APPLICATION FOR THE APPROVAL OF SPOROPOLLENIN EXINE CAPSULES FROM *LYCOPODIUM CLAVATUM* SPORES

Under

Regulation (EC) No 258/97 of the European Parliament and of the Council of 27th January 1997 Concerning Novel Foods and Novel Food Ingredients

January 2013

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Table of Contents

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ADMINISTRATIVE DATA

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Sporomex Ltd proposes to license the production of food items containing active ingredients encapsulated within the sporopollenin exines (exoskeletal shells), acting as capsules, from plant spores or pollens: for consistency, in this proposal the sporopollenin exine capsules will be abbreviated to as SEC. Compositionally, the SEC powder is comprised of carbon, hydrogen and oxygen and is terpene in nature. Approval is sought under Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients (hereafter referred to as EC 258/97), and accordingly, this submission has been prepared pursuant to the Commission Recommendation of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients (hereafter referred to as the Commission Recommendation of 1997) (European Parliament and the Council of the European Union, 1997).

Article 1(2.) of EC 258/97 states that the regulation "…shall apply to the placing on the market within the Community of foods and food ingredients which have not hitherto been used for human consumption to a significant degree within the Community and which fall under the following categories…(e) foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating and breeding practices and which have a history of safe food use;". Naturally occurring SEC are only present as extracted shells in relatively small amounts in food and thus could be considered a novel food/food ingredient due to their extraction from spores and pollens (many of which are consumed in large quantities).

Section 4 of the Commission Recommendation of 1997 outlines recommendations made by the Scientific Committee on Food (SCF) pertaining to the "Scientific Classification of Novel Foods for the Assessment of Wholesomeness", which facilitates the safety and nutritional evaluation of a given novel food/food ingredient.

Of the 6 classes identified, SEC would be classified as Class 2 "Complex Novel Food from non-GM source", since their production is by conventional techniques, and with no use of genetic modification.

Since the proposed use of SEC to encapsulate and thereby protect/preserve currently permitted food ingredients/additives has not been introduced to the community, SEC can be further allocated under Sub-Class 2.2: "the source of the novel food has no history of food use in the Community". The essential information requirements corresponding with this classification are outlined in a detailed list below, and are expanded upon in separate sections throughout the document, forming the basis of the application (Recommendation 97/618/EC - Commission of the European Communities, 1997).

Sporomex Ltd, January 2013

Summary

Sporopollenin is the major component of the tough outer (exine) walls of spores and pollen grains. This application outlines the proposed use of ‘sporopollenin exine capsules’ (SEC) as an enhanced delivery vehicle for oils consumed in the diet. These oils may currently occur naturally, be found as additives or consumed as supplements.

The SEC is made from a unique polymer, known as sporopollenin, which possess polycarotenoid like characteristics and is constructed only of carbon, hydrogen and oxygen. As such it is extremely tough, resistant to acid and alkali degradation and has been shown to pass unchanged though the digestive system of animals. To produce SEC, the spores from *Lycopodium clavatum* are initially emptied of all their genetic/protein material. The empty SEC can then be loaded with the ‘active’, for example fish oil or a vitamin. The filled SEC is a powder, which could potentially be consumed by the individual as a supplement or incorporated into a food or drink by a manufacturer to produce a functional food.

Release of the active ingredient (i.e. at which stage during transition through the GI tract) is dependant primarily upon the chemical properties of active ingredient itself, but enhanced delivery of actives has been proven.

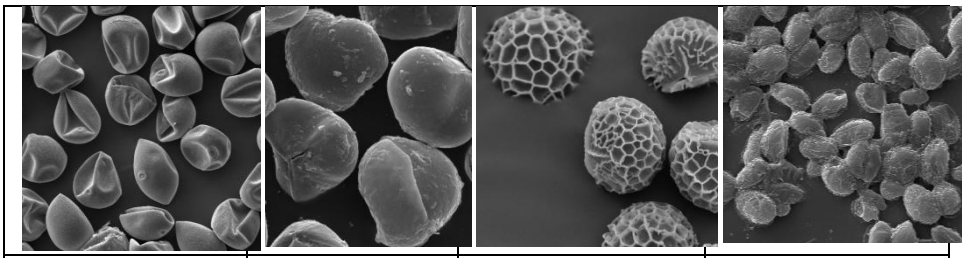
There is no potential for adverse effects from consumption of the SEC as they pass through the digestive tract unchanged and lack of allergenic potential is proven by the absence of protein and %N in both samples of SEC.

As such it is suggested that SEC provide a novel and enhanced delivery method for commonly consumed oil or other lipophilic products.

General Introduction

Sporopollenin is the major component of the tough outer (exine) walls of spores and pollen grains. Physically and chemically it is extremely stable and it is usually well preserved in soils and sediments. The exine layer is often intricately sculptured in species-specific patterns (hence their use in archaeology and forensic science).

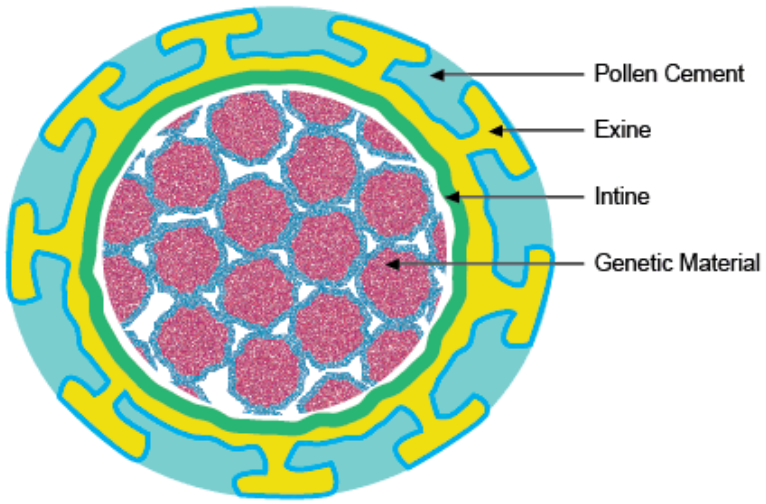
Figure 1: Sporopollenin exine capsules (SEC) of different sizes from commercially available pollens and spores of different plants.



