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Effects of lipid emulsions on parenteral nutrition associated liver dysfunction (PNALD)

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Memòria presentada per Ana Novak per optar al títol de doctor per la Universitat de Barcelona

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- Llop Talaveron J, Leiva Badosa E, Novak A, Sanmartí-Martinez N, Jódar Masanes R, Suñé Negre JM, Ticó Grau JR, Badia Tahull MB: ***Selección de emulsiones lipídicas en nutrición parenteral: parámetros bioquimicos y hematológicos***; Nutrición Hospitalaria. 2017; 34(4):767-775.

Oral communication:

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- Sanmartí Martinez N, Novak A, Esteve Pitarch E, Leiva Badosa E, Badia Tahull M, Llop Talaveron J, Jodar Masanes, R: ***Nutrición parenteral: lípidos administrados en los hospitales de Catalunya***; 31 Congreso nacional Sociedad Española de nutrición parenteral y enteral SENPE (May 2016).

ABBREVIATIONS

ACN	acetonitrile
ALT	alanine transferase
AP	alkaline phosphatase
AOAC	Association of Official Analytical Chemists
ASPEN	American Society for Parenteral and Enteral Nutrition
AST	aspartate transferase
BIL	bilirubin
CAD	charged aerosol detection
DAD	diode array detector
ECD	electrochemical detection
ELSD	evaporative light scattering detection
GGT	gamma-glutamyl transferase
EMA	European Medicine Agency
ESPEN	European Society for Clinical Nutrition and Metabolism
EtOH	ethanol
FDA	Food and Drug Administration
FXR	farnesoid X receptor
HPLC	high performance liquid chromatography
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IUPAC	International Union of Pure and Applied Chemistry
k’	capacity factor
LCT	long-chain triglycerides
LDL	low-density lipoprotein
MCT	medium-chain triglycerides
MeOH	methanol
MS	mass spectrometry
MUFA	monounsaturated fatty acid
N	number of theoretical plates
NP-HPLC	normal phase high performance liquid chromatography

Ph. Eur.	European Pharmacopoeia
p	probability value
PN	parenteral nutrition
PNALD	parenteral nutrition associated liver dysfunction
PUFA	polyunsaturated fatty acid
PXR	pregnane X receptor
R	correlation coefficient
R^2	coefficient of determination
RI	refractive index
RP-HPLC	reverse phase high performance liquid chromatography
R_s	resolution between peaks
RSD	relative standard deviation
RRT	relative retention time
SD	standard deviation
S/N	signal-to-noise ratio
Tf	tailing factor
TNF-α	tumour necrosis factor-α
t_R	retention time
UHPLC	ultra-high performance liquid chromatography
UHPLC-MS/MS	ultra-high performance liquid chromatography-tandem mass spectrometer
USP	United States Pharmacopeia
UV	ultraviolet

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INTRODUCTION

Parenteral nutrition (PN) is for patients unable to receive nutrition via enteral administration. During the years, lipid emulsions were developed as a good source of energy and macronutrients, available in vegetable oils and fish oils, which represent one of the main constituents.

However, it was observed that long-term administration of lipid emulsions in critically ill patients had resulted in alterations of hepatic function. Although the aetiology is still unclear, different studies have attributed the elevated plasmatic concentrations of phytosterols to the higher incidence of complication known as parenteral nutrition associated liver disease (PNALD). It was indicated that phytosterols, present in lipid emulsions with vegetable oils, started to accumulate in liver, leading to hepatotoxic effects. There were conducted several studies on infant and adult patients, as well as on animals, in order to explain the mechanism of PNALD and the possibilities of its prevention. Among them, the use of α-tocopherol as antioxidant was investigated for hepatoprotective effects.

On the other hand, only few studies of lipid emulsions, available on pharmaceutical market, were performed. Developed methods for determination of phytosterols, cholesterol, squalene and tocopherols require liquid chromatography with specific detectors and chromatographic conditions or gas chromatography. Moreover, the proposed methods are able to determine only specific phytosterols and there is not any available simple method to identify and quantify the entire profile of phytosterols, cholesterol, squalene and tocopherols in lipid emulsions with different composition.

OBJECTIVES

The main objective of this study is to determine daily dose of phytosterols and to assess their clinical effect on patients receiving lipid emulsions as a form of PN.

The research work is based on the following goals:

- A novel analytical method will be developed to simultaneously determine phytosterol, cholesterol, squalene and tocopherols fractions in commercially available lipid emulsions for PN. Validation of the proposed method will be performed to ensure its suitability for routine use.

- Determined concentrations of phytosterols and tocopherols will be evaluated as possible preventive in PNALD.

- The extent of use of lipid emulsions, obtained by evaluation of PN protocols in various hospitals in Catalonia, will be studied.

- A clinical study will be conducted on hospitalised adult patients, treated with PN. In order to compare the influence of phytosterols, one group of patients will receive lipid emulsion, based on vegetable oil, whereas another group will receive one based on fish oil. Changes of biochemical parameters will be monitored and alterations of hepatic function, leading to the damaging effects of phytosterols will be confirmed, as observed in previous studies.

- PN will be evaluated considering plasmatic phytosterol levels and the possibilities of preventive for PNALD.

BIBLIOGRAPHIC PART

1 PARENTERAL NUTRITION

Parenteral nutrition (PN) is intravenous administration of nutrients and it is intended for patients who are unable to receive enteral feeding. Commercially available preparations consist of different proportions of amino acids, carbohydrates, lipids and electrolytes. Lipid emulsions are currently the most widely used preparations in PN (1–3).

Dosage of PN is individualised to each patient in order to ensure sufficient caloric intake. Moreover, vitamins and trace elements are added to cover the deficit of specific macronutrients, according to the pathology. Guidelines are prepared for various patient status with specified dosage (1–3).

1.1 Parenteral lipid emulsions

Lipid emulsions for PN are dispersions of oil in water, which comply the requirements for parenteral administration, therefore, they are sterile, stabile, with isotonic pH and compatible with the application site, in order to avoid local irritation. During the formulation, impurities and incompatibilities of excipients must be controlled so as to prevent especially chronic toxicity, as the administration is in large volumes and over a long-term (1–4).

In PN, lipid emulsions were developed to ensure sufficient daily caloric intake and as a source of essential fatty acids. However, with time beneficial effects on inflammatory pathways, immune response and recovery of patients, were observed (5,6).

During the years, various formulations of parenteral lipid emulsions were developed and improved. Vegetable and fish oils are used as a lipid component of the emulsion. Phospholipids, derived from egg yolk, emulsify the triglycerides from oils and form particles similar to chylomicrons. Higher concentrations of emulsifier are added to

preparations with lower percentage of lipids in emulsion, which may influence on lipid and lipoprotein metabolism, as well as alter the lipid composition of cell membranes. Lipid emulsions with 20% of lipids have demonstrated the optimal stability (7,8). Combinations of various vegetable and fish oils are used in parenteral lipid emulsions as a source of essential fatty acids, as well as vitamins E and K. Essential fatty acids regulate cell membrane properties, such as fluidity and permeability, cell metabolism and cell response. Arachidonic and eicosapentaenoic acid are involved in inflammatory responses and their administration is particularly important for the recovery of patients after surgical interventions (7–9).

It was observed that soya bean based lipid emulsions contain vitamin K in concentrations not only to cover daily doses, but also alter the coagulation process. Therefore, the use of soya bean based emulsions should be restricted for patients after surgeries and on anticoagulant therapy. Moreover, manufacturers should control and declare the content of vitamin K in lipid emulsion (10,11).

Vitamin E is naturally present in vegetable oils and some manufacturers use the active isomer, α-tocopherol, as antioxidant in lipid emulsions to protect against peroxidation and ensure the stability of preparations. Antioxidant effects were also observed in patients. Manufacturers declare the content of added α-tocopherol (7,8).
Parenteral lipid emulsions are usually divided into three generations, according to the development of formulation and used lipid compound (5).

1.1.1 Lipid emulsions of first generation

Lipid emulsions of first generation consist of soya bean and/or safflower oil as a lipid component.
Soya bean based lipid emulsions contain long-chain triglycerides (LCT) and are rich in ω-6 polyunsaturated fatty acids (PUFA) and linoleic acid, which are metabolised into prostaglandins and leukotrienes with pro-inflammatory response. Consequently, immunosuppression is promoted and systemic inflammatory reactions is altered (5,7,8,12). Furthermore, high concentrations of unsaturated fatty acids are more susceptible to lipid peroxidation, which results in less stable lipid emulsion (12,13).

Long-term administration of soya bean based lipid emulsions resulted in alterations of hepatic function, which was identified as PNALD. Several studies have investigated their correlation in order to explain aetiology of PNALD. The results showed a higher content of phytosterols in soya bean oil, compared to other vegetable oils, which accumulate in liver and interfere in hepatic function (5,7,8,14,15).

1.1.2 Lipid emulsions of second generation

Lipid emulsions of second generation are mainly based on medium-chain triglycerides (MCT), derived from coconut oil, and olive oil and were developed to reduce undesirable effects of soya bean oil and to obtain more stable antioxidants in parenteral preparations (13).

Shorter triglyceride chains of MCT, compared to LCT, result in more desirable physicochemical properties and clinical effects. MCT have better solubility in water and form more stable emulsions. Clinically, faster clearance from blood stream as well as no accumulation in liver, no storage in adipose tissue and no protein loss, were observed. MCT have demonstrated better control of immune response, compared to soya bean oil, such as decrease in pro-inflammatory response, chemotaxis, phagocytosis and functions of polymorphonuclear cells (5,7,8,13,14,16–18).

Olive oil is rich in ω-9 medium-chain monounsaturated acids (MUFA), particularly oleic acid, which is more stable for peroxidation, compared to PUFA and results as less immunosuppressive and may inhibit pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukins (5,7,8,13,14,16,19,20).

1.1.3 Lipid emulsions of third generation

Lipid emulsions of third generation incorporated fish oil to the mixture of vegetable oils. Fish oil is rich in ω-3 PUFA, which are involved in lipid metabolism, blood coagulation, immune response, inflammation and endothelial function. Compared to ω-6 PUFA, inflammatory response is reduced. High levels of PUFA require addition of antioxidants, especially in lipid emulsions with pure fish oil (5,7,8,14,16,21,22).

Lipid emulsion based only on fish oil in several studies reversed the altered hepatic function in PNALD. The effect was attributed to the absence of phytosterols and fish oil was considered for prevention of PNALD (23–26).

1.2 Guidelines on parenteral nutrition

European (ESPEN) and American (ASPEN) societies for parenteral and enteral nutrition have prepared guidelines with nutrition requirements for adult and paediatric patients in order to unify and improve PN in various countries.
Recommendations for PN were divided into different specialities, among them, renal failure, cardiology and pneumology, gastroenterology, geriatrics, hepatology, intensive care, non-surgical oncology, and surgery, so as to ensure specific requirements of each patient status and to enhance treatment (27,28).

Generally, guidelines recommend limited use of PN when enteral nutrition is not possible. Long-term PN results in alterations of gastrointestinal tract. There were changes observed in mucosa, reduced permeability and enteric function, leading to damaging flora and the immune response (3).

ESPEN and ASPEN established caloric and macronutrients' intake, specific for each pathology, based on patient body weight and clinical parameters (27,28). Below, a summary of administration of lipid emulsions, according to ESPEN, for each patient status is presented.

For adult patients with renal failure, a dosage of 0.8-1.2 g lipids/kg/day, up to maximally 1.5 g lipids/kg/day is recommended according to ESPEN and ASPEN (29).

In Cardiology and Pneumology, administration of lipid emulsions is preferred to glucose based nutrition, due to lower arterial concentrations of CO_2. The use of lipid emulsions based on soya bean oil may reduce mechanical ventilation, although according to ESPEN, it was not confirmed (30).

Adult patients in Gastroenterology may receive up to 1 g lipids/kg/day. Combination with glucose PN is recommended in order to avoid cholestasis (31).
Geriatric patients may receive lipid emulsions in higher percentage of lipids, as the insulin resistance is common (32).

In Hepatology, lipid emulsions with lower content of 6-ω-PUFA are recommended to avoid pro-inflammatory response, particularly in alcoholic steatohepatitis and liver cirrhosis. The dosage for acute liver failure is established at 0.8-1.2 g lipids/kg/day (33).

Long-term administration of home PN in adults requires substitution of essential fatty acids and the dosage may be up to 1 g lipids/kg/day. Higher doses resulted in chronic cholestasis and liver disease (34).

For adult patients in Intensive Care, lipid emulsions are an important source of essential fatty acids. It is recommended to administer 0.7-1.5 g lipids/kg over 12 to 24 h. The use of olive oil based lipid emulsions is particularly beneficial for critically ill patients, due to the decrease of inflammatory cytokines, whereas, fish oil based lipid emulsions have demonstrated faster recovery (35).

In patients of Non-surgical oncology, cachexia and insulin resistance are common, therefore high percentage of lipid emulsions is recommended. High lipid clearance and oxidation rate was observed in cancer patients, therefore high doses, 0.7-1.9 g lipids/kg/day, are administered. Whereas, dosage of LCT higher than 2.6 g lipids/kg/day showed adverse effects (36).

In acute and chronic pancreatitis, the use of lipid emulsions is adequate in doses 0.8-1.5 g lipids/kg/day. However, persistent hypertriglyceridemia, as a result of alteration of lipid metabolism, should be avoided. Temporary discontinuation of lipid emulsion administration should be employed to lower triglycerides (37).

ESPEN guidelines for Surgery alert about higher incidence of cholestasis and hypertriglyceridemia in long-term PN and recommend lower percentage of lipid emulsions in combination with glucose nutrition. On the other hand, in critically ill patients, ω-3 fatty

acids from PN demonstrated anti-inflammatory function, improved organ function and a faster recovery of patients (38).

Lipid emulsions are administered to paediatric patients primarily to cover high energy needs and to supply essential fatty acids. Doses are higher than in adult patients and may reach up to 3-4 g lipids/kg/day in infants and 2-3 g lipids/kg/day in older children. Occurrence of hyperlipidaemia should be avoided by dose adjustments (39).

2 PARENTERAL NUTRITION ASSOCIATED LIVER DYSFUNCTION (PNALD)

Parenteral nutrition associated liver dysfunction (PNALD) is defined as one of the complications of PN and has been observed particularly after long-term administration of lipid emulsions (40–43).

2.1 Aetiology

Although the exact aetiology of PNALD is unknown, various risk factors are being investigated, from changes in cholesterol metabolism and bile system, lack of gastrointestinal stimulation, to direct hepatotoxicity such as with phytosterols.

One of the main risk factors for development of PNALD in children is underdeveloped liver function, biliary system and short bowel due to prematurity (44,45).

The absence of enteral nutrition and gastrointestinal stimulation reduces production of bile acids and gallbladder activity, which leads to formation of biliary sludge and consequently hepatotoxicity (46,47).

Another important risk factor is selection of lipid emulsion for PN. It was observed that metabolism and elimination of lipid emulsions based on MCT is faster, compared to LCT based ones. Consequently, MCT are less likely to accumulate in liver and produce less hepatotoxic effects (46,48).

One of the most investigated risk factor is the intake of phytosterols. Lipid emulsions based on soya bean oil, resulted in higher concentrations of phytosterols, compared to other vegetable oils. Long-term constant intake of phytosterols resulted in alterations of hepatic function. It was proposed that high concentrations of phytosterols inhibit enzymes involved in cholesterol and bile acid synthesis and metabolism. As a result, phytosterol elimination is limited and they begin to accumulate in liver, which gradually leads to liver dysfunction (46,49,50).

2.2 Mechanism

Phytosterols are investigated to explain the exact mechanism of action and clinical effects. According to available studies, phytosterols interfere with cholesterol pathways and functions, due to similarities in chemical structure.

Intravenous administration enables incorporation of phytosterols into circulatory system without barriers, which results in higher plasmatic concentrations of phytosterols compared to concentrations after peroral or enteral administration. Phytosterols are poorly absorbed from gastrointestinal tract due to ABCG5/8 transporters, which prevent accumulation of sterols in enterocytes (40,51).

Phytosterols, as exogenous compounds, have no specific pathway for their efficient elimination. Moreover, their sterol structure enables interference in synthesis and metabolism of cholesterol and bile acids. It was observed that phytosterols, especially β-sitosterol and cholestenol, inhibit enzyme cholesterol-7α-hydroxylase, responsible for cholesterol metabolism to bile acids. Decreased levels of bile acids lead to decrease in elimination of phytosterols and consequently their accumulation in liver, which leads to cholestasis and liver injury. Furthermore, it was observed, -particularly in paediatric patients- that phytosterols may precipitate and form sludge or stones due to lower solubility of phytosterols in sparse quantity of bile (40,49,52,53).

Studies showed that stigmasterol is an antagonist of farnesoid X receptor (FXR) and pregnane X receptor (PRX), two nuclear receptors responsible for homeostasis of bile acids that consequently prevent hepatic injury. FXR controls synthesis and flow of bile acids in hepatobiliary system , whereas PRX limits the levels of bile acids in liver (40,49,54).

Similar chemical structure of phytosterols enables their inclusion in cells instead of cholesterol, which may influence on cell membrane fluidity, interfere with cell functions, such as signalling, endocytosis and exocytosis. It was observed that β-sitosterol and campesterol replaced up to 60% of microsomal cholesterol in hepatocytes (52).

2.3 Liver function

PNALD begins with mild elevation of hepatic transaminases, continues to steatosis and cirrhosis, up to end-stage liver failure. Cholestasis is typical for paediatric patients, whereas, in adults, alterations of hepatic function is presented as steatosis, steatohepatitis, fibrosis and cirrhosis (55,56).

Alteration of liver function is defined as increased plasmatic concentration of alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), and conjugated (direct) bilirubin (BIL) (56–58).

GGT and AP are sensitive, although unspecific indicators, that may alter under various physiological and pathological conditions, such as sepsis, malnutrition and inflammatory bowel disease. It was observed that GGT was moderately elevated after application of parenteral nutrition and decreased after adjustment of caloric intake, use of enteral nutrition or use of cyclic PN (55,59,60).

In more advanced alteration of hepatic function, after three weeks of lipid emulsion administration, plasmatic concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are elevated. Increased ALT was established as an indicator of hepatic injury. Alterations of AP, ALT and total BIL were associated to septic shock, hypoprealbuminemia, hyperglycemia or hypoglycemia and elevated creatinine (46,56–58).

2.4 Prevention

Mechanisms of PNALD are closely studied to establish an effective prevention of PNALD. Several strategies have been proposed to prevent alterations of liver function in long-term PN.

2.4.1 Dose reduction

Studies compared liver function after administration of lipid emulsions at high dose (3 g lipids/kg/day) and low dose (1 g lipids/kg/day). The results showed a higher incidence

of cholestasis with elevated GGT, AP and total BIL in groups receiving higher dose, whereas the lower dose did not demonstrate hepatic alterations (61–63).
Administration of lipid emulsions at lower dose was also attributed to lower intake of phytosterols and therefore reduced possibility for liver damage (39,49,64).

2.4.2 Change of lipid emulsion

The highest content of phytosterols was found in soya bean oil, therefore lipid emulsions of second and third generation were designed to reduce its content in the mixture of vegetable oils. Studies mainly reported improved hepatic function after administration of lipid emulsions with lower content of soya bean oil (15,64–66).

On the other hand, the use of fish oil based lipid emulsions with ω-3 fatty acids resulted as good prevention for PNALD. Studies have shown that initially elevated liver function test significantly decrease after administration of fish oil. The effect was attributed to lack of phytosterols in fish oil, which resulted in more effective elimination of latter (23,64,67,68).

2.4.3 Enteral feeding

Studies have demonstrated an importance of peroral or enteral feeding after gradually discontinuing PN. It was noticed that stimulation of gastrointestinal tract activates bile acid system and restoration of hepatobiliary system, which results in improved liver function parameters, particularly of BIL and elimination of phytosterols. Therefore, it is a good strategy for PNALD prevention (44,46,64).

2.4.4 Addition of tocopherols

Addition of α-tocopherol to lipid emulsion was initially intended for better stability of lipid emulsions. However, initial clinical parameters of cholestasis and lipidemia improved after administration of tocopherol rich lipid emulsions. The effect was attributed to activation of bile acid and fatty acid oxidation pathways. Moreover, a decreased accumulation of phytosterols in liver was observed and due to its hepatoprotective function, it is being investigated as potential preventive for PNALD (7,69).

3 STEROLS AND SQUALENE

Sterols are defined as steroid alcohols, with hydroxyl group on C3 on cholestane ring, according to IUPAC. Trivial nomenclature is used for identification of cholesterol and phytosterols (70). On the other hand, squalene is classified as acyclic terpene, a chain of unsaturated hydrocarbons (71,72).

