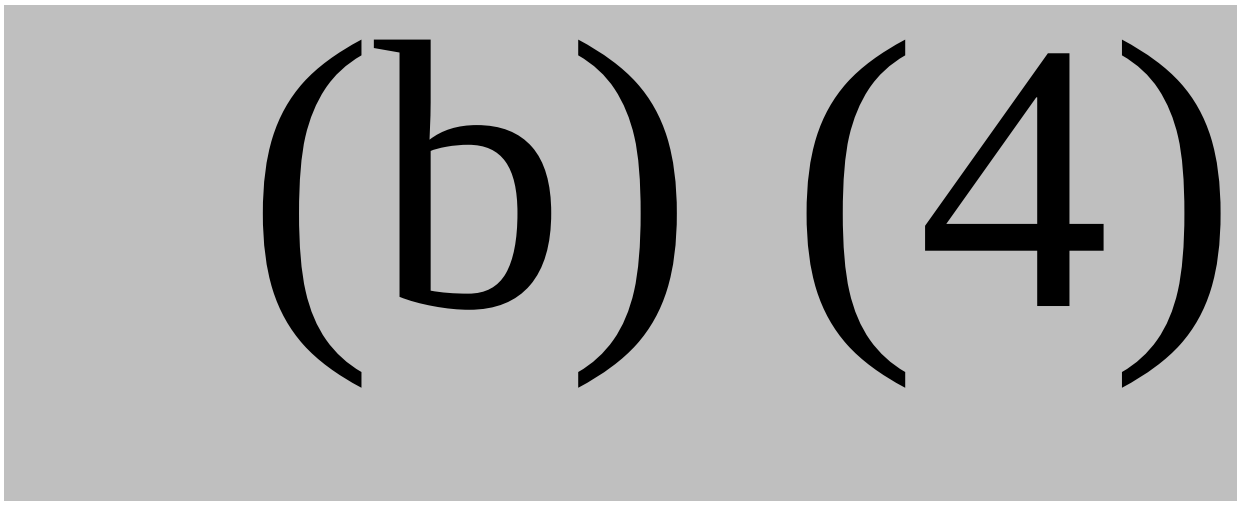
RESPONSIBLE PERSONNEL

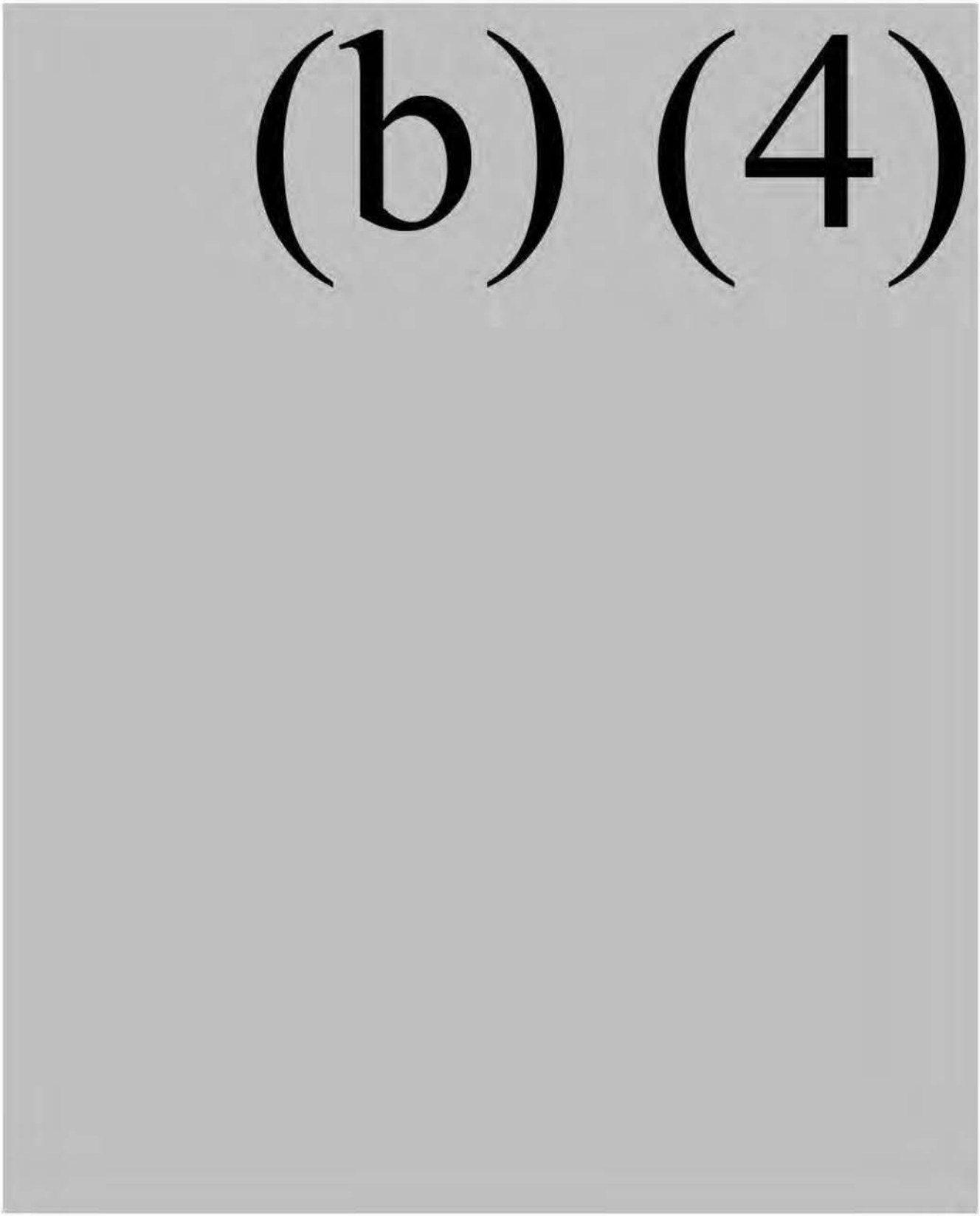
Study scientist(s)	:	[redacted]
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY

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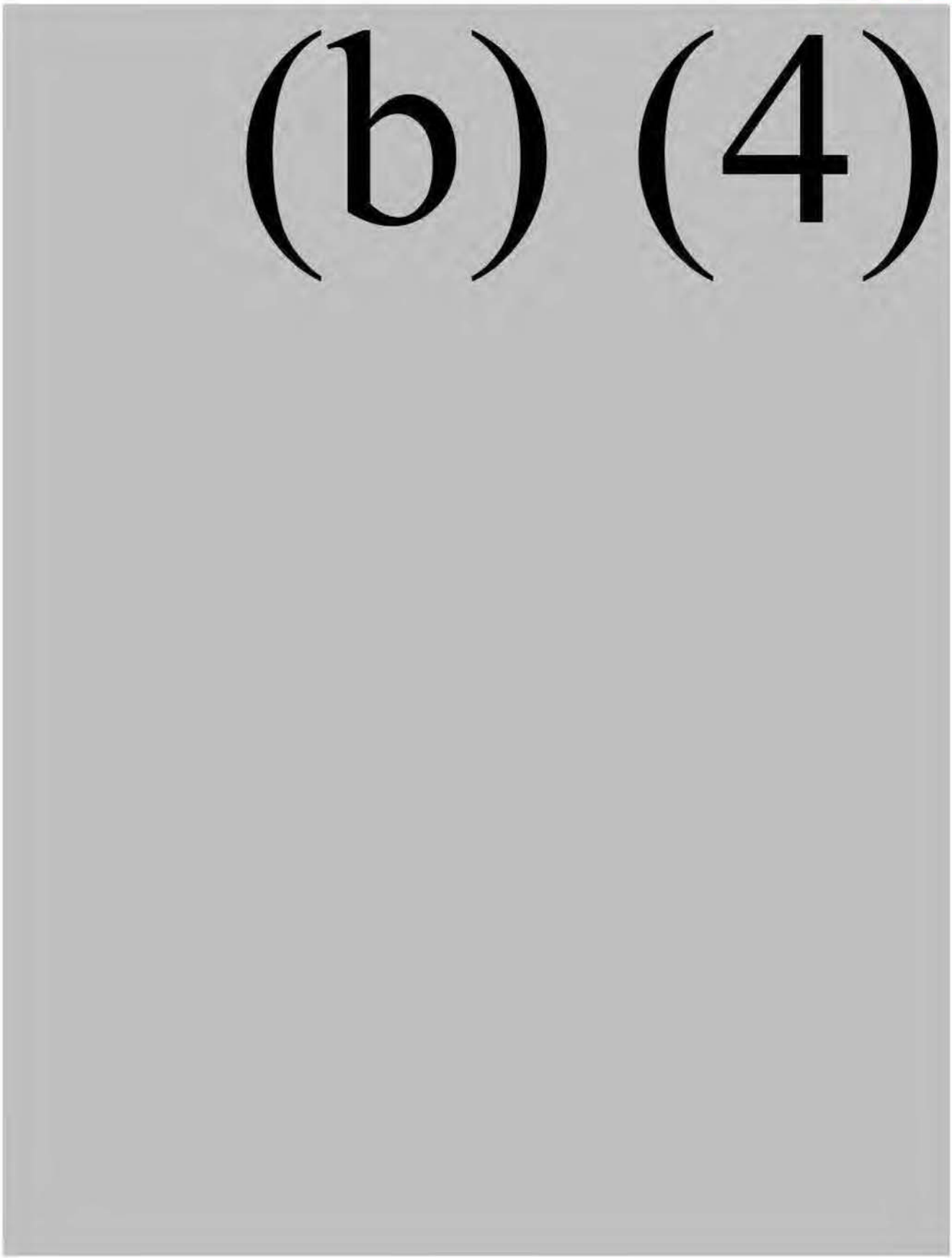
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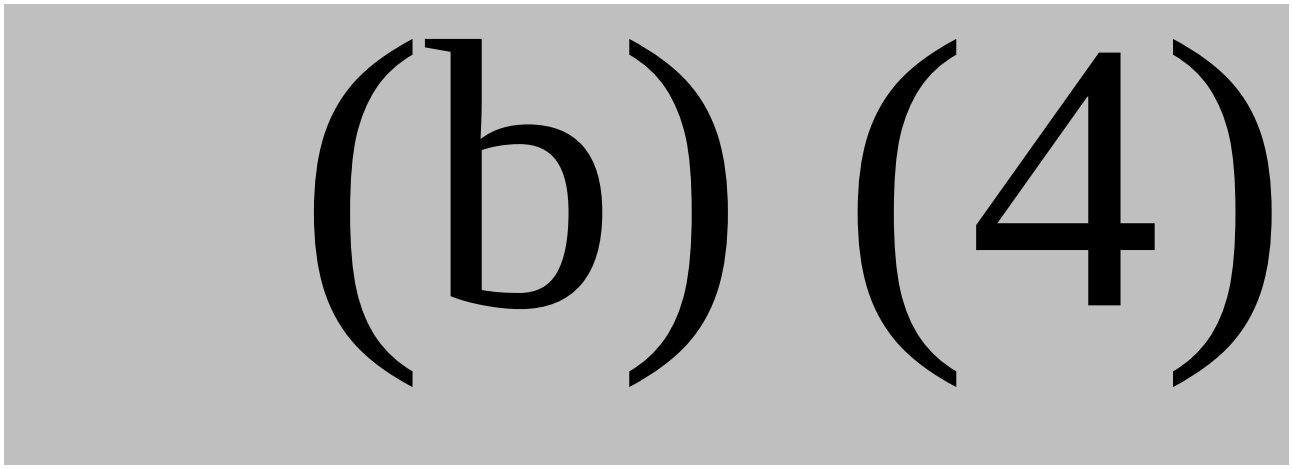
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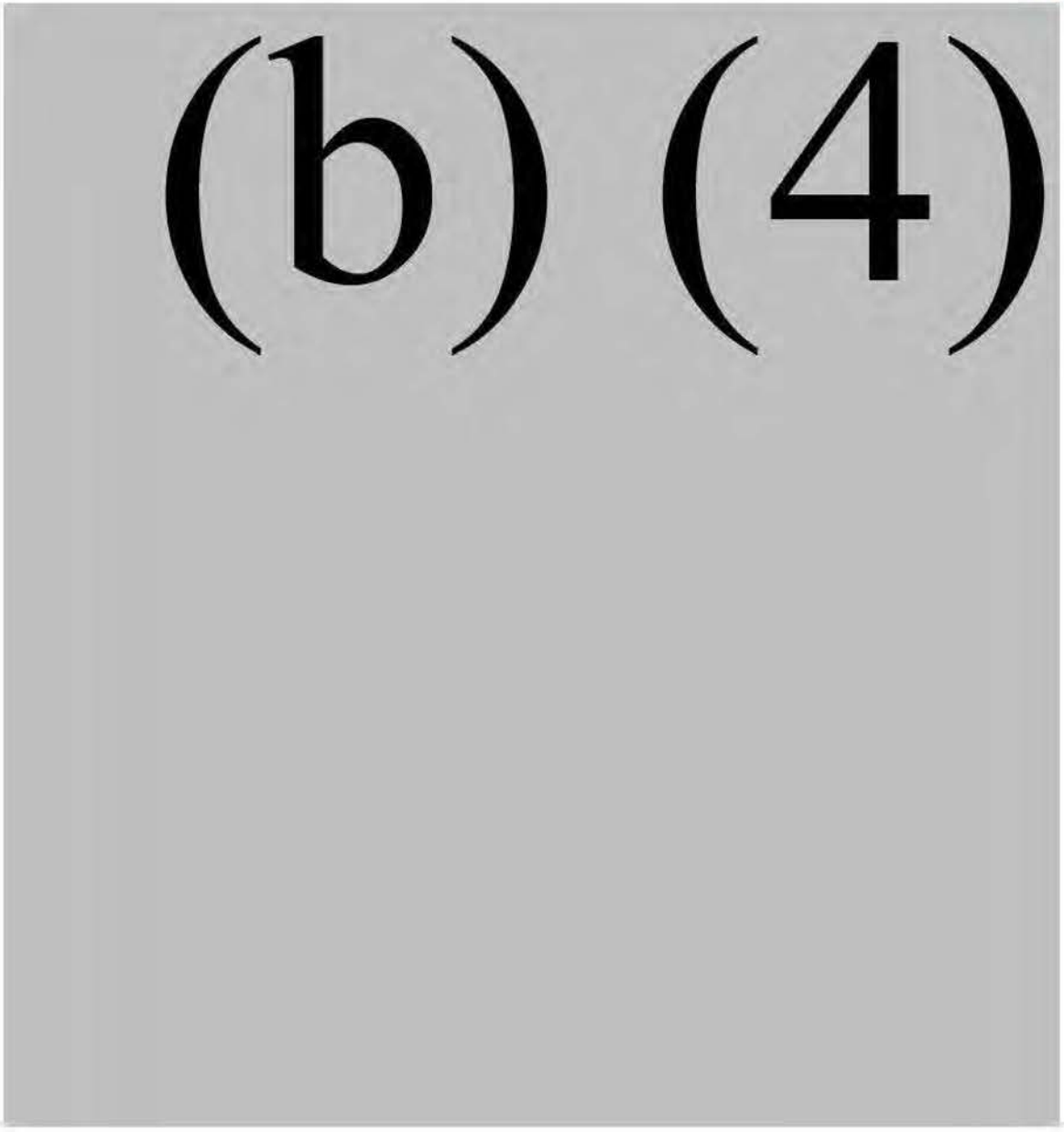
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Analyse

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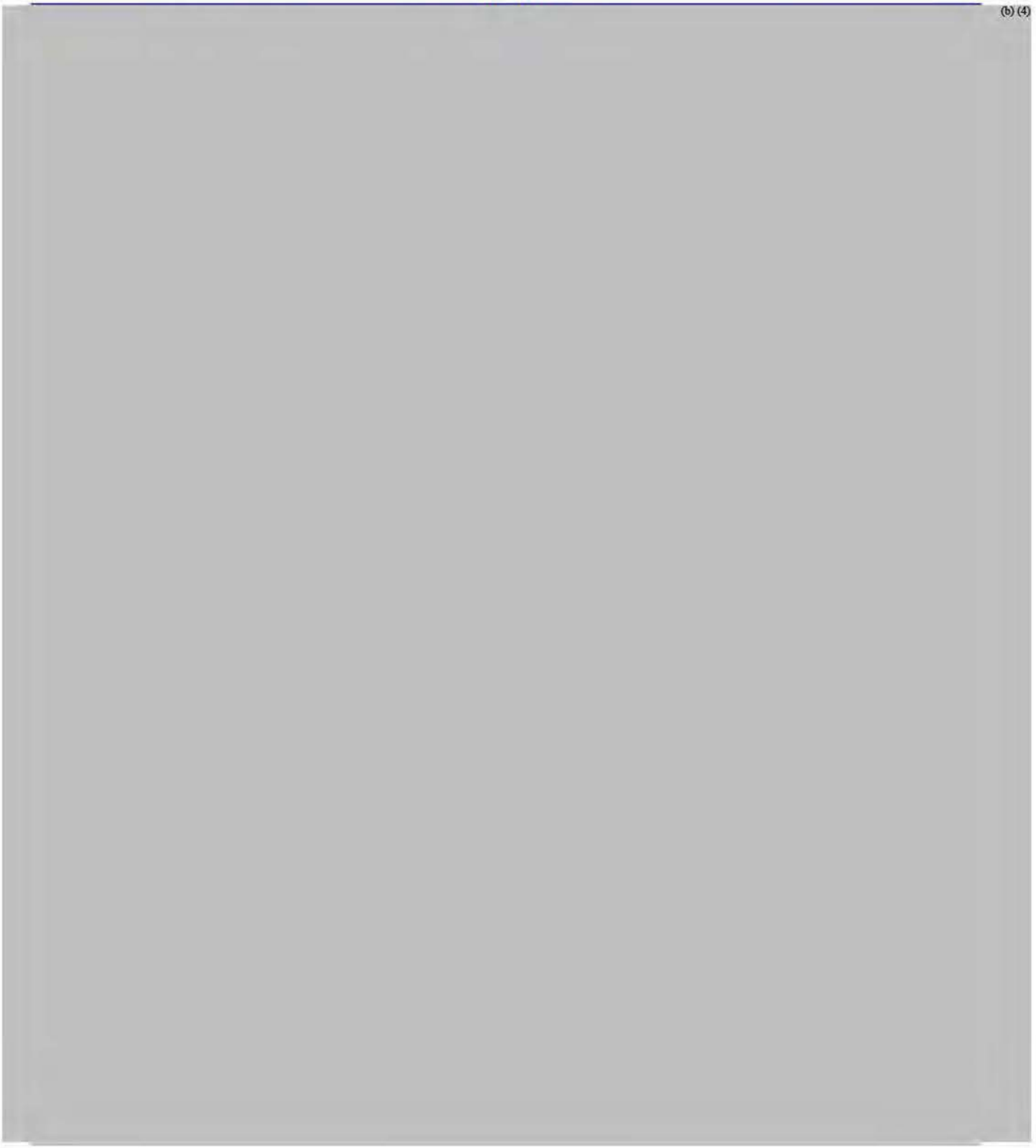

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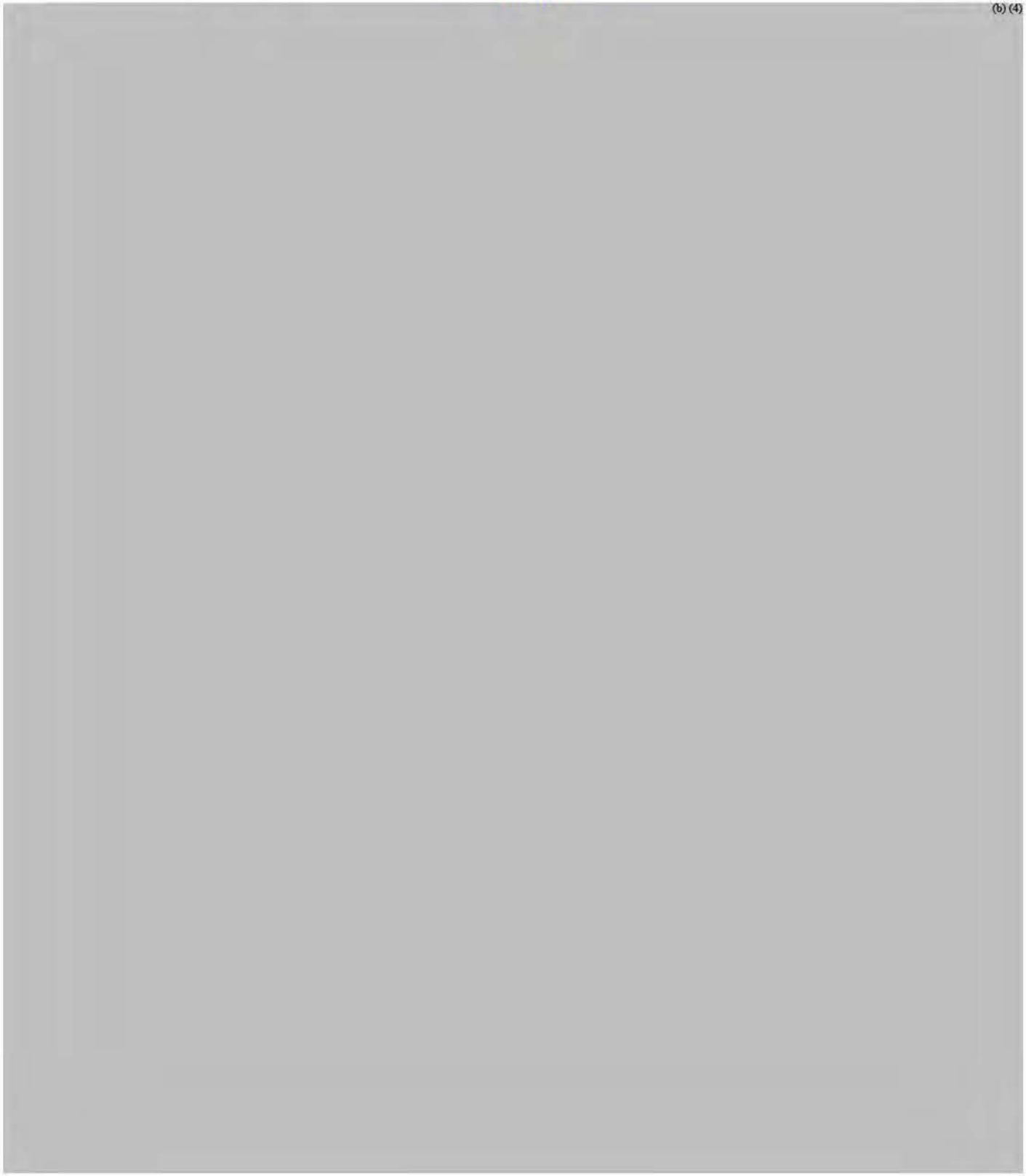

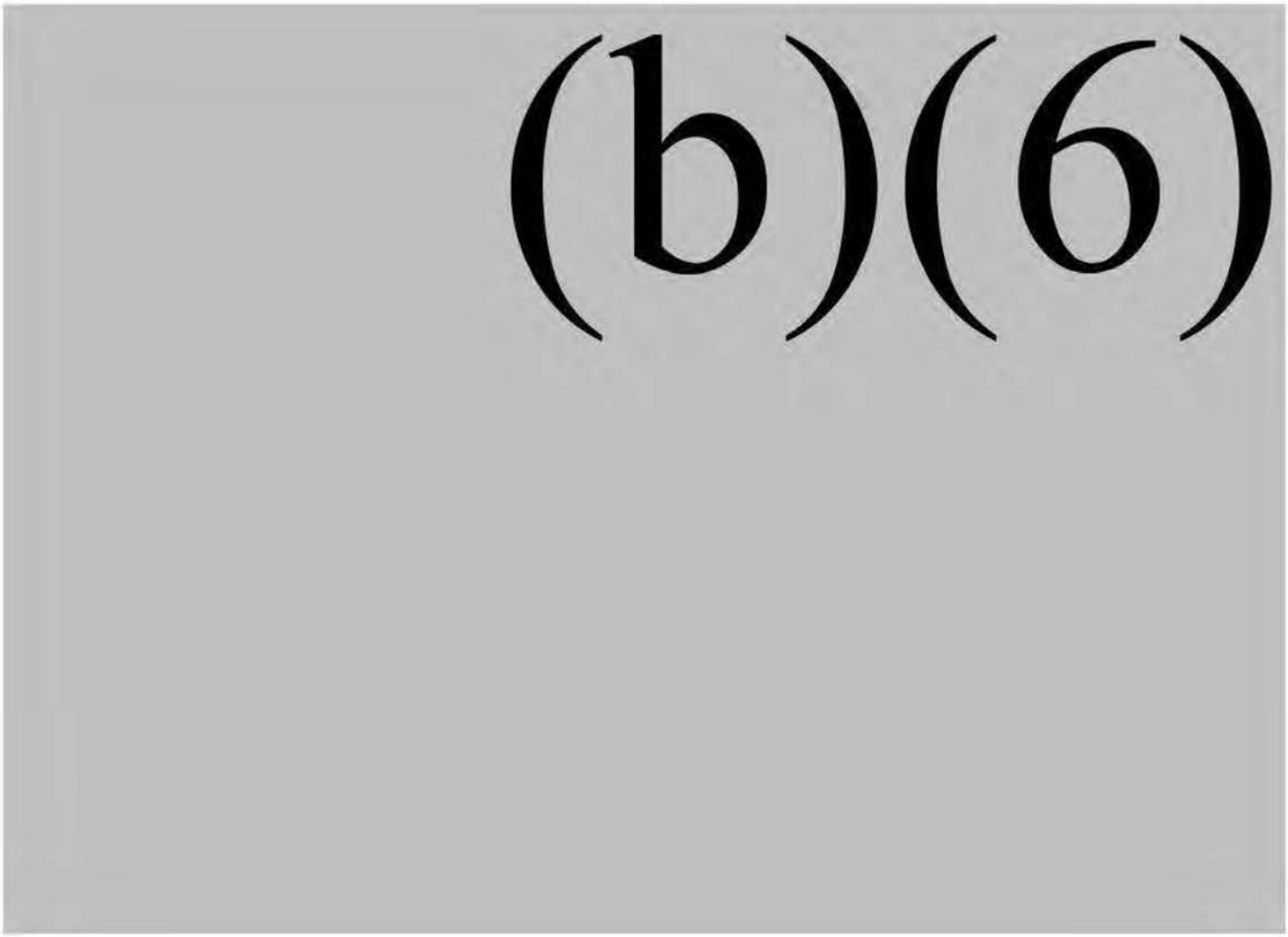
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REPORT

ID: 23612-2/2020

Stability of "Axtra® PHY GOLD 30 L" in pelleted feed for Poultry, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature:… (b) (6) …… Date:29/09/2020……..

Study Scientist:

(b)(6)

Signature:… (b) (6) … Date:27/09/2020……..

This report is approved by:

Product Manager:

Arno de Kre

Signature:… (b) (6) Date:28/09/2020……..

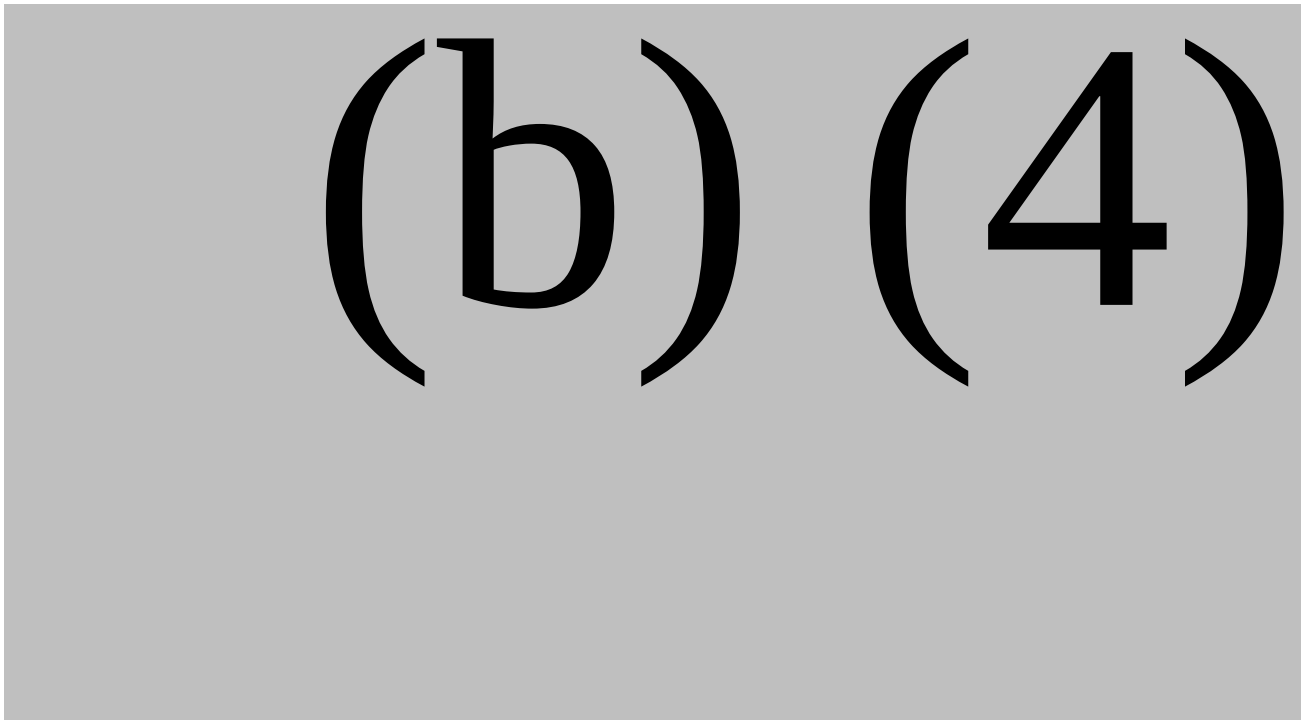
RESPONSIBLE PERSONNEL

Study Scientist(s)	:	[redacted]
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY



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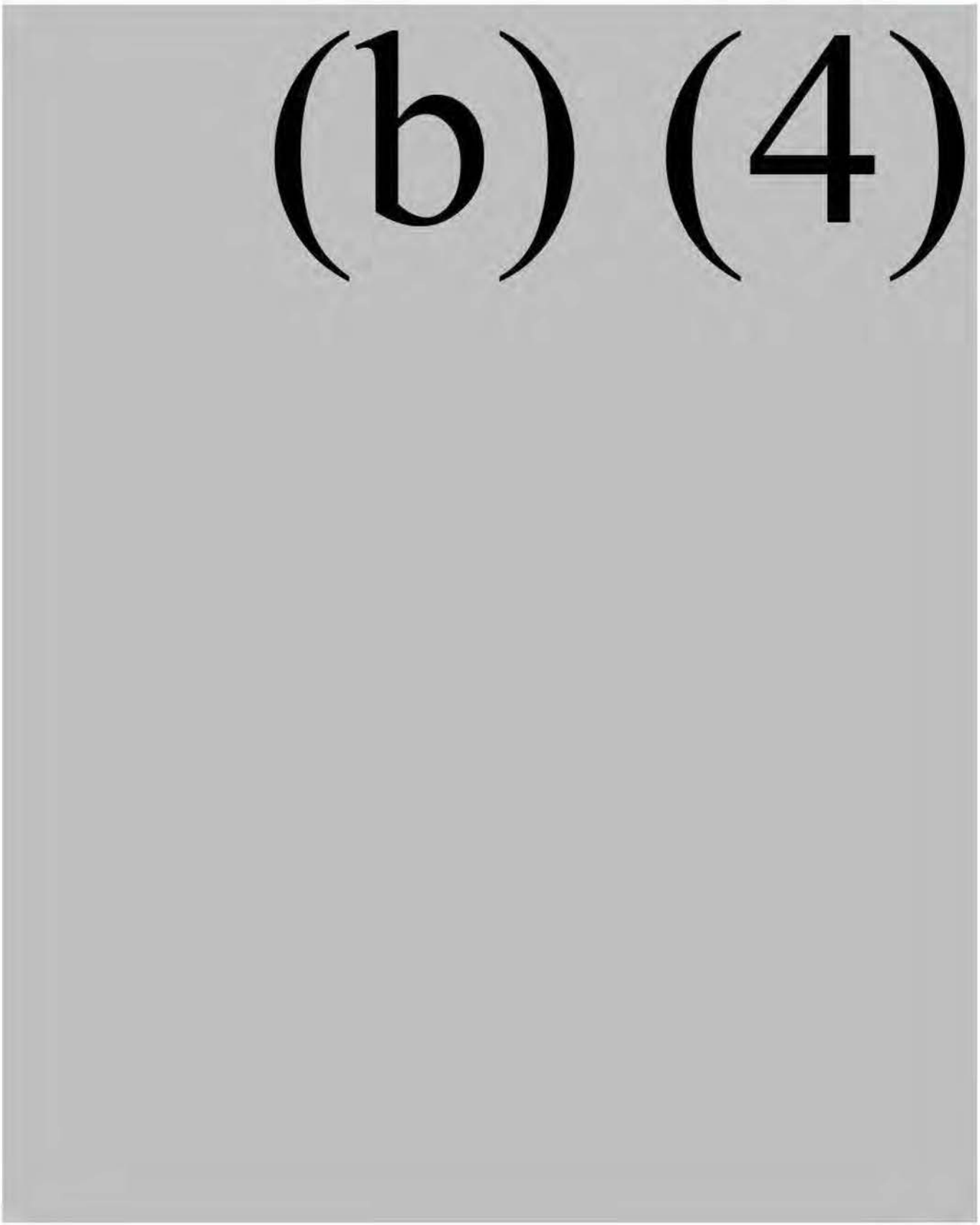
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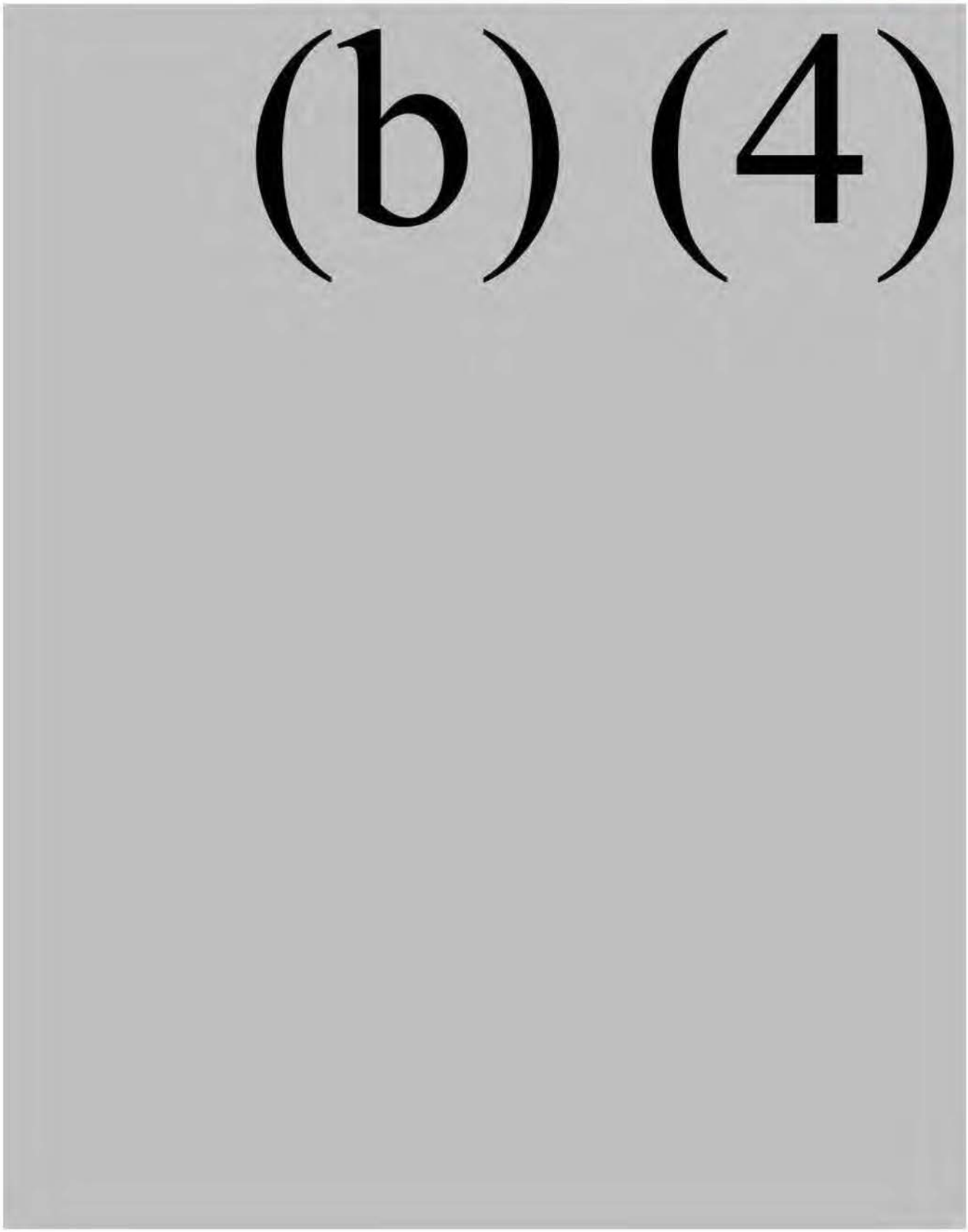
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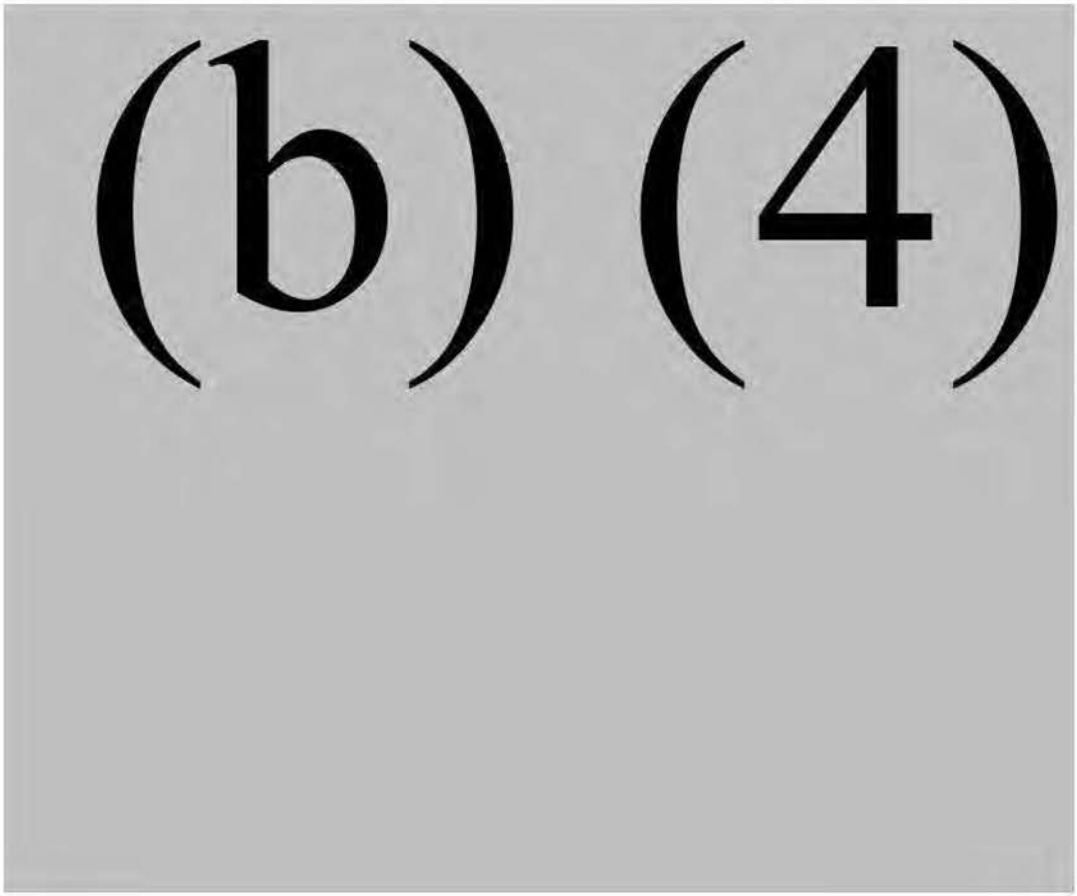
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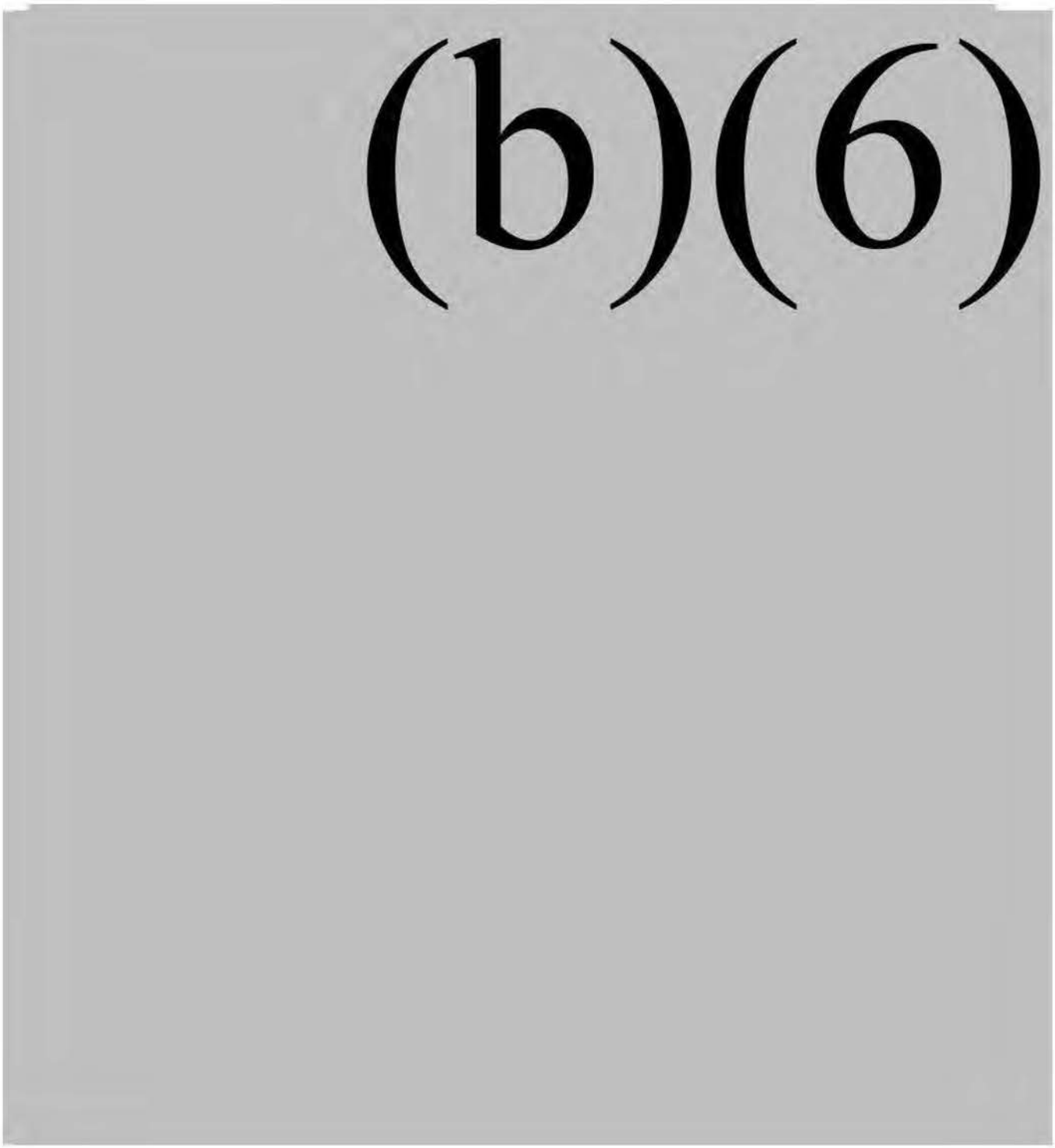
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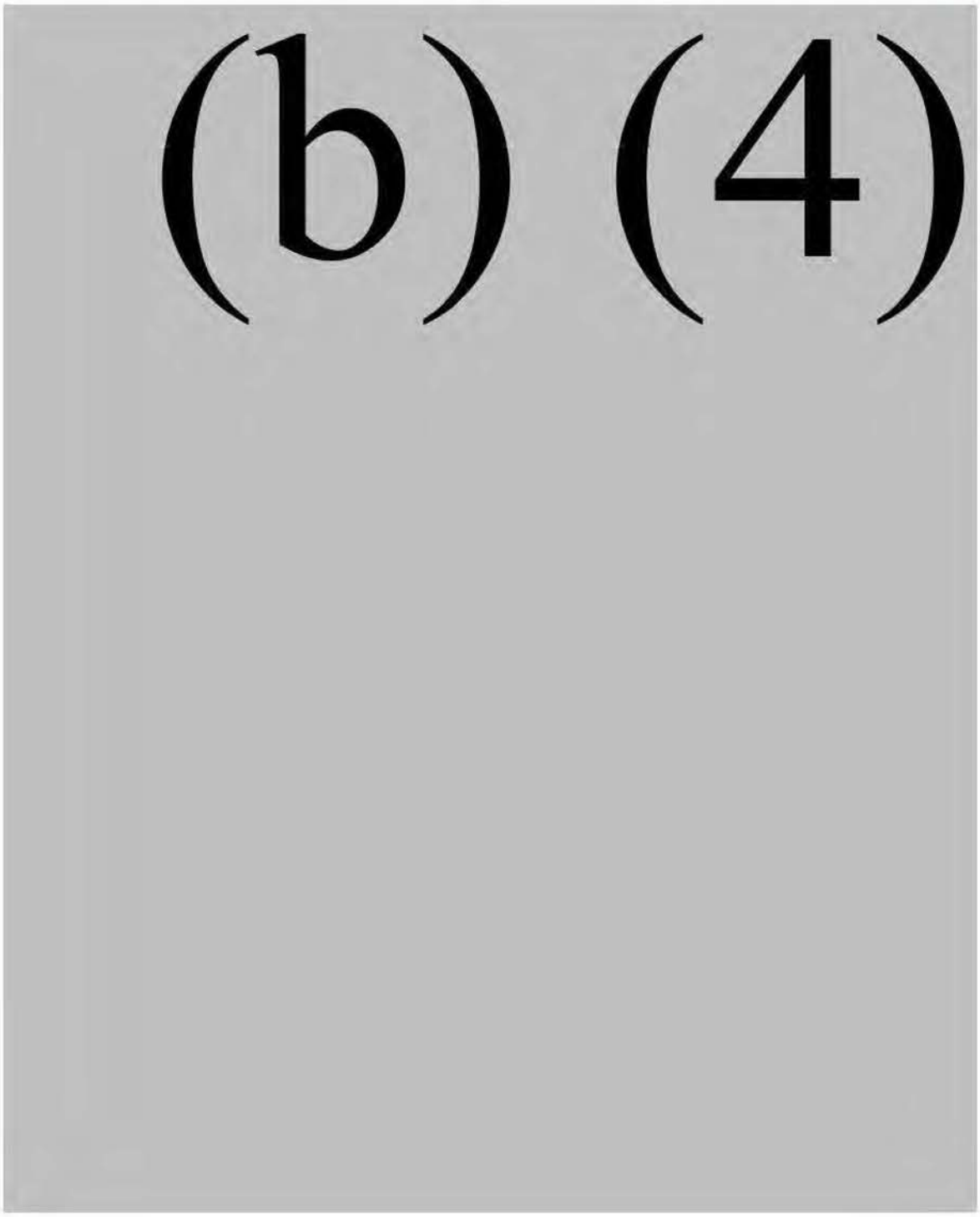