Zea maize (80µm)	*L.clavatum* (minor species) (40µm)	*Lycopodium clavatum* (major species) (25µm)	*Chlorella vulgaris* (8µm)

All pollens/spores possess an outer shell called an exine that protects the genetic material and nutrients [(see Figure 2)]. The exine is made from a unique polymer, known as sporopollenin, which possess polycarotenoid like characteristics and is constructed only of carbon, hydrogen and oxygen.

Figure 2: Schematic diagram of a spore/pollen particle.



SEC are elastic and can be compressed under many tons of pressure but still return to their original form after compression. The shells are non-toxic and have been shown to possess taste-masking properties in two human trials (Barrier, S. *et al*., 2010; Diego-Taboada, A. *et al.,* 2013).

Sporopollenin is composed of carbon, hydrogen and oxygen, though the exact chemical composition is not known, due to its unusual chemical stability and resistance to degradation by enzymes and strong chemical reagents. Analyses have revealed a mixture of biopolymers, containing mainly long chain fatty acids, phenylpropanoids, phenolics and traces of carotenoids.

To produce 'sporopollenin exine capsules' (SEC) the spores or pollens are initially emptied of all their genetic, lipid and protein material to leave the empty SEC. This SEC has anti-oxidant properties and can be used at low levels (< 1%) in oils or other foods to increase shelf-life. The effectiveness of the exine depends upon any other anti-oxidants that are present.

What is important to this application is that the SEC can be filled with an active additive such as a functional ingredient, for example fish oil or vitamins. The filled SEC is a powder: for consumption this powder could potentially be incorporated into a food or drink, by the consumer or by a manufacturer.

The benefit of encapsulation in SEC can take two forms. Firstly, some beneficial additives such as fish oil or vitamin B can have a very unpleasant taste. This unpleasantness can be masked in the mouth by the sporopollenin exine capsules, leaving the additive to be released in the intestine. An alternative application is that of retention of volatile materials. In the food product the SEC will retain the volatile material until it is eaten; upon consumption the volatile material is then released in the mouth.

Some functional ingredients such as probiotics and some vitamins are most effective when released in the lower intestine, as they are destroyed by acids in the upper gastro intestinal (GI) tract, and as such their effectiveness is low. SEC can be used to protect this type of ingredient against acids in the upper GI tract; the active ingredient is then released lower down the GI tract under alkaline conditions.

Release of the active ingredient (i.e. at which stage during transition through the GI tract) is dependant primarily upon the chemical properties of the active ingredient itself and affected to a lesser degree by the shape and size of the SEC employed as a carrier. Non-volatile hydrophobic molecules encapsulated within SEC will not be released into an aqueous environment without harsh physical agitation or pressing.

Volatile molecules can be released readily from the SEC in air but if they are hydrophobic they will not be released rapidly into an aqueous environment. However, if the aqueous environment possesses a surfactant then release of the hydrophobic volatile can be rapid. Thus, the release of the active ingredient can be tailored to the need of the functional food.

SPECIFICATIONS OF SPOROPOLLENIN EXINE CAPSULES (SEC) AS A NOVEL FOOD

Based on Commission Recommendation 97/618/EC decision trees the following questions must be addressed pertaining to the specifications of the novel food (European Commission 1997):

- “Depending on the derivation and composition of the Novel Food, is appropriate analytical information available on potentially toxic inherent constituents, external contaminants and nutrients?”

- “Is the information representative of the novel food when produced on a commercial scale?”

- “Is there an appropriate specification (including species, taxon, etc for living organisms) to ensure that the novel food marketed is the same as that evaluated?”

These questions have been addressed collectively in Sections 1.a through 1.d

1.a Description

A sporopollenin exine (SEC) is the outer shell of a spore, made from a unique polymer, known as sporopollenin. It possesses polycarotenoid like characteristics and is constructed only of carbon, hydrogen and oxygen.

1.b Empirical Formula

Sporopollenin is a complex framework of mainly cross-linked saturated aliphatic chains, with some being un-saturated. Attached to the chains there are oxygenated functional groups including carboxylic acids, lactones, hydroxyls and phenols, the latter of which are in conjugation with the carbon framework: the empirical formula is of the order Carbon 3 : Hydrogen 5 : Oxygen 1 (Gooday *et al*., 1974, Barrier, S., PhD Thesis, 2008, Ariizumi, T. and Toriyama, 2011)

1.c Structural Formula

The chemical structure of sporopollenin is still controversial. In summary, all of the features of sporopollenin structure that have been established without ambiguity are listed as follows:

- Sporopollenin is composed of only three elements, carbon, hydrogen and oxygen, with a C/H ratio of 5/8 (mol/mol) like terpenes; nitrogen and metals are absent.

- Sporopollenin is constituted of an aliphatic matrix, common to most species of vascular plants (ferns, gymnosperms and angiosperms); the carbon skeleton is cross-linked by various side-groups whose exact nature, position and number are directly species-dependant.

- Certain chemical functions are present on sporopollenin different species: carbon chains; conjugated unsaturated systems; hydroxyls; ethers; methyls. Others are only confirmed in some species (like *Lycopodium clavatum*) such as, carbonyls (ketones) and carboxyls (acids, esters).

- Aromaticity of sporopollenin is strongly debated, although it seems reasonable to link the aromatic/aliphatic ratio to each species; for instance, phenyls and phenols are recognised features in sporopollenin from *Lycopodium clavatum.*

1.d Applicability to All Spore or Pollen Sources

The genetic basis of sporopollenin synthesis and exine formation is explored in a recent review by Ariizumi and Toriyama (2011). Although the evolution of sporopollenin in land plants is not clear, sporopollenin is known to be found in fossil green algae, moss, ferns, liverwort, bryophytes, and even fungi. The exine structure, composed of sporopollenin is constitutionally the same regardless of source, but as illustrated in Figure 1, will vary in shape and size depending upon its origin.

2. EFFECT OF THE PRODUCTION PROCESS APPLIED TO SEC

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient information pertaining to the effect of the production process applied to the novel food:

- “Does the novel food undergo a production process?”
- “Is there a history of use of the production process for the food?” If no, “does the process result in a significant change in the composition or structure of the novel food compared to its traditional counterpart?”
- “Is information available to enable identification of the possible toxicological, nutritional and microbiological hazards arising from use of the process?”
- “Are the means identified for controlling the process to ensure that the novel food complies with its specification?”
- “Has the process the potential to alter the levels in the novel food of substances with an adverse effect on public health?”
- “After processing is the novel food likely to contain microorganisms of adverse public health significance?”

