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Paulette M. Gaynor, Ph.D.
Office of Food Additive Safety (HFS-200),
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

April 15, 2019

Re: GRAS Notice for phospholipase A1 produced by a genetically engineered strain of *Aspergillus niger*

Dear Dr. Gaynor:

On behalf of DSM Food Specialties (DSM), I am submitting under cover of this letter one paper copy and one digital copy of a generally recognized as safe (GRAS) notice for a phospholipase A1 enzyme produced by a pure culture of a strain of *Aspergillus niger* (PLN) genetically engineered for more efficient expression of the native gene encoding this enzyme.

The electronic copy is provided on a virus-free CD and is an exact copy of the paper submission.

DSM has determined through scientific procedures that this substance is GRAS when used in the processing of edible crude oils and fats, including vegetable oil (*e.g.*, soybean oil, rapeseed oil, canola oil, sunflower oil), algal oil, animal fats, and fish oil. As such, the notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

If you have any questions regarding this notification, or require any additional information to aid in the review of DSM's conclusion, please do not hesitate to contact me.

Sincerely,

[redacted]

Katherine Vega, Ph.D.
Sr. Manager Regulatory Affairs

GRAS Notice

Phospholipase A1 produced by *Aspergillus niger*

April 2019

DSM Food Specialties
PO Box 1
2600 MA Delft
The Netherlands
Tel: 31 611377088

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1.0 SIGNED STATEMENTS AND CERTIFICATIONS

1.1. Submission of GRAS Notice

DSM Food Specialties (DSM) is hereby submitting a Generally Recognized as Safe (GRAS) notice in accordance with the provisions of 21 CFR part 170, subpart E.

1.2. Name and Address of Notifier

DSM Food Specialties
PO Box 1
2600 MA Delft
The Netherlands

1.3. Name of Notified Substance

The common or usual name of the notified substance is phospholipase A1 (also referred to as PLA1 in this dossier). It consists of phospholipase A1 produced by submerged fed-batch fermentation using a selected, pure culture of *Aspergillus niger*; the strain used to produce PLA1, designated as PLN, was genetically engineered to overexpress the native PLA1. The phospholipase A1 preparation is produced and sold in liquid form, standardized with glycerol.

1.4. Intended Conditions of Use

The phospholipase A1 preparation is intended for use in the processing of edible crude oils and fats, including vegetable oil (*e.g.*, soybean oil, rapeseed oil, canola oil, sunflower oil), algal oil, animal fats, and fish oil. It is not expected to be present in the finished refined oil or fat and is therefore best characterized as a processing aid.

1.5. Statutory Basis for GRAS Conclusion

DSM's GRAS conclusion is based upon scientific procedures in accordance with § 170.30(a) and (b).

1.6. Exclusion from Premarket Approval Requirements

The notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, based on the conclusion by DSM that the phospholipase A1 is GRAS under the conditions of its intended use in the processing of edible oils and fats.

1.7. Availability of Information for FDA Review

The complete data and information that are the basis of the GRAS conclusion will be made available to FDA. Upon request, DSM will provide access to review and copy the data during

customary business hours at its facility in Parsippany, New Jersey, or, upon request, will provide copies in electronic format or on paper.

1.8. Exemptions from FOIA Disclosure

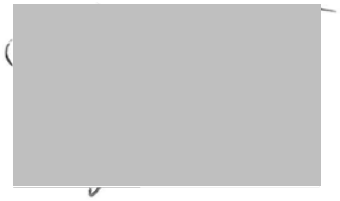
This notification does not contain confidential data or proprietary information, and therefore no FOIA exemptions are claimed.

1.9. Authorization to share trade secrets with FSIS

DSM does not anticipate that FSIS consultation will be required. However, should FDA find the need to share the information in this application with FSIS, DSM has no objections.

1.10. Certification

On behalf of DSM Food Specialties, I certify that, to the best of my knowledge, the GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and DSM Food Specialties, and pertinent to the evaluation of the safety and GRAS status of a phospholipase A1 produced by a genetically engineered strain of *Aspergillus niger* (PLN) for use in the processing of edible oils and fats.



_____

Katherine Vega, PhD
Senior Manager Regulatory Affairs
DSM Nutritional Products North America

2.0 IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1. Identity of Notified Substance

Systematic name:	Phosphatidylcholine 1-acylhydrolase
Other names:	PLA1
Accepted name:	Phospholipase A1
IUPAC/IUB Number:	EC 3.1.1.32
Amino acid sequence:	MFLRREFGAVAALSVLAHAAPAPAPMQRRDISSTVLDNID LFAQYSAAAYCSSNIESTGTTLTCDVGNCPLVEAAGATTID EFDDSSSYGDPTGFIAVDPTNELIVLSFRGSSDLSNWIADLD FGLTSVSSICDGCEMHKGFYEAWEVIADTITSKVEAAVSSY PDYTLVFTGHSYGAALAAVAATVLRNAGYTLDLYNFGQP RIGNLALADYITDQNMGSNYRVTHTDDIVPKLPPELL GYHHFSPEYWITSGNDVTVTTSDVTEVVGVDSTAGNDGTL LDSTTAHRWYTIYISECS
Molecular mass:	32 kDa (298 aa; 279 aa without signal sequence)

2.1.1. Principal Enzymatic Activity

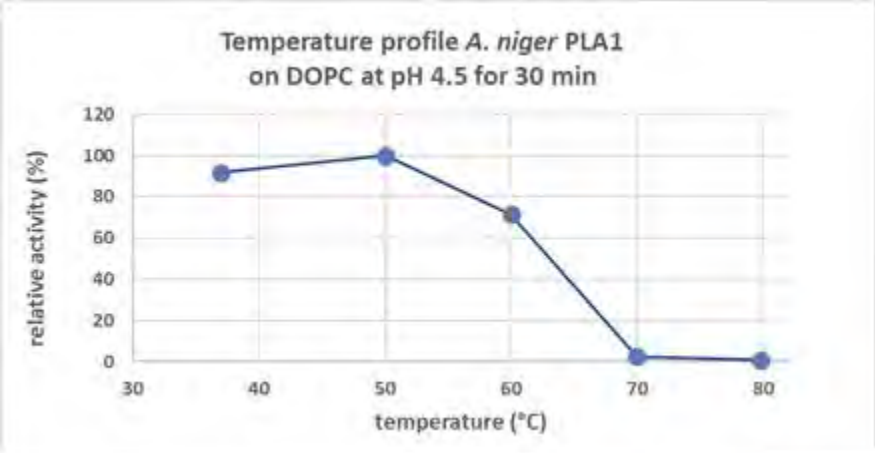
Phospholipase A1 is an enzyme that cleaves phospholipids at the SN1 position, forming lysophospholipids and a fatty acids, according to enzyme classification E.C. 3.1.1.32. Typical substrates include, but are not limited to, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) or phosphatidic acid (PA).

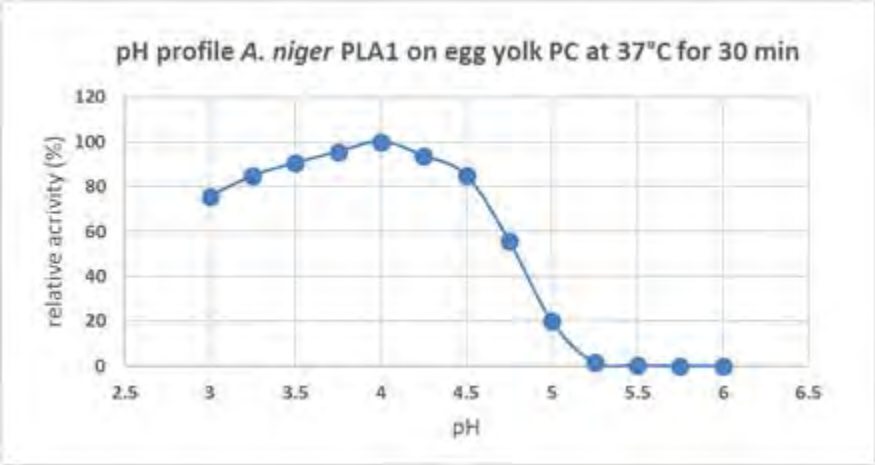
The activity of DSM's PLA1 is determined using a spectrophotometric assay. It uses 1,2-dioleoyl-sn-glycero-3-phosphocholine as substrate. A mix of enzyme and substrate dissolved in acetate buffer (pH 4.5) is incubated at 37ºC. During the 30-minute incubation, free fatty acids (FFA) are produced because of an enzymatic reaction with the substrate. The amount of formed free fatty acids is determined enzymatically with the NEFA kit of WAKO Diagnostics. The PLA1 assay is relative, an enzymatic standard is used. For calibration of the enzyme standard a solution of oleic acid is used as standard to calculate the amount of free fatty acids formed. The determination of the free fatty acid concentration is performed on the Konelab Analyzer and the intensity of the colour is measured at 540 nm.

The activity is expressed in PhosphoLipase A1 Units (PLAU). One PLAU is defined as the amount of enzyme that liberates 1.0 mM free fatty acid/1.4 under the conditions of the assay (see Appendix 1).

2.1.2. Activity under various conditions

The activity of the phospholipase A1 described in this GRAS notice was measured under various pH and temperature conditions. The results are presented in the figures below.





As is evident from these figures, phospholipase A1 from genetically engineered *Aspergillus niger* strain PLN exhibits an optimal activity around pH 3.0 to 4.5, whereas the optimum temperature range is between 40 and 55 °C. No enzyme activity is left at temperatures above 70 °C.

2.2. Specifications for Food-Grade Material

The common starting material of the formulation is the ultra-filtration (UF) concentrate. Typically, its composition falls within the following ranges:

Enzyme activity	280000 - 510000 PLAU/g UF
Water (%)	80 - 95
Ash (%)	0 - 1

Proteins (%) 5 - 15

Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the production microorganism and the fermentation medium. These constituents consist of organic material (proteins, peptides, amino acids, carbohydrates, lipids) and inorganic salts.

To obtain a final formulation, the stabilized food enzyme is formulated as a liquid preparation. To ensure the stability of the enzyme preparation, information about special conditions of storage and/or use appears on DSM's product label and/or other accompanying documentation.

The Total Organic Solids (TOS, calculated as dry matter minus ash) and the activity/TOS ratio of the phospholipase A1 preparation were determined for 3 different batches and are included in the table below.

Batch number	Water (%)	Ash (%)	TOS (%)	Activity (PLAU/g)	PLAU/mg TOS
PLN 618092701	87.2	0.62	12.2	447700	3670
PLN 618095801	91.9	0.31	7.8	287500	3695
PLN 618097901	84.9	0.57	14.6	506500	3474
Mean					3613

The finished product is subjected to extensive controls and complies with the following JECFA and FCC purity specifications:

Parameter	**Norm**
Lead	≤ 5 mg/kg
Coliforms	≤ 30 CFU/g
Salmonella	0 per 25g
Escherichia coli	0 per 25g
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

2.3. Description of the Production Organism

2.3.1. Production organism

The strain used to produce the phospholipase A1 enzyme, *Aspergillus niger* PLN, was genetically engineered by DSM for more efficient expression of the *Aspergillus niger* gene encoding the native enzyme protein. Integration of multiple copies of the phospholipase A1

expression cassette resulted in a strain that produces higher concentrations of phospholipase A1 enzyme.

An ancestor of the production strain PLN has been genome sequenced and identified as *Aspergillus niger* (Pel *et al*., 2007).

2.3.2. Host and donor organism

Aspergillus niger represents both the host and donor organism, since the genes introduced encode the native phospholipase A1.

