ORIGINAL SUBMISSION

September 10th, 2014

FYTOZIMUS

GRN 000551

VIA FEDERAL EXPRESS
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Attn: Dr. Antonia Mattia

Re: ***GRAS Notification for Cynzime® a Milk Clotting Enzyme (Ciprosin) Derived From Cynara cardunculus - L***

Dear Dr. Antonia Mattia:

Fytozimus Biotech, Inc. (Fytozimus, a Canadian Corp.), on behalf of itself and Enzyme Development Corp.*, is submitting under cover of this letter three paper copies and one eCopy of a joint GRAS notification for a milk-clotting enzyme preparation (Ciprosin), trade name, Cynzime®, derived from the dried flowers of the plant Cynera cardunculus - L. Fytozimus has determined, through scientific procedures, that its Ciprosin is generally recognized as safe ("GRAS") for use as a milk-clotting enzyme for the production of cheeses. Cynzime is particularly useful in the production of cheese and cheese by-products from the milk of Ewes, as well as other species, including Mares, Camels and Cows, but not restricted to these.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, this use of Ciprosin is exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act, because the notifier has determined that such use is GRAS.

If you have any questions regarding this notification, or require any additional information to aid in the review of this conclusion, please do not hesitate to contact me via email at : aurelio@fytozimus.com

Sincerely,

(b) (6)

Aurélio Fernandes,
Managing Director,
Fytozimus Biotech, Inc.
13915 - 96 Street, Edmonton, Alberta, Canada T5E5Z1

==========

*Enzyme Development Corporation
505 Eighth Avenue, 15th Floor
New York, NY 10018
Attn.: Mr. Peter Moodie

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FOOD ADDITIVE SAFETY

Notice:

The enclosed disk contains an electronic copy of the GRAS Notification from Fytozimus Biotech, Inc. It has been scanned for viruses using Kaspersky software and is believed to be free from viruses or malware.

Gerald Mercer

10/27/14

GRN 000551

GRAS Notification for Cynzime® Milk Clotting Enzyme Preparation Derived from Cynara cardunculus Flowers

SUBMITTED BY:

FYTOZIMUS BIOTECH, INC.
13915 – 96 STREET
EDMONTON, ALBERTA, CANADA
T5E5Z1

Document prepared by:
Gerald D. Mercer, Consultant
10/24/2014

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Table of Contents

1. GRAS Exemption Claim 2

1.1 NAME AND ADDRESS OF NOTIFIER.....2
1.2 NAME OF GRAS SUBSTANCE.....2
1.3 INTENDED USE AND CONSUMER EXPOSURE.....2
1.4 BASIS FOR GRAS DETERMINATION.....3
1.5 AVAILABILITY OF INFORMATION.....3
1.6 ABBREVIATIONS.....3

2. IDENTITY OF THE SUBSTANCE.....3

2.1 SUBSTANCE NAME.....3
2.2 TRADE OR COMMON NAMES.....3
2.3 BOTANICAL IDENTIFICATION OF SOURCE AND CLOSELY RELATED PLANTS.....3
2.4 MANUFACTURING PROCESS.....4
2.5 PHYSICAL AND CHEMICAL PROPERTIES.....7
2.6 COMPOSITION OF COMMERCIAL PRODUCT.....8
2.6.1 ALLERGENICITY.....10
2.7 SPECIFICATIONS OF FOOD-GRADE PRODUCT.....10
2.8 CONTAMINANTS.....11
2.9 STABILITY.....13

3. INTENDED USE AND CONSUMER EXPOSURE.....13

3.1 INTENDED TECHNICAL EFFECT.....14
3.2 RESIDUAL AMOUNTS OF ENZYME IN CHEESE.....14
3.3 ESTIMATED LIFETIME EXPOSURE TO PHYTOCHEMICALS.....16

4. REVIEW OF SAFETY DATA.....17

4.1 SCIENTIFIC INFORMATION DEVELOPED AS BASIS FOR GRAS.....17
4.2 SUPPORTING INFORMATION REGARDING HISTORY OF USE.....17

5. SAFETY ASSESSMENT AND EXPERT PANEL REPORT.....19

5.1 REPORT OF THE PANEL OF EXPERTS (EPR).....19
5.2 CONCLUSIONS.....19

6. LIST OF APPENDICES.....21

7. LITERATURE CITED.....74

1 GRAS EXEMPTION CLAIM

1.1 NAME AND ADDRESS OF NOTIFIER

Fytozimus Biotech, Inc.
13915- 96 Street
Edmonton, Alberta, Canada
T5E5Z1
Main Contact: Mr. Aurelio Fernandes, Managing Director

1.2 NAME OF GRAS SUBSTANCE

Cyprosin, Cardoon, Cynzime®, Extract of *Cynera cardunculus* Flowers

1.3 INTENDED USE AND CONSUMER EXPOSURE

Fytozimus Biotech, Inc. has developed a concentrated, standardized coagulant for use in the production of a variety of cheeses and cheese by-products. This product, named Cynzime®, is derived from the flowers of *Cynara cardunculus* L. (*Cc-L).* Cc-L, and related varieties of this thistle are cultivated and grow wild in the Mediterranean regions where it is used as a source of milk-clotting enzyme for cheese production from ewes milk as well as others. Consumer exposure calculations and examples are detailed below in Table 1 (Appendix A) and 3.2 below. Cyprosin, the milk-clotting enzyme in Cynzime® is currently used in artisanal cheese production of ewe milk cheeses such as Serra da Estrela, or Serpa in Portugal and Manchego, La Serena in Spain, some of them having PDO (Protected Designation of Origin) status (Sousa and Malcata 2002; Roseiro et al. 2003a; Prados et al. 2007). These cheeses are highly appreciated for their unique flavour, and aroma. It can also be used for production of cream cheeses, gouda, cheddar, romano among others. Fytozimus, as noted above, has developed technology to produce a more uniform, standardized product, which will make it more useful in the production of a wider variety of cheeses.

1.4 BASIS FOR GRAS DETERMINATION

This claim is based upon scientific information gathered from published articles as well as analytical data gathered by Fytozimus. These data and calculations regarding potential human exposure to the enzyme preparation and trace components derived from the *Cynara cardunculus* plant characterize the product and clearly demonstrate the safety of the Cynzime® milk clotting enzyme preparation for use in the manufacture of a variety of cheeses and cheese products.

Supporting the scientific evidence of the safety of Cynzime® is a very large body of information regarding the history of safe use of the enzyme preparation derived from the flowers of *Cynara cardunculus* – L, primarily in the region of southwestern Europe, Spain, Portugal, southern France and the Mediterranean. This information also details the safety of the *C. cardunculus* – L plant itself as a source of the milk-clotting enzyme preparation.

1.5 AVAILABILITY OF INFORMATION

All information pertaining to the present GRAS Notification will be available for FDA during normal business hours at the address noted above (Fytozimus Biotech, Inc., Edmonton, Alberta, Canada

1.6 ABBREVIATIONS

Cc-L: *Cynara cardunculus* – L

2 IDENTITY OF THE SUBSTANCE

2.1 SUBSTANCE NAME

Milk – clotting enzyme preparation, aqueous extract of flowers of *Cynara cardunculus* – L.

2.2 TRADE OR COMMON NAMES

Cynzime®, Cardoon, Cyprosin, ENZECO® ASPARTIC PROTEASE

2.3 BOTANICAL IDENTIFICATION OF SOURCE AND CLOSELY RELATED PLANTS

Cynara cardunculus – L, the cultivated cardoon (var. *altilis* DC.) and the wild cardoon [var. *sylvestris* (Lamk) Fiori]. The three types (wild cardoon, cultivated cardoon and globe artichoke) are fully cross-compatible and inter-fertile and their F1 hybrids are fully fertile (Basnizki and Zohary, 1994; Mauromicale and Ierna, 2000).

2.4 MANUFACTURING PROCESS

Brief description of the process for production of Cynzime®

A schematic of the production process for Cynzime® is shown in Figure 1.

The enzymatic extract, in which the cyprosins are present, is obtained from *Cynara cardunculus* (cardoon) flower, a plant widely distributed in southern Europe (Mediterranean area), Spain and Portugal and is largely used for milk-clotting (Heimgartner et al. 1990).

The enzymatic extract is obtained according to an optimized extraction process carried out by Fytozimus Biotech. The production procedure is the following:

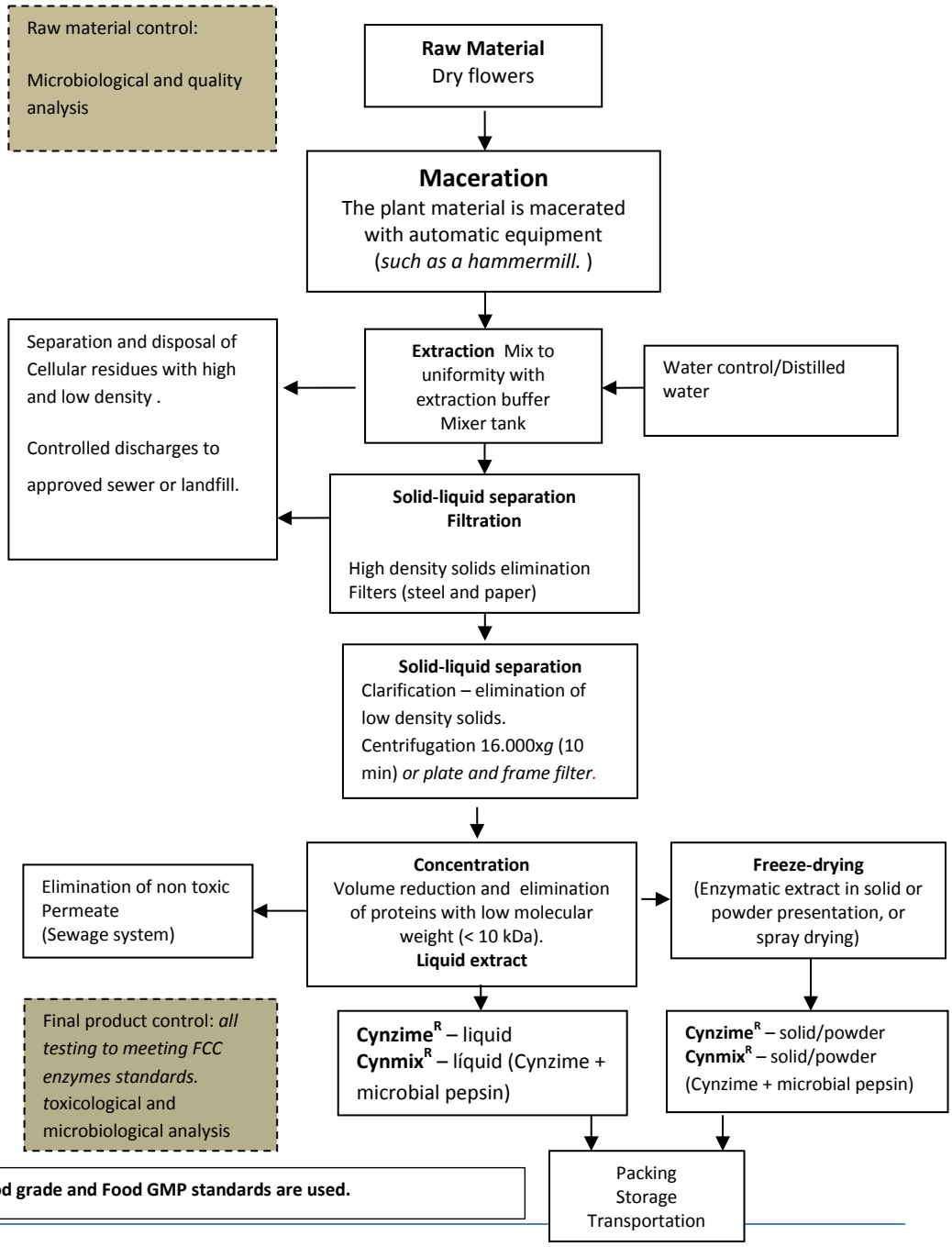
- The dried flowers of *Cynara cardunculus* are macerated using a grinder.
- The powder obtained is added to a sodium phosphate buffer pH 7.4 for extraction and chemical stabilization of the enzymatic extract. This first extract is maintained at low temperature (4ºC) during at least 12 hours with mild agitation to ensure a uniform extraction.
- After that, the extract is filtered using a metal filter with a low cut-off for removing the high density solid compounds.
- The liquid phase, obtained after filtration, is centrifuged at 16.000×g during 10 minutes at 4ºC for removing the low density solid compounds. *An alternative process is to use a plate and frame filter press with diatomaceous earth.* The liquid phase obtained is filtered using a pre-filter to remove large molecular

weight material such as a 1000K or 800K dep filter. The liquid and concentrated using an ultrafiltration cartridge with a 10 kDa cut-off.

- The stabilizer (NaCl 10%) and the preservative (Sodium benzoate 0.1%) are added to the final product.
- The extract is then standardized and prepared for use by cheese makers in a product called Cynzime®. The product can be presented as liquid or solid. The solid is obtained from the liquid extract by freeze drying or spray drying.

Figure 1

Schematic of the complete process by which cyprosin is made from cardoon to the finished product:



2.5 PHYSICAL AND CHEMICAL PROPERTIES

Description and Characterization of Cyprosin

Cyprosin is a heterodimeric aspartic protease with a molecular weight of about 49 kDa. Cyprosins correspond to previous cynarases renamed as cyprosins due to the IUPAC rules. They have three isoforms (1, 2 and 3) and each isoform has two sub-units (large sub-unit ≈ 33kDa; a small sub-unit ≈ 16kDa). Both subunits of cyprosins 1 and 2 are glycosylated, while in cyprosin 3 only the large subunit is glycosylated. The differences of about 2 kDa existing between the large and the small subunits of the different isoforms suggest the presence of one N-linked mannose chain (Heimgartner et al. 1990).

According to the Enzyme Commission of the International Union of Biochemistry and Molecular Biology, Cyprosin and its three isoforms, can be classified as EC3.4.23. The terminology is based on the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) list of commercial enzymes registered as an enzymatic extract constituted by similar enzymes or isoforms.

Empirical formula, structural formula, molecular formula and molecular weight:

FIGURE 2 Cyprosin is an heterodimeric aspartic protease with molecular weight of 49 kDa.

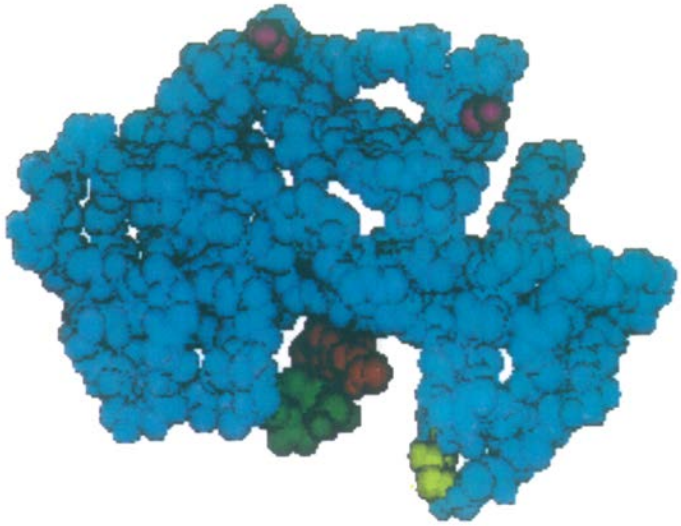


Figure 2-Schematic 3D representation of cyprosin B protein structure.