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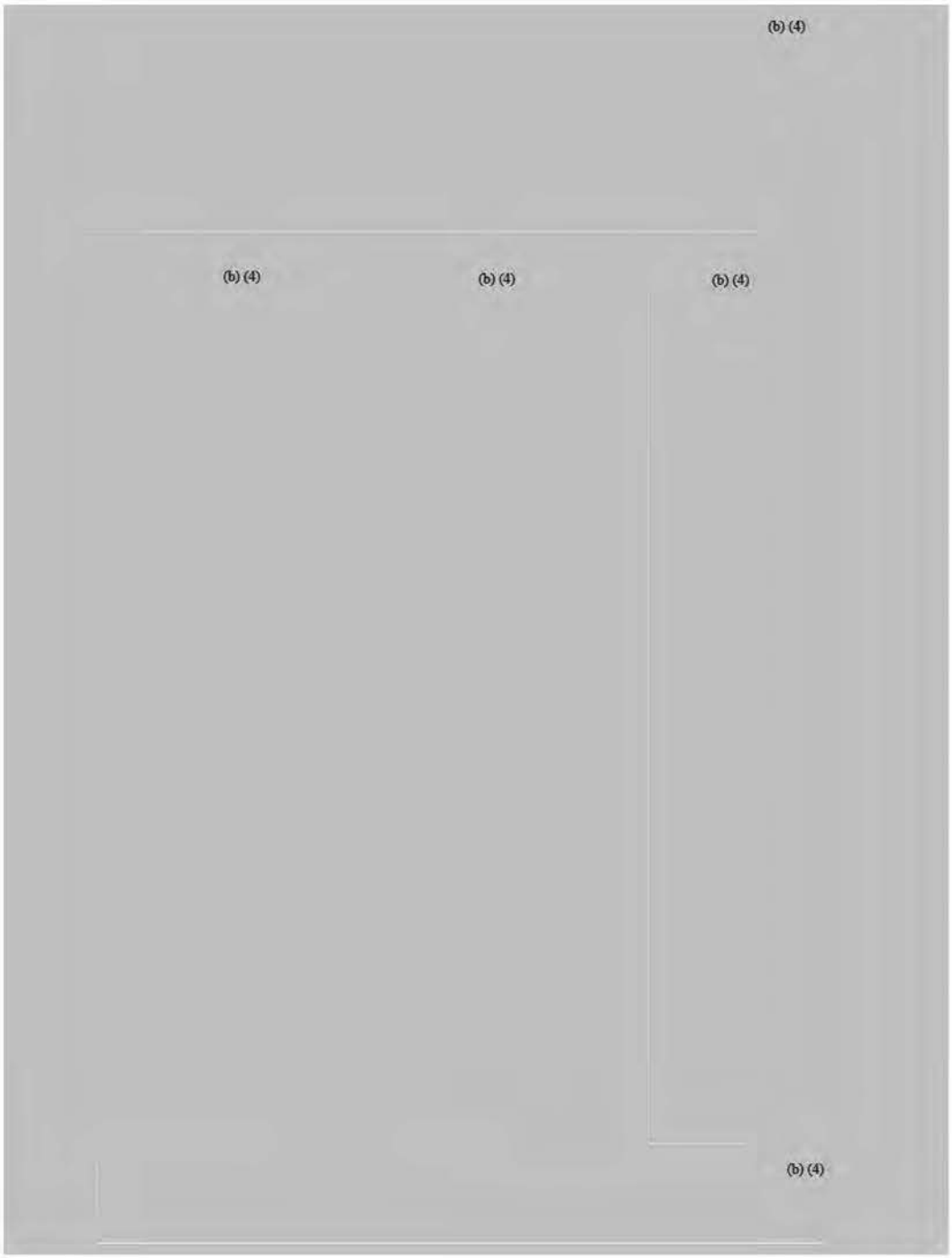

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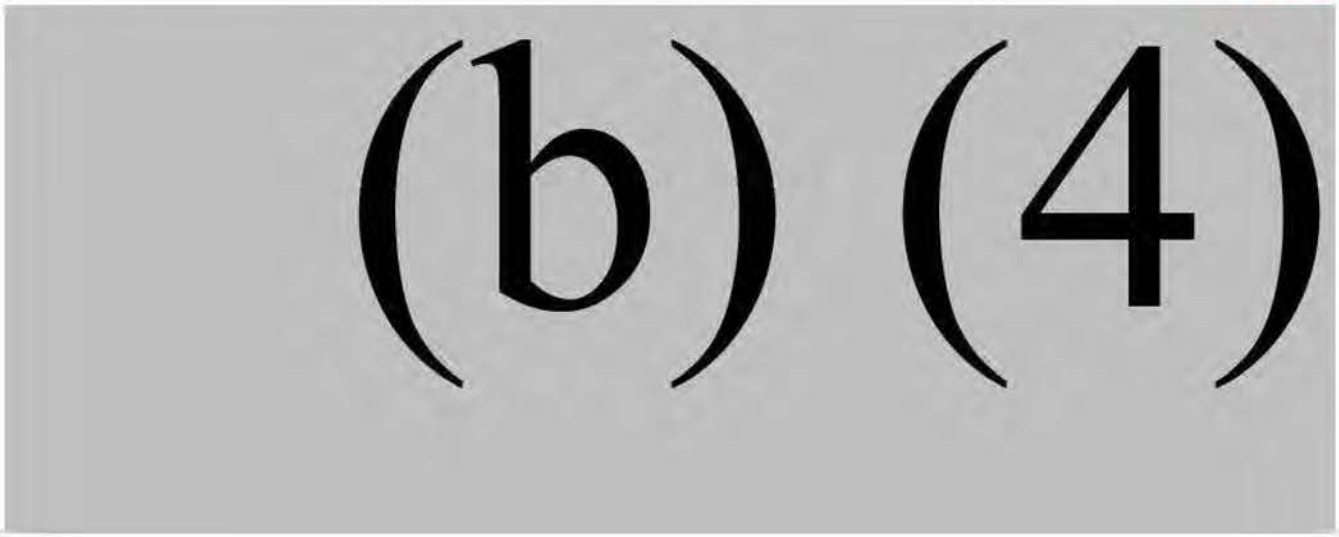

(b) (4)

REPORT

ID: 23567-3/2020

Stability of "Axtra® PHY GOLD 65 G" in pelleted feed for Swine, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs Manager:

Alessandra Tossoli

Signature: (b) (6) …… Date: 29/09/2020……..

Study scientist:

(b)(6)

Signature:… (b) (6) … Date: 27/09/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

Signature:… (b) (6) Date: 28/09/2020……..

(b) (4)

RESPONSIBLE PERSONNEL

Study Scientist(s)	:	[redacted]
Product manager	:	Arno de Kreij
Regulatory manager	:	Alessandra Tossoli

ARCHIVES

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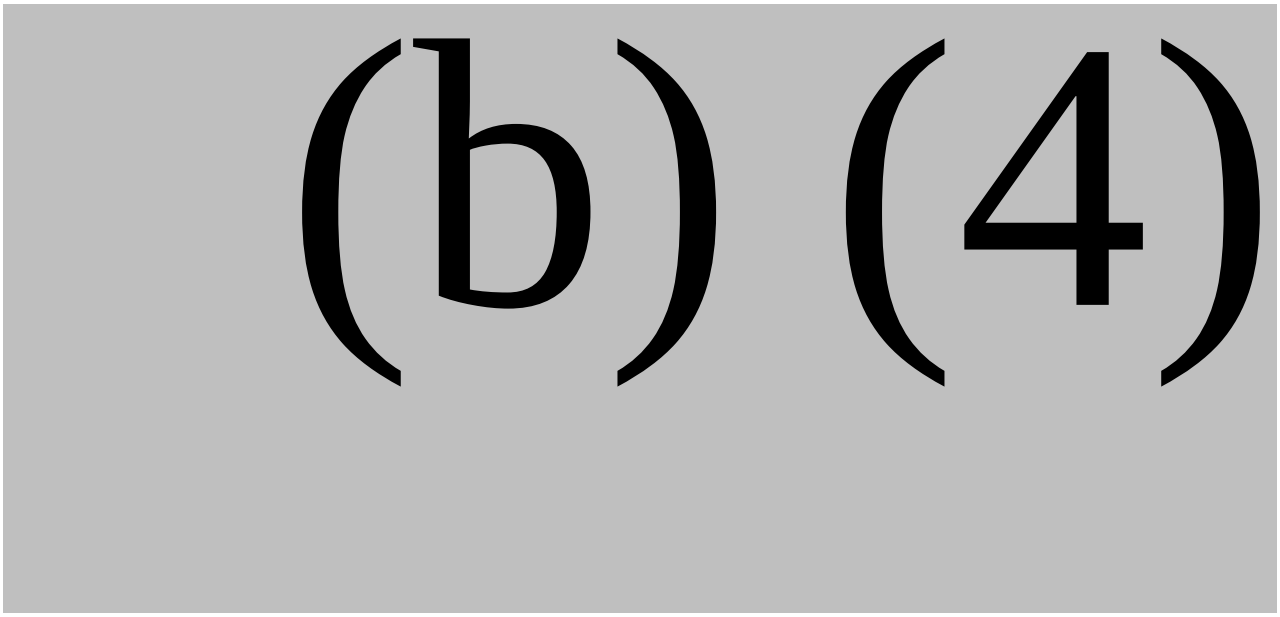
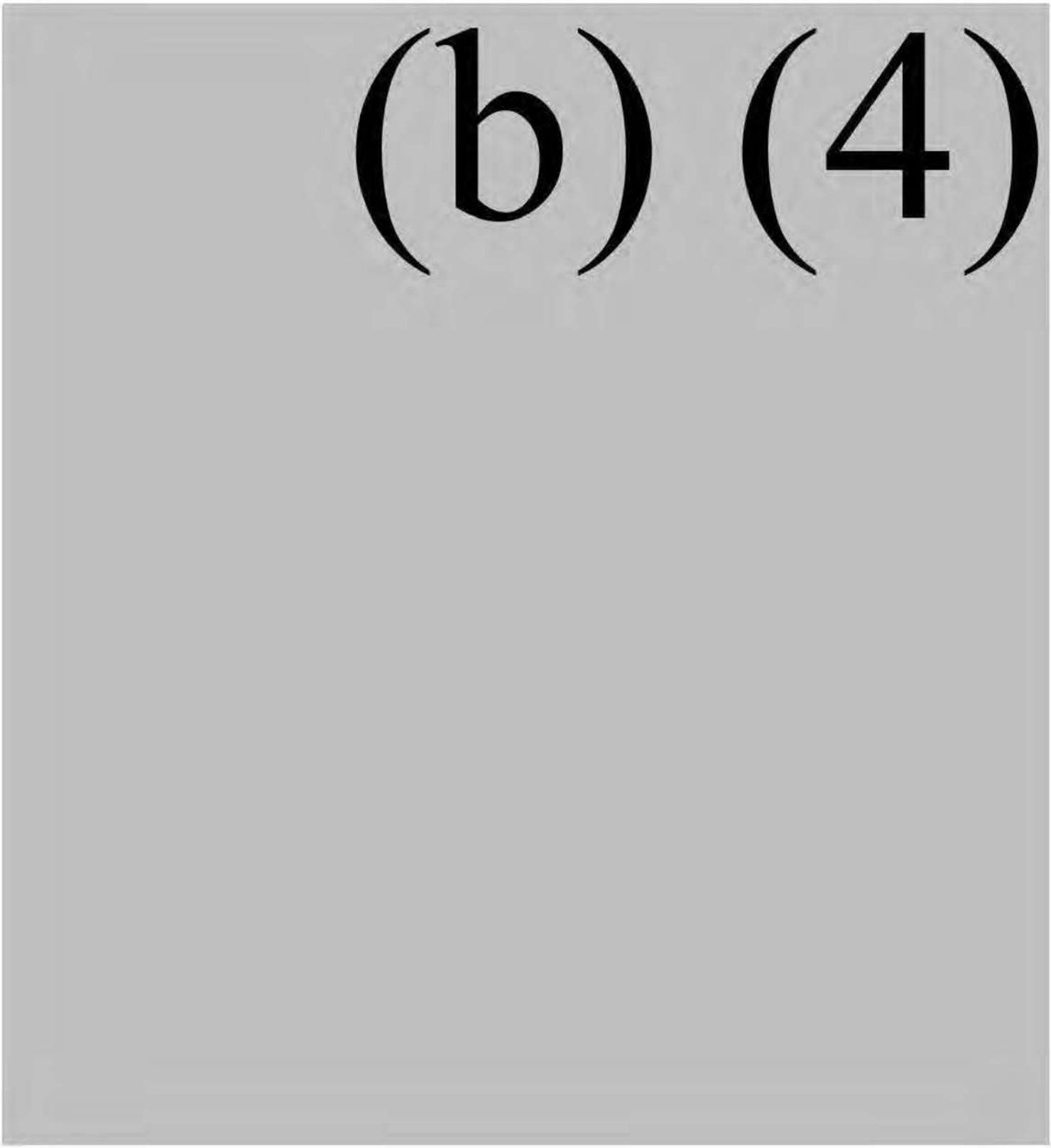
1. SUMMARY

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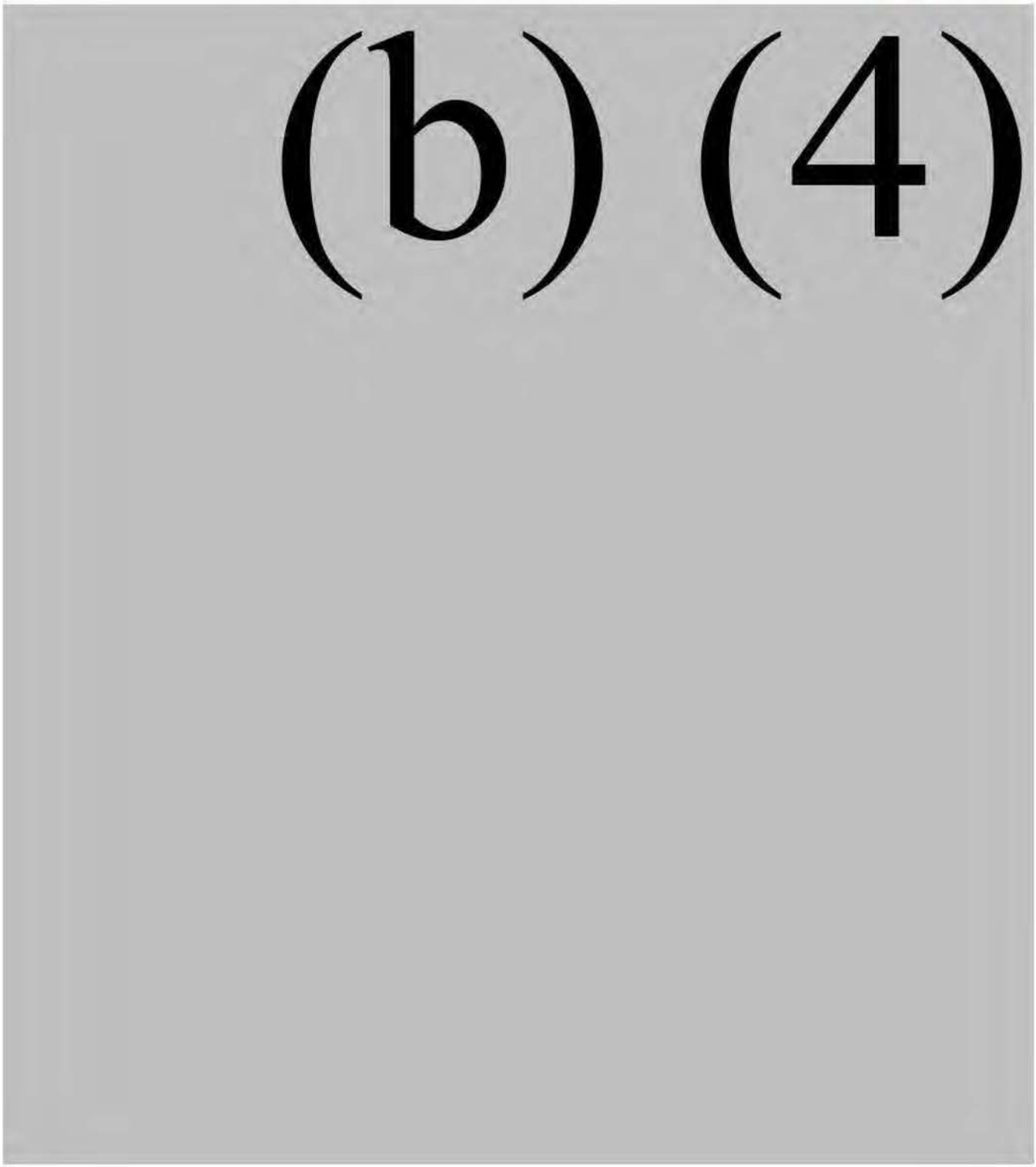

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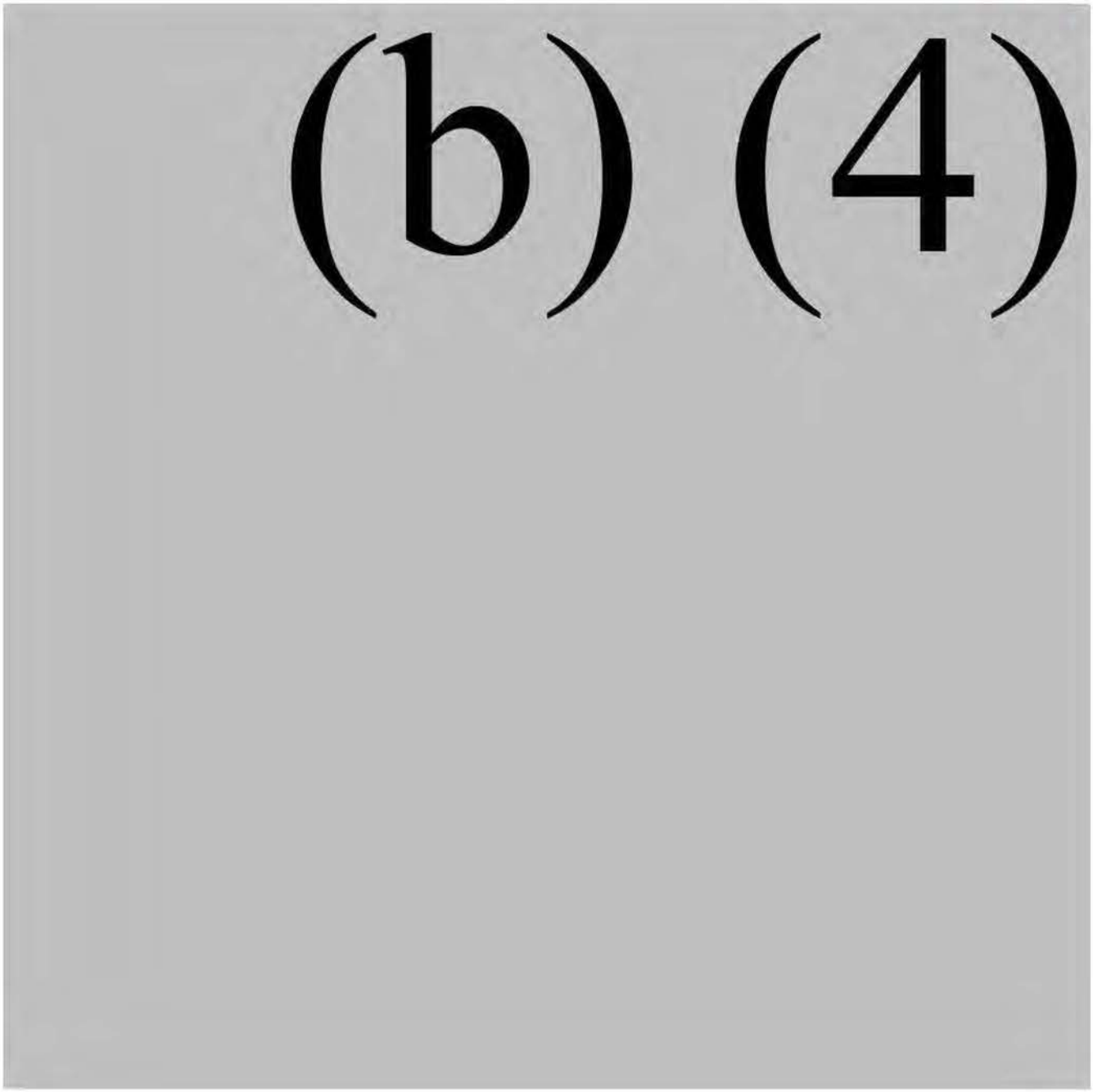

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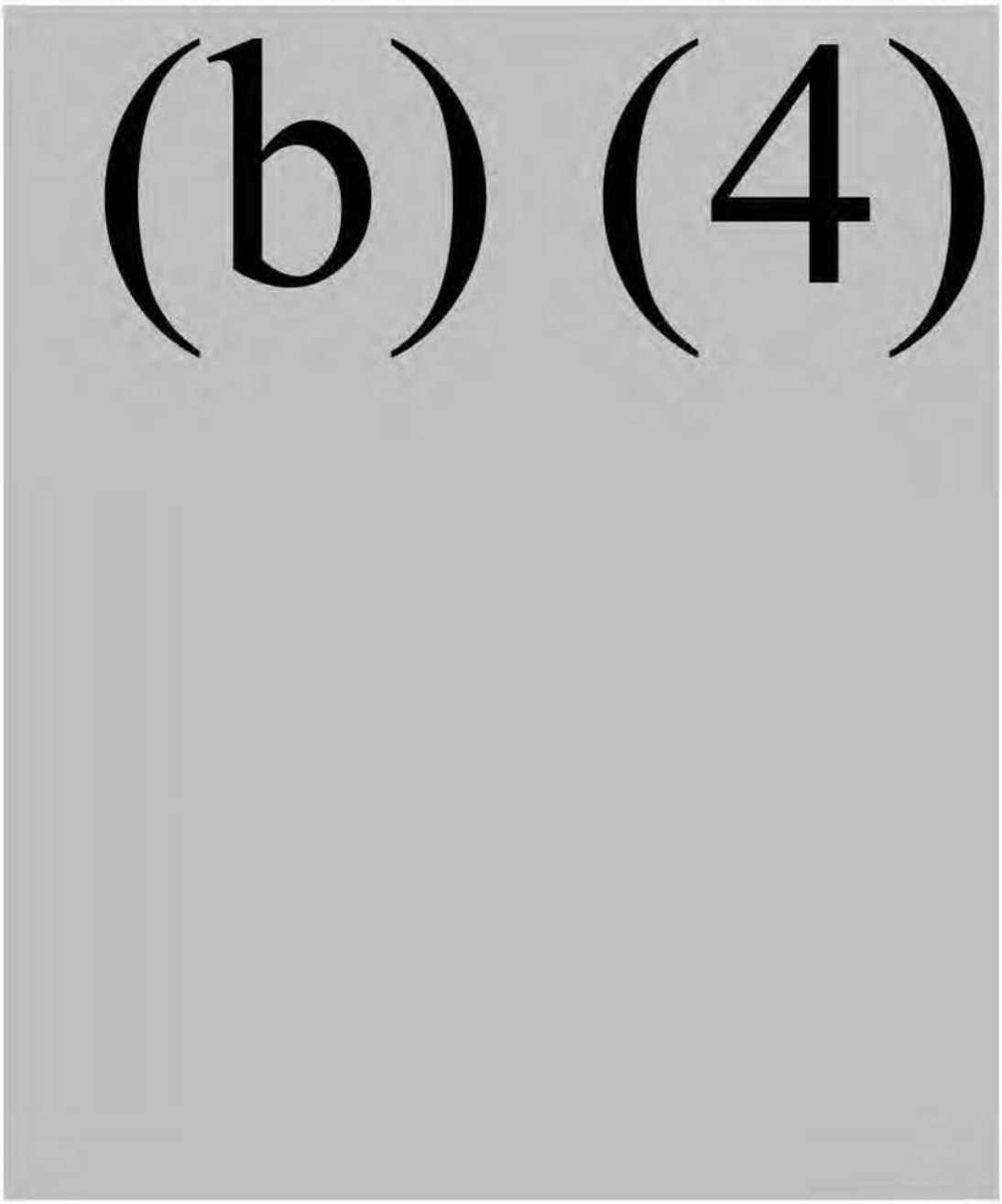
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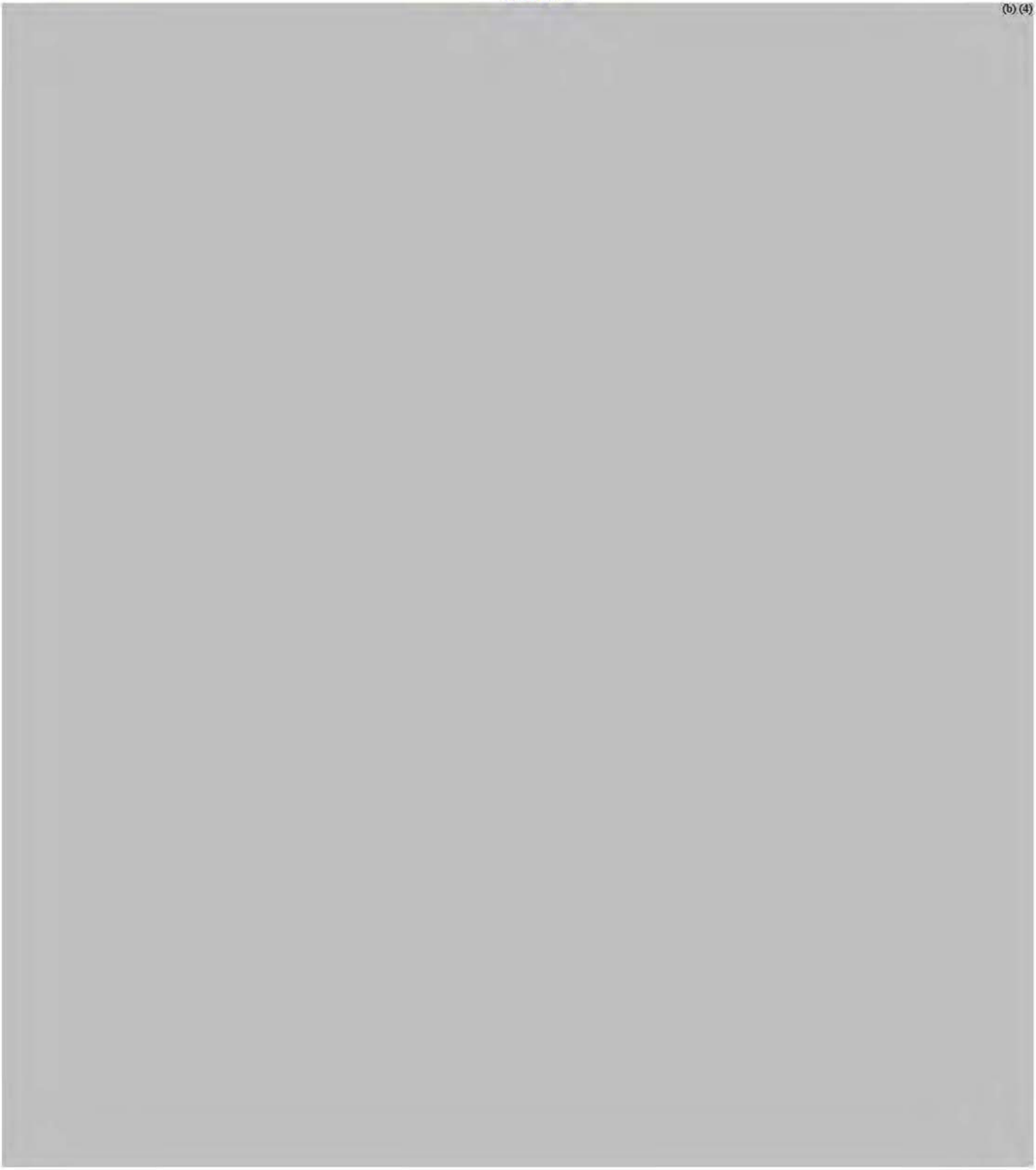

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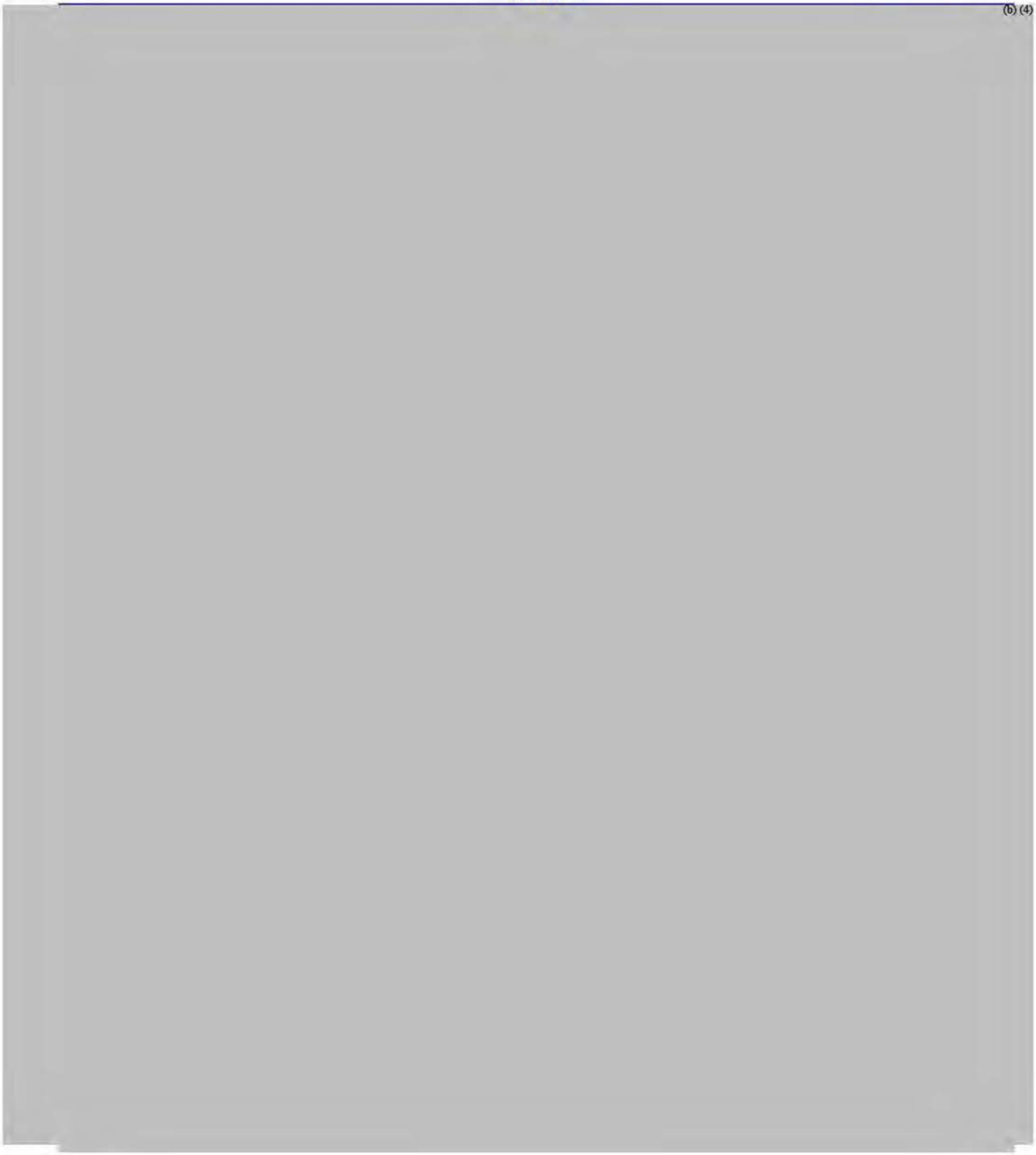

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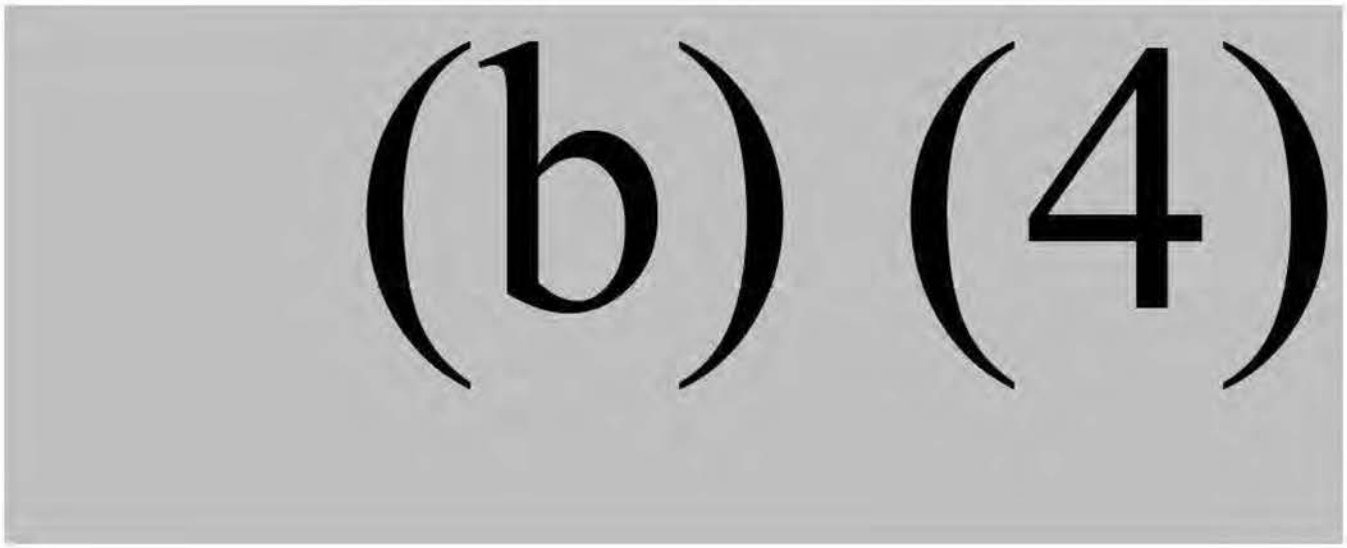

(b) (4)

REPORT

ID: 23567-1/2020

Stability of "Axtra® PHY GOLD 65 G" in pelleted feed for poultry, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs Manager:

Alessandra Tossoli

Signature: (b)(6)..... Date: 29/09/2020.....

Study scientist:

(b)(6)

Signature:... (b)(6) Date:27/09/2020.....

This report is approved by:

Product Manager:

Arno de Kreij

Signature:... (b)(6) Date:28/09/2020.....

RESPONSIBLE PERSONNEL

Study Scientist(s)	:	[redacted]
Product manager	:	Arno de Kreij
Regulatory manager	:	Alessandra Tossoli

ARCHIVES

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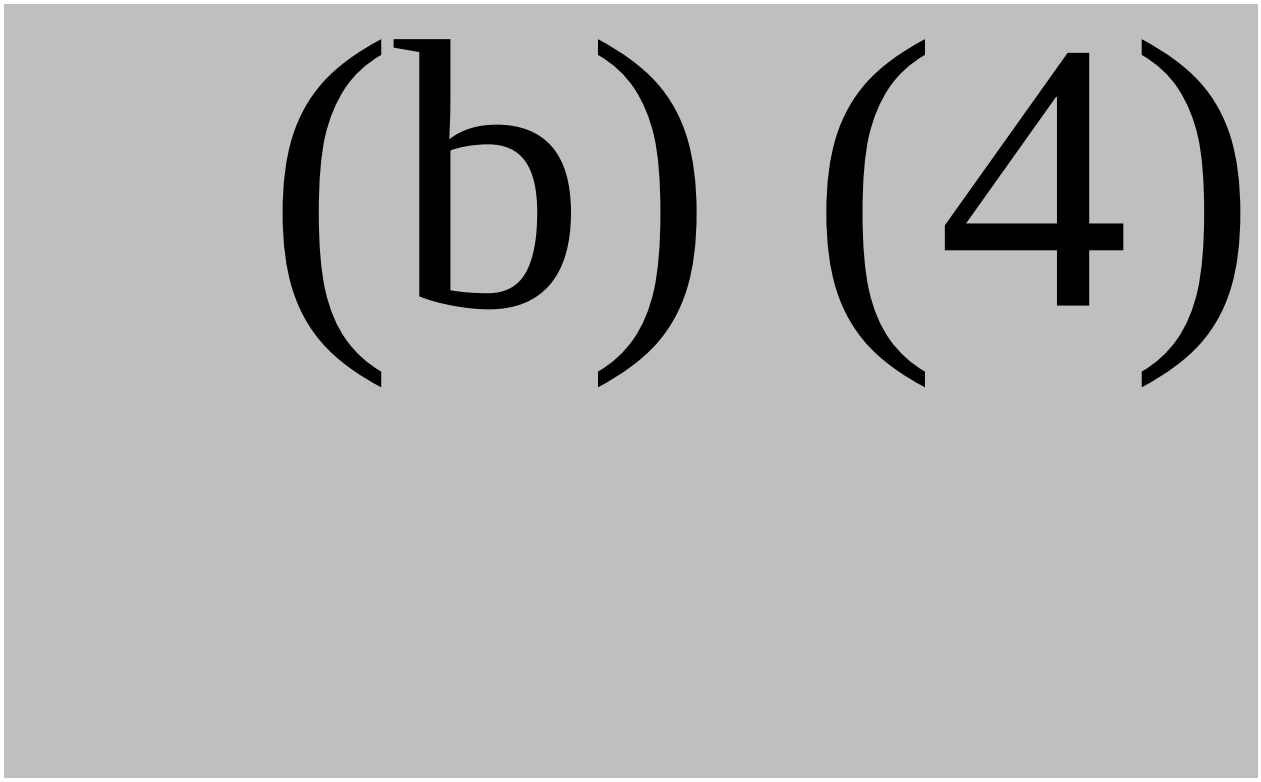
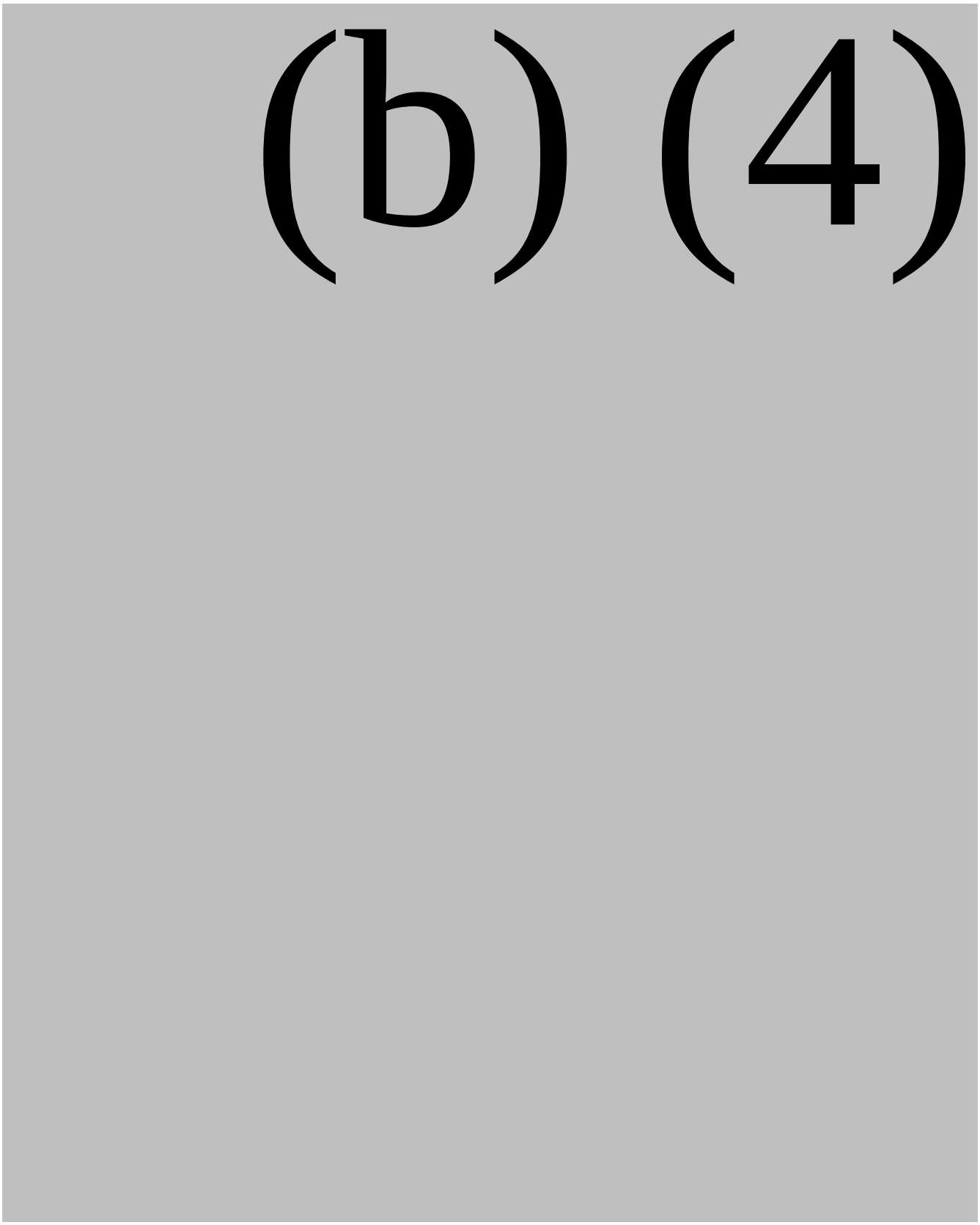
1. SUMMARY