Phytosterols are substances of plant origin, considered as cholesterol equivalents, owing to similar sterol structure and analogous functions in cell membrane regulation.

Phytosterols are commonly determined in biology and food sciences, due to their abundance in plants (73–78), fungi (79–82) and additions to food as cholesterol lowering compounds (83–89). Recently, their clinical importance has increased due to their beneficial effects in reducing cholesterol when administered perorally (90–92). Phytosterols are being investigated in different applications for various clinical effects.

3.1 β-sitosterol

β-sitosterol (Figure 1) is one of the most common phytosterols (86). It may be incorporated into the cell membranes as it resembles cholesterol and may alter signal transduction. Recent studies have demonstrated anti-inflammatory, anti-bacterial, anti-fungal and anti-tumoral effects (93–95). Moreover, β-sitosterol and campesterol were investigated for use as cancer biomarkers (96). Due to its abundance in vegetable oils and its accumulation in liver, β-sitosterol is studied as one of the possible factor for development of PNALD (52,97).

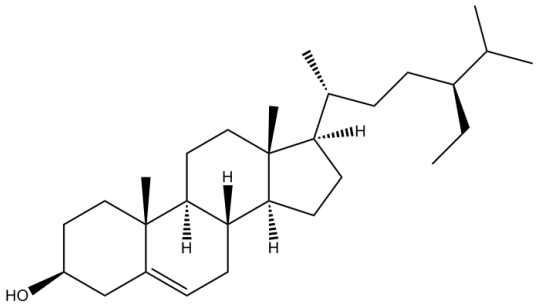

Figure 1: β-sitosterol chemical structure.

3.2 Brassicasterol

Brassicasterol (Figure 2) is present in low concentrations in vegetable oils and is mainly found in Brassicaceae (86,89). It is being investigated as a potential biomarker in Alzheimer's disease, based on its pass of blood-brain barrier (98).

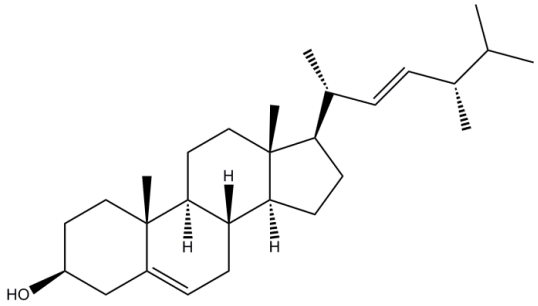

Figure 2: Brassicasterol chemical structure.

3.3 Campesterol

Campesterol (Figure 3) is widely present in plants (86). In the studies, diet rich in phytosterols, especially campesterol and β-sitosterol, resulted in reduced cell proliferation, which may be used in anti-cancer treatment (94,95,99). Together with β-sitosterol showed good potential for use as biomarker in cancer diagnostics (96). Furthermore, it is studied for possible effects in development of PNALD (97).

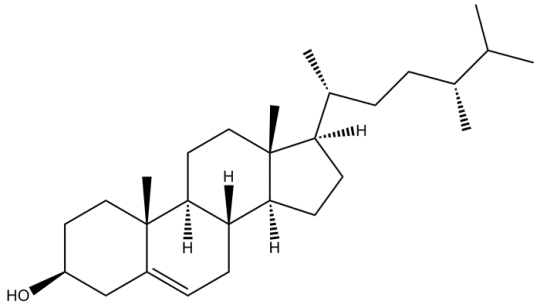

Figure 3: Campesterol chemical structure.

3.4 Cholesterol

Cholesterol (Figure 4) is the essential structural component of all cells of animal origin as well as precursor of steroid hormones and bile acids (100).

Exogenous cholesterol contributes to hypercholesterolemia and increases the risk for cardiovascular disease (101).

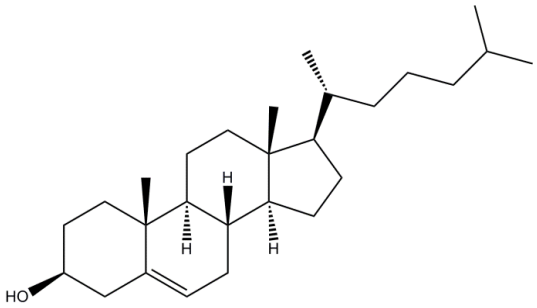

Figure 4: Cholesterol chemical structure.

3.5 Desmosterol

Desmosterol (Figure 5) is the direct precursor of cholesterol (102). High levels of desmosterol were related to non-alcoholic steatohepatitis (103). Its anti-inflammatory and anti-viral functions are studied (104,105).

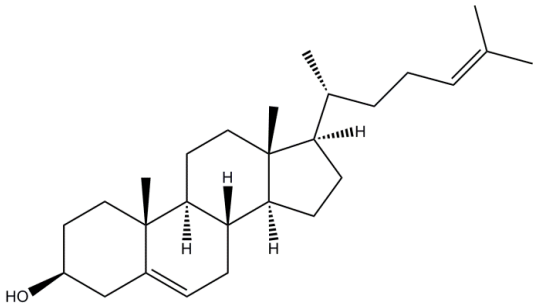

Figure 5: Desmosterol chemical structure.

3.6 Ergosterol

Ergosterol (Figure 6) is D2 provitamin, present in yeasts and fungi, which is studied for antirachitic and anti-fungal effects (87,106). Its unsaturated sterol ring enables selectivity in identification from other sterols (85,86,107).

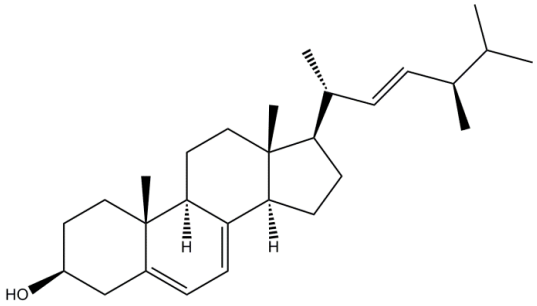

Figure 6: Ergosterol chemical structure.

3.7 Lanosterol

Lanosterol (Figure 7) is one of cholesterol precursors, present mainly in fungi (106,107). It has demonstrated potential in treatment of cataracts (108).

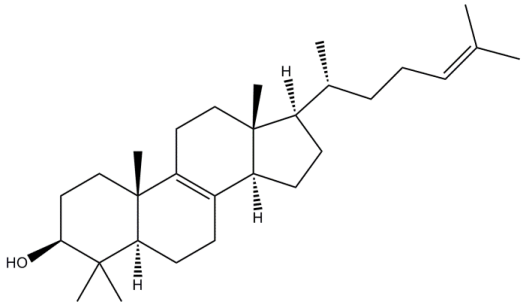

Figure 7: Lanosterol chemical structure.

3.8 Lathosterol

Lathosterol (Figure 8) is one of cholesterol precursors, present in vegetable oils at low concentrations. It is an important indicator of lipid lowering effects of phytosterols (109).

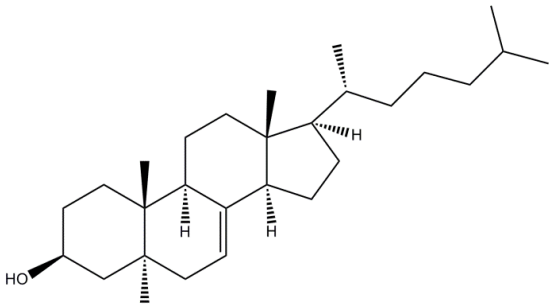

Figure 8: Lathosterol chemical structure.

3.9 Stigmasterol

Stigmasterol (Figure 9) is one of the majorly occurring phytosterols in plants (86). It is majorly found in soya bean oil and is studied as a possible factor for hepatic damage, due to alteration of bile acids (54,64).

On the other hand, its potential anti-inflammatory effects were investigated for anti-osteoarthritic therapy (110).

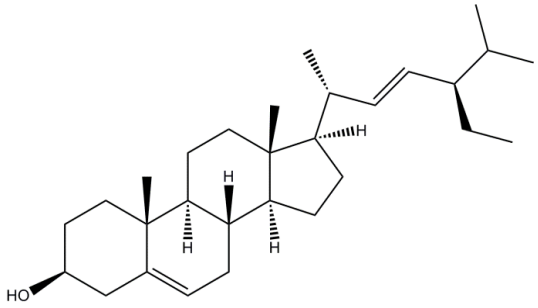

Figure 9: Stigmasterol chemical structure.

3.10 Squalene

Squalene (Figure 10) is a triterpene precursor of sterols, particularly abundant in olive oil (78,106,111). It has demonstrated good antioxidant activity and it is being studied for anti-cancerous effects (72).

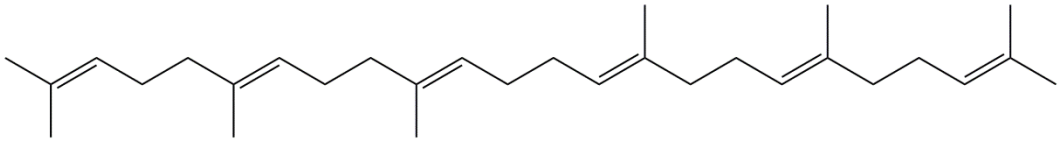

Figure 10: Squalene chemical structure.

4 TOCOPHEROLS

Vitamin E is comprised of tocopherols and tocotrienols. Tocopherols, with more potent biological activity, are methyl-substituted chroman-6-ol ring attached at C2 to a saturated isoprenoid side chain. According to the position of methyl substituents in the chromanol ring, there are α-, β-, γ-, and δ-isomer. Naturally occurring isomer of α-tocopherol is identified as RRR-α-tocopherol to distinguish from the synthetic one, all-rac-α-tocopherol (112–115).

Vitamin E is a lipid soluble antioxidant of plant origin, found especially in nuts, fruits and vegetables. Mammals are unable to synthesise it, therefore its intake is essential for proper functioning of the cells. Antioxidant properties prevent oxidation of unsaturated lipids and stabilize biological membranes (112,113).

Lately, in clinical studies, tocopherols have been investigated for their potential to prevent PNALD, among others. Addition of vitamin E, up to 9.1 mg of α-tocopherol per day, is believed to have hepatoprotective effects due to its antioxidant activity as well as activation of enzymes and transporters in liver cells (65,67,69,116–118).

Antioxidant activity is based on donating phenolic hydrogen to free radicals. The most abundant and the strongest antioxidant is α-tocopherol, other isomers are also investigated for their antioxidant potential as well as other clinical effects (119–122).

4.1 α-tocopherol

Isomer α-tocopherol (Figure 11) is the most studied and most widely used tocopherol. In pharmaceutical and food industry, it is added as lipid antioxidant in form of acetate ester to ensure better stability, compared to free alcohol form (112,121,122).

Studies have demonstrated the addition of α-tocopherol to parenteral lipid emulsions improves stability of pharmaceutical product as well as effects clinically. Addition of vitamin E was found to reduce peroxidation in lipoproteins and in endothelium (116,118,122,123), as well as to interfere in the activity of protein kinase C and

prostaglandin metabolism (113). Apart of antioxidant function, it is involved in proliferation and differentiation of smooth muscle cells, platelets and monocytes. Moreover, deficiency of α-tocopherol affected neuromuscular diseases, immune system and anaemia (119). Improved hepatic function in PNALD, attributed to α-tocopherol (69), is yet to be confirmed with additional studies (124).

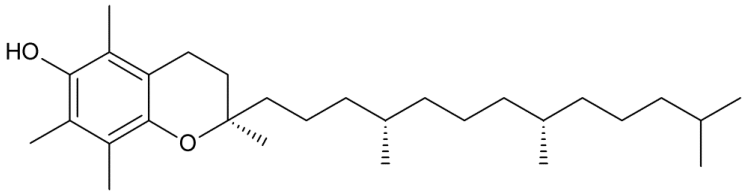

Figure 11: α-tocopherol chemical structure.

4.2 β-tocopherol

Contribution of isomer β-tocopherol (Figure 12) to antioxidant activity of vitamin E is minor compared to α-tocopherol and it is present in low concentrations. Clinically, its modulation of inflammatory pathways was studied (21,122,125).

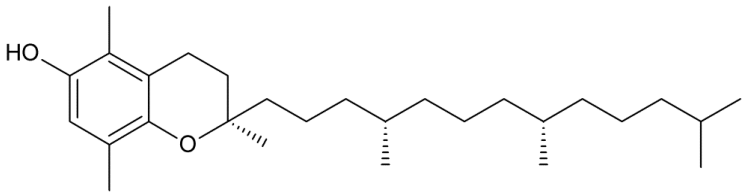

Figure 12: β-tocopherol chemical structure.

4.3 γ-tocopherol

Isomer γ-tocopherol (Figure 13) is the second most abundant naturally occurring isomer of vitamin E. According to the studies, γ-tocopherol acts as nucleophile and its antioxidant effect might be more effective compared to α-tocopherol. Moreover, antioxidant activity is maintained with increasing concentration, whereas in α-tocopherol, pro-oxidant activity was observed (121,122,126,127).

In clinical studies, γ-tocopherol resulted to be more effective in decrease platelet aggregation and LDL oxidation, compared to α-tocopherol (125,128). Moreover, its potential for anti-inflammatory and antineoplastic effects is studied (122).

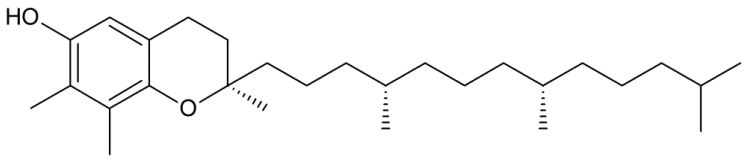

Figure 13: γ-tocopherol chemical structure.

4.4 δ-tocopherol

Isomer δ-tocopherol (Figure 14) has similar antioxidant potential as α-tocopherol.

Recent studies have suggested that δ-tocopherol is involved in pro-inflammatory response, reduction of lipid accumulation and angiogenesis. Furthermore, it is investigated as a possible prevention for various cancers, such as hormone-dependent breast cancer, colon, lung and prostate cancer (113,122).

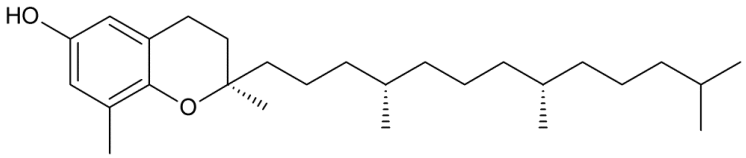

Figure 14: δ-tocopherol chemical structure.

5 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Liquid chromatography represents separation on solid stationary phase and liquid mobile phase, based on different affinity of analytes to both phases. Distribution of analytes depends on their physicochemical properties (1,2,129).

High-performance liquid chromatography (HPLC) uses packed stationary phase and chromatographic system, which enables a separation under high pressures (up to 400 bar).

HPLC chromatographic system (Figure 15) consists of a deposit of mobile phase, a pump, which distributes the mobile phase through the system, an injector to introduce the sample into the system, a column compartment with column as stationary phase, a detector to detect the signal of analyte, and a collection of data via software on computer (1,2,129).

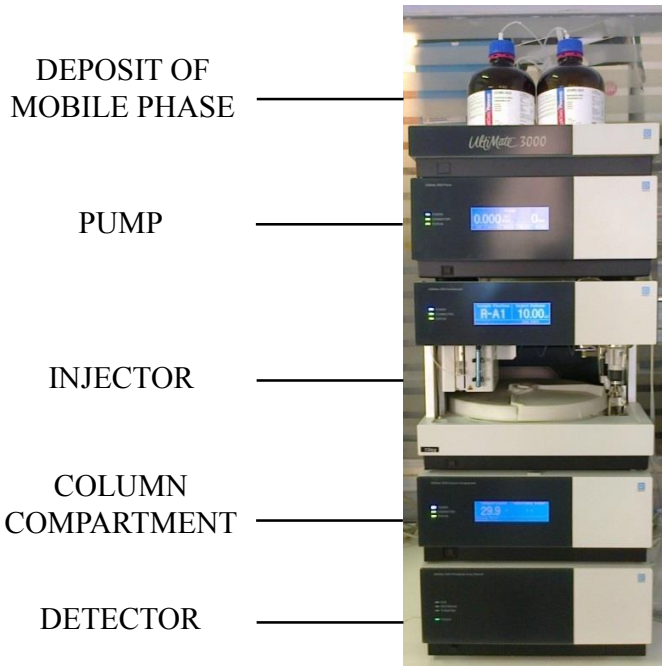

Figure 15: HPLC chromatographic system.

Ultra-performance liquid chromatograph (UPLC) is based on the same principle of separation as HPLC. However, smaller particles of stationary phase and finer capillary system in chromatographic system are used, which enable pressures up to 1000 bar. Therefore, the separation of analytes is faster, with better resolution and more sensitive (1,2,129,130).

5.1 Parameters of HPLC analytical method

During the development of analytical method for HPLC, stationary phase, mobile phase, flow rate, injection volume, column temperature and detection parameters are defined and adjusted in order to obtain optimal separation of analytes.

5.1.1 Stationary phase

On the stationary phase, separation of analytes occurs as a result of different partition, adsorption, or ion-exchange processes. It represents a column, packed with chemically treated porous material in order to obtain different chemical properties for separation. Columns differ in dimensions (length, inner diameter), pore size and chemistry of the packaging. Larger columns retain analytes longer, compared to the shorter ones, whereas larger pore size results in faster elution of the analyte (129,130).

Normal-phase chromatography uses polar stationary phases with unmodified silica, porous graphite or polar chemically modified silica, e.g. cyanopropyl or diol. It is suitable for the separation of highly lipophilic compounds, soluble in nonpolar solvents (hexane, heptane, etc.). Polar interactions retain compounds, with more hydrophilic properties, on the column, whereas more lipophilic compounds elute with mobile phase (1,2,129,131).

Reverse-phase chromatography (RP-HPLC), as the name suggests, is reverse to normal-phase and uses non-polar stationary phases with chemically modified porous silica. The majority of the analyses are RP-HPLC, as the technique is more selective compared to NP-HPLC and the low-molecular analytes are soluble in aqueous solvents. More polar compounds, dissolved in mobile phase elute faster than the less polar compounds, which interact with the column (1,2,129,131).

Modifying the column package material, by adding specific chemical groups, enables additional interaction between the analytes and the column, which results in more selective analysis. The most common substitutions are presented in Table 1.
Alkyl chains (C_8-C_{30}) provide dispersion interactions, increasing with the length of the alkyl chain, and are used in separation of lipophilic compounds. Amino group (-NH_2) forms weak ionic interactions and it is common in analyses of saccharides and vitamins.

Ciano and diol substitutions form hydrogen bonds and are typical for separation of polar compounds. Phenyl (-Ph) group enables a formation of π-π bonds between phenyl ring and aromatic compounds, which improves the separation of latter (1,2,129,131–136).

Table 1: Column functional groups and corresponding typical analytes.

Column functional groups	Typical analytes
alkyl chains (C_8-C_{30})	lipophilic compounds
amino group (-NH_2)	saccharides, vitamins
ciano (-CN)	polar compounds
diol (-OH-OH_2)	polar compounds
phenyl group (-Ph)	aromatic compounds

5.1.2 Mobile phase

Mobile phase is a solvent or a mixture of solvents, which passes through the chromatographic system and elutes the analytes from the stationary phase. Variation of concentration, polarity, pH and ionic strength of the mobile phase results in different interaction with the analyte and changes in its elution. Stronger interaction of analytes with mobile phase results in faster elution and minimum retention on the stationary phase. On the other hand, weak interactions prolong the elution, which results in longer retention of the analyte on the stationary phase. Solvents, used as mobile phase, are adapted according to the stationary phase. In normal-phase chromatography, non-polar solvents are used, such as hexane, heptane and octane. On the other hand, reverse-phase chromatography uses polar solvents, usually aqueous solutions (1,2,131,136).

Isocratic elution uses the constant composition of mobile phase, whereas, gradient elution is based on the variation of the solvents during the analysis. Combination of different solvents is used in gradient elution to separate analytes with different solubility (1,2,131,136).

5.1.3 Flow rate

Pump distributes the solvents through the chromatographic system, according to the set flow rate. Mobile phase is delivered as isocratic elution -a constant composition of eluents- or gradient elution, with varying composition of solvents throughout the analysis. Quaternary pump mixes the solvents in the established proportions, prior to the contact with analyte. Flow rate may vary during the analysis to change the retention or elution of the analytes (1,2,131).