The amino acids sequence of cyprosin is represented below:

```
  1   mgtaikasvl alflffllsp tafsvsnggl lrvglkkrkv dqinqlsghg vsmeakarkd
 61   fgfggalrds gsdiialkny mdaqyygeig igsppqkftv ifdtgssnlw vpsakcyfsv
121   lfhskyks shsstykkng tsaaiqygtg sisgfvsqds vklgdlvvke qdfieatkep
181   tflaakfd gilglgfqei svgksvplwy nmvnqglvqe pvfsfwfnrn adeeeggelv
241   gvdpnhfk gkhtyvpvte kgywqfdmgd vliedkttgf csdgcaaiad sgtsllagpt
301   iteinhai gakgvmsqqc ktlvsqygkt miemllseaq pdkicsqmkl ctfdgardas
361   iesvvden ngksssgvhd emctfcemav vwmqnqikrn etedniinyv nelcdrlpsp
421   esavdcns lssmpniaft iggkvfelcp eqyilkigeg eaaqcisgft amdvapprgp
481   lwilgdvfmg ryhtvfdygk lrvgfaeaa
```

2.6 COMPOSITION OF COMMERCIAL PRODUCT

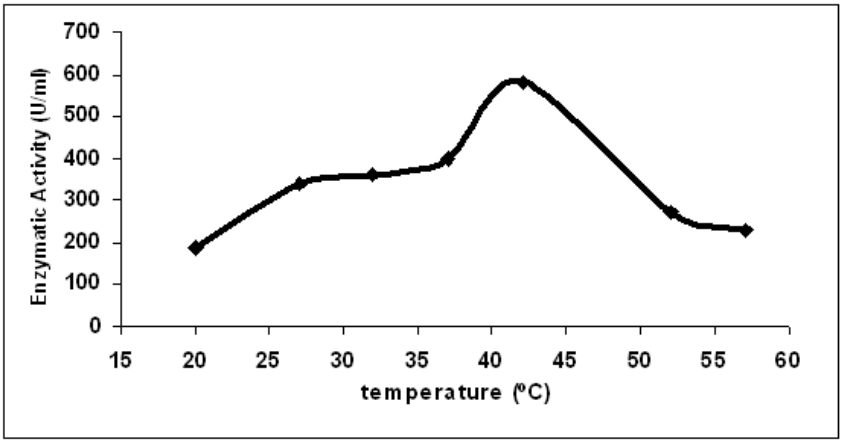
Cyprosin's chemical structure and properties (ie. color, powder, crystal, particle size, odor, taste)

- The enzymatic extract is a dark brown liquid or solid, with a fresh plant odour, and the powder is brown with a slight smell of artichoke.

- The enzymatic extract prepared from *Cynara cardunculus* flower, in which are present the three isoforms of the aspartic protease cyprosin (corresponding to the trade mark Cynzime®) is 100% natural. The final product contains 10.0% (w/v) NaCl and 1.0% (w/v) sodium benzoate as stabilizers.

Physico-chemical properties to identify cyprosin:

- Optimum Temperature: 42ºC (see Figure 2)
- Optimum pH 5.2 (see Figure 3)
- Activity inhibitor : Pepstatin A
- Activity inhibition temperature: 55ºC
- IP (isoelectric point): 5.0



- **Figure 2 -**Evaluation of optimum temperature for proteolytic/clotting activity (P. Sampaio, PhD thesis, 2009)

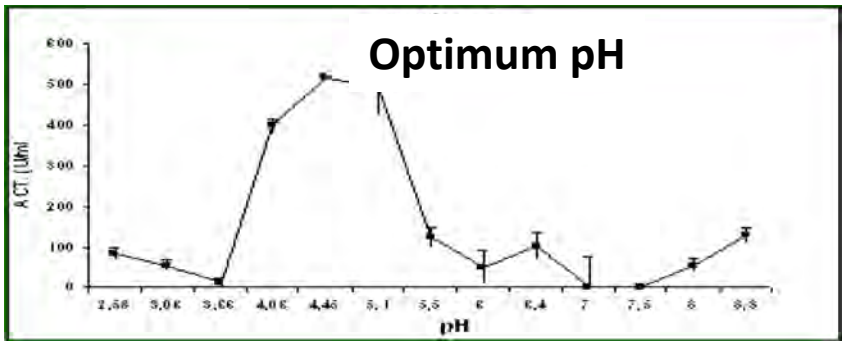


Figure 3 -Evaluation of optimum pH for proteolytic/clotting activity (P. Sampaio, PhD thesis, 2009)

Biological properties – how the enzyme reacts to break specific bonds:

Cyprosin is an aspartic protease that hydrolyzes the peptide bond Met[105]-Phe[106] of α-, β-and *k*-caseins caseins present in the milk from cow, goat, sheep, buffalo and camel, promoting their coagulation. The enzyme is added to milk at 32-36°C, at specific pH 6.2-6.5 and in a ratio enzyme/milk that promotes milk coagulation and further cheese production. The enzyme residues, to a great extent, are removed in whey during cheese production and ripening.

2.6.1 ALLERGENICITY

The potential for Cynzyme® to cause an allergic response via ingestion as a result of consuming cheese made with this milk-clotting enzyme is addressed in Appendix J.

2.7 SPECIFICATIONS OF FOOD-GRADE PRODUCT

Product Specifications: Cynzime®

Cynzime® meets the general specifications as set forth by the FAO WHO Joint Expert Committee on Food Additives (JECFA)/

Product: Liquid milk clotting enzyme -Natural source
Appearance: Liquid, light brown

Strength: 1:3000 in European Commercial Units (according to the Berridge method). Analysis error: +/-5%
Stability : <10% loss of activity in six months when stored at 4 - 8°C
Composition: Water, Enzyme, Sodium Chloride and Sodium Benzoate
Storage: Store at 4 – 8°C
Use: Food industry (cheese and milk-derived products)
Shelf life: Twelve months after shipment to end user.
Origin: Enzyme from plant origin, obtained by extraction and purification of proteases from *Cynara cardunculus* –L flowers

DETERMINATION	METHOD*	RESULT / UNIT
Coliforms	NP-3788	$< 1\times 10^1$ CFU/g
Yeasts & Molds	prNP-3277-1	$< 1\times 10^1$ CFU/g
Salmonella	PAM 55	Neg. in 25 g
Staphylococcus coagulase	NP-4400-2	$< 1\times 10^1$ CFU/g
Listeria monocytogenes	PAM 56	Neg. in 25 g
Halophillic bacteria	PAM 44	$< 1\times 10^1$ CFU/g
Lead (as Pb):	**USP**	NMT 5 mg/kg
E. coli	**USP**	absent in 25g sample

* Future testing will be conducted using USP, BAM methods

Characteristics of the enzyme preparation:

% Total Organic Solids = 100 – (% ash + % water + % diluents / additives)= 58.5 g/liter.

Milk Clotting Activity:
Results after extraction:

Protein concentration: 8-15 mg/ml
Proteolytic activity: 6.000 – 10.800 U/ml. Proteolytic activity is determined by Twining method (Twining 1984).
Specific activity: 1328 U/mg

Clotting activity: 25 – 58 IMCU/ml - Cynzime® (liquid)

Clotting activity: 175 – 220 IMCU/ml – Cynzime® (solid/powder)

2.8 CONTAMINANTS

2.8.1 Phyto-chemicals from source plant

Due to the purification and reduction of lower molecular weight constituents during the production process, phyto-chemicals, such as polyphenolic compounds are shown to be present at less than 560 ppm in the worst case. Even if present in the enzyme product at this level, lifetime exposure to these phyto-chemicals would be = 3.8661×10^{-6} grams of phenolics in a lifetime.

Calculation of lifetime exposure to polyphenolic compounds known to be present in *Cynara sp.*

Samples sent for analysis were determined to contain 560 ppm total phenolics (worst case). Based upon dosing of Cynzime® at a rate of 1.0 -1.2 ml/gallon of milk, resulting in the production of 1 pound of hard cheese (e.g. aged cheddar) or two pounds of soft cheese (mozzarella or ricotta) and assuming 100% of the total phenolics remained in the cheese, and that per capita consumption of cheese in the US is 33.5 pounds (source: Wisconsin dairy industry statistics) :

(560/1,000,000*1.5 grams)/33.5*454)

0.00084/15209

$5.523*10^{-8}$

Assume 70 years of eating cheese

$5.523 * 10^{-8} *70 = 3.8661 * 10^{-6}$ grams of total phenolics in 70 years

2.8.2 Mycotoxins

Mycotoxins have been tested for, with the following results:

Aflatoxin, Ochratoxin A and Zearalenone *

B1 <0.500 ppb

B2 <0.500 ppb

G1 <0.500 ppb

G2 <0.500 ppb

Ochratoxin A <1.00 ppb

Zearalenone <10.0 ppb

2.8.3 Pesticide residues (See Appendix F)

A wide range of pesticides were tested for and results indicate that all were below the levels of quantification (LOQ). These results are detailed in Appendix F.

2.9 STABILITY

The enzyme stability study over time, showed that the enzymatic activity /proteolytic activity is stable after 1 year storage at 4ºC - 8ºC (Fig. 4).

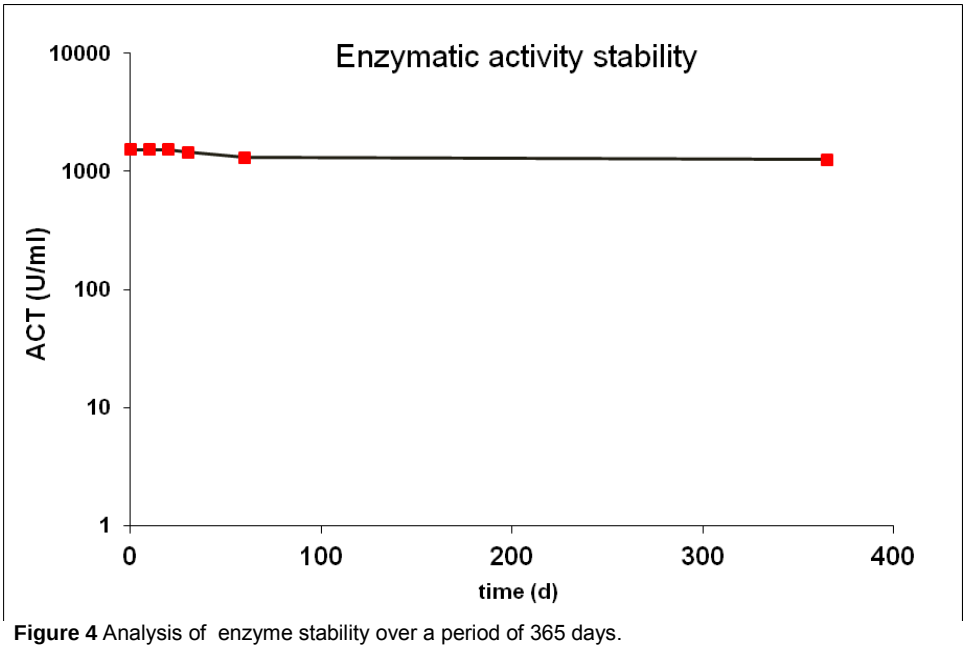


Figure 4 Analysis of enzyme stability over a period of 365 days.

3 INTENDED USE AND CONSUMER EXPOSURE

3.01 Portuguese cheeses are already sold in the USA. During 2013 there were 22 container shipments from Portugal to the USA listed as "cheese, butter, and dairy products." The shipments were to freight forwarders so an individual breakdown of the container load is not available. However, the cardoon produced cheeses are offered on various websites in the USA when they are seasonally available.

Grupo Seabra is the largest importer of Queijo da Serra in the US. They import close to $ 40 Million US on a yearly basis and Queijo da Serra cheese, alone, is in the range of 20 - 30 Tons. There is a yearly Queijo da Serra festival in New Jersey where the association representatives (from Portugal) usually attend. It is clear then, that US consumers are currently consuming significant quantities of cheese

produced using *Cynara cardunculus* – derived milk clotting enzyme (Cardoon).

3.1 INTENDED TECHNICAL EFFECT

The intended technical effect of the use of Cynzime® is to coagulate/ hydrolyze the caseins in milk, leading to its use as a process step in the production of cheeses of various types.

3.2 RESIDUAL AMOUNTS OF ENZYME IN CHEESE

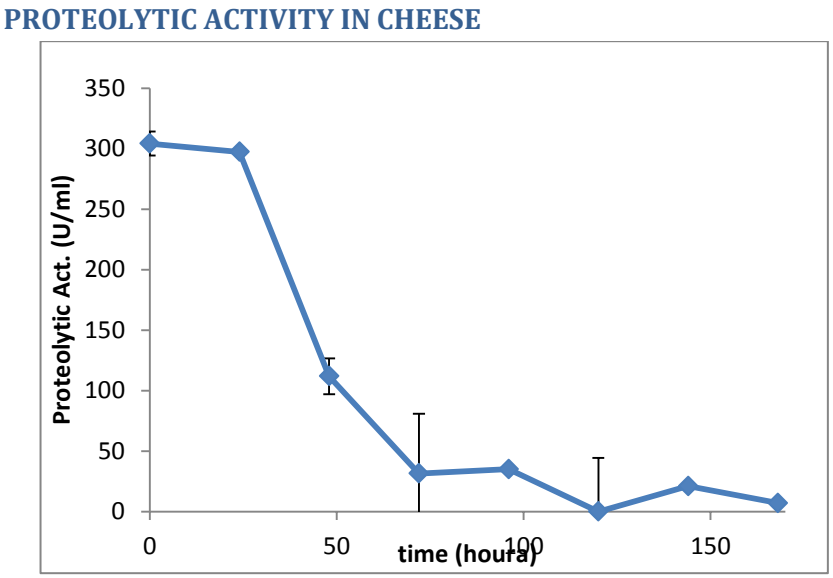


Figure 5 Evolution of cyprosin proteolytic activity in cheese along (160 hours) of cheese ripening at 4ºC.

The enzyme loses its activity in the first 160 hours of cheese ripening at 4C (Fig. 5). Any trace amount of the enzyme activity remaining after 160 hours is lost by the time the cheese reaches the distributor.

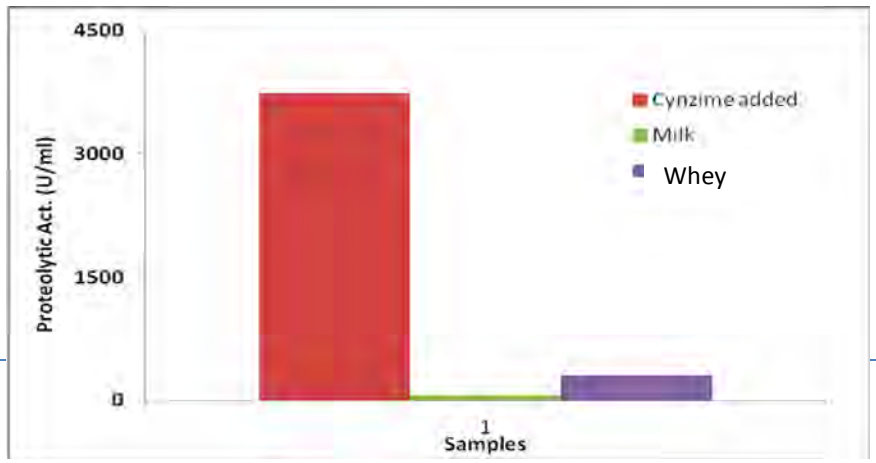


Figure 6 -Analysis of proteolytic activity in milk with Cynzime® added, in control (milk without adding Cynzime®), and in whey obtained after clotting step.