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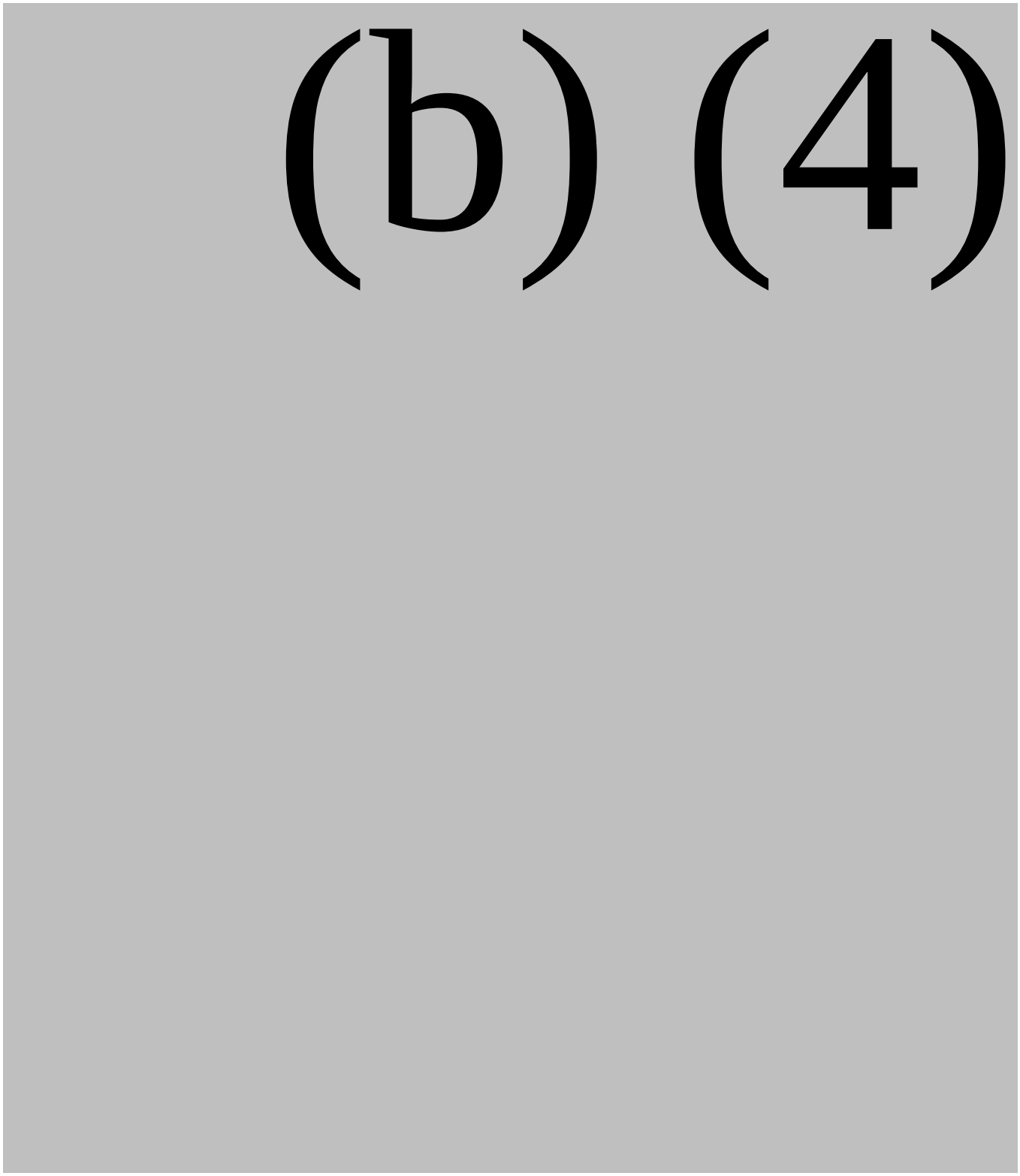
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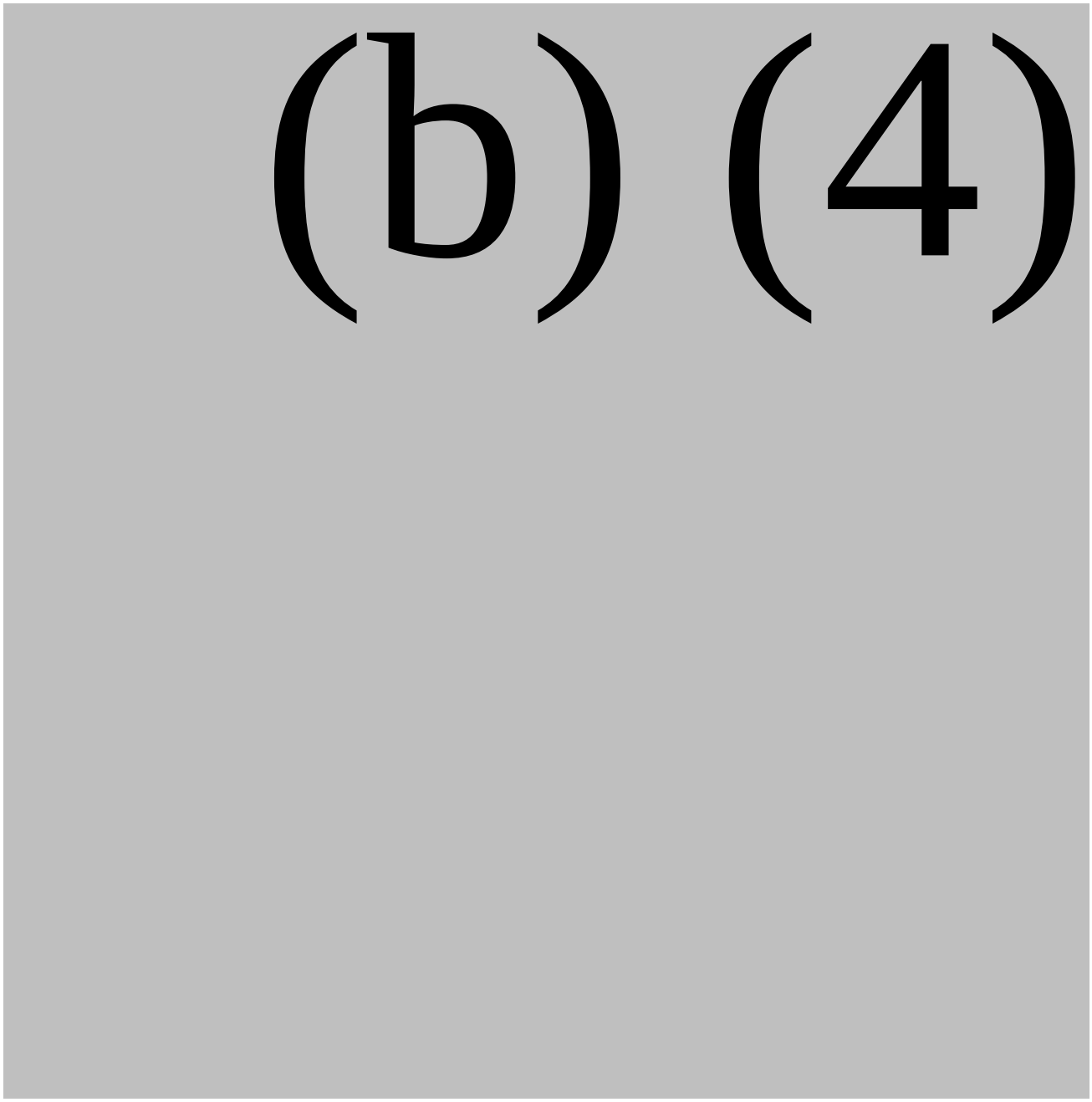
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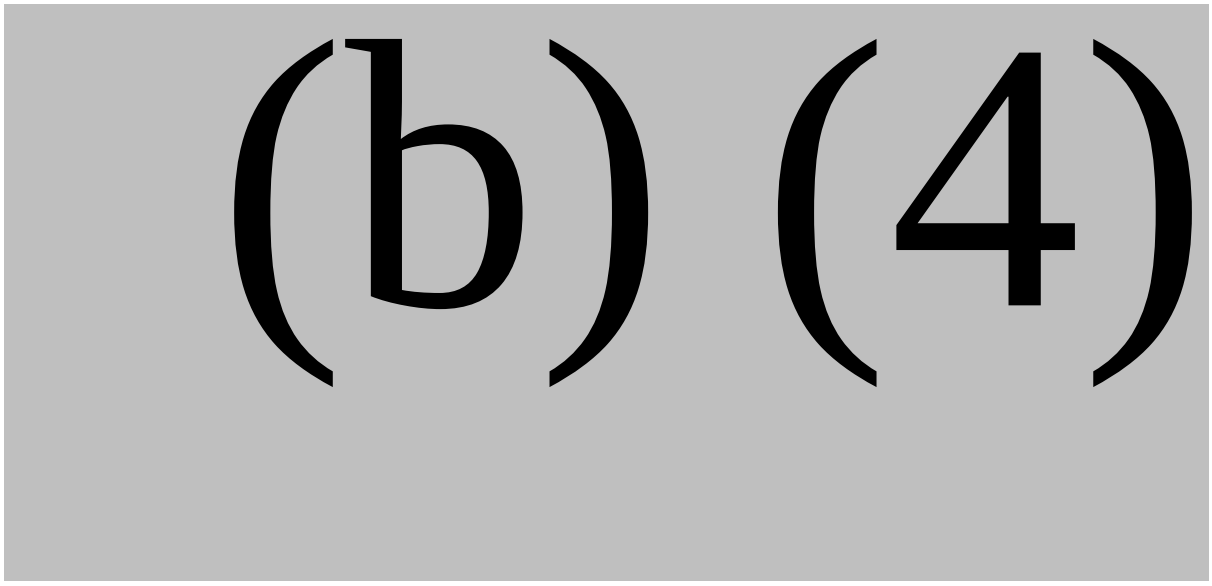
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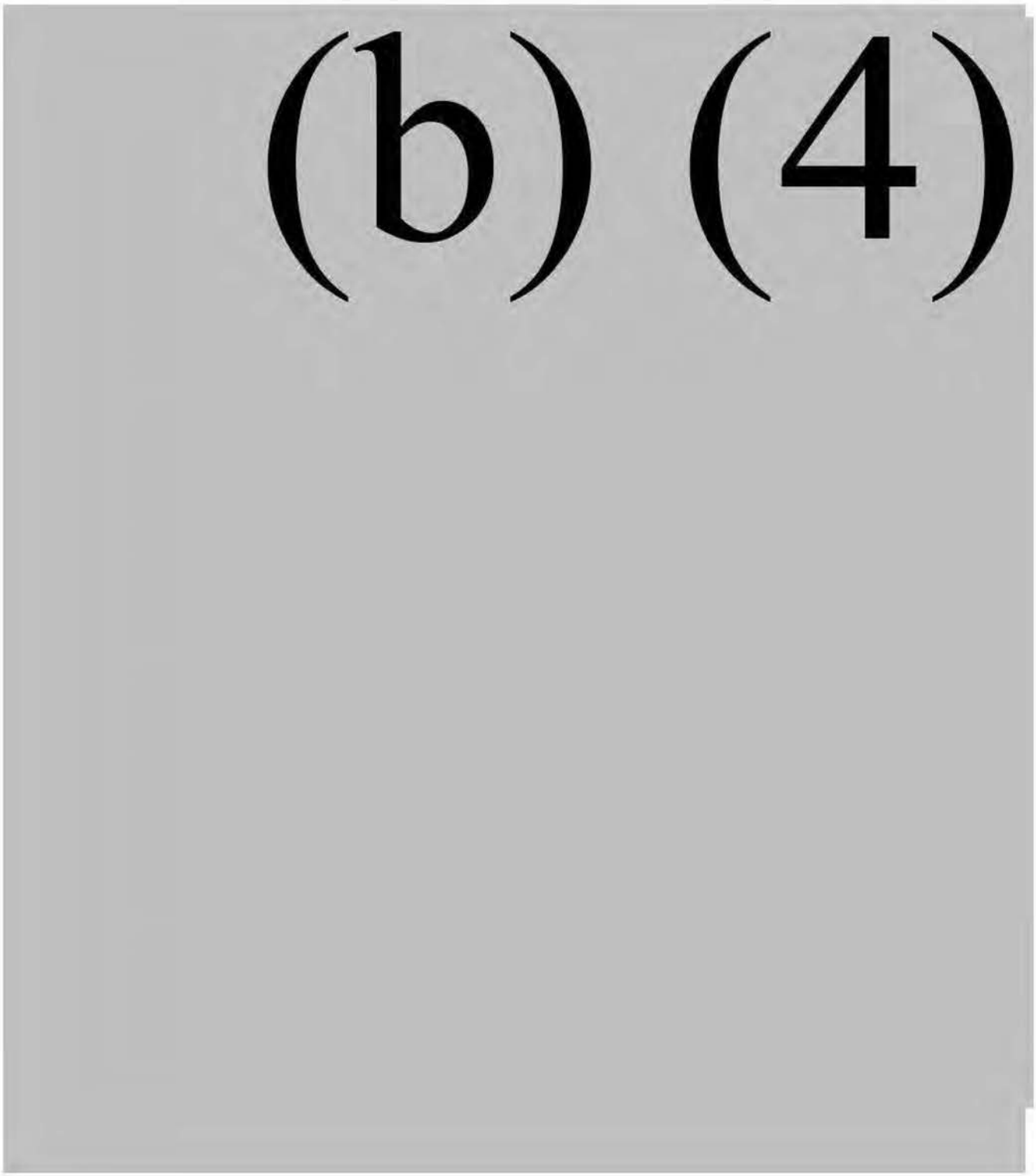

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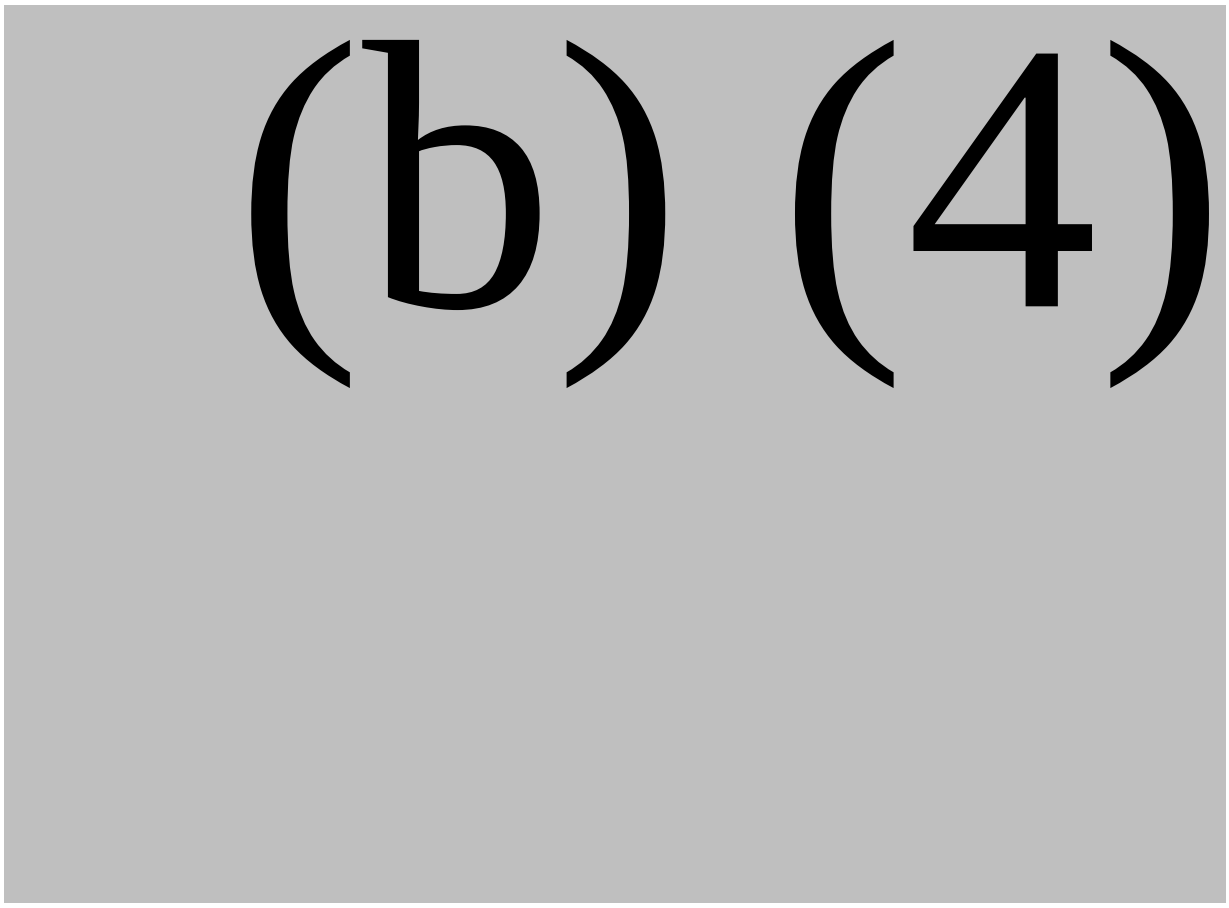
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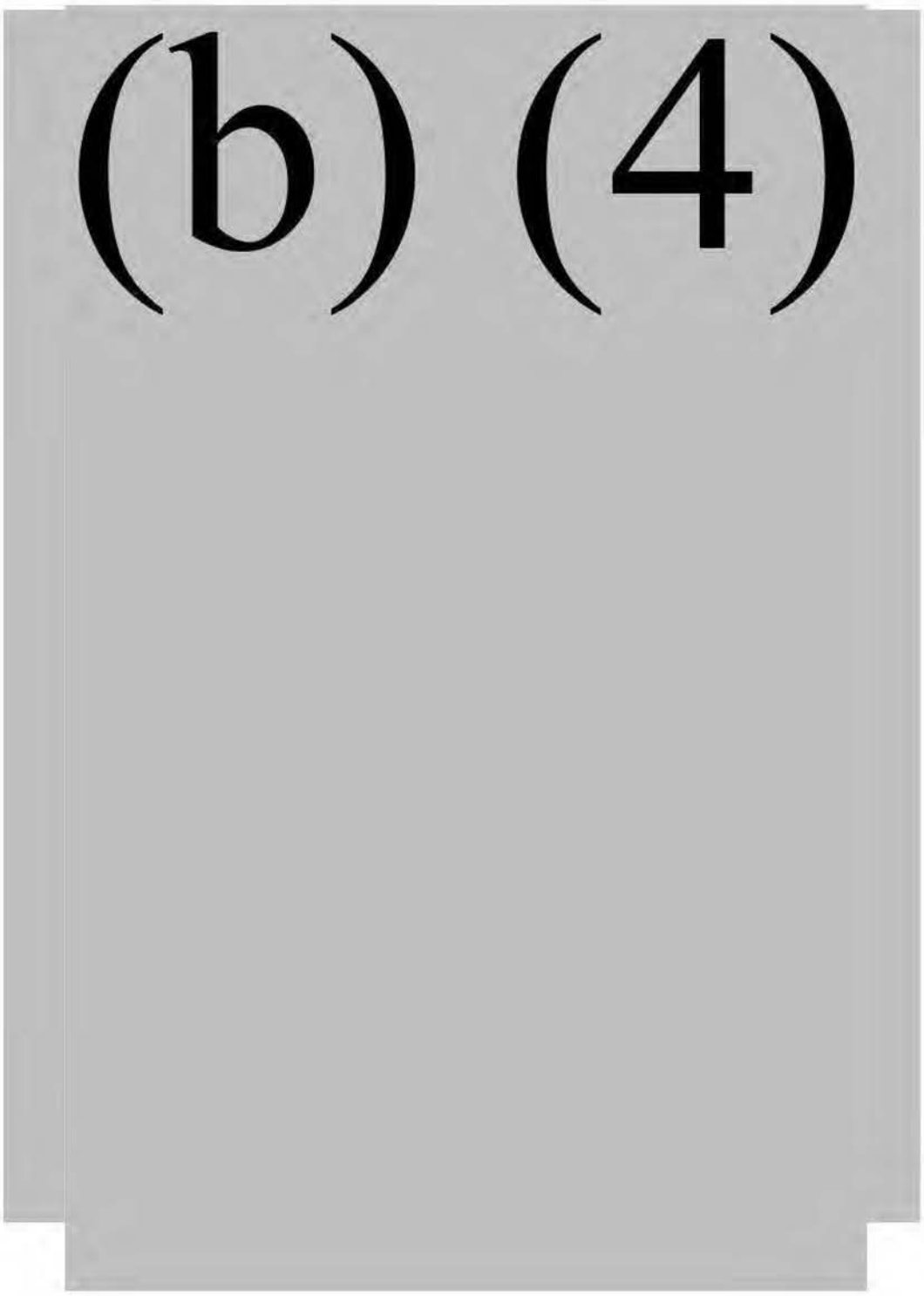

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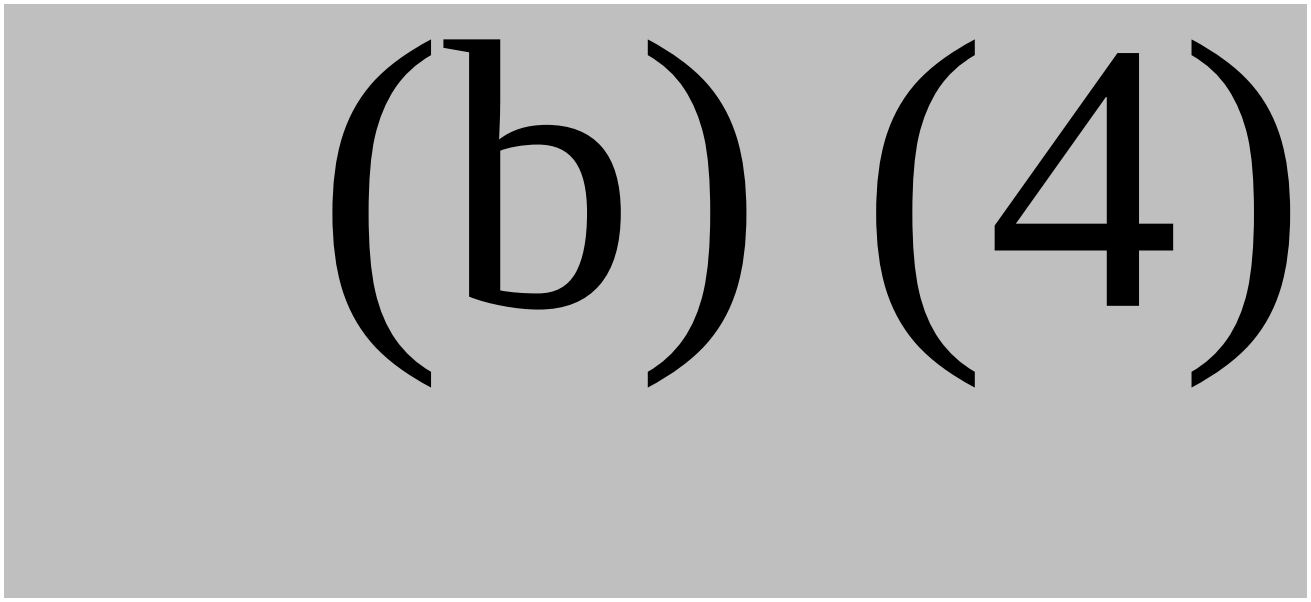


(b) (4)

REPORT

ID: 23566-4/2020

Homogeneity and Stability of "Axtra® PHY GOLD 30 T" in mash feed for Swine, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

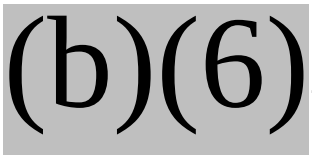
There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

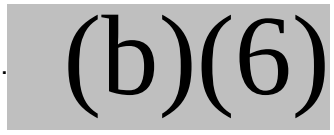
Regulatory Affairs:

Alessandra Tossoli

Signature:  … Date: 5/10/2020……..

Study scientist:

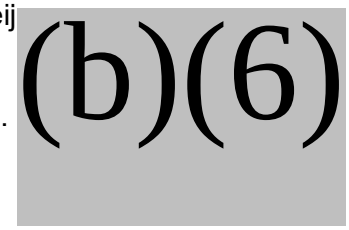
(b)(6)

Signature:…  Date: 5/10/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

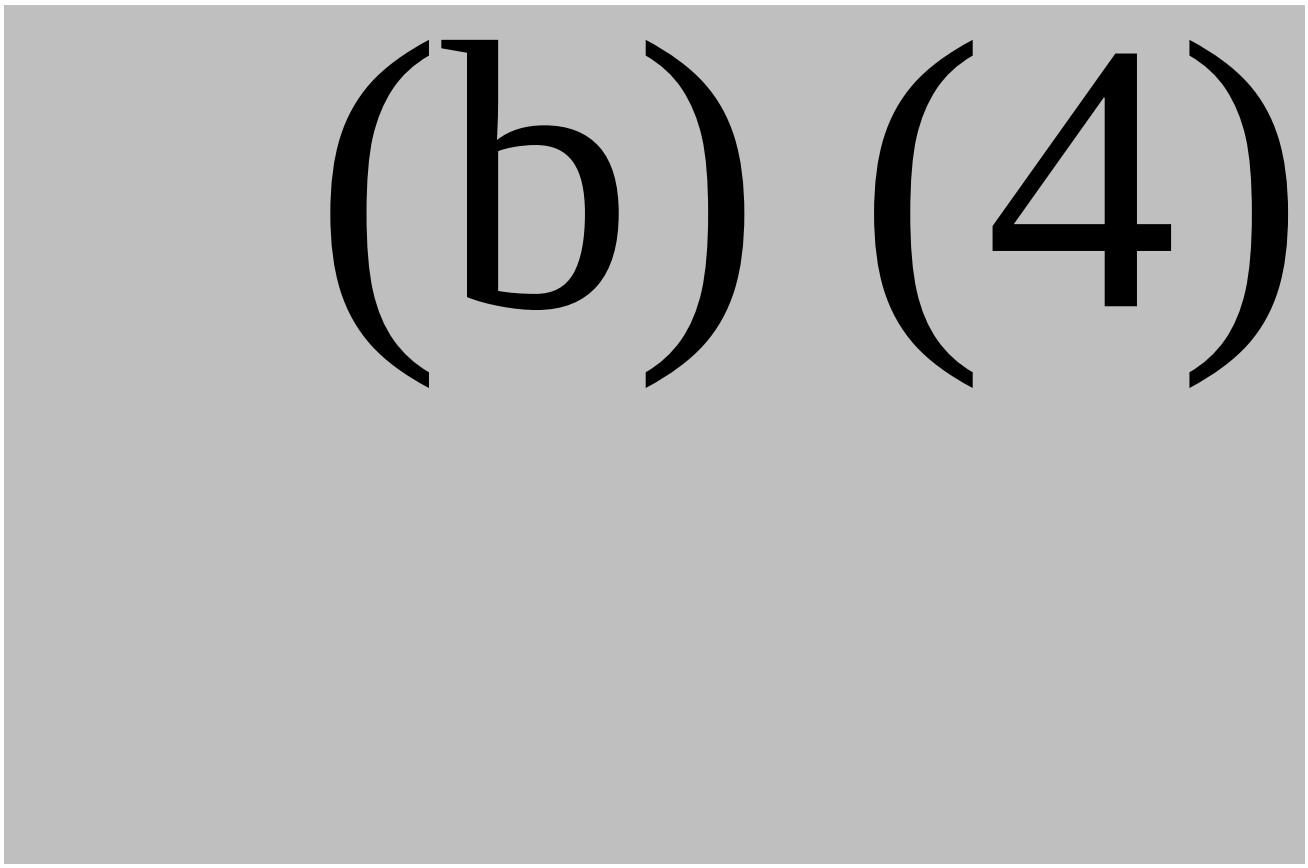
Signature:…  Date: 5/10/2020……..

RESPONSIBLE PERSONNEL

Study scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.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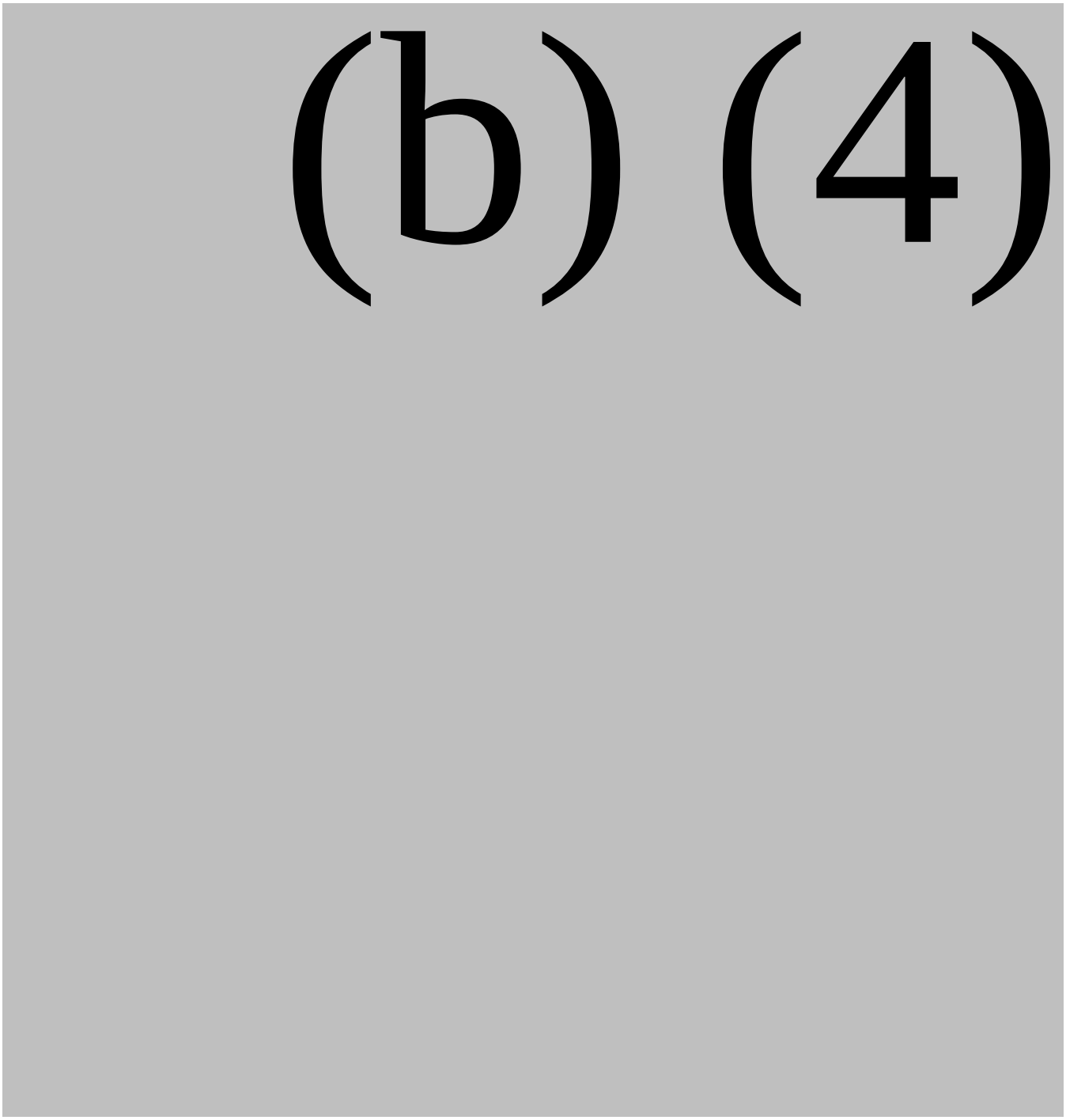
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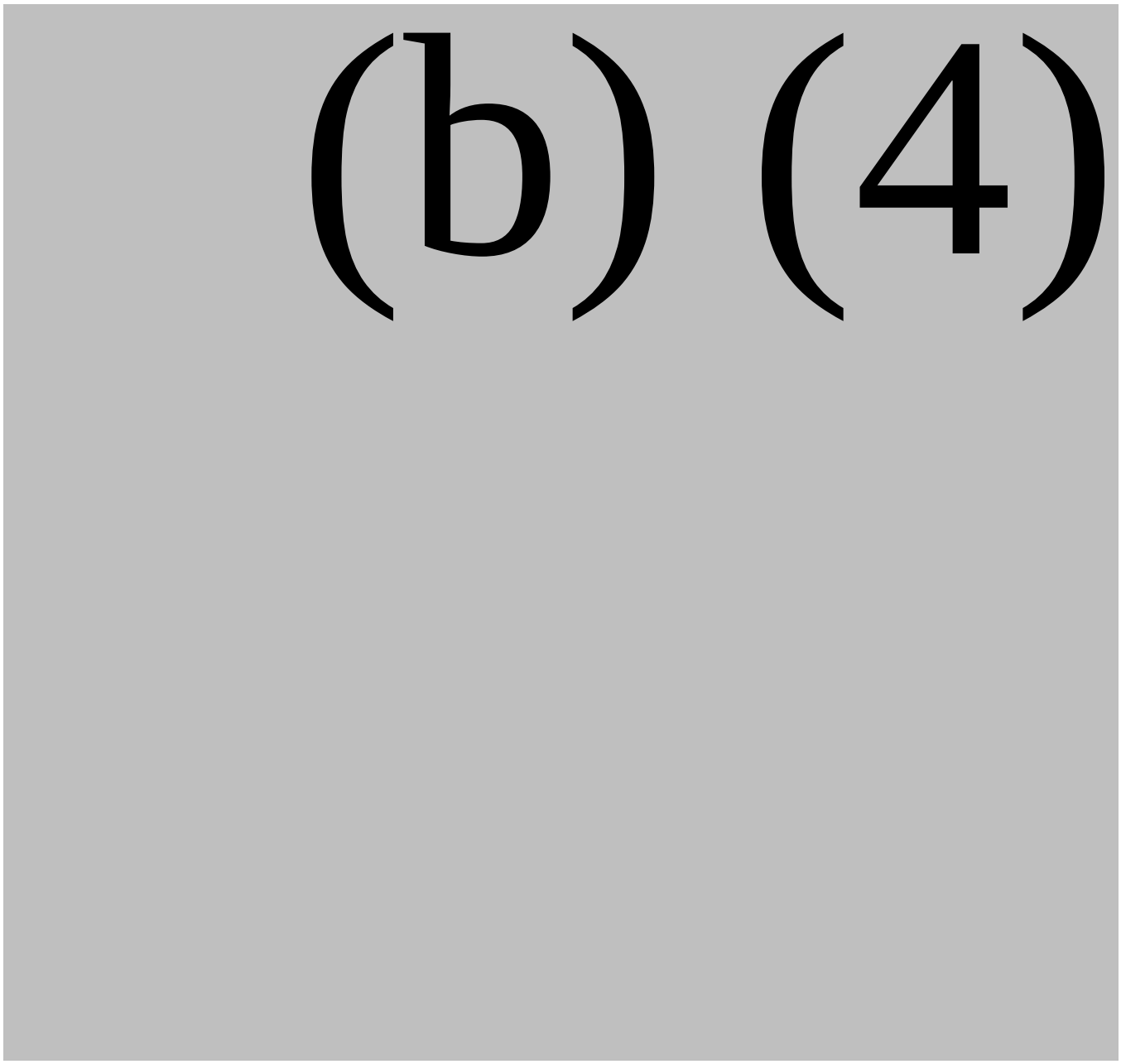

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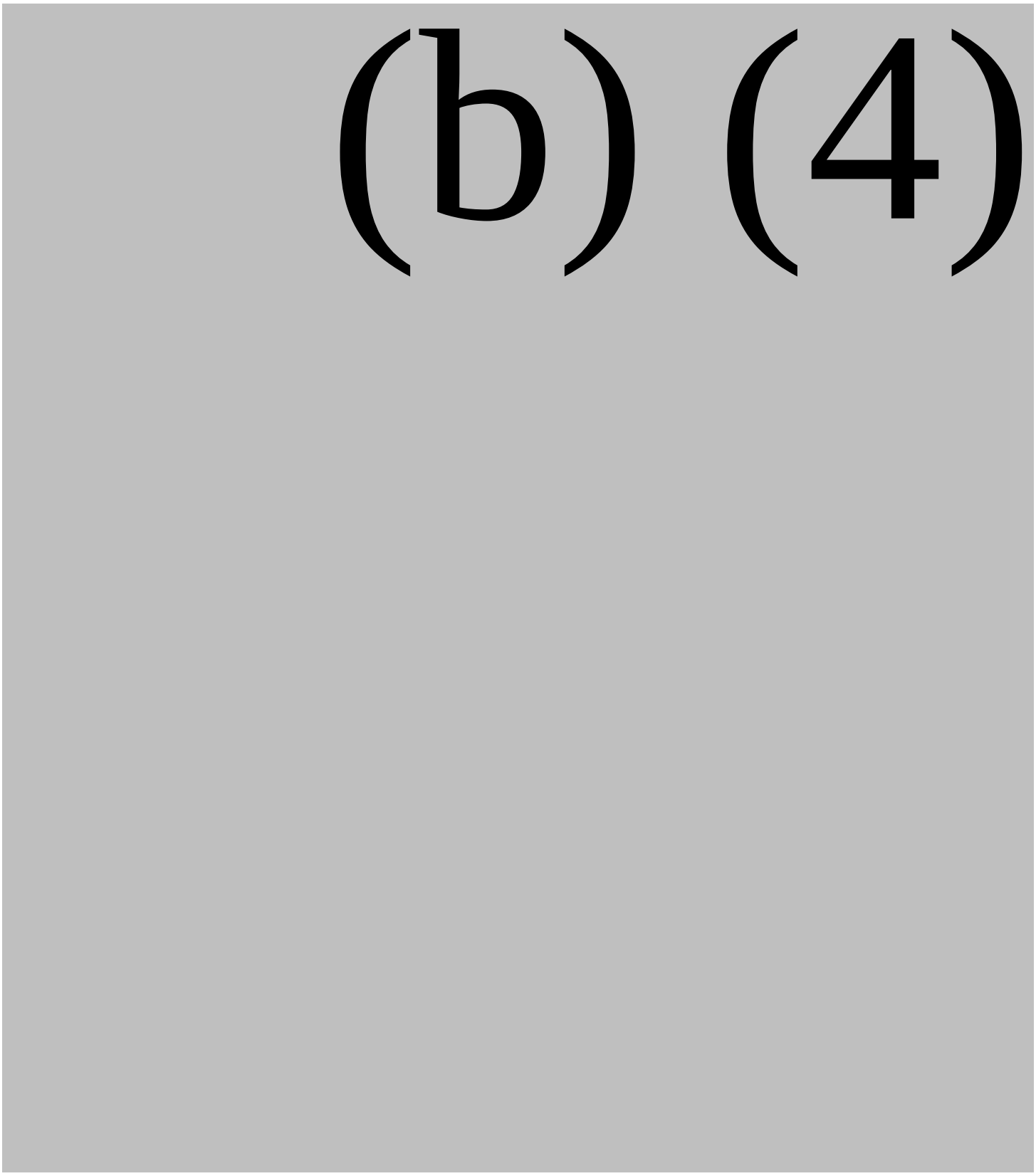
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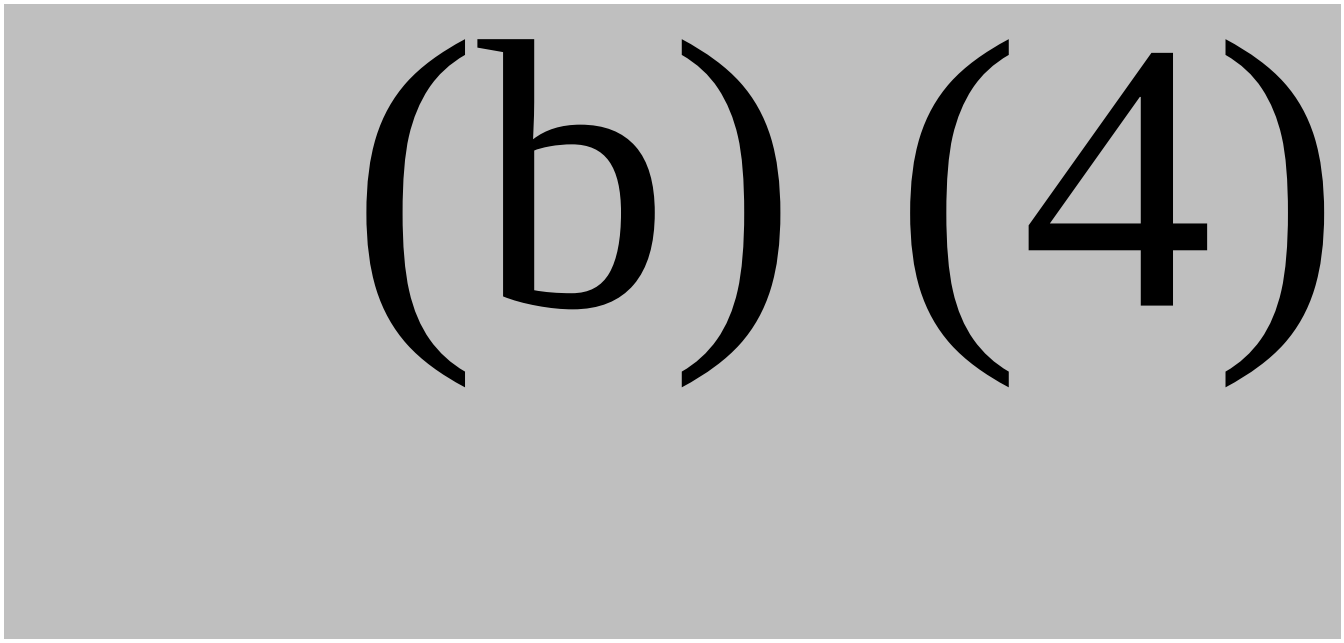

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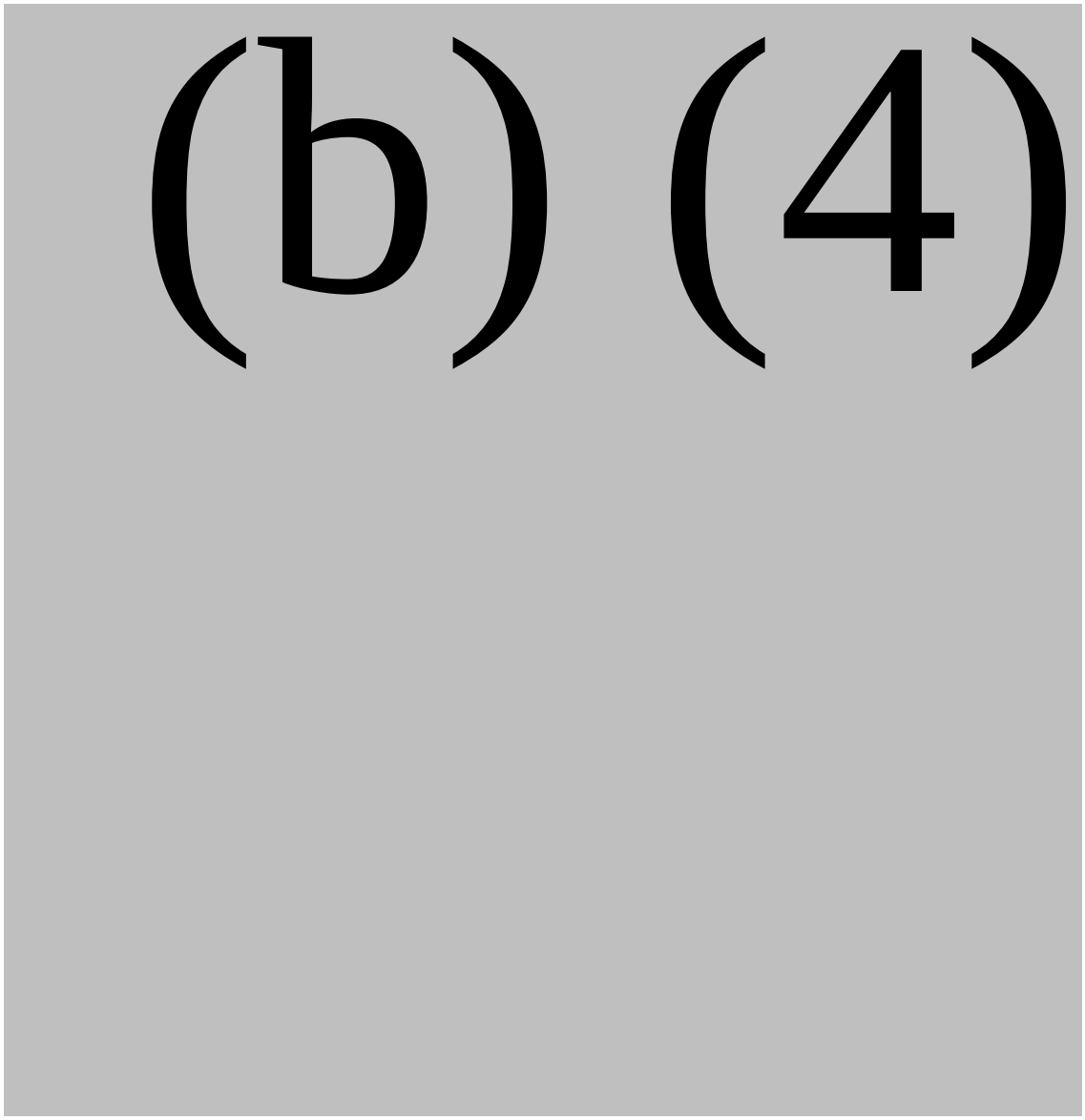