5.1.4 Injection volume

Injector regulates the volume of the sample that is entering into the chromatographic system. Injection is performed manually, with higher deviation of volume precision, or using an auto-sampler. Higher injection volume results in higher response (1,136).

5.1.5 Column temperature

Constant temperature in the column compartment enables even distribution of the sample through the column. Higher temperatures are used to accelerate the elution, as the viscosity of the mobile phase and sample is reduced (1).

5.1.6 Detection

Detector detects the analytes after their separation on the column. There are selective detectors, which measure the physicochemical properties of the analyte, and universal detectors, which measure the properties of the eluent.

The most common is ultraviolet (UV) detector with monochromatic light of fixed wavelength in the UV or visible wavelength range or Diode array detector (DAD), which enables detection of the analyte with various wavelengths at once. To improve the specificity and sensitivity of the detection, fluorescence spectrophotometry, refractive index detection (RI), electrochemical detection (ECD), evaporative light scattering detection (ELSD), charged aerosol detection (CAD), mass spectrometry (MS), radioactivity and others, are usually used. Each technique requires a specific sample preparation in order to obtain proper detection (1,2,131).

5.2 Validation of analytical method

Validation is a process of testing a developed analytical method to prove its selectivity linearity, precision, accuracy and robustness, in order to ensure correct results during the routine use. It is one of the requirements of national and international agencies for registration of medicines.

There are available ICH guidelines and guidance prepared by official organisations (FDA, EMA, AOAC) with recommendations and specifications for correct performance of validation (1,2,137,138).

5.2.1 System suitability

System suitability verifies proper functioning of the chromatographic system before performing the analysis. According to Ph. Eur and USP, precision of analyte (RSD $\leq$ 2%) must be ensured after a replicate of injections of a standard solution. Other parameters established in the validation, such as peak symmetry and signal-to-noise (S/N) ratio, are also useful to determine suitability of the chromatographic system (1,2,137,138).

5.2.2 Stability of the solution

Stability of the solution is established as a period of time, when the integrity of the solution is ensured. Stability is studied under normal laboratory conditions and does not imply stress conditions, used in studies of related substances. The obtained data are used during the analytical procedure for proper storage time and conditions of prepared solutions (1,2,137,138).

5.2.3 Selectivity

Selectivity of the method enables identification of analytes from the remaining components (impurities, related substances and matrix), present in the analysed sample and comprises of identification, purity tests and assay of the analyte. Identification of analyte is confirmed by comparison with reference standard and absence of interaction with sample components or structurally similar compounds. Confirmation may be performed using another confirmative analytical method. In chromatography, resolution of two peaks (Rs), defined as a distance between two peaks, is used to ensure proper separation between analytes.

Peak purity proves the absence of coelution of other compounds from the sample. Assay ensures that the quantification of the analyte is correct and unaffected by presence of its impurities or compounds from the sample (1,2,137,138).

5.2.4 Linearity

Linearity is a parameter of analytical method which correlates concentration of analyte to the response obtained in the analysis. Statistical study using method of least squares is usually performed. Obtained data of y-intercept, slope of the regression line, coefficient of correlation (R) and coefficient of regression (R^2) are reported (1,2,137,138).

5.2.5 Precision

Precision of the method is the ability to obtain homogeneous results after several measurements of the same sample and covers repeatability, intermediate precision and reproducibility. Repeatability demonstrates precision of an analyst in one day, intermediate precision compares repeatability between different days and different analysts, whereas reproducibility ensures precision between laboratories. Precision is evaluated by comparing standard deviations (RSD) of measurements (1,2,137,138).

5.2.6 Accuracy

Accuracy is defined as the ability of the analytical method to provide results close to the true or reference value. Generally, it is performed as a method of standard addition, where a known concentration of standard is added to the sample at different concentration levels and the percentage of recovery is evaluated. Accuracy may be evaluated also from the linearity data comparing estimated and obtained concentrations. Acceptance criteria is set according to the sample (1,2,137,138).

5.2.7 Robustness

Robustness ensures that the analytical method remains unaffected under minor deliberate variations of method parameters.

In HPLC methods, changes in the percentage of major component of mobile phase composition, flow rate, injection volume, column temperature and detection wavelength may be applied. The results obtained under robustness conditions are compared to the ones obtained under normal conditions of the established analytical method (1,2,137,138).

EXPERIMENTAL PART

6 MATERIALS AND METHODS

6.1 Analyses of lipid emulsions for parenteral nutrition

6.1.1 Reagents

Brassicasterol (≥ 98% purity), campesterol (≥ 65% purity), desmosterol (≥ 84% purity), ergosterol (≥ 95% purity), lanosterol (≥ 93% purity), lathosterol (≥ 99% purity), β-sitosterol (≥ 85% purity), stigmasterol (≥ 95% purity), and squalene (≥ 98% purity) were purchased from Sigma Aldrich (St. Louis MO, USA). Cholesterol (≥ 97% purity) was obtained from Fagron (Barcelona, Spain). Acetonitrile and methanol, UHPLC grade, were acquired from Panreac (Darmstadt, Germany). Potassium hydroxide (KOH) from Fagron (Barcelona, Spain), 96% ethanol from Panreac (Darmstadt, Germany), pyrogallol (≥ 99% purity) from Sigma Aldrich (St. Louis MO, USA), and heptane from Panreac (Darmstadt, Germany) were used for sample preparation.

α-tocopherol (≥ 99.9% purity), γ-tocopherol (≥ 98% purity), and δ-tocopherol (≥ 92% purity), as well as ergosterol (≥ 95% purity) and β-sitosterol (≥ 70% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Acetonitrile and methanol, HPLC grade, were acquired from Fisher Scientific (Loughborough, United Kingdom) and Panreac (Darmstadt, Germany). Potassium hydroxide (KOH) from Riedel-de-Haën (Seelze, Germany), absolute ethanol from Fisher Scientific (Loughborough, United Kingdom), pyrogallol (≥ 99% purity) from Sigma Aldrich (St. Louis MO, USA), and heptane from Fisher Scientific (Leicestershire, United Kingdom) were used for sample preparation.

6.1.2 Samples of parenteral lipid emulsions

Lipid emulsions for PN, commercially available on Spanish pharmaceutical market, were used in the study. Each brand had different oil composition, declared by manufacturers, and three non-consecutive batches were analysed for each one, as presented in Table 2.

Table 2: Analysed lipid emulsions for PN.

Lipid emulsion	Composition	Batch number
ClinOleic 20% (Baxter)	80% olive oil 20% soya bean oil	14H29N30 15F15N31 15F15N31 (bottle 2) 16F22N30
Intralipid 20% (Fresenius Kabi)	100% soya bean oil	10HB3671 10IK7012 10KC3584
Lipofundina MCT 20% (Braun)	50% soya bean oil 50% MCT	143638082 144718082 154818081
Lipoplus 20% (Braun)	50% MCT 40% soya bean oil 10 % fish oil	144538082 153938083 160128082
Omegaven 10% (Fresenius Kabi)	100% fish oil	16H60131 16IE1319 16IE1319 (bottle 2) 16KF4628
SMOFlipid 20% (Fresenius Kabi)	30% soya bean oil 30% TCM 20% olive oil 15% fish oil	16IF1650 16HI0273 16IG1719 16IG1719 (bottle 2) 16K65043

6.1.3 Equipment

Method development and validation of phytosterols were performed on chromatograph Dionex UltiMate 3000, equipped with pump (LPG-3400 M), autosampler (WPS3000), thermostated column compartment (TCC-3100, 6P), DAD (PDA-3000), and software Chromeleon datasystem (version 6.80 SR15, Dionex).

Robustness was performed on Agilent 1100 with pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A), DAD (G1315A), and software ChemStation (version A.08.03, Agilent Technologies).

Validation of tocopherols was performed on VWR Hitachi ELITE LaChrom chromatograph, equipped with pump (L-2130), autosampler (L-2200), DAD (L-2450) and software EZChrom Elite (version 3.1.6, Agilent).

Robustness was performed on Dionex UltiMate 3000, equipped with pump (LPG-3400 M), autosampler (WPS3000), thermostated column compartment (TCC-3100, 6P), DAD (PDA-3000) and software Chromeleon datasystem (version 6.80 SR15, Dionex).

For sample preparation of phytosterols and lipid emulsions, there were used analytical balance Sartorius (BP211D), sonicator Ultrasons J.P. Selecta, vortex agitator Janke & Kunkel Ika-Labortechnik, heater SBS (A-64) and rotary evaporator Büchi (R).
Samples for validation of tocopherols were prepared using analytical balance Mettler Toledo (AG 245), heater Heidolph (MR 3002), vortex agitator Techmatic (TM1), and rotary evaporator Büchi (R-124).

6.1.4 Validation of phytosterols

Validation of phytosterols was based on ICH (137), USP (2) and AOAC International (138) guidelines and consisted of verification of system suitability, standard solution stability, selectivity, linearity, precision, accuracy, and robustness of developed analytical method.

6.1.4.1 Preparation of standard solutions

a) Preparation of stock solutions

For each sterol and squalene, a stock solution was prepared in order to facilitate the preparation of phytosterol standard solution. Prepared stock solutions were stored at 4-6°C.

Squalene stock solution 2500 µg/mL: 25 mg of squalene was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Cholesterol stock solution (2500 µg/mL): 25 mg of cholesterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

β-sitosterol stock solution (2500 µg/mL): 25 mg of β-sitosterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Ergosterol stock solution (2500 µg/mL): 25 mg of ergosterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Stigmasterol stock solution (2500 µg/mL): 25 mg of stigmasterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Campesterol stock solution (500 µg/mL): 5 mg of campesterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Lanosterol stock solution (500 µg/mL): 5 mg of lanosterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Brassicasterol stock solution (500 µg/mL): 5 mg of brassicasterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Desmosterol stock solution (500 µg/mL): 5 mg of desmosterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

Lathosterol stock solution (500 µg/mL): 5 mg of lathosterol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

b) Preparation of phytosterol standard solution

Phytosterol standard solution was prepared as mixture of phytosterol stock solutions in volumes as presented in Table 3. Obtained solution was mixed well, filtered through 0.45 µm PVDF filter and a vial for HPLC was prepared.

Table 3: Composition of phytosterol standard solution.

Phytosterol stock solutions	Volume	Final concentration
squalene (2500 µg/mL)	2 mL	500 µg/mL
cholesterol (2500 µg/mL)	1 mL	250 µg/mL
β-sitosterol (2500 µg/mL)	1 mL	250 µg/mL
ergosterol (2500 µg/mL)	0.5 mL	125 µg/mL
stigmasterol (2500 µg/mL)	0.5 mL	125 µg/mL
campesterol (500 µg/mL)	1 mL	50 µg/mL
lanosterol (500 µg/mL)	1 mL	50 µg/mL
brassicasterol (500 µg/mL)	1 mL	50 µg/mL
desmosterol (500 µg/mL)	1 mL	50 µg/mL
lathosterol (500 µg/mL)	1 mL	50 µg/mL

c) Preparation of system suitability solution

System suitability solution was prepared by weighing 10 mg of β-sitosterol (≥ 70% purity), dissolving it in 100.0 mL of MeOH, HPLC grade and sonicating for about 5 min to improve the dissolution. Solution was filtered through 0.45 μm PVDF filter and vial for HPLC was prepared.

6.1.4.2 System suitability

System suitability was evaluated with six consecutive injections of system suitability solution under chromatographic conditions described in analytical method. Software Chromeleon was used to define capacity factor (k'), tailing factor (Tf), number of theoretical plates (N) and resolution between peaks (R_s). Retention time (t_R) and RSD of peak areas were determined.

6.1.4.3 Standard solution stability

The stability of prepared phytosterol standard solution of phytosterols was analysed from 0 to 12 days. The solution was maintained at room temperature at 25 ± 2ºC in order to investigate the stability during analysis.

6.1.4.4 Selectivity

Standard solutions were characterised to obtain UV absorption maximums and relative retention times (RRT) for each phytosterol, cholesterol, and squalene. RRT was calculated according to USP (2), as RRT = t_{r1}/t_{r0}, where t_{r0} is retention time of ergosterol, set as internal standard, and t_{r1} retention time of other sterols and squalene.

6.1.4.5 Linearity

To establish the linearity of analytes, a phytosterol standard solution was prepared in triplicate, according to the following dilutions: 1/1, 1/2, 1/5, 1/10, 1/20, 1/50, 1/100, 1/200, and 1/500. From obtained calibration curves, mean linearity, and regression statistics were calculated. Response factor was calculated as RF = response/concentration.

6.1.4.6 Precision

Repeatability of the instrumental system was performed at different concentrations of phytosterol standard solution, corresponding to the dilutions 1/1, 1/5, and 1/20, prepared for linearity. Ten consecutive injections were performed at each concentration and the statistics of obtained response factors were determined. The procedure was repeated on different days to investigate inter-day precision.

6.1.4.7 Accuracy

Accuracy was determined from the data, obtained from the linearity, corresponding to the dilutions 1/1, 1/20, and 1/500. Percentage of recovery was calculated and statistically evaluated.

6.1.4.8 Robustness

The phytosterol standard solution was analysed under minor variations in chromatographic conditions, such as detection wavelength, column temperature, injection volume, and the use of different HPLC equipment. The investigated variations can be critical in simultaneous separation of several analytes. The use of two different chromatographs ensures the method transferability between HPLC equipment. Recovery was calculated and statistically significant differences were investigated by ANOVA and t-student test.

6.1.4.9 Acceptance criteria

Acceptance criteria for validation of phytosterols, presented in Table 4, was established according to the guidelines ICH Q2(R1) (137), USP 39-N34 (2) and AOAC (138).

Table 4: Acceptance criteria for validation of phytosterols.

System suitability:

- capacity factor: ≥ 1.5
- symmetry factor: 0.8-1.5
- number of theoretical plates: > 2000
- resolution: ≥ 1
- retention time: ~43 min

Standard solution stability:

- to be determined in the study

Selectivity:

- resolution of analysed peaks
- no interference of solvents

Linearity:

- response factor RSD < 2%
- R > 0.999
- R^2 > 0.990

Precision:

- instrumental day precision: RSD < 1%
- instrumental inter-day precision: RSD < 2%
- method precision: RSD < 11%

Accuracy:

- recovery = 80-110%

Robustness:

- ANOVA: $F_{calc} < F_{tab}$
- t-student test: $t_{calc} < t_{tab}$

6.1.5 Validation of tocopherols

Validation of tocopherols was based on previously established validation for phytosterols.

6.1.5.1 Preparation of standard solutions

a) Preparation of stock solutions

For each tocopherol, a stock solution was prepared in order to facilitate the preparation of tocopherol standard solutions. Prepared stock solutions were stored at 4-6°C.

α-tocopherol (10 mg/mL): 100 mg of α-tocopherol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

γ-tocopherol (2.5 mg/mL): 25 mg of γ-tocopherol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

δ-tocopherol stock solution (10 mg/mL): 100 mg of δ-tocopherol was dissolved in 10.0 mL of MeOH, HPLC grade and sonicated for about 5 min to improve the dissolution.

b) Preparation of tocopherol standard solutions

Two tocopherol standard solutions, in concentration 500 μg/mL and 300 μg/mL, were prepared as mixture of tocopherols. Volumes of tocopherol stock solutions, presented in Table 5, were diluted to 100.0 mL with MeOH, HPLC grade. Obtained solutions were mixed well, filtered through 0.45 μm PVDF filter and vials for HPLC were prepared.

Table 5: Composition of tocopherol standard solutions.

Tocopherol stock solutions	Volume	Final concentration
Standard solution 500 µg/mL		
α-tocopherol (10 mg/mL)	5.0 mL	500 µg/mL
γ-tocopherol (2.5 mg/mL)	4.0 mL	100 µg/mL
δ-tocopherol (10 mg/mL)	5.0 mL	500 µg/mL
Standard solution 300 µg/mL		
α-tocopherol (10 mg/mL)	3.0 mL	300 µg/mL
γ-tocopherol (2.5 mg/mL)	1.0 mL	25 µg/mL
δ-tocopherol (10 mg/mL)	3.0 mL	300 µg/mL

c) Preparation of system suitability solution

System suitability was prepared as previously established in validation of phytosterols.

6.1.5.2 System suitability

System suitability was the same as previously established in validation of phytosterols.

6.1.5.3 Standard solution stability

The stability of prepared tocopherol standard solution was analysed from 0 to 7 days. The solution was maintained at room temperature at 25 ± 2ºC in order to investigate the stability during analysis.

6.1.5.4 Selectivity

Standard solutions were characterised to obtain UV absorption maximums and RRTs, relative to ergosterol, for each tocopherol.

6.1.5.5 Linearity

To establish the linearity of analytes, tocopherol standard solution of 500 μg/mL (with dilutions 1/1, 1/2, 1/5, and 1/25) and 300 μg/mL mL (with dilutions 1/1, 1/2, 1/5, 1/10 and 1/20), were prepared in triplicate. From obtained calibration curves, mean linearity, and regression statistics were calculated. Response factor was calculated as RF = response/concentration.

6.1.5.6 Precision

Repeatability of the instrumental system was performed at different concentrations of tocopherol standard solution, corresponding to the concentrations: 500 μg/mL, 100 μg/mL and 15 μg/mL, prepared for linearity. Ten consecutive injections were performed at each concentration and the statistics of obtained response factors were determined. The procedure was repeated on different days to investigate inter-day precision.

6.1.5.7 Accuracy

Accuracy was determined from the data, obtained from the linearity, corresponding to the concentration 500 μg/mL, 100 μg/mL, and 15 μg/mL. Percentage of recovery was calculated and statistically evaluated.

6.1.5.8 Robustness

The tocopherol standard solution was analysed under minor variations in chromatographic conditions, such as detection wavelength, column temperature, injection volume, flow rate, the use of different column batches, and different HPLC equipment.

Recovery was calculated and statistically significant differences were investigated by ANOVA and t-student test.

6.1.5.9 Acceptance criteria

Acceptance criteria for validation of tocopherols were the same as previously established for phytosterols.

6.2 Clinical study

6.2.1 Survey on use of lipid emulsions in Catalan hospitals

To investigate the extent of use and the preferences in selection of type of lipid emulsion, a survey on use of parenteral lipid emulsions in different Catalan hospitals, was employed. Parameters, presented in Table 6, included size of hospital, according to the number of beds, brand and type of parenteral lipid emulsion and administration protocol. Gathered data were statistically evaluated by ANOVA.

Table 6: Investigated parameters in survey on use of lipid emulsions in hospitals.

Parameter	Definition
Hospitals	group I (≥ 500 beds) group II (200-499 beds) group III (< 200 beds)
Lipid emulsions	Brand
	Presentation: one-chamber bag multi-chamber bag
	Source of lipids: first generation (soya bean oil) second generation (MCT and olive oil) third generation (fish oil)
Administration protocol	g of lipids/kg of patient/day

6.2.2 Clinical trial

Clinical trial, referenced with EudraCT Number: 2014-003597-17, was project of Institute of Health Carlos III (PI14/00706, AES 2014) and was supported by the Investigation Agency of Spanish Society of Hospital Pharmacy (AISEFH 2014). It was conducted at Bellvitge University Hospital, under supervision of Prof. Josep Manuel Llop Talaveron.
Clinical trial of phase IV was designed as unicentric, double-blinded and randomised in two groups.

6.2.2.1 Selection of patients

Selection of patients suitable for clinical trial was carried out, among hospitalised adults in unit of General and Digestive Surgery at Bellvitge University Hospital. Established inclusion and exclusion criteria are presented in Table 7 and Table 8.

Table 7: Inclusion criteria.

Inclusion criteria:
- significant increase of GGT after 7-day administration of PN
- adult patients (18 years and older) of both sexes and any ethnicity
- willing and able to give informed consent for participation in the trial

Table 8: Exclusion criteria.

Exclusion criteria:
- history of hypersensitivity of type I or idiosyncratic reactions to the composition of parenteral lipid emulsions
- pregnancy and lactation
- plasmatic triglycerides > 3 mmol/L
- chronic treatment with corticosteroids or recent immunosuppressive treatment (one month prior to the clinical trial)
- patients with AIDS
- patients after transplantation
- contraindications of parenteral lipid emulsions, according to the manufacturer

The most important and limiting inclusion criterion was the change in liver function test GGT after seven day administration of PN. Patients with initially normal liver function tests were administered lipid emulsion ClinOleic 20%, based on soya bean and olive oil, at dose 0.8 g/kg/day for seven days and parameters of hepatic function, such as GGT, ALT, AP, and total BIL, were monitored. Criteria of alteration of liver function, previously established at Bellvitge University Hospital (56), are presented in Table 9.
Patients, who showed significant increase of GGT, double or more than initial normal levels, entered the study.