These questions have been addressed collectively in Sections 2.a through 2.c

2.a Raw Materials Used in the Manufacturing Process

2 a i Spores

Lycopodium clavatum (club moss) spores are widely available from a number of commercial companies, including Tibrewala International (Nepal) currently one of the main suppliers to Sporomex.

2 a ii Extraction process reagents

The reagents (acids, alkalis, bleaching agents, ethanol) used in the extraction process are food/medical grade.

2.b Exine Manufacturing Process

2 b i Preparation of Sporopollenin Exine Capsules (SEC)

The specific conditions of mineral acid and alkali used to free SEC from other organic materials (leaving pure SEC) in *Lycopodium clavatum* L. spores are described in detail:

Preparation of sporopollenin exine capsules: Raw *Lycopodium clavatum* L. spores (loose powder, 250 g) are suspended in acetone (750 cm^3) and stirred under reflux for 4 h. The defatted spores are filtered and dried overnight in open air. They are suspended in 6% (w/v) potassium hydroxide aqueous solution (750 cm^3) and stirred under reflux for 6 h. After filtration, this operation is repeated with a new 6% (w/v) potassium hydroxide solution (750 cm^3). The suspension is filtered and washed with hot water (3 x 300 cm^3) and hot ethanol (2 x 300 cm^3) and dried overnight in open air. It is suspended in 85% (v/v) ortho-phosphoric acid (750 cm^3) and stirred under reflux for 7 days. The solid is filtered, washed with water (5 x 250 cm^3), acetone (250 cm^3), 2M hydrochloric acid (250 cm^3), 2M sodium hydroxide (250 cm^3), water (5 x 250 cm^3), acetone (250 cm^3) and ethanol (250 cm^3) and dried in an oven at 60 °C. The SEC (75 g) obtained by the foregoing protocol were nitrogen-free, by combustion elemental analysis and devoid of cellulose (main component of intine). The combustion elemental analysis was typically C, 68.9; H, 7.9; N, 0.0. Combustion elemental analyses of sporopollenin and derivatives were performed

on a Fisons instrument Carlo Erba EA 100 CHNS analyser: the analysis reported is based upon three repeats.

2 b ii Encapsulation of products

All the oils that it is anticipated will be encapsulated in SEC are food or medical grade products and are already widely consumed by the general public.

2.c i Stability of SEC

SEC are remarkably resistant to physical, biological and chemical non-oxidative attack having been described as "*one of the most extraordinary resistant materials known in the organic world*" (Kettley 2001, Brooks and Elsik, 1974, Shaw, 1970). Methods developed to isolate sporopollenin as SEC from *Lycopodium clavatum* give evidence of its exceptional stability and chemical inertness. Indeed, it is highly resistant to a variety of hot strong acids (including phosphoric acid, sulphuric acid and hydrofluoric acid), alkalis (e.g. concentrated sodium or potassium hydroxides) and organic solvents (e.g. acetone, methanol or dichloromethane) (Zetzsche and K. Huggler 1928, Zetzsche *et al*., 1937, Zetzsche and Kälin 1931, Shaw, 1970,Shaw and Apperley 1996).

Hydrofluoric acid solution is reported to not modify the chemical composition of sporopollenin and hence be useful for intine removal (Domínguez *et al*., 1998). Additionally, the integrity of the SEC remains intact after stirring (2h or 24h) in DCM, ethanol, water, toluene, DMF or DMSO, at room temperature or 50°C [Kettley 2001, Boasman, 2003). SEM pictures revealed that sporopollenin isolated from *Lycopodium clavatum* L. (25µm) was not soluble in these solvents and remained undamaged (Barrier, S. *et al*., 2010).

The enzymatic extraction procedure developed by Wiermann *et al.* [Schulze Osthoff and R. Wiermann 1987, Herminghaus *et al*., 1998] has proved sporopollenin, as SEC, resistant to a wide range of enzymes (protease, amylase, lipase, cellulase and hemicellulase). This may explain why it does not easily submit to bacterial decomposition or to digestion.

Exines have also been shown to pass unchanged through the gut (see section 6.d.i); oil-filled SEC are empty upon recovery.

2.c ii Evidence for Release of Actives from SEC

It should be noted that, unlike intact pollen or spores, SEC are perforated shells (Rowley, J.R. *et al*., 2003) and do not have a complex lipid coating or internal organelles of the raw spores/pollen. This is likely to influence release characteristics of the internal components of the microcapsules (Barrier, S. *et al*., 2011 and Diego-Taboada, A *et al*., 2013).

Firstly, SEC are round microcapsules, which in the case of those extracted from *Lycopodium clavatum* are approximately 27 µm in diameter (Barrier, S. *et al*., 2011). The walls of the SEC microcapsules are only 2 µm thick and possess a myriad of channels, which are *ca* 40 nm in diameter connecting the inside chamber with the outer surface; hence the microcapsules are porous (Rowley, J.R. *et al*., 2003). This porosity allows the microcapsules to be loaded with an active and the active to be released. The SEC wall comprises sporopollenin which is hydrophobic, but with some amphiphilicty this enables lipids to be encapsulated preferentially and retained within the SEC (Wakil, A. *et al*., 2010).

In vitro evidence for release of an active:

Release of an active can take place by passive diffusion. For example, we have demonstrated that encapsulated enzymes can be released into a stirred buffer solution and still retain their catalytic activity (Barrier, S. *et al*., 2011). Similarly, it has been shown that a commercial magnetic resonance imaging agent can be released at different rates by passive diffusion into stirred blood plasma and buffer solution respectively, at the same pH (Lorch, M. *et al*., 2009). The amount of release can be monitored by solid state nuclear magnetic resonance and gravimetric means.

