<u>בחינה בכימיה לעורכי פטנטים</u>

קיבלתם מהממציאים תוצאות ניסויים, במסמך המהווה disclosure.

כמו כן קיבלתם:

1. פרטים של הסינתזות והניסויים שבוצעו, לרבות תוצאות השוואתיות.
2. תיאור כללי של תחום האמצאה והבעיה הטכנולוגית לה היא מציעה פתרון.

עליכם לערוך בקשה לפטנט <u>בישראל</u>, כולל תביעות, בהתאם לחוק ולתקנות הפטנטים.
אם יש צורך במידע נוסף מהממציאים, אנא הפנו אליהם שאלות מתאימות בגוף הטקסט או בתור מסמך נלווה המופנה לממציאים.
אנא הקפידו על תביעות בעלות היקף ראוי. אם יש לדעתכם צורך להרחיב מעבר לדוגמאות שסופקו, אנא ציינו זאת במקומות המתאימים בפירוט.

חלוקת הנקודות:

35% - פירוט

30% - תביעות

20% - מבנה וצורה

15% - הערכה כללית, ובכלל זאת שאלות לממציא והמלצות

בהצלחה !

Background

Phosphatidylserine (PS), a phospholipid nutrient, is active in cell membranes and is the major acidic phospholipid component in the membrane of brain cells. PS plays a crucial role in many membrane-associated nerve cell processes. The main purpose of PS is to help maintain proper membrane fluidity, which has major implications on most membrane functions.

PS has been the subject of numerous human clinical trials of memory loss, mood, cognitive performance and learning ability. Many of the studies show that PS can be helpful for those with age-related memory impairment, and can even help to optimize cognition in those with no cognitive impairment.

Dietary PS is efficiently and rapidly absorbed in the intestine, taken up into the blood, and readily crosses the blood-brain barrier to reach the nerve cells in the brain.

PS can be extracted from bovine brain or from plants, or it can be produced from lecithin (e.g. soybean or egg yolk) using biocatalysis. By using the transphosphatidylation reaction with phospholipases D (PLDs), the head group of phospholipids can be modified easily. Thus, PS can be produced from phosphatidylcholine or any other phospholipid mixture and serine through PLD catalysis.

Currently, PS is manufactured and marketed in powder and fluid forms, at different concentrations, ranging from 10% to 90% (weight percent, w/w). The fluid form of the PS commonly consists of a clear and transparent solution of PS, usually in oily media of medium-chain triglycerides (MCT) or soy triglycerides. This form is commonly used for dietary supplements in the form of soft gel capsules. PS supplements fall within the category of nutraceuticals, which are defined as any substance that is a food, or part of a food and provides medical and/or health benefits, including the prevention and treatment of disease. In the broad definition, both dietary supplements and functional foods are considered nutraceuticals.

One of the main difficulties with PS preparations, especially in liquid form, is low stability of PS, due to rapid decomposition. The exact cause of this decomposition is not fully understood but the common belief is that decomposition is caused mainly by residual biocatalytic activity and/or side reactions with water or glycerol or other alcohol moieties, however removal of residual enzymatic activity is difficult, if at all possible. Decomposition may be particularly significant when the final PS preparation is fluid, and is encapsulated in soft gel capsules. Soft gel encapsulation usually results in the migration and subsequent incorporation of low levels of water and/or glycerol into the capsule content.

Decomposition of the PS in PS preparations, especially fluid preparations, removes the serine head group, resulting in loss of activity of the PS active ingredient. Hydrolysis of the PS, utilizing water present in the fluid PS preparation itself or in the fluid preparation following encapsulation will lead to the formation of phosphatidic acid (PA). Where glycerol or other alcohol moieties are present in the fluid PS preparation itself or in the fluid preparation following encapsulation they may lead to the replacement of the serine head group with other alcohols, yielding phosphatidylglycerol (PG) or other corresponding phospholipid derivatives. Degradation may all occur due to for example decarboxylation of the serine carboxylic group, yielding products such as phosphatidyl-ethanolamine (PE) or other derivatives. Lipid peroxidation may also play a role in PS degradation. PS can be degraded by full or partial hydrolysis of the phospholipid fatty acids, yielding de-acylated PS (GPS) or lyso-PS (LPS), correspondingly. In case of PS phosphate removal, either enzymatically or chemically, diglycerides can be formed, also resulting in reduction of the PS active ingredient.