Table 9: Alteration of liver function criteria.

Parameter	Concentration
GGT	> 1 μkat /L (60 UI/L)
AP	> 1.5 μkat/L (269 UI/L)
ALT	> 0.83 μkat/L (49 UI/L)
total BIL	> 25 mol/L (1.4 mg/dL)

Selection lasted from March 2015 until March 2017 in order to include 20 patients, a minimum for statistical significance of obtained results, which had been previously approved by Spanish Agency of Medicine and Medical Devices (AEMPS).

6.2.2.2 Administered treatment and sampling

Clinical trial was based on two study arms, presented in Table 10, one using lipid emulsion with phytosterols and another without phytosterols. The administered dose of lipid emulsion was reduced from 0.8 to 0.4 g/kg/day, for both groups, in order to prevent considerable alterations of hepatic parameters.

Table 10: Clinical trial study arms.

Study arms:

- **Group A**: provision of lipid emulsion with phytosterols: **ClinOleic 20%**, based on olive and soya bean oil, in the dose of 0.4 g/kg/day
- **Group B**: provision of lipid emulsion without phytosterols: **Omegaven 10%**, based on fish oil, in the dose of 0.4 g/kg/day

Patients were randomly (1:1) included into defined groups. Lipid emulsions were blinded to patients and personnel included in sampling.

Blood sampling was programmed on Day 0 and Day 7 in order to determine levels of phytosterols and hepatic function, presented in Table 11. During the clinical study, nutritional parameters, inflammatory parameters and renal function were monitored to prevent complications.

Table 11: Investigated plasmatic parameters.

Investigated clinical parameters:

- concentration of phytosterols in plasma
- levels of enzymes of hepatic function:
  - GGT
  - AP
  - ALT
  - total BIL

Blood samples were collected into heparinised tubes at the beginning of clinical trial and after 7 days of lipid emulsion administration. After centrifugation at 2000 g and 4°C during 10 min, supernatant was collected and stored at -80°C until the analysis.

6.2.2.3 Analyses of phytosterols in plasma

Collected plasma samples were analysed in biochemical laboratory of Bellvitge University Hospital, according to procedure developed by Dr. Raül Rigo Bonnin and his research group.

Procedure for determination of phytosterols in plasma is described in Annex 1. Briefly, sample preparation included saponification, liquid-liquid extraction with hexane, drying of collected supernatant with nitrogen and reconstitution of dry residue with MeOH. Samples were analysed by UHPLC-MS/MS under established chromatographic and mass spectrometry conditions.

Descriptive statistics were carried out using frequency tables of all the variables. For continuous variables, descriptive statistics (n, mean and standard deviation) were used. We analysed the values of the variables studied on Day 0 and Day 7 post-randomisation and difference between groups were analysed by a t-student test.

In order to provide an adequate and unbiased estimate of the true effect of our intervention, efficacy analyses were performed on the population per protocol. Data analyses were performed using IBM-SPSS (version 22).

7 RESULTS AND DISCUSSION

7.1 Analyses of lipid emulsions for parenteral nutrition

7.1.1 Development of analytical method

Development of analytical method was based on simultaneous separation of all analytes, using reverse phase HPLC to enable simple use in routine.

Separation of phytosterols, cholesterol and squalene was based on available bibliographic data and preliminary tests. Described analytical methods were specific for separation of one or only few phytosterols and there was no method for HPLC able to separate all phytosterols, cholesterol and squalene in one analysis. Simultaneous separation of phytosterols and cholesterol, slightly differing in substitutions of sterol ring and with similar physicochemical properties, was challenging. Meanwhile, squalene, with its triterpene structure, demonstrated a good separation from sterols under various chromatographic conditions.

Determination of sterols was described using UV detection at wavelengths 202-215 nm (74,78,139–141) or employing special detectors, such as evaporative light scattering detector (ELSD) (76,142), charged aerosol detector (CAD) (143) or coupled with mass spectroscopy (MS/MS) (144), to increase the sensitivity and specificity of detection.

Preliminary tests of solubility and UV absorbance maxima were performed to characterise analytes. Solubility of phytosterols was investigated in various mixtures of reverse phase solvents to establish composition of mobile phase and conditions for sample preparation. Solubility increased with more non-polar solvents, as expected due to sterol chemical structure. Predominantly used reverse phase solvent ACN was unable to dissolve phytosterols completely, whereas MeOH demonstrated good dissolution and was chosen as solvent for standard solutions and samples. Precipitation of phytosterols was observed with increasing addition of H_2O to the mixture of ACN and MeOH, which was considered in mobile phase for elution.

UV detection was chosen to ensure applicability on commonly equipped HPLC systems. Phytosterols, cholesterol and squalene had UV maxima between 190 nm and 200 nm, with exception of ergosterol at 280 nm, and the solvent cut-off for MeOH was at 205 nm. Detection wavelength was set at 210 nm to obtain high absorbance suitable for quantification of all analytes and avoid interference of solvent absorbance.

In existing analytical methods, chromatographic columns with hydrophobic stationary phase and C_{18} (74,140–142,145,146), C_8 (78,139,143) and phenyl (76) chemical bonding, were commonly used and resulted in relatively good separation of investigated specific sterols. All three were chosen for further testing for simultaneous determination of sterols and squalene.

In search of optimal method, a mixture of sterols was tested on columns C_{18}, C_8 and phenyl under isocratic conditions, using various proportions of ACN, H_2O and MeOH as mobile phase. Other chromatographic conditions were set to achieve efficient analysis and remained fixed to facilitate the comparison of obtained chromatograms. Flow rate was 1.5 mL/min for faster elution of squalene and to prevent precipitation of sterols. Column temperature was maintained at 30ºC, which ensured proper fluidity of mobile phase and prevented sterol oxidation at higher temperatures. Injection volume was set to 30 µL to obtain quantifiable peaks, whereas detection was at 210 nm, as established in preliminary testing.

Various mixtures of mobile phase were tested for each column and the composition with the best separation of sterols is presented in Table 12.

Table 12: Chromatographic conditions suitable for optimisation.

	C_{18}	**Phenyl**	**C_8**
Column	Symmetry C18, 150 x 3.9 mm, 5 µm, Waters	Zorbax SB-Phenyl, 150 x 4.6 mm, 5 µm, Agilent	Zorbax XDB-C8, 150 x 4.6 mm, 5 µm, Agilent
Mobile phase composition (v/v/v)	ACN-MeOH = 98:2	ACN-MeOH-H_2O = 48:22.5:29.5	ACN-MeOH-H_2O = 80:0.5:19.5

Corresponding chromatograms (Figure 16) are focused on separation of sterols, in order to evaluate the potential for further optimisation.

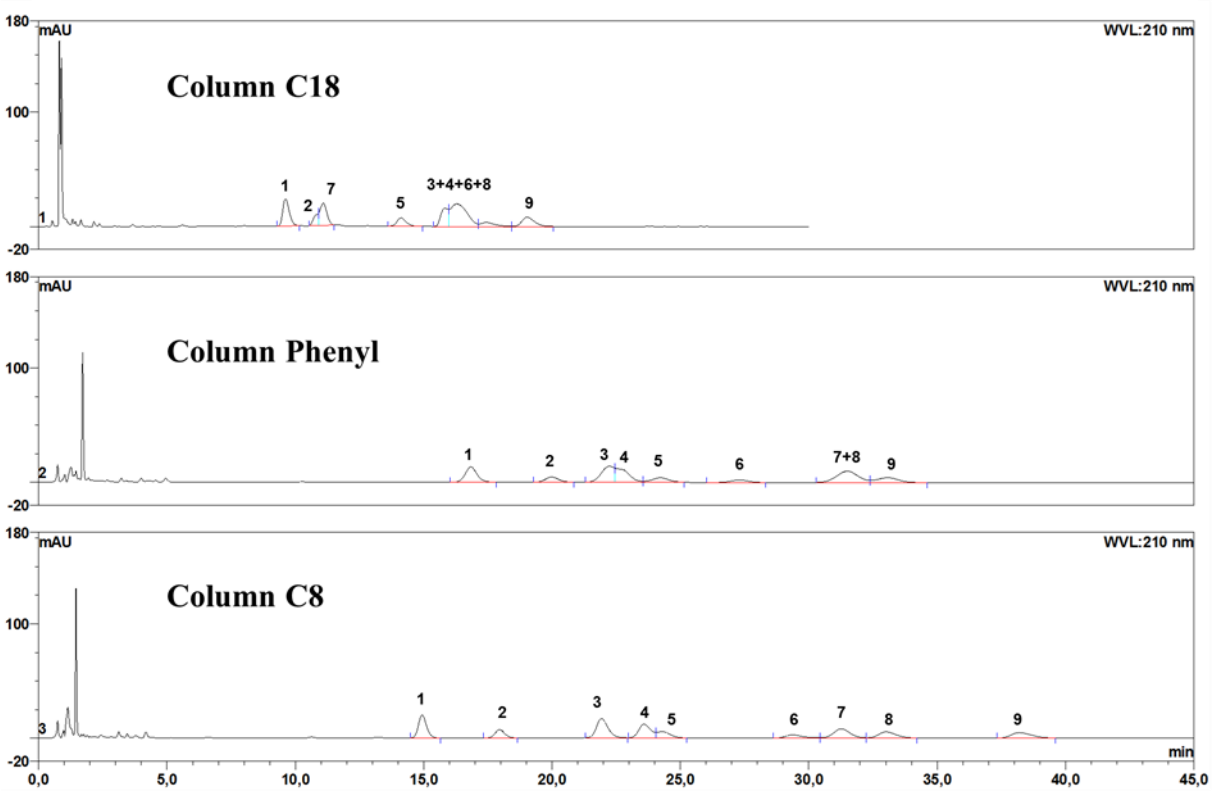

Figure 16: Comparison of chromatograms of sterol mixture obtained by columns C_{18}, phenyl and C_8. Identified peaks are: 1 – desmosterol, 2 – ergosterol, 3 – lathosterol, 4 – cholesterol, 5 – brassicasterol, 6 – campesterol, 7 – lanosterol, 8 – stigmasterol and 9 – β-sitosterol.

The shortest time of elution of sterols, less than 25 min, was observed with the most hydrophobic column C_{18} and mobile phase ACN-MeOH = 98:2 (v/v). There was observed faster elution of lanosterol and brassicasterol (Figure 16, peaks 2 and 7), compared to phenyl and C_8 columns, attributed to higher percentage of ACN in mobile phase. In spite of adjustments of chromatographic conditions, coelution and peak overplapping impeded adequate separation of analysed sterols. Stigmasterol and campesterol (Figure 16, peak 7), phytosterols interesting for their clinical effects, were unable to separate, despite the difference in alkyl side chain. Difficulties with selectivity of column C_{18} were previously described in the literature under various chromatographic conditions (74,85,142).

Coelution was also observed between cholesterol and lathosterol (Figure 16, peak 6), differing in position of double bond in sterol ring. Peak overlapping between ergosterol and lanosterol (Figure 16, peaks 2 and 3) was present although the sterol rings have different positions of double bond and alkyl side chain. Selectivity of column C_{18} was limited and unable to determine analysed sterols, therefore, it was discarded for further optimisation.

Separation of sterols improved using phenyl end-capped column, which provided additional interactions with analytes (76). Zorbax SB-Phenyl column and mobile phase ACN-H_2O-MeOH = 48:29.5:22.5 (v/v/v) prolonged the retention of sterols to more than 60 min, with elution of squalene after 100 min, and demonstrated better selectivity than C_{18} column. Addition of H_2O increased the polarity of elution mixture and sterols were retained longer on the stationary phase, which can be observed in brassicasterol and lanosterol (Figure 16, peaks 2 and 7). Coelution of stigmasterol and lanosterol was close to the peak of β-sitosterol (Figure 16, peaks 7 and 8). Peaks of cholesterol and lathosterol (Figure 16, peaks 3 and 4) were separated better than on C_{18} column, although impeded their quantification. Adjustments of chromatographic conditions did not improve the separation of analysed compounds.

Column C_8, as less hydrophobic column than C_{18} and without added functional groups, enabled the simultaneous identification of all sterols (147). Zorbax Eclipse XDB C_8 and mobile phase ACN-H_2O-MeOH = 80:19.5:0.5 (v/v/v) retained sterols for about 40 min, whereas squalene eluted after 120 min. Analytes were well separated, with no coelution, although peak overlapping between cholesterol and brassicasterol (Figure 16, peaks 4 and 5) was observed. The order of eluted phytosterols was the same as on phenyl column. Higher percentage of ACN in mobile phase accelerated elution of desmosterol and ergosterol, whereas interaction with stationary phase retained longer other sterols and squalene, which enabled good separation. The method was selected for further optimisation with gradient elution for faster elution of squalene and to shorten the time of analysis.

Gradient elution was based on previously studied isocratic conditions on column C_8 and shorter retention of squalene in 100% ACN. To establish conditions of gradient elution,

100% ACN (component A) and H_2O-MeOH (component B) = 95:5 (v/v) were mixed under different initial percentages and increments of organic solvent. Generally, higher percentage of ACN resulted in faster elution of all analytes and shorter time of analysis. However, there was observed coelution of campesterol and stigmasterol.

On the other hand, for efficient elution of squalene, with the narrowest peak and the shortest retention time, use of 100% ACN, was required. After adjustments, initial percentage of ACN was set to 75% with increase up to 90% to separate all sterols in 45 min, followed by 15 min in 100% of ACN to elute squalene.

Optimal chromatographic conditions for determination of phytosterols, cholesterol and squalene are summarised in Table 13.

Table 13: Optimal chromatographic conditions.

Column:	Zorbax Eclipse XDB C_8 (150 x 4.6 mm, 5 μm; Agilent Technologies)
Mobile phase:	A: MeOH-H_2O = 5:95 (v/v) B: 100% ACN

Gradient:

Time	Component A	Component B
0 min	25%	75%
45 min	10%	90%
50 min	0%	100%
65 min	0%	100%

Flow rate:	1 mL/min
Injection volume:	30 μL
Temperature:	30°C
UV detection:	210 nm

Figure 17 shows the chromatogram obtained under optimised chromatographic conditions. Complete elution of all analytes was achieved in 65 min, with retention times of sterols

between 23 min and 45 min and 56 min for squalene. Chromatogram included a 10 min of conditioning and re-equilibration to initial composition of mobile phase.

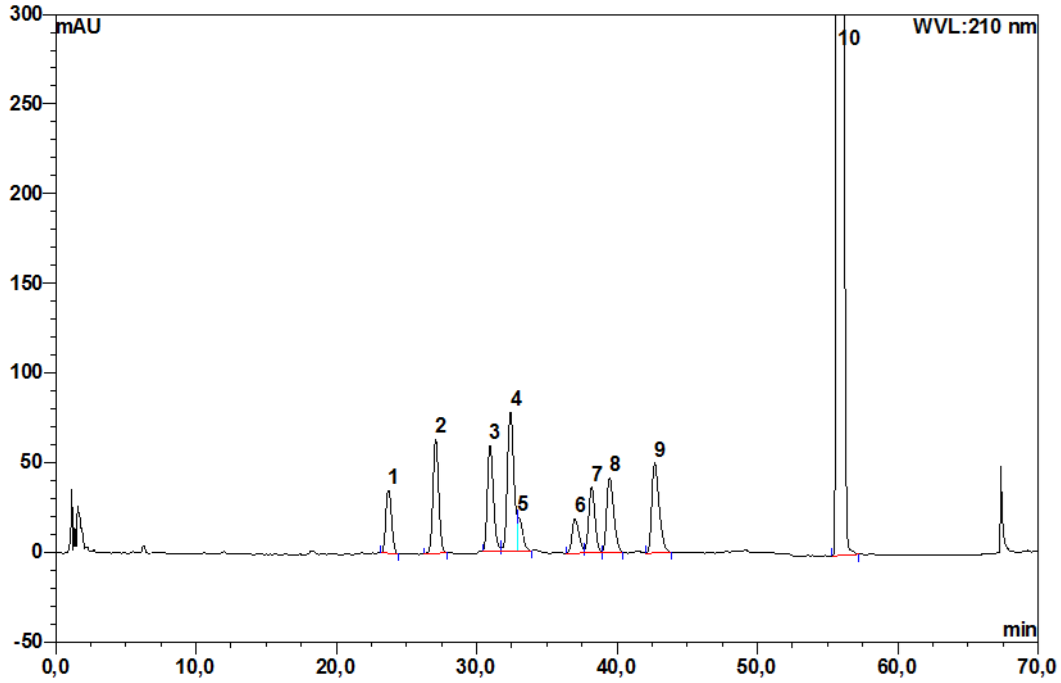

Figure 17: Chromatogram of optimised method with all separated phytosterols. Identified peaks are: 1 – desmosterol, 2 – ergosterol, 3 – lathosterol, 4 – cholesterol, 5 – brassicasterol, 6 – campesterol, 7 – lanosterol, 8 – stigmasterol, 9 – β-sitosterol and 10 – squalene.

From the obtained chromatogram, there were observed similarities in order of elution of sterols, previously described in separation on GC. Differences in substitutions on the side chain and the presence of double bonds were correlated with retention time on the stationary phase (84,148). Sterols with double bonds in side chain eluted faster compared to the saturated ones, which is seen in brassicasterol and campesterol as well as stigmasterol and β-sitosterol. Retention on the column was longer according to the larger side chain. Increase of retention time was observed comparing cholesterol, with no substitution on the side chain, campesterol, with methyl substitution, and β-sitosterol, with ethyl substitution.

Differences in saturation of sterol ring demonstrated minor changes in retention time. More saturated sterols were retained longer. Ergosterol, with two double bonds on sterol ring, eluted faster than brassicasterol, with only one double bond.

Developed analytical method was considered for possible determination of tocopherols to enable more complete analysis of lipid emulsions in evaluation of their clinical effects.

Method selectivity was investigated by analysing a mixture of sterols, squalene and tocopherols. Obtained chromatogram (Figure 18) demonstrated good separation of analysed compounds, due to differences in chemical structure.

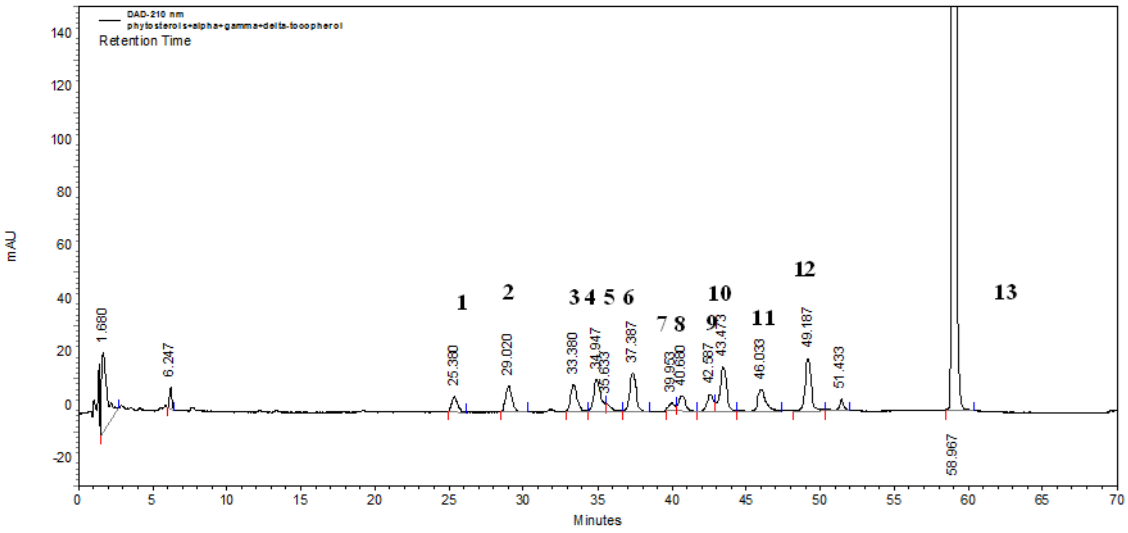

Figure 18: Chromatogram of the mixture of phytosterols and tocopherols. Identified peaks are: 1 – desmosterol, 2 – ergosterol, 3 – lathosterol, 4 –cholesterol, 5 – brassicasterol,6 – δ-tocopherol, 7 – campesterol, 8 – lanosterol, 9 – stigmasterol, 10 – γ-tocopherol, 11 – β-sitosterol, 12 – α-tocopherol, 13 – squalene.

Tocopherols eluted between approximately 37 and 50 min, among phytosterols. The order of elution followed the number of substitutions on chromanol ring, as previously described (76,146). The shortest retention time was observed in δ-tocopherol, with one methyl substitution, followed by γ-tocopherol, with two methyl substitutions and the longest retention was in α-tocopherol, with three methyl substitutions.