- The maximum level of use can be slightly higher than what you might recommend just to cover for any fluctuations in raw material that might affect the clotting. However, dosing of the enzyme preparation beyond the range of 1.0 – 1.2 ml/liter of milk, becomes economically infeasible and provides no benefit to the cheese-making process schedule.

Activity of the enzyme when the cheese is sold to the consumer.

The graph in fig. 7 shows the proteolytic activity values of the enzyme (in red), the proteolytic activity in the milk without adding Cynzime (in green) and the proteolytic activity in the whey after clotting (in violet).

From fig.5 (pg.19) it can be seen that the activity of the enzyme in the cheese is very nearly 0.0 U/ml at 160h of ripening

Analytical method of food additive detection

The Cynzime® activity is evaluated according to Heimgartner et al. (1990) using flourescein isothiocyanate-labelled K-casein (FITC-casein) as the substrate, prepared according to Twining (1984). One unit of proteolytic activity was defined as the amount of enzyme that increases one unit of emitted fluorescence at 525 nm (excitation at 495 nm) after 60 min hydrolysis at 37°C (Heimgartner et al. 1990). Fluorescence intensity was measured using a spectrofluorimeter (Shimadzu RF-1501, Kyoto, Japan).

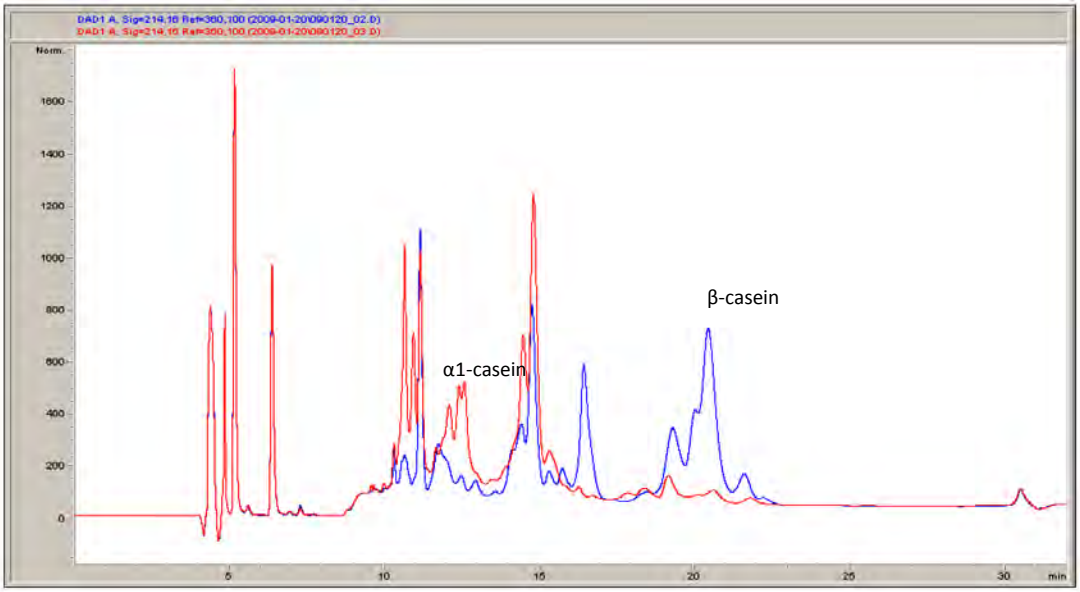


Figure 7 –Proteolytic action of Cynzime® over different caseins of milk, assayed using a HPLC analyzer (from DANISCO, 2009).

In figure7, it can be seen the caseins cleavage by Marzyme (blue) and the Cynzime® + Marzyme (red)

3.3 ESTIMATED LIFETIME EXPOSURE TO PHYTOCHEMICALS

While certain phytochemicals are known to be present in the tissues of *C. cardunculus* – L, including polyphenols, and this is seen as a benefit to users who consume the plant for that reason. However, the presence of such phytochemicals in the extract of the mature flowers for use as a milk-clotting enzyme preparation, while clearly not harmful, may be unwanted. In order to dispel any concern about the presence of trace amounts of these polyphenolic compounds in the commercial milk-clotting enzyme preparation Cynzime®, we have estimated and calculated the potential exposure of consumers of cheeses made with Cynzyme®. We determined that a lifetime exposure to such polyphenols would be 3.8661×10^{-6} grams of phenolics in a lifetime. We believe that such low exposure, as compared to the consumption of the plants themselves, present a negligible risk.
(See Sect. 2.8.1 above.)

Further discussion regarding the chemical characterization and toxicological properties of *Cynara cardunculus* – L may be seen in Appendix C.

4 REVIEW OF SAFETY DATA

As noted above, a considerable amount of published literature discusses the various uses of the Cardoon plant and its constituents for cheese-making, direct consumption as part of the cuisine and as folk remedies. In no instance does this body of information implicate Cardoon or the enzyme preparation derived from its flowers, in any adverse events, toxicity or other adverse effects. Several of these articles were reviewed and summarized in Table 3 (Appendix J).

4.1 SCIENTIFIC INFORMATION DEVELOPED AS BASIS FOR GRAS

Presented herein are data collected via a number of sources, including published literature, analytical measurements of the nature and characteristics of Cynzime®. Characterization of the enzyme protein, total organic solids (TOS), possible co-extracted chemicals or other contaminants and antioxidant polyphenolic compounds has also been conducted.

In addition to the product specifications and characteristics, analytical measurements of a number of parameters were conducted to assure the enzyme preparation is not, nor does it contain any poisonous or deleterious substance which would render it unsafe.

4.2 SUPPORTING INFORMATION REGARDING THE HISTORY OF USE OF *CYNARA CARDUNCULUS – L* PLANT, MILK-CLOTTING ENZYME AND PRODUCTS DERIVED THEREFROM

Key to the determination of the general recognition of safety of Cynzime® is the extensive documented evidence in published literature of the origin, ecology and cultivation of the plant *Cynara cardunculus* and related species, as food. In her Doctoral Thesis, Ciancolini (11) presents evidence that the genus *Cynara* was known by the Greeks and Romans, as reported by Columella in *De Rustica* and by Plinio in *Naturalis Historia* 371-287 BC. According to Ciancolini, the species *Cynara cardunculus* L. (*Cc-L)* is native to the Mediterranean Basin, where its commercial production makes a significant contribution to the agricultural economy. (2). *Cynara cardunculus* L. is a complex species consisting of the globe artichoke [var. *scolymus* (L.) Fiori], the cultivated cardoon (var. *altilis* DC.) and the wild cardoon [var. *sylvestris* (Lamk) Fiori]. The three types (wild cardoon, cultivated cardoon and globe artichoke) are fully cross-compatible and inter-fertile and their F1 hybrids are fully fertile (Basnizki and Zohary, 1994; Mauromicale and Ierna, 2000). The *Cynara* L. is a relatively small

genus taxa (Wiklund, 1992) of the daisy family Asteraceae (ex Compositae), native to the Mediterranean region, sharing its distribution with the olive (*Olea europea*). *C. cardunculus* is a hemicryptophyte, perennial rosette plant, which renews itself year after year by means of lateral shoots arising just below the soil surface. The wild type is a robust thistle with a characteristic rosette of large spiny leaves, branched flowering stems, and blue-violet flowers. In Sicily there are a few areas where wild cardoon plants have white and pink flowers. The wild cardoon is distributed over the west and central part of the Mediterranean Basin (from Portugal to west Turkey), as well as The Canary Islands and Madeira. In post-Columbian times, it colonized some parts of the New World and is a weed in parts of Argentina, Chile and California (Basnizki and Zohary, 1994).

Globe artichoke is an important vegetable in Mediterranean countries, where about 80% of the land surface (128 Kha***) is cultivated. Italy (50 Kha), Spain (13 Kha), Egypt (9 Kha) and France (9 Kha) are the leading countries. In the other parts of the world, Peru (7 Kha), Chile (5 Kha), Argentina (4 Kha) and USA (3 Kha, mainly in California) are the main producers (FAO, 2012). For the first time, it has been reported in China in 2001, where 9 Kha are cultivated. The globe artichoke is widely consumed raw, boiled, steamed or fried, and used in many recipes, since it tastes good and is perceived as a healthy and nutritious vegetable, but other possible uses are: fresh and dry biomass and forage for livestock feed, preparation of alcoholic beverages and medications from leaves dry extracts and inulin extraction from roots (Foti *et al*., 1999; Maccarone *et al*., 1999; Gominho *et al*., 2001; Fernández *et al*., 2006; López-Molina *et al*., 2006). Globe artichoke is described in the pharmacopaea and health-promoting functions are currently reported. Cardoon has been cultivated as a vegetable since ancient times but the land area devoted to this crop has never been large (2-3000 ha), localised in Spain, Italy, France and Greece (Ierna *et al*., 2012). The commercial product is the enlarged petioles, central rib and a small part of the leaf border (two thirds of the inferior part of the leaf is utilized) harvested at the beginning of the winter after a bleaching period, which usually takes about one month. The plant, which can grow up to 2 m high, has the leaves of the basal rosette petiolata, very large, coriaceous, bright green and tomentose** above and white-tomentose beneath. The stem is the floral scape as a corymbose cyme*. The flowers are grouped in large globular non-edible capitula with the colour of corolla blue, lilac or whitish.

**Corymbose cyme: A usually flat-topped flower cluster in which the individual flower stalks grow upward from various points of the main stem to approximately the same height.*

** *Tomentose refers to plant hairs that are flattened and matted.[1] The Latin word meaning 'cushion stuffing,' tomentum, is used to describe a woolly coating formed by the 'tomentose' hairs*

****Kha = Kilo-hectare*

Cc-L is known to produce, in various parts of the plant, a variety of Polyphenols, Flavonoids and other compounds, believed to impart value to its use in food, feed and other applications. (Pandino, et.al (). The flowers, however are of particular interest in the present case, because of their value for use as a coagulant, primarily for ewe's milk in the production of a variety of cheese products consumed widely in Portugal, Spain, and throughout the Mediterranean region. Traditionally, crude aqueous extracts of the flowers of var. *sylvestris* are used by local farmers and small-scale, artisans in the production of a variety of cheeses and cheese by-products in Portugal, Spain and Italy and is a successful replacement for rennet in Cheese making.

Cc-L is also widely used as food, animal feed, as a home remedy and folk medicine and as a source of antioxidant polyphenols sold in the health food market. Fytozymus is herewith, presenting information to support a claim that Cynzime®, in its various formats, is exempt from pre-market approval and that Cynzime® enzyme preparation is Generally Recognized as Safe by experts qualified by education, training and experience, for its intended use as a milk clotting enzyme preparation. The scientific information is supported by an extensive body of evidence that the enzyme preparation, derived from the flowers of the Cc-L plant, and the cheese produced therefrom, and the Cc-L plant itself, have a history of use dating to Roman times. This history of use information is supported by a body of published information, and historical documents, which convincingly support the GRAS status of the product based on history of use and consumption by humans and animals.

5 SAFETY ASSESSMENT AND GRAS PANEL REPORT

5.1 GRAS PANEL REPORT

The report of the GRAS Panel, its members and conclusions are attached as Appendix H.

5.2 CONCLUSIONS

Based upon scientific information developed by Fytozimus Biotech, Inc. regarding the nature and characteristics of the aqueous extracts of dried flowers of the thistle *Cynara cardunculus* – L, such information consisting of thorough characterization of the enzyme preparation, the nature and quantities of potential contaminants, Fytozimus Biotech, Inc. hereby claims that the use of the milk-clotting enzyme preparation Cynzime® is safe and suitable for its intended use and is Generally Recognized as GRAS by qualified experts.

Supporting this claim is the information cited herein, which clearly demonstrates not only the safe use of the extract of the flowers as a milk clotting enzyme by artisanal producers of cheeses therefrom, but also the wide practice of consumption of the plant itself and of close relatives by humans and animals since Roman times. These facts are clearly stated in historic as well as current scientific literature.

6 LIST OF **APPENDICES**

Appendix A: Table 1 and Discussion

Appendix B: Cynzyme® Safety: Review of published information regarding *C. cardunculus*

Appendix C: Chemical characterization and toxicological properties of *C. cardunculus*

Appendix D: Certificate of Analysis – Product Specifications

Appendix E: Certificate of Proximate Analysis for Cynzime

Appendix F: Pesticide Analysis

Appendix G: Protein Profile and Polyphenol Analysis

Appendix H: Expert Panel Report

Appendix I: Additional Scientific Safety Documentation

Appendix J: Allergenicity potential of Cynzyme®

7 **LITERATURE CITED**

APPENDIX A.

TABLE 1 AND DISCUSSION

Appendix A

Table I - an analysis of the potential human exposure to *Cc-L* total organic solids (TOS)

In Table I an analysis of the potential human exposure to *Cc-L* total organic solids (TOS) as the result of consuming products made with extracts of the Cc-L flowers, is presented. As can be seen from this analysis, it is clear that, given the fact that the plant itself is safely and historically consumed by humans and animals as food or feed, the exposure to any residue of an enzyme preparation prepared from portions of the plant and used in the preparation of a variety of cheeses, should present a negligible safety concern. The historical use of the plant is described in nearly every published scientific paper or article regarding the plant, its applications in the diet of the region and its use in the production of cheeses since at least Roman times. Unlike certain other (microbial) enzyme preparations, which may have been mentioned in passing in writings from Biblical times, the thistle, *Cynara cardunculus* (Cardoon) is specifically mentioned as having been consumed since at least the fourth century BC (Columella in *De Rustica* and by Plinio in *Naturalis Historia 371-287* BC).

Human exposure to Cc-L enzyme or residuals thereof, may be calculated in several ways, i.e. 1.) based upon total consumption of cheeses made using the enzyme preparation by the population of a region; 2.) based upon estimates of the total enzyme protein consumed in cheeses made using the enzyme. Direct statistical data regarding the usage of Cardoon flowers in the artisanal production of cheeses must be inferred by estimates of cheese production or consumption in the regions where it is commonly made. In the case of the commercial product described herein (Cynzime®), exposure based upon use of the product at the recommended rates may be calculated using Total Organic Solids content of the enzyme preparation (TOS). This is the means by which potential worst-case exposure is calculated in Table I.

.