Aspergillus niger is a fungus that produces large black or brown conidia by phialids [a bottle-shaped structure within or from which conidia (conidiospores) are formed]. The fungus is a saprophyte able to grow on a wide variety of complex substrates. It is ubiquitous in soil and is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles and other decaying vegetation. Consequently, it is also known to naturally occur in foods such as rice, seeds, nuts, olives, and dried fruits.

The formal classification of *Aspergillus niger* strain PLN is as follows:

Kingdom:	FUNGI
Division:	EUMYCOTA
Subdivision:	DEUTEROMYCOTINA
Class:	HYPHOMYCETES
Order:	Moniliales
Family:	Moniliaceae
Genus:	*Aspergillus*
Sub-genus:	Circumdati
Section:	Nigri (= *Aspergillus niger* group)
Species:	*Aspergillus niger*
Strain:	PLN

For several decades, *Aspergillus niger* has been safely used in the commercial production of food substances. For example, industrial production of citric acid by *Aspergillus niger* has taken place since 1919 (Schuster *et al*., 2002). In the U.S. citric acid recovered from *Aspergillus niger* fermentation liquor is GRAS-affirmed under 21 CFR 184.1033.

Since the 1960s, *Aspergillus niger* has also been widely used in the food industry to produce many food enzymes (Bennett, 1985a, 1985b; Schuster *et al.*, 2002). These food enzymes, including those derived from recombinant *Aspergillus niger* strains, have been evaluated by JECFA and many countries that regulate the use of food enzymes, such as the U.S., France, Denmark, Australia, and Canada.

Examples of enzymes derived from *Aspergillus niger* in the U.S. food regulations include carbohydrase and cellulase for use in clam and shrimp processing (21 CFR 173.120) and chymosin preparations to coagulate milk in cheeses and other dairy products (21 CFR 184.1685). As the table below illustrates, there are also several notices in the GRAS Notice Inventory for substances derived through use of classical *Aspergillus niger* strains (*e.g.*, GRN Nos. 89, 111, 132, 750), along with others where *Aspergillus niger* was used as the host and/or donor organism in the development of genetically engineered strains. The U.S. FDA indicated it had no questions about the GRAS conclusion in these notices.

GRN No.	Substance	Date of closure	FDA Response
801	Chymosin enzyme from *Camelius dromedarius* produced in *Aspergillus niger*		Pending
783	*Triacylglycerol lipase* from *Rhizopus oryzae* produced in *Aspergillus niger*		Pending
750	Beta-glucosidase from *Aspergillus niger*		Pending
739	Mannanase enzyme from *Talaromyces leycettanus* produced in *Aspergillus niger*		Pending
703	Alpha-glucosidase from *Aspergillus niger* produced by *Trichoderma reesi*	Nov 9, 2017	FDA has no questions
699	Trehalase from *Myceliophthora sepedonium* produced by *Aspergillus niger*	Nov 13, 2017	FDA has no questions
657	Glucoamylase from *Penicillum oxalicum* produced in *Aspergillus niger*	Nov 23, 2016	FDA has no questions
651	Phospholipase A1 from *Talaromyces leycettanus* produced in *Aspergillus niger*	Nov 23, 2016	FDA has no questions
589	Xylanase from *Aspergillus niger* [carrying a endo-1,4-β-xylanase gene synthesized in vitro from a cDNA coding sequence obtained from *Rasamsonia emersonii*]	Sep 17, 2015	FDA has no questions
510	Acid lactase from *Aspergillus oryzae* expressed in *Aspergillus niger*	Sep 29, 2014	FDA has no questions
428	Asparaginase enzyme preparation from genetically modified *Aspergillus niger*	Nov 26, 2012	FDA has no questions
412	Chitin-glucan from *Aspergillus niger*	Jun 18, 2012	FDA has no questions
402	Peroxidase enzyme preparation derived from a genetically modified strain of *Aspergillus niger*	Nov 23, 2012	FDA has no questions
397	Chitosan from *Aspergillus niger*	Dec 19, 2011	FDA has no questions
345	Carboxypeptidase enzyme preparation from modified *Aspergillus niger*	Dec 22, 2010	FDA has no questions
315	Transglucosidase enzyme preparation from *Trichoderma reesei* expressing the gene encoding transglucosidase from *Aspergillus niger*	May 5, 2010	FDA has no questions
296	Lipase enzyme preparation from a genetically modified strain of *Aspergillus niger*	Oct 1, 2009	FDA has no questions

GRN No.	Substance	Date of closure	FDA Response
214	Asparaginase enzyme preparation from *Aspergillus niger* expressing the asparaginase gene from *Aspergillus niger*	Mar 13, 2007	FDA has no questions
183	Phospholipase A2 enzyme preparation from *Aspergillus niger* expressing a gene encoding a porcine phospholipase A2	May 11, 2006	FDA has no questions
158	Lipase preparation from *Aspergillus niger* expressing a gene encoding a lipase from *Candida antartica*	Mar 16, 2005	FDA has no questions
132	Lactase enzyme preparation from *Aspergillus niger*	Dec 12, 2003	FDA has no questions
111	Lipase enzyme preparation from *Aspergillus niger*	Dec 20, 2002	FDA has no questions
106	Glucose oxidase enzyme preparation from Aspergillus oryzae carrying a gene encoding a glucose oxidase from *Aspergillus niger*	Oct 3, 2002	FDA has no questions
89	Five enzyme preparations from *Aspergillus niger*: Carbohydrase enzyme preparation, catalase enzyme preparation, glucose oxidase enzyme preparation, pectinase enzyme preparation, and protease enzyme preparation	Apr 3, 2002	FDA has no questions (additional correspondence available)
32	Pectin lyase derived from *Trichoderma reesei* carrying a gene encoding pectin lyase from *Aspergillus niger*	Apr 20, 2000	FDA has no questions
Page Last Updated: 02/26/2019 Accessed online in February 2019 through: https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices			

The long experience of industrial use has resulted in extensive knowledge of the characteristics of *Aspergillus niger* and an understanding of its metabolic reactions. The nonpathogenic nature of the organism has been confirmed by several experimental studies (Schuster *et al*., 2002). *Aspergillus niger* is therefore generally accepted as a nonpathogenic organism, as supported by the following:

- *Aspergillus niger* can be used under the lowest containment level at Good Industrial Large Scale Practice (GILSP), as defined by the Organisation for Economic Co-operation and Development (OECD, 1992);

- In the U.S., *Aspergillus niger* is not listed as a Class 2 or higher Containment Agent under the National Institutes of Health (NIH) Guidelines for Recombinant DNA Molecules (USA, 2013);

- The U.S. Environmental Protection Agency (EPA) has exempted *Aspergillus niger* from review by the agency, due to its extensive history of safe use (USA, 1997);

- *Aspergillus niger* is classified as a low-risk-class microorganism, as exemplified by the listing as Risk Group 1[1] in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BauA) (Germany, 2016), the German Central Commission for Biological Safety (ZKBS) (Germany, 2018), and the Dutch Commission on Genetic Modification (Netherlands, 2011).

- *Aspergillus niger* does not appear on the list of pathogens in the Belgian Biosafety Server (Belgium, 2010), or the list of pathogens in Annex III of Directive 2000/54/EC (EU, 2000) on the protection of workers from risks related to exposure to biological agents at work, as it is globally regarded as a safe microorganism.

Although *Aspergillus niger* is known to produce ochratoxins and fumonisins (Palencia *et al*., 2010; Frisvad *et al*., 2011; Blumenthal, 2004), toxin production by industrial strains under the routine conditions of industrial submerged fermentations has not been reported, despite the long history of use.

In 1988, JECFA allocated a numerical Acceptable Daily Intake (ADI) to enzyme preparations produced by *Aspergillus niger*, based on the concern that some strains may produce unknown toxins (JECFA, 1988). However, in 1990, JECFA revised the ADI to "not specified," based on the long history of use of the organism for enzyme production, numerous toxicological studies, and two expert reports that concluded that the production of toxins was highly unlikely (JECFA, 1990).

DSM has established specifications to ensure the absence of mycotoxins in the enzyme preparation (see section 2.2).

2.3.3. Development of the Production Strain

The parental strain of *Aspergillus niger*, NRRL 3122, was obtained from the Culture Collection Unit of the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, USA. The parental strain is one of the most common industrial *Aspergillus niger* strains used (Frisvad, 2011).

For increased production of a required protein (such as an enzyme), it is necessary that the genome of the production organism contain more than one copy of the gene encoding the protein. In classical production strains, such 'gene multiplication' is achieved by classical mutation-selection techniques. Starting from the parental strain, these classical techniques were used to obtain mutants with an enhanced production capacity for the enzyme glucoamylase (GAM). When the molecular biological techniques were developed for *Aspergillus niger*, it was

[1] In the German lists, *Aspergillus niger* is listed as Risk Group 2, with the exception of well-defined production strains with a long history of use, which are classified as Risk Group 1.

shown that one of these classical mutants, deposited as DS 03043, contains 7 copies of the gene encoding glucoamylase (the *gla*A gene).

Subsequently, *Aspergillus niger* strain DS 03043 was used as parental strain in a series of genetic modifications, including the deletion of the seven *gla*A loci (*i.e.*, the promoter and the coding sequences) and the inactivation of a major protease (*pep*A), resulting in the recipient strain DS 38556. The purpose of the genetic modifications was to create a safe standard recipient strain in which any desired gene can be integrated into predefined loci of the genome, resulting in a genetically well-characterized engineered production strain for any protein of interest (van Dijck *et al*., 2003).

In the recipient strain DS 38556, multiple expression cassettes encoding the *Aspergillus niger* phospholipase A1-encoding *pln*A gene were inserted, resulting in *Aspergillus niger* strain PLN, which produces higher concentrations of the native (*Aspergillus niger)* phospholipase A1. Correct integration of the expression cassettes in the *Aspergillus niger* genome was verified *via* Southern blotting and hybridization.

Due to the targeted integration of the expression cassettes, there is no risk of disturbing other parts of the genome that might lead to the accidental activation of *e.g.*, mycotoxin genes.

The final production strain, *Aspergillus niger* PLN, does not contain any selection markers or heterologous DNA.

2.3.4. Stability of the genetic traits

The genotypic and phenotypic stability of the *Aspergillus niger* PLN production strain was tested. The phenotypic stability of the strain is proven by its capacity to produce a constant level of the phospholipase A1 enzyme. This was assessed by measuring the enzyme activity in relation to the Total Organic Solids (TOS) in three independent batches of the food enzyme, as summarized in the table below.