In continuation, developed analytical method was validated for phytosterols and tocopherols separately.

7.1.2 Sample preparation of lipid emulsions

Commercially available lipid emulsions for PN comprise of various constituents that enable proper consistency and stability. However, excipients had similar physicochemical properties as sterols and interfere with determination of phytosterols, which required a special sample preparation.

Based on existing published procedures for analysis of lipid emulsions, saponification and extraction is required to simultaneously remove lipophilic and hydrophilic excipients (40,149). Described procedures were adapted to enable preparation of lipid emulsions with different composition and suitable for analysis on HPLC.

The first step in sample preparation (Table 14) was saponification of lipid emulsion. To emulsion was added internal standard to determine percentage of extraction. In described GC procedures, 5α-cholestane, was used, although due to the lack of chromophores, it was not suitable for UV detection. Phytosterol ergosterol, unlikely to be found in parenteral lipid emulsion, was added in concentration 100 μg/mL as internal standard. To discard possible ergosterol assay in lipid emulsions, samples with and without internal standard were prepared simultaneously.

Strong alkaline medium, 7% KOH in 96% ethanol, was used to hydrolyse triglycerides and therefore, convert them into water-soluble forms which enabled their separation from lipophilic phytosterols. Antioxidant pyrogallol was added as 1% solution to prevent phytosterol oxidation during the preparation. Saponification mixture was heated for 20 min at temperature of 60°C to facilitate alkaline hydrolysis and was cooled down before extraction.

Liquid-liquid extraction included addition of H_2O, to dissolve saponifiable compounds, and addition of heptane, to extract phytosterols and other unsaponifiable lipid compounds. After separation of phases, heptane (upper) layer was collected and dried on rotary evaporator at temperature of 20°C to remove heptane.

Obtained dry residue was dissolved in 2 mL MeOH, to dissolve phytosterols without incompatibilities with the mobile phase and to obtain sufficient sample for analysis.

Solution was filtered through PDVF filter 0.45 μm and a vial was prepared for HPLC analysis.

Table 14: Sample preparation procedure.

SAPONIFICATION
1 mL lipid emulsion + 1 mL 100 μg/mL ergosterol + 10 mL 7% KOH in EtOH + 3 mL 1% pyrogallol + heating 20 min at 60°C
↓
EXTRACTION
5 mL H_2O + 2 x 5 mL heptane
↓
DRYING
heptane layer by rotavapor at 20°C
↓
HPLC ANALYSIS
dry residue reconstitution in 2 mL MeOH
filtration PVDF filter 0.45 μm

Established sample preparation procedure was submitted to minor variations in order to determine critical parameters and to investigate its robustness. Tested parameters were addition of reagents (KOH, pyrogallol, H_2O, and heptane), heating time and temperature, and drying temperature. Lipid emulsion samples were prepared modifying only one parameter at a time. Mean recovery of total phytosterols was calculated and statistically evaluated, using ANOVA study.

Results are presented in Table 15. Recoveries, obtained at optimal conditions were set to 100% to facilitate comparison.

Table 15: Robustness data for sample preparation.

Addition of KOH	**9 mL**	**10 mL**	**11 mL**
Average recovery (%)	90.55 ± 8.96	100.00	94.50 ± 3.68
ANOVA	F_{exp} = 4.319	$F_{crit.}$ = 3.682	$p < 0.05$

Addition of pyrogallol	**2.5 mL**	**3 mL**	**3.5 mL**
Average recovery (%)	100.94 ± 6.53	100.00	98.38 ± 10.69
ANOVA	F_{exp} = 0.193	$F_{crit.}$ = 3.682	$p < 0.05$

Heating temperature	**55°C**	**60°C**	**65°C**
Average recovery (%)	97.57 ± 14.18	100.00	98.03 ± 3.96
ANOVA	F_{exp} = 0.138	$F_{crit.}$ = 3.682	$p < 0.05$

Heating time	**15 min**	**20 min**	**25 min**
Average recovery (%)	90.67 ± 16.17	100.00	97.56 ± 5.48
ANOVA	F_{exp} = 1.446	$F_{crit.}$ = 3.682	$p < 0.05$

Addition of H_2O	**4 mL**	**5 mL**	**6 mL**
Average recovery (%)	103.49 ± 6.30	100.00	104.08 ± 5.11
ANOVA	F_{exp} = 1.327	$F_{crit.}$ = 3.682	$p < 0.05$

Addition of heptane	**2 x 4 mL**	**2 x 5 mL**	**2 x 6 mL**
Average recovery (%)	99.51 ± 4.83	100.00	93.74 ± 14.57
ANOVA	F_{exp} = 0.925	$F_{crit.}$ = 3.682	$p < 0.05$

Drying temperature	**20°C**	**30°C**	**40°C**
Average recovery (%)	100.00	98.48 ± 5.23	93.62 ± 6.81
ANOVA	F_{exp} = 2.709	$F_{crit.}$ = 3.682	$p < 0.05$

Addition of KOH resulted to be the most critical step in sample preparation. Between calculated recoveries at studied volumes of KOH, a statistically significant difference ($F_{exp} > F_{crit.}$) was observed. According to the obtained recoveries, volume of 9 mL of KOH was not sufficient for proper saponification, whereas 11 mL influenced on phytosterol

stability, which resulted in low recovery. Therefore, an exact volume of 10 mL of KOH was required for optimal saponification.

Antioxidant pyrogallol was added in volumes 2.5-3.5 mL and no statistically significant difference was observed between obtained recoveries ($F_{exp} < F_{crit.}$). Comparing phytosterol recoveries, it was observed slightly higher percentage in 2.5 mL compared to 3 mL of pyrogallol. However, the addition of antioxidant remained at 3 mL, in order to facilitate sample preparation.

Heating temperature during saponification was studied from 55°C up to 65°C and there was observed no statistically significant difference between obtained recoveries ($F_{exp} < F_{crit.}$). Increasing or decreasing of heating temperature resulted in lower recoveries due to insufficient or excessive saponification conditions. Optimal heating temperature for saponification remained at 60°C.

Heating time during saponification varied from 15 min to 25 min and no statistically significant difference was observed between obtained recoveries ($F_{exp} < F_{crit.}$). Longer or shorter times did not improve the percentage of recovery, therefore, previously established 20 min were optimal for saponification.

Addition of H_2O in extraction in volumes 4-6 mL did not have statistically significant influence ($F_{exp} < F_{crit.}$) on recovery of phytosterols. Lower volume of H_2O enabled easier extraction and sample manipulation, whereas higher volume demonstrated better removal of hydrophilic constituents from the sample. Despite of slightly higher recoveries in addition of 4 and 6 mL, volume of H_2O in extraction remained at 5 mL in order to achieve good extraction with simple preparation.

Volume of heptane used for extraction of phytosterols varied from 4 to 6 mL and no statistically significant difference ($F_{exp} < F_{crit.}$) was observed between obtained recoveries. Increasing or decreasing addition of heptane resulted in lower recoveries due to difficult manipulation of sample or inefficient exaction.

Drying temperature of collected heptane layer varied from 20°C up to 40°C and had no statistically significant influence ($F_{exp} < F_{crit.}$) on recovery of phytosterols. Increasing temperature of drying resulted in lower percentages of recovery and established temperature of 20°C was sufficient to evaporate volatile heptane.

The study of robustness demonstrated that established sample preparation procedure is robust, enables good recoveries of phytosterols, and simple sample manipulation. Minor variations in preparation conditions did not have statistically important influence on recovery, except of addition of KOH, which was established as critical step.

Summarised conditions of sample preparation are presented in Table 16.

Table 16: Optimal sample preparation conditions.

SAPONIFICATION	Addition of lipid emulsion:	1.0 mL
	Addition of internal standard:	1.0 mL
	Addition of KOH:	10.0 mL
	Addition of pyrogallol:	3 mL
	Heating temperature:	60°C
	Heating time:	20 min
EXTRACTION	Addition of H_2O:	5 mL
	Addition of heptane:	2 x 5 mL
DRYING	Drying temperature:	20°C
HPLC ANALYSIS	Addition of MeOH:	2.0 mL
	Filtration:	PVDF filter 0.45 μm

Developed sample preparation successfully removed the effect of matrix, which enabled identification and quantification of phytosterols, cholesterol and squalene under conditions that did not alter their chemical structure. Procedure was found efficient also for determination of tocopherols, which enabled their simultaneous analysis with phytosterols.

7.1.3 Validation study of phytosterols

Developed analytical method was validated according to ICH guidelines (137) to ensure its suitability for routine use. System suitability, stability of standard solution, selectivity, linearity, precision, accuracy and robustness were tested and compliance with established acceptance criteria was proven.

Validation of phytosterols was performed in the laboratory of SDM at University of Barcelona on chromatograph Dionex UltiMate 3000 with software Chromeleon and chromatograph Agilent 1100 with software ChemStation.

7.1.3.1 System suitability

System suitability was tested to investigate the appropriateness of chromatographic system to perform analytical method and to work in routine.

System suitability solution was analysed and with software Chromeleon chromatographic parameters: capacity factor, tailing factor, number of theoretical plates, resolution between peaks, and retention time of β-sitosterol, were studied. The obtained results are presented in Table 17. The chromatographic system complied the established acceptance criteria and was considered suitable for method validation.

Table 17: System suitability data for validation of phytosterols.

Chromatographic parameter	Acceptance criteria	Result
Capacity factor	≥ 1.5	84.15
Tailing factor	0.8-1.5	1.05
Theoretical plates	> 2000	28496
Resolution	≥ 1	37.71
Retention time of β-sitosterol	~43 min	43.26 min

7.1.3.2 Stability of the standard solution

Stability of the standard solution was studied to determine the possible degradation under normal working conditions in the laboratory.

Standard solution of phytosterols was maintained at room temperature (25 ± 2ºC) and analysed according to established times of analysis, from 0 to 12 days. Results obtained on Day 0 were considered as 100% recovery.

The obtained data are presented in Table 18 and Figure 19. Percentage of recovery remained within the established limits (80-110%) for all phytosterols, cholesterol and squalene.

Phytosterol standard solution was considered stable for at least 12 days at room temperature.

Table 18: Stability data of phytosterol standard solution.

Phytosterols	**Recovery (%)**			
	Day 0	**Day 1**	**Day 8**	**Day 12**
β-sitosterol	100.00	98.73	98.67	98.47
brassicasterol	100.00	99.56	96.96	94.49
campesterol	100.00	104.85	103.57	105.59
cholesterol	100.00	99.76	99.50	101.59
desmosterol	100.00	97.86	98.30	99.99
ergosterol	100.00	104.54	103.79	103.87
lanosterol	100.00	96.18	99.94	98.67
lathosterol	100.00	94.59	100.54	101.77
squalene	100.00	97.90	98.68	97.70
stigmasterol	100.00	91.52	94.11	94.69

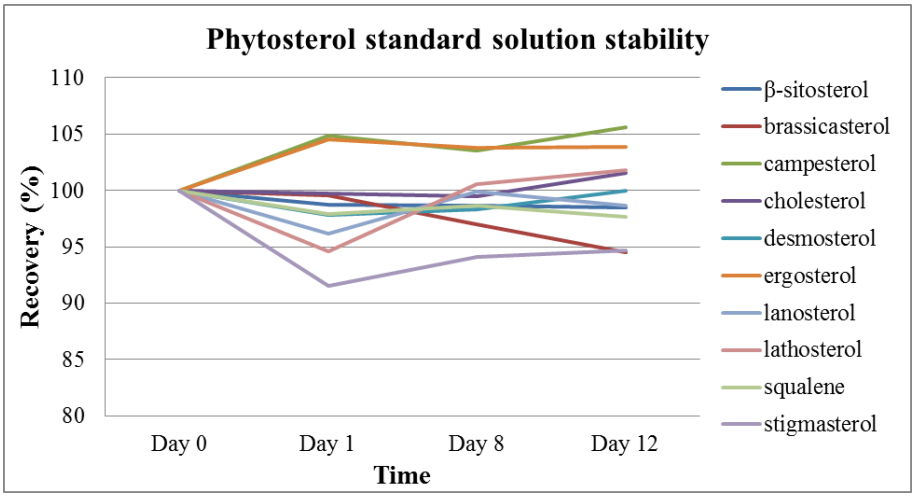

Figure 19: Stability of standard solution of phytosterols.

7.1.3.3 Selectivity

Selectivity was tested to ensure proper separation and identification of analysed compounds.

Blank, individual solutions of phytosterols, cholesterol and squalene and mixture of all compounds were analysed to study the separation, absence of interaction and establish UV maxima and relative retention time (RRT). Obtained chromatograms are presented from Figure 20 to Figure 31.

Phytosterols, cholesterol and squalene demonstrated good separation that enabled identification and quantification of analytes. Furthermore, there was not observed any interference with blank sample (MeOH). For identification of each phytosterol, UV maxima and relative retention times, presented in Table 19, were determined. Absorption maxima varied from 190.4 nm, in brassicasterol, up to 281.2 nm, in ergosterol, depending on chromophores. Relative retention times were calculated relative to ergosterol, which was used as internal standard in analytical method. The shortest retention time was observed in desmosterol (RRT = 0.88), whereas the longest retention was in squalene (RRT = 2.05) due to its long chain alkene structure.

Table 19: UV maxima and relative retention times of phytosterols.

Phytosterol	UV maximum (nm)	Relative retention time
β-sitosterol	193.4	1.57
brassicasterol	190.4	1.22
campesterol	192.6	1.37
cholesterol	193.5	1.20
desmosterol	193.9	0.88
ergosterol	281.2	1.00
lanosterol	194.3	1.41
lathosterol	190.6	1.15
squalene	199.4	2.05
stigmasterol	193.0	1.46

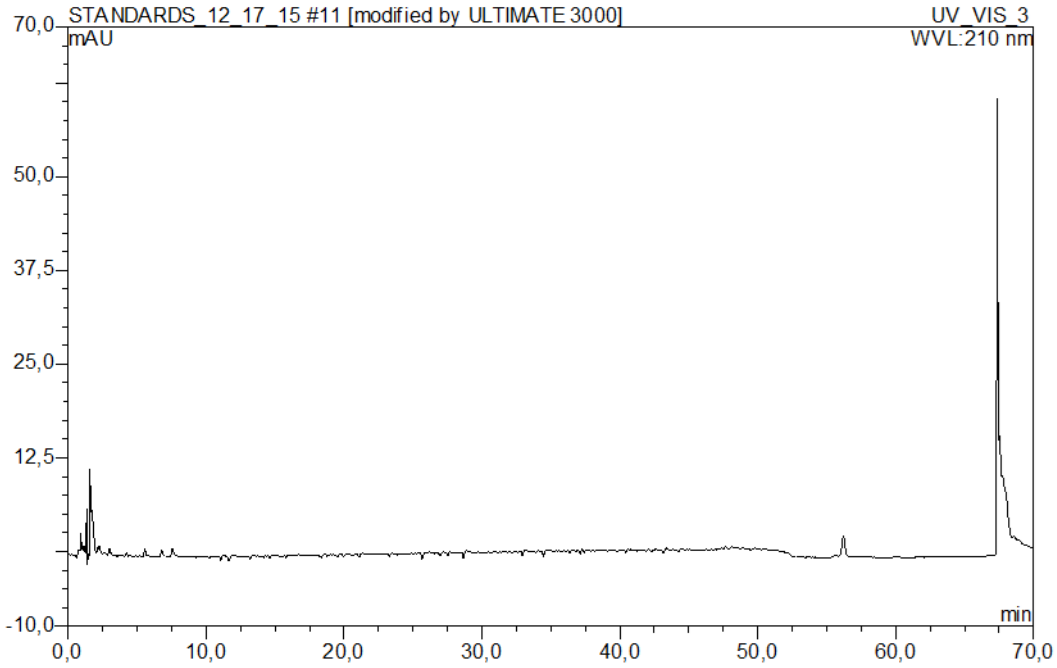

Figure 20: Chromatogram of blank (MeOH).

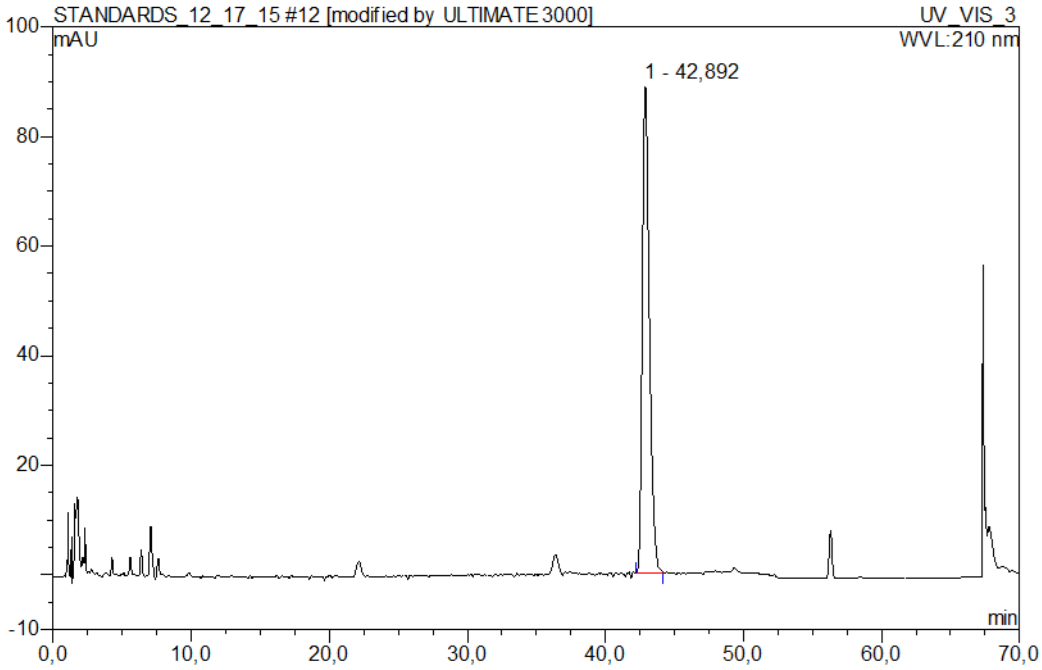

Figure 21: Chromatogram of β-sitosterol.

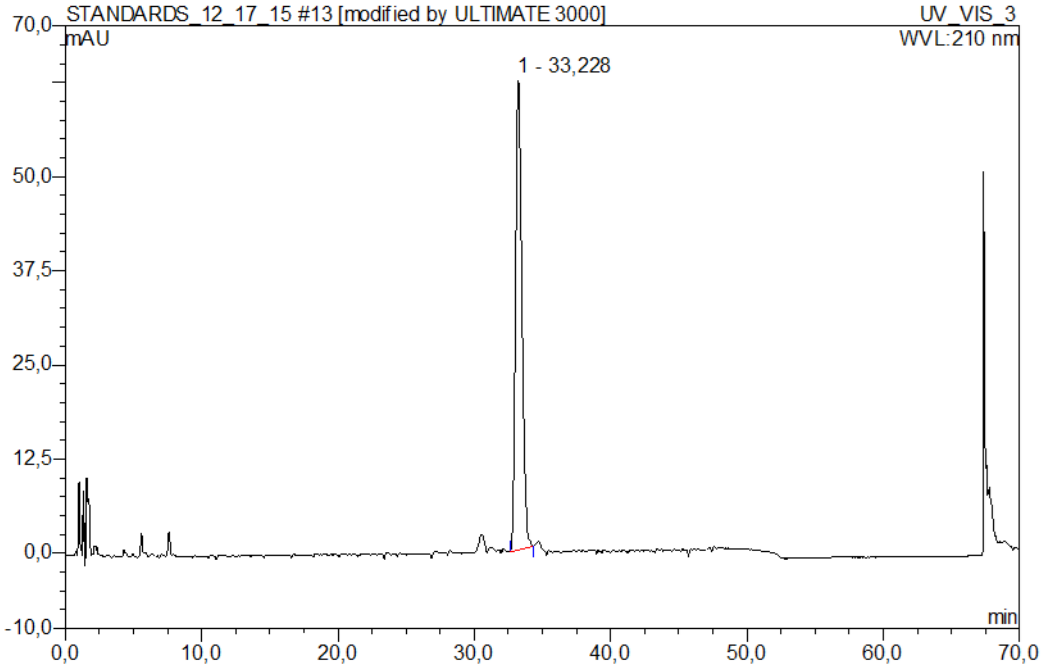

Figure 22: Chromatogram of brassicasterol.