Table I - Calculation of exposure to Cynzime® TOS resulting from consumption of cheese and other products

Typical dosing rate of Cynzime = 1 liter / 3000 liters of milk ……	58.5	grams of TOS/3000 liters
Assuming that all portions of milk are consumed as cheese,whey, or requeijão and that the TOS is evenly distributed among the products, consumers eating the cheese resulting from 3000 liters of milk, would be exposed to :…….	19.5	milligrams TOS/kg of cheese
Since a typical person consumes cheese in e.g., Portugal …….	10	kg/capita/year
Total exposure to TOS would be …….	195	mg/person/yr
or, for a 70 kg (BW) eater …….	2.785714	mg/kg body weight/year
In the extreme, supposing that information were available for a 90th Percentile eater ……	100	kg cheese/yr
exposure would be: …….	27	mg TOS/kg body weight/yr
Since there have been no reports in the literature of adverse effects from eating the plant or products produced therefrom, it is extremely unlikely that adverse effects could result from the use and consumption of Cynzime or products produced therefrom.	>0	risk

Notes, regarding Table I: There are several methods by which potential human exposure to the milk-clotting enzyme preparation derived from *C. cardunculus* flowers may be determined. These methods, detailed in Appendix A, include: 1.) calculating the exposure to the enzyme preparation currently used in traditional cheeses based upon the amount of cheeses and by-products consumed by the population of the region where these cheeses are consumed; 2.) basing exposure to the Total Organic Solids in Cynzyme® as determined by the standard method (100% of solids – water – diluents – Ash). Appendix A also contains details of the amount of cheeses produced using coagulating enzymes other than *C. cardunculus* for comparison. Unlike in the US, where consumption data are provided in food consumption tables by USDA, no such data are available in Portugal, Spain, France and other regions where *C. cardunculus* cheeses are traditionally consumed. Therefore, an estimate is made based on total cheese consumed per capita of the entire population of the region.

Cyprosin is currently used in artisanal cheese production of ewe milk cheeses such as Serra da Estrela, or Serpa in Portugal and Manchego, La Serena in Spain, some of them having PDO (Protected Designation of Origin) status (Sousa and Malcata 2002; Roseiro et al. 2003a; Prados et al. 2007). These cheeses are highly appreciated for their unique flavor, and aroma characteristics. Cyprosin can also be used for production of cream cheeses, gouda, cheddar, and romano among others.). The enzyme extract has been used since the Roman era for cheese production for different milk types.

Fytozimus Biotech, Inc. has developed technology, wherein the active coagulant principles of the Cc-L flower, that is, the aspartic protease, is extracted from the flower. The enzyme is referred to as cyprosin and is marketed under the trade name Cynzyme®.
Cynzyme® will bring value to the technology of cheesemaking for these types of cheeses and perhaps others, as its characteristics become more widely known and appreciated. A standardized enzyme preparation, versus the current “art” of using Cc-L flowers, will help both technically and economically, by preventing cheese making failures and standardizing the cheese making process. The process for production of Cynzyme also reduces the amount of unwanted co-extractants such as the poly-phenolic compounds mentioned above.

APPENDIX B.

Cynzyme® SAFETY

Review of Published Information

APPENDIX B. Cynzime® SAFETY: Review of published information regarding *C. cardunculus*

Table of Selected Published Information on Safety and Characterization of *Cyneria cardunculus*

No.	Source Reference	Characteristics Studied	Safety Relationship	How related
1.	Velez, et. al. (2012)	Potential pharmacological use of plant or parts thereof	May demonstrate usefulness of plant as sources of bioactive components. Active components mainly from parts other than flowers.	References long history of food and medical food uses. Mentions no adverse effects of such use.
2	Pandino, et. al, (2012)	Review of Polyphenols and Flavones and their value in human diet	Plant or portions thereof are widely used as part of the cuisine of the region	References wide use as food and reports no adverse effects.
3	Roseiro, et.al., (2003)	Review of use of Vegetable Coagulants in Cheese Making. Focus on Cynera spp. Proteases.	Many references to consumption of plant parts, leaves and flowers	Anecdotal reports of benefits to liver function, lowering cholesterol. No reports of adverse effects.
4	Dahl, et. al., (2000)	Review of Characteristics of Cheeses made with C. cardunculus and importance in diet of region	Artisanal use of C. cardunculus flowers for coagulant	Demonstrates wide use and agricultural importance
5	Christaki, et. al. (2012)	Review use of Cardoon and Globe Artichoke	Use of flowers in cheesemaking and consumption of plants in human and animal nutrition	Reports growing use and cultivation for biofuel, human and animal nutrition, no adverse characteristics
6	Ciancolini, Anna; Doctoral Thesis, (This review is the most comprehensive treatise on Cynara spp. and is pivotal to the safety analysis of the plant and its uses.)	Extensive review of C. cardunculus origins, history, and uses	Describes domestication, farming, breeding, uses and consumption and	Review contains no evidence of toxic components associated with milk clotting

			the importance to the region.	enzyme extract.
7	Sousa and Malcata (2001	Review of the role of C. cardunculus during ripening of cheeses from several milk species.	Notes that Cc flower extract used for centuries. Paper compares effect of milk type on proteolysis, lipolysis, vs. animal rennet.	Detailed characterization of Cc -L proteinases, specificity, mode of action, peptides produced from different milk species. No mention of toxic effects.

APPENDIX C

Chemical characterization and

Toxicological properties of

C. cardunculus

APPENDIX C - Chemical Characterization and Toxicological Properties of *Cynara cardunculus*

Toxicological Safety

No direct toxicology studies have been conducted on Cynzime for a number of reasons, including those noted above regarding the history and level of use as human and animal feed of the *C cardunculus* plant itself. The destruction of animals for such studies would be unnecessary and unjustified.
While, to our knowledge, no direct animal toxicology studies have been conducted on Cynzyme®, the enzyme is almost completely inactive when the consumer eats the cheese. The toxicological safety of Cynzyme®, enzyme preparations relies, primarily, on the long history of safe use as food, folk medicine and, employing its enzymatic characteristics, its milk-clotting, activity.

- The cheeses made with the coagulant from cardoon date back to the Roman time on the Iberian peninsula. After thorough searches of the existing literature on cardoon, no reports of toxic effect have been discovered. Cheese made with this coagulant is eaten at all meals including breakfast and alone, as a snack or appetizer. Cardoon is the same species as the edible artichoke and is considered to be a very healthy plant used in daily intake.

- Cyprosin clots different types of milk and has been used since the Roman era for cheese production. The cardoon flower extract has been used for centuries in artisanal production of ewe milk cheeses such as Serra da Estrela, or Serpa in Portugal and Manchego, La Serena in Spain having PDO status (Sousa and Malcata
2002; Roseiro et al. 2003a; Prados et al. 2007). These cheeses are highly appreciated for their unique flavor, and aroma, are often consumed daily with meals and no cases of toxicity have been reported. (Valentão, et. al., 2002)

Despite exhaustive evidence of the history of safe use of the C. cardunculus plant and its close relatives as part of the diet and cuisine of Southeastern Europe, it is reasonable to consider the chemical makeup of these plants and discuss what is known about the nature of phyto-chemicals produced by these plants and their various portions. A further search of the literature focusing on this aspect was conducted by Fytozimus Biotech Inc. and a quite large body of information was collected.

As noted above, the Cynara spp. has been used for centuries for its beneficial health characteristics in addition to its use as a source of milk clotting enzyme, Cynarins. These plants have been known as a source of phenolic compounds believed to impart beneficial effects as antioxidants or digestive aids. Scientific studies to analyze the number and variety of these compounds have been undertaken by several researchers.

The methods for identification and quantification as well as methods of isolation and purification have been developed. Some studies of the physiological effects of these chemicals have been undertaken and beneficial effects have been described. Although the literature has not revealed an abundance of specific toxicological studies on the plants or the isolated phyto-chemicals therefrom, it is reasonable to extend from these present studies, that Cynara plants themselves are non-toxic and safe for consumption by humans and animals. As noted above, the primary focus of this notification is the safety of the use of the enzymatic component of the flowers, specifically of *Cynara cardunculus – L*, as a milk clotting enzyme preparation, it is reasonable to consider that small or trace amounts of the phytochemicals present in other parts of the plant could become contaminants or otherwise incorporated into the Cynzime® enzyme preparation. Herein, is a discussion regarding that potential and any associated risk.

Schutz, et al (2004) reported on methods to identify and quantify phenolic compounds from C. scolymus L. heads, juice and pomace. HPLC and mass spectrometric methods were developed and used to detect and identify 22 major compounds, among them 11 caffeoylquinic acids and 8 flavonoides. Apigenin-7-O-glucuronide was found to be the major flavonoid and 1,5-Di-O-caffeoylquinic acid the major hydroxycinnamic acid. Although an array of these and related compounds were identified and quantified, all were present in the heads, pomace and juice in the parts per million (mg/kg) range. The isolation, study and use of these compounds may be of interest to development of functional foods or other health promoting uses, but appear to be of little concern to the extraction and use of Cynzime®, which is extracted from the mature flowers of C. cardunculus L.

Wang, et al (2003), used a fractionation procedure to purify antioxidative components from aqueous methanol extractions of artichoke heads and leaves of C. scolymus L., a

close relative of C. cardunculus L. They identified two compounds unique to the heads of these plants. Apigenin-7-rutinoside and narirutin, were found in three varieties of the plant: Imperial Star, Green Globe and Violet, where they observed significant differences by variety and organ. They noted that, while extensive studies have shown these plants to be rich sources of polyphenolic compounds, studies of the edible heads were sparse. Their study results also showed that the leaves contained the highest concentration of flavonoids and caffeic acid derivatives, which ranged, in total, up to 7% in the leaves, the amounts found in mature and young heads were considerably lower.

Valentao, et al (2002) reported that polyphenols are able to act as antioxidants and that C. carduculus L. may contain these in the form of flavonoids and phenolic acids. Their study of the antioxidant activity in preparations from Cc-L showed promising antioxidant characteristics and may be useful as replacements for use as natural antioxidants to replace BHA and BHT, which compounds have come under scrutiny because of possible toxicity. There is also interest for use in functional foods as natural antioxidants from botanical sources, particularly edible plants. These authors note that Cc-L is widely used as a source of proteases from the flowers, Cardoon leaves are traditionally used for their diuretic, choleretic and hepatoprotective effects.

Ramos, et al (2013) studied lipophilic extracts of *C. cardunculus L* for the presence of terpenic compounds which may be important, high value, bioactive compounds based upon observed anti-inflammatory and tumor antiproliferative properties. Again, it must be noted that the source of these compounds is primarily the leaves of the plant, not the flowers, and despite their presence in the plant, have not been demonstrated to be of toxicological significance after centuries of inclusion in the diets of humans and animals in the region.

Mennen, et al (2005) discussed the risks and safety of polyphenol consumption. Potential hazards, benefits and risks were discussed in a round-table discussion at the 1st International Conference on Polyphenols and Health held in Vichy, France, November, 2003. Reports of beneficial effects of polyphenols were discussed and it was noted that in some countries intake levels may be in the range of 100 – 300 mg/day, in the form of grape seed extracts, soy products or wine. Risk assessment was thought to be difficult because of the lack of reliable intake and food composition data. Most studies of polyphenols have been aimed at determining the protective effects of polyphenols against disease or toxins. Reliable hazard identification proved difficult because of the wide variety of potential benefits under study and lack of cohesive research results with which to compile a reliable database and a means to model the human situation more closely. The authors also pointed out the need for safety

assessment, and for accumulation of *in vitro*, animal and epidemiological studies on the nature and uses of these compounds.

Three other publications report on studies identifying potential therapeutic effects of extracts of C. cardunculus L or relatives: Miadokova, et al, (2007) reported antigenotoxic effects of extracts from C. c L. Yasukawa, et al (2010) discussed the inhibitory effect of C. c L on induced inflammation and tumor promotion in studies in mouse skin. Heidarian, et al (2013), studied the protective effect of C. scolymus leaf extract against lead toxicity in rats. None of these reports discussed or reported toxic effects associated with extracts of these plants.

CONCLUSIONS

A number of researchers have studied the *Cynara cardunculus L* (Cardoon) plant and its close relatives, seeking to identify and quantify a variety of compounds, believed to be related to activities as anti-oxidants, hypocholesterolemic agents, or imparting other beneficial effects to the consumption of the plants by humans and animals. While a number of such compounds have been shown to be present, and beneficial effects of consuming the plants have been reported, evidence of or reports of toxicity related to this consumption have not been found. One study reported Grandinin (the major compound) showed radical scavenging properties by its reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [EC(50) = 4.3 +/- 0.3 micro g mL(-1)]. It was found to be relatively nontoxic in mice [LD(50) = 316 mg Kg(-1) b.wt.].

Our review of the relevant literature has failed to reveal any significant toxicological evidence related to the use of the Cynara plant varieties reported, but in particular, any evidence whatsoever of toxicity associated with the specific subject of this notice, i.e., *Cynara cardunculus L,* also known as Cardoon, its flowers, extracts of mature flowers, being the source of the milk clotting enzyme Cynarase, which Fytozimus Biotech, Inc. has developed into a commercial enzyme preparation planned for sale into the enzyme marketplace for use in cheese making using the milk of Ewes, Cows, Goats and other species. It is our belief and to the best of our knowledge, we have demonstrated that the source plant, its flower, the enzyme extract therefrom and the enzyme preparation to be marketed are Generally Recognized as Safe. This conclusion has been reviewed

by a panel of experts knowledgeable in the areas of food safety, toxicology, risk assessment and dairy science. Their review and conclusions therefrom will be summarized in an expert opinion document authored by the panel.

Table 3 – Phytochemicals commonly found in *Cardunulus spp.*			
Species (var.)	**Plant Portion**	**Chemical**	**Comment**
C. scolymus	Leaf	caffeic acid, Chlorogenic acid, Cynarin, luteolin	Study of lead toxicity in rats Heidarian, et al, (2013)
C. cardunculus	Flowers	Caffeic acid, flavonoids	Study of effect on induced inflammation and tumor promotion in mice – Yasukawa, et al (2010)
C. cardunculus	Flowers	Bioflavonoids, hydroxyl-cinnamic acid (as chlorogenic acid	Study of antigenotoxic effect of aqueous extract Miadokova, et al, (2007), In study, both clastogenicity and anticlastogenicity seen, speculate interference with metabolic activation in test.
C. cardunculus, var. altilis(DC)	Lipophylic extracts of several plant parts	Sesquiterpene lactones and pentacyclic triterpenes (families). Cynaropicrin main lactone of all but florets. Full analysis identified large number of compounds. Interest relates to tumor antiproliferative and anti-inflammatory effects	Ramos, P. et al (2013) Study to identify wide variety of phytochemical of possible interest in various therapeutic applications. Presence of some may explain folk medicine benefits.
C. cardunculus –L	Leaves, infusion	Flavonoids and phenolic acids.	Valentao, et al(2002) Study of antioxidant properties of plant extracts. Possible interest as replacements for synthetic BHA and BHT as preservatives.
C. scolymus L	Leaves and heads	Wide variety of antioxidant	Wang, et al (2003) Analyzed

		flavonoids and caffeic compounds	number and type of antioxidant phenolic compounds. Highest concentration found in leaves, less in heads.
C. scolymus L	Heads, juice and pomace	Numerous caffeoylquinic acids, flavones and flavonoids. Method may find use in screening, process and quality control	Schutz et al (2004) Study method to characterize and quantify phenolic constituents.

APPENDIX D.

CERTIFICATE OF ANALYSIS

PRODUCT SPECIFICATIONS

3

Enzyme Development Corporation
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New York, New York 10018-6505
Tel: (212) 736-1580 | e-mail: info@EnzymeDevelopment.com | Laboratories and Plant
Fax: (212) 279-0056 | web: www.EnzymeDevelopment.com | Scranton, PA 18504

October 1, 2013

CERTIFICATE OF ANALYSIS
CYNZIME™

CUSTOMER NAME: FYTOZIMUS BIOTECH, INC.