In a further experiment (Diego-Taboada, A, *et al*,. 2013) ibuprofen was loaded into SEC extracted from *Lycopodium clavatum*. Release of the ibuprofen into agitated environments of simulated gastric fluid and phosphate buffer (pH 7.4) to mimic the change from the gastric environment was observed. In simulated gastric fluid an overall loss of 12 ± 1% of the drug from the microcapsules was observed, indicating the feasibility of the SEC to retain the majority of the drug during transit through the stomach. In contrast, when SEC at the same loading level of the drug were agitated in an excess of phosphate buffer (pH 7.4) to

mimic the change from the gastric environment upon entry into the GI tract, most (85 ± 2%) of the load was released in 5 min.

Additionally, it has also been shown that oil filled SEC can release oil gradually as they are rubbed between two surfaces due to the elastic nature of the exines (Diego-Taboada, A. *et al*., 2012).

In vivo evidence for release of an active:

In vivo evidence in humans that actives are released from SEC is presented in Section 4.a; Figure 3, which illustrate increased bioavailability of fish oil and in serum when delivered by SEC compared to un-encapsulated oils. The exact mechanism of release has not been elucidated; however, there are consistent factors that suggest a reasonable putative mechanism of release of the lipid actives. Firstly, the lipid actives are released post the gastric environment of the stomach, based on the time at which the maximum absorption of the lipid is seen in the bloodstream. This indicates that the mechanism of release is polar and pH dependent. The gastric environment of the stomach is highly polar and would repel a lipid, preventing it from exiting from the relatively lipophilic microcapsules. The slightly alkaline region of the intestines will be less polar and conducive to allowing release of a lipid.

A further action to the mechanism could be the physical pressures, such as by peristalsis that would be exerted upon the SEC in the intestinal region of the gastrointestinal tract (GIT), especially against the GIT wall. Such physical action could well play a role in releasing the lipid active in this part of digestion. Lastly, the size and morphology of the SEC may well be important in delivery. We have shown by taking sections of mouse intestinal villi following ingestion of SEC that the SEC can fit neatly between the villi. These data suggest that the exines interact with intestinal mucus positioning them favourably at the intestinal surface to facilitate absorption. Such close contact with the villi may be important in delivery of the active directly into the blood circulation of the villi.

A preliminary study has been undertaken to compare mucoadhesion properties of SEC with known naturally occurring mucoadhesion polymers, namely chitosan. Such properties were assessed in vitro, using Peak Detachment Force (PDF), Work of Adhesion (WA) and Differential Scanning Calorimetry (DSC). All of the resulting data were

supportive of SEC possessing mucoadhesive characteristics and in excess of chitosan (Thompson, 2012).

3 HISTORY OF THE SOURCE OF SPOROPOLLENIN EXINES

Based on the Commission Recommendation 97/618/EC decision trees the following questions must be addressed pertaining to the history of the source organism (European Commission 1997):

- “Is the Novel Food obtained from a biological source, i.e. a plant, animal or microorganism?”
- “Has the organism used as the source of the Novel Food been derived using GM?”
- “Is the source organism characterised?”
- “Is there information to show that the source organism and/or food obtained from it are not detrimental to human health?”

These questions have been addressed collectively in Sections 3.a through 3.b

3.a *Lycopodium* spore source and GM status

Lycopodium clavatum is not Genetically Modified (GM) nor are the exines derived from *Lycopodium clavatum* obtained from GM sources.

3.b Information on detrimental health effects

There is no evidence of adverse health effects resulting from consumption of *Lycopodium clavatum* exines.

4 INTAKE/EXTENT OF USE OF SPOROPOLLENIN EXINES (SEC)

Based on Commission Recommendation 97/618/EC decision trees the following questions must be addressed regarding to intake/extent of use of the Novel Food (European Commission 1997):

- “Is there information of the anticipated uses of the Novel Food based on its properties?”
- “Is there information to show anticipated intakes for groups predicted to be at risk?”
- “Will introduction of the novel food be restricted geographically?”
- “Will the Novel Food replace other foods in the diet?”

These questions have been addressed collectively in Sections 4.a through 4.f

4 a Intended Uses

The intention is to use the encapsulation technology of SEC to deliver a variety of oils already regularly consumed in the diet. One anticipated use is the encapsulation of EPA-fish oils. Within this section these are used as examples to illustrate the reasons for employing SEC for delivery and anticipated intake.

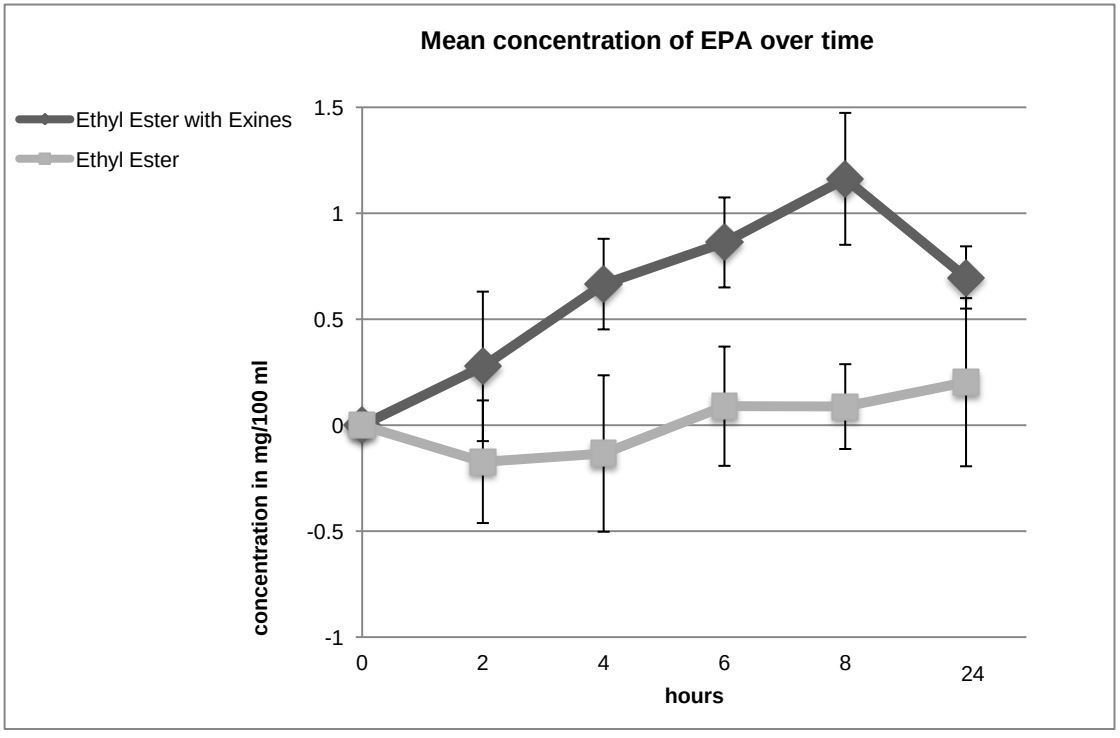
It is anticipated that exine encapsulated oils may be used in various food categories and as such permission is sought for use in the following food categories; food supplements, dietary foods for special medical purposes, foods intended for use in energy-restricted diets for weight reduction, other foods for particular nutritional uses (PARNUTS), as defined in Directive 2009/39/EC, bakery products, breads and rolls, sweet biscuits, breakfast cereals, cooking fats, dairy analogues (except drinks), dairy products (except milk-based drinks), non-alcoholic beverages (including dairy analogue and milk-based drinks), cereal/nutrition bars and spreadable fats and dressings.