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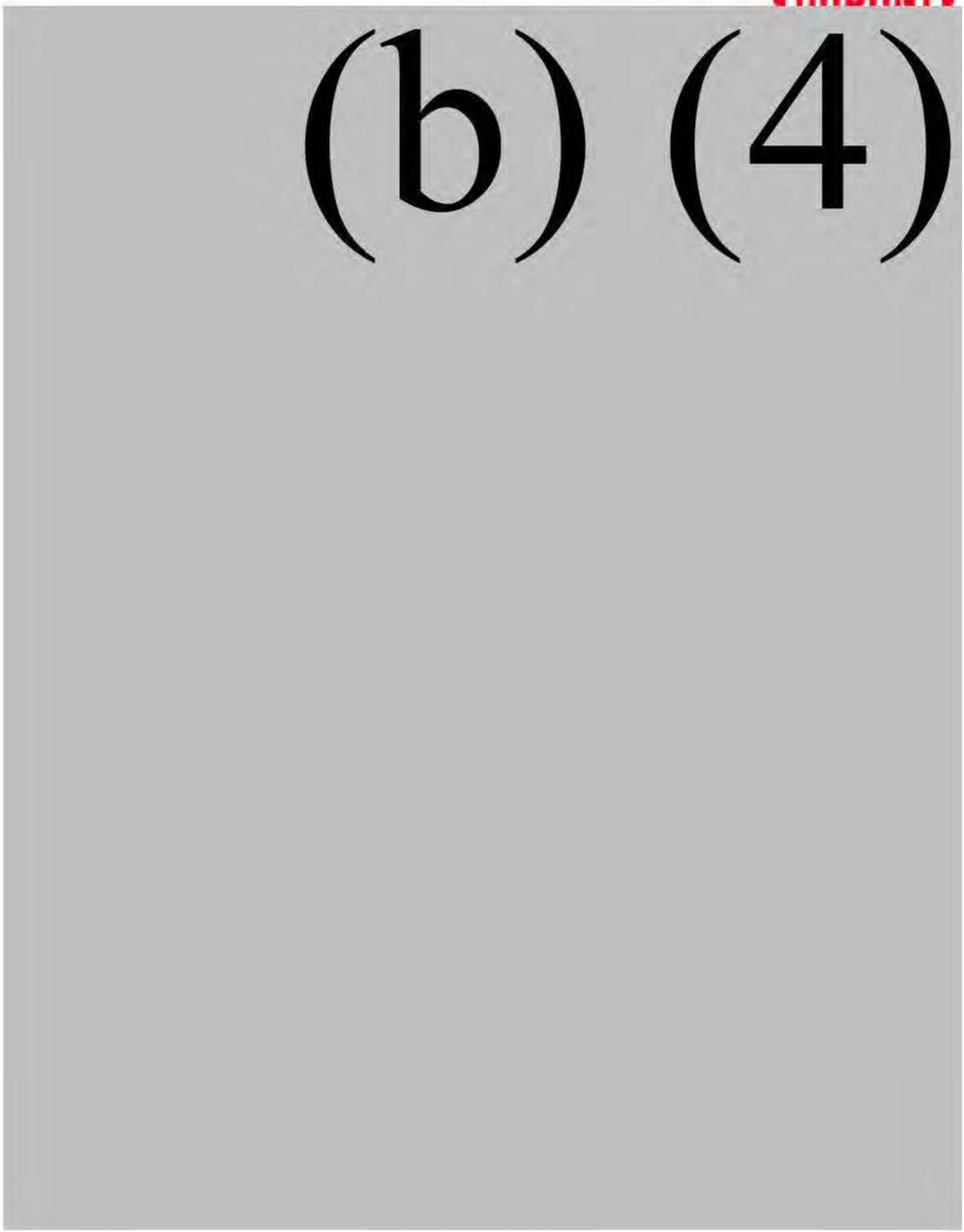

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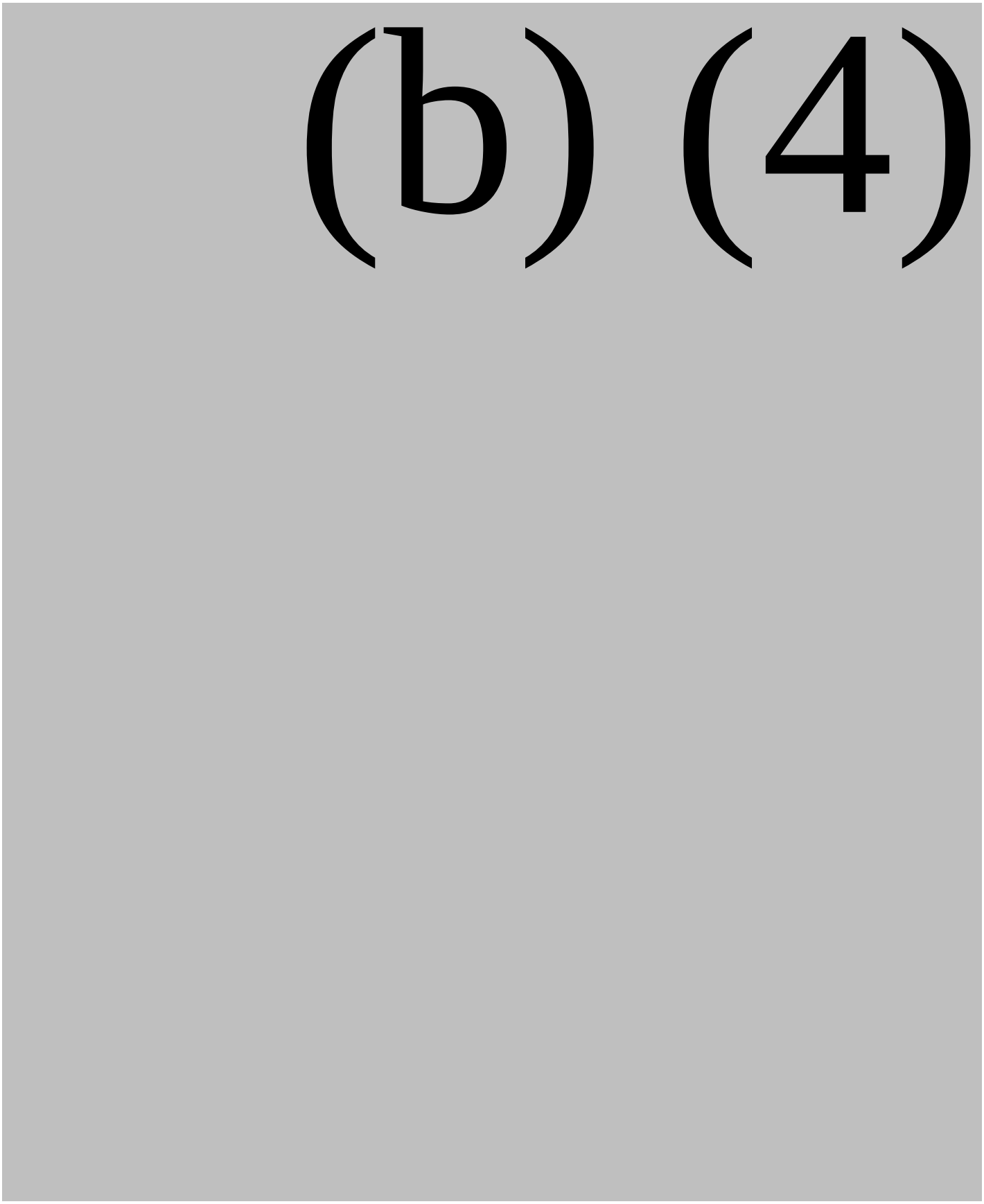
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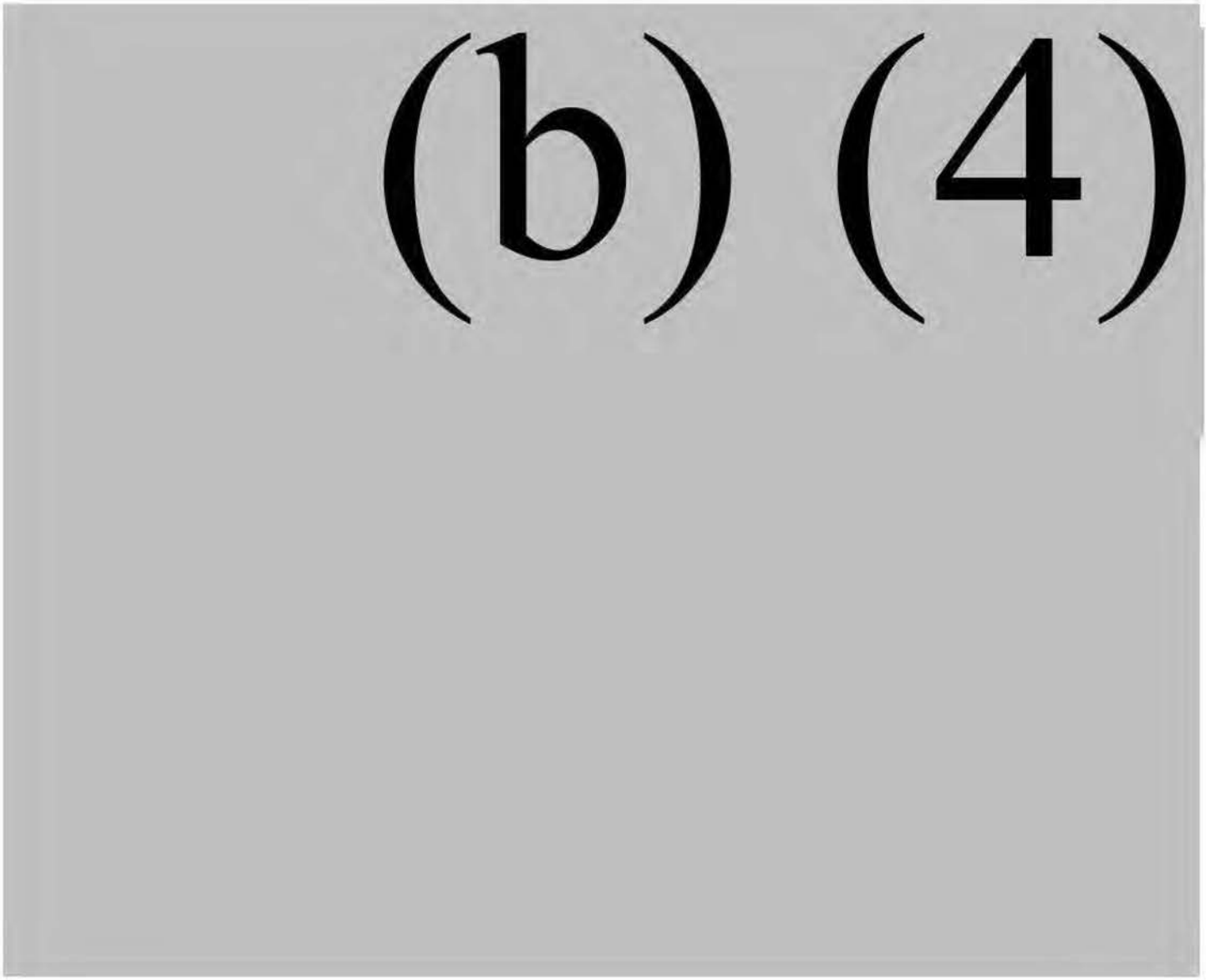


REPORT

ID: 23566-2/2020

Homogeneity and Stability of "Axtra® PHY GOLD 30 T" in mash feed for Poultry, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature: (b)(6) …… Date: 5/10/2020

Study Scientist:

(b)(6)

Signature:… (b)(6) Date: 5/10/2020

This report is approved by:

Product Manager:

Arno de Kreij

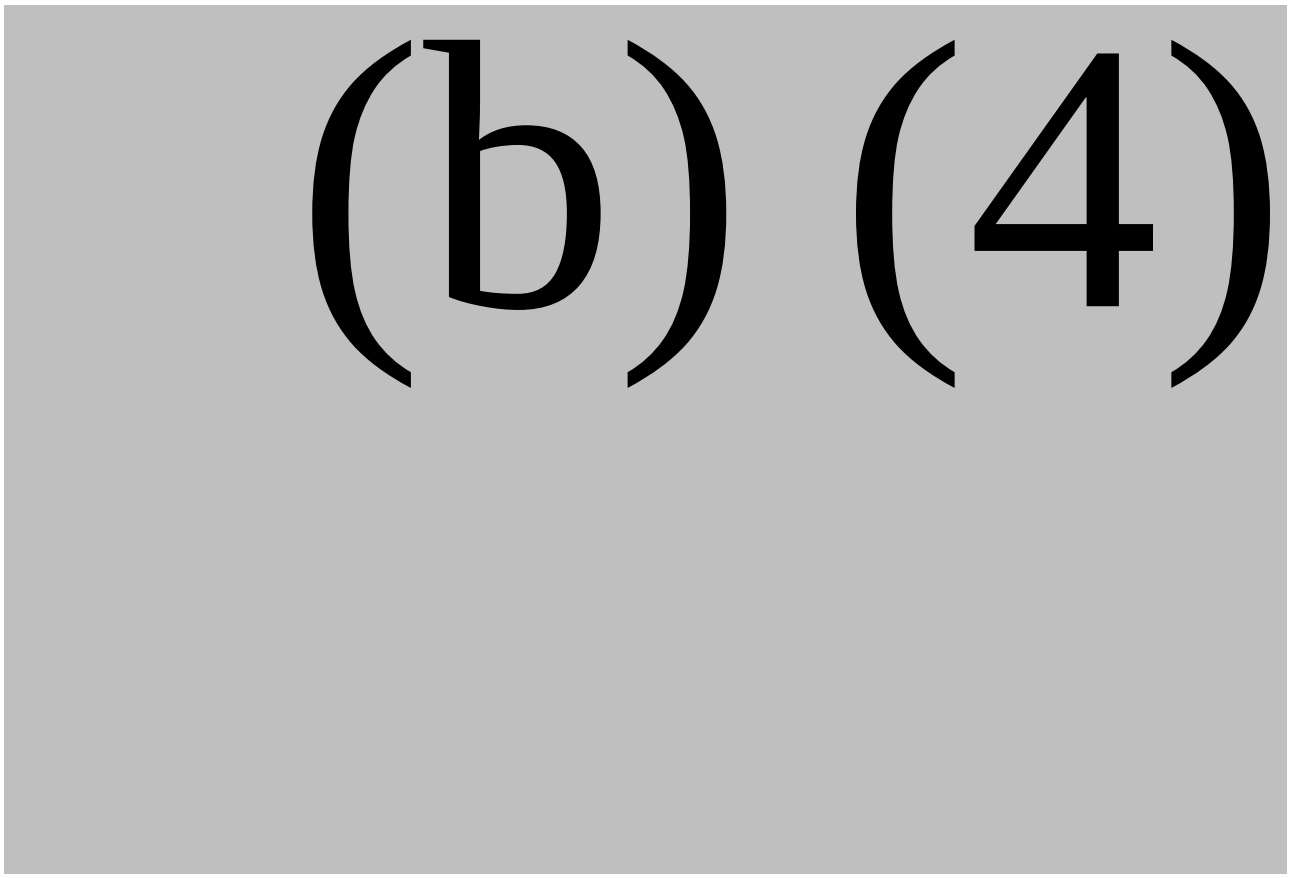
Signature:… (b)(6) Date: 5/10/2020

RESPONSIBLE PERSONNEL

Study scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.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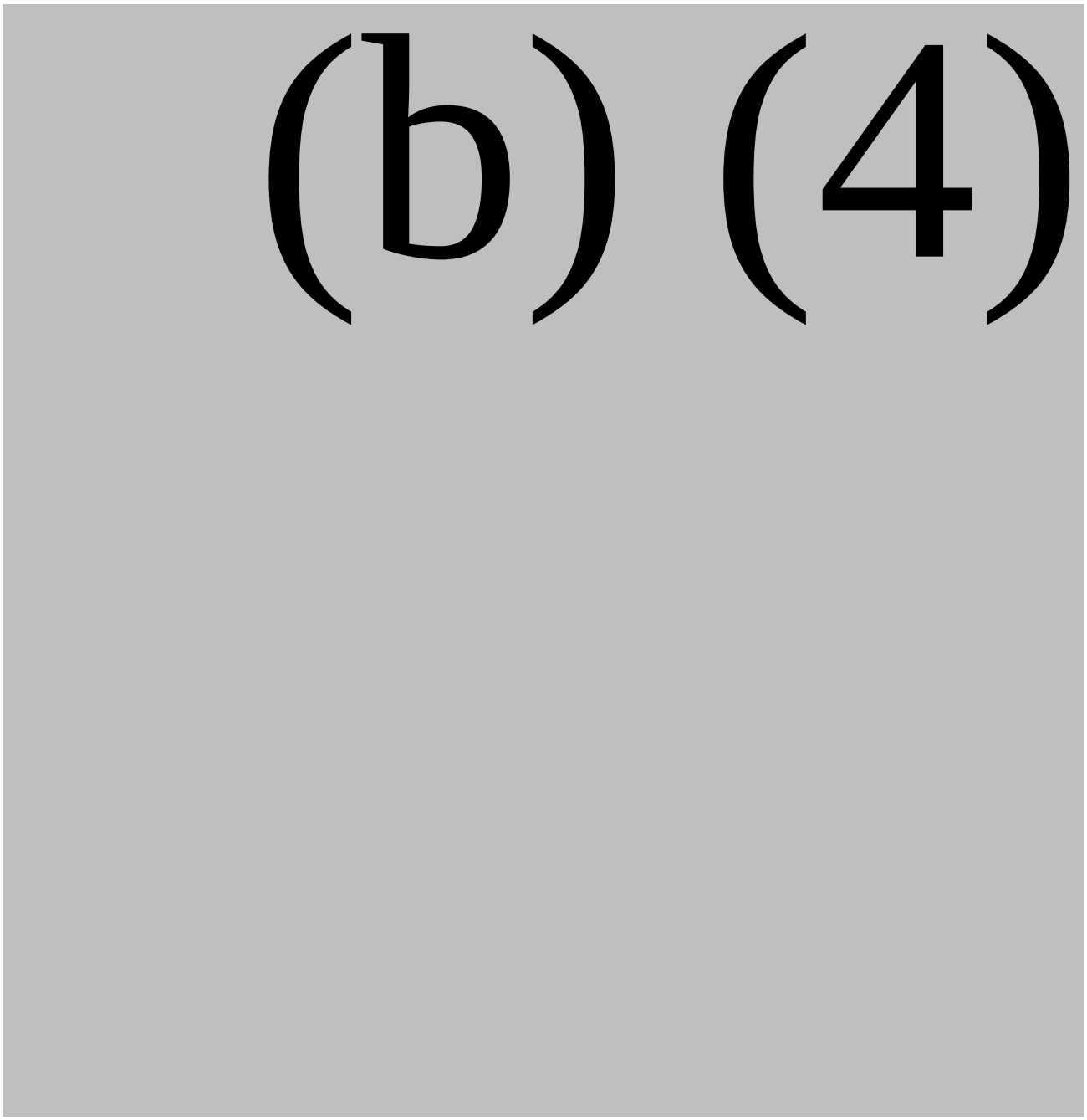
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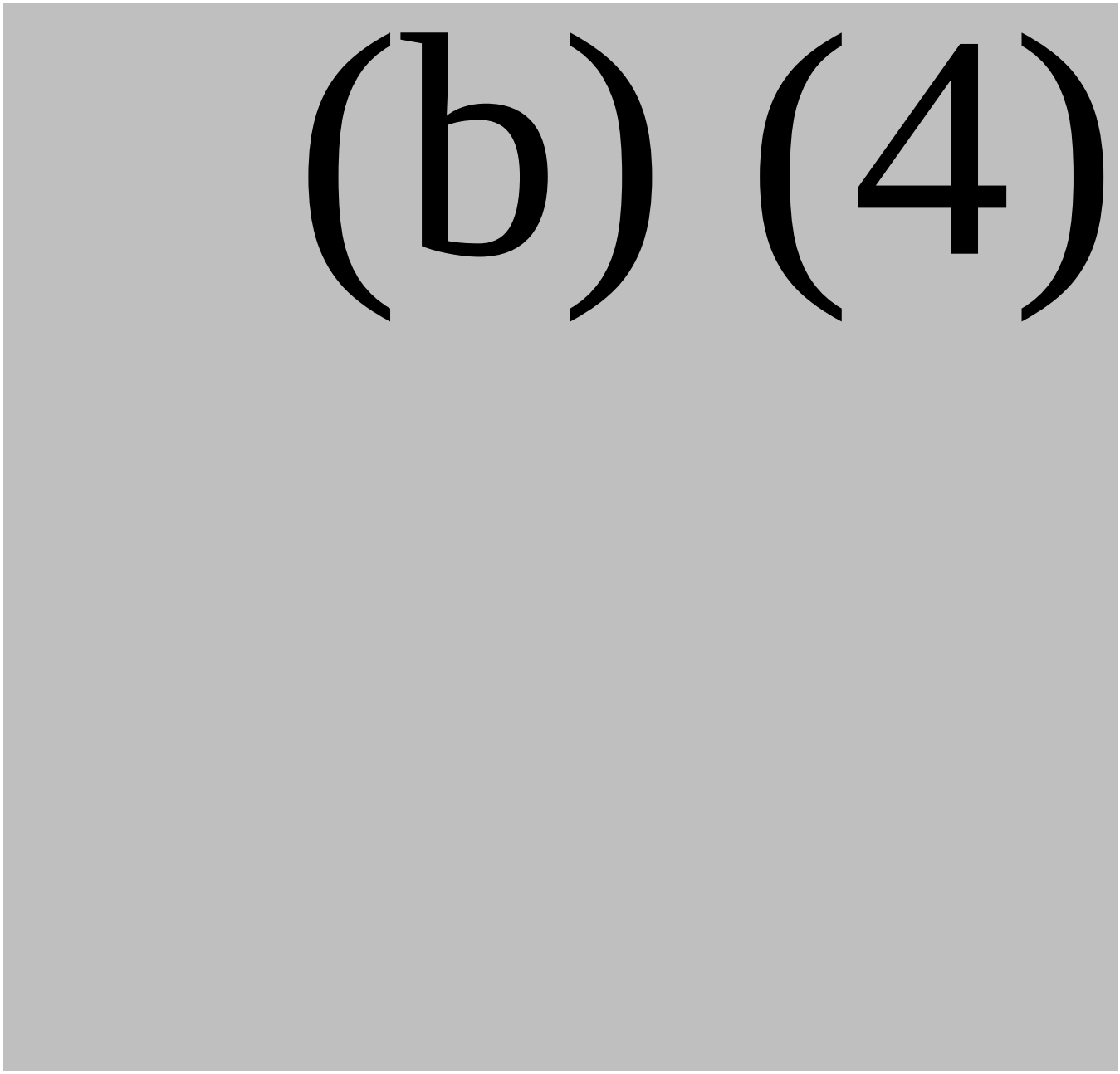

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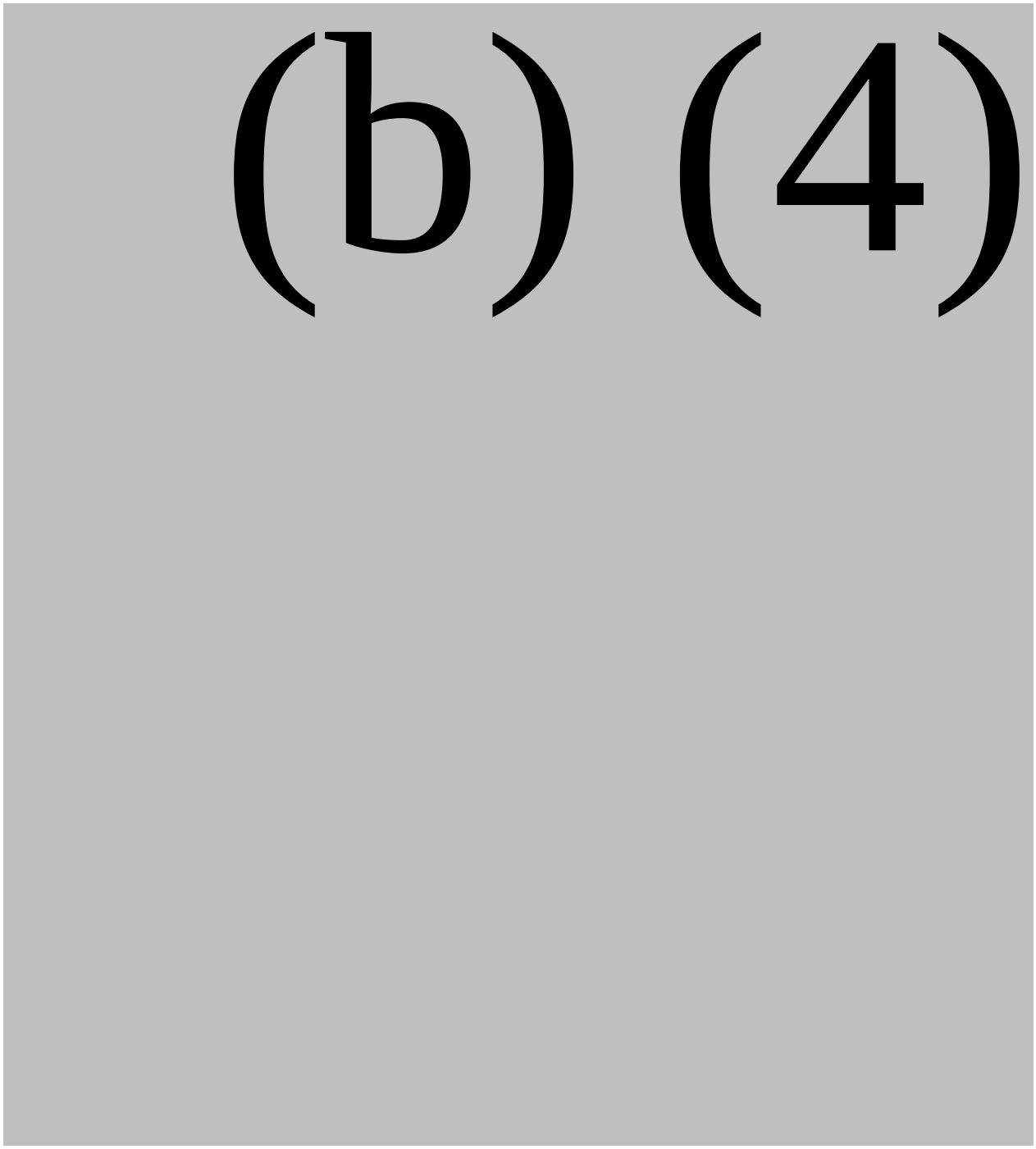
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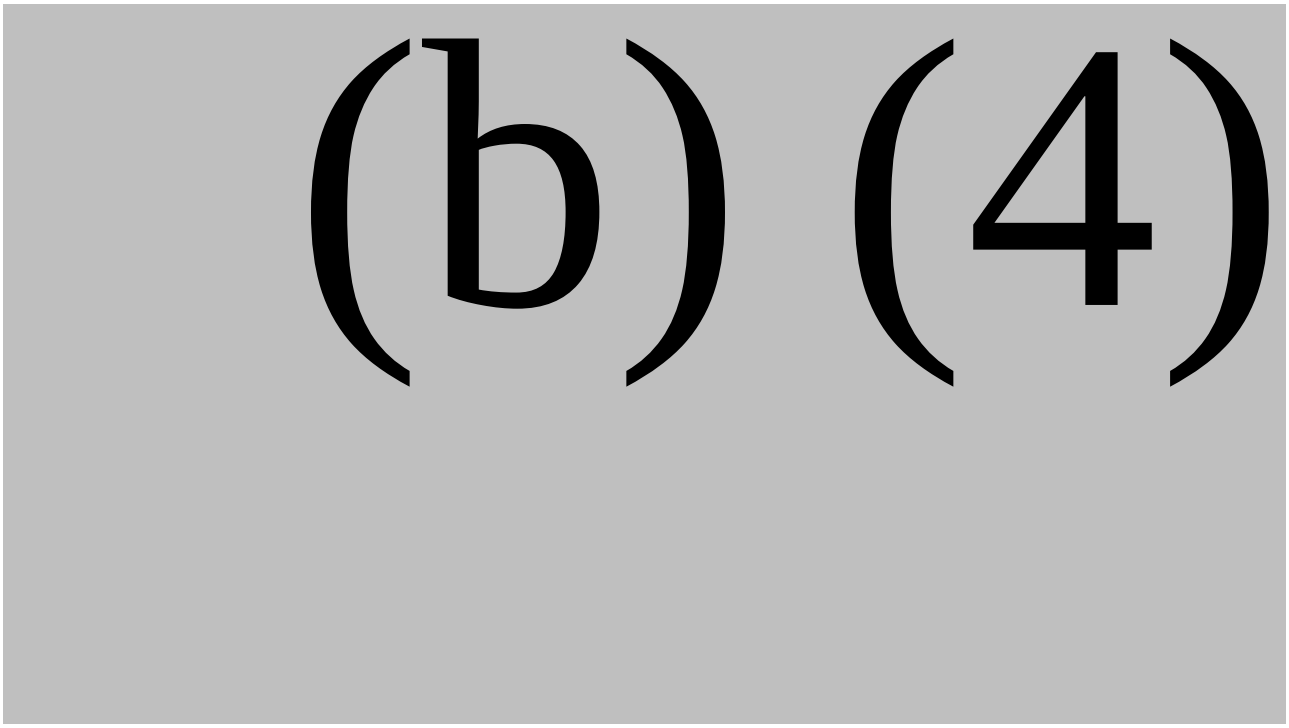

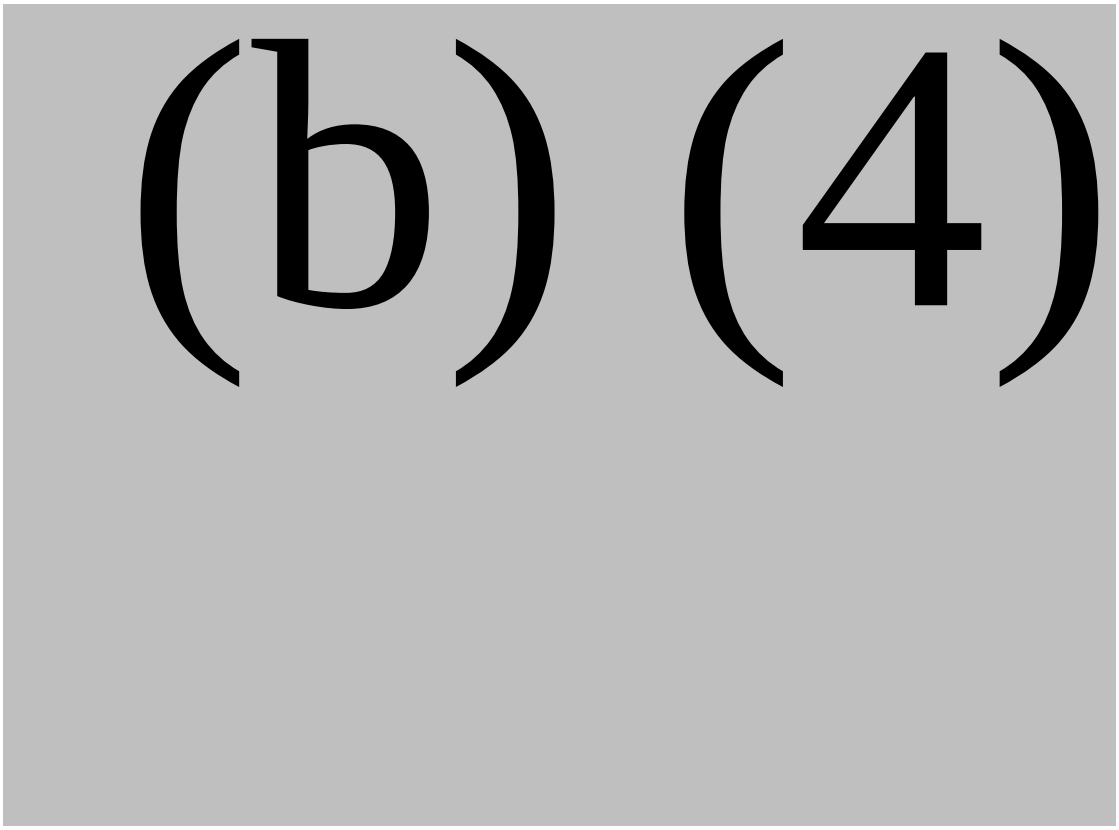

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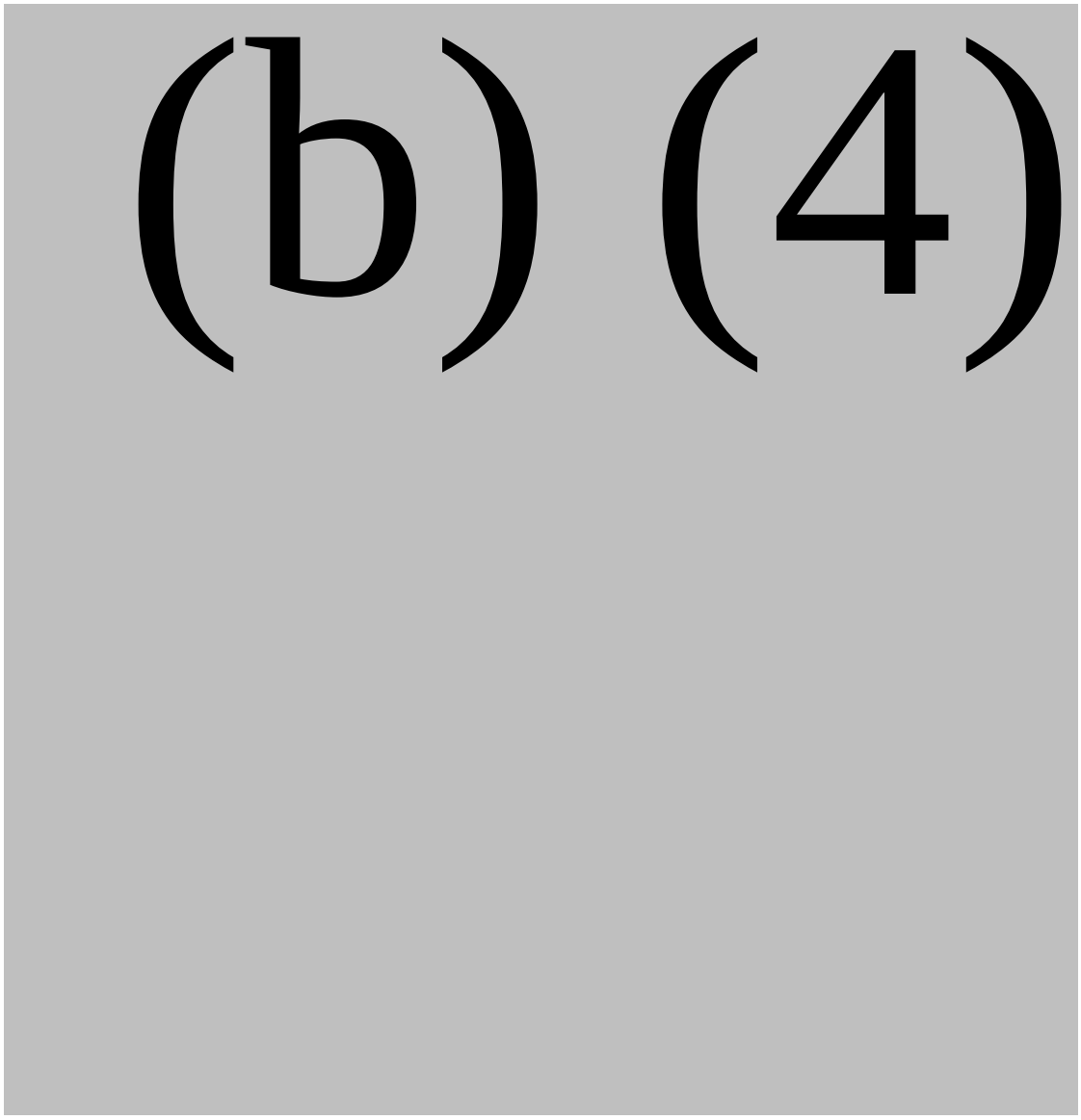
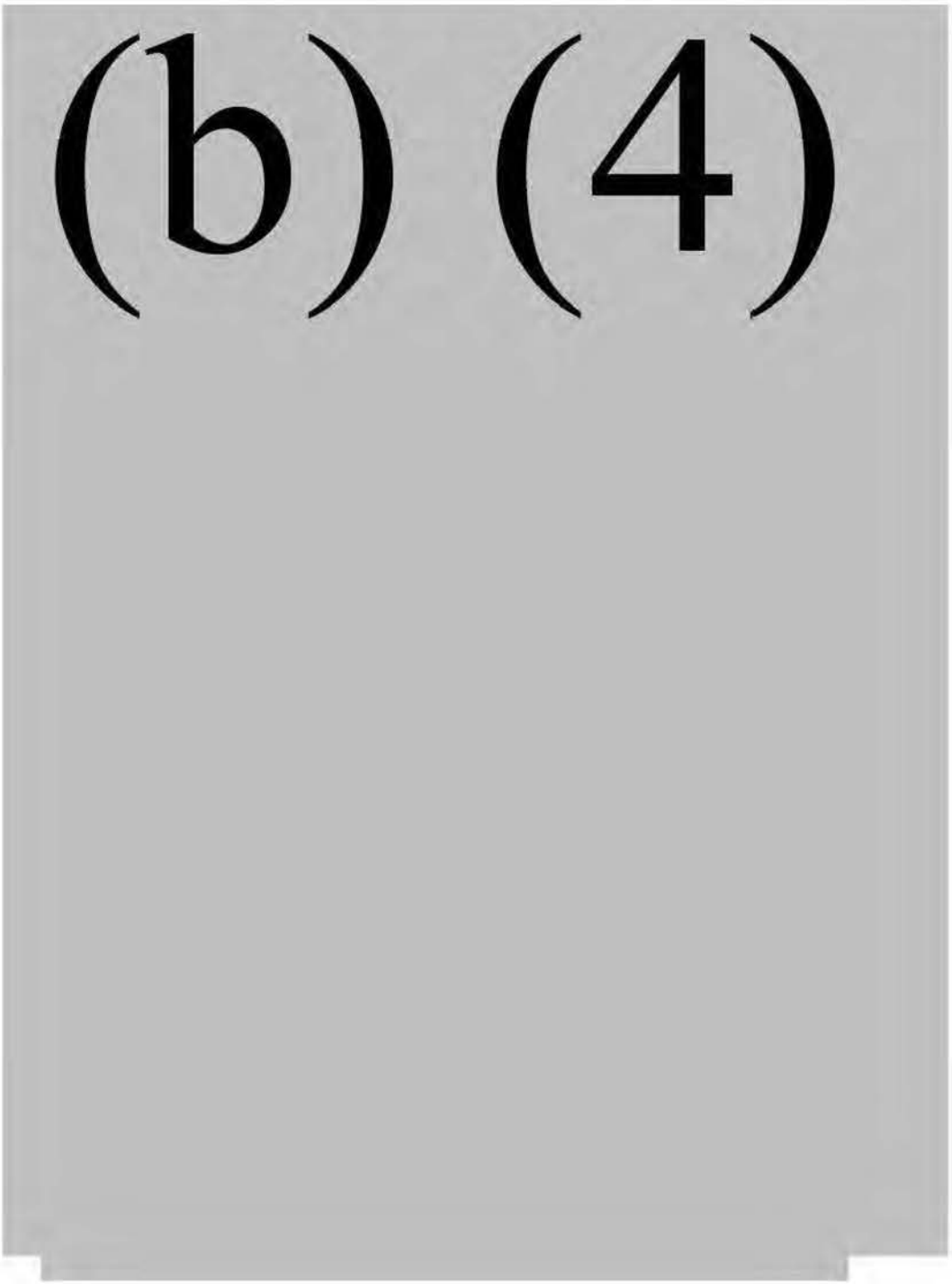
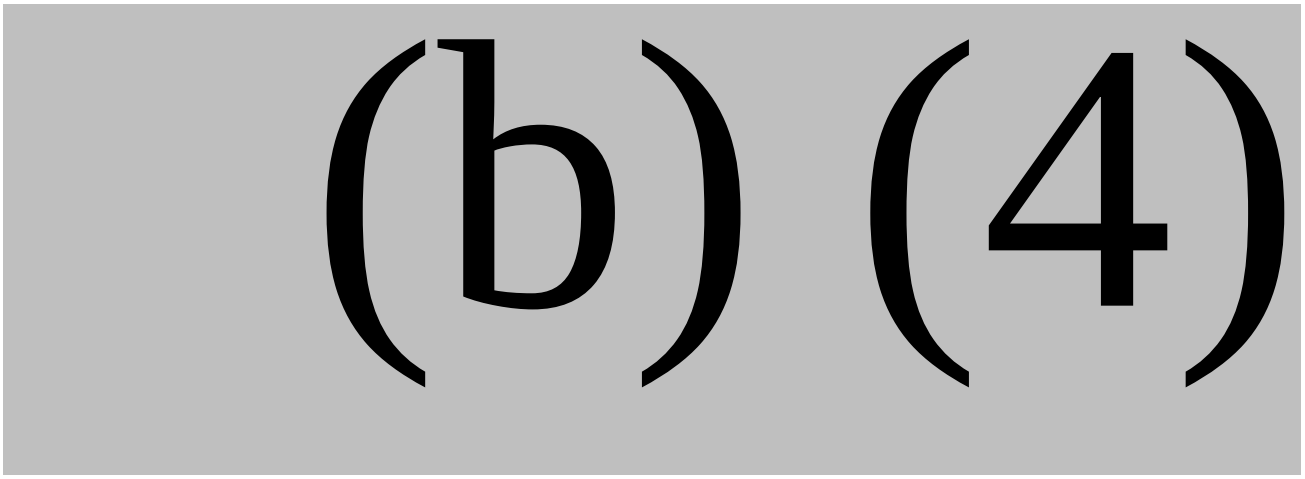






(b) (4)

REPORT

ID: 23567-4/2020

Homogeneity and Stability of "Axtra® PHY GOLD 65 G" in mash feed for Swine, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs Manager:

Alessandra Tossoli

Signature:… (b)(6) …… Date:29/09/2020……..

Study scientist:

(b)(6)

Signature:… (b)(6) Date:27/09/2020……..

This report is approved by:

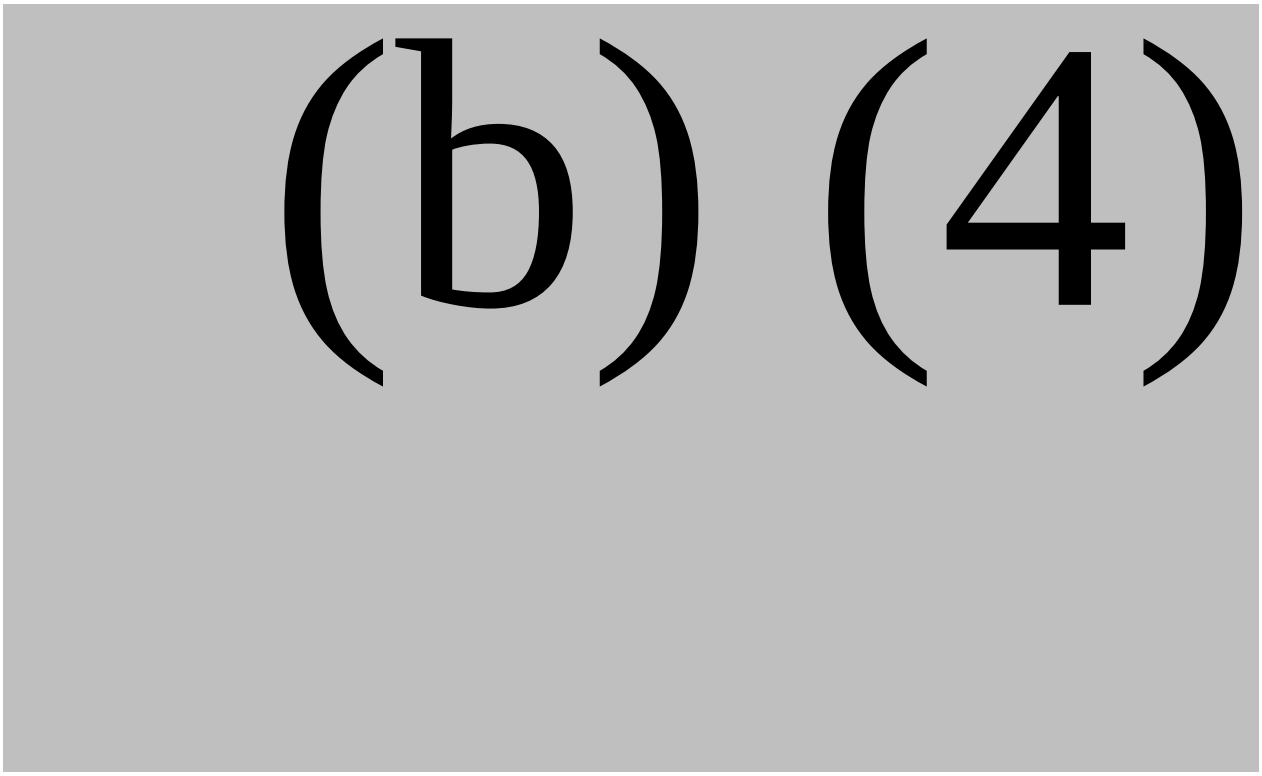
Product Manager:

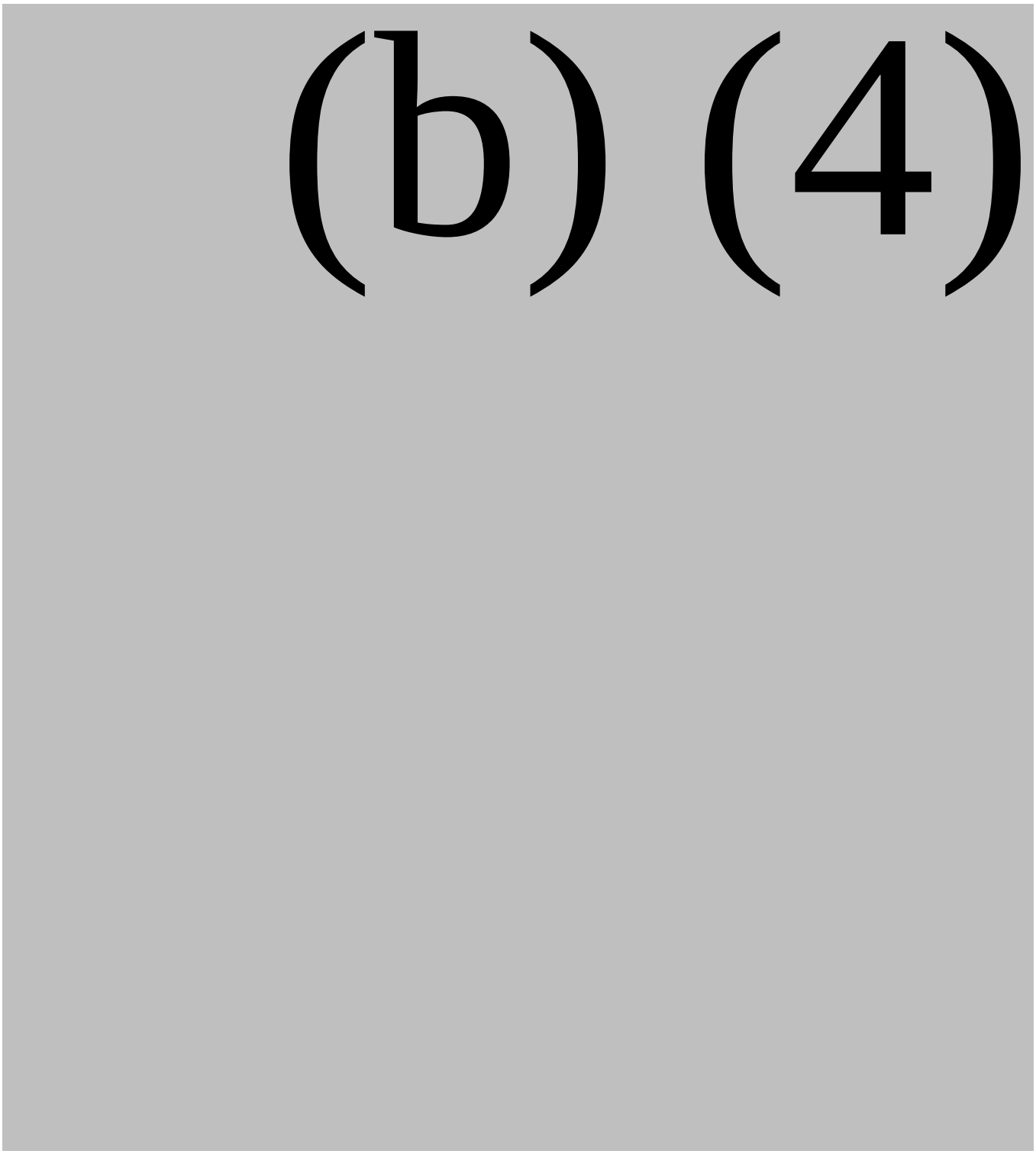
Arno de Krei

Signature:… (b)(6) Date:28/09/2020……..