PRODUCT CODE: 3-0146-000

LOT: (b) (6)

		SPECIFICATIONS
DESCRIPTION:	Conforms	
ACTIVITY:	34 MCU/g	~30 MCU/g
ASH:	8.04%	
pH:	4.67	
SPECIFIC GRAVITY:	1.07	
APC:	1,000/ml	
YEAST & MOLD:	<10/ml	
E. coli:	Negative/25ml	
COLIFORMS:	<10/ml	
SALMONELLA:	Negative/25ml	
LEAD:	NMT 5mg/kg *(Tested Annually)*	
TEST:	September 25, 2013	
RETEST:	September 25, 2014	

(b) (6)

George E. Holmon
Quality Assurance Manager

APPENDIX E.

CERTIFICATES OF PROXIMATE ANALYSIS

COVANCE

Report Number:	931551-0
Report Date:	27-Dec-2013
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Sample Name:	Cynzime	Covance Sample:	2631307
Project ID	ENZY_DEV-20131217-0026	Receipt Date	17-Dec-2013
PO Number	L1284	Receipt Condition	Ambient temperature
Sample Serving Size		Login Date	17-Dec-2013
		Storage Condition	5 (+/- 3) degrees Celsius
		Online Order	10

Analysis	Result
Calories	
Calories	27.4 Cal/100g
Calories from Fat	
Calories	<1.00 Cal/100g
Fatty Acid Profile *	
Saturated Fatty Acids (Acid Form)	0.036 g/100g
Total Cis Unsaturated Fatty Acids (Acid Form)	0.034 g/100g
Monounsaturated Fatty Acids (Acid Form)	0.010 g/100g
Polyunsaturated Fatty Acids (Acid Form)	0.024 g/100g
Trans Fatty Acids (Acid Form)	<0.002 g/100g
Total Fatty Acids	0.077 g/100g
Cholesterol	
Cholesterol	<1.00 mg/100g
Carbohydrates	
Total Carbohydrates	5.2 g/100g
Total Dietary Fiber	
Total Dietary Fiber	<0.75 g/100g
Sugar Profile	
Fructose	0.2 g/100g
Glucose	0.1 g/100g
Sucrose	<0.1 g/100g
Lactose	<0.1 g/100g
Maltose	<0.1 g/100g
Galactose	<0.1 g/100g
Total Sugar	0.3 g/100g
Protein (N x 6.25) Dumas Method	
Protein	1.47 g/100g
Vitamin A from Carotenes *	
Vitamin A From Carotene	<35.0 IU/100g
Carotenes	
Beta Carotene	<0.0200 mg/100g
Vitamin C	
Vitamin C	<1.0 mg/100g
Elements by ICP Emission Spectrometry	

*** This analysis is not ISO accredited.**

Printed: 27-Dec-2013 3:01 pm Page 1 of 5

COVANCE

Report Number:	931551-0
Report Date:	27-Dec-2013
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation

314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Sample Name:	Cynzime	Covance Sample:	2631307
Project ID	ENZY_DEV-20131217-0026	Receipt Date	17-Dec-2013
PO Number	L1284	Receipt Condition	Ambient temperature
Sample Serving Size		Login Date	17-Dec-2013
		Storage Condition	5 (+/- 3) degrees Celsius
		Online Order	10

Analysis	Result
Elements by ICP Emission Spectrometry	
Calcium	16.6 mg/100g
Iron	0.373 mg/100g
Sodium	2780 mg/100g
Ash	
Ash	7.62 %
Moisture	
Moisture	85.6 g/100g
Elements by ICP Mass Spectrometry	
Arsenic *	<10.0 ppb
Cadmium *	<10.0 ppb
Lead	0.0172 ppm
Mercury *	<10.0 ppb
Antimony *	<10.0 ppb
Heavy Metals *	
Heavy Metals	<5 ppm
Aflatoxin, Ochratoxin A and Zearalenone *	
B1	<0.500 ppb
B2	<0.500 ppb
G1	<0.500 ppb
G2	<0.500 ppb
Ochratoxin A	<1.00 ppb
Zearalenone	<10.0 ppb
Multi-Residue Screen for spices, botanicals, harvested products	
All tested pesticides	Below LOQ

Method References	Testing Location
Aflatoxin, Ochratoxin A and Zearalenone (AHMF_OZ_S:4)	**Covance Laboratories - Madison**
Official Method No. 2008.02, Official Methods of Analysis of AOAC INTERNATIONAL (modified), 18th Ed., AOAC INTERNATIONAL: Gaithersburg, Maryland (2005).	
Ash (ASHM_S:7)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 923.03, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	

*** This analysis is not ISO accredited.**

Printed: 27-Dec-2013 3:01 pm Page 2 of 5

COVANCE

Report Number:	931551-0
Report Date:	27-Dec-2013
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Method References	Testing Location
Calories (CALC:6)	**Covance Laboratories - Madison**
Code of Federal Regulations, Title 21, Part 101.9, pp. 24-25.	
Calories from Fat (CFAT:4)	**Covance Laboratories - Madison**
Code of Federal Regulations, Title 21, Part 101.9, pp. 24-25.	
Carbohydrates (CHO:8)	**Covance Laboratories - Madison**
United States Department of Agriculture, "Energy Value of Foods", Agriculture Handbook No. 74, pp. 2-11, (1973).	
Carotenes (CAR1_S:19)	**Covance Laboratories - Madison**
Official Methods of Analysis, Method 2005.07, AOAC INTERNATIONAL, (modified). Quackenbush, F. W., "Reverse Phase HPLC Separation of cis- and trans-Carotenoids and Its Application to Beta Carotenes in Food Materials," *Journal of Liquid Chromatography*, 10: 643-653 (1987) (modified).	
Cholesterol (CHOK_S:11)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005), Official Method 994.10. (Modified)	
Elements by ICP Emission Spectrometry (ICP_S:20)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 984.27 and 985.01, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	
Elements by ICP Mass Spectrometry (ICP_MS_S:14)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	
Fatty Acid Profile (FALT_S:22)	**Covance Laboratories - Madison**
Official Method No. 996.06, Official Methods of Analysis of the AOAC INTERNATIONAL (modified), 19th Ed., AOAC INTERNATIONAL: Gaithersburg, Maryland (2012).	
Official Methods and Recommended Practices of the AOCS, Official methods Ce 2b-11 (2011), Ce 1h-05 (2009), Ce 1j-07 (2013), Ce 2-66 (2009),The American Oil Chemists' Society, Champaign, IL (modified).	
Heavy Metals (HYMT_S:7)	**Covance Laboratories - Madison**
The United States Pharmacopeia, 32nd Revision, Method 231, United States Pharmacopeial Convention, Inc.: Rockville, Maryland (2009). (Modified)	
Moisture (M100T100_S:6)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 925.09 and 926.08, AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005). (Modified).	

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Printed: 27-Dec-2013 3:01 pm Page 3 of 5

COVANCE

Report Number:	931551-0
Report Date:	27-Dec-2013
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Method References	Testing Location
Multi-Residue Screen for spices, botanicals, harvested products (PS03_SPL:6)	**Covance Laboratories - Greenfield**

[1] AOAC Official Method 2007.01
[2] CEN Standard Method EN 15662
[3] Anastassiades, M.; Lehotay, S.J.; Stajnbaher, D.; Schenck, F.J. J. AOAC Int. **2003**, 86, 412-431.
[4] Lehotay, S.J.; Mastovska, K; Lightfield, A.R. J. AOAC Int. **2005**, *88*, 615-629.
[5] Lehotay, S.J.; Mastovska, K.; Yun, S. J. J. AOAC Int. **2005**, *88*, 630-638.
[6] Mastovska, K.; Dorweiler, K.; Lehotay, S.J.; Wegscheid, J.; Szpylka, K. J. Agric. Food Chem., 2010, 58, 5959-5972.

List of the tested pesticides and their limits of quantification (LOQs) are available upon request.
Please contact us if you want a complete listing of all compounds determined during testing.

Protein (N x 6.25) Dumas Method (DGEN_S:6) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 968.06 and 992.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Sugar Profile (SUGN_S:10) — **Covance Laboratories - Madison**

Mason, B. S., and Slover, H. T., "A Gas Chromatographic Method for the Determination of Sugars in Foods," Journal of Agricultural and Food Chemistry 19(3):551-554 (1971). (Modified)

Brobst, K. M., "Gas-Liquid Chromatography of Trimethylsilyl Derivatives, Methods in Carbohydrate Chemistry," 6:3-8, Academic Press, New York, NY, (1972). (Modified)

Total Dietary Fiber (TDFL_S:16) — **Covance Laboratories - Madison**

Vitamin A from Carotenes (BCAV_S:9) — **Covance Laboratories - Madison**

Calculation on request.

Vitamin C (VCF_S:8) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 967.22, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

*** This analysis is not ISO accredited.**

Printed: 27-Dec-2013 3:01 pm Page 4 of 5

COVANCE

Report Number:	931551-0
Report Date:	27-Dec-2013
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Testing Location(s)	Released on Behalf of Covance by
Covance Laboratories - Greenfield 671 S. Meridian Road Greenfield IN 46140 866-964-2034	**Sharon McKilligin - Associate Director** ACCREDITED Testing Cert #2918.06
Covance Laboratories - Madison 3301 Kinsman Blvd Madison WI 53704 608-242-2712 x4170	**Lori Ross - Associate Director** ACCREDITED Testing Cert #2918.01

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COVANCE

Report Number:	954231-0
Report Date:	11-Feb-2014
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Sample Name:	**Cynzime R6801-1**	**Covance Sample:**	**2707738**
Project ID	ENZY_DEV-20140124-0003	**Receipt Date**	23-Jan-2014
PO Number	L1296	**Receipt Condition**	Ambient temperature
Sample Serving Size		**Login Date**	24-Jan-2014
Description	27116	**Storage Condition**	5 (+/- 3) degrees Celsius
		Online Order	10

Analysis	Result
Calories	
Calories	14.8 Cal/100g
Calories from Fat	
Calories	<1.00 Cal/100g
Fatty Acid Profile *	
Saturated Fatty Acids (Acid Form)	0.002 g/100g
Total Cis Unsaturated Fatty Acids (Acid Form)	0.004 g/100g
Monounsaturated Fatty Acids (Acid Form)	<0.002 g/100g
Polyunsaturated Fatty Acids (Acid Form)	0.002 g/100g
Trans Fatty Acids (Acid Form)	<0.002 g/100g
Total Fatty Acids	0.004 g/100g
Cholesterol	
Cholesterol	<1.00 mg/100g
Carbohydrates	
Total Carbohydrates	2.3 g/100g
Total Dietary Fiber	
Total Dietary Fiber	<0.75 g/100g
Sugar Profile	
Fructose	0.2 g/100g
Glucose	<0.1 g/100g
Sucrose	<0.1 g/100g
Lactose	<0.1 g/100g
Maltose	<0.1 g/100g
Galactose	<0.1 g/100g
Total Sugar	0.2 g/100g
Protein (N x 6.25) Dumas Method	
Protein	1.42 g/100g
Total Vitamin A *	
Total Vitamin A	<0.35 IU/g
Vitamin A as Retinol	
Vitamin A from Retinol	<100 IU/100g
Vitamin A from Carotenes *	
Vitamin A From Carotene	<35.0 IU/100g
Carotenes	

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Printed: 11-Feb-2014 8:05 am Page 1 of 5

COVANCE

Report Number:	954231-0
Report Date:	11-Feb-2014
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Sample Name:	Cynzime R6801-1	Covance Sample:	2707738
Project ID	ENZY_DEV-20140124-0003	Receipt Date	23-Jan-2014
PO Number	L1296	Receipt Condition	Ambient temperature
Sample Serving Size		Login Date	24-Jan-2014
Description	27116	Storage Condition	5 (+/- 3) degrees Celsius
		Online Order	10

Analysis	Result
Carotenes	
Beta Carotene	<0.0200 mg/100g
Vitamin C	
Vitamin C	<1.0 mg/100g
Elements by ICP Emission Spectrometry	
Calcium	12.5 mg/100g
Iron	0.110 mg/100g
Sodium	2670 mg/100g
Ash	
Ash	7.52 g/100g
Moisture	
Moisture	88.8 g/100g
Elements by ICP Mass Spectrometry	
Arsenic *	<0.0100 ppm
Cadmium *	<0.0100 ppm
Lead	0.00569 ppm
Mercury *	<0.0100 ppm
Heavy Metals *	
Heavy Metals	<5 ppm
Aflatoxin, Ochratoxin A and Zearalenone *	
B1	<0.500 ppb
B2	<0.500 ppb
G1	Matrix Interference
G2	Matrix Interference
Ochratoxin A	<1.00 ppb
Zearalenone	<10.0 ppb
Multi-Residue Screen for spices, botanicals, harvested products	
All tested pesticides	Below LOQ

Method References	Testing Location
Aflatoxin, Ochratoxin A and Zearalenone (AHMF_OZ_S:4)	**Covance Laboratories - Madison**
Official Method No. 2008.02, Official Methods of Analysis of AOAC INTERNATIONAL (modified), 18th Ed., AOAC INTERNATIONAL: Gaithersburg, Maryland (2005).	

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Printed: 11-Feb-2014 8:05 am Page 2 of 5

COVANCE

Report Number:	954231-0
Report Date:	11-Feb-2014
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
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Scranton Pennsylvania 18504 United States

Method References	Testing Location
Ash (ASHM_S:7)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 923.03, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	
Calories (CALC:6)	**Covance Laboratories - Madison**
Code of Federal Regulations, Title 21, Part 101.9, pp. 24-25.	
Calories from Fat (CFAT:4)	**Covance Laboratories - Madison**
Code of Federal Regulations, Title 21, Part 101.9, pp. 24-25.	
Carbohydrates (CHO:8)	**Covance Laboratories - Madison**
United States Department of Agriculture, "Energy Value of Foods", Agriculture Handbook No. 74, pp. 2-11, (1973).	
Carotenes (CAR1_S:20)	**Covance Laboratories - Madison**
Official Methods of Analysis, Method 2005.07, AOAC INTERNATIONAL, (modified). Quackenbush, F. W., "Reverse Phase HPLC Separation of cis- and trans-Carotenoids and Its Application to Beta Carotenes in Food Materials," *Journal of Liquid Chromatography*, 10: 643-653 (1987) (modified).	
Cholesterol (CHOK_S:12)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005), Official Method 994.10. (Modified)	
Elements by ICP Emission Spectrometry (ICP_S:21)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 984.27 and 985.01, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	
Elements by ICP Mass Spectrometry (ICP_MS_S:15)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	
Fatty Acid Profile (FALT_S:25)	**Covance Laboratories - Madison**
Official Method No. 996.06, Official Methods of Analysis of the AOAC INTERNATIONAL (modified), 19th Ed., AOAC INTERNATIONAL: Gaithersburg, Maryland (2012).	
Official Methods and Recommended Practices of the AOCS, Official methods Ce 2b-11 (2011), Ce 1h-05 (2009), Ce 1j-07 (2013), Ce 2-66 (2009),The American Oil Chemists' Society, Champaign, IL (modified).	
Heavy Metals (HYMT_S:7)	**Covance Laboratories - Madison**
The United States Pharmacopeia, 32nd Revision, Method 231, United States Pharmacopeial Convention, Inc.: Rockville, Maryland (2009). (Modified)	
Moisture (M100T100_S:7)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 925.09 and 926.08, AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005). (Modified).	