Batch no:	PLN 618092701	PLN 618095801	PLN 618097901	Mean
Ash (%)	0.62	0.31	0.57	0.50
Water (%)	87.2	91.9	84.9	88.0
TOS (%)	12.2	7.8	14.6	11.5
Activity (PLAU/g)	447700	287500	506500	413900
Activity/TOS ratio (PLAU/mg TOS)	3670	3695	3474	3613

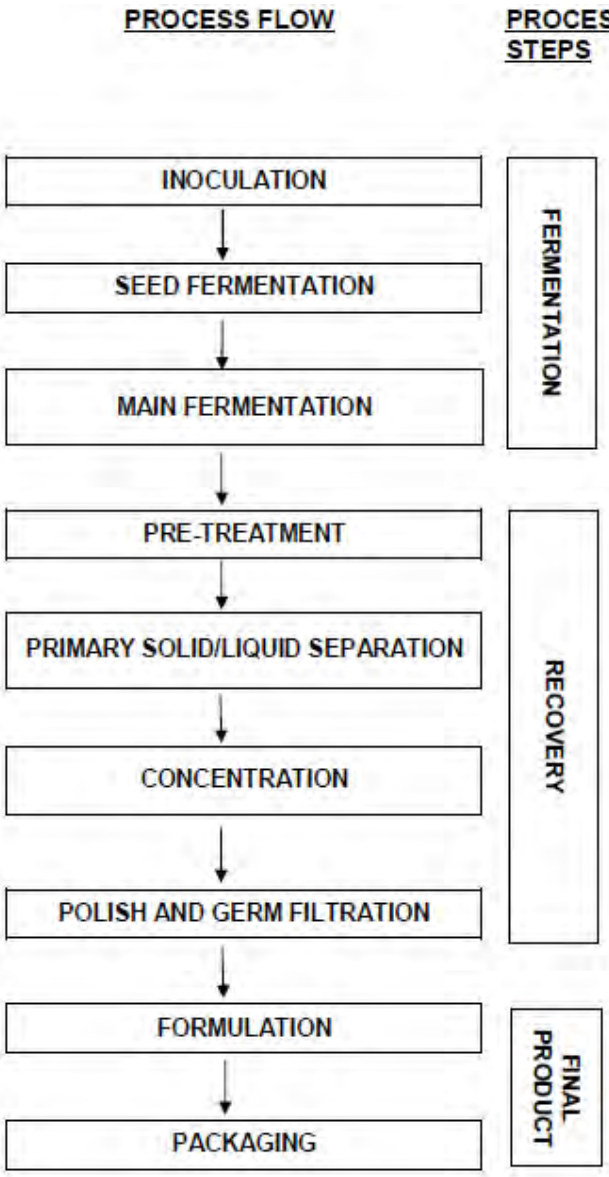
The genotypic stability of the PLN strain was tested by comparing the genotype of the strain at the end of the fermentation with the strain of the original working cell bank (WCB) that was used as inoculation material for the fermentation, as well as with the recipient strain DS 38556. For three independent ‘end of fermentation’ batches, the DNA from the biomass at the end of the fermentation was isolated and used as template for the PCR amplification of the characteristic Δ*gla*A loci and the P*gla*A-*pln*A promoter-gene insert.

The results showed no changes in the genetic structure of the phospholipase A1 expression cassettes between the WCB (start of fermentation) and the *Aspergillus niger* PLN strain at the end of the fermentation. These data thus confirm the genetic stability of the strain.

2.4. Method of Manufacture

2.4.1. Overview

Phospholipase A1 is produced by DSM in a controlled submerged fed-batch fermentation of a selected, pure culture of *Aspergillus niger* (PLN). The production process includes the fermentation, recovery (downstream processing) and formulation of the product. Production is in accordance with current Good Manufacturing Practices (GMP) for human food, with adequate controls. An overview of the different steps involved is provided in the figure below.


PROCESS FLOW
PROCESS STEPS
INOCULATION
SEED FERMENTATION
MAIN FERMENTATION
PRE-TREATMENT
PRIMARY SOLID/LIQUID SEPARATION
CONCENTRATION
POLISH AND GERM FILTRATION
FORMULATION
PACKAGING
FERMENTATION
RECOVERY
FINAL PRODUCT

2.4.2. Raw Materials

Raw materials used in the fermentation include carbon and nitrogen sources, vitamins, salts, and minerals. The raw materials used for the media are of food-grade quality and meet predefined quality standards that are strictly monitored and controlled by the Quality Assurance Department of DSM Food Specialties. The same applies to all substances used as processing aids (*e.g.*, pH and foam control agents, filter aids).

2.4.3. Fermentation Process

Phospholipase A1 is produced by DSM in a controlled submerged fed-batch fermentation of a pure culture of *Aspergillus niger*. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken, and microbiological analyses are done to ensure absence of foreign microorganisms.

The fermentation process consists of three steps: inoculum, followed by the seed fermentation and the main fermentation. The whole process is performed in accordance with Good Manufacturing Practices (GMP) for food.

Biosynthesis of phospholipase A1 occurs during the main fermentation. To produce the enzyme of interest, a submerged, aerobic fed-batch fermentation process is employed, using a stirred tank fermenter. To control the growth of the production organism and the enzyme production, the feeding rate of the feed medium is based:

- upon a predetermined profile; or
- on deviation from defined set points for pH or dissolved oxygen concentration.

Growth of the production organism and the level of enzyme production are checked at the end of the main fermentation by analysis of aseptically collected samples.

2.4.4. Recovery Process

During fermentation, the enzyme protein is excreted by the production organism, *Aspergillus niger*¸ into the fermentation medium. After fermentation, the biomass is killed by the addition of sodium benzoate and incubation over a prolonged period at adequate pH and temperatures. This treatment effectively kills the *Aspergillus niger* cells. The cell material is separated from the enzymes by means of a simple membrane filtration process. Subsequently, the remaining particles are removed with a polish filtration and a germ filtration, and the enzyme solution then concentrated by ultrafiltration (UF).

2.4.5. Formulation and Standardization Process

To obtain a liquid enzyme preparation, the ultrafiltration (UF) concentrate is standardized with glycerol to the desired final enzyme activity.

2.4.6. Quality Control of Finished Product

The final phospholipase A1 preparation is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO in 2006 and the FCC (10^{th} edition). These specifications are described in Section 2.2.

2.5. General Production Controls (Good Manufacturing Practice)

Commercial demands require a strictly controlled production process.

The enzyme manufacturing site in France, is in operation since 1922 and has received ISO 9001-2000 certification.

Optionally, the fermentation and recovery steps of the phospholipase A1 manufacturing process can be outsourced to other manufacturing facilities located in the major industrial markets, provided that they operate in compliance with GMP standards and HACCP principles, have FSSC or other applicable third-party certification, and can meet the established product specifications.

2.5.1. Technical Measures

The batches of primary seed material are prepared, preserved, and stored in such a way that contamination and degeneration are avoided, and genetic stability is secured. The vials are clearly labeled, and strict aseptic techniques are applied during the recovery of the culture.

Only sterilized raw materials are used to prepare the nutrient medium for the fermentation.

The fermenter is a contained system. Only sterilized air is used in the fermentation. Membrane valves, air filters and seals are regularly checked, cleaned, and replaced if necessary. Prior to inoculation, the fermenter is cleaned, rinsed, and sterilized. The sterilized nutrient medium and the complete biomass broth are transferred aseptically to the main fermenter. The methods used effectively prevent microbial contamination during fermentation.

The preparation of sterile media and the cleaning of the equipment are laid down in Quality Assurance documents and strictly followed.

Microbial contamination is prevented during downstream processing by several germ filtration steps. The filters are thoroughly cleaned for each production run.

2.5.2. Control Measures

After preparation of a new batch of primary seed material, samples are checked for identity, viability, and microbial purity. If these parameters meet the internal specifications, the strain is tested for production capacity. Only if the productivity and the product quality meet the required standards, the new batch of primary seed material will be accepted for further production runs.

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

At regular intervals during the seed fermentation samples are taken aseptically for analysis of pH and microbiological quality.

During the main fermentation the dissolved oxygen content, pH, temperature, viscosity and microbial quality are monitored. If microbial controls show that significant contamination has occurred, the fermentation will be discontinued.

Also, during downstream processing samples are being taken and checked for the level of microbial contamination. If these checks show that significant contamination has occurred, the downstream processing will be discontinued.

The consistency of the manufacturing process was confirmed by analytical results obtained from consecutive 3 batches of enzyme. As the data from the table below show, the product conforms to the current edition of USP FCC monograph, and to JECFA specifications.

Batch number:	**PLN 618092701**	**PLN 618095801**	**PLN 618097901**
Lead (mg/kg)	< 0.01	< 0.01	< 0.01
Salmonella per 25g	absent	absent	absent
Coliforms (CFU/g)	< 1	< 1	< 1
Escherichia coli per 25g	absent	absent	absent
Antimicrobial activity	not detected	not detected	not detected
Mycotoxins	not detected	not detected	not detected

3.0 DIETARY EXPOSURE

3.1. Proposed Food Uses

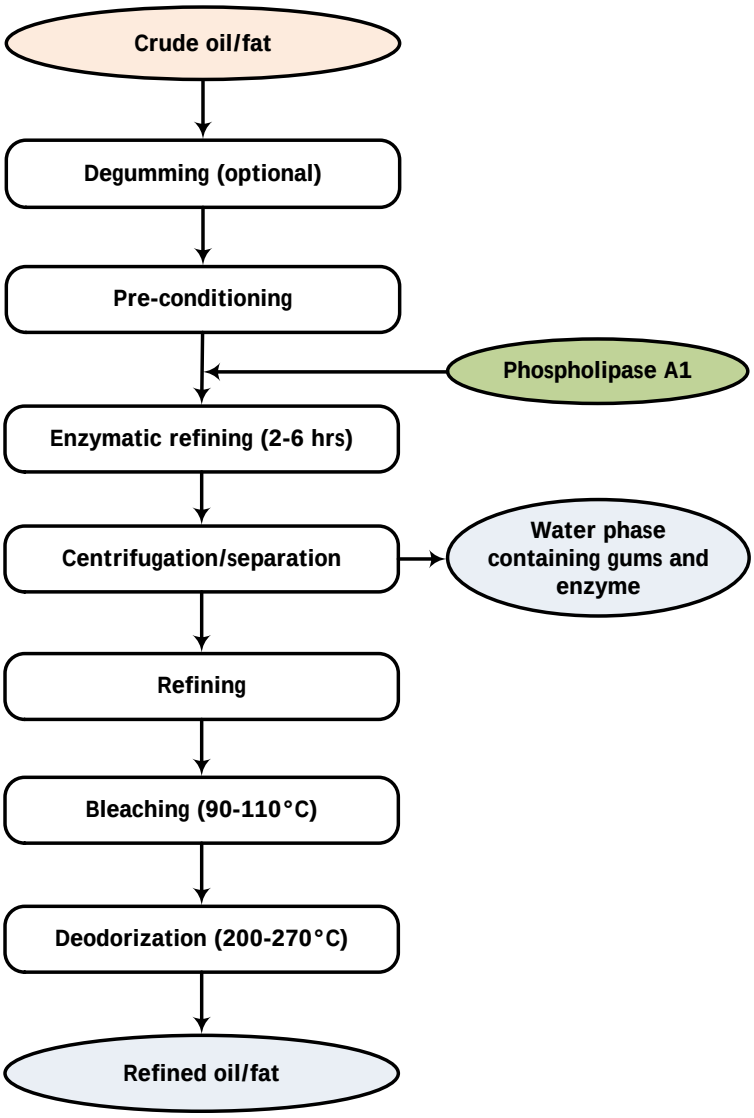
DSM's phospholipase A1 catalyzes the hydrolysis of phospholipids present in oils and fats, at the SN-1 position, producing free fatty acids and lysophospholipids. By doing so, the enzyme reduces the emulsion-forming capacity of phospholipids, improving the separation between the gum phase (containing mainly phospholipids) and the oil phase in the degumming/refining process.

In general, phospholipase A1 is used for the following technical effects:

- To reduce the emulsion-forming capacity of phospholipids, therefore increasing oil yield; and
- To reduce the phosphorus content in the refined oil, minimizing the use of chemicals in refining process.

DSM intends to promote phospholipase A1 from genetically engineered *Aspergillus niger* strain PLN for use in the processing (water/enzymatic degumming) of edible crude oils and fats, including vegetable oil (*e.g.*, soybean oil, rapeseed oil, canola oil, sunflower oil), algal oil, animal fats, and fish oil.

An overview of the steps involved in crude oil processing is shown below.