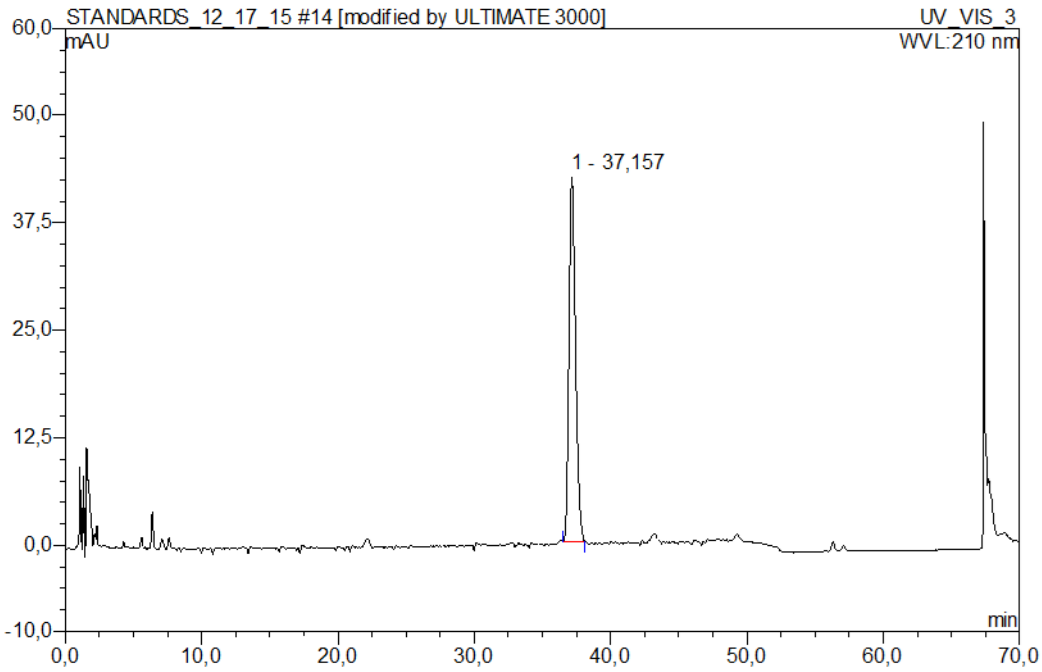

Figure 23: Chromatogram of campesterol.

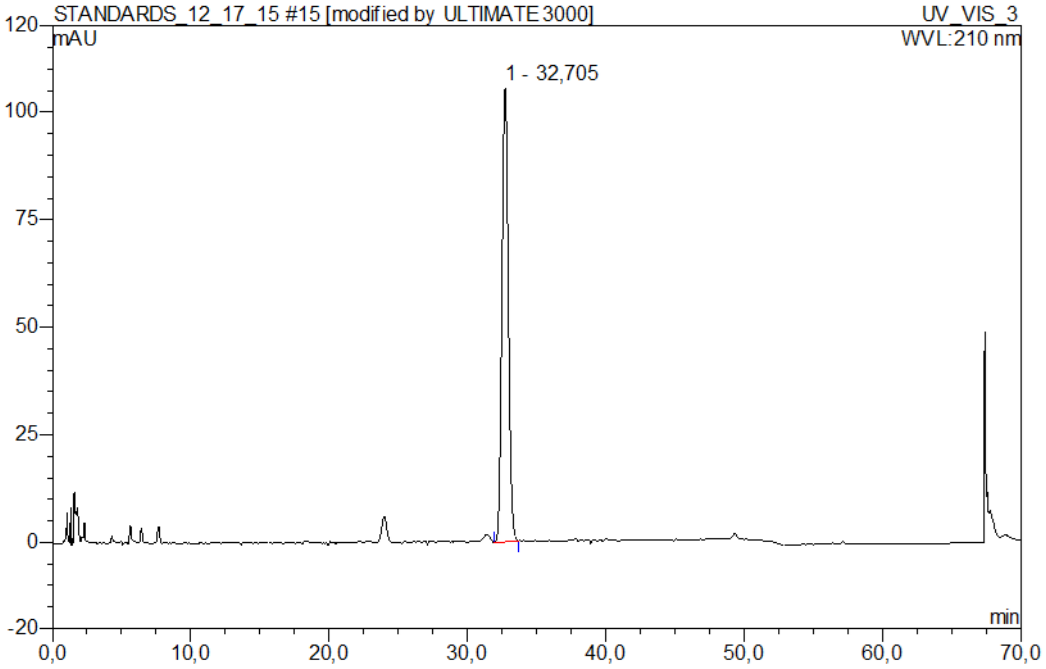

Figure 24: Chromatogram of cholesterol.

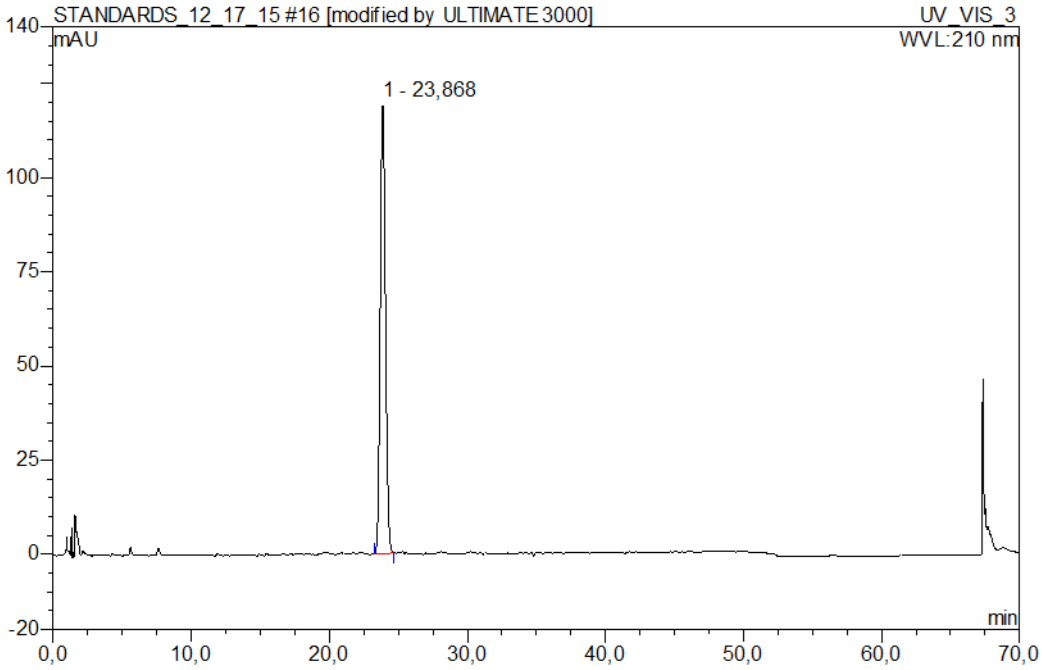

Figure 25: Chromatogram of desmosterol.

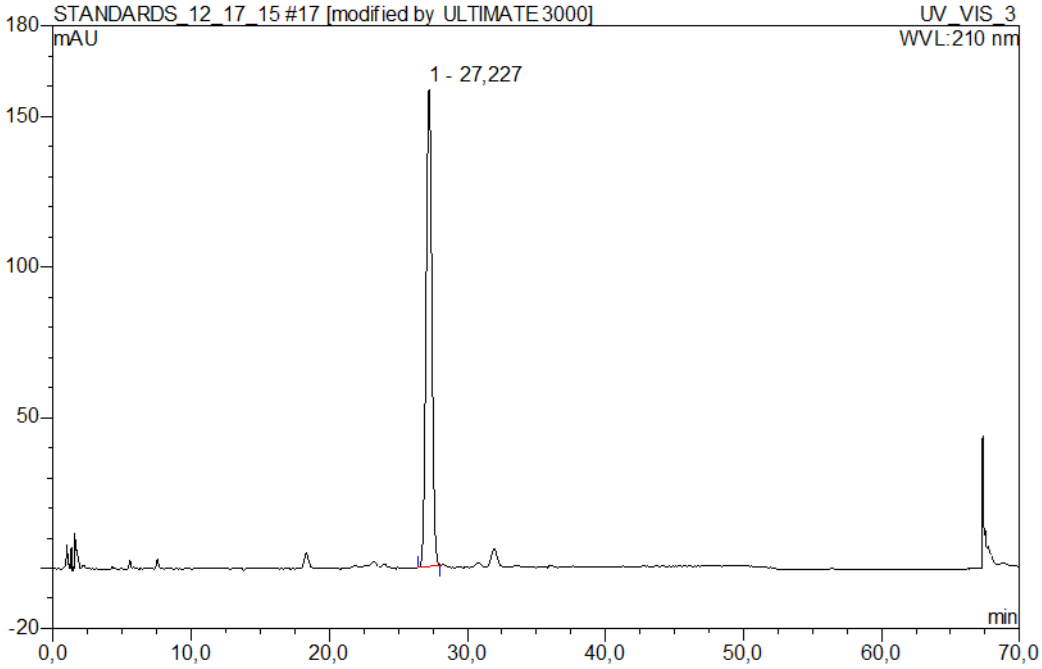

Figure 26: Chromatogram of ergosterol.

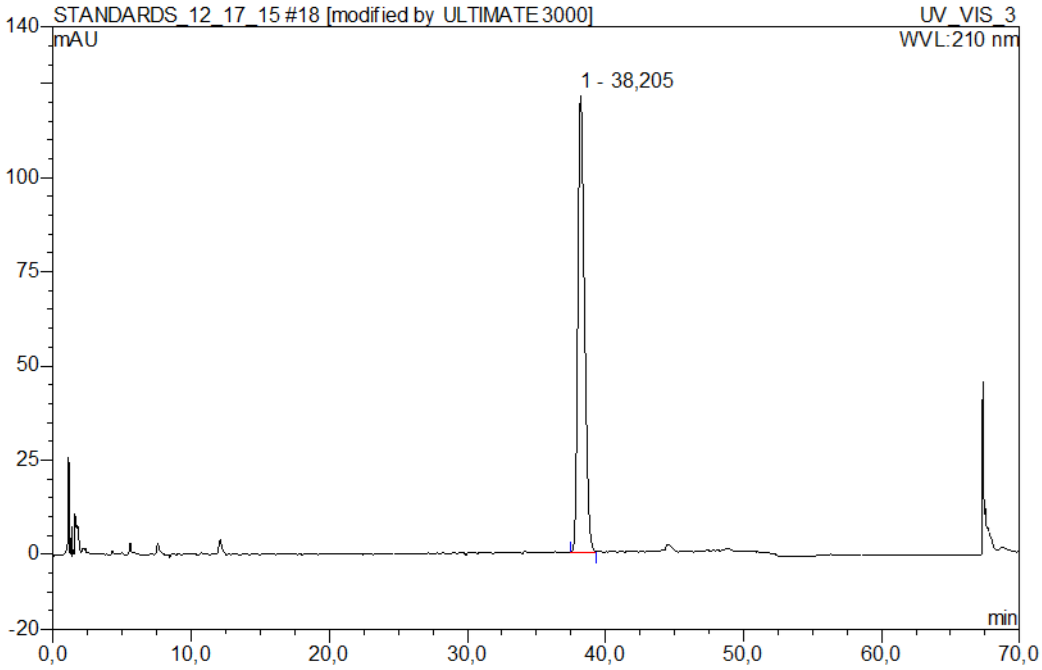

Figure 27: Chromatogram of lanosterol.

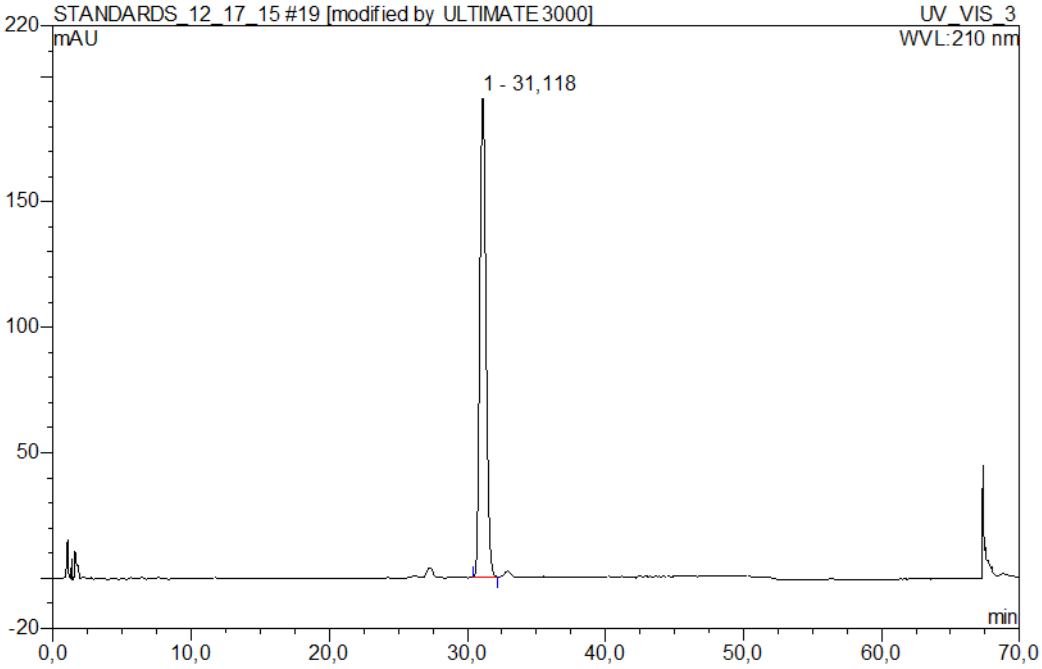

Figure 28: Chromatogram of lathosterol.

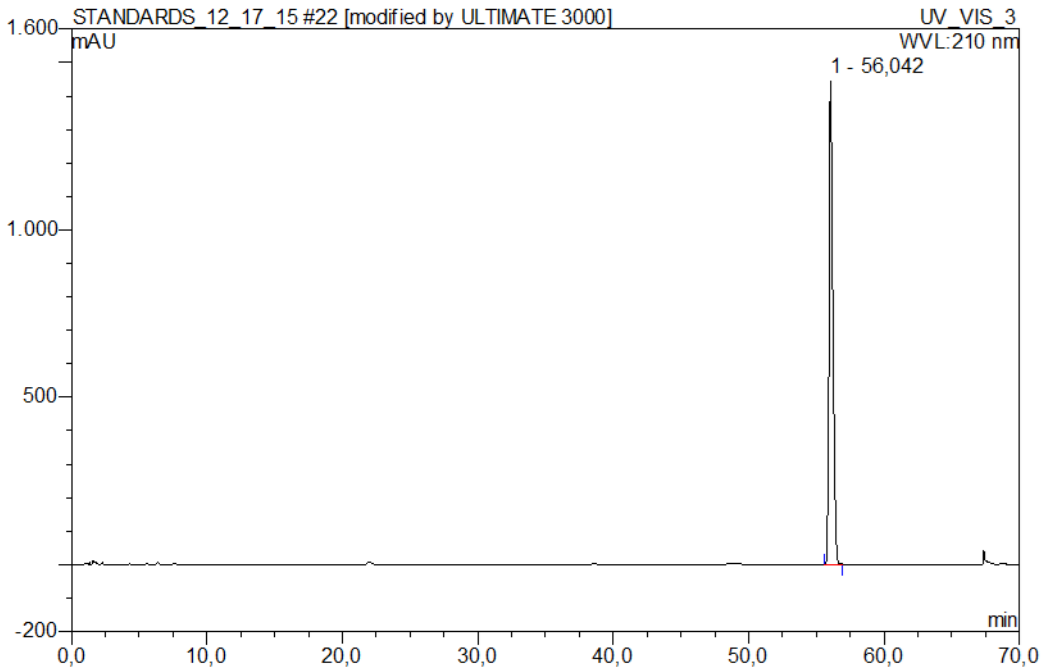

Figure 29: Chromatogram of squalene.

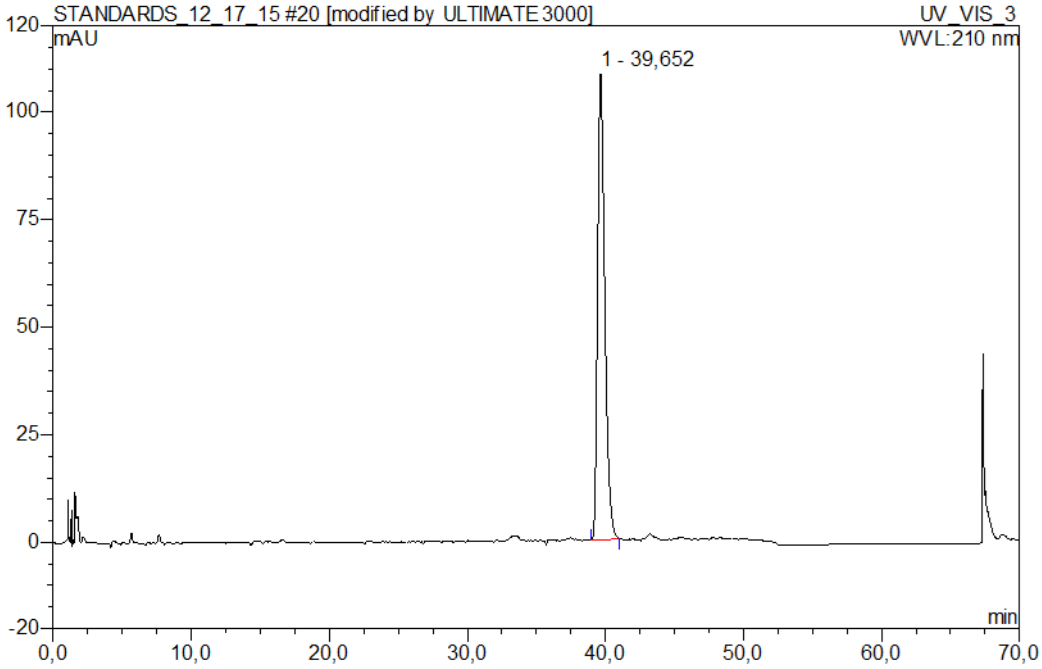

Figure 30: Chromatogram of stigmasterol.

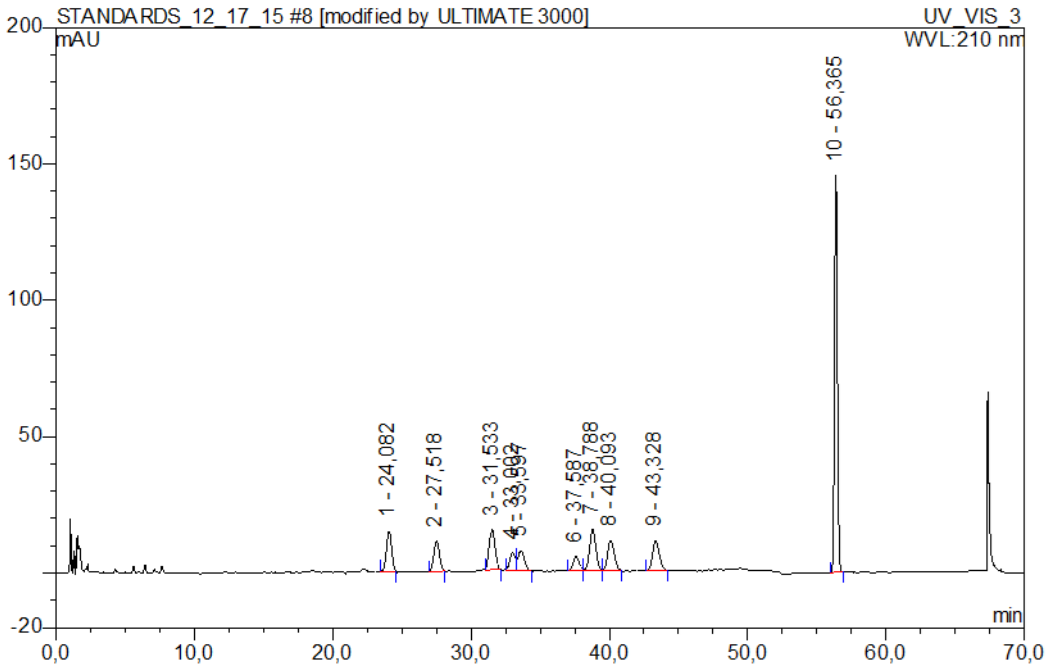

Figure 31: Chromatogram of mixture of phytosterols. Identified peaks are: 1 – desmosterol, 2 – ergosterol, 3 – lathosterol, 4 – cholesterol, 5 – brassicasterol, 6 – campesterol, 7 – lanosterol, 8 – stigmasterol, 9 – β-sitosterol, 10 – squalene.