Considering polyunsaturated oils such as fish oils as an example. Fish oils are currently added to a small number of products such as bread, dairy spreads and baby foods. The application methods employed currently are limited in terms of loading and shelf life and they are not appropriate in longer life products such as bread and breakfast cereals.

Demand exists but no efficient solution has yet been found, to provide high performing microencapsulated formulations for such oils with the required properties of long-term protection and efficient release. SEC encapsulation with its inherent (i) high antioxidant properties, (ii) acid, base and heat resistance (iii) ultra violet light protection and (iv) high loading potential overcomes these issues. Unlike most other encapsulating materials, SEC are able to withstand both acid and alkali conditions as well as most food processing temperatures, because of this and the fact that loaded SEC microcapsules can be free flowing powders and confer enhanced stability, they are extremely easy to incorporate into food products, and as such produce higher added value products.

Sporomex has shown in a human oral intake trial that the ethyl ester of EPA (eicosapentaenoic acid), encapsulated into sporopollenin exine microcapsules (SEC) is absorbed more rapidly, and in considerably higher amounts, than when taken simply as the free oil (Figure 3) (Wakil 2010).

Fig. 3 The change in mean EPA serum level in human volunteers over time obtained from the ethyl ester of EPA administered orally with and without SEC



4.b Anticipated intake

It is anticipated that SEC filled with oils will primarily be consumed as food supplements as the resulting product is a free-flowing powder that the consumer can ingest as preferred, for example, stirred into a drink or yoghurt or sprinkled on breakfast cereal. Using SEC loaded with fish oil (omega 3) is a novel way to disguise the unpleasant taste associated with their intake (Barrier *et al*, 2010).

The SEC can be filled with an active ingredient on a weight to weight ratio (exine:ingredient) between 1:1 and 1:4. Continuing to use the case of fish oil (omega 3) as an example, a ratio of 1:1 is optimal (Barrier, S. *et al*., 2010) so the weight of SEC consumed is the same as that of the active ingredient. Using this ratio also has the benefit of being a worst-case scenario, as higher loading of product to SEC would result in lower SEC consumption to deliver the required dose of fish oil. Normal supplemental intake of cod liver oil (omega 3) is 0.5 – 1.5 g per day, thus consumption of SEC-based cod liver oil supplements would result in an equivalent intake of SEC, i.e. 0.5-1.5 g per day.

Intake data for these food groups in the UK population (from UK NDNS) are presented in [Table 1]. Intakes for the average consumer and the high level (95th percentile) consumer are included. The UK NDNS provides consumption data for the average UK consumers, including between the ages of 19-64 (Table 1, Henderson *et al*. 2001). In order to assess the potential intake of SEC for the average and 95th percentile UK consumer an exposure assessment has been undertaken.

Calculating the daily consumption of each product category by the average adult consumer, then multiplying this by the proposed inclusion % of SEC, (as provided in Table 2) we provide an estimate of the potential consumption of SEC by the 95th percentile UK consumer. This is a worst-case scenario, making the assumption that all the food consumed in these categories was a functional food containing SEC.

Not all consumers consume a product from each category every day, and therefore this statistical representation has a very low probability of occurring.

Table 1. Consumption of bread and pasta by UK consumers (taken from UK NDNS, 2001).

	Age Groups										
	19 – 24		25 - 34		35 – 49		50 - 64		All consumers		
	Consumption										
Food Group	**Mean ($g\cdot person^{-1}\cdot d^{-1}$)**	**P_{95} ($g\cdot person^{-1}\cdot d^{-1}$)**	**Mean ($g\cdot person^{-1}\cdot d^{-1}$)**	**P_{95} ($g\cdot person^{-1}\cdot d^{-1}$)**	**Mean ($g\cdot person^{-1}\cdot d^{-1}$)**	**P_{95} ($g\cdot person^{-1}\cdot d^{-1}$)**	**Mean ($g\cdot person^{-1}\cdot d^{-1}$)**	**P_{95} ($g\cdot person^{-1}\cdot d^{-1}$)**	**Mean ($g\cdot person^{-1}\cdot d^{-1}$)**	**P_{95} ($g\cdot person^{-1}\cdot d^{-1}$)**	**Percentage consumers**
Bread	96.9	188.4	103.4	215.7	103.2	203.9	102.7	200.1	102.3	206.3	99%
Pasta	27.3	64.3	26.2	67.4	25.6	69.3	20.3	47.5	24.9	63.7	22%
Bread and Pasta[1]	103.2	194.4	110.5	227.9	109.0	212.8	105.6	210.7	107.7	215.9	99%

[1]This is the integrated consumption of both bread and pasta, not a simple summation of the two.

Source: Henderson, L., Gregory J. and Swan, G. (2002) National Diet and Nutrition Survey: Adults Aged 19 to 64 years. Volume 1: Types and Quantities of Foods Consumed. HMSO, London.

Table 2. Inclusion of SEC in functional food products and potential consumption by 95th percentile UK consumer.