The enzyme is expected to be removed from the process with the separated gum after centrifugation; any trace amounts that might remain, including any water-soluble total organic solids (TOS) components, would be removed during subsequent refining (washing, bleaching, deodorization) steps. Therefore, no enzyme would remain in the refined edible oil or fat.

3.2. Anticipated Consumer Intake

As noted above, DSM's phospholipase A1 produced by *Aspergillus niger* strain PLN is intended for use in the processing of edible crude oils and fats, and is not expected to be present in the final refined oil or fat. Therefore, no or negligible dietary exposure is expected.

Nevertheless, in order to provide a “worst-case” scenario for assessing consumer intakes, DSM calculated possible daily human exposures by assuming that all of the enzyme product would be retained in the refined oil or fat.

Refined edible oils and fats are used in a wide variety of food applications (*e.g.*, cooking oil, dressings, margarines, bakery products). A use level of 25-100 ppm phospholipase A1 (formulated enzyme) is recommended for edible oil/fat processing, with an enzyme activity of at least 50,000 PLAU/g formulated enzyme product. The formulated enzyme product contains 3474 PLAU/mg TOS (mean value), as shown in section 2.2. This would result in a range of 0.35-1.38 mg TOS/kg oil or fat. Assuming a mean daily intake of oils and solid fats of 53 g/person/day for U.S. males and females aged 2 and older (USDA, 2014) and a body weight of 60 kg, this would lead to an estimated daily intake (EDI) of 0.31-1.22 μg TOS /kg bw/day.

4.0 SELF-LIMITING LEVELS OF USE

The amount of an enzyme required to accomplish a specific technical or functional effect will depend in part on the type and quality of the raw material being treated. DSM's enzyme preparation containing phospholipase A1 produced by *Aspergillus niger* strain PLN is intended to be used in a manner consistent with current GMP. Use of excessive amounts would likely be associated with undesirable technological effects.

5.0 COMMON USE IN FOOD PRIOR TO 1958

The elements of this section do not apply because DSM's GRAS conclusion is based on scientific procedures. However, it is important to note that phospholipases occur naturally in the human body and in various organisms that are part of the human diet.

6.0 NARRATIVE OF THE BASIS FOR THE GRAS CONCLUSION

6.1. Overview

DSM has determined that its phospholipase A1 enzyme from a genetically engineered strain of *Aspergillus niger* (PLN) is GRAS when used in the processing of crude edible oils and fats.

The primary consideration in DSM's safety assessment was that use of this enzyme in the degumming of edible oils and fats is more aptly characterized as a processing aid. At the end of the oil/fat refining process, no enzyme is expected to remain, having been removed with the separated gum upon centrifugation; any trace amounts that might remain would be removed during subsequent refining steps (washing, bleaching, deodorization). Therefore, no or negligible consumer exposure is expected.

Regardless, DSM considered the totality of available information, discussed below and elsewhere in this document, to be sufficient to support a GRAS conclusion.

In particular, DSM considered that:

- The phospholipase A1 enzyme that is the subject of this GRAS notice is produced *via* controlled submerged fed-batch fermentation using a strain of *Aspergillus niger* (PLN) developed by DSM. At the end of fermentation, the cell material is separated from the enzyme by means of filtration, followed by concentration through ultrafiltration (UF), and formulation using ingredients adequate for use in human foods.
- DSM's phospholipase A1 is manufactured under current GMP, using food-grade materials and under adequate controls. The enzyme preparation meets the specifications established by DSM, including JECFA and USP FCC purity criteria (see section 2.2).
- *Aspergillus niger* represents the host and donor organism. This microorganism has a long history of use in the production of food ingredients, including food enzymes.
- The genetic modifications made to *Aspergillus niger* to make the production strain have been well characterized and do not lead to production of harmful or toxic substances. Multiple copies of the phospholipase A1 expression cassette results in a strain that produces higher concentrations of the native (*Aspergillus niger*) phospholipase A1 enzyme. The strain does not contain antibiotic resistance markers or heterologous DNA, and lacks the genes encoding for glucoamylase and a pepsin-like protease (see section 6.3).
- Phospholipase A1 is widely present in nature including humans and in various organisms that are part of the human diet. The substrates (phospholipids) and the reaction products (fatty acids, lysophospholipids) are themselves present in food.

- A comparison of DSM's phospholipase A1 amino acid sequence to known food allergens did not reveal any significant matches (see section 6.4.1).

6.2. Safety of the host organism

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the International Food Biotechnology Council, food or food ingredients are safe to consume if they have been produced according to current Good Manufacturing Practices from a nontoxigenic and nonpathogenic organism (Coulston and Kolbye, 1990). A nontoxigenic organism is defined 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 nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza and Foster, 1983).

As discussed previously, *Aspergillus niger* is widely distributed in nature and is generally considered as nonpathogenic (Schuster *et al*., 2002).

Some *Aspergillus niger* strains are capable of mycotoxin production, with ochratoxin A and fumonisin B2 being of most concern in terms of human and animal safety (Nielsen *et al*., 2009; Frisvad *et al*., 2018). However, ochratoxin A and fumonisin production by *Aspergillus niger* was not observed under standard industrial submerged fermentation conditions.

The safety of *Aspergillus niger* as a production organism for food enzymes and as a host for recombinant strains is well-documented. Therefore, *Aspergillus niger* would be considered a safe and appropriate host and donor organism, further supported by its classification as a low-risk organism in the U.S. and other countries (see section 2.3.2).

6.3. Safety of the Production Strain

The PLN production strain was derived from DSM's safe strain lineage of *Aspergillus niger* recipient and production strains (Van Dijck *et al*., 2003). DSM has used *Aspergillus niger* strains from this lineage for more than 30 years for food enzyme production and has performed a number of safety studies on different enzyme products produced by strains from this lineage. A series of classical strain improvements and selection steps were employed, along with genetic modifications that integrated multiple expression cassettes of the *Aspergillus niger* phospholipase A1 gene at defined locations, and deleted genes coding for a glucoamylase and a protease.

Southern blot analysis and PCR analysis of the final production strain confirmed the integration of 20 copies of the phospholipase A1 gene at the targeted integration sites, as well as the deletion of the glucoamylase and protease genes. No selection markers or vector sequences are present in the final production strain.

The genotypic stability of *Aspergillus niger* strain PLN was confirmed by comparing the genetic structure of (i) the phospholipase A1 expression cassettes of the working cell bank (WCB) at the start of fermentation with (ii) samples at the end of fermentation. The ability of *Aspergillus niger* strain PLN to produce a constant level of the phospholipase A1 enzyme provides evidence of its phenotypic stability (see section 2.3.4).

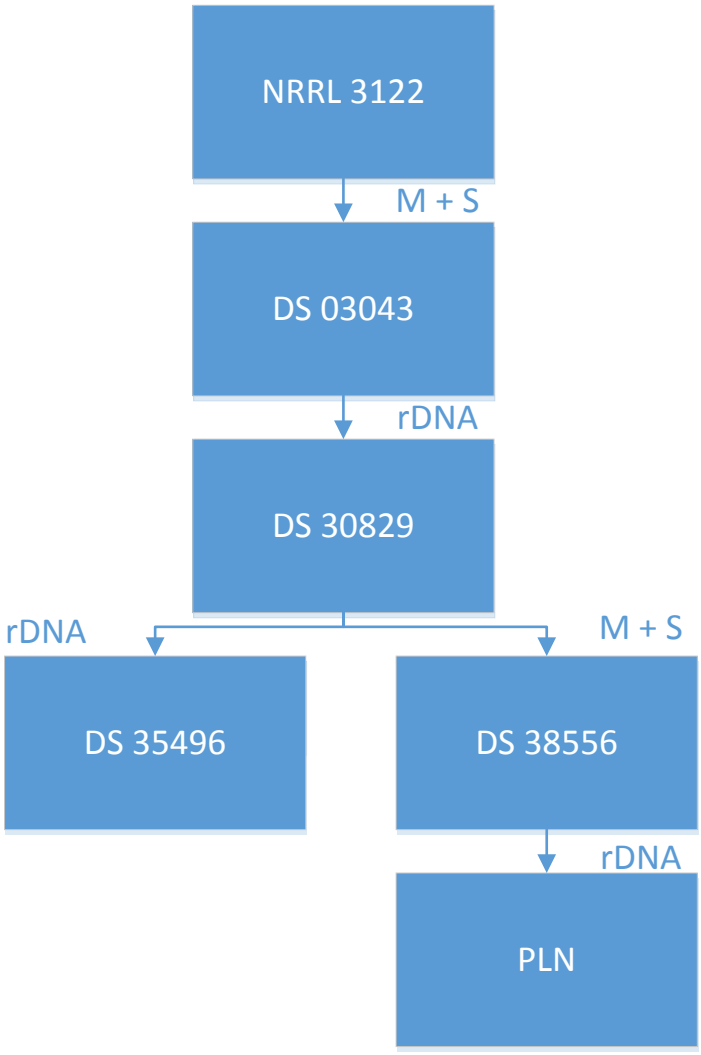
6.3.1. Safe strain lineage approach

DSM considered in its safety assessment that *Aspergillus niger* PLN would qualify as an organism whose safety can be established based on the "safe strain lineage" approach, as supported by several publications (Pariza and Foster, 1983; Pariza and Johnson, 2001; and Pariza and Cook, 2010; Sewalt *et al*., 2016). Specifically, DSM considered that the following conditions described by Pariza and Johnson (2001) would be met: (1) thorough characterization of the host organism as nonpathogenic and nontoxigenic; (2) determination of the safety of all new DNA that has been introduced into the host organism; and (3) use of modification procedure(s) appropriate for food use.

This safe strain lineage approach is in line with the EFSA CEF guidelines (EFSA, 2009, § 4.1.2), where it is mentioned that a full testing battery may be waived in specific cases. It has also been used in several GRAS notices that have generated no question from U.S. FDA (for *Aspergillus niger*: US FDA GRAS notification GRN 000183, GRN 000214, GRN 000296 and GRN 000345; for *Aspergillus oryzae*: US FDA GRAS notification GRN 000008, GRN 000034, GRN 000043, GRN 000075, GRN 000103, GRN 000106, GRN 000142 and GRN 000201; for *Trichoderma reesei*: US FDA GRAS notification GRN 000230, GRN 000315, GRN 000333 and GRN 000372).

Strains derived from the same strain lineage behave in the same way with respect to their growth requirements and metabolism. Consequently, food enzymes derived from the same strain lineage and produced under similar fermentation conditions are expected to have a comparable background of impurities that may originate from the production strains, and would thereby have comparable safety profiles. As such, toxicity testing of phospholipase A1 produced using *Aspergillus niger* PLN was not considered necessary, because sufficient information is available otherwise (as specified above and elsewhere in this dossier) to establish the safety of this enzyme under the conditions of use.

Further support is provided by toxicity studies of a phospholipase A2 (PLA2) enzyme produced by an *Aspergillus niger* strain (PLA) from the same lineage, described briefly by van Dijck *et al.* (2003) in a discussion of the safety of a new generation of DSM *Aspergillus niger* production strains. The relationship between these strains is shown in the figure below. Although PLA1 and PLA2 cleave phospholipids at different positions (SN-1 *vs*. SN-2), the shared lineage in this instance and similarities in the production methods and materials would suggest safety information about one provides insight into the safety of the other.



DSM's lineage of safe *Aspergillus niger* production strains: DS number = deposition number in DSM's internal strain collection; M + S = mutation and selection; rDNA = use of recombinant DNA techniques; DS 35496 = production strain for phospholipase A2; PLN = production strain for phospholipase A1 (this dossier).