One way to overcome degradation has been proposed in WO 03/088949, wherein the phospholipid is embedded in a hard or paste-like matrix.

EP 0922707 discloses a process for the purification of phosphatidylserines in diphasic organic solvents systems. PS calcium salt is subjected to crystallization from heptane/acetone, and may subsequently be converted into any other desired salt. The lecithin starting material is dissolved in the organic phase before the

reaction with the serine is initiated. The resulting calcium salt proved to be soluble in oils, forming a clear solution.

Conventionally, soluble PS salts were more appealing, yielding clear solutions, and were easier to handle when preparing liquids for encapsulation in soft gel capsules.

It is known that commercially available PS preparations lose a major part of the PS biological activity after relatively short storage periods.

Disclosure

The following abbreviations are employed

- EDTA: ethylenediaminetetraacetic acid
- GC: gas chromatography
- GPS: de-acylated PS
- HPLC: high performance liquid chromatography
- HPTLC: high performance thin layer chromatography
- LPS: lyso-PS
- MCT: medium chain triglycerides
- NMR: nuclear magnetic resonance
- PA: phosphatidic acid
- PC: phosphatidylcholine
- PE: phosphatidylethanolamine
- PG: phosphatidylglycerol
- PI: phosphatidylinositol
- PLC: phospholipase C
- PLD: phospholipase D
- PS: phosphatidylserine
- RH: relative humidity
- RT: room temperature

Phosphatidylserine (PS) is an essential component of cell membranes, which is particularly important in the well-functioning of brain cells, with a known link to memory, mood, cognitive performance and learning ability. For all its important

functions, it is desirable to supplement PS in the human diet. Although supplements do exist in the market, they are defective with regard to the amount that is *de facto* present in preparations that reach the consumer, since there is an inherent problem of degeneration and decomposition of PS in the currently available compositions.

PS is generally known to be unstable. Even pure dry powders stored under cold conditions are prone to high rates of degradation. Furthermore, compositions of high concentrations of PS, as well as pure PS, are usually more prone to instability problems. It has been described that pure PS is prone to degradation at the rate of 0.5%w/w per day [Sigma Catalog]. The exact cause or mechanism of this degradation is not fully known. In many cases, decomposition is attributed to hydrolysis or transphosphatidylation reactions; however, products of such reactions can rarely, if ever, be isolated.

In order to overcome this instability problem, we have developed and present herein a PS composition which is more stable. Method of producing such stable PS is also presented.

We provide a stable PS composition of matter comprising desired levels of which can vary. Exemplary PS content in the composition generally exceeds 15%. Higher levels are desirable.

The PS composition may also comprise other functional ingredients, such as other phospholipids (lecithin, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and/or phosphatidylnositol (PI)), Omega-3 and Omega-6 fatty acid or their derivatives such as esters, plant sterols or sterol esters, vitamins and antioxidants. Phosphatidic acid (PA) may also be present. It is desirable that the PA content is below 10%. The PS composition of matter may further optionally comprise physiologically acceptable free-flow agents, emulsifiers, stabilizers, as well as diluents, excipients, and carriers. The PS composition of matter of the invention is suitable for use as a dietary supplement, nutraceutical food and/or as a drug additive.

The PS composition of matter exhibits a stability such that at least 95% of the original PS content, and as high as 97% and even 99% of the original content are preserved after a storage period of at least 6 to 12 months, and even after storage for 24 months.