Product categories	**All consumers P_{95} ($g \cdot person^{-1} \cdot d^{-1}$)**	**SEC - % inclusion**	**Grams of SEC consumed per day**
Bread	206.3	0.005	1.0
Pasta	63.7	0.005	0.3
Bread and pasta	215.9	0.005	1.1

4.c Intake for Groups Predicted to be at Risk

There are no groups for whom intake of SEC is predicted to be a risk.

4.d Geographic restriction of SEC release

Sporomex do not believe that the release of SEC products into the EU should be in anyway restricted due to: (i) the product delivering nutritional benefits to consumers: there being sufficient evidence of lack of potential for ingestion to give rise to adverse effects to human health.

4.e Will SEC replace other foods in the diet?

SEC will not replace other foods in the diet. SEC will be used to enhance palatability of food products e.g. masking flavor of fish oils primarily in food supplements, and potentially to enhance delivery of beneficial ingredients (oils already consumed in the diet) that would be consumed in addition to a standard diet.

4.f Labelling

SEC will be displayed on the labelling of the food product as such or in the list of ingredients of foodstuffs containing it, in accordance with the requirements of Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs (as amended).

5 INFORMATION FROM PREVIOUS HUMAN AND ANIMAL EXPOSURE

Based on Commission Recommendation 97/618/EC decision trees, the following questions must be addressed regarding the previous human exposure to the Novel Food (European Commission 1997):

- "Is there previous information from previous direct, indirect, intended, or unintended human exposure to the Novel Food or its source which is relevant to the Community situation with respect to production, preparation, population, lifestyles, and intakes?"

- "Is there information to demonstrate that exposure to the Novel Food is unlikely to give rise to nutritional, microbiological, toxicological and/or allergenicity problems?"

These questions have been addressed collectively in Sections 5.a through 5.c.

5.a Current Dietary Exposure to Spores and Pollens

Many types of spores/pollens (all of which have the same fundamental chemical structure, but differ in physical appearance depending upon their source) are consumed as part of a normal diet. Intact pollen itself though not the empty pollen shells, i.e., SEC, has in fact been sold as a health food supplement for over 20 years (www.beepollensecrets.com) and may be consumed at levels as high as 35g per day (www.nutritional-supplements-health-guide.com).

Pollen is also consumed in regular food products. One reference states that the number of pollen grains in honey depends on a number of factors, but can range from less than 20,000 to over 100,000 pollen grains per 10g of honey (Linskens and Jorde , 1997).

More recent Australian research on one type of honey showed the pollen content to vary between 0.15% and 0.44% by weight and also quotes Canadian figures of 0.2% to 0.24% (Australian Government Report 2004).

Other foods contain pollen from the plants from which they were produced, for example one study (http://www.burstscience.com/Studies/study82.php) showed that 70 particles of pollen were present in 1g of rye bread.

In the UK in 2008 Moody Muesli Limited launched a range of breakfast cereals that contained super-fruits, minerals, vitamins and bee pollen. The cereals were also marketed in France and Japan:

http://www.toutpourlesfemmes.com/conseil/Des-mueslis-bons-pour-les-rides-et.html
http://www.cosmeticsbusiness.com/technical/article_page/Nutricosmetics__beauty_goes_deeper/59562

Leatherhead Food Research "The UK Food and Drinks Report 2009" listed the Moody Muesli pollen containing products under its Significant New Cereals Launches, 2008-2009 (page 106).

http://s3.amazonaws.com/zanran_storage/www.leatherheadfood.com/ContentPages/800677035.pdf

The consumption of spores is high, particularly in Asia where *Chlorella* in particular is widely eaten and over 4000 tonnes are consumed in South Korea alone (Tamiya *et al.*,1954, Tokuyasu, M.,1983). Whatever the plant source of the spore the composition of the SEC is fundamentally the same, it varies principally in physical shape.

The structure of the exine shell (SEC) of all spores is largely composed of sporopollenin. This is found in significant amounts in the spores of fungi such as mushrooms, which are eaten in relatively large amounts.

As shown, this encapsulating material that Sporomex proposes to use is already present in common everyday foods that are regarded as being safe. The extraction method used to prepare the SEC for use involves treating the spores with acid. This is similar to what occurs naturally in the stomach and also during many cooking processes, e.g. if vinegar is present, so the pollen is not being treated unusually.

5.b Legislation Regarding Consumption of Spores and Pollens

No specific information relating to *Lycopodium clavatum* spores is available.

A paper published in 2005 stated that, “apart from Brazil, only a few countries such as Switzerland and Argentina have legally recognised pollen as a food additive and established official quality standards and limits” (Almeida-Muradian *et al*., 2005).

The United States FDA opinion on the safety of pollen consumption is that pollen particles can be regarded as GRAS. The United States FDA states that, “though sold in many health food stores, pollen is not considered an additive.....does not have to comply with special standards”. Larkin (1984), a former commissioner of the FDA, also commented within the FDA Consumer journal that, “since the pollen has not been shown to be harmful other than to those suffering allergy, bee pollen may be marketed as a food”.

5.c Comment upon current consumption of sporopollenin and sporopollenin exine capsules (SEC)

Sporopollenin will be consumed regularly as part of a normal diet, in the form of naturally occurring spores and pollen grains. Some of these will be empty (comprising SEC) at the time of consumption; others will be emptied in the acidic environment of the stomach in a manner similar to the preparation of SEC for loading with actives undertaken by Sporomex.

6 TOXICOLOGICAL INFORMATION SPOROPOLLENIN EXINES

Based on Commission Recommendation 97/618/EC decision trees the following questions must be addressed regarding the toxicological information of the Novel Food (European Commission 1997):

- “Is there a traditional counterpart to the Novel Food that can be used as a baseline to facilitate the toxicological assessment?” If no, “is there information from a range of toxicological studies appropriate to the Novel Food to show that the Novel Food is safe under anticipated conditions of preparation and use?”

- “Is there information which suggests that the Novel Food might pose an allergic risk to humans?”

These questions have been addressed collectively in [Sections 6.a through 6.d.]

6 a Sporopollenin exine capsules (SEC) are inert and pass unchanged through the gut

As described earlier (see Section 1) SEC are inert, comprising carbon, hydrogen and oxygen. The studies below illustrate that the SEC pass unchanged through the body.