6.3.2. Toxicity studies of phospholipase A2 derived from a related *Aspergillus niger* strain

Van Dijck *et al*. (2003) briefly described toxicity studies (genotoxicity and 90-day rat oral toxicity) performed with phospholipase A2 produced using a different strain from this lineage, *Aspergillus niger* PLA. The studies were also described in detail in a GRAS notice for phospholipase A2, filed as GRN No. 183. The agency indicated (May 11, 2006) it had no questions about DSM's GRAS conclusion.

Briefly, the studies, which were carried out in accordance with OECD guidelines and in compliance with the principles of Good Laboratory Practice (GLP), showed no evidence of genotoxicity (*in vitro* reverse mutation assay; *in vitro* chromosome aberration test; *in vivo* mouse micronucleus test), and a no-observed-adverse-effect level (NOAEL) of 10,000 mg enzyme preparation/kg bw/day, or 1350 mg TOS/kg bw/day, was proposed based on the results of a 90-day rat oral toxicity study.

DSM considered that the NOAEL value of 1350 mg TOS/kg bw/day from the 90-day study above might serve as a useful reference for context with respect to consumer exposures, since the two enzymes are produced by organisms developed by DSM that have common ancestry and are produced under similar fermentation conditions, with comparable impurity profiles. Applying the outcome of the study on the phospholipase A1 enzyme, DSM's intake estimates would be at least 1000 times lower than the NOAEL of 1350 mg TOS/kg bw/day. In actuality, because the enzyme is expected to be removed with the separated gum, no or negligible consumer exposure is expected.

6.4. Safety of phospholipase A1

Phospholipases are part of the digestive enzymes present in pancreatic juice of mammals including humans (Rossiter, 1968; Johnson and McDermott, 1974). They are enzymes that catalyze the hydrolysis of phospholipids into fatty acids and other lipophilic substances. These enzymes are responsible for fast turnover rates of cellular phospholipids.

Phospholipase A1, specifically, cleaves phospholipids at the SN-1 position, forming lysophospholipids and free fatty acids, each of which occurs naturally in foods and in the human body. Phospholipase A1 is conserved in a wide range of organisms including humans and has a variety of cellular functions that include regulation and facilitation of the production of lysophospholipid mediators (Franson 1971; Aoki 2007; Richmond 2011).

No safety concern has been identified in literature from the use of phospholipase A1 enzyme[2].

The safety of phospholipase A1 has been discussed in other GRAS notices, listed in the table below. The discussion in these notices regarding the natural occurrence of phospholipase A1 is considered relevant in supporting the safety and GRAS status of DSM's phospholipase A1 produced by *Aspergillus niger* PLN.

GRN No.	Substance	Date of closure	FDA response
811	Phospholipase A1 produced by *Aspergillus oryzae*		Pending

[2] Literature search conducted in Pubmed in January 2019 with the following search terms: "phospholipase and safe*", "phospholipase A and safe*", "phospholipase and tox*", "phospholipase A and tox*".

651	Phospholipase A1 from *Talaromyces leycettanus* produced in *Aspergillus niger*	Nov 23, 2016	FDA has no questions
142	Phospholipase enzyme preparation from *Aspergillus oryzae* expressing the gene encoding a phospholipase A1 from *Fusarium venenatum*	Jun 23, 2004	FDA has no questions
Page Last Updated: 02/26/2019 Accessed online in February 2019 through https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices			

The safety of phospholipase A1 from *Fusarium venenatum* expressed in *Aspergillus oryzae* has also been evaluated by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO and received an ADI "not specified" (68th JECFA meeting, 2008).

In its evaluation of the safety of phospholipase A1, DSM also considered that, in the *Federal Register* notice on the GRAS affirmation of enzyme-modified lecithin (61 FR 45886-45889, Aug. 30, 1996), FDA addressed concerns regarding possible effects of lysophospholipids on the stomach mucosa under certain pathologic conditions. The agency concluded based on how enzyme-modified lecithin is consumed and other evidence that such effects were not likely to occur.

While lysophospholipids are also products of phospholipase A1 reactions, use of phospholipase A1 in food processing as proposed by DSM is not expected to have such effect either, especially considering that the enzyme and lysophospholipids are removed from the finished refined oil or fat.

Since it is generally known that commercial enzyme preparations of *Aspergillus niger* are not toxic and phospholipase A1 is a natural constituent of many organisms, including human, it is not expected that the proposed use of DSM's phospholipase A1 will be associated with any adverse health effects.

6.4.1. Allergenicity

As proteins, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens.

Enzymes have a long history of safe use in food. Since new enzymes are generally (based on) existing enzymes, it is very unlikely that a new enzyme would be a food allergen. Moreover, exposure to an enzyme associated with ingestion is typically very low and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system (Grimble, 1994). To our knowledge, no reports exist on sensitization to enzyme products in the final commercial food after ingestion. Bindslev-Jensen *et al*. concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindslev-Jensen *et al*., 2006).

To assess potential allergenicity, DSM compared the amino acid sequence of the phospholipase A1 protein produced by the genetically engineered strain of *Aspergillus niger* (PLN) with the amino acid sequences of known (food) allergens stored in the database AllergenOnline™ (available at http://www.allergenonline.org/, last updated February 2019). AllergenOnline™ allows the search in NCBI, SwissProt, PIR, PRF, PDB and the WHO-IUIS databases using a FASTA algorithm. The comparison was done in June 2018.

The search was performed following the guidelines developed by EFSA in the safety evaluation document of the newly expressed proteins in genetically modified plants (EFSA, 2006) and the guidelines from the FAO/WHO consultation (FAO/WHO, 2001, 2009). According to the guidelines, cross-reactivity between the expressed protein and a known allergen has to be considered when there is:

- more than 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty; or
- identity of short contiguous amino acid segments (*i.e.*, at least 8 contiguous amino acids). Although the 2001 WHO/FAO consultation suggested searching for matches of 6 identical amino acid segments or longer, it is recognized that a search for such small sequences would lead to the identification of too many false positives (EFSA, 2006; FAO/WHO, 2009). It has been reported that an immunologically significant sequence similarity requires a match of at least 8 contiguous identical residues (Metcalfe *et al*., 1996; Fuchs and Astwood, 1996).

The amino acid sequence comparison did not reveal any relevant matches with known food allergens. Based on these results, DSM concluded that the phospholipase A1protein is not likely to produce an allergenic or sensitization response upon oral consumption.

6.5. Safety of the manufacturing process

The manufacture of DSM's phospholipase A1 is performed under current food GMP requirements and with adequate controls. Moreover, it is indicated that ingredients are used that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

The phospholipase A1 preparation meets purity specifications consistent with those of JECFA for chemical and microbiological purity of food enzymes and the USP Food Chemicals Codex (FCC) monograph for Enzyme Preparations.

6.6. Substantial equivalence

Several expert groups have discussed the concept of substantial equivalence relative to food safety assessment. Essentially, all these groups conclude that if a food ingredient is substantially

equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA appears to have accepted this concept in the determination that several enzyme preparations are safe for use in food. In particular, FDA has considered differences in glycosylation between enzyme proteins. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to chemical modifications, or site-directed mutagenesis, would not raise safety concerns.

There are no agreed-upon criteria by which substantial equivalence is determined. With respect to enzymes produced by microorganisms, the enzyme activity and intended use, the production organism and the process conditions should be taken into account.

Phospholipase A1 (EC 3.1.1.32) belongs to the carboxylic ester hydrolases (EC 3.1.1). As noted previously, the safety of this enzyme, including its natural occurrence, has been discussed in other GRAS notices. These discussions are considered relevant in supporting the safety and GRAS status of DSM's phospholipase A1 produced by *Aspergillus niger* PLN because the similarities outweigh the differences; *i.e.*, once separated from the production organism, these enzymes would have comparable compositions (cellular fractions containing enzyme activity with residual amounts of fermentation ingredients and processing aids) and functions (phospholipid hydrolysis). These constituents and the products of the reactions they mediate also represent substances normally consumed by humans, *i.e.*, proteins, peptides, amino acids, carbohydrates, and lipids.

In addition to the safety of the phospholipase A1 enzyme itself, DSM's production organism is a specific, genetically engineered *Aspergillus niger* strain that has multiple copies of the native phospholipase A1 gene and overexpresses the enzyme. *Aspergillus niger* has a long history of use in the production of food ingredients, and as a safe host for enzyme-encoding genes (Olempska-Beer *et al*., 2006).

Consequently, the *Aspergillus niger* strain used to produce phospholipase A1 is considered as safe as the production strains that have produced other enzymes considered GRAS. Accordingly, the resulting enzyme product from DSM's production strain can be considered as safe as other enzymes produced by strains from this species.

6.7. Summary of the basis for a GRAS conclusion

Combined, the elements described in this dossier support DSM's conclusion that: (1) there is sufficient information available to support the safety of DSM's phospholipase A1 produced by a genetically engineered strain of *Aspergillus niger* (PLN) when used in the degumming/refining of crude edible oils and fats; (2) there is a basis to conclude that this technical evidence of safety would be generally known and accepted by qualified experts.

7.0 SUPPORTING DATA AND INFORMATION

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APPENDIX 1: Method of analysis for phospholipase A1 activity

No.: DBC-ABC-A-10367
Version: 1.0

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

PRODUCT	VALIDATED METHOD
Phospholipase A1	NO

Primary author	Approvers	Status & Dates	Content owner
Wim Bijleveld	Laumen, Frank Chantal Christis	**Status:** **Effective:** **Next Review Date:**	Optional
Reviewers: Bijleveld, Wim, Laumen, Frank			

1. SAFETY, HEALTH AND ENVIRONMENT

Information about hazard symbols, description of H phrases, risk class information, handling conditions and additional PPE's for working with the chemicals mentioned in this method of analysis can be found in the table in annex 1.

Combination of chemicals and reagents
The combination of chemicals and reagents used in this method of analysis has been considered not to cause safety risks.

Chemical spills
See DBC-SLD-P-10052 "SHE Book Service Lab Delft".
The spills that may occur in the analysis performed according to this method do not require additional measures.

(Set-up of) equipment
The equipment used in this method of analysis, in combination with the chemicals and reagents used, has been considered not to cause safety risks.

Waste
See DBC-SLD-P-10052 "SHE Book Service Lab Delft".
Waste generated in the analysis performed according to this method does not require additional measures.

2. PRINCIPLE

a. Application

This method is applicable for the determination of *A. niger* phospholipase A1 (PLA1) activity in fermentation broth, ccUF, filtrate and final product (50% glycerol formulation).

b. Description of the method

This method describes a spectrophotometric assay for the determination of phospholipase activity. This activity is determined using 1,2-dioleoyl-sn-glycero-3-phosphocholine as substrate. A mix of enzyme and substrate dissolved in acetate buffer (pH 4.5) is incubated at 37ºC. During the 30 minutes incubation, free fatty acids (FFA) are produced because of an enzymatic reaction with the substrate. The amount of formed free fatty acids

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is determined enzymatically with the NEFA kit of WAKO Diagnostics (see figure 1). The PLA1 assay is relative, an enzymatic standard is used. For calibration of the enzyme standard a solution of oleic acid is used as standard to calculate the amount of free fatty acids formed. The determination of the free fatty acid concentration is performed on the Konelab Analyzer and the intensity of the colour is measured at 540 nm.