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Printed: 11-Feb-2014 8:05 am Page 3 of 5

COVANCE

Report Number:	954231-0
Report Date:	11-Feb-2014
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Certificate of Analysis

Enzyme Development Corporation
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Scranton Pennsylvania 18504 United States

Method References	Testing Location
Multi-Residue Screen for spices, botanicals, harvested products (PS03_SPL:6)	**Covance Laboratories - Greenfield**

[1] AOAC Official Method 2007.01
[2] CEN Standard Method EN 15662
[3] Anastassiades, M.; Lehotay, S.J.; Stajnbaher, D.; Schenck, F.J. J. AOAC Int. **2003**, 86, 412-431.
[4] Lehotay, S.J.; Mastovska, K; Lightfield, A.R. J. AOAC Int. **2005**, *88*, 615-629.
[5] Lehotay, S.J.; Mastovska, K.; Yun, S. J. J. AOAC Int. **2005**, *88*, 630-638.
[6] Mastovska, K.; Dorweiler, K.; Lehotay, S.J.; Wegscheid, J.; Szpylka, K. J. Agric. Food Chem., 2010, 58, 5959-5972.

List of the tested pesticides and their limits of quantification (LOQs) are available upon request.
Please contact us if you want a complete listing of all compounds determined during testing.

Protein (N x 6.25) Dumas Method (DGEN_S:6) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 968.06 and 992.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Sugar Profile (SUGN_S:10) — **Covance Laboratories - Madison**

Mason, B. S., and Slover, H. T., "A Gas Chromatographic Method for the Determination of Sugars in Foods," Journal of Agricultural and Food Chemistry 19(3):551-554 (1971). (Modified)

Brobst, K. M., "Gas-Liquid Chromatography of Trimethylsilyl Derivatives, Methods in Carbohydrate Chemistry," 6:3-8, Academic Press, New York, NY, (1972). (Modified)

Total Dietary Fiber (TDFL_S:16) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL 18th Ed., Method 991.43, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Total Vitamin A (VAT_CALC:12) — **Covance Laboratories - Madison**

Calculation on request.

Vitamin A as Retinol (VALC_S:13) — **Covance Laboratories - Madison**

Official Methods of Analysis, Methods 992.04, 992.06, and 2001.13, AOAC INTERNATIONAL (Modified).

Vitamin A from Carotenes (BCAV_S:9) — **Covance Laboratories - Madison**

Calculation on request.

Vitamin C (VCF_S:9) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 967.22, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

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Printed: 11-Feb-2014 8:05 am Page 4 of 5

COVANCE

Report Number:	954231-0
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314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Testing Location(s)	Released on Behalf of Covance by
Covance Laboratories - Greenfield 671 S. Meridian Road Greenfield IN 46140 866-964-2034	**Sharon McKilligin - Associate Director** ACCREDITED Testing Cert #2918.06
Covance Laboratories - Madison 3301 Kinsman Blvd Madison WI 53704 608-242-2712 x4170	**Lori Ross - Associate Director** ACCREDITED Testing Cert #2918.01

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COVANCE

Report Number:	953796-0
Report Date:	10-Feb-2014
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Sample Name:	Cynzime R6801-3	Covance Sample:	2707739
Project ID	ENZY_DEV-20140124-0003	Receipt Date	23-Jan-2014
PO Number	L1296	Receipt Condition	Ambient temperature
Sample Serving Size		Login Date	24-Jan-2014
Description	27117	Storage Condition	5 (+/- 3) degrees Celsius
		Online Order	10

Analysis	Result
Calories	
Calories	14.6 Cal/100g
Calories from Fat	
Calories	<1.00 Cal/100g
Fatty Acid Profile *	
Saturated Fatty Acids (Acid Form)	<0.002 g/100g
Total Cis Unsaturated Fatty Acids (Acid Form)	0.004 g/100g
Monounsaturated Fatty Acids (Acid Form)	<0.002 g/100g
Polyunsaturated Fatty Acids (Acid Form)	<0.002 g/100g
Trans Fatty Acids (Acid Form)	<0.002 g/100g
Total Fatty Acids	<0.002 g/100g
Cholesterol	
Cholesterol	<1.00 mg/100g
Carbohydrates	
Total Carbohydrates	1.7 g/100g
Total Dietary Fiber	
Total Dietary Fiber	<0.75 g/100g
Sugar Profile	
Fructose	0.2 g/100g
Glucose	<0.1 g/100g
Sucrose	<0.1 g/100g
Lactose	<0.1 g/100g
Maltose	<0.1 g/100g
Galactose	<0.1 g/100g
Total Sugar	0.2 g/100g
Protein (N x 6.25) Dumas Method	
Protein	1.97 g/100g
Total Vitamin A *	
Total Vitamin A	<0.35 IU/g
Vitamin A as Retinol	
Vitamin A from Retinol	<100 IU/100g
Vitamin A from Carotenes *	
Vitamin A From Carotene	<35.0 IU/100g
Carotenes	

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Printed: 10-Feb-2014 3:33 pm Page 1 of 5

COVANCE

Report Number:	953796-0
Report Date:	10-Feb-2014
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Sample Name:	Cynzime R6801-3	Covance Sample:	2707739
Project ID	ENZY_DEV-20140124-0003	Receipt Date	23-Jan-2014
PO Number	L1296	Receipt Condition	Ambient temperature
Sample Serving Size		Login Date	24-Jan-2014
Description	27117	Storage Condition	5 (+/- 3) degrees Celsius
		Online Order	10

Analysis	Result
Carotenes	
Beta Carotene	<0.0200 mg/100g
Vitamin C	
Vitamin C	<1.0 mg/100g
Elements by ICP Emission Spectrometry	
Calcium	13.1 mg/100g
Iron	<0.0992 mg/100g
Sodium	2790 mg/100g
Ash	
Ash	7.56 g/100g
Moisture	
Moisture	88.8 g/100g
Elements by ICP Mass Spectrometry	
Arsenic *	<0.0100 ppm
Cadmium *	<0.0100 ppm
Lead	0.00548 ppm
Mercury *	<0.0100 ppm
Heavy Metals *	
Heavy Metals	<5 ppm
Aflatoxin, Ochratoxin A and Zearalenone *	
B1	<0.500 ppb
B2	<0.500 ppb
G1	Matrix Interference
G2	Matrix Interference
Ochratoxin A	<1.00 ppb
Zearalenone	<10.0 ppb
Multi-Residue Screen for spices, botanicals, harvested products	
All tested pesticides	Below LOQ

Method References	Testing Location
Aflatoxin, Ochratoxin A and Zearalenone (AHMF_OZ_S:4)	**Covance Laboratories - Madison**
Official Method No. 2008.02, Official Methods of Analysis of AOAC INTERNATIONAL (modified), 18th Ed., AOAC INTERNATIONAL: Gaithersburg, Maryland (2005).	

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Printed: 10-Feb-2014 3:33 pm Page 2 of 5

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Report Number:	953796-0
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Scranton Pennsylvania 18504 United States

Method References	Testing Location
Ash (ASHM_S:7)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 923.03, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	
Calories (CALC:6)	**Covance Laboratories - Madison**
Code of Federal Regulations, Title 21, Part 101.9, pp. 24-25.	
Calories from Fat (CFAT:4)	**Covance Laboratories - Madison**
Code of Federal Regulations, Title 21, Part 101.9, pp. 24-25.	
Carbohydrates (CHO:8)	**Covance Laboratories - Madison**
United States Department of Agriculture, "Energy Value of Foods", Agriculture Handbook No. 74, pp. 2-11, (1973).	
Carotenes (CAR1_S:20)	**Covance Laboratories - Madison**
Official Methods of Analysis, Method 2005.07, AOAC INTERNATIONAL, (modified). Quackenbush, F. W., "Reverse Phase HPLC Separation of cis- and trans-Carotenoids and Its Application to Beta Carotenes in Food Materials," *Journal of Liquid Chromatography*, 10: 643-653 (1987) (modified).	
Cholesterol (CHOK_S:12)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005), Official Method 994.10. (Modified)	
Elements by ICP Emission Spectrometry (ICP_S:21)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 984.27 and 985.01, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)	
Elements by ICP Mass Spectrometry (ICP_MS_S:15)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	
Fatty Acid Profile (FALT_S:25)	**Covance Laboratories - Madison**
Official Method No. 996.06, Official Methods of Analysis of the AOAC INTERNATIONAL (modified), 19th Ed., AOAC INTERNATIONAL: Gaithersburg, Maryland (2012).	
Official Methods and Recommended Practices of the AOCS, Official methods Ce 2b-11 (2011), Ce 1h-05 (2009), Ce 1j-07 (2013), Ce 2-66 (2009),The American Oil Chemists' Society, Champaign, IL (modified).	
Heavy Metals (HYMT_S:7)	**Covance Laboratories - Madison**
The United States Pharmacopeia, 32nd Revision, Method 231, United States Pharmacopeial Convention, Inc.: Rockville, Maryland (2009). (Modified)	
Moisture (M100T100_S:7)	**Covance Laboratories - Madison**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 925.09 and 926.08, AOAC INTERNATIONAL, Gaithersburg, MD, USA,(2005). (Modified).	

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COVANCE

Report Number:	953796-0
Report Date:	10-Feb-2014
Report Status:	Final

Certificate of Analysis

Enzyme Development Corporation
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Scranton Pennsylvania 18504 United States

Method References	Testing Location
Multi-Residue Screen for spices, botanicals, harvested products (PS03_SPL:6)	**Covance Laboratories - Greenfield**

[1] AOAC Official Method 2007.01
[2] CEN Standard Method EN 15662
[3] Anastassiades, M.; Lehotay, S.J.; Stajnbaher, D.; Schenck, F.J. J. AOAC Int. **2003**, 86, 412-431.
[4] Lehotay, S.J.; Mastovska, K; Lightfield, A.R. J. AOAC Int. **2005**, *88*, 615-629.
[5] Lehotay, S.J.; Mastovska, K.; Yun, S. J. J. AOAC Int. **2005**, *88*, 630-638.
[6] Mastovska, K.; Dorweiler, K.; Lehotay, S.J.; Wegscheid, J.; Szpylka, K. J. Agric. Food Chem., 2010, 58, 5959-5972.

List of the tested pesticides and their limits of quantification (LOQs) are available upon request.
Please contact us if you want a complete listing of all compounds determined during testing.

Protein (N x 6.25) Dumas Method (DGEN_S:6) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 968.06 and 992.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Sugar Profile (SUGN_S:10) — **Covance Laboratories - Madison**

Mason, B. S., and Slover, H. T., "A Gas Chromatographic Method for the Determination of Sugars in Foods," Journal of Agricultural and Food Chemistry 19(3):551-554 (1971). (Modified)

Brobst, K. M., "Gas-Liquid Chromatography of Trimethylsilyl Derivatives, Methods in Carbohydrate Chemistry," 6:3-8, Academic Press, New York, NY, (1972). (Modified)

Total Dietary Fiber (TDFL_S:16) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL 18th Ed., Method 991.43, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

Total Vitamin A (VAT_CALC:12) — **Covance Laboratories - Madison**

Calculation on request.

Vitamin A as Retinol (VALC_S:13) — **Covance Laboratories - Madison**

Official Methods of Analysis, Methods 992.04, 992.06, and 2001.13, AOAC INTERNATIONAL (Modified).

Vitamin A from Carotenes (BCAV_S:9) — **Covance Laboratories - Madison**

Calculation on request.

Vitamin C (VCF_S:9) — **Covance Laboratories - Madison**

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 967.22, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005). (Modified)

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Printed: 10-Feb-2014 3:33 pm Page 4 of 5

COVANCE

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Certificate of Analysis

Enzyme Development Corporation
314 South Sherman Avenue
Scranton Pennsylvania 18504 United States

Testing Location(s)	Released on Behalf of Covance by
Covance Laboratories - Greenfield 671 S. Meridian Road Greenfield IN 46140 866-964-2034	**Sharon McKilligin - Associate Director** ACCREDITED Testing Cert #2918.06
Covance Laboratories - Madison 3301 Kinsman Blvd Madison WI 53704 608-242-2712 x4170	**Lori Ross - Associate Director** ACCREDITED Testing Cert #2918.01

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APPENDIX F.

PESTICIDE ANALYSIS

Labiagro

TagusPark - Av. Prof. Cavaco Silva, nº 33 • Edifício C
2740- 22 Oeiras
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Assinatura do autor por LABIAGRO
<labiagro@isq.pt> Validade desconhecida
Digitally signed by LABIAGRO
Date: 2011.12.05 15:22:18 WET

IPAC acreditação L0298 Ensaios

TEST REPORT 12596.Q/11

Sample Num :12596.Q/11

Porto Salvo, 05 of December of 2011

Final Report

Version 2

Client identification

Name: Fytozimus Biotech

Address: Edificio ICAT, Campus da Faculdade de Ciências 1749-016 Campo Grande, Lisboa

Sample Identification

Sample type: Extracto Phytozimus

Sample reception: The client is responsible for the sample collection

Laboratory sample identification: Packaged in a 500 ml plastic container.. Conservation state: Refrigerada.

Client sample identification: Extracto Phytozimus

Reception date	Date of test(s)	Completion test(s) date
08 of November of 2011	09 of November of 2011	17 of November of 2011

TEST RESULT(S)

Tests/Methods	Results	Units	Limit of quantification (L.Q.)
Determination of pesticide residue by QuECHERs method High performance liquid chromatography Mass spectrometry (LC-MS/MS) PI.LQ.12, Edição B			
- Acephate	< L.Q.	mg/kg	0,01
- Acetamiprid	< L.Q.	mg/kg	0,01
- Alachlor	< L.Q.	mg/kg	0,01
- Amitraz (*)	< L.Q.	mg/kg	0,01
- Atrazine	< L.Q.	mg/kg	0,01
- Azinphos-ethyl	< L.Q.	mg/kg	0,02
- Azinphos-methyl	< L.Q.	mg/kg	0,03
- Azoxystrobin	< L.Q.	mg/kg	0,01
- Benalaxyl (*)	< L.Q.	mg/kg	0,01
- Bitertanol	< L.Q.	mg/kg	0,01
- Boscalid (*)	< L.Q.	mg/kg	0,01
- Bupirimate	< L.Q.	mg/kg	0,01
- Buprofezin	< L.Q.	mg/kg	0,01
- Carbaryl	< L.Q.	mg/kg	0,01
- Carbendazim	< L.Q.	mg/kg	0,01
- Carbofuran	< L.Q.	mg/kg	0,01
- Chlorfenvinphos	< L.Q.	mg/kg	0,01

(b) (6)

Tânia Azevedo
Técnica do Laboratório de Química

(b) (6)

Ana Trindade
Substituta do Responsável Técnico de Química

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Page 1 of 6

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TEST REPORT 12596.Q/11

TEST RESULT(S)