7.1.3.4 Linearity

Linearity was investigated to determine the relationship between the concentration of analysed compounds and chromatographic response.

Phytosterol standard solution was prepared in triplicate and diluted 1/1, 1/2, 1/5, 1/10, 1/20, 1/50, 1/100, 1/200, and 1/500 to obtain calibration curve with at least five points for each phytosterol. Mean linearities and corresponding statistical evaluations are presented from Table 20 to Table 39 and from Figure 32 to Figure 41.

In all phytosterols was observed a good correlation ($R > 0.999$ and $R^2 > 0.990$) between concentration and mean peak area. Differences in linearity curves were attributed to physicochemical properties of sterols and squalene. Positive intercept in squalene was explained with higher absorption maximum due to its triterpene structure. Brassicasterol interaction with cholesterol resulted in its slightly positive intercept, meanwhile, other sterols demonstrated negative intercepts.

Confidence intervals of intercept (lower and upper 95.0%) showed proportionality, passing the zero, in all phytosterols. Test of slope, where confidence levels (lower and upper 95.0%) of slope were evaluated, demonstrated slopes different from zero. Both tests confirmed good fitness of data for linearity of phytosterols.

Response factors were calculated to correlate mean peak area with concentration of phytosterols. Variation of calculated response factors was higher than established in the acceptance criteria (response factor RSD < 2%). Deviations were attributed to preparation errors and detection at low concentrations.

Method was proved linear for determination of phytosterols.

Table 20: Data of β-sitosterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
β-sitosterol	3.57	0.557	1.56E-01
	8.93	1.521	1.70E-01
	17.86	3.222	1.80E-01
	35.71	6.108	1.71E-01
	89.29	14.954	1.67E-01
	178.57	30.716	1.72E-01
R	0.9999	**Average RF**	1.70E-01
R^2	0.9998	**SD**	7.9E-03
intercept	-0.0240	**RSD**	5%
slope	0.1714		

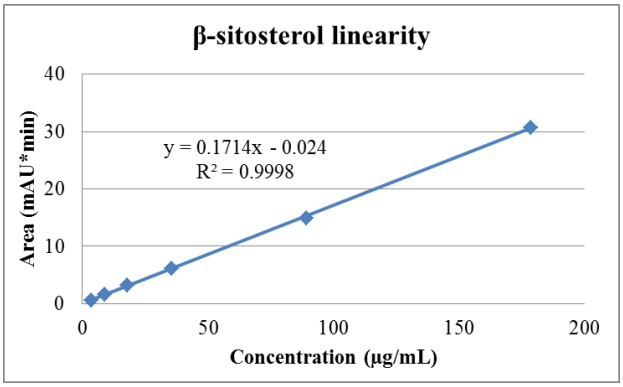

Figure 32: Graph of β-sitosterol linearity.

Table 21: Statistical data of β-sitosterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99988213
R Square	0.999764275
Adjusted R Square	0.999705343
Standard Error	0.199358028
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	674.246452	674.246452	16964.89632	2.08391E-08
Residual	4	0.158974494	0.039743623		
Total	5	674.4054265			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.023979331	0.109476844	-0.219035646	0.837344816	-0.327935777	0.279977115	-0.327935777	0.279977115
X Variable 1	0.171360001	0.00131563	130.2493621	2.08391E-08	0.167707226	0.175012776	0.167707226	0.175012776

Table 22: Data of brassicasterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Brassicasterol	2.45	0.367	1.50E-01
	4.90	0.812	1.66E-01
	9.80	1.566	1.60E-01
	24.50	4.394	1.79E-01
	49.00	8.087	1.65E-01
R	0.9987	**Average RF**	1.64E-01
R^2	0.9974	**SD**	1.1E-02
intercept	0.0138	**RSD**	7%
slope	0.1672		

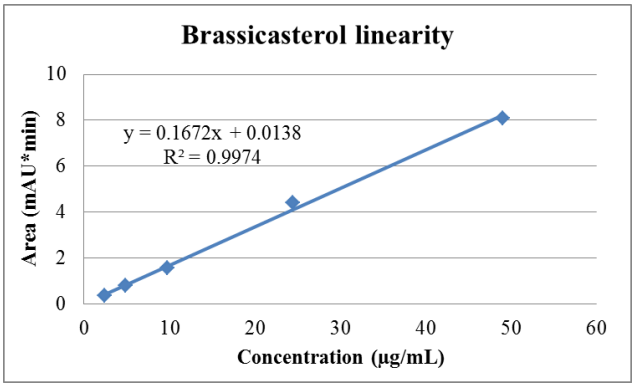

Figure 33: Graph of brassicasterol linearity.

Table 23: Statistical data of brassicasterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998723959
R Square	0.997449546
Adjusted R Square	0.996599395
Standard Error	0.188041047
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	41.48585089	41.48585089	1173.261126	5.47076E-05
Residual	3	0.106078306	0.035359435		
Total	4	41.5919292			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	0.01376726	0.122084994	0.112767831	0.917337149	-0.374761678	0.402296198	-0.374761678	0.402296198
X Variable 1	0.167209013	0.004881602	34.25289953	5.47076E-05	0.151673578	0.182744448	0.151673578	0.182744448

Table 24: Data of campesterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Campesterol	1.63	0.472	2.90E-01
	3.25	0.961	2.96E-01
	6.50	2.029	3.12E-01
	16.25	5.219	3.21E-01
	32.50	10.617	3.27E-01
R	1.0000	**Average RF**	3.09E-01
R^2	1.0000	**SD**	1.6E-02
intercept	-0.0989	**RSD**	5%
slope	0.3292		

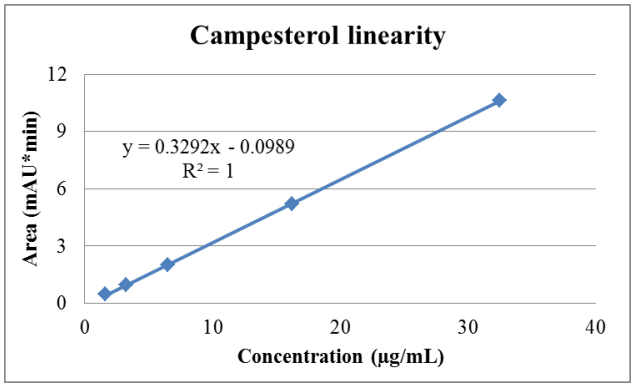

Figure 34: Graph of campesterol linearity.

Table 25: Statistical data of campesterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999980297
R Square	0.999960593
Adjusted R Square	0.999947458
Standard Error	0.030482808
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	70.73677382	70.73677382	76126.40317	1.0499E-07
Residual	3	0.002787605	0.000929202		
Total	4	70.73956142			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.098895092	0.019790857	-4.997009123	0.015417704	-0.161878431	-0.035911753	-0.161878431	-0.035911753
X Variable 1	0.329188781	0.001193101	275.910136	1.0499E-07	0.3253918	0.332985762	0.3253918	0.332985762

Table 26: Data of cholesterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Cholesterol	4.97	0.683	1.37E-01
	12.44	1.909	1.53E-01
	24.87	4.181	1.68E-01
	49.75	7.832	1.57E-01
	124.37	20.200	1.62E-01
	248.73	41.607	1.67E-01
R	0.9999	**Average RF**	1.58E-01
R^2	0.9997	**SD**	1.1E-02
intercept	-0.2430	**RSD**	7%
slope	0.1674		

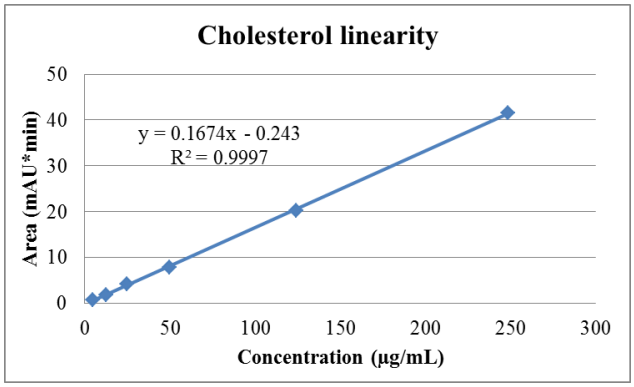

Figure 35: Graph of cholesterol linearity.

Table 27: Statistical data of cholesterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999867373
R Square	0.999734763
Adjusted R Square	0.999668454
Standard Error	0.287784415
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1248.664281	1248.664281	15076.86853	2.63838E-08
Residual	4	0.331279477	0.082819869		
Total	5	1248.995561			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.24297545	0.15803592	-1.537469779	0.19899584	-0.681753506	0.195802605	-0.681753506	0.195802605
X Variable 1	0.167415211	0.00136345	122.7879006	2.63838E-08	0.163629666	0.171200756	0.163629666	0.171200756

Table 28: Data of desmosterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Desmosterol	0.84	0.316	3.76E-01
	2.10	0.761	3.62E-01
	4.20	1.711	4.07E-01
	8.40	3.336	3.97E-01
	21.00	8.259	3.93E-01
	42.00	16.967	4.04E-01
R	0.9999	**Average RF**	3.90E-01
R^2	0.9998	**SD**	1.7E-02
intercept	-0.0593	**RSD**	4%
slope	0.4037		

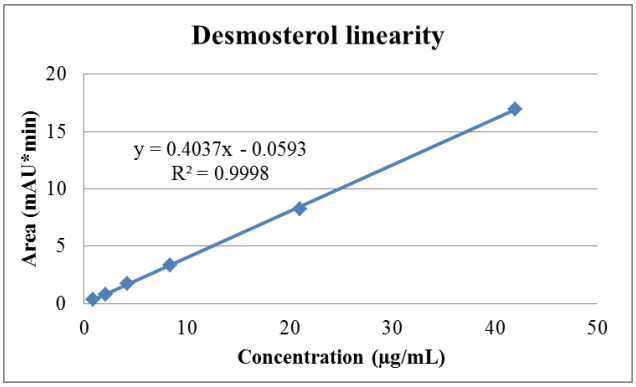

Figure 36: Graph of desmosterol linearity.

Table 29: Statistical data of desmosterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99990798
R Square	0.999815968
Adjusted R Square	0.99976996
Standard Error	0.097597157
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	206.9951435	206.9951435	21731.30557	1.27012E-08
Residual	4	0.038100821	0.009525205		
Total	5	207.0332443			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.059253219	0.053595177	-1.105569996	0.330932382	-0.208057285	0.089550847	-0.208057285	0.089550847
X Variable 1	0.403682022	0.002738398	147.4154184	1.27012E-08	0.396079011	0.411285032	0.396079011	0.411285032

Table 30: Data of ergosterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Ergosterol	2.41	0.542	2.24E-01
	6.04	1.446	2.40E-01
	12.07	2.916	2.41E-01
	24.15	5.784	2.39E-01
	60.37	14.451	2.39E-01
	120.75	29.206	2.42E-01
R	1.0000	**Average RF**	2.38E-01
R^2	1.0000	**SD**	6.6E-03
intercept	-0.0429	**RSD**	3%
slope	0.2418		

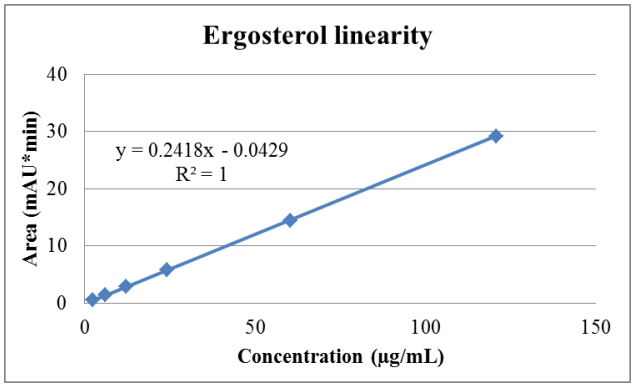

Figure 37: Graph of ergosterol linearity.

Table 31: Statistical data of ergosterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999986801
R Square	0.999973602
Adjusted R Square	0.999967002
Standard Error	0.063652247
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	613.9080368	613.9080368	151522.0506	2.61325E-10
Residual	4	0.016206434	0.004051609		
Total	5	613.9242432			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.042915913	0.034954434	-1.227767333	0.286843349	-0.13996498	0.054133155	-0.13996498	0.054133155
X Variable 1	0.241819392	0.000621231	389.2583341	2.61325E-10	0.240094577	0.243544206	0.240094577	0.243544206

Table 32: Data of lanosterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Lanosterol	2.33	0.889	3.83E-01
	4.65	1.787	3.84E-01
	9.30	3.815	4.10E-01
	23.25	9.547	4.11E-01
	46.50	19.604	4.22E-01
R	0.9999	**Average RF**	4.02E-01
R^2	0.9999	**SD**	1.7E-02
intercept	-0.1614	**RSD**	4%
slope	0.4237		

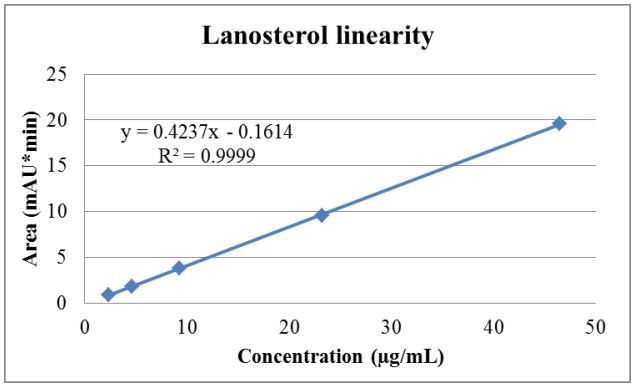

Figure 38: Graph of lanosterol linearity.

Table 33: Statistical data of lanosterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999936456
R Square	0.999872917
Adjusted R Square	0.999830556
Standard Error	0.100813574
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	239.8918935	239.8918935	23603.56237	6.08048E-07
Residual	3	0.03049013	0.010163377		
Total	4	239.9223836			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.161394283	0.065452861	-2.465809436	0.09039991	-0.369694499	0.046905933	-0.369694499	0.046905933
X Variable 1	0.42370208	0.002757857	153.634509	6.08048E-07	0.414925346	0.432478813	0.414925346	0.432478813

Table 34: Data of lathosterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Lathosterol	0.99	0.573	5.79E-01
	2.48	1.310	5.29E-01
	4.95	2.722	5.50E-01
	9.90	5.591	5.65E-01
	24.75	14.655	5.92E-01
	49.50	29.250	5.91E-01
R	1.0000	**Average RF**	5.68E-01
R^2	0.9999	**SD**	2.5E-02
intercept	-0.1516	**RSD**	4%
slope	0.5943		

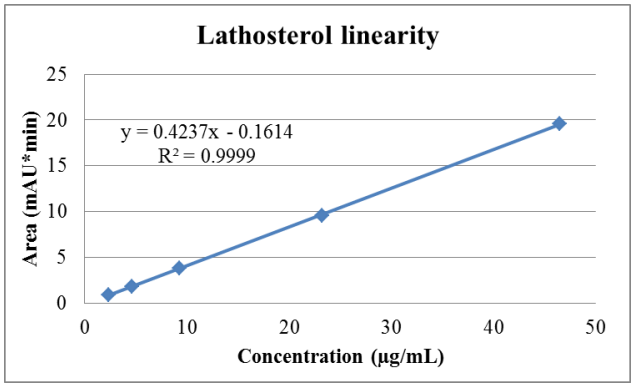

Figure 39: Graph of lathosterol linearity.

Table 35: Statistical data of lathosterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999957582
R Square	0.999915165
Adjusted R Square	0.999893956
Standard Error	0.114966218
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	623.1453684	623.1453684	47146.43757	2.69893E-09
Residual	4	0.052868925	0.013217231		
Total	5	623.1982373			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.151568985	0.063133342	-2.400775557	0.074293811	-0.326855244	0.023717274	-0.326855244	0.023717274
X Variable 1	0.594289569	0.002736993	217.1323043	2.69893E-09	0.586690458	0.601888679	0.586690458	0.601888679

Table 36: Data of squalene linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Squalene	1.00	1.959	1.97E+00
	2.49	4.997	2.01E+00
	4.98	9.989	2.01E+00
	9.96	19.888	2.00E+00
	24.90	49.301	1.98E+00
	49.80	98.176	1.97E+00
	99.59	191.376	1.92E+00
	248.99	446.867	1.79E+00
	497.97	824.413	1.66E+00
R	0.9990	**Average RF**	1.92E+00
R^2	0.9980	**SD**	1.2E-01
intercept	8.8607	**RSD**	6%
slope	1.6678		

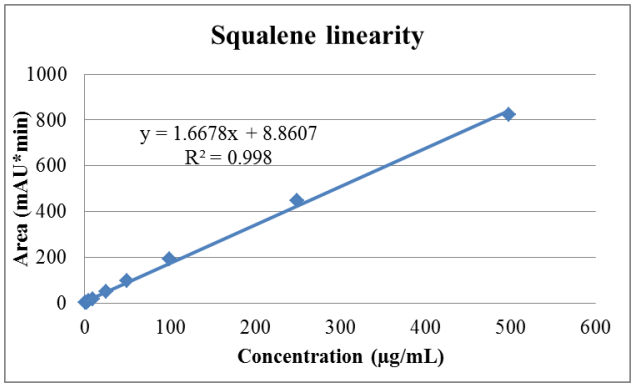

Figure 40: Graph of squalene linearity.

Table 37: Statistical data of squalene linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998985885
R Square	0.997972799
Adjusted R Square	0.997683199
Standard Error	13.47703149
Observations	9

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	625905.0458	625905.0458	3446.03724	1.09248E-10
Residual	7	1271.412645	181.6303778		
Total	8	627176.4585			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	8.860660659	5.383361117	1.645934662	0.143772591	-3.868965593	21.59028691	-3.868965593	21.59028691
X Variable 1	1.667840559	0.028411525	58.70295768	1.09248E-10	1.600657978	1.73502314	1.600657978	1.73502314

Table 38: Data of stigmasterol linearity.

	Concentration (µg/mL)	Mean area (mAU*min)	Response factor
Stigmasterol	2.44	0.483	1.98E-01
	6.10	1.222	2.00E-01
	12.20	2.431	1.99E-01
	24.41	4.983	2.04E-01
	61.01	12.487	2.05E-01
	122.03	25.880	2.12E-01
R	0.9999	**Average RF**	2.03E-01
R^2	0.9997	**SD**	5.2E-03
intercept	-0.1509	**RSD**	3%
slope	0.2121		

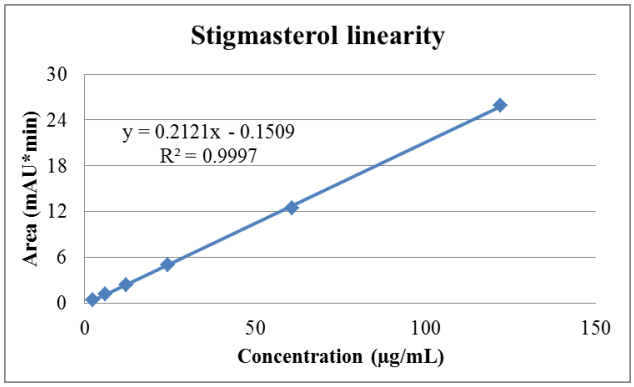

Figure 41: Graph of stigmasterol linearity.

Table 39: Statistical data of stigmasterol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999859198
R Square	0.999718416
Adjusted R Square	0.99964802
Standard Error	0.18426825
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	482.204532	482.204532	14201.37072	2.97363E-08
Residual	4	0.135819152	0.033954788		
Total	5	482.3403511			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	-0.150856992	0.101190339	-1.490824065	0.210267532	-0.431806412	0.130092428	-0.431806412	0.130092428
X Variable 1	0.212063805	0.001779514	119.1695042	2.97363E-08	0.207123082	0.217004528	0.207123082	0.217004528

Concentration range of validated analytical method for determination of phytosterols was based on linearity data (Table 40). Lower limit was considered as limit of quantification (LOQ), whereas, upper limit was established according to their potential assay in lipid emulsions.

Table 40: Concentration range of phytosterols.

Phytosterols	**Concentration interval (µg/mL)**
β-sitosterol	9-179
brassicasterol	3-49
campesterol	2-32
cholesterol	12-249
desmosterol	2-42
ergosterol	6-121
lanosterol	2-46
lathosterol	2-49
squalene	25-498
stigmasterol	6-122

7.1.3.5 Precision

Precision testing consisted of repeatability of the instrumental system and repeatability of the method at different concentration levels and performed on different days.