(b) (4)

1. SUMMARY





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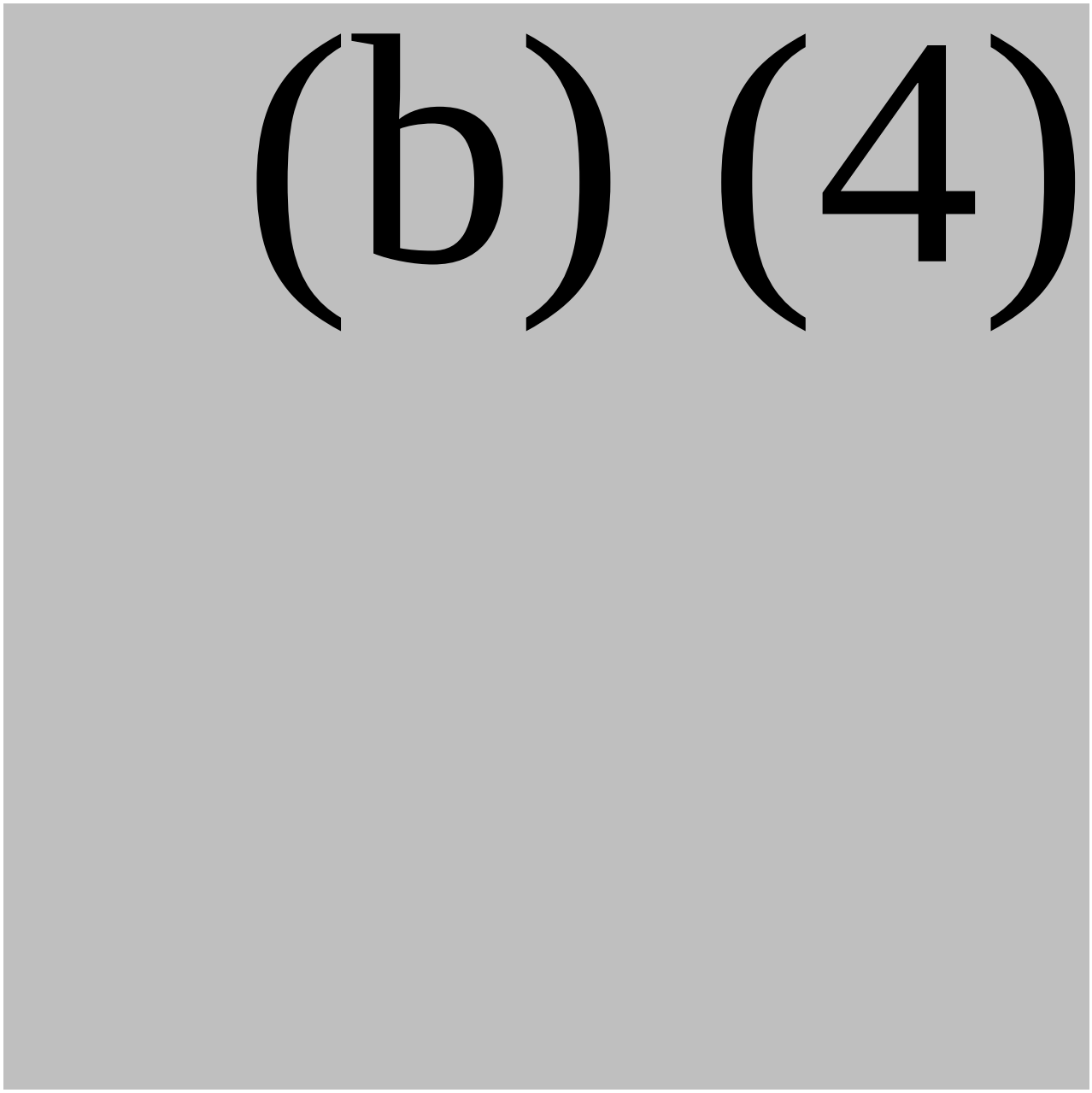
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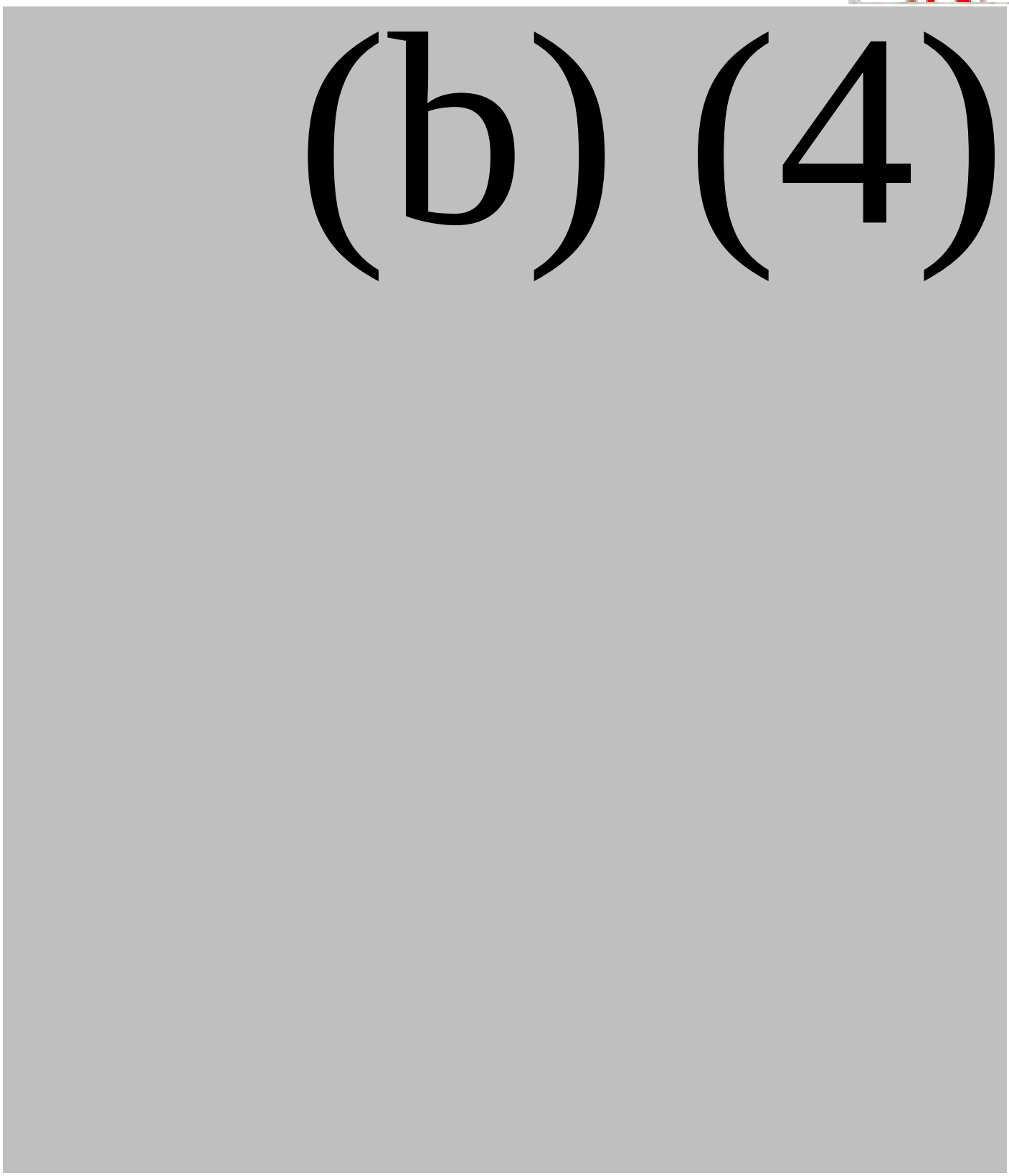
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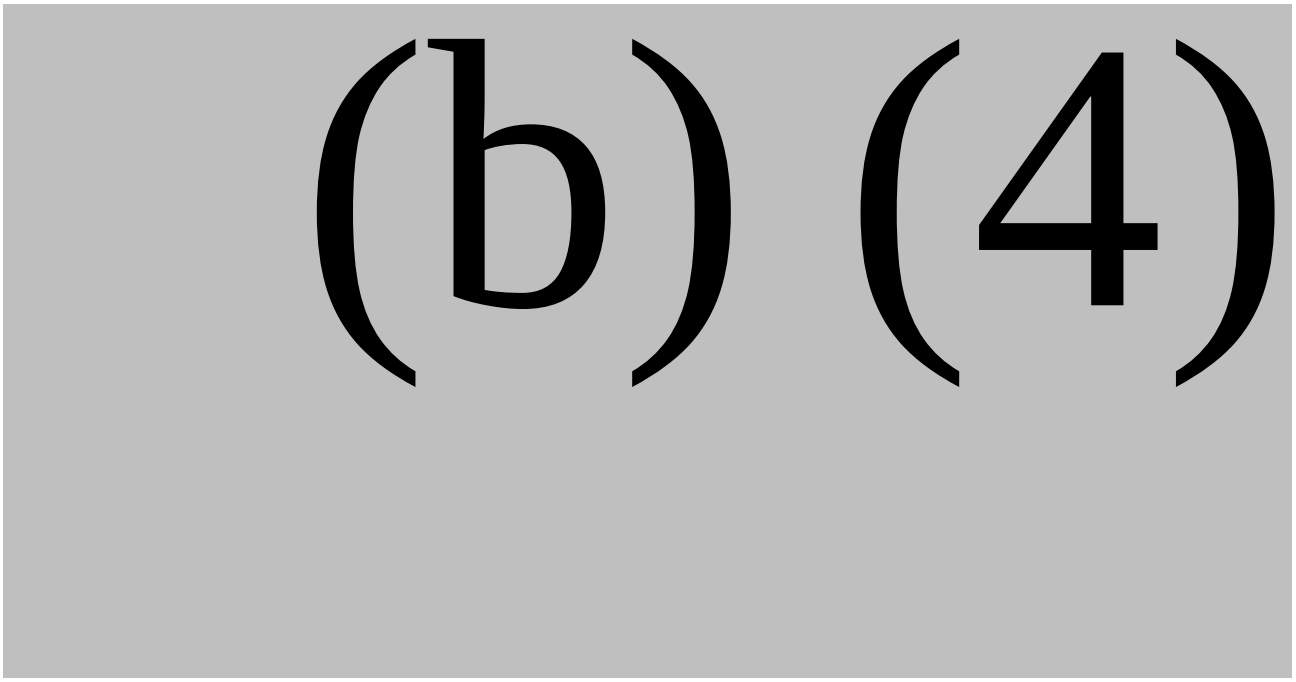
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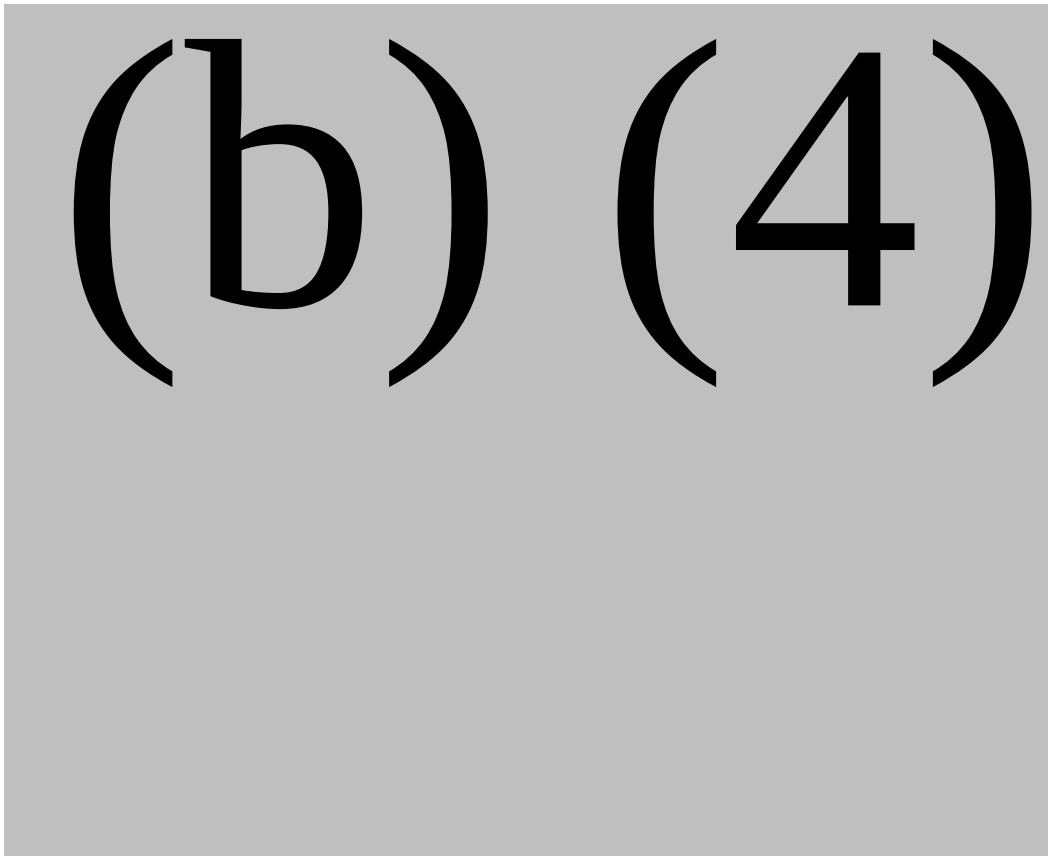
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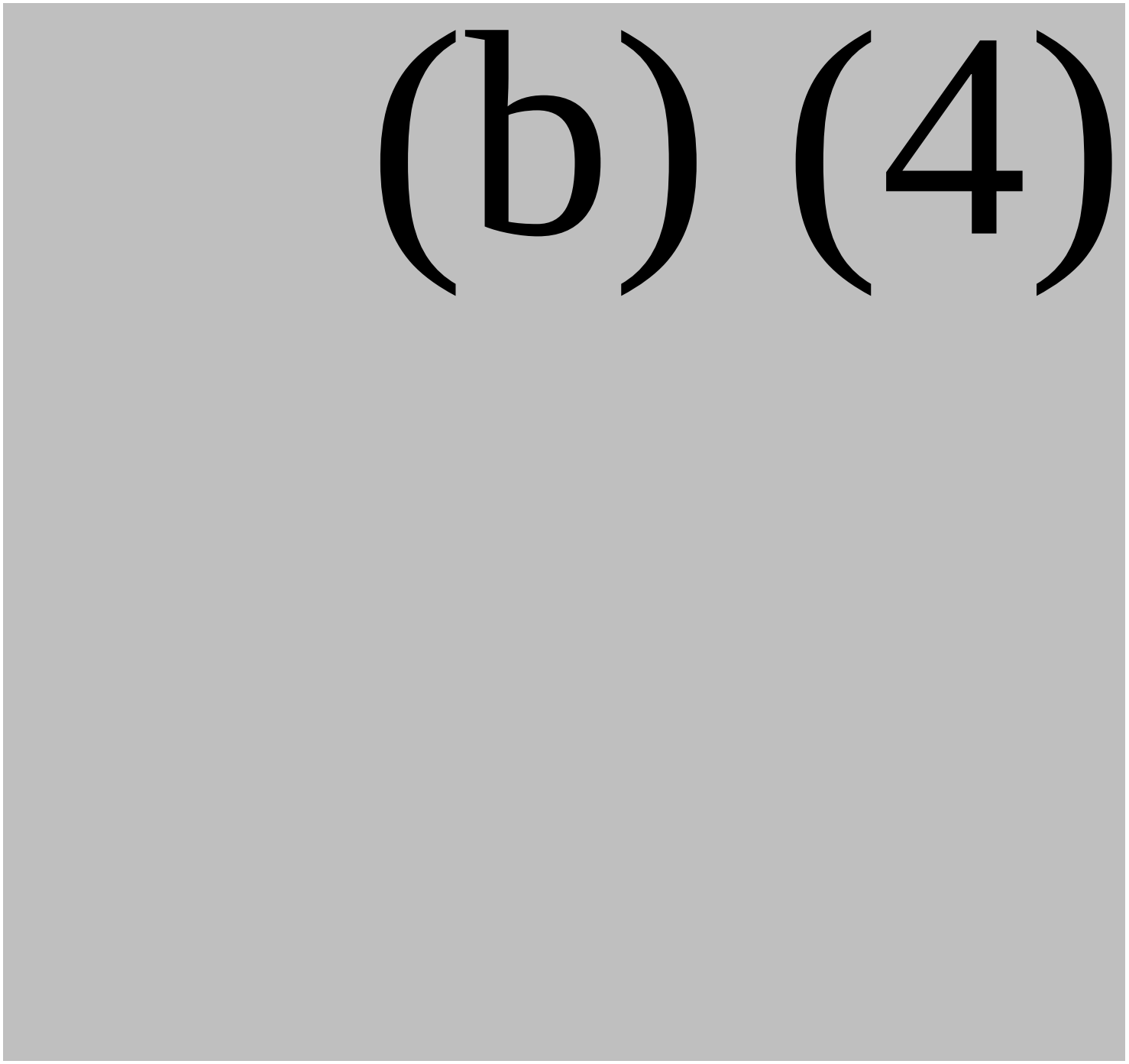

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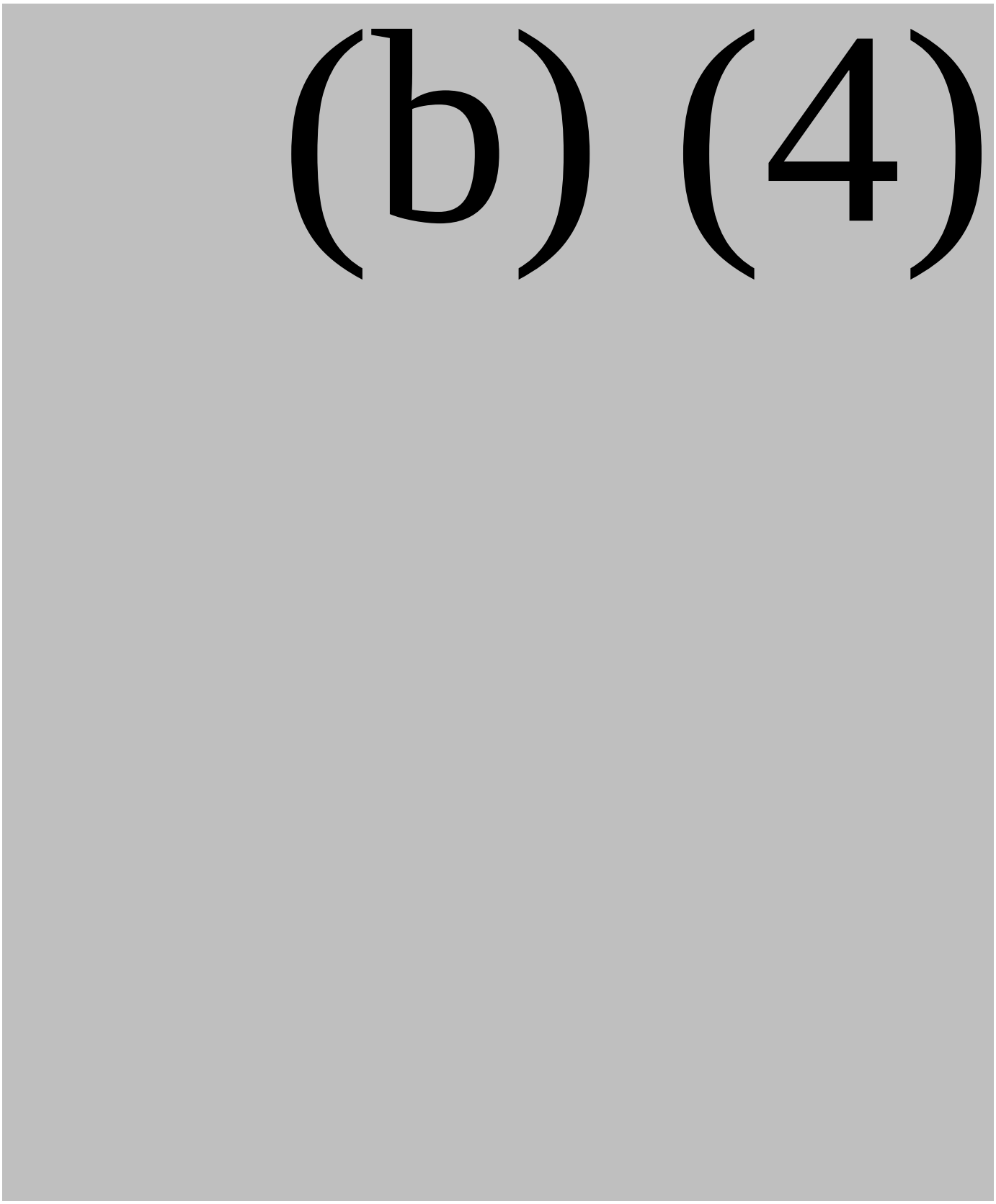
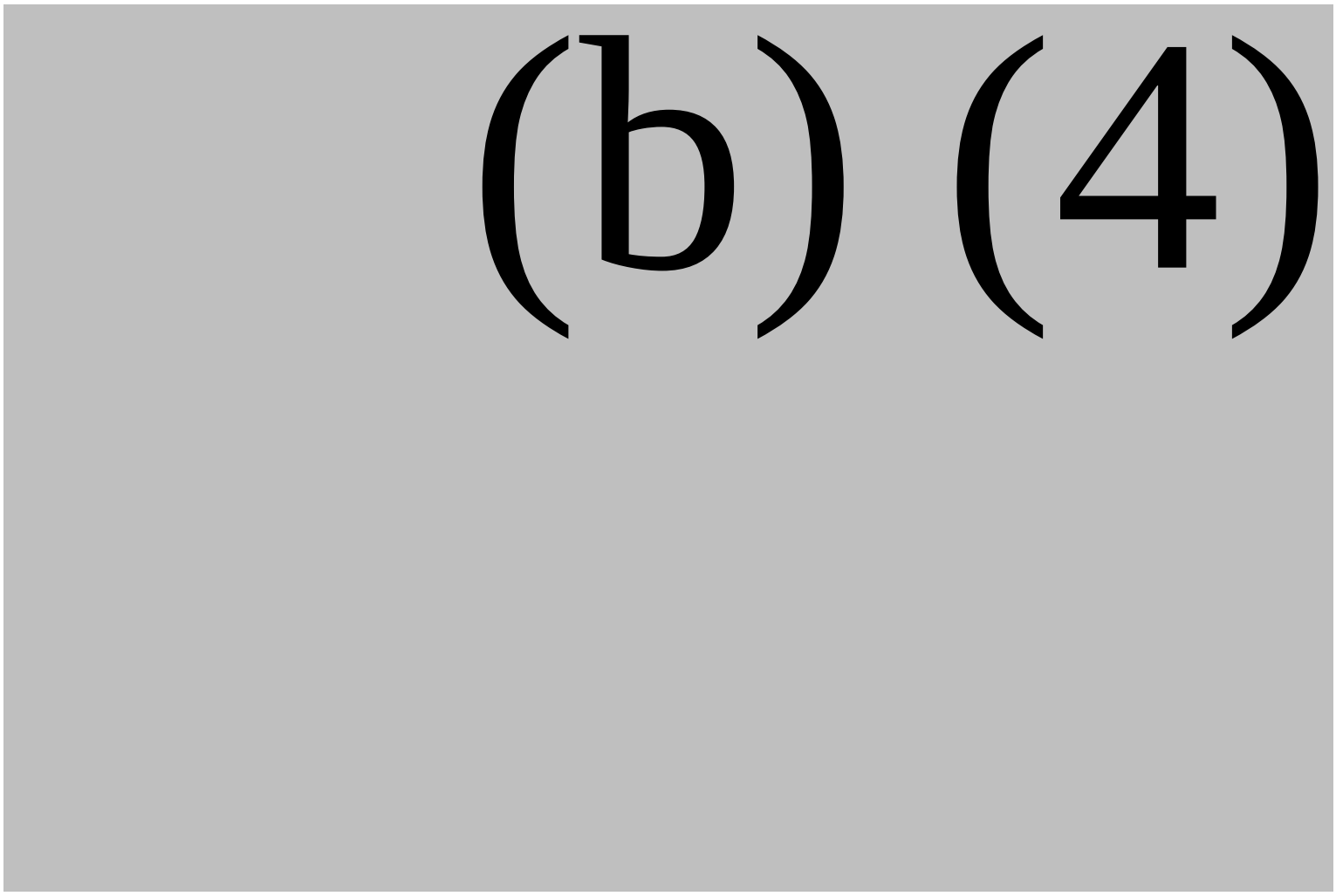




REPORT

ID: 23567-2/2020

Homogeneity and Stability of "Axtra® PHY GOLD 65 G" in mash feed for poultry, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

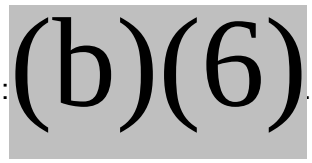
There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs Manager:

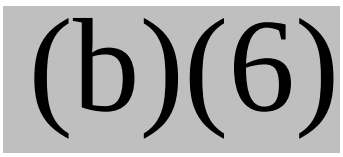
Alessandra Tossoli

Signature: 

Date: 29/09/2020

Study scientist:

(b)(6)

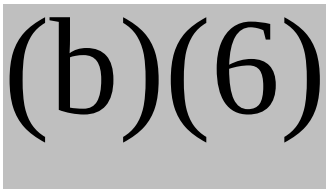
Signature: 

Date: 27/09/2020

This report is approved by:

Product Manager:

Arno de Kreij

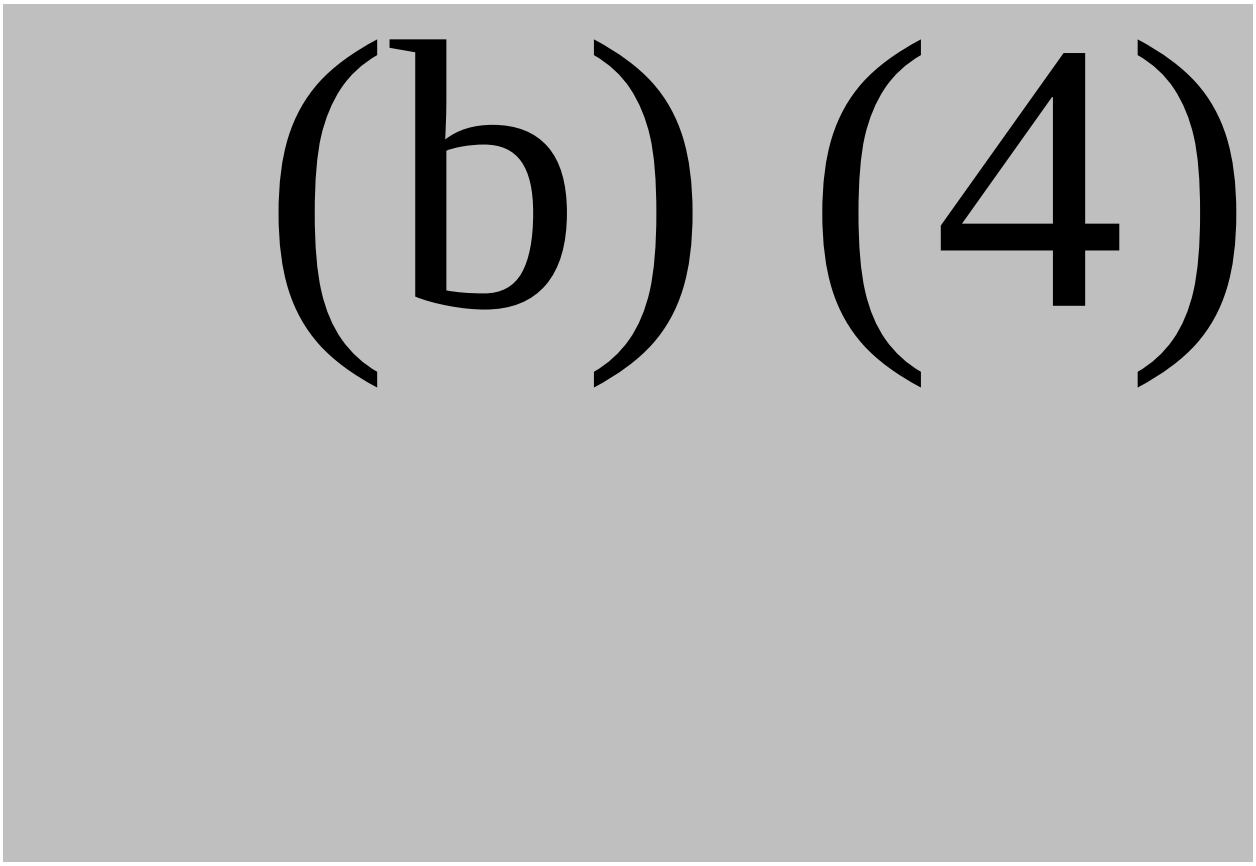
Signature: 

Date: 28/09/2020

(b) (4)

(b) (4)

1. SUMMARY



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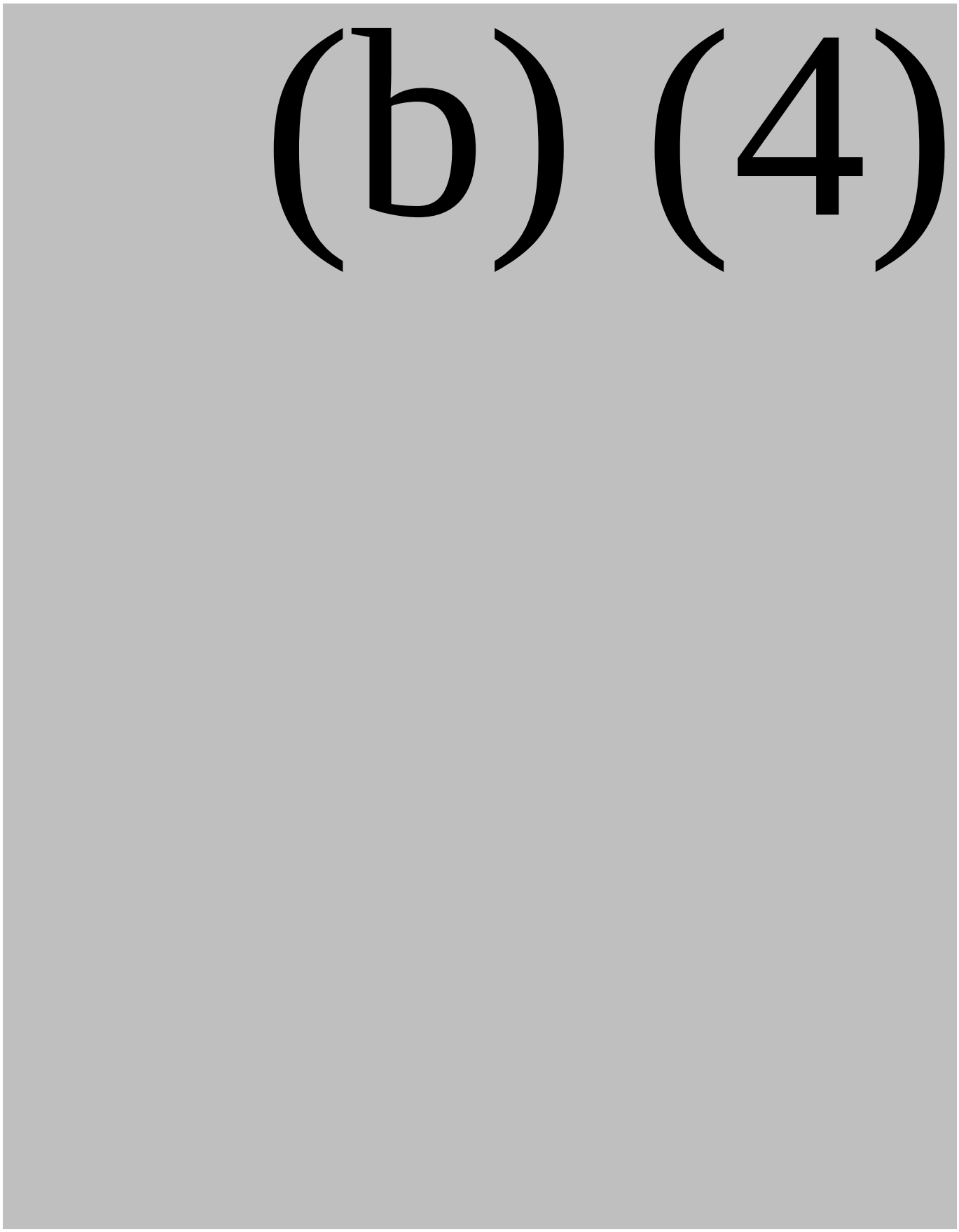
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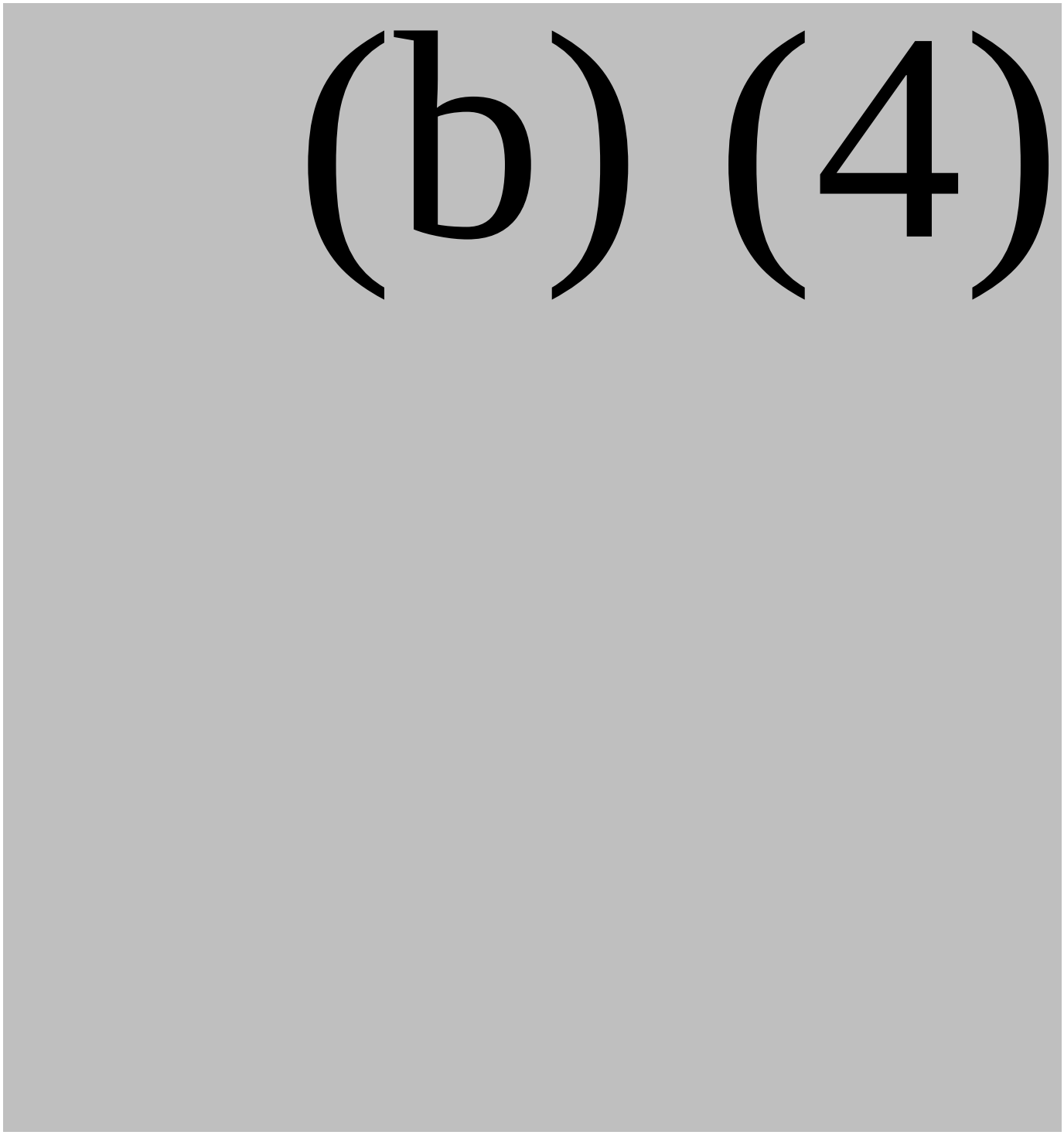
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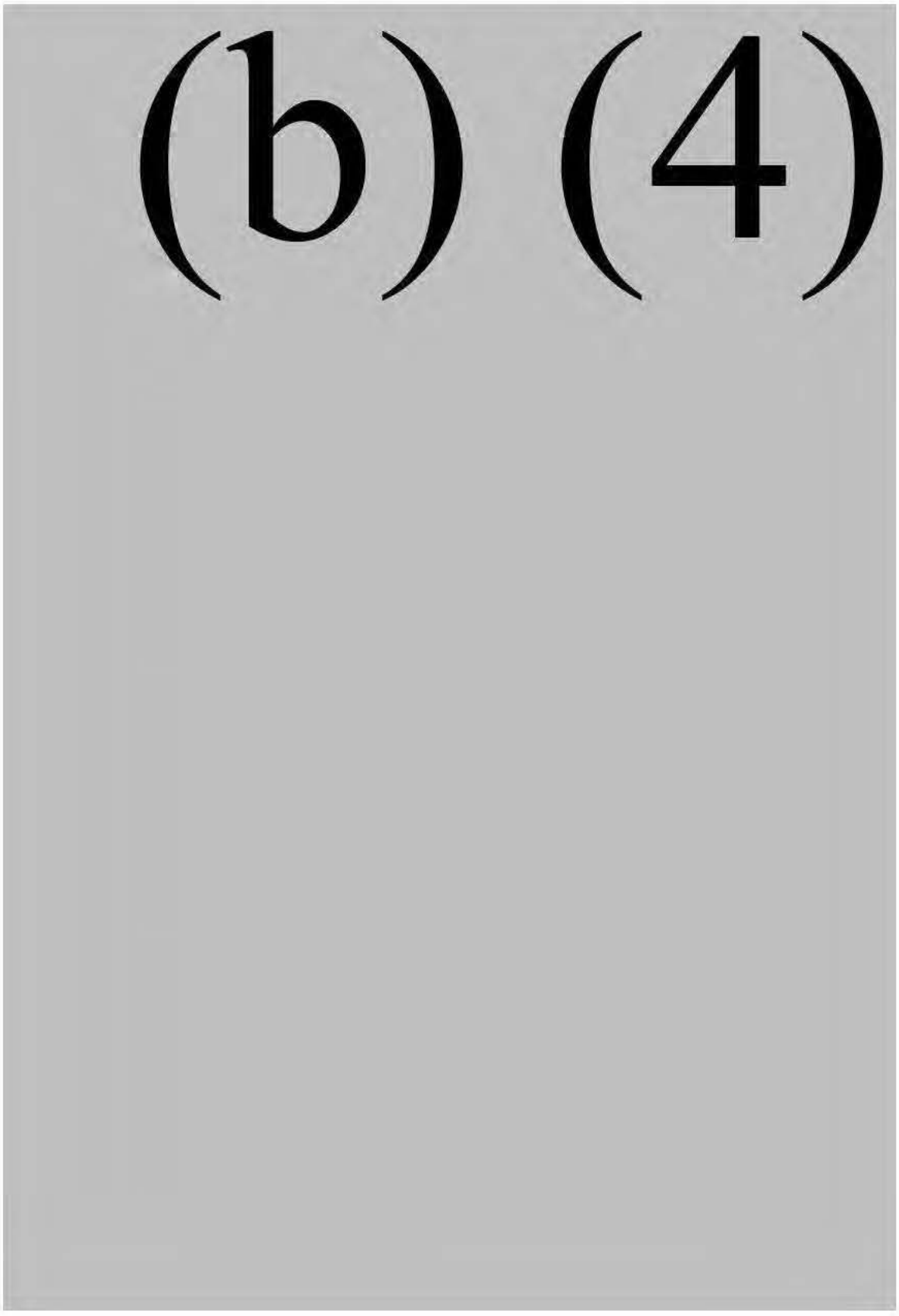
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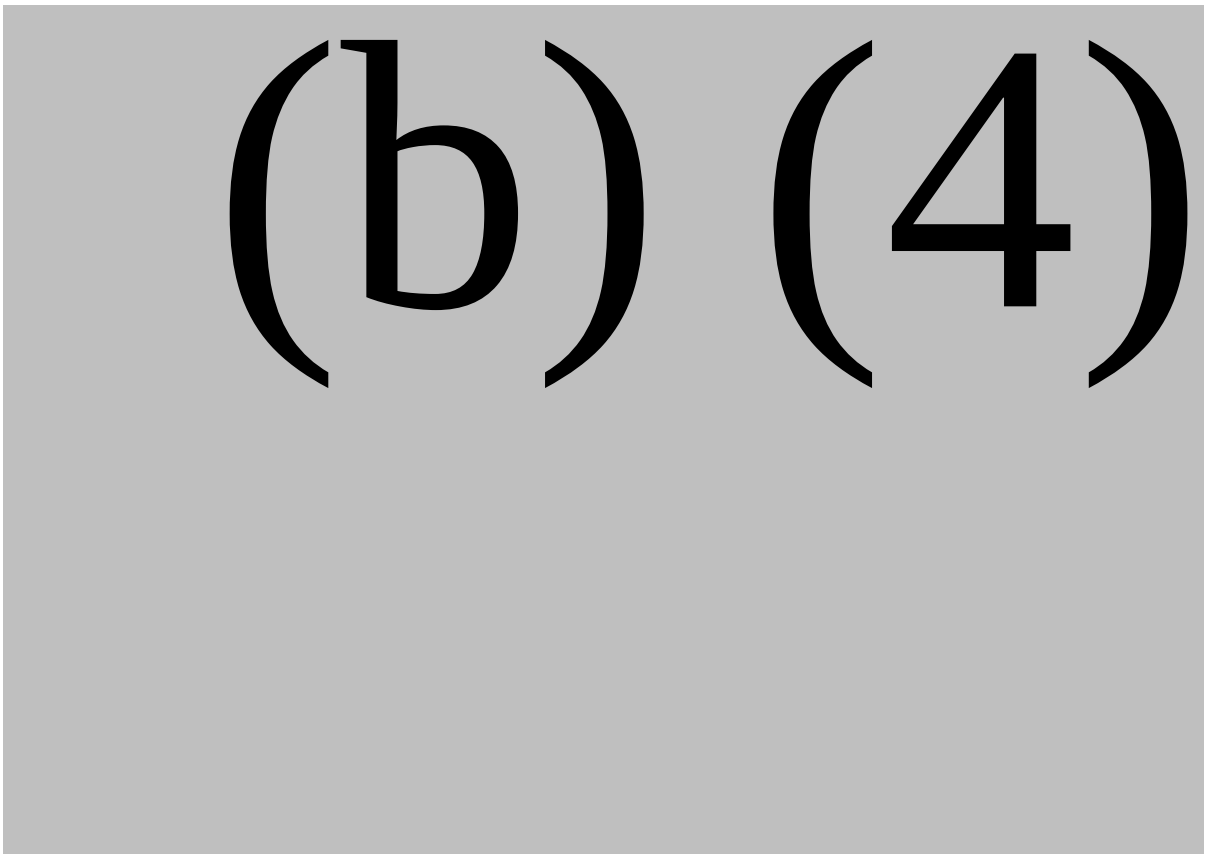
(b) (4)

(b) (4)

[redacted]

Inmengingspercentage: 1.0000%

Naam	Per kg premix		Per kg eindvoer	
[redacted] (b) (4)	[redacted] (b) (4)	kg	[redacted] (b) (4)	kg
		l		l
		IE		IE
		IE		IE
		IE		IE
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mcg		mcg
		mcg		mcg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		KCal		KCal
		g		g
		g		g
		g		g
		g		g
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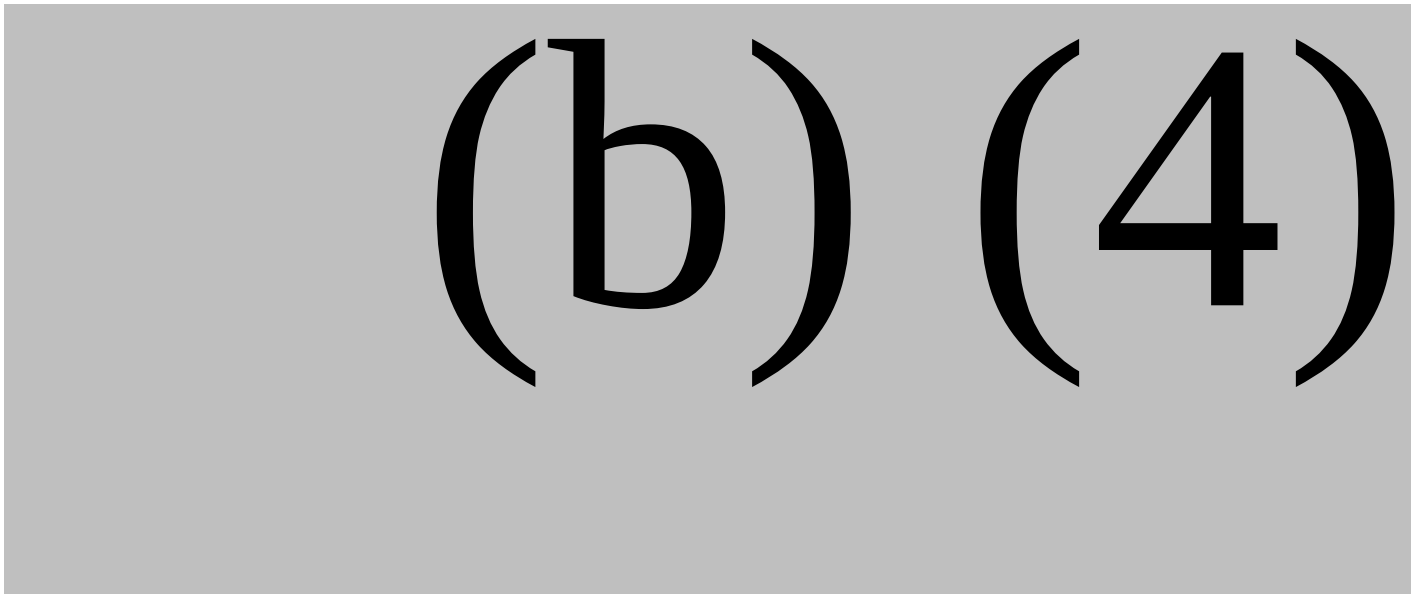
		g		g
		g		g
		g		g

(b) (4)

[redacted]

[redacted] (b) (4)



(b) (4)

REPORT

Determination of feed processing stability of "Axtra® PHY GOLD 30 T" in pelleted feed

Author: A. Tossoli

Study completed: Aug 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature:… (b) (6) …… Date:29/09/2020……..