Tests/Methods	Results	Units	Limit of quantification (L.Q.)
- Chlorpyrifos	< L.Q.	mg/kg	0,01
- Chlorpyrifos-methyl	< L.Q.	mg/kg	0,01
- Chlortoluron	< L.Q.	mg/kg	0,01
- Clofentezine	< L.Q.	mg/kg	0,01
- Cyazofamid	< L.Q.	mg/kg	0,01
- Cymoxanil	< L.Q.	mg/kg	0,02
- Cyproconazole (*)	< L.Q.	mg/kg	0,01
- Cyprodinil	< L.Q.	mg/kg	0,01
- Diazinon	< L.Q.	mg/kg	0,01
- Dichlorvos	< L.Q.	mg/kg	0,01
- Difenoconazole	< L.Q.	mg/kg	0,01
- Diflufenican	< L.Q.	mg/kg	0,01
- Dimethoate	< L.Q.	mg/kg	0,01
- Dimethomorph	< L.Q.	mg/kg	0,01
- Diuron	< L.Q.	mg/kg	0,01
- Epoxyconazole (*)	< L.Q.	mg/kg	0,01
- Ethion	< L.Q.	mg/kg	0,01
- Ethoprophos	< L.Q.	mg/kg	0,01
- Etrinphos	< L.Q.	mg/kg	0,01
- Famoxadone	< L.Q.	mg/kg	0,02
- Fenamidone	< L.Q.	mg/kg	0,01
- Fenamiphos	< L.Q.	mg/kg	0,01
- Fenarimol	< L.Q.	mg/kg	0,02
- Fenhexamid	< L.Q.	mg/kg	0,01
- Fenitrothion	< L.Q.	mg/kg	0,01
- Fenoxaprop-p-ethyl	< L.Q.	mg/kg	0,01
- Fenoxycarb	< L.Q.	mg/kg	0,01
- Fenpiclonil	< L.Q.	mg/kg	0,02
- Fenpropimorph	< L.Q.	mg/kg	0,01
- Fenpyroximate	< L.Q.	mg/kg	0,01
- Fenthion (*)	< L.Q.	mg/kg	0,01
- Fentoate	< L.Q.	mg/kg	0,01
- Flufenoxuron (*)	< L.Q.	mg/kg	0,01
- Fluquinconazole	< L.Q.	mg/kg	0,01
- Flusilazole	< L.Q.	mg/kg	0,01
- Fonophos	< L.Q.	mg/kg	0,01

(b) (6)

Tânia Azevedo
Técnica do Laboratório de Química

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Page 2 of 6

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Laboratório Químico, Agroalimentar e Microbiológico

TEST REPORT 12596.Q/11

TEST RESULT(S)

Tests/Methods	Results	Units	Limit of quantification (L.Q.)
- Fosalone	< L.Q.	mg/kg	0,01
- Heptenophos	< L.Q.	mg/kg	0,01
- Hexaconazole	< L.Q.	mg/kg	0,01
- Hexythiazox	< L.Q.	mg/kg	0,01
- Imazalil	< L.Q.	mg/kg	0,01
- Imidacloprid	< L.Q.	mg/kg	0,02
- Indoxacarb (*)	< L.Q.	mg/kg	0,01
- Iprodione (*)	< L.Q.	mg/kg	0,01
- Iprovalicarb	< L.Q.	mg/kg	0,01
- Isoproturon (*)	< L.Q.	mg/kg	0,01
- Linuron	< L.Q.	mg/kg	0,01
- Malathion	< L.Q.	mg/kg	0,01
- Mecarbam	< L.Q.	mg/kg	0,01
- Mepanipyrim	< L.Q.	mg/kg	0,01
- Metalaxyl	< L.Q.	mg/kg	0,01
- Metalaxyl-M	< L.Q.	mg/kg	0,01
- Methacrifos (*)	< L.Q.	mg/kg	0,01
- Methamidophos (*)	< L.Q.	mg/kg	0,01
- Methidathion	< L.Q.	mg/kg	0,02
- Methiocarb	< L.Q.	mg/kg	0,01
- Metholachlor	< L.Q.	mg/kg	0,01
- Methomyl	< L.Q.	mg/kg	0,01
- Metribuzin	< L.Q.	mg/kg	0,01
- Mevinphos	< L.Q.	mg/kg	0,01
- Molinate	< L.Q.	mg/kg	0,01
- Myclobutanyl	< L.Q.	mg/kg	0,01
- Nuarimol	< L.Q.	mg/kg	0,01
- Ofurace	< L.Q.	mg/kg	0,01
- Omethoate	< L.Q.	mg/kg	0,01
- Oxadiazon	< L.Q.	mg/kg	0,01
- Oxadixyl	< L.Q.	mg/kg	0,01
- Parathion-ethyl	< L.Q.	mg/kg	0,01
- Parathion-methyl	< L.Q.	mg/kg	0,01
- Penconazole	< L.Q.	mg/kg	0,01
- Pendimethalin	< L.Q.	mg/kg	0,01
- Phosfamidon	< L.Q.	mg/kg	0,01

(b) (6)

Tânia Azevedo
Técnica do Laboratório de Química

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TEST REPORT 12596.Q/11

TEST RESULT(S)

Tests/Methods	Results	Units	Limit of quantification (L.Q.)
- Phosmet	< L.Q.	mg/kg	0,01
- Piperonyl-butoxide (*)	< L.Q.	mg/kg	0,01
- Pirimicarb	< L.Q.	mg/kg	0,01
- Pirimiphos-ethyl	< L.Q.	mg/kg	0,01
- Pirimiphos-methyl	< L.Q.	mg/kg	0,01
- Profenofos	< L.Q.	mg/kg	0,01
- Propachlor	< L.Q.	mg/kg	0,01
- Propamocarb (*)	< L.Q.	mg/kg	0,01
- Propanil	< L.Q.	mg/kg	0,01
- Propiconazole	< L.Q.	mg/kg	0,01
- Propoxur	< L.Q.	mg/kg	0,01
- Propylene thiourea (*)	< L.Q.	mg/kg	0,01
- Propyzamide	< L.Q.	mg/kg	0,01
- Pyraclostrobin (*)	< L.Q.	mg/kg	0,01
- Pyrazophos	< L.Q.	mg/kg	0,01
- Pyridaben (*)	< L.Q.	mg/kg	0,01
- Pyrimethanil	< L.Q.	mg/kg	0,01
- Quinalphos	< L.Q.	mg/kg	0,01
- Quinoxyfen (*)	< L.Q.	mg/kg	0,01
- Simazine	< L.Q.	mg/kg	0,01
- Spinosad	< L.Q.	mg/kg	0,01
- Spirodiclofen (*)	< L.Q.	mg/kg	0,01
- Spiroxamine (*)	< L.Q.	mg/kg	0,01
- Sulphotep	< L.Q.	mg/kg	0,02
- Tebuconazole	< L.Q.	mg/kg	0,01
- Tebufenozide (*)	< L.Q.	mg/kg	0,01
- Tebufenpyrad (*)	< L.Q.	mg/kg	0,01
- Teflubenzuron (*)	< L.Q.	mg/kg	0,01
- Terbufos	< L.Q.	mg/kg	0,01
- Terbuthylazine	< L.Q.	mg/kg	0,01
- Tetrachlorvynphos	< L.Q.	mg/kg	0,01
- Tetraconazole	< L.Q.	mg/kg	0,01
- Thiabendazole	< L.Q.	mg/kg	0,01
- Thiacloprid	< L.Q.	mg/kg	0,01
- Thiamethoxam	< L.Q.	mg/kg	0,01
- Thiodicarb (*)	< L.Q.	mg/kg	0,01

(b) (6)

Tânia Azevedo
Técnica do Laboratório de Química

(b) (6)

Ana Trindade
Substituta do Responsável Técnico de Química

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TEST REPORT 12596.Q/11

TEST RESULT(S)

Tests/Methods	Results	Units	Limit of quantification (L.Q.)
- Thiophanate-methyl (*)	< L.Q.	mg/kg	0,01
- Triadimefon	< L.Q.	mg/kg	0,01
- Triadimenol (*)	< L.Q.	mg/kg	0,01
- Triazophos	< L.Q.	mg/kg	0,01
- Tricyclazole	< L.Q.	mg/kg	0,01
- Trifloxystrobin	< L.Q.	mg/kg	0,01
- Triticonazole (*)	< L.Q.	mg/kg	0,01
Determination of pesticides residues by QuEChERS method. Gas chromatography with mass spectrometry (GC-MS) PI.LQ.13, Edição A (*)			
- alpha-Endosulfan	< L.Q.	mg/kg	0,02
- beta-Cyfluthrin	< L.Q.	mg/kg	0,02
- beta-endossulfan	< L.Q.	mg/kg	0,02
- Bifenthrin	< L.Q.	mg/kg	0,01
- Bromopropylate	< L.Q.	mg/kg	0,02
- Captan	< L.Q.	mg/kg	0,02
- Chlorpropham	< L.Q.	mg/kg	0,02
- Cypermethrin	< L.Q.	mg/kg	0,05
- Deltamethrin	< L.Q.	mg/kg	0,05
- Diclofluanid	< L.Q.	mg/kg	0,01
- Dicofol	< L.Q.	mg/kg	0,02
- Disulfoton	< L.Q.	mg/kg	0,01
- Flucythrinate	< L.Q.	mg/kg	0,05
- Folpet	< L.Q.	mg/kg	0,02
- lambda-Cyhalothrin	< L.Q.	mg/kg	0,01
- Oxyfluorfen	< L.Q.	mg/kg	0,02
- Permethrin	< L.Q.	mg/kg	0,02
- Procymidone	< L.Q.	mg/kg	0,02
- sulphate-endossulfan	< L.Q.	mg/kg	0,02
- tau-Fluvalinate	< L.Q.	mg/kg	0,01
- Tetradifon	< L.Q.	mg/kg	0,02
- Tolyfluanid	< L.Q.	mg/kg	0,05
- Trichlorfon	< L.Q.	mg/kg	0,05
- Trifluralin	< L.Q.	mg/kg	0,01
- Vinclozolin	< L.Q.	mg/kg	0,05

(b) (6)

Tânia Azevedo
Técnica do Laboratório de Química

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APPENDIX G.

PROTEIN PROFILE AND

POLYPHENOL ANALYSIS

Enzyme Development Corporation

505 8th Street, Suite 1500 New York, New York 10008-6505
Tel: (212) 736-1580 | E-Mail: info@EnzymeDevelopment.com | Laboratories and Plant
FAX: (212) 279-0056 | Web: www.EnzymeDevelopment.com | Scranton, PA 18504

September 18, 2014

Intertek: American Analytical Chemistry Laboratories
JMT Technology Center:
711 Parkland Court
Champaign IN 61821

TEST RESULTS:

Protein Profile: (NuPAGE Mini Gel and NuPAGE Bis-Tris Mini Gel) (Sample size: 1g)

SIZE	Cynzime R6763	Cynzime R6801-1	Cynzime R6801-3
10 kDa	1%	1%	1%
15 kDa	1%	1%	1%
20 kDa	8%	85	8%
38 kDa	75%	76%	76%
45 kDa	12%	12%	12%
Between 60-80 kDa	3%	2%	2%

Polyphenols (HPLC-UV) (Sample size: 1ml)

SIZE	Cynzime R6763	Cynzime R6801-1	Cynzime R6801-3
3,5 diCQA	12.7 mcg/ml	26.5 mcg/ml	26.9 mcg/ml
3-CQA	2.1 mcg/ml	5.1 mcg/ml	6.1 mcg/ml
4-CQA	2.5 mcg/ml	6.9 mcg/ml	7.3 mcg/ml
5-CQA	9.15 mcg/ml	54.0 mcg/ml	54.0 mcg/ml
Others	631.3 mcg/ml	474.7 mcg/ml	466.7 mcg/ml
Standardized to Gallic Acid	0.29 % (w/w)	0.13 % (w/w)	0.13 % (w/w)

Bonita Russell
Laboratory Manager
Enzyme Development Corporation

APPENDIX H.

EXPERT PANEL REPORT

Expert Panel Consensus Opinion Concerning the Generally Recognized as Safe (GRAS) Status of the Proposed Use In Cheese Making of a Milk Clotting Enzyme Preparation Derived From *Cynara cardunculus-L* (Cynzime®)

1 **08 May 2014**

INTRODUCTION

Fytozimus Biotech, Inc. (Fytozimus) has developed concentrated, standardized milk clotting enzyme preparation for use in cheese making (i.e., the production of a variety of cheeses and cheese by-products.) This product, named Cynzime®, is derived from the flowers of *Cynara cardunculus* L. (Cc-L). Cc-L, and related varieties of this thistle, are cultivated and grow wild in the Mediterranean regions where it is used as food, animal feed, as a home remedy and folk medicine

Fytozimus convened a panel of independent scientists ("Expert Panel"), qualified by their scientific training and relevant extensive national and international experience to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available data and information on the Cynzime® milk clotting enzyme preparation and determine if the proposed use of Cynzime® milk clotting enzyme preparation in cheese making is safe, suitable, and Generally Recognized As Safe (GRAS), based on scientific procedures. The Expert Panel consisted of Professor Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Professor Robert J. Nicolosi, Ph.D. (University of Massachusetts- Lowell) and Professor Michael W. Pariza, Ph.D. (University of Wisconsin- Madison).

The Expert Panel defined safe or safety as "a reasonable certainty of no harm under the proposed conditions of use of the ingredient in foods" (21 C.F.R. 170.3; U.S.FDA 2013).

The Expert Panel, independently and collectively, critically examined a dossier (Documentation Supporting the Safety of Cynzime® Milk Clotting Enzyme Preparation Derived from *Cynara cardunculus* Flowers", 26 September 2013), prepared by Gerald D. Mercer based on a comprehensive review of the scientific literature through 01 September 2013 by Fytozimus and Gerald Mercer. The dossier included information on the botanical source of the enzyme; manufacturing details; analyses for phytochemicals including polyphenols and other phenolic antioxidants; analyses for nutrients including sugars, fatty acids, fiber, carotenoids, and vitamins; analyses for heavy metals, mycotoxins, and pesticide residues; microbiological analysis; specifications for the finished enzyme product; information on stability and batch analyses; the proposed conditions of use; consumer exposure estimates; and an assessment of

the safety of Cynzime® milk clotting enzyme preparation including history of safe use of Cc-L flowers for human food and animal feed. The Expert Panel also evaluated other information that it deemed appropriate or necessary.

Following its independent and critical evaluation of the data and information provided and other information deemed appropriate, the Expert Panel convened via teleconference on November 26, 2013 with Gerald D. Mercer and representatives of Fytozimus. Following discussion, the Expert Panel requested additional information from Fytozimus. Following receipt and review of this additional information, the Expert Panel unanimously concluded that the proposed use in cheese making of Cynzime® milk clotting enzyme preparation, meeting appropriate food grade specifications presented in the dossier and manufactured consistent with current Good Manufacturing Practice (cGMP), is safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures (the substantial chemical and functional equivalency of Cynzime® milk clotting enzyme preparation to the centuries-old practice of using aqueous extractions of mature Cc-L flowers for cheese making), corroborated by a history of safe use of Cc-L flowers as human food and animal feed.

2 BASIS FOR SAFE AND SUITABLE AND GRAS STATUS OF CYNZIME® MILK CLOTTING ENZYME PREPARATION

Cynzime® milk clotting enzyme preparation is prepared from dried Cc-L flowers grown in the Mediterranean basin (Italy, Portugal and Spain). Fytozimus has developed a proprietary extraction process for removing the active milk-coagulating enzyme from the Cc-L flower (i.e. aspartic protease, cyprosin). Through a series of aqueous extractions, filtrations (centrifugations), separations and concentrations, both a liquid form and a solid form (powder) are prepared. Sodium chloride (10%) is added as a stabilizer and sodium benzoate (1%) is added as a preservative to the final product**.** All processing aids used are approved for use and are safe and suitable. Analysis of three batches confirms the consistency of production (i.e. meet the specifications presented in the dossier). Stability data for the 1:3000 liquid product show a <10% loss of activity in six months when stored at 4^0-8^0 C and shelf life is reported to be 12 months after shipment to the end user. The product in powder form is considered inherently stable and is distributed with the recommendation that the user store it at 4^0-8^0 C and re-test the activity yearly.