We have prepared a PS composition of matter in the form of a salt which is insoluble in organic solvents, particularly salts of divalent ions such as calcium salt and/or magnesium salt. This salt can be dispersed in a lipid or oil base to provide a stable solid-in-oil dispersion of PS calcium salt or magnesium salt. The PS dispersions are prepared with the kind of carrier that does not enable full solubilization of the PS. Such a carrier can be an edible oil (e.g., triglyceride-based product such as vegetable oil, fish oil, etc.).

The PS divalent metal salts that are insoluble in organic solvents, specifically oils and lipids are prepared as described in the experimental section. We surprisingly found that PS divalent metal salts prepared by this method, from non-solubilized lecithin, in a substantially aqueous system (monophasic) cannot be dissolved in oils, and thus when introduced to an oil, in order to prepare a fluid for encapsulation, form a dispersion, not a solution. These dispersions surprisingly proved more stable, as such or when encapsulated in soft gel capsules. The stability of the PS dispersion of the invention is presented in the experimental section.

It may be suggested that when the lecithin starting material is dissolved in a non-polar organic solvent, the phospholipids tend to form micelles where their non-polar side (the fatty acids "tails") turn toward the surrounding medium (the non-polar organic solvent in this case). When the reaction is a mono-phasic reaction in water, which is a polar medium, the lecithin is not solubilized which may cause the phospholipids to form the opposite arrangement, and turn their polar head group (the "phosphoserine") outside, toward the medium (which is water, i.e. polar), while their fatty acid tails (non-polar) turn away from the medium. Those two forms of arrangement of the molecules in the formed PS are believed to be retained after the PS is isolated. The produced PS has a form of a "polar arrangement", where the fatty acids tails are turned to the inner side of the micelles, and the polar side of the PS is turned outwards, i.e. toward the non-

polar medium, and it is therefore not soluble in such media, e.g. oil. This PS "polar arrangement" will form a dispersion, rather than a solution in oil. The PS dispersion exhibits improved stability.

The PS which is not soluble in the carrier is found in a crystalline form, at different particle sizes. It is suggested that at this form, the PS is less accessible to decomposing factors, such as water, glycerol, residual enzyme, and any other factor which requires reaction on a molecular level with the PS molecule or one of its substituents.

The PS dispersion may also be semi-solid form (extremely high viscous form, achieved with low quantities of the carrier). The solid nature further inhibits or delays any chemical or enzymatic degradation processes that might result in the reduction of levels of the PS active ingredient, merely due to the fact that the kinetic profile of such processes in solid phase have substantially lower rate coefficients.

A common product for dietary supplements is PS in MCT oil with at least 20%w/w of PS. This allows the production of 500mg soft gel capsules with 100 mg PS, the standard and most common daily serving of PS currently available in the market. For encapsulation in soft gel capsules, which is one of the most popular forms of capsules today, it is necessary to have a fluid preparation at ambient conditions or at temperatures not exceeding 35°C, although not necessarily a liquid. These limitations arise from the soft gel encapsulation technique and machinery. Until now, it had not been possible to produce fluid PS with over 20% of PS content. It is advantageous to produce fluid PS with higher concentrations of PS, which will allow smaller capsule sizes or the addition of other ingredients to the capsule.

The PS dispersion in oil/lipid base can be used as a dietary supplement, nutraceutical food and/or drug additive, and can also be incorporated into food articles.

PS has been correlated with the improvement of mood and memory, as well as cognitive performance and learning ability. PS is considered useful in preventing memory loss, particularly age-related memory loss.

Experimental

Preparation of stable phosphatidylserine in powder form using an immobilized enzyme preparation

Commercially available immobilized PLD was used. The PLD exhibited high reactivity and high quality PS was synthesized. Most importantly, the levels of phosphatidic acid were usually low.