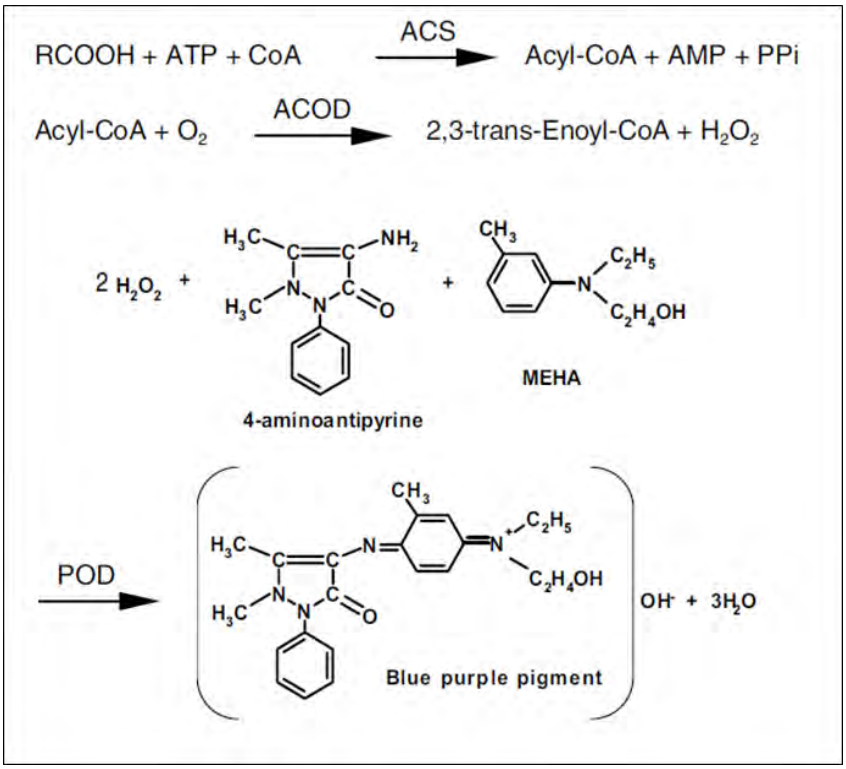

Figure 1. Reaction scheme for the determination of free fatty acids (FFA). ACS = acyl-CoA synthetase, ACOD = Acyl-CoA oxidase, POD = Peroxidase

c. Unit definition

The activity is expressed as NPLAU per g or mL. 1 NPLAU (**N**ew **P**hospho**L**ipase **A**1 **U**nit) is defined as the amount of enzyme that liberates 1 mM of free fatty acid per minute under the defined assay conditions.

PLAU = DPLAU = NPLAU/1.4

PLAU = activity unit used on Certificate of Analysis of the final product.
DPLAU = **D**evelopment **PLAU** according to method DBC-ABC-A-10333 using phosphatidylcholine as substrate at pH 4.5 and 37°C.
NPLAU = **N**ew **PLAU** according to method DBC-ABC-A-10367
1.4 = conversion factor between PLAU and NPLAU [1]

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

d. Measuring range

The measuring range of this method is 0.1-0.5 NPLAU/mL.

e. Summary of the validation report

No official validation study was done for this method. After development, qualification of the method was performed for fermentation broth EOK, ccUF and final product in 50% glycerol [1]. The following data were found:

	Broth	ccUF	Product
average (PLAU/g)	101537	467158	67722
overall	5.4%	6.4%	4.9%
within days	4.8%	5.2%	4.1%
between days	3.0%	4.2%	3.0%
intermediate single (n=1)	5.6%	6.7%	5.1%
intermediate duplicate (n=2)	4.5%	5.6%	4.2%
intermediate triplicate (n=3)	4.1%	5.2%	3.8%

3. APPARATUS AND CONDITIONS

a. Apparatus

- Konelab Arena 30 : Thermo, adjusted at 540 nm
- Konelab sample cups : Content 1.5 mL
- Konelab reagent vessels : 20 mL and 60 mL
- Water bath adjusted at 37.0 ± 0.1°C : Julabo 19
- Balance, accurately to 0.0001 g. : Mettler AJ100
- Balance, accurately to 0.001 g : Mettler PM400
- pH-meter : Radiometer PHM 82
- Diluter, provided with 0.5 and 5.0 mL cylinders : Dilutrend, Boehringer
- Vortex : Genie-2, Scientific Industries
- Magnetic stirrer : Variomag, multipoint HP15
- Disposable culture tubes (glass), 16x100mm : VWR, 212-0016
- Pipetman 1mL : VWR Research Plus
- Multipipette : Eppendorf Multipipette plus

Or equivalent apparatus.

b. Conditions

Not applicable.

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

4. MATERIALS

a. Chemicals

- Triton X-100 : Sigma, X-100
- Hydrochloric acid solution (2 mol/L) : Merck 1.09063
- Sodium Hydroxide (2 mol/L) : Merck 1.09136
- Acetic acid (glacial) 100% : Merck 1.00063
- TRIS [tris(hydroxidymethyl)aminomethane] : Merck 1.08387
- DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) : Avanti 850375P-1g
- NEFA-HR R1 set (Konelab reagent) : VWR, WAKO434-91795
- NEFA-HR R2 set (Konelab reagent) : VWR, WAKO436-91995
- NEFA standard (1.0 mM oleic acid) : VWR, WAKO270-77000

b. References, standards and controls

Standard for absolute method:
For micro calibration, a 1.0 mM oleic acid solution from Wako Chemicals is used as standard. Use the exact value from the certificate of analysis from supplier.

Standard for relative method:
Use an enzyme standard with an officially assigned activity. Store the stock and amounts for daily use under the conditions mentioned on the Certificate of Analysis.

Control:
Use a control preparation with an officially assigned activity. Store the stock and amounts for daily use under the conditions mentioned on the Certificate of Analysis.

c. Reagents

- Water:
Ultra High Quality (UHQ) water, conductivity ≤ 0.10 µS/cm

- **Stop solution**: 2 M tris pH 8.3 ± 0.1
Dissolve 12.1 g Tris in approximately 10 mL water and 10 mL 2 M HCl in a 50 mL measuring cylinder. Adjust the pH with 2 M HCl to 8.3 ± 0.1 and make up to volume. This solution is stable for 1 month, when stored in the refrigerator.

- **Dilution buffer**:100 mM acetate pH 4.50/ 0.2% (w/v) Triton X-100
Dissolve 6.0 g glacial acetic acid and 2 g Triton X-100 in approximately 800 mL water in a 1 L measuring cylinder and stir on a magnetic stirrer until completely dissolved. Adjust the pH to 4.50 ± 0.05 with 2 M sodium hydroxide. Make up volume with water and mix. This solution is stable for 1 month when stored in the refrigerator.

-**Substrate buffer**: 100mM acetate buffer pH 4.50/ 2% (w/v) Triton X-100

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

Dissolve 3.0 g glacial acetic acid and 10 g Triton X-100 in approximately 400 mL water in a 500 mL measuring cylinder and stir on a magnetic stirrer until completely dissolved. Adjust to pH 4.50 ± 0.05 with 2 M sodium hydroxide. Make up volume with water and mix. This solution is stable for 1 months when stored in the refrigerator.

Substrate solution: 0.625% (w/w) DOPC (7.95 mM) 100mM acetate buffer 2% (w/v) Triton X-100 pH 4.50
Prepare a DOPC solution of precisely 1.00 g DOPC per 160.0 g substrate buffer. Correct for inaccuracy in DOPC weighing, for example dissolve 1.02 g DOPC with 1.02 x 160 = 163.2 g substrate buffer.

NEFA reagents
- **R1 (Konelab NEFA-R1)**
Transfer 1 bottle with liquid R1 to the sealed glass bottle R1 and mix. Transfer this solution into a 60 mL Konelab vessel. This solution R1 is stable for 1 month when kept in the refrigerator.

- R2 (Konelab NEFA-R2)
Transfer 1 bottle with liquid R2 to the sealed glass bottle R2 and mix. Transfer this solution into a 20 mL Konelab vessel. This solution R2 is stable for 1 month when kept in the refrigerator.

5. PROCEDURE

a. Preparation

Turn on the water bath at 37.0°C ± 0.1°C.

b. Pretreatment reference

Not applicable.

c. Pretreatment enzymatic standard for relative method

Weigh an amount of standard equivalent to approximately 50 000 NPLAU accurately to within 0.001 g in a 50 mL volumetric flask, note the weight and make up to volume with dilution buffer.
The standard is diluted with dilution buffer according to Table 1.

Table 1 Dilution scheme for enzymatic standard. Final activity can deviate, depending on weighing of standard

Code	df1	df2	df3	tdf	NPLAU/mL
ES0	-	-	-	-	0
ES1	20	25	20	500 000	0.100
ES2	20	25	10	250 000	0.200
ES3	25	15	9	168 750	0.296
ES4	25	10	10	125 000	0.400
ES5	20	10	10	100 000	0.500

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

d. Pretreatment standard for absolute method

A standard solution of oleic acid (1.0 mmol/L) is used as standard. The standard is diluted with water according to Table 2 with the aid of a pipetman.

Table 2. Dilution scheme of oleic acid standard

Code	FFA (mM)	Volume of standard solution [mL]	volume of water [mL]	total dilution factor
S1	1.00	1.00	0.00	1
S2	0.75	0.75	0.25	1.33
S3	0.50	0.50	0.50	2
S4	0.25	0.50	1.50	4
S5	0.125	0.25	1.75	8
S6	0.063	0.125	1.875	16
S7	0	0	1.00	blank

e. Pretreatment control

Weigh from the control preparation, in duplicate, approximately 250 000 NPLAU accurately to within 0.001 g in 50 mL volumetric flasks, note the weight and make up to volume with dilution buffer and gently mix on a magnetic stirrer. Dilute the control to a final concentration of 0.25 NPLAU/ml (tdf 1 000 000), first 50 times (100 µL+4900 µL) then 20 times (250 µL+4750 µL) and finally 20 times (250 µL+4750 µL).

f. Pretreatment samples (fermentation samples, ccUF, liquids and products):

Suspend from the sample at least 1.0 g, weighed accurately to within 1 mg, in approximately 20 mL dilution buffer in a 25 mL volumetric flask. Make up to volume with the dilution buffer and mix using a magnetic stirrer. Subsequently dilute the sample solution with the dilution buffer to a final concentration of approximately 0.25 NPLAU/mL.

g. Preparation measurement

Microcalibration absolute Standard:
Introduce 1 mL of each dilution of the oleic acid standards into sample cups and place them in the Konelab sample segments and take notice of the used segments and positions (see par. 5.8).

Enzymatic standard, Samples and Blanks:
Treat enzyme standards and samples the same. For every standard and sample a blank is included. For each enzyme standard and sample, fill two tubes with 1.6 mL substrate solution with a multipipette.
Preheat the test tubes with substrate solution for 10 minutes in a water bath at 37.0 ± 0.1°C.

At time t = 0 minutes, in the order of the series and with regular time intervals, add 200 µl enzyme solution (=enzymatic standard/ diluted sample) to the tubes containing substrate. Vortex tubes and re-place in the water bath. At time t = 30.0 minutes, in the same order and with the same regular time intervals, add 200 µL stop reagent to all the tubes, including the blanks using a multipipette and vortex.
Add 200 µl enzyme solution to the **blanks** using a pipetmen and vortex.

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

Introduce approximately 1 mL of the final solution into sample cups and place them in the Konelab sample segments and take notice of the used segments and positions (see par. 5.8).

h. Measurement

Using the Konelab Arena 30 analyzer:

- Log in the Konelab software.
- A yellow flagged message stating "startup needed" should be visible in the main screen. Press F1-startup to perform startup operations and press OK.
- When startup is completed, check the water blanks by pressing F8, F2, F8 and F1, check water blank. Press F5 to show all tested wavelengths. If outliers are seen (above 2 mA), then repeat the water blank by pressing F1 twice.