6 a ii Human Studies

6.b Allergenicity

6.b.i Use of aggressive extraction procedures to obtain sporopollenin exine capsules (SEC)

SEC are extracted using aggressive procedures of concentrated mineral acid and alkali known to denature and destroy proteins. These aggressive procedures comprehensively remove all protein from the exines (SEC), protein being the only component of intact pollen that may potentially cause allergenicity.

6.b.ii Determination of protein in sporopollenin exine capsules (SEC)

Samples of SEC from *Lycopodium clavatum* have been analysed by an independent laboratory (Chemistry Department, Memorial University of Newfoundland) who attempted to extract proteins from the sample of SEC supplied to them by Sporomex, namely by using detergent-based lysis and the sonication methods. Obtained extracts were analysed by the Lowry method, which indicated a total absence of proteins.

In accordance with the ACNFP Guidance ‘Proteins in novel foods: issues for consideration’ further analysis of the extract from the SEC samples was undertaken using MALDI-ToF (ESI-QqToF-MS) and CID-MS/MS; the analysis indicated the complete absence of any protein or peptide (Diego-Taboada, A., 2013).

Electrophoretic separation of proteins was also attempted using Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE) at different acrylamide / bisacrylamide concentrations (usually 12% resolving / 4% stacking gels) using a vertical slab gel electrophoresis apparatus. Each lane was loaded with 50 µl of the extract from the SEC. Electrophoresis was conducted at 50-100 V.

Proteins were detected in the gels by staining with coomassie brilliant blue R250 for 2 to 4 hours. Then the gels were detained in de-staining solution for 1 to 2 hours until clear background was obtained. No proteins were detected in the SEC samples by SDS-PAGE (Diego-Taboada, A., 2013).

Details of these protein analyses (MALDI-ToF and SDS-PAGE) can be found in the report from the independent laboratory appended at Annex A.

Analysis of %N in sporopollenin exine capsules (SEC) using a Fisons instrument Carlo Erba EA 100 CHNS analyser which is a combustion method in the fashion of the Dumas method has been undertaken (the details of this analysis can e found in Annex B), again a negative result was obtained.

Samples were also negative investigated by the Bradford assay or a ninhydrin colorimetric tests but it is accepted that these are not accepted in accordance with the ACNFP's Guidance 'Proteins in novel foods: issues for consideration'.

Using a weight of evidence approach it was concluded that the absence of detectable protein by all methods employed (including those recommended in the ACNFP Guidance) suggests minimum risk of allergenicity resulting from ingestion of SEC obtained from *Lycopodium clavatum* spores.

6 c Microbiological hazards

Certificates for microbial batch testing are attached in Annex C. These provide evidence that no microbiological contamination was found in SEC samples (up to 3 years old) tested. Samples were tested using a methodology designed to comply with EU GMP, as outlined by the Medicines and Healthcare products Regulatory Agency (MHRA) of the UK.

CONCLUSION

Approval is sought under Regulation (EC) No 258/97 of the European Parliament and of the Council of 27th January 1997 concerning novel foods and novel food ingredients, for the approval of sporopollenin exine capsules (SEC) extracted from *Lycopodium clavatum* spores, as a food ingredient. Sporomex intends to market SEC as a vehicle for enhanced delivery of oils (already consumed regularly in the diet) as food supplements or for manufacturers to incorporate the loaded SEC into novel foods.

The type of oils that would be delivered *via* the SEC are consumed at low levels, up to 1 g per day. The oils are loaded at a ratio of 1:1 – 1:4 into the SEC and the worst-case scenario estimate of intake included in this application, based on the highest combined exposures for the 95th centile consumer consuming two example products fortified with oils delivered *via* SEC resulted in an intake of 1.1 g per day.

The safety of SEC extracted from *Lycopodium clavatum* has been evaluated and as it has been proven that the SEC pass unchanged through the digestive tract, it is anticipated that supplements and novel foods containing low levels of SEC would be well tolerated. Particular attention has been given to the potential for SEC to induce allergenicity; lack of allergenic potential is proven by the absence of protein and %N (measured using methods recommended by ACNFP in their Guidance document) in both samples of SEC.

From a critical evaluation of the data and information included in this Application, it is concluded that the use of sporopollenin exine capsules (SEC) extracted from *Lycopodium clavatum* spores loaded with food or medical grade oils (as described herein) and manufactured in accordance with Good Manufacturing Practices, is safe and suitable for the proposed uses.

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Annex A

These data are now published in Diego-Taboada, A., Maillet, L., Banoub, J., Lorch, M., Rigby, A. S., Boa, A. N., Atkin S. L. and Mackenzie, G. Protein free microcapsules obtained from plant spores as a model for drug delivery: ibuprofen encapsulation, release and taste masking *Journal of Materials Chemistry* ***B*****, 2013, 1 (5), 707 - 713**(DOI:10.1039/C2TB00228K)

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April 5, 2012

Dear Professor Grahame Mackenzie
Department of Chemistry
The University of Hull,
Kingston upon Hull,
HU6 7RX, UK

RE: Attempts to Extract Protein from Sporopollenin Sample and Mass Spectrometric Analyses.

Dear Professor Mackenzie,

Enclosed for your perusal is the list of our attempts to extract proteins from the sample of sporopllenin that you sent us by courier post and that we received on March 24th, 2012.

We have attempted to extract the total protein content from your sporopollenin (lysis), and to remove any interfering or contaminating substances. We have used for the extraction of the protein from sporopollenin two different methods namely the detergent-based lysis and the sonication methods. These are good techniques for the disruption of the cells. Please note that the experiments were repeated at least four times and that the results obtained were indeed reproducible.

1) Detergent-Based Lysis Protein Extraction of sporopollenin
Thus, 1 gram of sporopollenin was extracted in 100 mL of ice-cold buffer containing 50 mM sodium acetate (pH 5.2), 1 mM ascorbic acid, 0.5 mM phenylmethylsulfonyl fluoride and 0.5% polyvinyl polypyrrolidone.
Subsequent to centrifugation, the supernatant was precipitated with 30% ammonium sulphate, at 4 °C, overnight. The pellet was discarded and the supernatant was desalted using a Sephadex G-50 column and the eluent (acetonitrile: water) was evaporated, the residue dissolved in water, lyophilized and was used for protein identification. [M. Ghosh et al. Acta Bot. Croat. 63 (2), 75–81, 2004].