Study Scientist:

(b)(6)

Signature: (b) (6) ….. Date:27/09/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

Signature: (b) (6) Date:28/09/2020……..

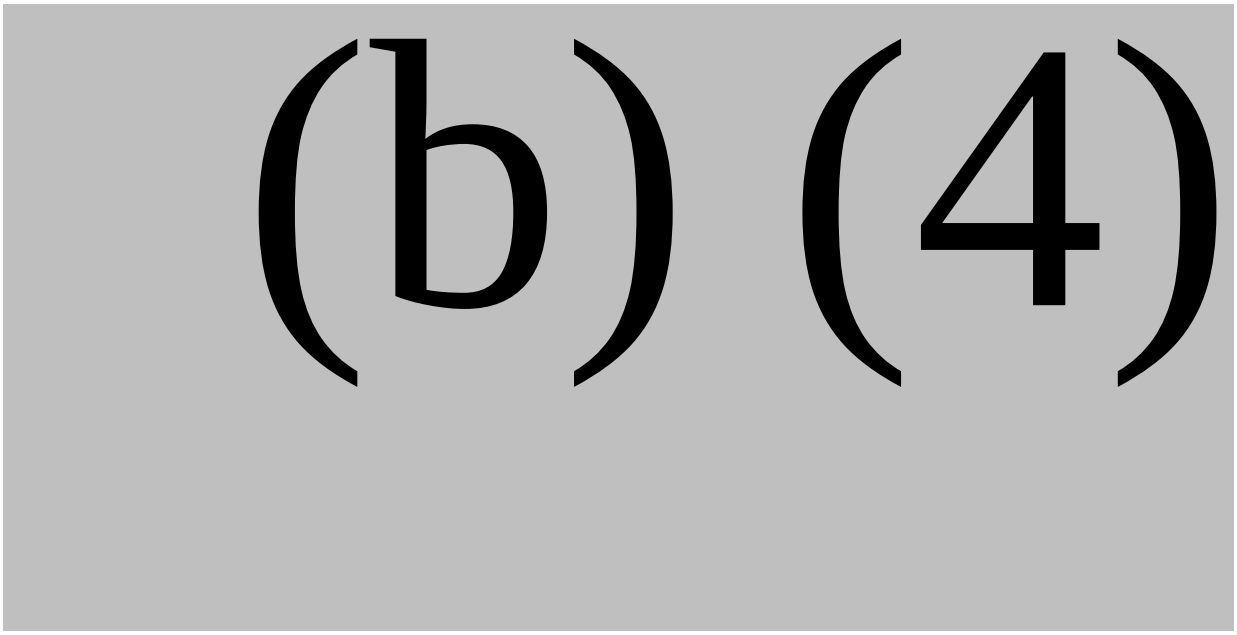
RESPONSIBLE PERSONNEL

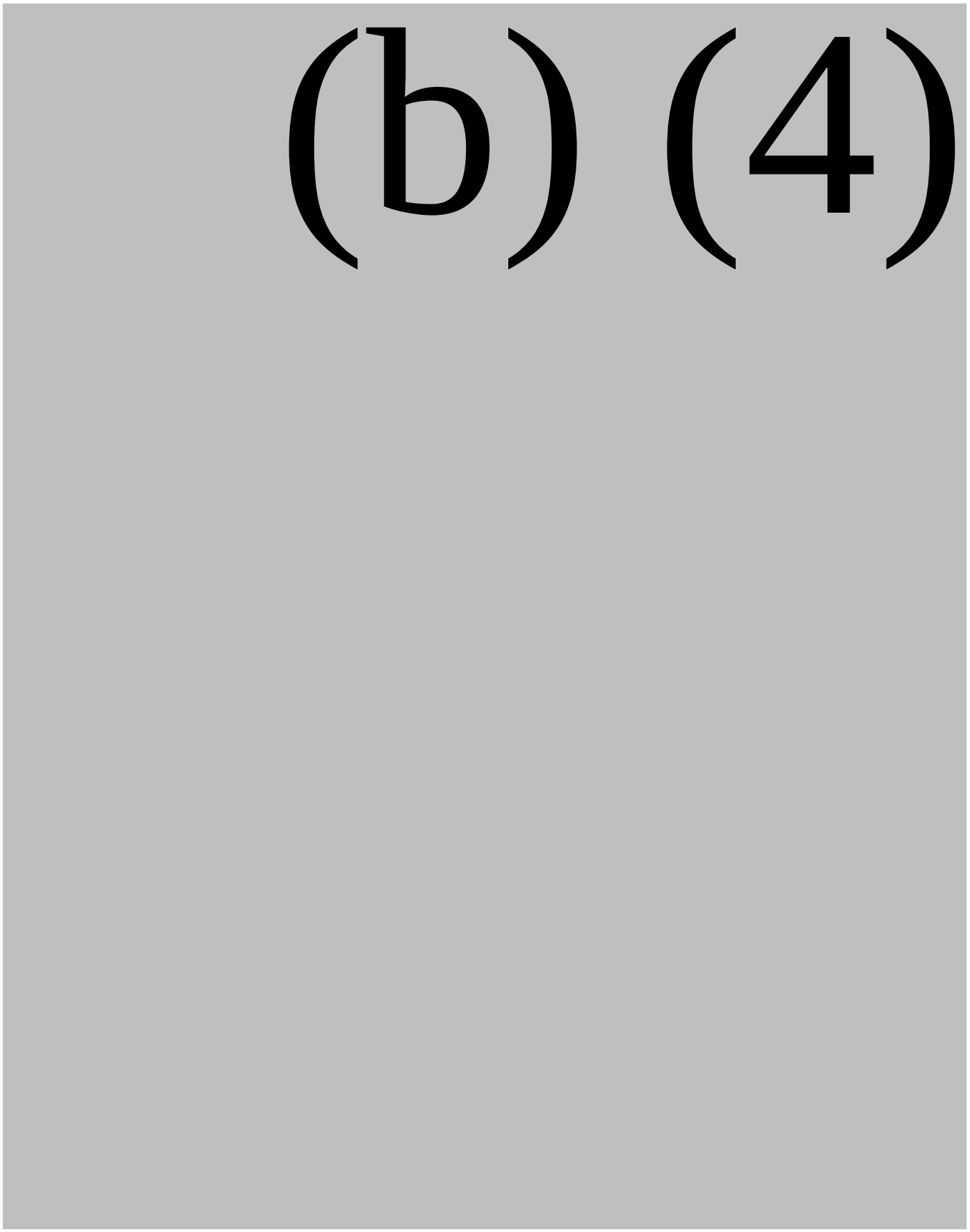
Study scientist(s)	:	[redacted]
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY

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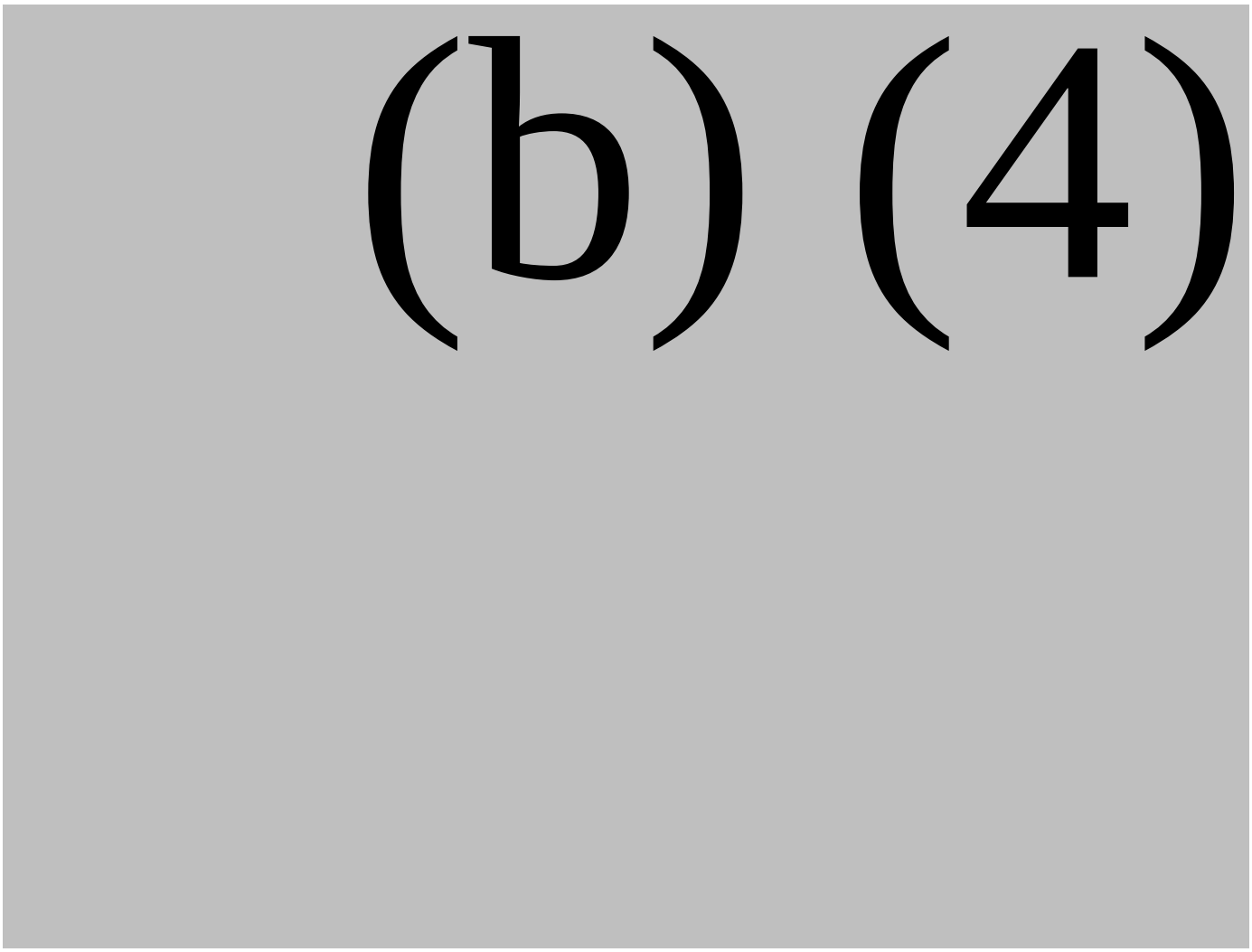

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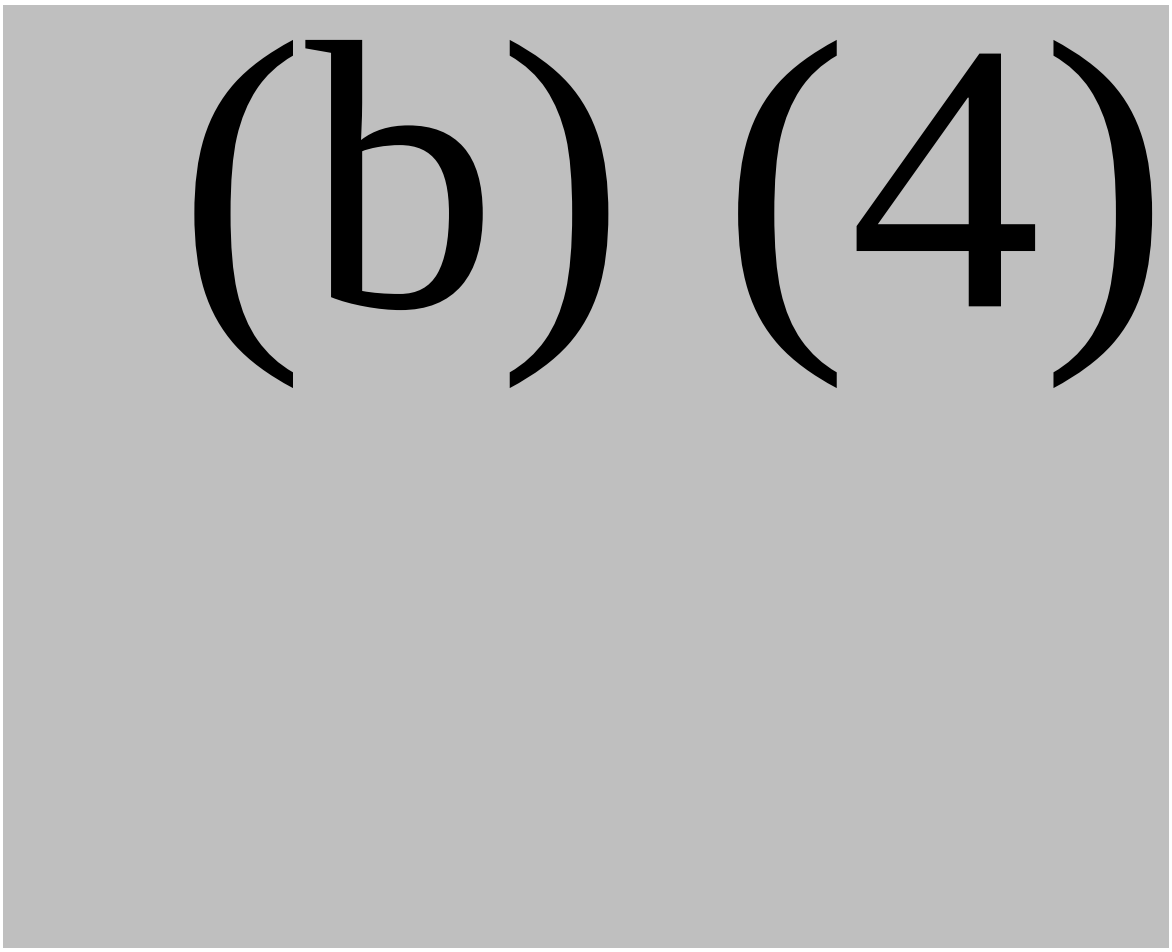
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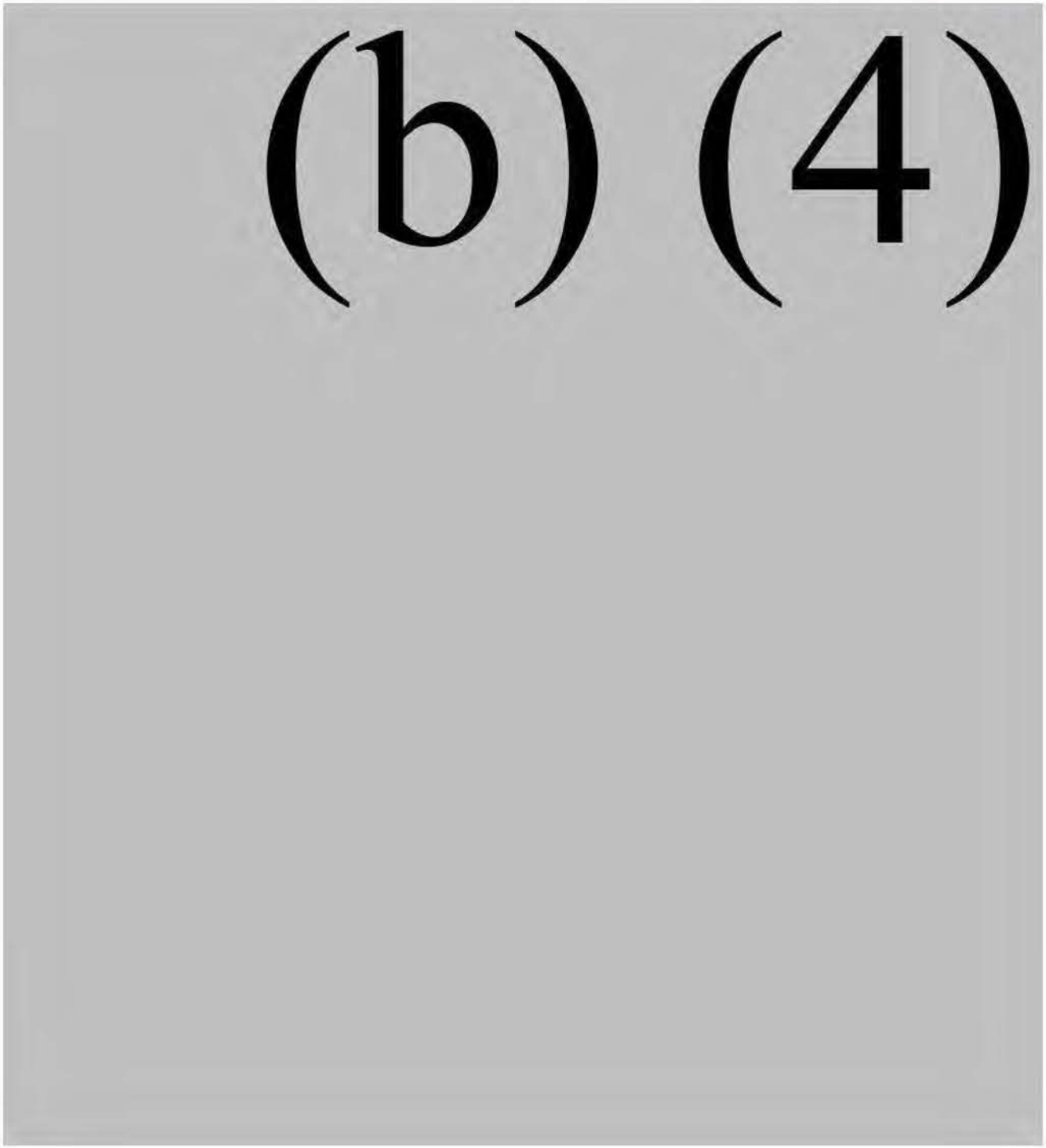

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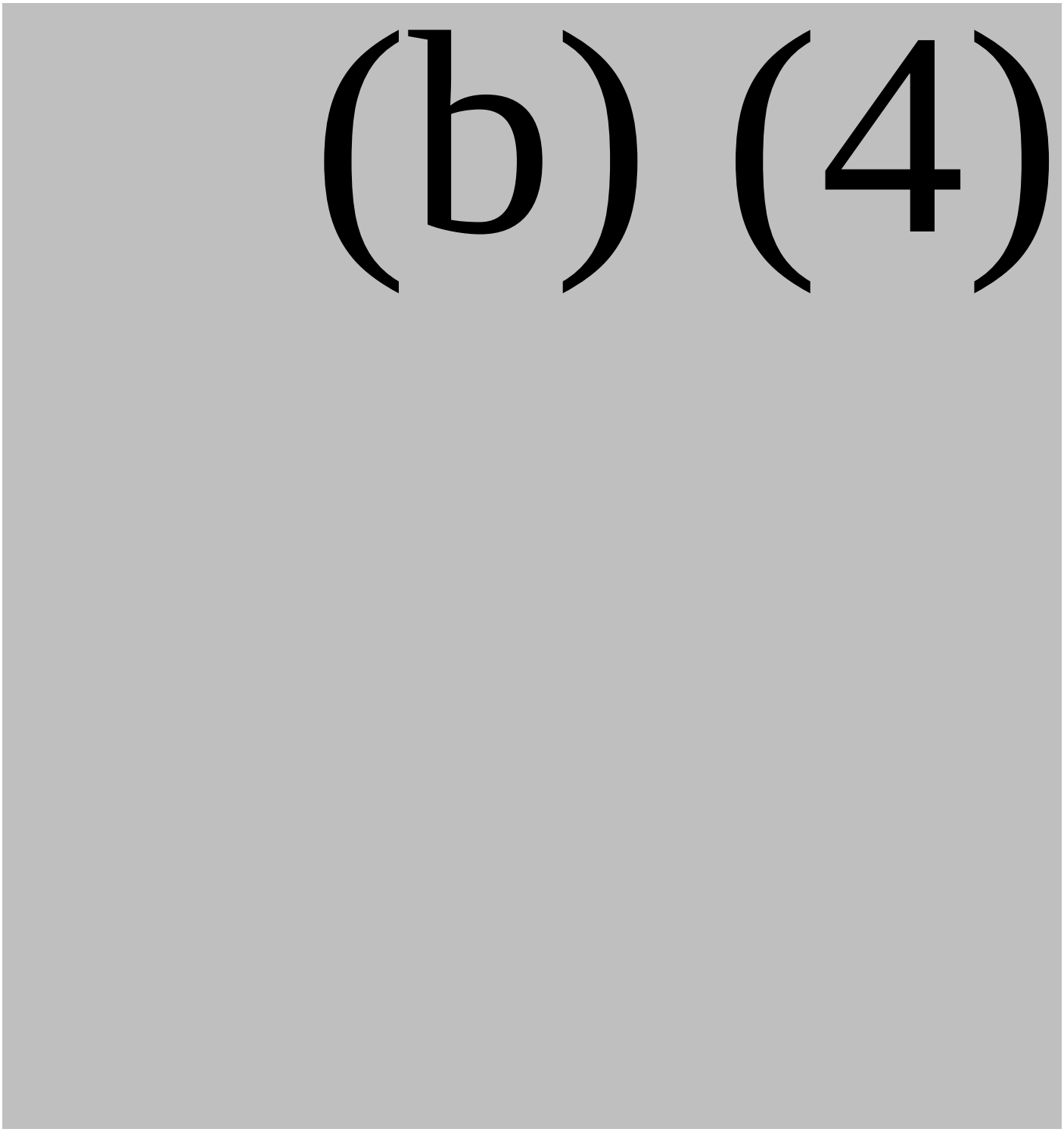

(b) (4)

(b) (4) [redacted]

Inmengingspercentage: 1.0000%

Naam	Per kg premix		Per kg eindvoer	
(b) (4) [redacted]	(b) (4) [redacted]	kg	(b) (4) [redacted]	kg
		l		l
		IE		IE
		IE		IE
		IE		IE
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mcg		mcg
		mcg		mcg
		mg		mg
		mg		mg
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		g		g
		g		g
		g		g
		g		g
		g		g
		KCal		KCal
		g		g
		g		g
		g		g
		g		g
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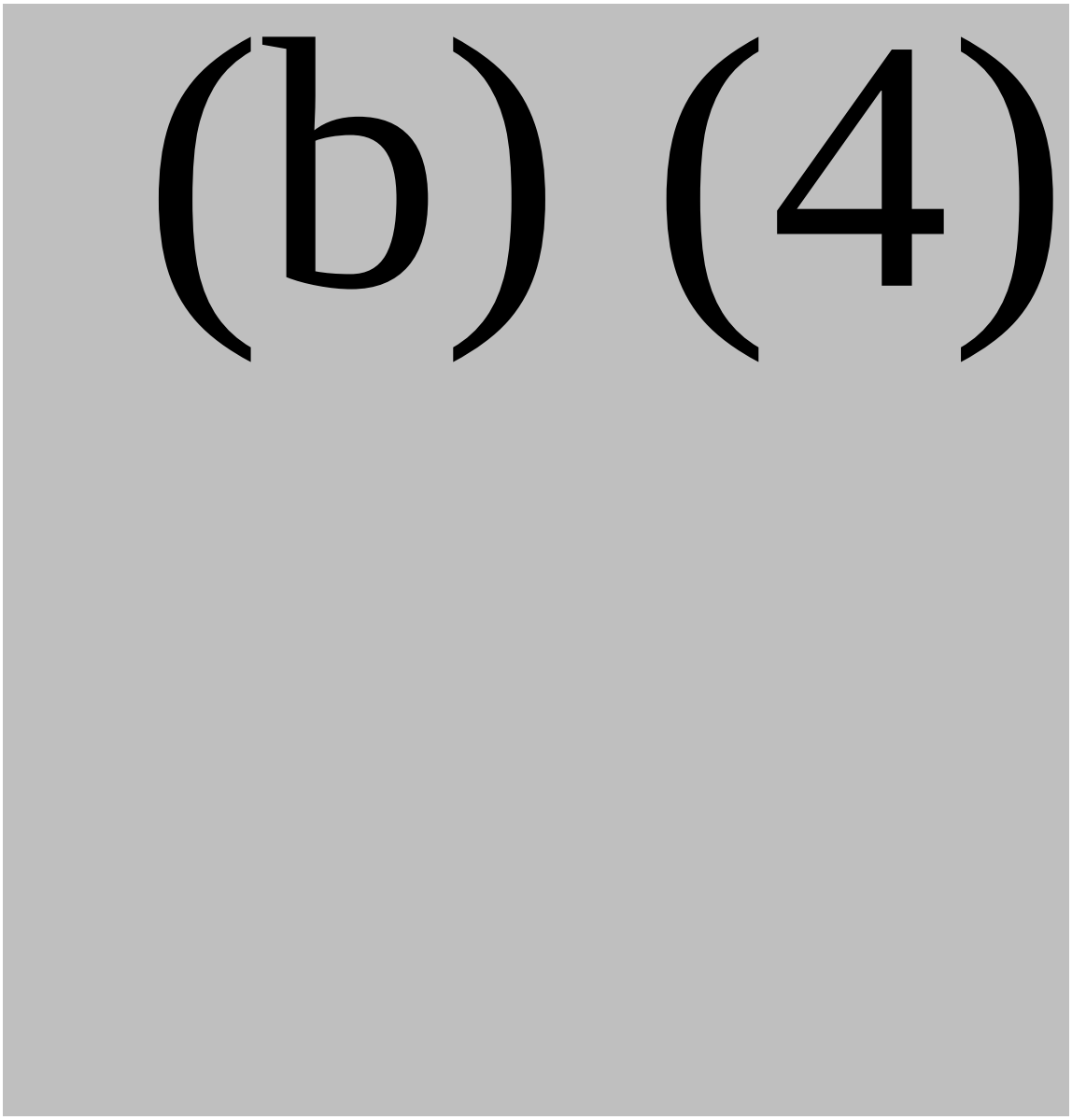
(b) (4) [redacted]

[redacted] (b) (4)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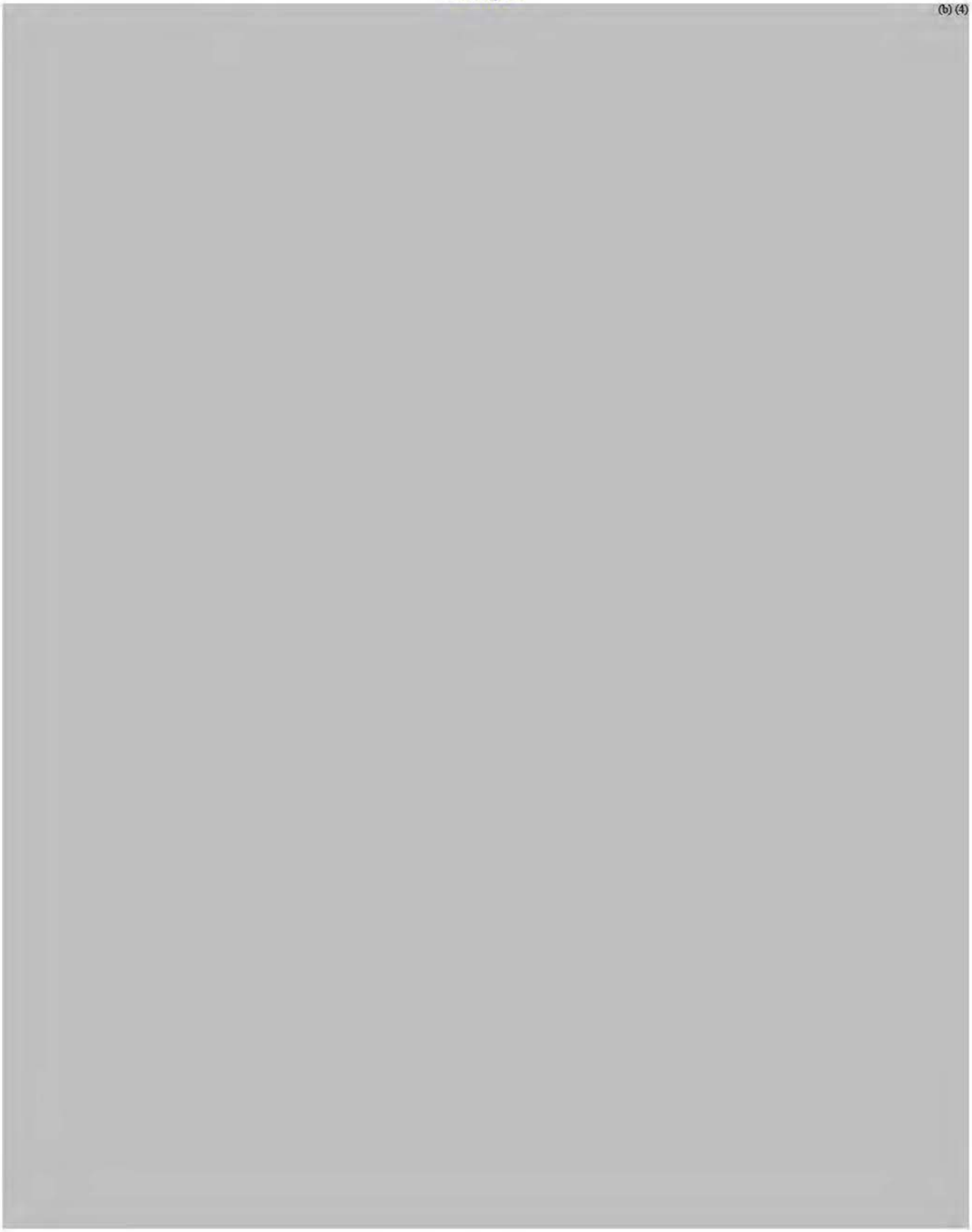
Analyse

(b) (4)



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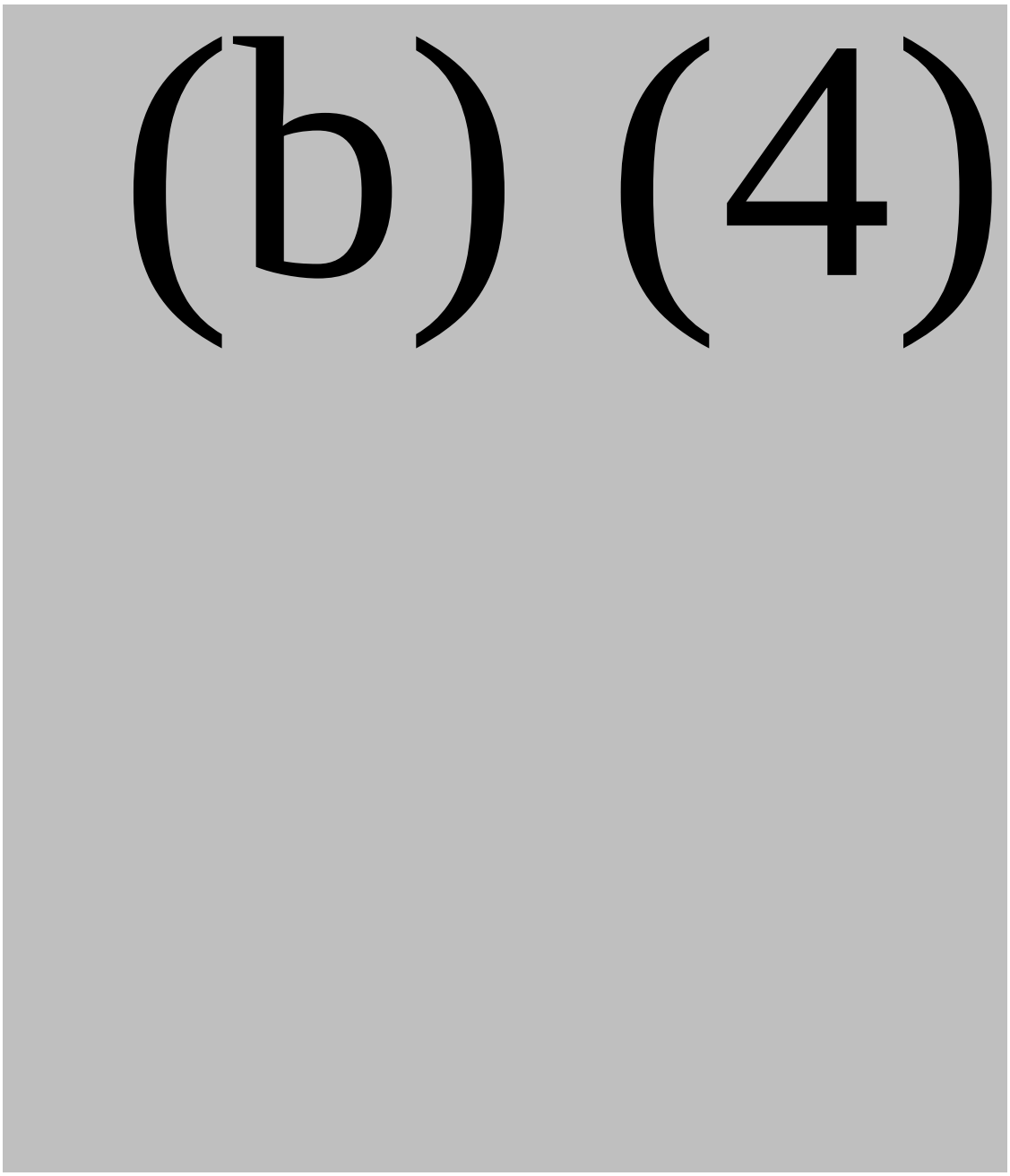
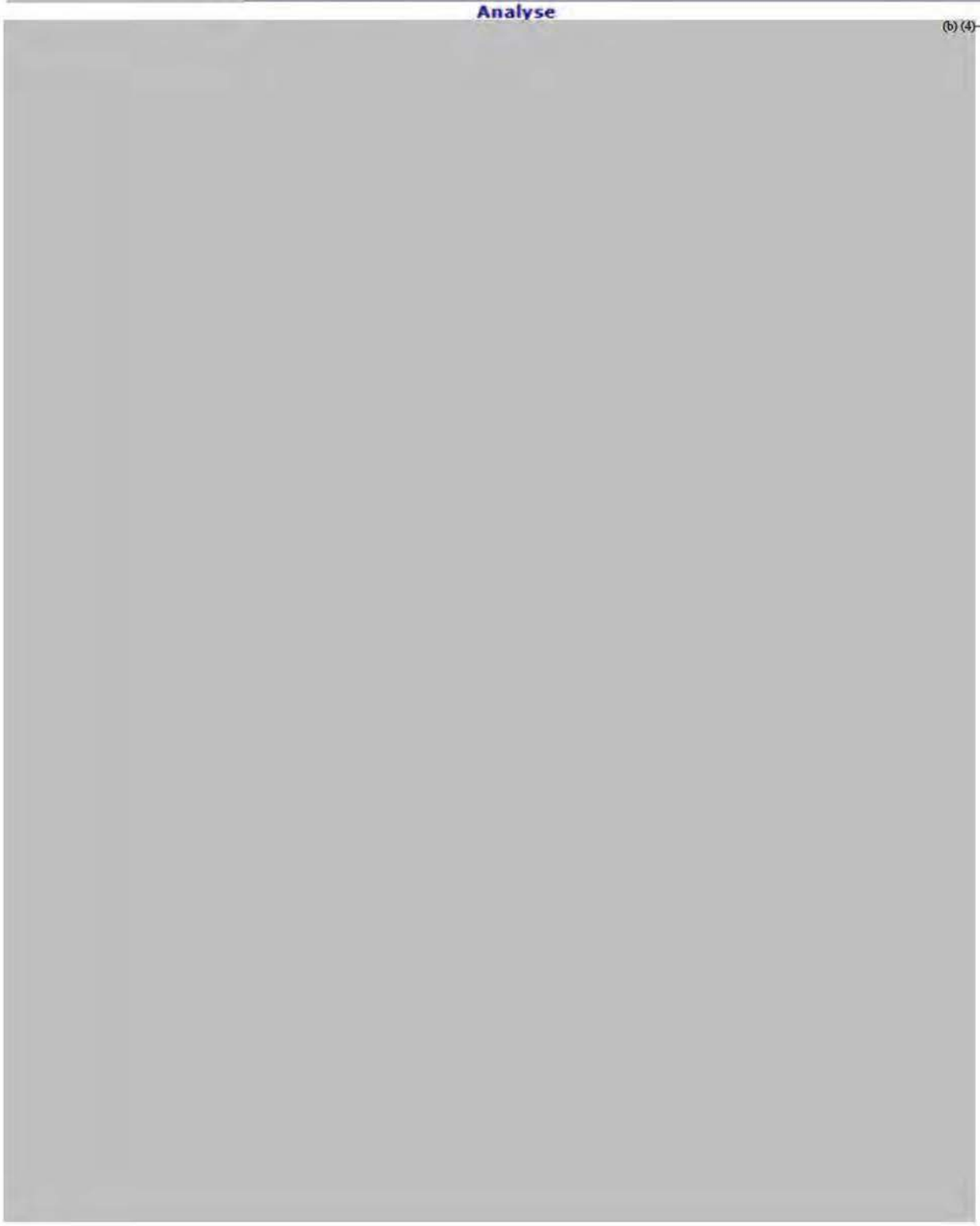


(b) (4)

(b) (4)

Analyse

(b) (4)



(b) (4)

(b) (4)

REPORT

Determination of feed processing stability of "Axtra® PHY GOLD 65 G" in pelleted feed

Author: A. Tossoli

Study completed: Aug 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

(b) (4)

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature: (b)(6) …… Date: 16/09/2020……..

Study Scientist:

(b)(6)

Signature:… (b)(6) … Date: 24/09/2020……..

This report is approved by:

Product Manager:

Arno de Kreij

Signature:… (b)(6) Date: 25/09/2020……..