Cynzime® milk clotting enzyme preparation is proposed for use as a coagulant in cheese making (cheese and cheese by-products) at a typical use rate of 1 liter of Cynzime®milk clotting Cynzime® enzyme preparation/ 3000 liters of milk (58.5 grams of TOS/ 3000 liters of milk. Consumption of all the cheese made from 3000 liters of milk would result in the consumption of 19.5 mg TOS/ kg of cheese. Data from the USDA food consumption tables[‡] indicate that US per capita consumption of all cheese products is 33.2 lb/capita/year (15.1 kg). Assuming all cheeses produced in the United States were made using Cynzime® milk clotting

enzyme preparation, exposure to TOS would be 294.3 mg TOS/person/year or for a 70 kg. person, 4.2 mg TOS/kg body wt./year. On a daily basis, this would amount to 0.012 mg TOS/kg bw/day.

There are no known or suspected reasons that the use of Cynzime® milk clotting enzyme preparation would be self-limiting (e.g. unpleasant organoleptic effects or color changes at higher levels of use). However, using more than is needed to produce the intended clotting time would be economically infeasible.

‡ http://www.ers.usda.gov/data-products/dairy-data.aspx

No formal safety/toxicological studies on Cynzime® milk clotting enzyme preparation or on the enzyme, aspartic protease, were identified during a comprehensive search of the scientific literature. However, traditional aqueous extractions of mature Cc-L flowers have been safely used since antiquity for cheese making. Results of chemical analyses comparing the active proteolytic principal in crude aqueous extracts of Cc-L flowers with Cynzime® milk clotting enzyme preparation demonstrate essential chemical equivalency. Danisco conducted a cheese-making trial with Cynzime® milk clotting enzyme preparation and reported no measurable enzymatic activity after 160 hours of ripening.

The basis for determining that the proposed use of Cynzime® milk clotting enzyme preparation (as a coagulant for cheese-making) is safe and suitable and GRAS is as follows:

1. The essential chemical and functional equivalence of Cynzime® milk clotting enzyme preparation and the active proteolytic principal in crude aqueous extracts of Cc-L flowers that has a long and documented history of safe use in cheese-making;

2. A long and documented history of the safe use of Cc-L and other members of the genus *Cynara* as human food and animal feed;

3. Results of chemical and biochemical analyses of the Cynzime® milk clotting enzyme preparation failed to identify the presence of potentially toxic phytochemicals.

4. Product Specifications for the final Cynzime® milk clotting enzyme preparation include testing for and demonstrating non-detectable levels of microbial pathogens, mycotoxins, pesticide residues and heavy metals in the final product.

3 CONCLUSION

We, the Expert Panel have, independently and collectively, critically evaluated the data and information summarized above and unanimously conclude that the proposed use as a coagulant in cheese making of Cynzime® milk clotting enzyme preparation, manufactured consistent with cGMP and meeting the food grade specifications presented in the dossier, is safe and suitable.

We, the Expert Panel have, independently and collectively, critically evaluated the data and information summarized above and unanimously conclude that the proposed use as a coagulant in cheese making of Cynzime® milk clotting enzyme preparation, manufactured consistent with cGMP and meeting the food grade specifications presented in the dossier, is Generally Recognized As Safe (GRAS) based on scientific procedures (the essential chemical and functional equivalence of Cynzime® milk clotting enzyme preparation and the active proteolytic principal in crude aqueous extracts of Cc-L flowers that has a long and documented history of safe use in cheese-making) and corroborated by a documented history of safe use.

It is our unanimous opinion that other qualified experts would concur with these conclusions.

_____ _____

Professor Michael W. Pariza, Ph.D.; Panel Chair Date

University of Wisconsin- Madison

_____ _____

Professor Joseph F. Borzelleca, Ph.D. Date

Virginia Commonwealth University School of Medicine

_____ _____

Professor Robert J. Nicolosi, Ph.D. Date

University of Massachusetts-Lowell

CONCLUSION

We, the Expert Panel have, independently and collectively, critically evaluated the data and information summarized above and unanimously conclude that the proposed use as a coagulant in cheese making of Cynzime® milk clotting enzyme preparation, manufactured consistent with cGMP and meeting the food grade specifications presented in the dossier, is safe and suitable.

We, the Expert Panel have, independently and collectively, critically evaluated the data and information summarized above and unanimously conclude that the proposed use as a coagulant in cheese making of Cynzime® milk clotting enzyme preparation, manufactured consistent with cGMP and meeting the food grade specifications presented in the dossier, is Generally Recognized As Safe (GRAS) based on scientific procedures (the essential chemical and functional equivalence of Cynzime® milk clotting enzyme preparation and the active proteolytic principal in crude aqueous extracts of Cc-L flowers that has a long and documented history of safe use in cheese-making) and corroborated by a documented history of safe use.

It is our unanimous opinion that other qualified experts would concur with these conclusions.

(b) (6)

Professor Michael W. Pariza, Ph.D.; Panel Chair
University of Wisconsin- Madison

6/30/2014
Date

(b) (6)

Professor Joseph F. Borzelleca, Ph.D.
Virginia Commonwealth University School of Medicine

Date 12 July 2014

(b) (6)

Professor Robert J. Nicolosi, Ph.D.
University of Massachusetts-Lowell

08/July/2014
Date

APPENDIX I.

ADDITIONAL SCIENTIFIC SAFETY DOCUMENTATION

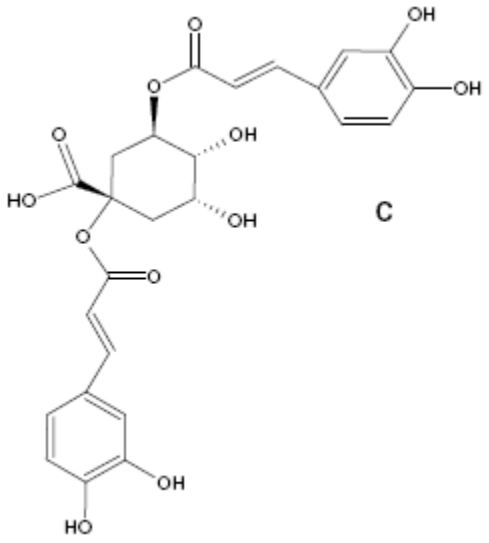
ADDITION TO DOCUMENTATION SUPPORTING THE SAFETY OF C. CARDUNCULUS MILK-CLOTTING ENZYME – CHEMICAL SAFETY

Although the main body of evidence relating to the safe use of C. cardunculus milk clotting enzyme preparation is related to the long history of safe use as documented in many published articles and scientific papers, this information is in addition to analytical characterization of the C. c – L plant and identification of chemical components found in various parts and tissues of the plant. In this analysis, we are including information about C.c-L an related varieties, all of which are used as food or as sources of chemical entities which find uses as healthful parts of the cuisine of the region of Southwest Europe discussed above, or as sources of folk remedies or medicinal foods used by the populations of the region. This would include, of course, the crude, or more refined enzyme preparations containing Ciprosin protease, the primary component of the subject enzyme preparation, Cynzime®.

Numerous scientific articles included in the bibliography, discuss the chemical components of the plants and their potential uses and characteristics. This portion of the documentation supporting the GRAS status of Cynzyme discusses these components and their potential impact on the safety and usefulness of Cynzyme.

1. In a treatise on the “Artichoke Thistle” by Amr Majul ( ), the uses of these plants, notably in this case, the “globe Artichoke” are discussed. Majul notes that these plants are widely used as part of the cuisine of the region, it is a poor choice for medicinal uses. The plants contain a component called “Cynerin”. Cynarin, the active compound in Cardoon, is what gives the plant its distinctive taste. It is also the active compound that is getting so much attention because of it's detoxifying and cholestrol lowering effects. Recently there has been an explosion of cynarin suplement products, mostly garnered from globular artichoke.

The Antioxidant abilities of Cynarin are being extensively studied. The structure of Cynarin is as follows:-



The cynarin is extracted from the flower part of the artichoke.

> Cynarin is the substance that imparts the distinctive flavor to the plant. Cynarin is getting much attention for its potential health benefits as an antioxidant, "detoxifier" and its purported hypocholesterolemic effect and is receiving attention among supplement producers for these characteristics. (Majul, 2013)

2. A method for the identification and quantification of phenolic compounds from artichoke (*Cynara scolymus* L.) heads, juice, and pomace by HPLC with diode array and mass spectrometric detection was developed. Among the 22 major compounds, 11 caffeoylquinic acids and 8 flavonoids were detected. Quantification of individual compounds was carried out by external calibration. Apigenin 7-*O*-glucuronide was found to be the major flavonoid in all samples investigated. 1,5-Di-*O*-caffeoylquinic acid represented the major hydroxycinnamic acid, with 3890 mg/kg in artichoke heads and 3269 mg/kg in the pomace, whereas in the juice 1,3-di-*O*-caffeoylquinic acid (cynarin) was predominant, due to the isomerization during processing. Total phenolic contents of ~12 g/kg on a dry matter basis revealed that artichoke pomace is a promising source of phenolic compounds that might be recovered and used as natural antioxidants or functional food ingredients.

BIBLIOGRAPHY (SUPPLEMENT)

Artichoke Thistle; Amr Majul; (2013), Source unknown

Identification and Quantification of Caffeoylquinic Acids and Flavonoids from Artichoke (*Cynara scolymus* L.) Heads, Juice, and Pomace by HPLC-DAD-ESI/MS^n ; Katrin Schütz , Dietmar Kammerer , Reinhold Carle , and Andreas Schieber *Institute of Food Technology, Section Plant Foodstuff Technology, Hohenheim University, August-von-Hartmann-Strasse 3, D-70599 Stuttgart, Germany *J. Agric. Food Chem.*, 2004, 52 (13), pp 4090–4096 **DOI:** 10.1021/jf049625x Publication Date (Web): June 4, 2004 Copyright © 2004 American Chemical

APPENDIX J

ALLERGENICITY POTENTIAL OF

Cynzime®

BY INGESTION

The potential of Cynzyme® cause an allergic reaction by ingestion was addressed as follows:

1. In an email communication from Jannavi Srinivasan to Gerald Mercer, et al, September 25, 2014, subject "Presubmission GRAS meeting," FDA stated it is understood that the likelihood of enzymes as allergens is low, but since enzymes are proteins the aspect needed to be addressed for safety in food uses., and further stated "one route is to conduct an amino acid sequence homology search for the enzyme of interest, against sequences of known allergens stored in publicly available data bases." FDA listed Allermatch and two other possible databases as options. FDA further stated "absence of an amino acid overlap of >= 35% using a window [of] 80 amino acids, or matches of contiguous stretches of eight or more amino acids, with the enzyme of interest, would predict the low potential for allergenicity using bioinformatics."

2. The amino acid sequence for Cynzime was provided.

3. An amino acid sequence homology comparison against Allermatch, one of the available databases suggested by FDA in above mentioned email, was conducted and used both the 80 amino acid sliding window and the 8 amino acid word match were run.

4. No matches were identified in the 8 contiguous amino acid word match (Attachment 1). Further, no matches were identified in the 80 amino acid sliding window using the UnitPro database (Attachment 2). However, one match above 35% overlap threshold was identified in the 80 amino acid sliding window against the WHO-ISUS database (Attachment 3). The match was to Asp f 10 from *Aspergillus fumigatus*. (Further attachments 4 and 5 provide the detailed information available via the search results.)

5. A match to the same allergen, Asp f 10, from the same source organism, *Aspergillus fumigatus*, was addressed in an agency response to a GRAS notice for a different enzyme. In the email of 9-25-2014, FDA also included a link to recent enzyme notices that had received 'no questions' response from the agency. A review of the GRAS Notices for enzymes that have included allergen information showed typically 'no identity matches greater than 35% overlap and no matches of 8 contiguous amino acids. However, one of the agency responses to a GRAS notice included in the above mentioned link, included GRN 333, which in fact identified the same identity match of the Asp f 10 (*Aspergillus fumigatus*), for an acid fungal protease (AFP) from *Trichoderma reesei*. The agency response letter of 9-24-10 to Genencor, included the following: "Genencor identified a significant homology (>35% ID within multiple 80 amino acid windows with the respiratory allergen Aspergillopepsin (Asp f 10) from Aspergillus fumigatus." Additional information was included about the hydrophobicity of the allergen and lack of exposure on the surface of the protein, that it is not a recognized food allergen, and further mentioned negligible amount of carry-over into product. (See attachments 6 & 7 for a screen shot of the relevant information; the full text of the agency response letter on GRN333 is on the FDA website.)

Thus, it is our opinion that, though one match did surface in the amino acid sequence comparison, it is a match that has previously been identified and indicated as not a recognized food allergen and should not be cause for concern about potential for food allergenicity of Cynzime®. This, along with the low potential for exposure to the enzyme protein comprising Cynzime®, provides assurance that there should be very little concern for the potential of allergenicity.

The kind assistance of Ms. Rachel Montgomery (Simple Compliance Solutions LLC) is acknowledged.

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ONLINE ABSTRACT:

Moharram FA, Marzouk MS, El-Toumy SA, Ahmed AA, Aboutabl EA.

Polyphenols of Melaleuca quinquenervia leaves--pharmacological studies of grandinin. **Phytother Res. (2003)** Aug;17(7):767-73.

Source: Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

Abstract:

Four polyphenolic acid derivatives and three ellagitannins were isolated from the leaves of Melaleuca quinquenervia (Clav.) S. T. Blake for the first time. Their structures were elucidated as gallic acid (1), ellagic acid (2), 3-O-methylellagic acid (3), 3,4,3'-tri-O-methylellagic acid (4), 2,3-O-hexahydroxydiphenoyl-(alpha/beta)-D-(4)C(1)-glucopyranose (5), castalin (6) and grandinin (7) on the basis of chemical, mass spectrometric (-ve ESI-MS) and spectroscopic (UV, (1)H-, (13)C NMR, (1)H,(1)H-, (1)H,(13)C-COSY, (1)H,(1)H-TOCSY and HMBC) analyses. Grandinin (the major compound) showed radical scavenging properties by its reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [EC(50) = 4.3 +/- 0.3 micro g mL(-1)]. It was found to be relatively nontoxic in mice [LD(50) = 316 mg Kg(-1) b.wt.]. It exhibited a significant dose-dependent (35-70 mg Kg(-1)) hypoglycemic effect by significantly reducing blood glucose level in basal condition and after heavy glucose load in normal mice. Moreover, it reduced the elevated blood glucose level in STZ-induced diabetic mice. In addition, grandinin reduced the elevated blood urea nitrogen and serum lipid peroxides in STZ-induced diabetic mice.

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ONLINE REVIEW:

Sahelian, Ray (Online Review of numerous articles) This review, although not published entirely in peer-reviewed journals, does point out some of the sources, uses and benefits of polyphenolic compounds. Many are found in commonly consumed foods, drinks, fruits and vegetables. They are commonly found in tea, coffee and wine. The review notes intake by humans from these and other sources, such as food supplements in the range from 200 – 1000 mg/day.

SUBMISSION END