2) Sonication Lysis Protein Extraction of sporopollenin
Sonication is another class of physical disruption which uses pulsed, high-frequency sound waves to agitate and lyse the sporopollenin (100 mgs). The following protocol was used. In short, 100 mgs of sporopollenein were pulverized in liquid nitrogen using a mortar and pestle, sonicated in 10% (w/v) TCA/acetone solution in a sonic water bath, washed successively with methanol and acetone, and, finally, phenol partitioned and ammonium acetate-methanol precipitated. [Appendix A: Bruggeman FJ, Westerhoff HV. The nature of systems biology. Trends Microbiol. 2007;15:45-50].

3) Electrophoretic Separation of Proteins:
Attempts to separate the proteins was made using Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) at different acrylamide / bisacrylamide concentrations (usually 12% resolving / 4% stacking gels) using a vertical slab gel electrophoresis apparatus. Each lane received 50 µl of the so-called protein extract. Electrophoresis was conducted at 50-100 V.

4) Staining:
The alleged proteins were detected in the gels by staining with coomassie brilliant blue R250 for 2 to 4 hours. Then the gel was detained in destaining solution for 1 to 2 hours until clear background was obtained.

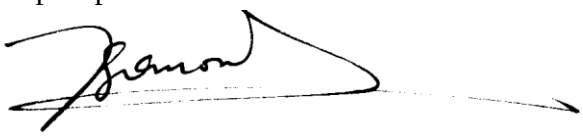
5) The obtained extracts were analyzed by the Lowry method which indicated a total absence of proteins.

6) ESI-QqTOF-MS and CID-MS/MS analysis of the so-called protein extract of sporopollenin:
The so-called protein extract was dissolved in acetonitile: water (7:3) and was injected directly in the ESI source of the ESI-QqTOF-MS was recorded in the positive ion mode using an Applied Biosystems API-QSTAR XL QqTOF-MS/MS hybrid tandem mass spectrometer (Applied Biosystems International-MDS Sciex, Foster City, CA, USA) equipped with a nano-electrospray source (Protana XYZ manipulator) which produces the electrospray through a PicoTip needle (10 mm i.d., New Objectives, Wobum, MA, USA) carrying a voltage of 2400 V. The mass spectra were acquired from m/z 100 to m/z 2000. As neither no single fragment ions nor multi-charged ions were noted in the ESI-MS, it became apparent that this sample did not contain any protein or peptide at all.

7) MALDI-TOF-MS analysis the so-called protein extract of sporopollenin:

Mass spectrometric analysis was carried out on a 4700 Proteomics analyzer with TOF-TOF optics (Applied Biosystems Foster City, CA, USA) and a 200-Hz frequency-tripled Nd: YAG laser.α-Cyano-4-hydroxycinnamic acid (α-CHCA) was used as matrix for the analysis of the so-called protein extract with an average of 5000 to 8000 laser shots per spectra.
Briefly, 1 mL of a 20 mg/mL solution of α-CHCA (dissolved in acetone, 0.1% trifluoroacetic acid (TFA); the use of acetone allowed good homogeneity of the matrix) was spotted on the MALDI plate and dried at room temperature. Then, an aliquot of 1 mL of so-called protein sample was spotted on the top of the dried matrix and left to dry before the MALDI-MS experiments. The analysis was achieved in the linear mode and the MALDI-TOF mass spectrometer was calibrated using BSA.

CONCLUSION
All MS analyses performed indicated the complete absence of any protein or peptide in Sporopollenin



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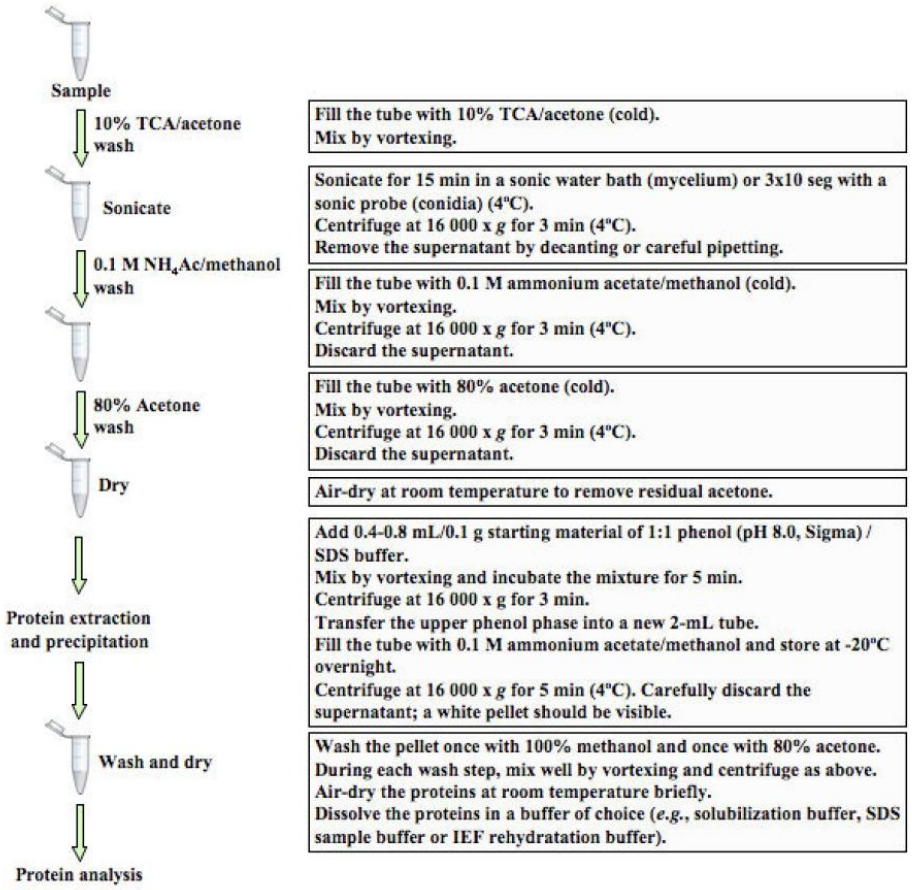
Appendix A:



TCA/acetone-phenol/methanol method for protein extraction and precipitation.

Annex B