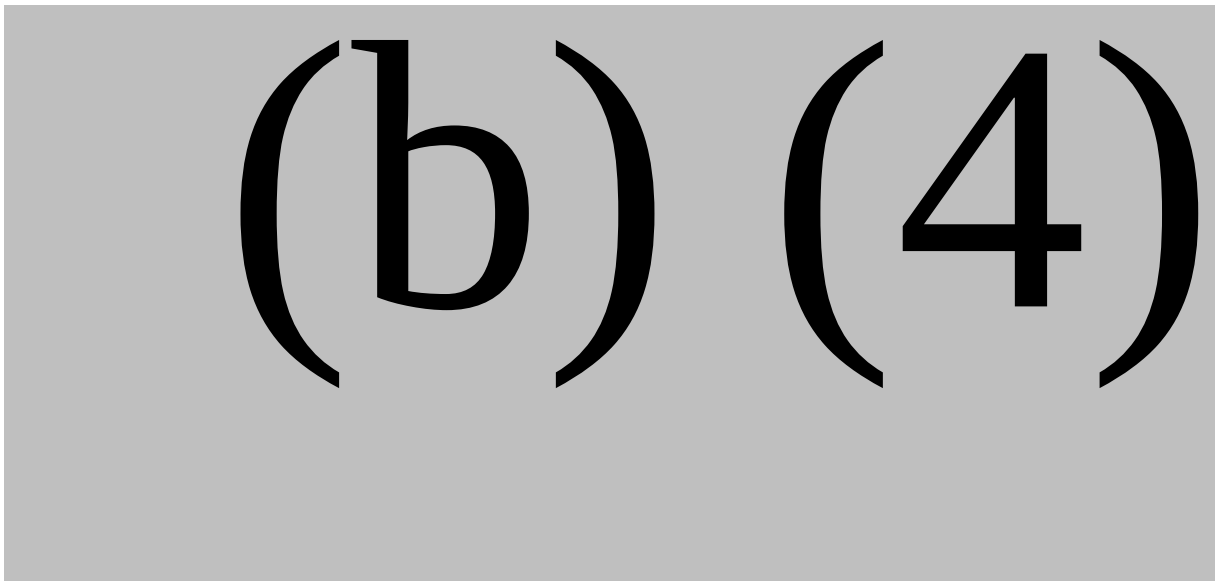
RESPONSIBLE PERSONNEL

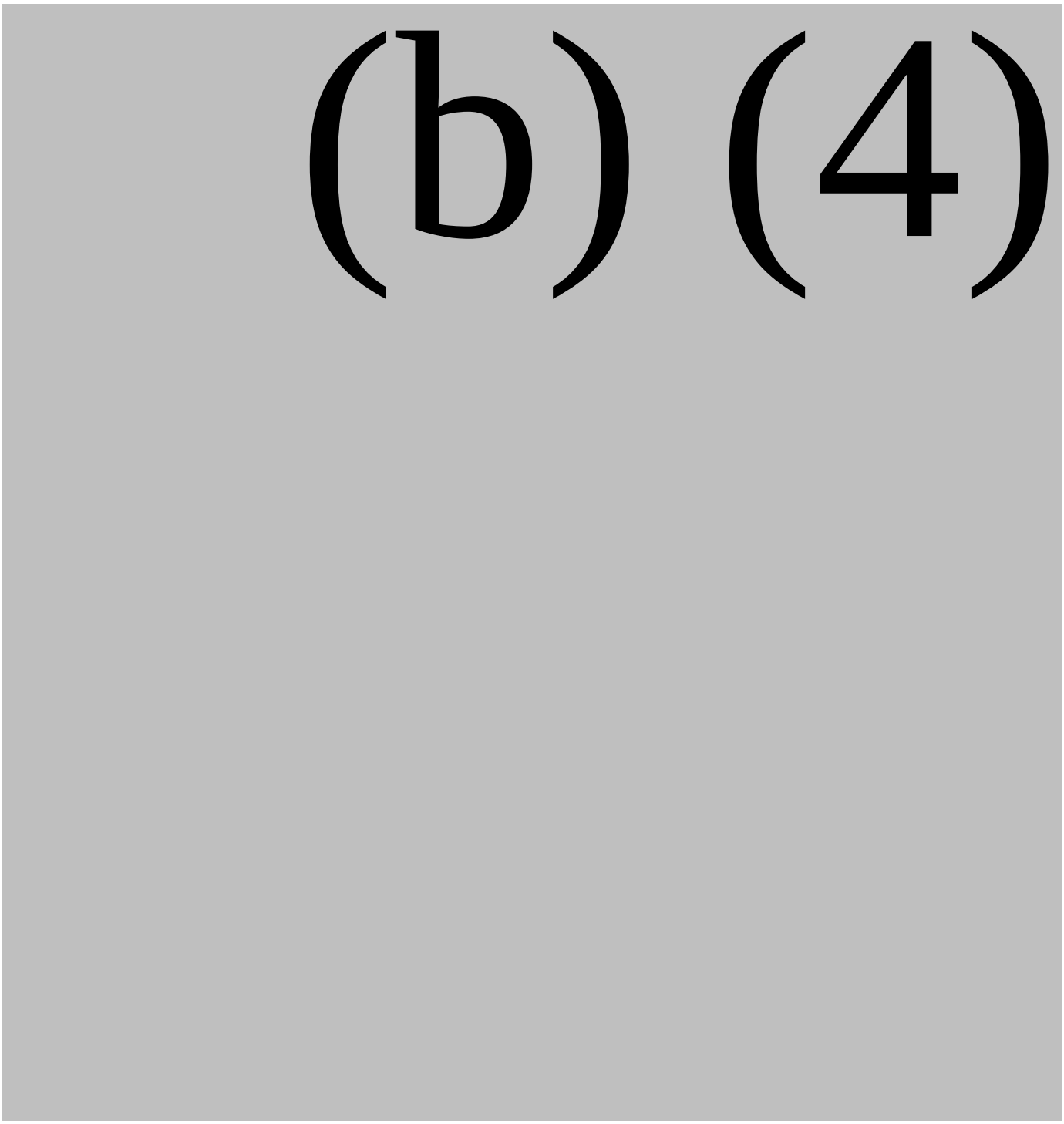
Study scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY

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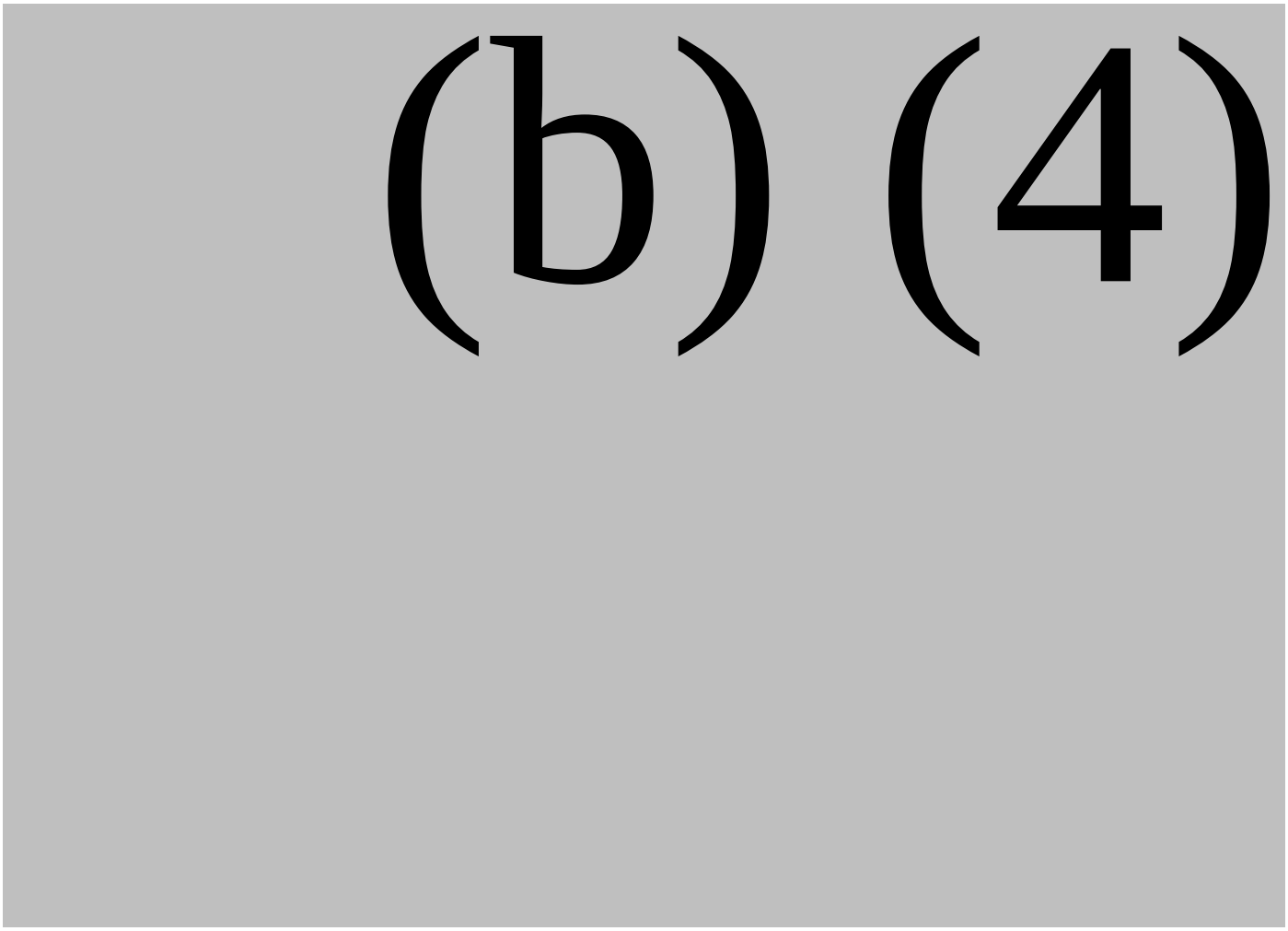
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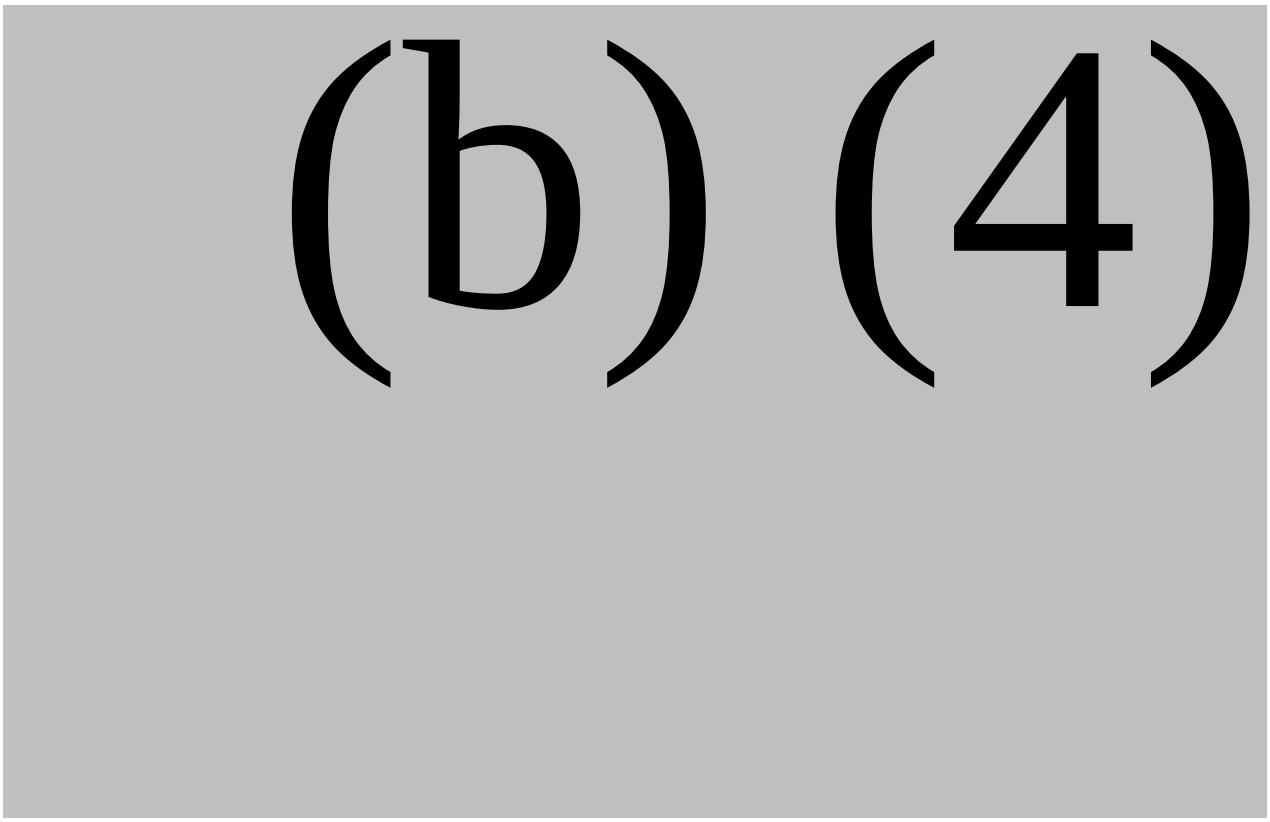
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(b) (4)

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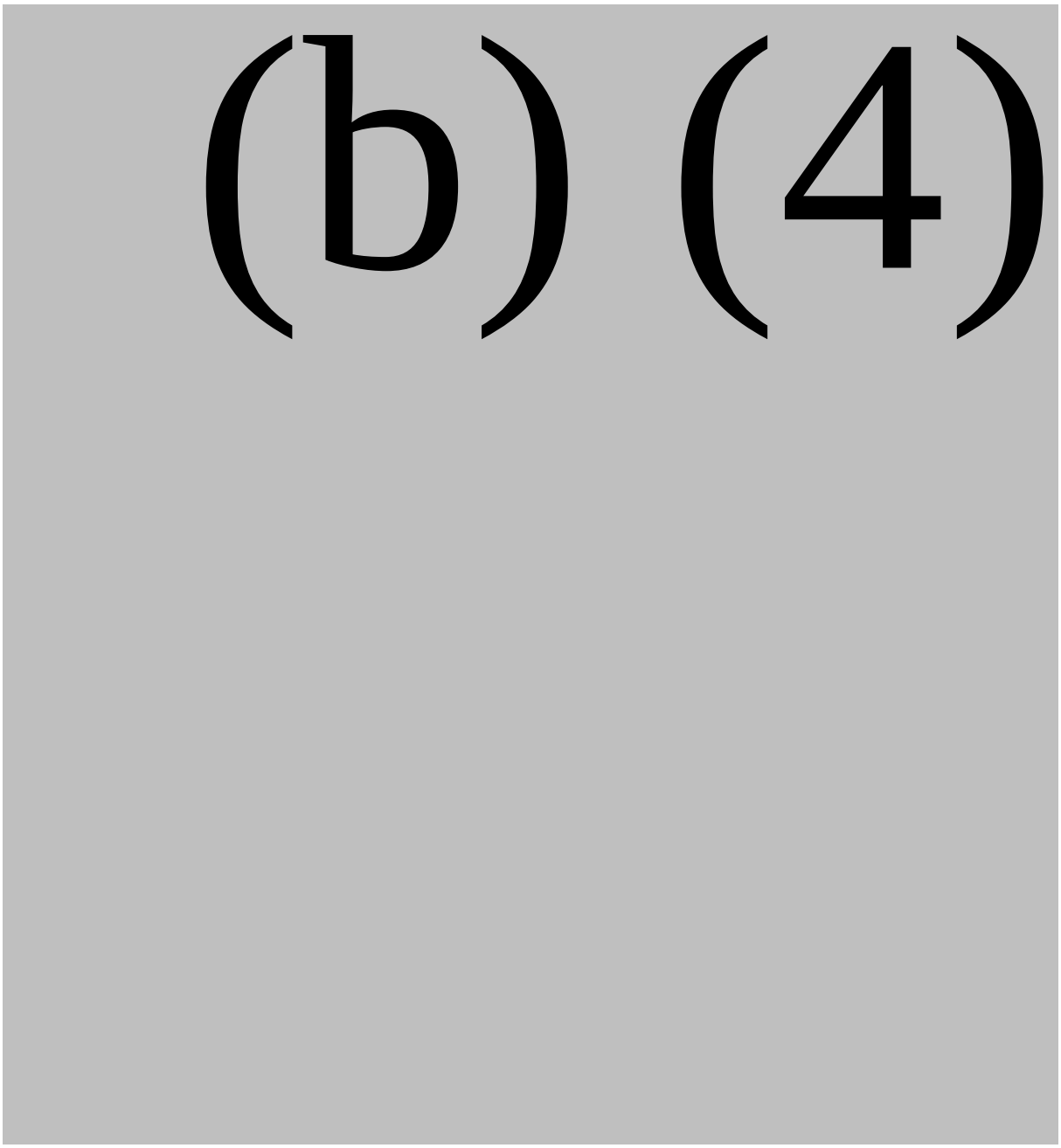
(b) (4)


(b) (4)

[redacted] (b) (4)

Inmengingspercentage: 1.0000%

Naam	Per kg premix		Per kg eindvoer	
[redacted] (b) (4)	[redacted] (b) (4)	kg	[redacted] (b) (4)	kg
		I		I
		IE		IE
		IE		IE
		IE		IE
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mg		mg
		mcg		mcg
		mcg		mcg
		mg		mg
		mg		mg
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		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		g		g
		KCal		KCal
		g		g
		g		g
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		g		g
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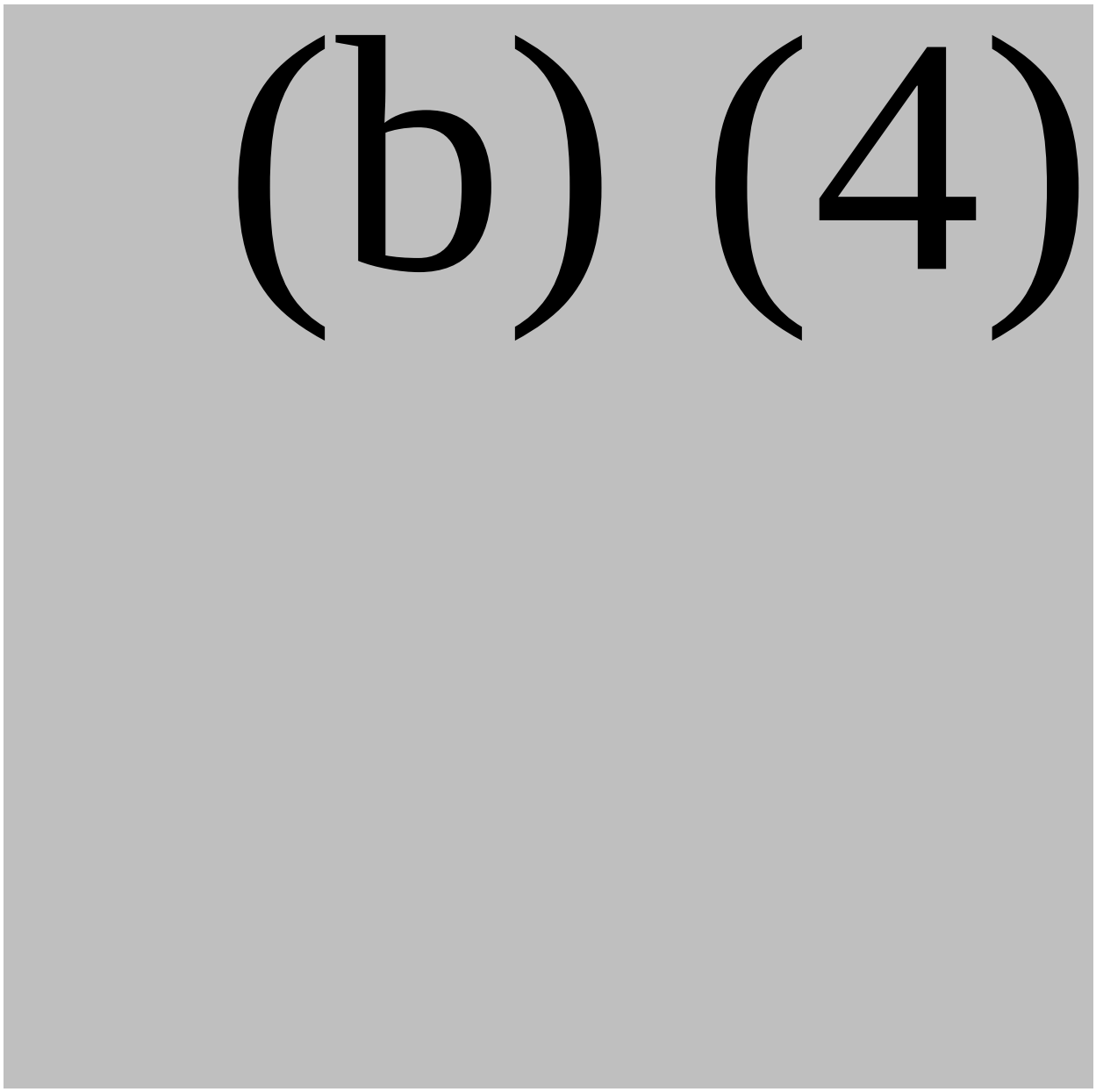
		g		g

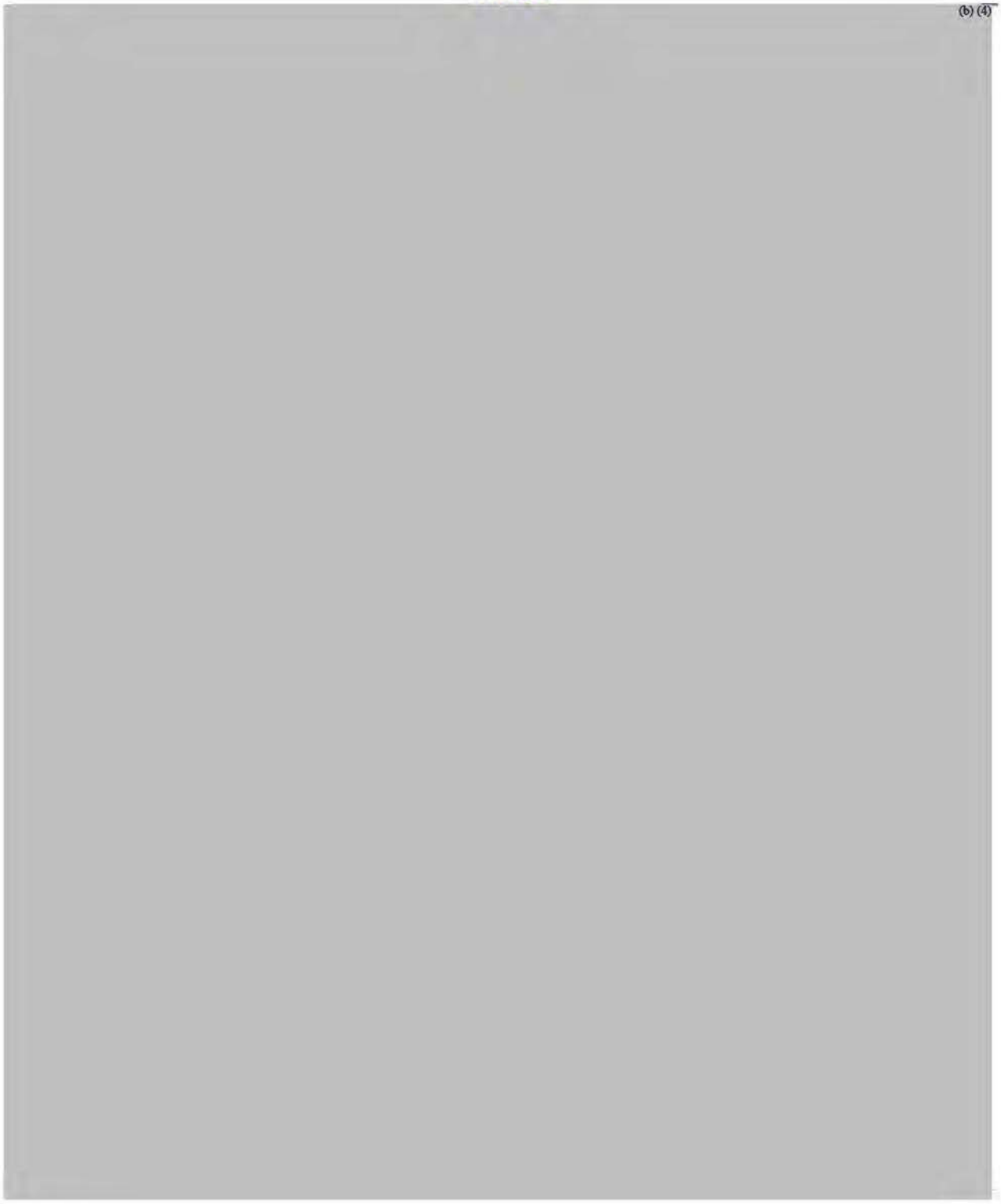
[redacted] (b) (4)

[redacted] (b) (4)


(b) (4)

Analyse

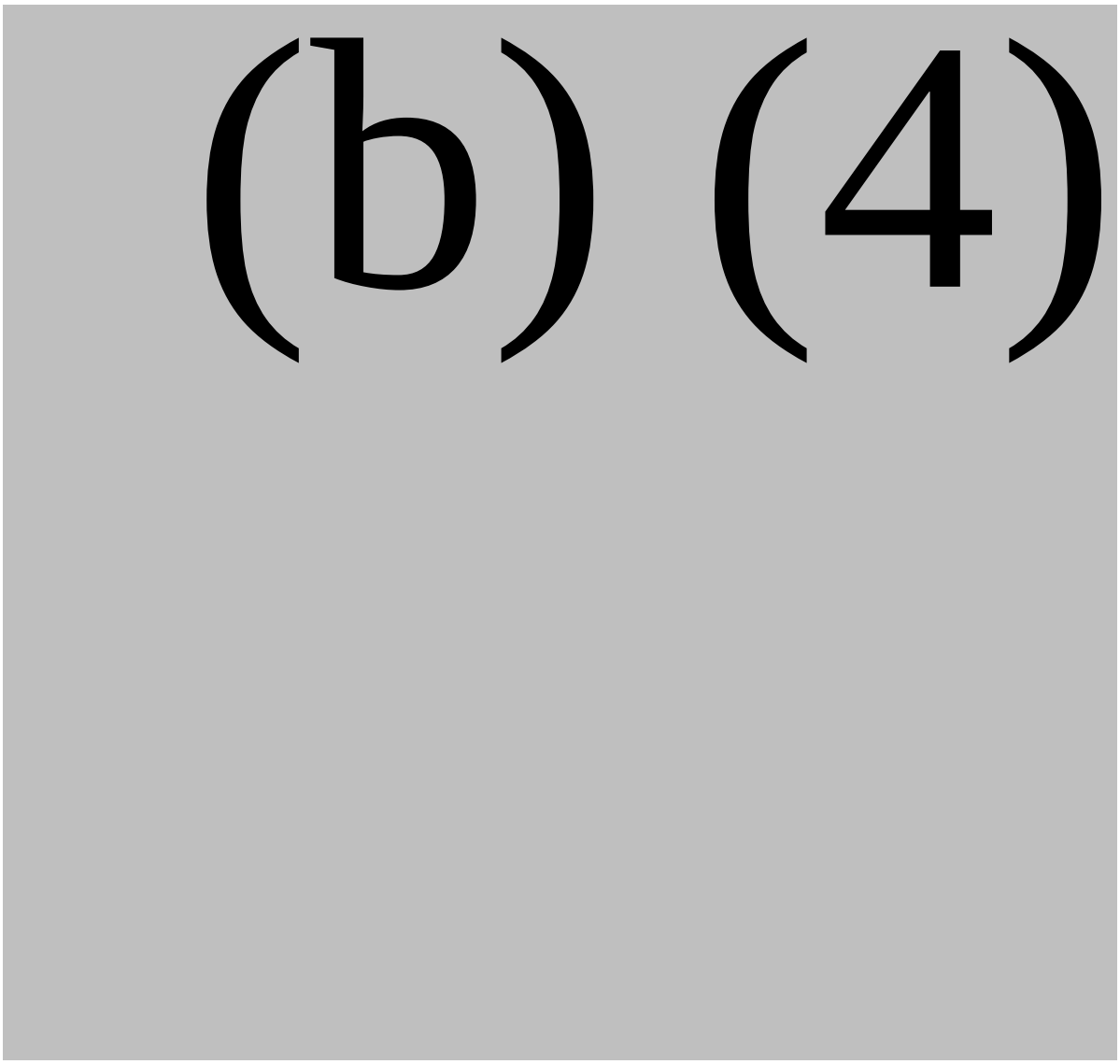
(b) (4)



(b) (4)

(b) (4)

(b) (4)



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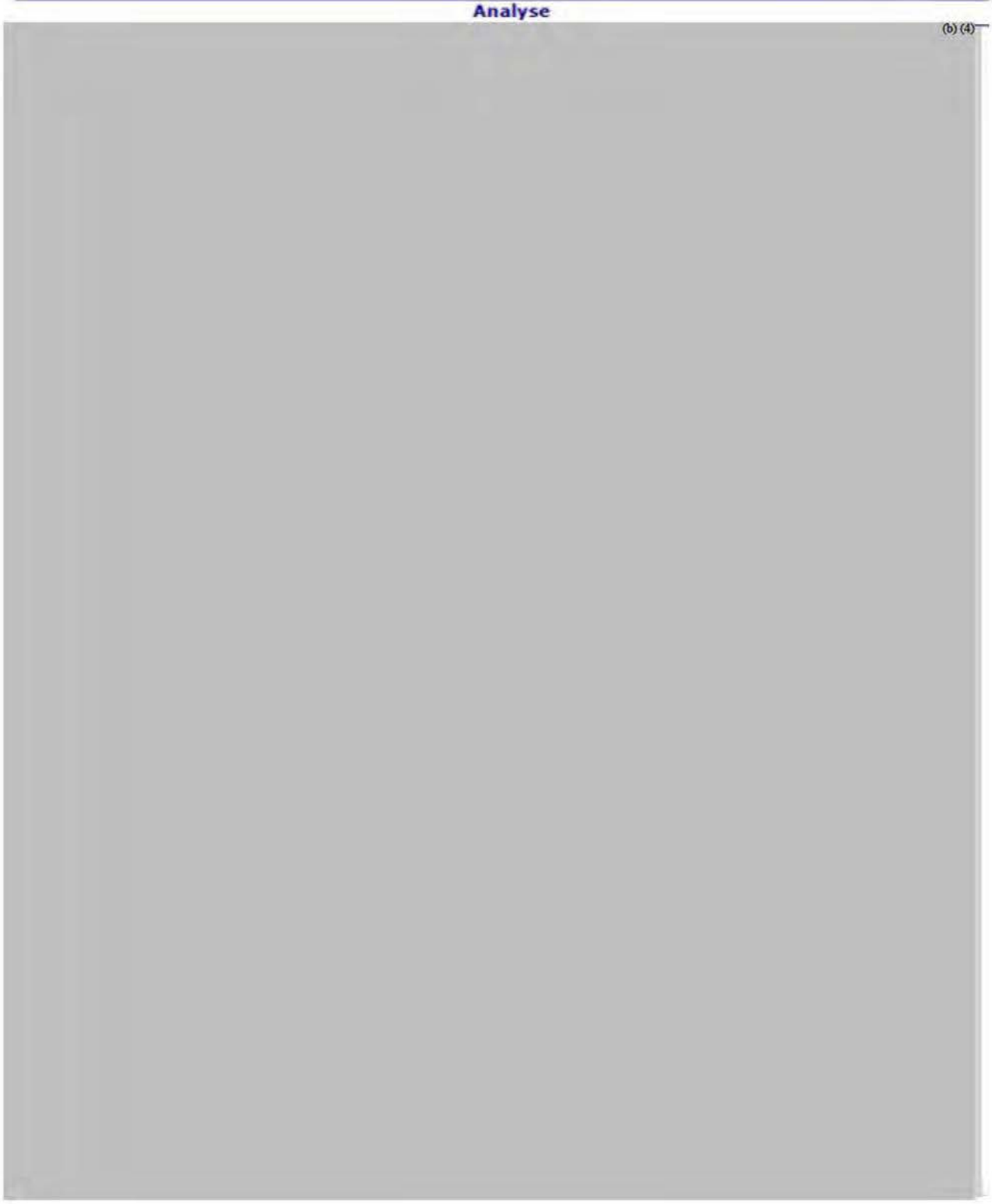


(b) (4)

(b) (4)

(b) (4)

REPORT

ID: 23566-2/2020

Homogeneity and Stability of "Axtra® PHY GOLD 30 T" in mash feed for Poultry, stored for 3 months at (b) (4)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs:

Alessandra Tossoli

Signature: (b) (6) …… Date: 5/10/2020

Study Scientist:

(b)(6)

Signature:… (b) (6) … Date: 5/10/2020

This report is approved by:

Product Manager:

Arno de Kreij

Signature:… (b) (6) .. Date: 5/10/2020

(b) (4)

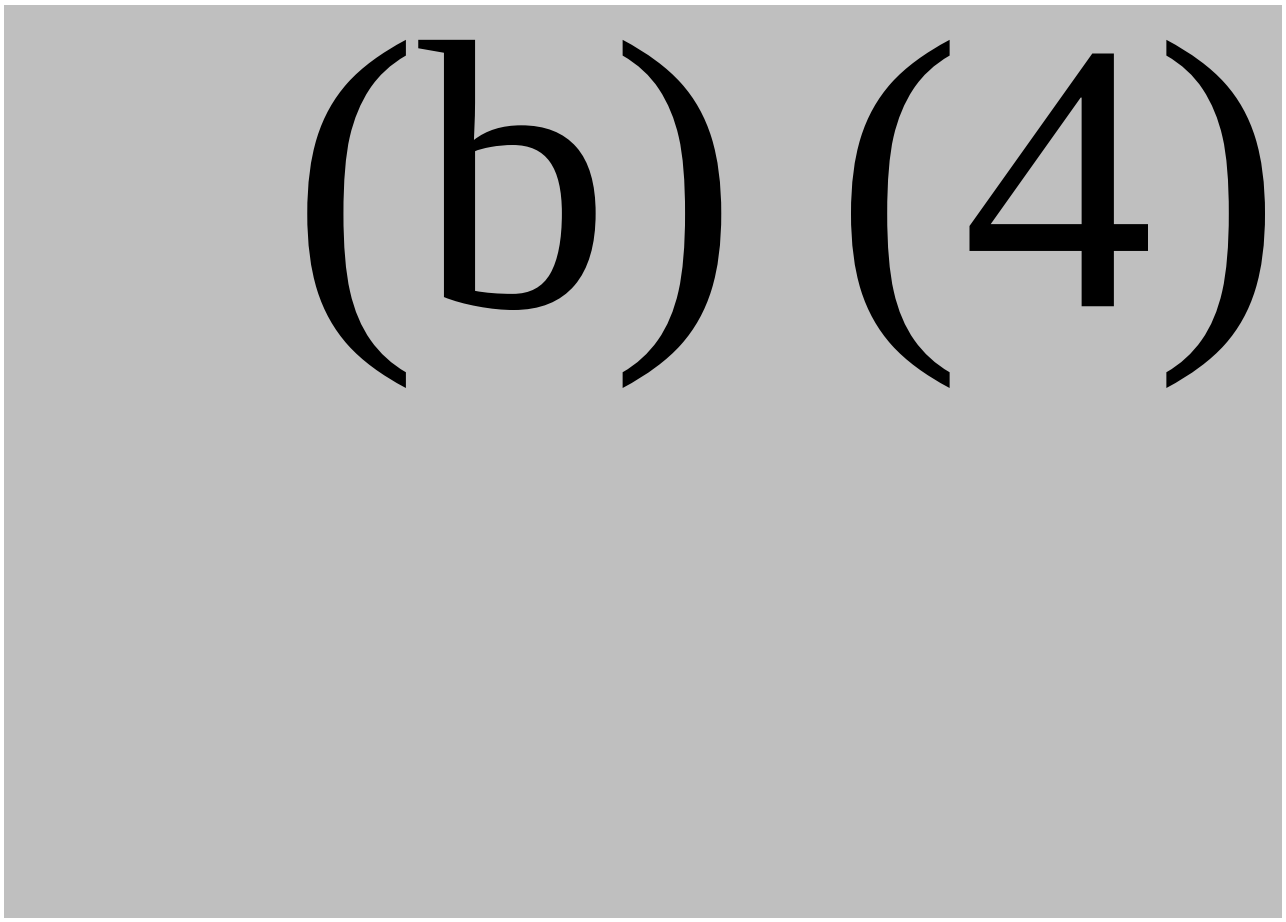
RESPONSIBLE PERSONNEL

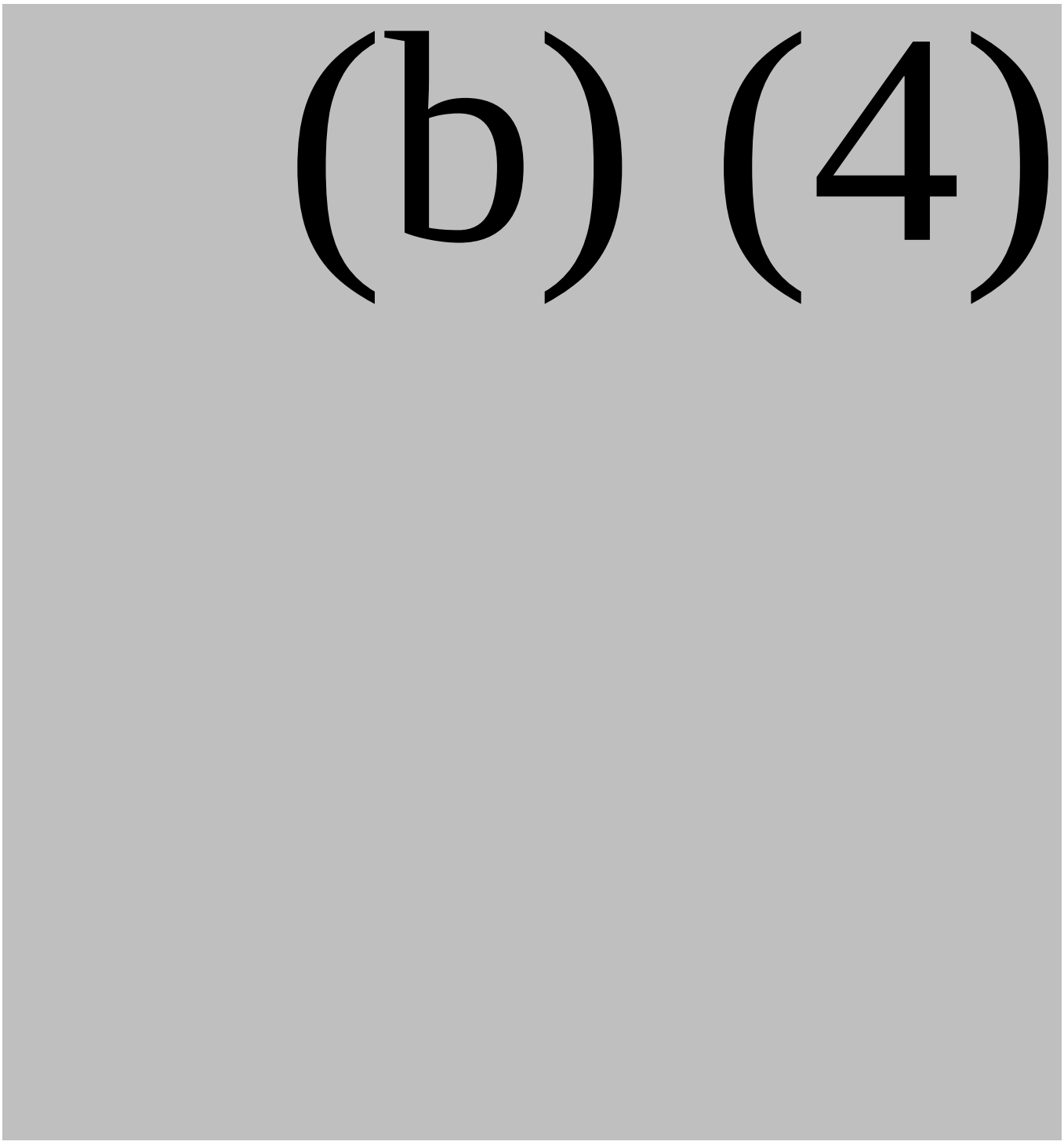
Study scientist(s)	:	(b)(6)
Product manager	:	Arno de Kreij
Regulatory affairs	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY

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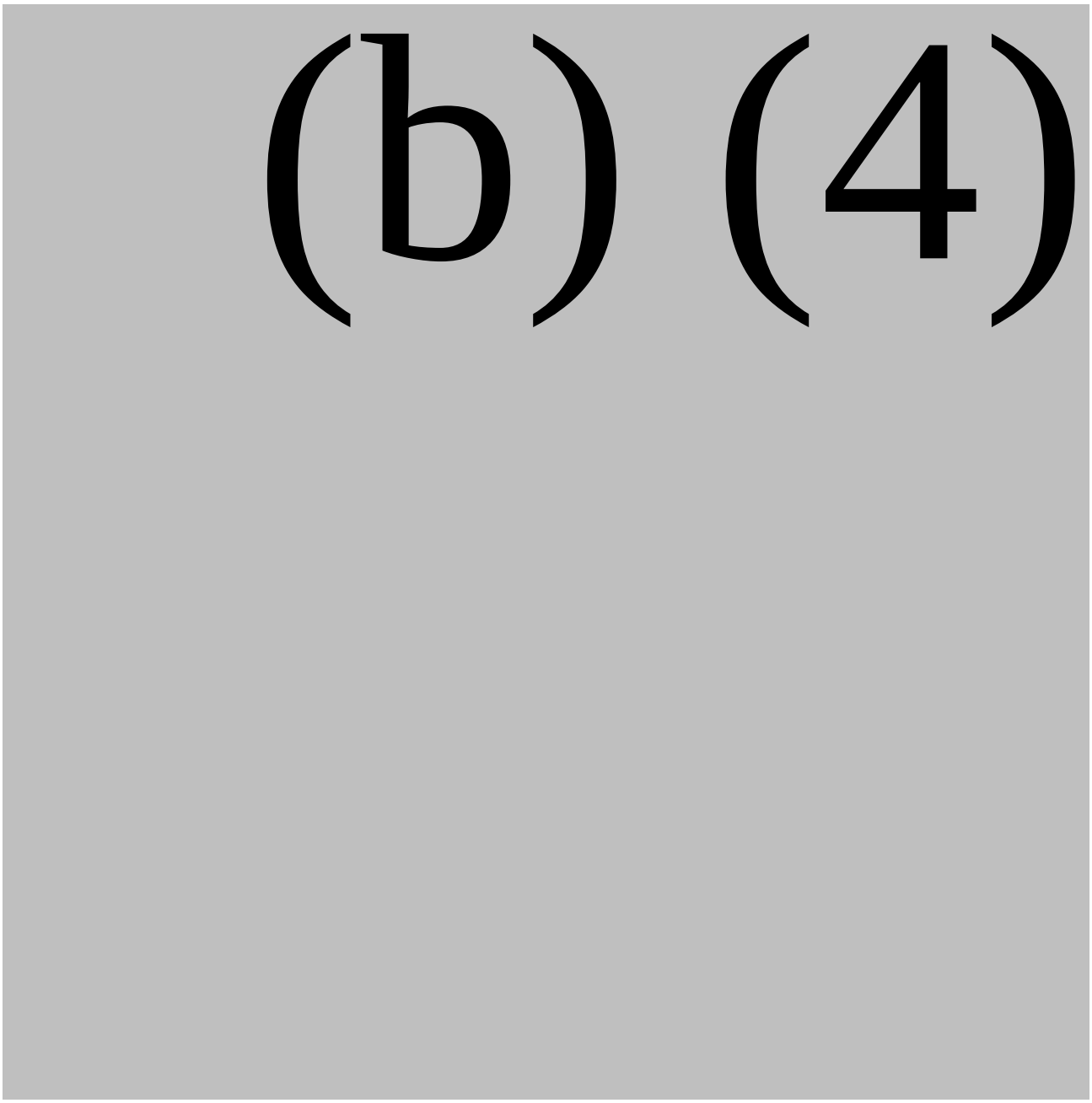
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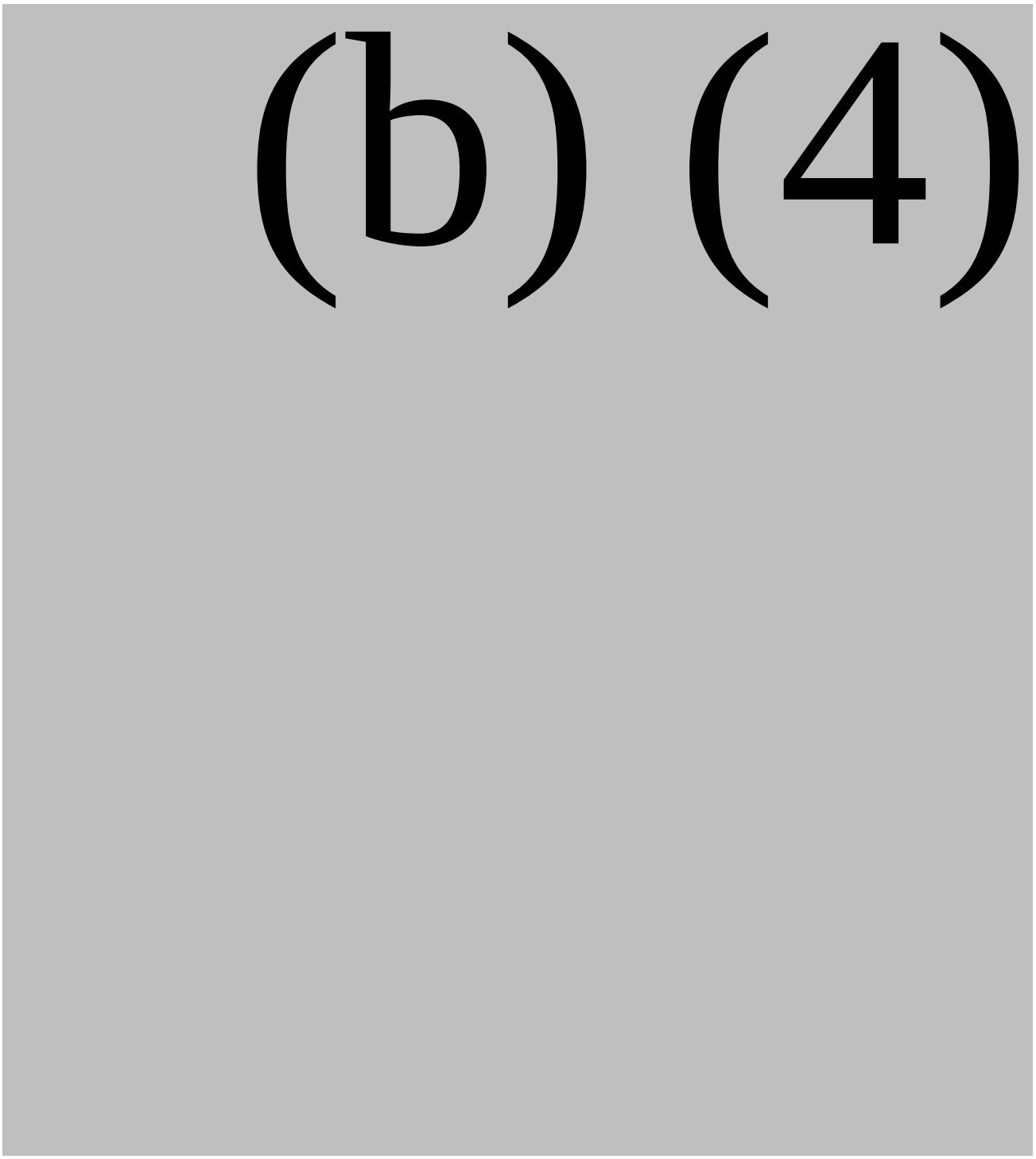
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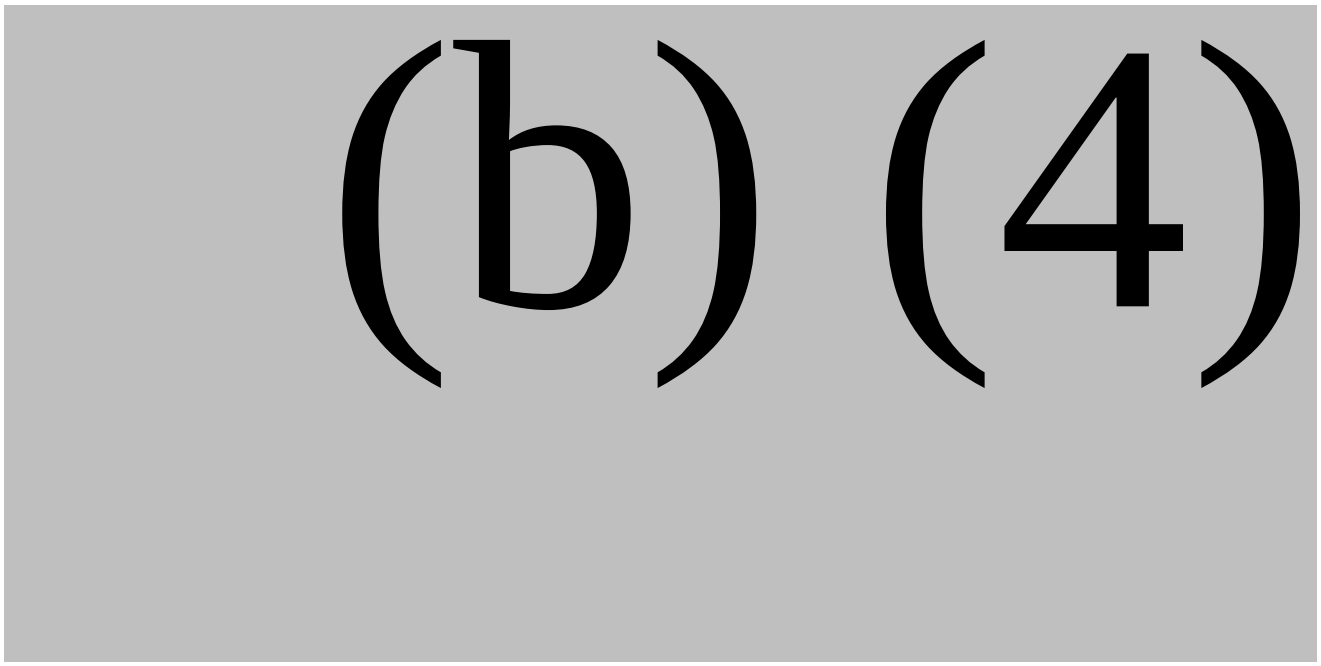
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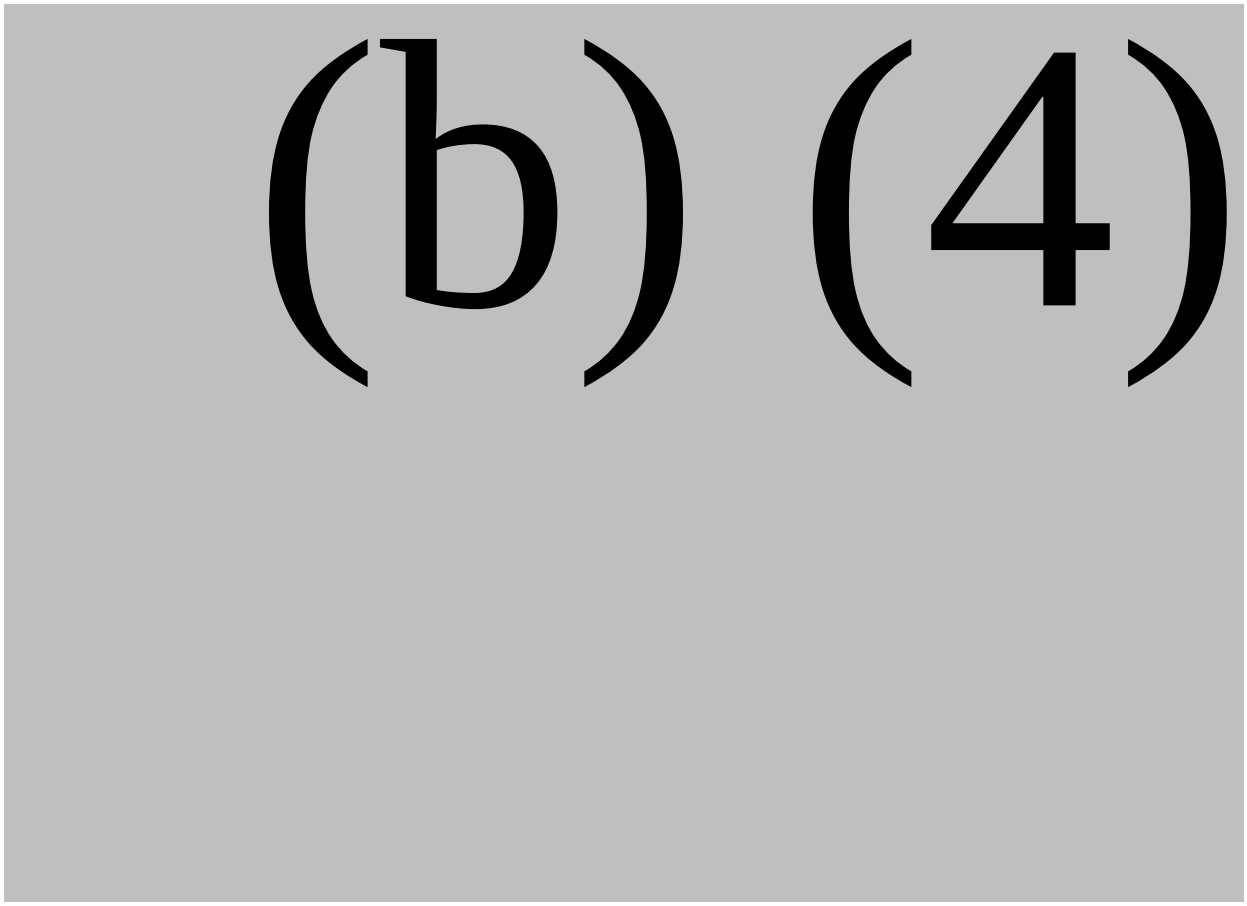
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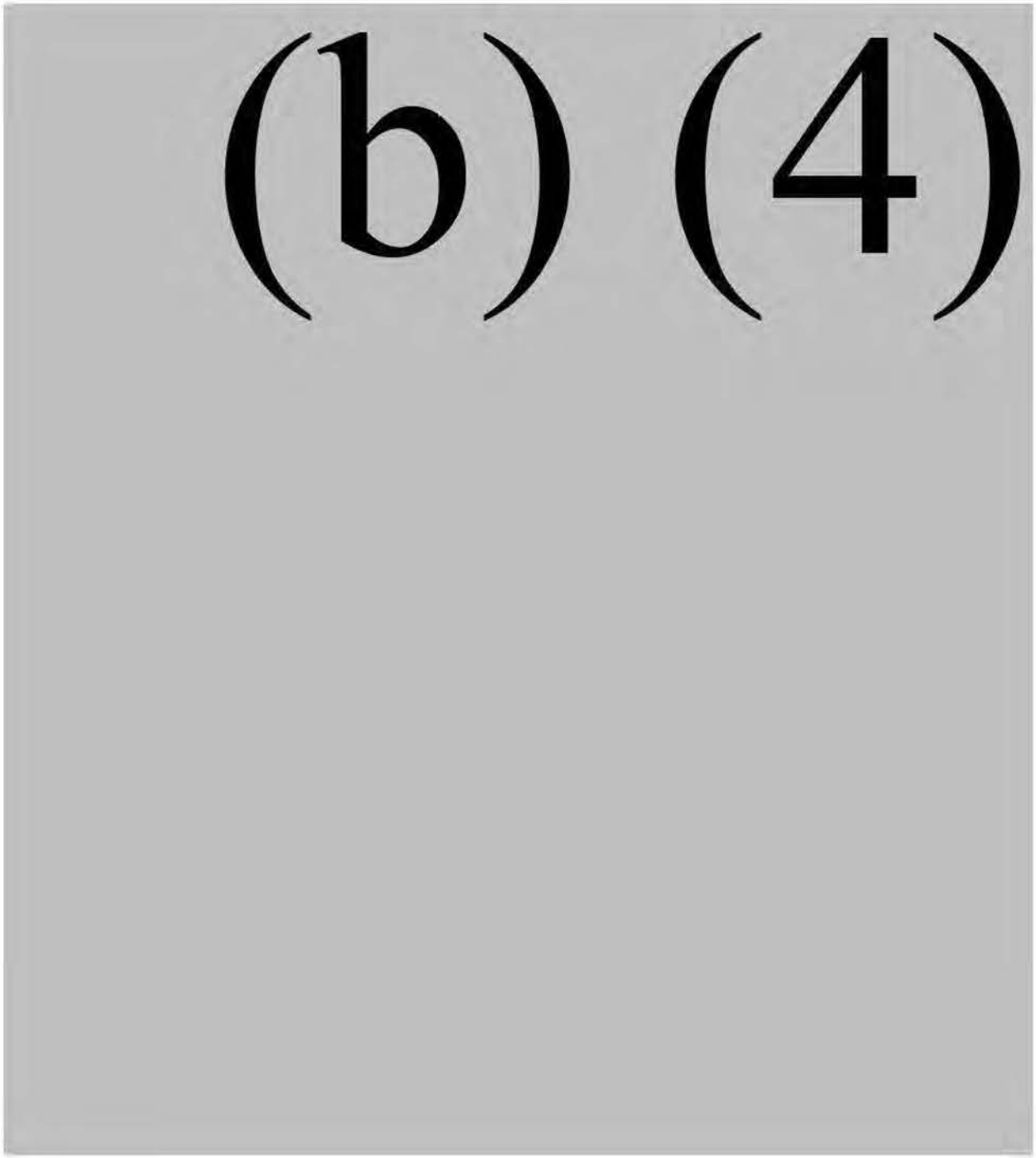
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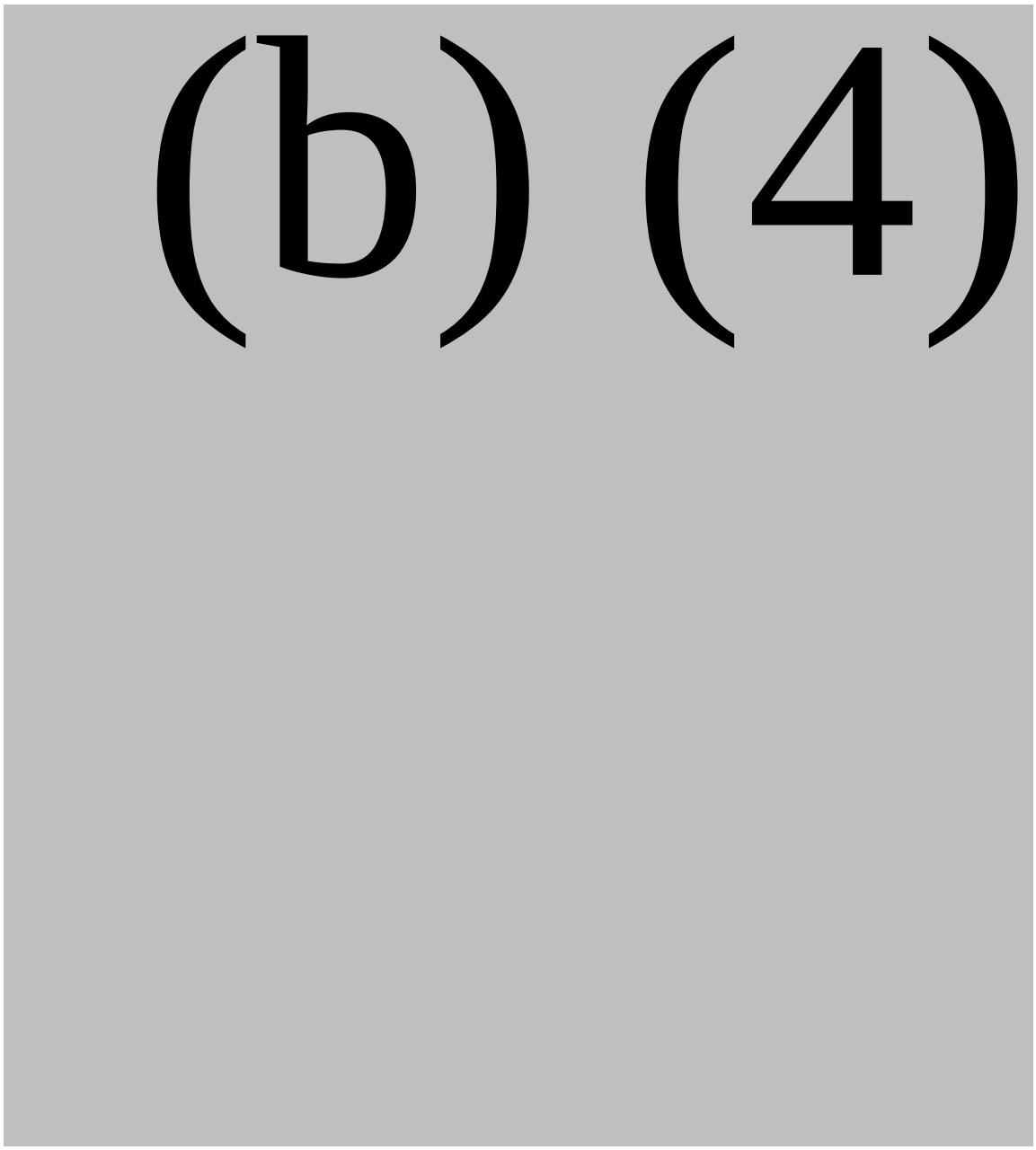

(b) (4)

[redacted] (b) (4)

Inmengingspercentage: 1.0000%

Naam	Per kg premix		Per kg eindvoer	
(b) (4) [redacted]	(b) (4) [redacted]	kg	(b) (4) [redacted]	kg
		l		l
		IE		IE
		IE		IE
		IE		IE
		mg		mg
		mg		mg
		mg		mg
		mg		mg
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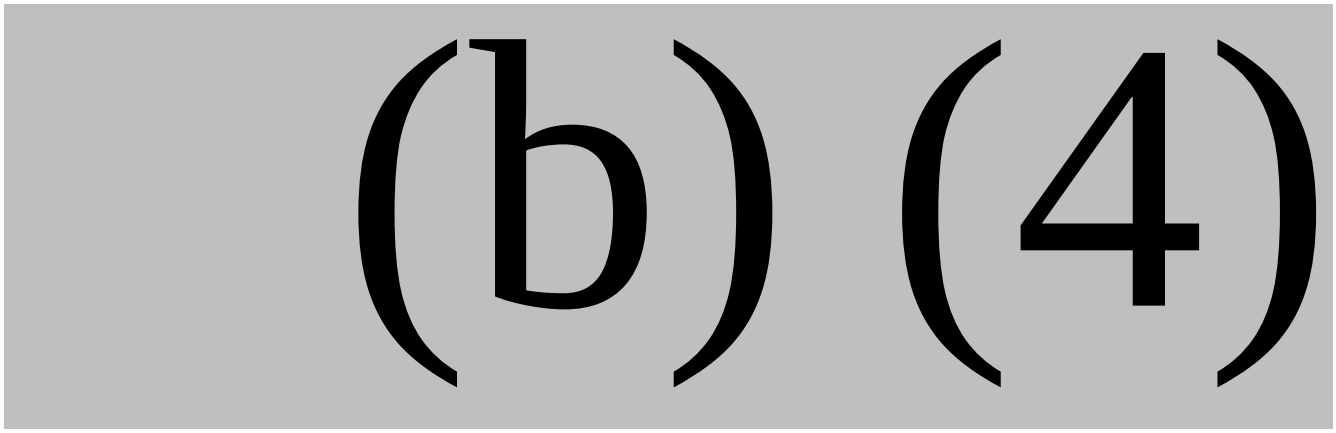
(b) (4) [redacted]

[redacted] (b) (4)



REPORT

ID: 23567-4/2020

Homogeneity and Stability of "Axtra® PHY GOLD 65 G" in mash feed for Swine, stored for 3 months at (b) (5)

Author: A. Tossoli

Study completed: July 2020

Test Laboratories:

DuPont Nutrition & Biosciences
Animal Nutrition
Feed Scientific Business Support
Edwin Rahrs Vej 38
DK-8220 Brabrand
Denmark

(b) (4)

CONTENTS:

LABORATORY PRACTICE

Protocol Deviations:

There are no protocol deviations, or other events that were considered to have affected the outcome of the study.

Acceptance of the Report:

I hereby declare that this report is an accurate reflection of the study conducted.

Regulatory Affairs Manager:

Alessandra Tossoli (b)(6)

Signature: (b)(6) ..... Date: 29/09/2020

Study scientist:

(b)(6)

Signature:... (b) (6) ... Date: 27/09/2020

This report is approved by:

Product Manager:

Arno de Kreij

Signature:... (b) (6) Date: 28/09/2020

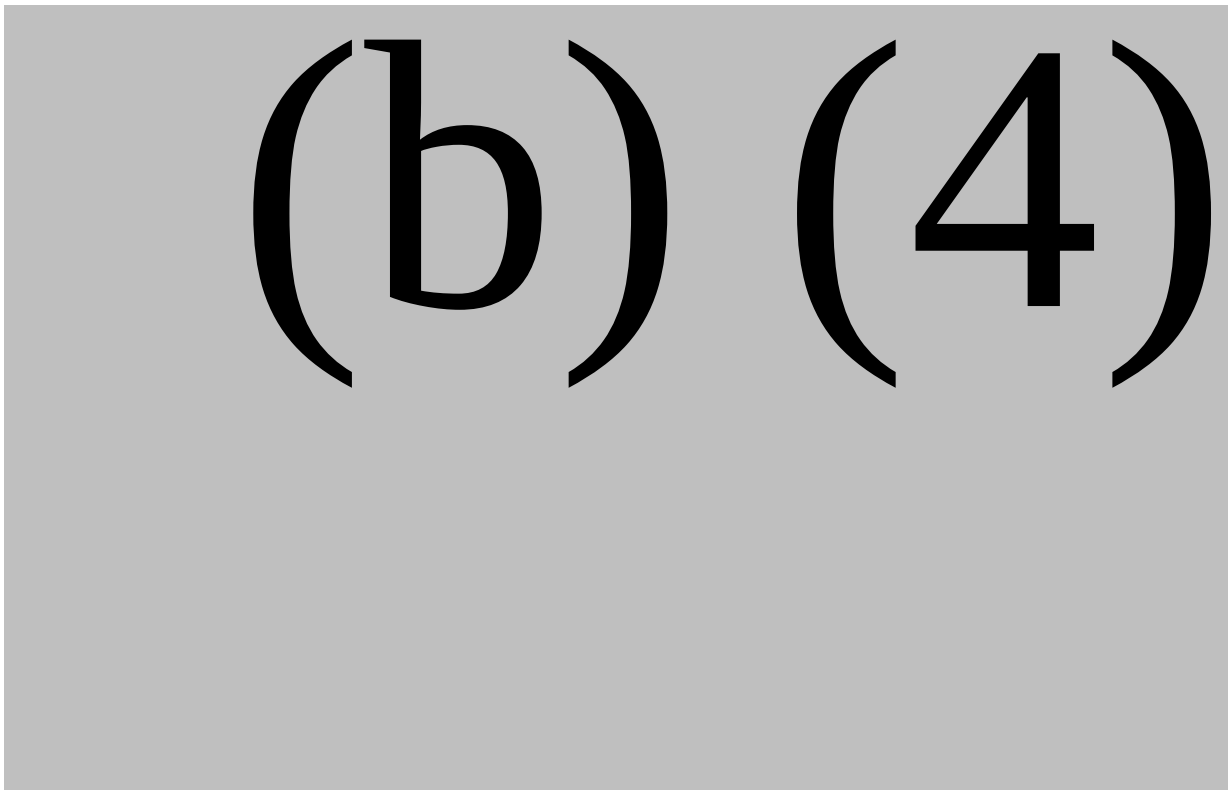
RESPONSIBLE PERSONNEL

Study Scientist(s)	:	[redacted]
Product manager	:	Arno de Kreij
Regulatory manager	:	Alessandra Tossoli

ARCHIVES

All raw data and documentation, a copy of the protocol and the final report will be retained for a period of ten years in the Danisco Animal Nutrition Archive under the appropriate reference.

1. SUMMARY



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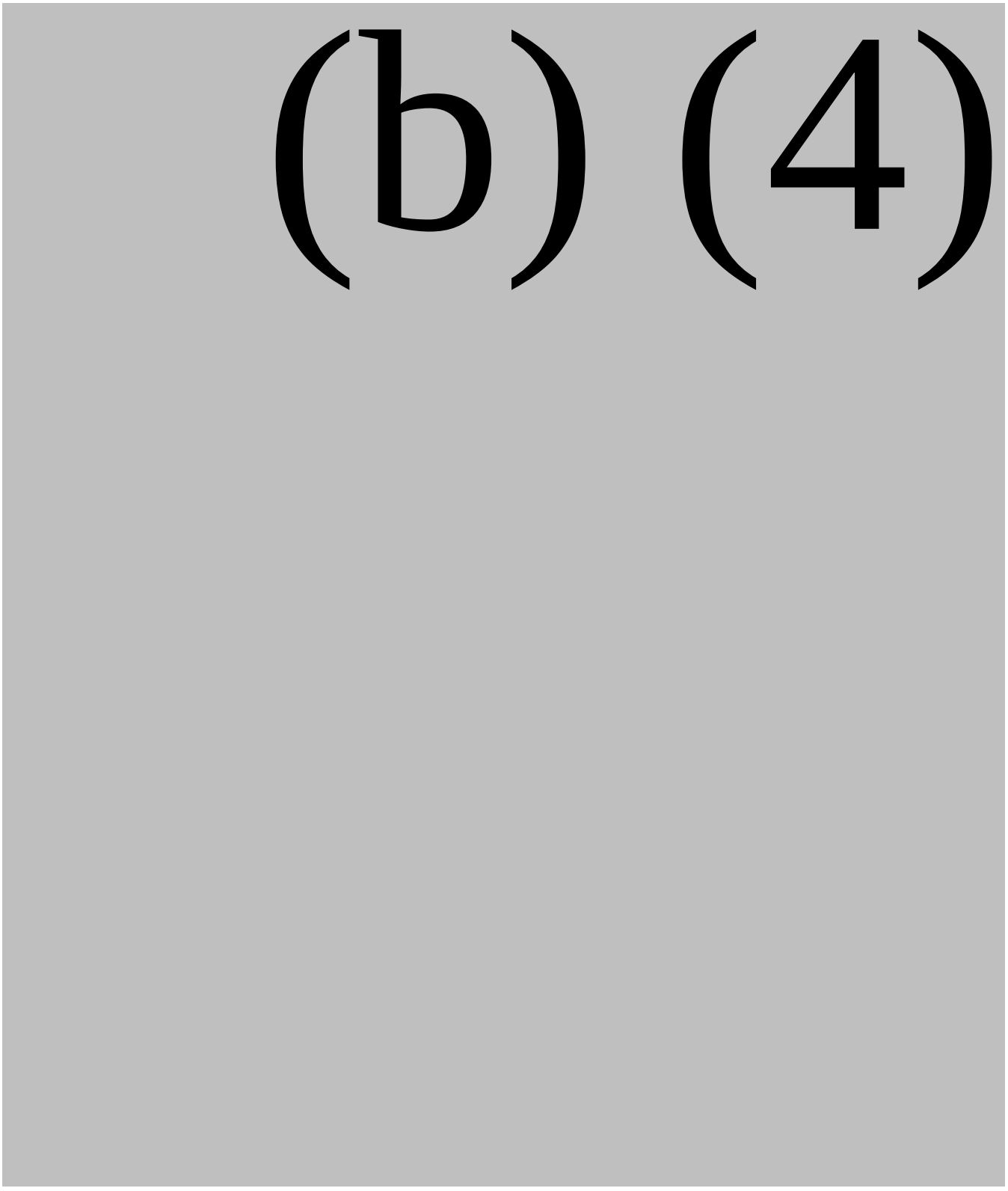
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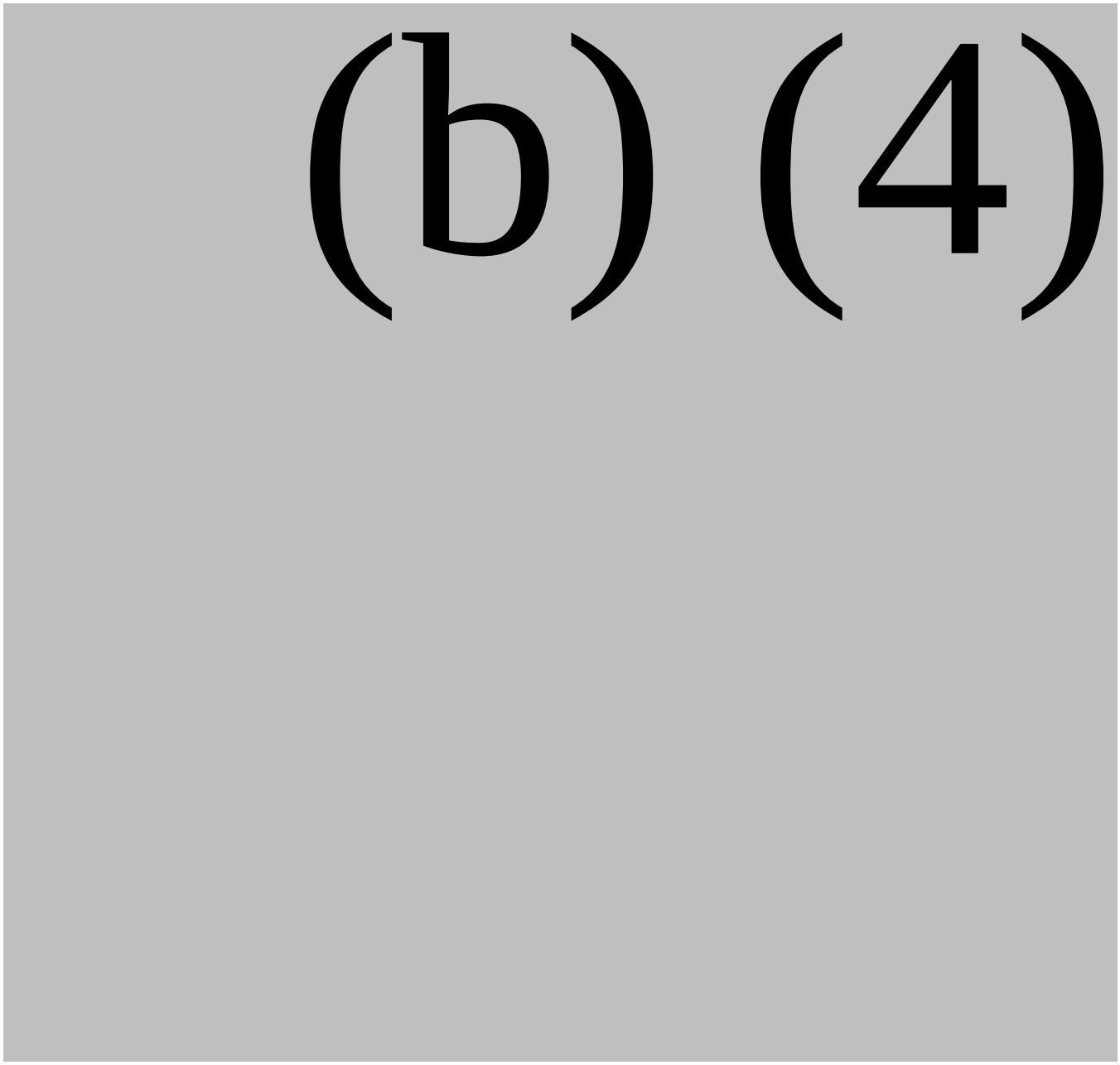
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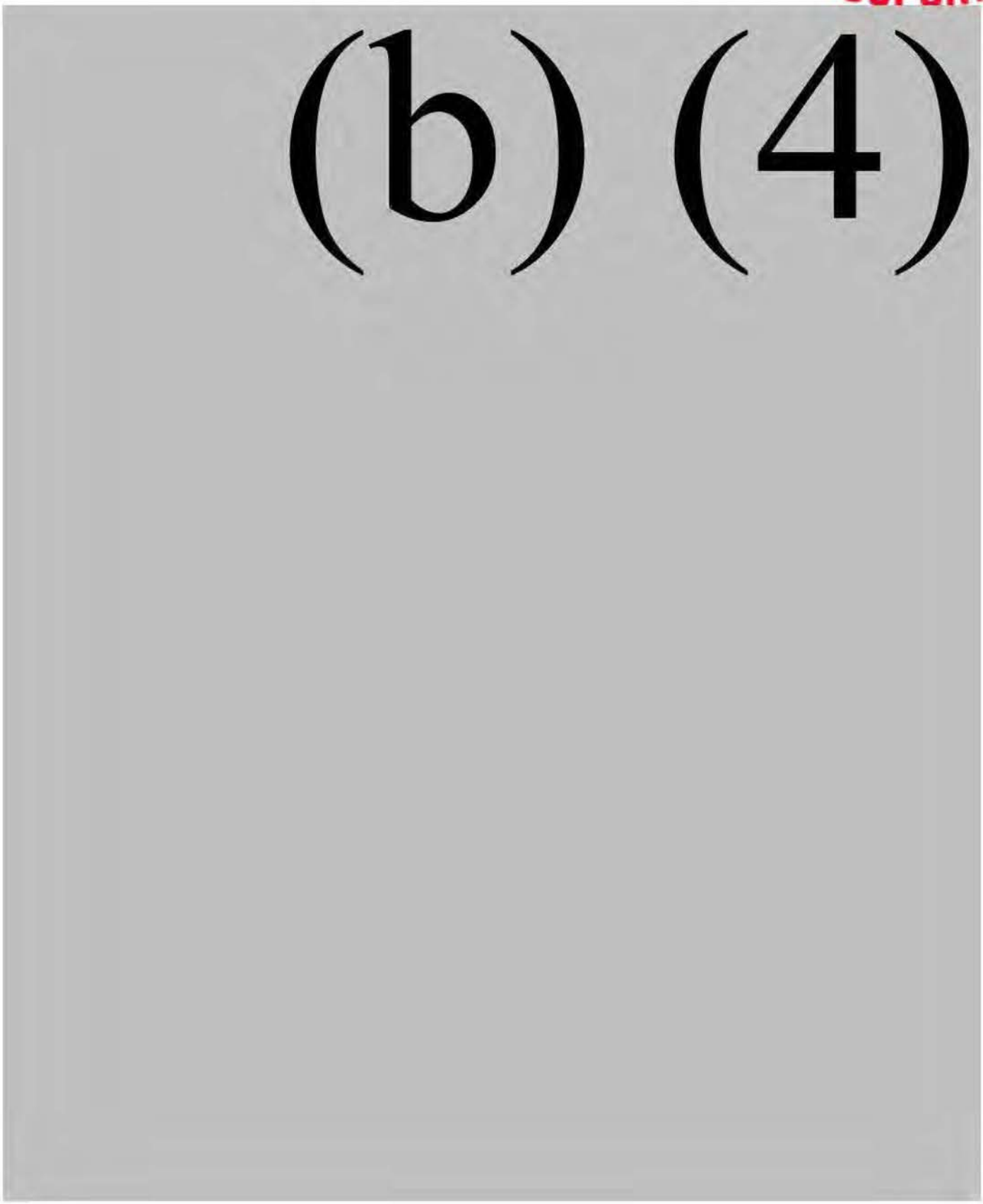

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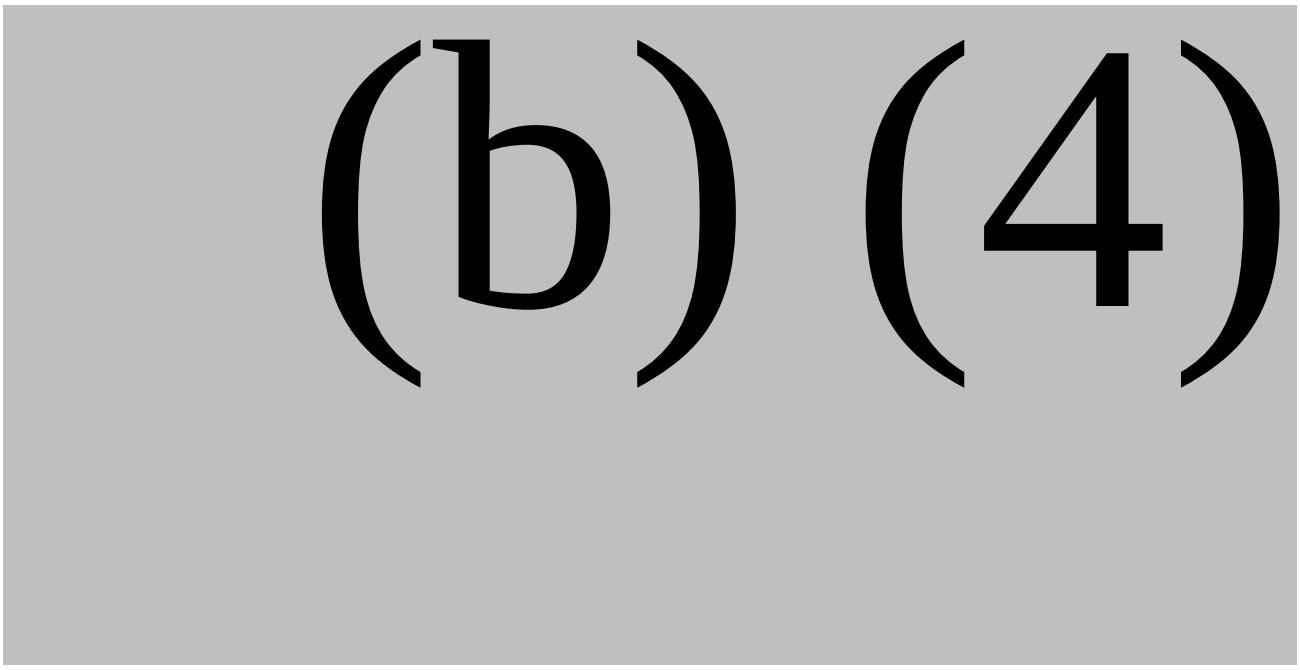

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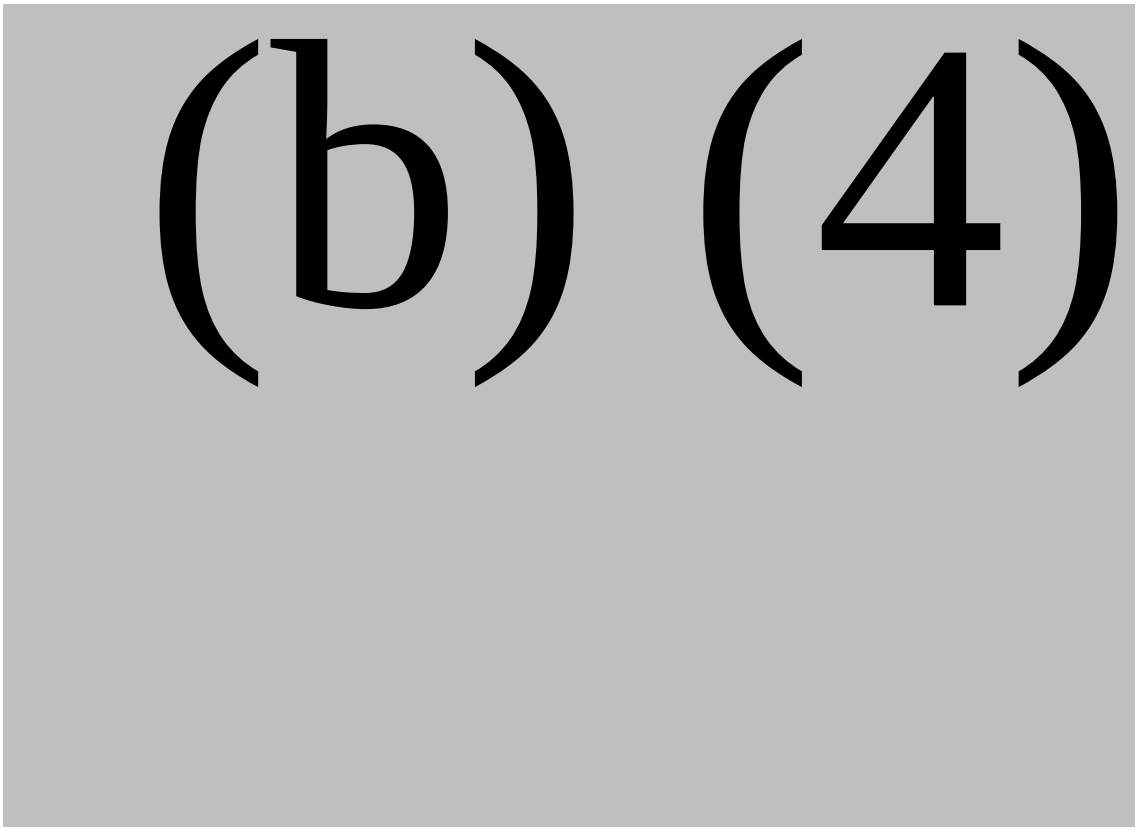
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Analyse

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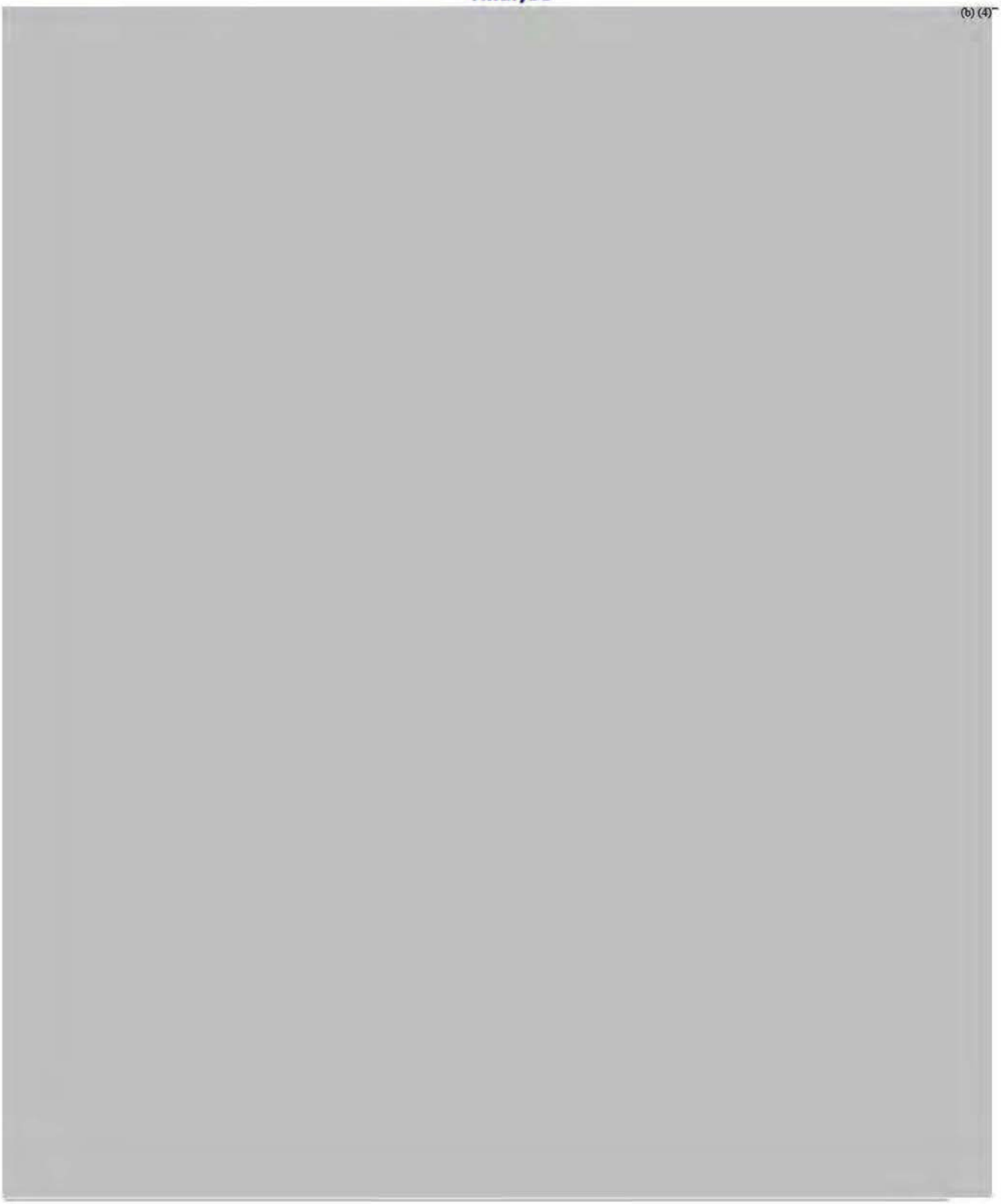

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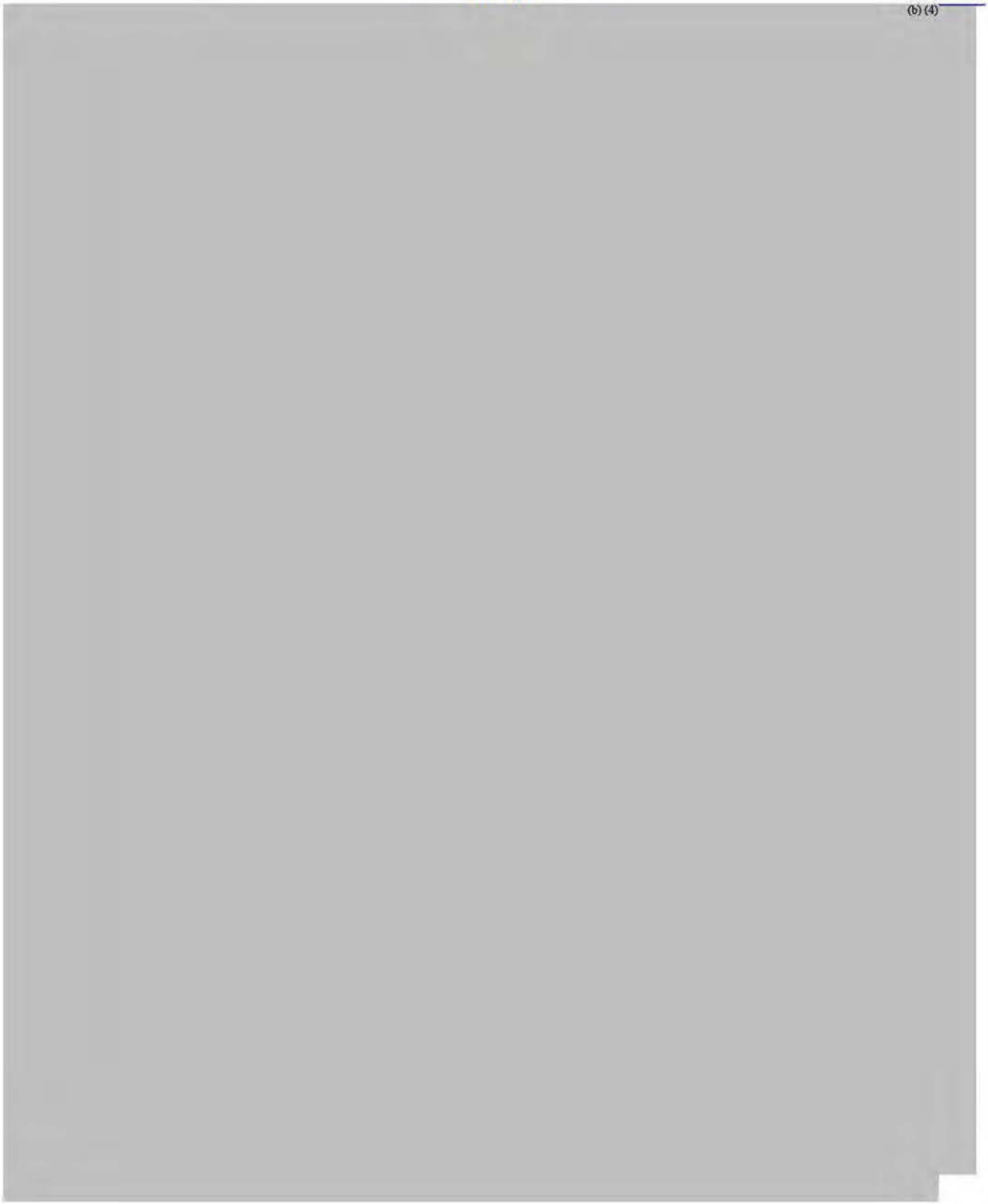
Analyse



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CONFIDENTIAL

Genencor International B.V.
Archimedesweg 30
2333 CN Leiden
The Netherlands

Open Reading Frame (ORF) analysis

(b) (4)

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Expression cassette insertion

(b) (4)

(b) (4)

ORF analysis

(b) (4)

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(b) (4)

CONFIDENTIAL

Genencor International B.V.
Health & Biosciences
Willem Einthovenstraat 4
2342 BH Oegstgeest
The Netherlands

Open Reading Frame (ORF) analysis

(b) (4)

Introduction

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Methods

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(b) (4)

(b) (4)

(b) (4)

Identification of Novel ORFs

(b) (4)

Theoretical peptide sequence

(b) (4)

Toxin Homology Search

(b) (4)

Allergen Homology Search

(b) (4)

(b) (4)

Results and Discussion

(b) (4)

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November 8, 2021

Megan Hall, M.S.
U.S. Food and Drug Administration
Center for Veterinary Medicine
Division of Animal Feeds, HFV-220
12225 Wilkins Avenue
Rockville, Maryland 20852

Where science
& creativity meet

REF: GRAS M000101Z0006

Re: AGRN #43 Phytase from *Trichoderma reesei* fed to increase nutritional availability of phosphate in swine and poultry diets

Dear Ms. Megan Hall,

Thank you for the discussion in the November 5th, 2021 teleconference between the Center for Veterinary Medicine and Danisco US Inc. to discuss questions identified in the GRAS Notice AGRN #43.

In response to the discussion we, Danisco US Inc., would like to formally request CVM to cease to evaluate the GRAS notice #43.

Sincerely,

(b)(6)

Vincent Sewalt, Ph.D.
Head, Regulatory Science & Advocacy
Global Regulatory Affairs
Danisco US Inc.
IFF
925 Page Mill Road
Palo Alto, CA 94304
Cell: (b)(6)
Email: Vincent.sewalt@iff.com