Requesting samples:

- Press the Samples button on top of the main screen.
- Press F8-more.
- Press F4-batch entry.
- Type the sample name using the A-number and press Enter.
- Select the used segments and used positions (see par. 5.6) from the pull-down menus.
- Select the test to run (**NEFA1**).
- Press F2-Save changes.
- Introduce the sample segments in the Konelab one by one by opening and closing the sample segment door. The Konelab automatically detects which segments are inserted.

Inserting reagents:

- Press the Reagents button on the top of the main screen.
- Press on a free reagent position number.
- Press F2-Insert reagent.
- Select **NEFA R1** reagent from the list.
- Press OK and follow on-screen instructions.
- Repeat the last 4 steps for the **NEFA R2** reagent.

Starting analysis:

- Go to the main screen and press the green start button, which is located on the keyboard at the right of the Home button. This is the only way the analyzer can be started.

Results:

- When analysis is complete, press the Results button on the top of the main screen.
- Press F8-more, F4 to see all analyzed samples.
- Press F8-more twice, F1 to see details of a selected sample. Press F1 once more to turn details off.
- Press F5-Print results to print the results.
-

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

Generating peak files:

- When the sample series is finished completely, go to the main screen and press F4-Reports and F8-more.
- Select items to report "samples" and "all" from the pull-down menus.
- Press F4-Results to file.
- Select "one row per result" and press OK.
- **Always** enter "results" as the filename.

Subsequently use the available program to generate the actual peak file.

Cleaning up:

- Remove the sample segments from the Konelab when the series is finished by pressing Sample disk in the main screen.
- Press one of the present segment numbers.
- Press F3-remove segment and remove the segment from the Konelab.
- Press F6-remove all samples to clear the samples from the memory.
- Select all your other segments one by one from the pull-down menu and repeat the two last steps until all segments are removed.
- Remove used reagents from the reagent tray by pressing Reagents on the main screen.
- Click on a reagent and then press F3-remove reagent and follow the on-screen instructions.
- Repeat this until all reagents are removed.

Standby:

After each analysis, a Standby should be done. The needles are then cleaned, and the tension is taken off the moving parts.

- Press F2-Standby in the main screen and follow the on-screen instructions.

6. CALCULATION

Use the computer program provided to calculate the PLA1 activity or if this is not available calculate as follows.

Relative calculation (enzymatic standard)

Correct the absorbance of each enzym standard for the absorbance of the corresponding blank ($A_s - A_b$) = ΔA.
Plot the exactly calculated PLA1 activity of the standard solutions against the ΔA.
The calibration curve is a second-degree polynomial function $y = ax^2 + bx + c$ (in which y = ΔA, x = PLA1 activity in NPLAU/mL). This curve is used to determine the amount of activity in unknown samples according to:

$$(NPLAU/mL)cc = -b+\sqrt{\{b^2 - 4a(c-\Delta A)\}}/2a$$

Calculate the PLA1 activity in the samples as follows:

$$NPLAU/g = (NPLAU/mL)cc \times Df/W$$

Method of Analysis

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ΔA	= ΔAbsorbance of sample
A_s	= absorbance of sample
A_b	= absorbance of corresponding blank
$(NPLAU/mL)_{CC}$	= activity obtained from calibration curve
NPLAU/g	= activity per g sample
Df	= total dilution factor of sample

To convert NPLAU to PLAU, use to following calculation factor.

PLAU = NPLAU/1.4

Micro calibration (oleic acid standard)

Determine the slope of the oleic acid calibration curve via linear regression.

Correct the absorbance of each enzyme standard for the absorbance of the corresponding blank:
$(A_s - A_b) = \Delta A$.

Calculate for each enzyme standard the amount of oleic acid formed in mM as follows:

[oleic acid] = ΔA/S

ΔA	= $(A_s - A_b)$
A_s	= absorbance enzyme standard
A_b	= absorbance corresponding blank
[oleic acid]	= amount of oleic acid formed after 30 minutes incubation (mM)
S	= slope of oleic acid calibration curve [L/mmole]

Calculate for each enzyme standard the amount of preparation added to the incubation tube in µg.

µg enzyme preparation = W x 1 000 000/ Df x V_s

W	= amount of standard weighed (g)
1 000 000	= conversion factor from g to µg (µg/g)
Df	= total dilution factor
Vs	= sample volume (0.2 mL)

Make a plot with the [oleic acid] on the X-axis and the amount of added enzyme on the y-axis.
Fit this curve with a second-degree polynomial function $y = ax^2 + bx + c$ (in which y = amount of enzyme in µg/tube, x = oleic acid concentration in mM). Use the coefficients of this curve to calculate the amount of enzyme that forms 1 mM oleic acid under the condition of the test.

$E = a^2 + b + c$

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

E = amount of enzyme that forms 1 mM oleic acid under the conditions of the test (µg)

Calculate the activity of the standard in NPLAU/g as follows:

$NPLAU/g = [oleic\ acid] \times V_t/(E \times t) \times 1\ 000\ 000$

[oleic acid]	= oleic acid formed = 1 mM
V_t	= incubation volume = 2 mL
t	= incubation time = 30 min
1 000 000	= conversion factor from µg E to g

7. ASSESSMENT

a. Requirements relative method

- A (diluted) sample solution must have an activity fitted within the measuring-range.
- Outliers can appear due to the shape of the calibration curve that cannot be fitted perfectly with a second-degree polynomial function. A slight S-shape can be observed in the calibration curve, where point 9 (ES1) is above the curve and point 12 (ES4) is below the curve, outliers (<25% deviation) are accepted for these points. Other points in the standard curve should not exceed 5% deviation (after fitting), they should be discarded and should be considered non-valid (= outlier). Refitting of the standard curve is then required.
- The fitted standard curve must consist of at least 80% of the standard curve points.
- The level of each control value must fit in the range: $C_{assigned} \pm 3 \times SD_{overall}$
  ($C_{assigned}$ = Assigned control value; $SD_{overall}$ = overall standard deviation of the average control value calculated from past series).
- The relative (absolute) difference in level between (duplicate) control values within a daily series is not allowed to exceed a value of $2.8 \times RSD_{within\ day}$.
  (Relative absolute difference in control values = (|control value 1 – control value 2|/Average control value) x 100%: $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past series using control values e.g. as determined in validation of the method).
- The relative (absolute) difference in level between (duplicate) sample values is not allowed to exceed a value of $2.8 \times RSD_{within\ day}$.
  (Relative absolute difference in sample values = (|sample value 1 – sample value 2|/Average sample value) x 100%; $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past sample series with a comparable type of matrix e.g. as determined in validation of the method).

The results of the control sample must be expressed as percentage of the assigned value.
The results of the control samples must be imported into the control charts available for this method of analysis.
All results must be evaluated.

b. Requirements micro-calibration

- A standard curve point of the oleic acid calibration line exceeding 5% deviation (after fitting) should be discarded and should be considered non-valid (= outlier). Refitting of the standard curve is required.
- The fitted standard curve must consist of at least 80% of the standard curve points.

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

- The curve used for micro-calibration must meet the same requirement as mention in paragraph 7.1

c. Actions

Relative method

- Repeat the analysis with an adjusted dilution when results (of the diluted sample) are out of the measuring range.
- Make a new calibration line when requirements are not met and repeat the series.
- When control values do not comply with the requirements repeat the series with new dilutions or weighing of the control sample.
- Repeat the analysis of a sample (new dilution/weighing) when exceeding the “difference of duplicate” requirement.

Micro-calibration

- Repeat the analysis when the slope of the oleic acid calibration line does not meet the requirements with new dilutions of the standard.

d. Authorization

After a training period by a for this method authorized laboratory technician, a technician will be authorized for this method when she/he succeeds in performing the test single-handed, whereby selected samples meet the criteria mentioned above.

8. REFERENCES

[1] Evelien de Weert (2018) Assay development and qualification for *A. Niger* PLA1 method DBC-ABC-A-10367

9. REMARKS

Before actual release or reporting of results, results should be checked by the administrator or responsible scientist of the method of analysis.

10. ANNEXES

Annex 1 Chemical table abstract
Annex 2 Konelab program
Annex 3 Example of an enzymatic standard calibration line
Annex 4 Example of a standard oleic acid solution calibration line

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

a. Annex 1: Chemical abstract table

Name	Hazard symbol(s)	Description of H phrases	Risk Class	Handling as such	Handling dilution or solution	Additional PPE's
Triton X-100		H302 Harmful if swallowed. H318 Causes serious eye damage. H411 Toxic to aquatic life with long lasting effects.	NA	Fume Hood	Lab Table	Nitrile Gloves
Hydrochloric acid 2.0 mol/L		H314 Causes severe skin burns and eye damage H335 may cause respiratory irritation H290 may be corrosive to metals	NA	Fume Hood	Lab Table	Nitrile Gloves
Sodium Hydroxide 2.0 mol/L		H314 Causes severe skin burns and eye damage H335 may cause respiratory irritation H290 may be corrosive to metals	NA	Lab Table	Lab Table	Nitrile Gloves
Acetic acid (glacial) 100%		H314 Causes severe skin burns and eye damage H226 Flammable liquid and vapor	NA	Fume Hood	Fume Hood	Nitrile Gloves
Tris(hydroxymethyl)aminomethane		H315 Causes skin irritation H319 Causes serious eye irritation H335 May cause respiratory irritation	NA	Lab Table	Lab Table	Nitrile Gloves
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)	NA	NA	NA	Lab Table	Lab table	-

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Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

Name	Hazard symbol(s)	Description of H phrases	Risk Class	Handling as such	Handling dilution or solution	Additional PPE's
NEFA-HR R1		H302 - Harmful if swallowed H402 - Harmful to aquatic life H412 - Harmful to aquatic life with long lasting effects	NA	Lab Table	Lab Table	Nitrile Gloves
NEFA-HR R2		H317 - May cause an allergic skin reaction	NA	Lab Table	Lab Table	Nitrile Gloves
NEFA standard (oleic acid)		NA	NA	Lab Table	Lab Table	Nitrile Gloves