250 g of L-serine (Rexim, France) were placed in a 1 Liter reactor filled with 750 ml of appropriate buffer (pH 3.5-7), for example citrate buffer, containing 200mM $CaCl_2$. After complete dissolution of the serine, 53 g of fractionated soy lecithin (Solae Company, USA) were added. The lecithin is not solubilized in this substantially aqueous reaction medium. Optionally a minor quantity of other organic solvents, such as hexane, ethyl acetate, diethyl ether, etc., may be added to assist the softening and dispersion of the lecithin starting material. The mixture was stirred at temperatures of 20-60°C for 0.5-2 hours, to homogeneously disperse the lecithin in the reaction medium. Immobilized PLD (1.25 g) was added to the reaction mixture, which was stirred for 24 hours and then left unstirred until the immobilized enzyme preparation precipitated. The upper layer, containing the phospholipid fraction, was removed. The PS was obtained from this fraction and washed with appropriate aqueous solutions to remove excess serine.

From this preparation, 47 g of PS divalent salts, predominantly calcium salt, in powder form were obtained with over 30% purity. Unexpectedly, the obtained PS divalent salt was not soluble in organic solvents, such as oils.

The immobilization of the enzyme reduces the amount of residual enzyme in the product. PS obtained was practically free of enzyme traces, mainly due to the fact that the enzyme was immobilized. Optional deactivation step may be used, to reduce even more any residual enzymatic activity.

PS dispersions

The dried PS salt powder was dispersed in MCT (by SternChemi, Germany) at a temperature of from room temperature up to 80°C to form a flowable liquid product, which resulted in solid phase particles of the PS divalent salt dispersed in the edible oil. The dispersion was achieved by rigorous stirring, homogenization, pressure-homogenization, and other industrial blending methods.

The dispersions of PS divalent salt, non-soluble in organic solvents, were checked for the stability of the phosphatidylserine as a bulk active ingredient and in soft gel capsules.

Preparation of soft get capsules

Dispersed PS divalent salt was prepared as described and used in the manufacturing of soft gel capsules, which were prepared by a routine method for soft gel capsules preparation.

The capsules containing the PS divalent salt were stored under three different conditions: (1) in sealed containers in a dark place at room temperature; (2) in sealed containers at 35°C and 60% RH (accelerated conditions); and (3) in open containers at 35°C and 60% RH (accelerated conditions).

The capsules stored at room temperature (condition 1) were tested for their PS concentration at the end of the manufacturing process and after a storage period of 4 weeks. The capsules stored at accelerated conditions (2 and 3) were tested for their PS concentration at the end of the manufacturing process and after storage periods of 1, 2, 3 and 4 weeks The PS concentration was analyzed using HPLC with ELS detector and/or HPTLC, and through ^{31}P NMR. The Table below shows the PS content in the different capsules (original content presented as 100%).

PS stability in soft gel capsules containing dispersed PS preparations:

Sample and storage conditions	Pre-storage PS content	Post-storage PS concentration after 4 weeks
Dispersion capsules at RT	100%	99%
Dispersion capsules accelerated conditions in sealed container	100%	100%
Non-stabilized liquid PS capsules at RT (commercial capsules)	100	94%
Non-stabilized liquid PS capsules accelerated conditions closed container (commercial capsules)	100%	81%
Non-stabilized liquid PS capsules accelerated conditions open container (commercial capsules)	100%	92% (after 3 weeks)

In addition to PS concentration, the water and glycerol content were measured in the capsule at the end of manufacture, since these may promote PS degradation. The water content was tested by a standard Karl- Fischer method. The glycerol content was tested by titration according to a standard AOCS (American Oil Chemists Society) method. The Table below shows the water and glycerol content in capsules manufactured from dispersion and capsules manufactured from fluid PS.

Water and glycerol content in capsules manufactured from dispersion or conventional liquid PS:

Capsule content	Water content (%)	Glycerol content (%)
PS dispersion	0.5	0.12
Liquid PS	1.7	1.43

The dispersion preparations were also effective in minimizing the migration and absorption of water and glycerol into the PS content of the capsule. The low levels of these reactants contribute to the shown storage stability of the PS dispersions capsules.