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

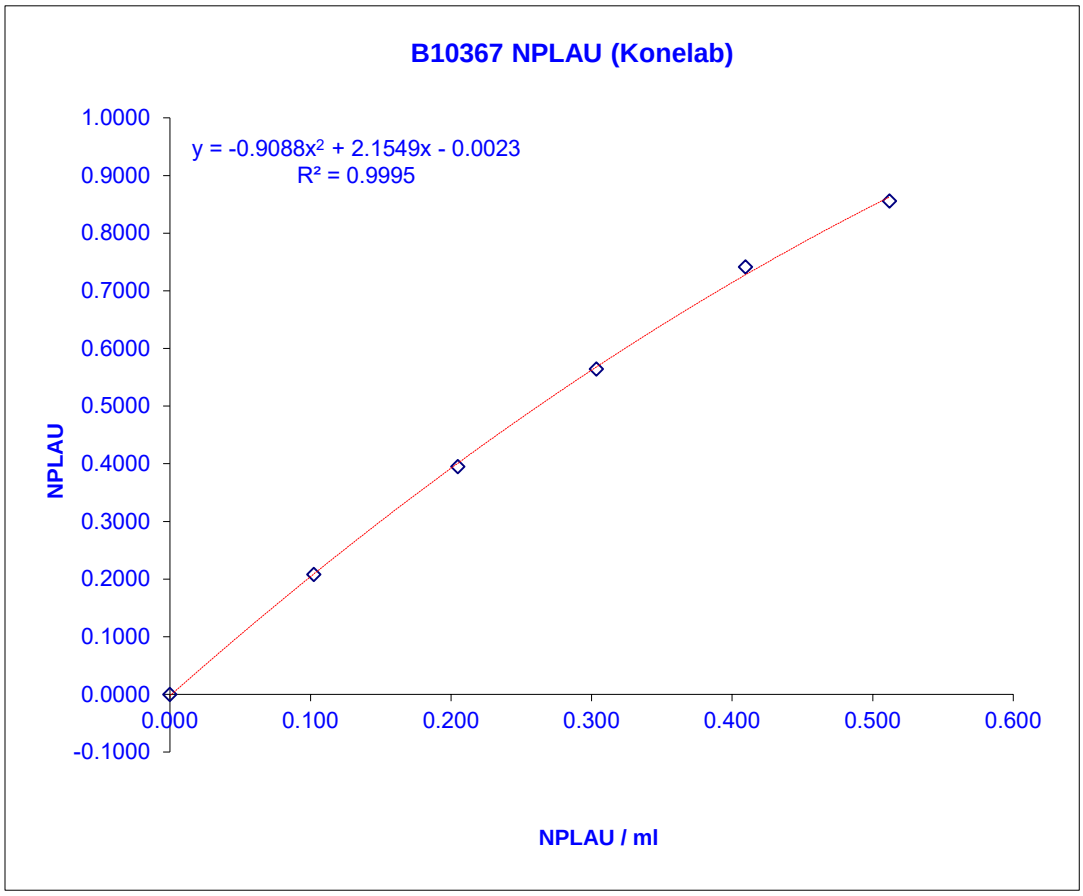
b. Annex 2: Konelab program NEFA1

Full name	NEFA-1 (fatty acids)				
Online Name	NEFA-1	Test In Use	YES		
Test type	Photometric		LOW	HIGH	
		Test limit	0.00000	*	abs
Result unit	abs	Initial absorbance	0.000	*	A
Number of Decim.	5	Dilution limit	*	*	abs
		Secondary dil 1+	0.0	0.0	
		Critical limit	*	*	abs
		Reflex test limit	*	*	abs
		Reflex test			
Acceptance	Automatic	Reference class	LOW	HIGH	In Use
Dilution 1+	0.0				
Sample type	Sample type 1	Correction factor	1.00		
		Correction bias	0.00	abs	
		Temperature	37.0	°C	
Calibration type	None				
Factor	1.00	Bias		0.00	
Bias correction in use	NO				
Manual QC in Use	NO	Routine QC in Use		NO	
Blank	None				
		Normal cuvette			
Reagent	NEFA-R1	Volume (ul)		150	
Disp. with	Extra	Add. Volume (ul)		30	
Wash reagent	None				
Incubation		Time (sec)		300	
Sample		Volume (ul)		10	
Disp. with	Extra	Add. Volume (ul)		30	
Dilution with	Water	Wash reagent		None	
Incubation		Time (sec)		180	
Reagent	NEFA-R2	Volume (ul)		75	
Disp. with	Extra	Add. Volume (ul)		30	
Wash reagent	None				
Incubation		Time (sec)		270	
Measurement	End point				
Wavelength (nm)	540 nm	Side wavel. (nm)		None	
Meas. type	Fixed timing				

Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

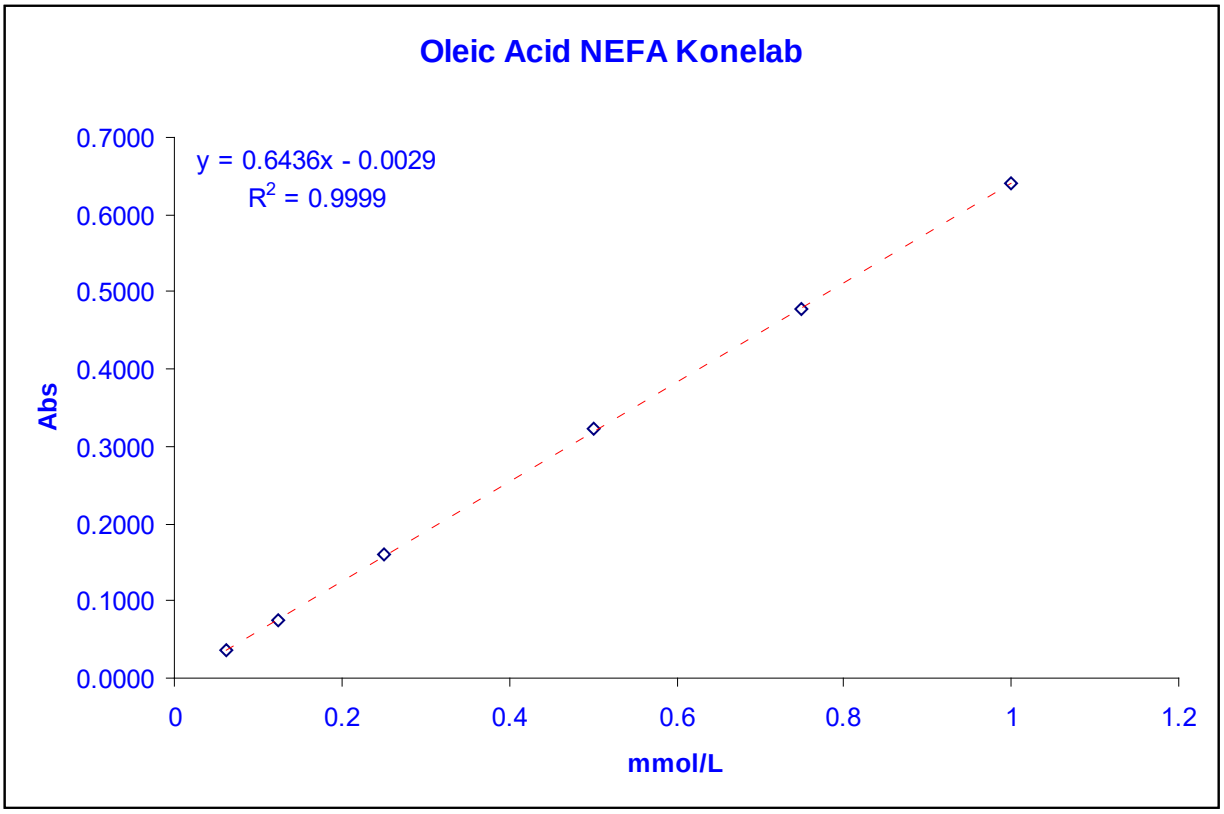
c. Annex 3. Example of a standard enzymatic calibration line.



Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

d. Annex 4. Example of a standard oleic acid solution calibration line.



Method of Analysis

Determination of A. niger Phospholipase A1 activity using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as substrate (pH 4.5) on the Konelab analyzer, relative method.

11. TRAINING

Is training of this document needed?
☒ no

☐ yes → select the type of training:
☐ Training Compass (default); see DBC-ALL-P-00046 mention roles beneath:
……
……..

☐ Classroom / On the job training

12. HISTORY

Version	Description of the modification
1	First version

DSM Nutritional Products
Regulatory Affairs NA

45 Waterview Blvd
Parsippany NJ 07054
USA

Jannavi R. Srinivasan Ph.D.
Team Lead, Chemistry Review Branch
Division of Food Ingredients
Office of Food Additive Safety
HFS255
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College Park, MD 20740

December 12, 2019

Subject: **Reply to questions regarding GRAS GRN No. 857 (phospholipase A1 produced by a genetically engineered strain of *Aspergillus niger*)**

Dear Dr. Srinivasan:

The following addresses questions DSM received on December 9, 2019 about the submission above.

1. **Please confirm the absence of the production strain in the final phospholipase A1 enzyme preparation.**

Answer: The absence of the production strain in the downstream processing steps of the phospholipase A1 production process is routinely checked and confirmed; it is part of the requirements for the operation license at the production sites.

DSM analyzed three batches of enzyme (PLN618092701, PLN618095801, PLN618097901) obtained after the lysis and filtration following the fermentation. Samples were run in triplicate using cultivation conditions that support the growth of *A. niger*. Positive controls with the production strain alone were run concurrently.

There was no evidence of *A. niger* growth in any enzyme sample, whereas growth was evident in the positive control samples. DSM therefore concludes that the lysis and filtration procedure effectively removes all cells and ensures that no viable cells of the *Aspergillus niger* production strain remain in later steps of downstream processing or the final enzyme preparation.

2. **Please confirm that the specifications in GRN 857 are consistent with most recent version of the Food Chemicals Codex (FCC). If yes, please update the FCC edition in your notice.**

Answer: We hereby confirm that the enzyme preparation complies with USP FCC, 11th edition. As far as we can tell, the GRAS notice makes reference to USP FCC in one form or another in pages 9, 18 (where it refers to 10th edition), 19, 25, and 31. Please indicate whether the agency wishes to have revised versions of these pages only (to specify that it is USP FCC, 11th edition) or the entire notice.

3. **Please provide a statement that the final phospholipase A1 enzyme preparation does not contain any major food allergens from the fermentation medium.**

Answer: As noted in section 6.4.1 of the GRAS notice, DSM did not consider the phospholipase A1 protein itself likely to produce an allergenic or sensitization response upon ingestion because a comparison to the amino acid sequence of known food allergens did not reveal any significant matches.

With regard to the possible presence of major food allergens in the enzyme preparation from use of certain allergen-derived ingredients in the fermentation medium, DSM considered that there is a considerable amount of information that would suggest otherwise.

For example, the following conclusions made by organizations with knowledge of industrial food enzyme use provide a reasonable basis for concluding that traces of fermentation ingredients in food enzyme preparations are unlikely to be associated with allergic responses.

- The Enzyme Technical Association in 2004 conducted a survey of its members, and collected information on the possible presence of protein from the fermentation media in the final enzyme product. ETA provided the supporting data and information to FDA in a letter in 2005, and sent an accompanying public statement which is posted on ETA's website (ETA, 2005). The statement concludes that no potentially allergenic protein from the fermentation medium has been found in the finished enzyme. Further, ETA points out that the typical manufacturing process of enzyme preparations includes a step to separate the biomass and fermentation media from the enzyme. This step ensures the enzyme product's purity and stability, and would likely remove most proteins present in the fermentation media.

- In addition, the Food Allergy Research and Resource Program (FARRP) issued a paper in August of 2013 which concluded that, because of the nature of enzymes as catalysts, they are used in very small amounts, and that the fermentation media are consumed during the enzymatic process (FARRP, 2013). It is clear that any *de minimis* amount of protein present in the fermentation media that survived the fermentation process will not cause a significant public health risk to the consumer. FARRP also underscored the fact that the proteins would likely be removed during the filtration of the enzyme product, as discussed by ETA. Further, FARRP indicated that there is no reliable assay that could be used to detect the presence of most allergenic proteins in the final enzyme products, as the proteins would likely be degraded into fragments that would not reach levels of quantitation accessible with current commercial ELISA assays. The full August 2013 statement clearly concludes that any protein allergen present in the final enzyme product would not be present at a level that requires labeling or raises a public health concern.

DSM also considered that this enzyme is intended for use in the degumming of edible oils and fats and no enzyme is expected to remain, having been removed with the separated gum upon centrifugation; any trace amounts that might remain would be removed during subsequent refining steps (washing, bleaching, deodorization). Therefore, no or negligible consumer exposure (to the enzyme and any traces of fermentation ingredients) is expected.

Therefore, DSM does not consider the phospholipase A1 preparation likely to produce an allergic or sensitization response upon ingestion.

I trust this information adequately addresses the questions. Please feel free to contact me with any other questions or concerns you might have.

Sincerely,

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