Repeatability of the instrumental system

To investigate the repeatability of the instrumental system, 10 consecutive injections of the standard solutions at dilutions: 1/20, 1/5 and 1/1, were analysed on two different days.

The results, RSD of peak areas, are presented in Table 41 for each phytosterol separately. Established acceptance criteria for instrumental day precision (RSD < 1%) and interday precision (RSD < 2%) complied for the phytosterols in highest concentrations, whereas at lowest concentrations, a higher variation of system repeatability, was observed due to peak integration.

Instrumental system was proven repeatable and precise for validation of phytosterols.

Table 41: Instrumental system repeatability data for phytosterols.

Phytosterol	Day precision RSD (%)			Interday precision RSD (%)		
	1/20	1/5	1/1	1/20	1/5	1/1
β-sitosterol	1.86	2.16	0.97	2.12	2.69	1.28
brassicasterol	16.90	6.96	2.79	15.51	13.10	6.79
campesterol	11.26	7.68	3.55	11.99	6.98	3.97
cholesterol	6.19	1.91	0.82	5.53	2.37	1.31
desmosterol	1.71	2.64	0.65	5.42	3.66	0.69
ergosterol	1.95	1.43	0.67	6.08	1.98	0.99
lanosterol	3.55	3.53	1.57	4.62	5.57	1.62
lathosterol	1.76	2.43	0.63	4.18	3.70	0.78
squalene	0.60	0.47	0.43	2.35	0.37	0.71
stigmasterol	4.50	2.98	1.22	6.50	3.32	1.19

Repeatability of the method

Method repeatability was investigated using phytosterol standard solution, corresponding to the dilutions 1/20, 1/5 and 1/1, and response factors were calculated. The results are presented from Table 42 to Table 51 for each phytosterol separately.

Variation of response factors was lower than 11%, which complied acceptance criteria. The method was proven repeatable and precise for determination of phytosterols.

Table 42: Standard solution repeatability for β-sitosterol.

β-sitosterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
Dilution 1/20	3.57	0.557	1.58E-01
	3.57	0.550	1.54E-01
	3.57	0.559	1.57E-01
Dilution 1/5	35.71	6.108	1.68E-01
	35.71	6.245	1.75E-01
	35.71	6.081	1.70E-01
Dilution 1/1	178.57	30.716	1.73E-01
	178.57	30.536	1.71E-01
	178.57	30.681	1.72E-01
		Average RF	1.66E-01
		SD	7.89E-03
		RSD	5%

Table 43: Standard solution repeatability for brassicasterol.

Brassicasterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
Dilution 1/20	2.45	0.379	1.55E-01
	2.45	0.358	1.46E-01
	2.45	0.365	1.49E-01
Dilution 1/5	9.80	1.703	1.74E-01
	9.80	1.499	1.53E-01
	9.80	1.495	1.53E-01
Dilution 1/1	49.00	8.197	1.67E-01
	49.00	8.161	1.67E-01
	49.00	7.904	1.61E-01
		Average RF	1.58E-01
		SD	9.40E-03
		RSD	6%

Table 44: Standard solution repeatability for campesterol.

Campesterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
	1.63	0.441	2.71E-01
Dilution 1/20	1.63	0.494	3.04E-01
	1.63	0.480	2.95E-01
	6.50	2.009	3.09E-01
Dilution 1/5	6.50	1.940	2.98E-01
	6.50	2.138	3.29E-01
	32.50	10.423	3.21E-01
Dilution 1/1	32.50	10.928	3.36E-01
	32.50	10.500	3.23E-01
		Average RF	3.10E-01
		SD	2.00E-02
		RSD	6%

Table 45: Standard solution repeatability for cholesterol.

Cholesterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
	12.44	1.989	1.60E-01
Dilution 1/20	12.44	1.879	1.51E-01
	12.44	1.859	1.49E-01
	49.75	8.000	1.61E-01
Dilution 1/5	49.75	7.943	1.60E-01
	49.75	7.554	1.52E-01
	248.73	41.585	1.67E-01
Dilution 1/1	248.73	41.487	1.67E-01
	248.73	41.750	1.68E-01
		Average RF	1.59E-01
		SD	7.19E-03
		RSD	5%

Table 46: Standard solution repeatability for desmosterol.

Desmosterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
	2.10	0.718	3.42E-01
Dilution 1/20	2.10	0.757	3.60E-01
	2.10	0.808	3.85E-01
	8.40	3.216	3.83E-01
Dilution 1/5	8.40	3.351	3.99E-01
	8.40	3.440	4.10E-01
	42.00	17.032	4.06E-01
Dilution 1/1	42.00	16.668	3.97E-01
	42.00	17.202	4.10E-01
		Average RF	3.88E-01
		SD	2.33E-02
		RSD	6%

Table 47: Standard solution repeatability for ergosterol.

Ergosterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
	6.04	1.475	2.44E-01
Dilution 1/20	6.04	1.362	2.26E-01
	6.04	1.502	2.49E-01
	24.15	5.701	2.36E-01
Dilution 1/5	24.15	6.026	2.50E-01
	24.15	5.624	2.33E-01
	120.75	28.790	2.38E-01
Dilution 1/1	120.75	30.097	2.49E-01
	120.75	28.730	2.38E-01
		Average RF	2.40E-01
		SD	8.30E-03
		RSD	3%

Table 48: Standard solution repeatability for lanosterol.

Lanosterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
	2.33	0.823	3.54E-01
Dilution 1/20	2.33	0.969	4.17E-01
	2.33	0.876	3.77E-01
	9.30	4.044	4.35E-01
Dilution 1/5	9.30	3.378	3.63E-01
	9.30	4.022	4.32E-01
	46.50	19.831	4.26E-01
Dilution 1/1	46.50	19.073	4.10E-01
	46.50	19.908	4.28E-01
		Average RF	4.05E-01
		SD	3.15E-02
		RSD	8%

Table 49: Standard solution repeatability for lathosterol.

Lathosterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
	2.48	1.313	5.31E-01
Dilution 1/20	2.48	1.334	5.39E-01
	2.48	1.284	5.19E-01
	9.90	5.526	5.58E-01
Dilution 1/5	9.90	5.815	5.87E-01
	9.90	5.432	5.49E-01
	49.50	29.691	6.00E-01
Dilution 1/1	49.50	28.085	5.67E-01
	49.50	29.973	6.06E-01
		Average RF	5.62E-01
		SD	3.08E-02
		RSD	5%

Table 50: Standard solution repeatability for squalene.

Squalene	Concentration (µg/mL)	Area (mAU*min)	Response factor
	24.90	49.299	1.98E+00
Dilution 1/20	24.90	49.430	1.99E+00
	24.90	49.173	1.97E+00
	99.59	191.669	1.92E+00
Dilution 1/5	99.59	190.408	1.91E+00
	99.59	192.051	1.93E+00
	497.97	834.550	1.68E+00
Dilution 1/1	497.97	817.049	1.64E+00
	497.97	821.640	1.65E+00
		Average RF	1.85E+00
		SD	1.50E-01
		RSD	8%

Table 51: Standard solution repeatability for stigmasterol.

Stigmasterol	Concentration (µg/mL)	Area (mAU*min)	Response factor
	6.10	1.228	2.01E-01
Dilution 1/20	6.10	1.274	2.09E-01
	6.10	1.165	1.91E-01
	24.41	5.434	2.23E-01
Dilution 1/5	24.41	4.747	1.95E-01
	24.41	4.769	1.95E-01
	122.03	26.794	2.20E-01
Dilution 1/1	122.03	24.522	2.01E-01
	122.03	26.324	2.16E-01
		Average RF	2.06E-01
		SD	1.16E-02
		RSD	6%

7.1.3.6 Accuracy

Accuracy was studied to confirm trueness of determined concentrations of phytosterols. Phytosterol standard solution at dilutions 1/20, 1/5 and 1/1 were analysed and mean percentage of recovery was calculated. The results are presented from Table 52 to Table 61 for each phytosterol separately.

Recoveries of all phytosterols were within established limits (80-110%) and statistically evaluated with t-student test and Cochran's Q test to study influence of different concentration levels on accuracy. There was observed no statistically significant difference between the recoveries ($t_{exp} < 2.306$ and $G_{exp} < 0.871$; $p < 0.05$) for both tests.

Method was confirmed accurate for determination of phytosterols.

Table 52: Accuracy for β-sitosterol.

β-sitosterol	**Added concentration (µg/mL)**	**Recovered concentration (µg/mL)**	**Recovery (%)**
Dilution 1/20	8.93	8.57	95.94
	8.93	8.97	100.46
	8.93	9.13	102.29
Dilution 1/5	35.71	35.04	98.12
	35.71	36.49	102.18
	35.71	35.53	99.49
Dilution 1/1	178.57	180.73	101.21
	178.57	178.43	99.92
	178.57	179.28	100.40
		Average recovery (%)	100.00
		SD	2.00
		RSD	2%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.695$	$G_{crit} = 0.871$	$p < 0.05$

Table 53: Accuracy for brassicasterol.

Brassicasterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	2.45	2.39	97.75
	2.45	2.26	92.34
	2.45	2.31	94.14
Dilution 1/5	9.80	10.76	109.81
	9.80	9.47	96.66
	9.80	9.45	96.40
Dilution 1/1	49.00	51.80	105.71
	49.00	51.57	105.25
	49.00	49.95	101.93
		Average recovery (%)	100.00
		SD	5.94
		RSD	6%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.832$	$G_{crit} = 0.871$	$p < 0.05$

Table 54: Accuracy for campesterol.

Campesterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	1.63	1.42	87.63
	1.63	1.60	98.16
	1.63	1.55	95.38
Dilution 1/5	6.50	6.49	99.80
	6.50	6.26	96.37
	6.50	6.90	106.21
Dilution 1/1	32.50	33.66	103.56
	32.50	35.29	108.57
	32.50	33.90	104.32
		Average recovery (%)	100.00
		SD	6.46
		RSD	6%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.480$	$G_{crit} = 0.871$	$p < 0.05$

Table 55: Accuracy for cholesterol.

Cholesterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	12.44	12.48	100.33
	12.44	11.79	94.78
	12.44	11.66	93.77
Dilution 1/5	49.75	50.19	100.88
	49.75	49.83	100.16
	49.75	47.39	95.26
Dilution 1/1	248.73	260.88	104.88
	248.73	260.26	104.63
	248.73	261.91	105.30
		Average recovery (%)	100.00
		SD	4.51
		RSD	5%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.568$	$G_{crit} = 0.871$	$p < 0.05$

Table 56: Accuracy for desmosterol.

Desmosterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	2.10	1.85	88.16
	2.10	1.95	92.95
	2.10	2.08	99.21
Dilution 1/5	8.40	8.29	98.72
	8.40	8.64	102.86
	8.40	8.87	105.60
Dilution 1/1	42.00	43.92	104.56
	42.00	42.98	102.33
	42.00	44.36	105.61
		Average recovery (%)	100.00
		SD	6.01
		RSD	6%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.675$	$G_{crit} = 0.871$	$p < 0.05$

Table 57: Accuracy for ergosterol.

Ergosterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	6.04	6.14	101.66
	6.04	5.67	93.88
	6.04	6.25	103.53
Dilution 1/5	24.15	23.72	98.24
	24.15	25.08	103.84
	24.15	23.40	96.91
Dilution 1/1	120.75	119.80	99.22
	120.75	125.24	103.72
	120.75	119.55	99.01
		Average recovery (%)	100.00
		SD	3.46
		RSD	3%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.560$	$G_{crit} = 0.871$	$p < 0.05$

Table 58: Accuracy for lanosterol.

Lanosterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	2.33	2.03	87.45
	2.33	2.39	102.97
	2.33	2.16	93.09
Dilution 1/5	9.30	9.99	107.43
	9.30	8.35	89.74
	9.30	9.94	106.85
Dilution 1/1	46.50	48.99	105.36
	46.50	47.12	101.34
	46.50	49.18	105.77
		Average recovery (%)	100.00
		SD	7.79
		RSD	8%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.599$	$G_{crit} = 0.871$	$p < 0.05$

Table 59: Accuracy for lathosterol.

Lathosterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	2.48	2.34	94.45
	2.48	2.37	95.96
	2.48	2.29	92.36
Dilution 1/5	9.90	9.84	99.37
	9.90	10.35	104.57
	9.90	9.67	97.68
Dilution 1/1	49.50	52.86	106.79
	49.50	50.00	101.01
	49.50	53.36	107.80
		Average recovery (%)	100.00
		SD	5.48
		RSD	5%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.454$	$G_{crit} = 0.871$	$p < 0.05$

Table 60: Accuracy for squalene.

Squalene	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	24.90	26.61	106.89
	24.90	26.68	107.17
	24.90	26.55	106.62
Dilution 1/5	99.59	103.47	103.89
	99.59	102.79	103.21
	99.59	103.68	104.10
Dilution 1/1	497.97	450.53	90.47
	497.97	441.08	88.58
	497.97	443.56	89.07
		Average recovery (%)	100.00
		SD	8.11
		RSD	8%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.767$	$G_{crit} = 0.871$	$p < 0.05$

Table 61: Accuracy for stigmasterol.

Stigmasterol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
Dilution 1/20	6.10	5.97	97.92
	6.10	6.20	101.59
	6.10	5.67	92.90
Dilution 1/5	24.41	26.44	108.33
	24.41	23.10	94.63
	24.41	23.20	95.07
Dilution 1/1	122.03	130.36	106.83
	122.03	119.31	97.77
	122.03	128.07	104.96
		Average recovery (%)	100.00
		SD	5.66
		RSD	6%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.591$	$G_{crit} = 0.871$	$p < 0.05$

7.1.3.7 Robustness

Robustness of analytical method was studied to determine the response of the instrumental system to variations of chromatographic conditions.

Phytosterol standard solution was analysed using small variations of detector wavelength, temperature of the column, injection volume and different HPLC instruments. Optimal conditions were fixed at wavelength of 210 nm, column temperature of 30°C, injection volume of 30 µL and the use of chromatograph Dionex UltiMate 3000. The obtained data were evaluated for statistically significant influence.

Variation of the wavelength

The detection wavelength was modified from 207 nm to 213 nm, with reference at 210 nm. Results are presented in Table 62 and statistical evaluation, using ANOVA test, showed no significant differences (F_{exp} = 1.427, F_{crit} = 2.456; $p < 0.05$) between the recoveries obtained under studied detector conditions.

Method was robust within the interval 210 ± 3 nm in detector wavelength for determination of phytosterols.

Table 62: Wavelength robustness data for phytosterols.

Phytosterols	**Recovery (%)**		
	207 nm	**210 nm**	**213 nm**
β-sitosterol	99.79	99.69	99.71
brassicasterol	99.12	100.40	100.01
campesterol	99.64	100.38	101.17
cholesterol	98.64	100.50	100.33
desmosterol	99.32	99.00	98.37
ergosterol	100.01	99.40	99.39
lanosterol	99.37	99.72	100.42
lathosterol	99.74	99.26	100.51
squalene	99.85	99.86	99.86
stigmasterol	99.54	99.91	100.63
Average recovery (%)	99.50	99.81	100.04
SD	0.41	0.51	0.78
RSD	0.4%	0.5%	0.8%
ANOVA	F_{exp} = 1.427	F_{crit} = 2.456	$p < 0.05$

Variation of the column temperature

The column temperature was modified from 27°C to 33°C, with reference at 30°C. Results are presented in Table 63 and statistical evaluation, using ANOVA test, showed no

significant differences between the obtained recoveries (F_{exp} = 1.423, F_{crit} = 2.456; $p < 0.05$), when minor variations to temperature were applied.

Method was robust within the interval 30 ± 3ºC in the temperature of the column for determination of phytosterols.

Table 63: Column temperature robustness data for phytosterols.

Phytosterols	**Recovery (%)**		
	27°C	**30°C**	**33°C**
β-sitosterol	99.86	99.69	99.87
brassicasterol	98.87	100.40	99.59
campesterol	100.70	100.38	100.57
cholesterol	101.49	100.50	100.38
desmosterol	101.45	99.00	100.07
ergosterol	100.02	99.40	99.94
lanosterol	99.91	99.72	100.22
lathosterol	100.19	99.26	99.98
squalene	100.24	99.86	100.00
stigmasterol	99.08	99.91	99.88
Average recovery (%)	100.18	99.81	100.05
SD	0.86	0.51	0.28
RSD	0.9%	0.5%	0.3%
ANOVA	F_{exp} = 1.423	F_{crit} = 2.456	$p < 0.05$

Variation of the injection volume

The volume varied from 25 µL to 35 µL, with reference at 30 µL. Results are presented in Table 64 and statistical evaluation, using ANOVA test, showed no significant differences between the obtained recoveries (F_{exp} = 1.207, F_{crit} = 2.456; $p < 0.05$), when minor variations in the volume of injection of the samples were applied.

Method was robust within the interval 30 ± 5 μL in the volume of injection of the samples for determination of phytosterols.

Table 64: Injection volume robustness data for phytosterols.

Phytosterols	**Recovery (%)**		
	25 μL	**30 μL**	**35 μL**
β-sitosterol	100.26	99.69	99.47
brassicasterol	99.77	100.40	98.66
campesterol	99.22	100.38	99.72
cholesterol	97.92	100.50	99.65
desmosterol	99.51	99.00	100.32
ergosterol	99.01	99.40	99.69
lanosterol	98.81	99.72	99.59
lathosterol	96.55	99.26	99.19
squalene	100.01	99.86	100.30
stigmasterol	99.82	99.91	99.45
Average recovery (%)	99.09	99.81	99.60
SD	1.12	0.51	0.49
RSD	1.1%	0.5%	0.5%
ANOVA	$F_{exp} = 1.207$	$F_{crit} = 2.456$	$p < 0.05$

Variation of the HPLC

Variation of HPLC instrument was performed on Dionex UltiMate 3000 (CQ 52) and Agilent 1100 (CQ 3). Results are presented in Table 65 and statistical evaluation, using t-student test, showed no significant differences between the obtained recoveries ($t_{exp} = 0.147$, $t_{crit} = 1.734$; $p < 0.05$), when analysis was performed at two different HPLC (Dionex UltiMate 3000 and Agilent 1100).

Method was robust to different HPLC equipment for determination of phytosterols.

Table 65: HPLC robustness data for phytosterols.

Phytosterols	**Recovery (%)**		
	Dionex UltiMate 3000 (CQ 52)		**Agilent 1100 (CQ 3)**
β-sitosterol	99.69		99.99
brassicasterol	100.40		99.34
campesterol	100.38		99.91
cholesterol	100.50		99.11
desmosterol	99.00		99.92
ergosterol	99.40		99.71
lanosterol	99.72		99.81
lathosterol	99.26		100.67
squalene	99.86		100.02
stigmasterol	99.91		99.95
Average recovery (%)	99.81		99.84
SD	0.51		0.42
RSD	0.5%		0.4%
t-student test	$t_{exp} = 0.147$	$t_{crit} = 1.734$	$p < 0.05$

7.1.4 Validation study of tocopherols

Validation of tocopherols was performed in the laboratory of Pharmaceutical Analysis at KU Leuven on chomatograph VWR Hitachi ELITE LaChrom with software EZChrom Elite to prove inter-laboratory precision and robustness of the method.
Analytical procedure and acceptance criteria were the same as in validation of phytosterols.

7.1.4.1 System suitability

System suitability was verified as established in validation of phytosterols and chromatographic parameters were studied with software EZChrom Elite. The obtained results are presented in Table 66.

The chromatographic system complied the established acceptance criteria and was considered suitable for method validation.

Table 66: System suitability data for validation of tocopherols.

Chromatographic parameter	Acceptance criteria	Result
Capacity factor	≥ 1.5	43.3
Tailing factor	0.8-1.5	1.5
Theoretical plates	> 2000	40220
Resolution	≥ 1	63.3
Retention time of β-sitosterol	~43 min	44.3 min

7.1.4.2 Stability of the standard solution

Standard solution of tocopherols was maintained at room temperature (25 ± 2ºC) and analysed according to established times of analysis, from 0 to 7 days. Results obtained at 0h were considered as 100% recovery.

The obtained data are presented in Table 67 and Figure 42.

Percentage of recovery of tocopherol standard solution remained within the established limits (80-110%) and was considered stable during a period of 7 days at room temperature.

Table 67: Stability data of tocopherol standard solution.

Tocopherols	Recovery (%)			
	0h	4h	8h	7 days
α-tocopherol	100.00	103.59	100.82	100.38
γ-tocopherol	100.00	104.45	102.09	103.66
δ-tocopherol	100.00	103.93	101.87	103.76

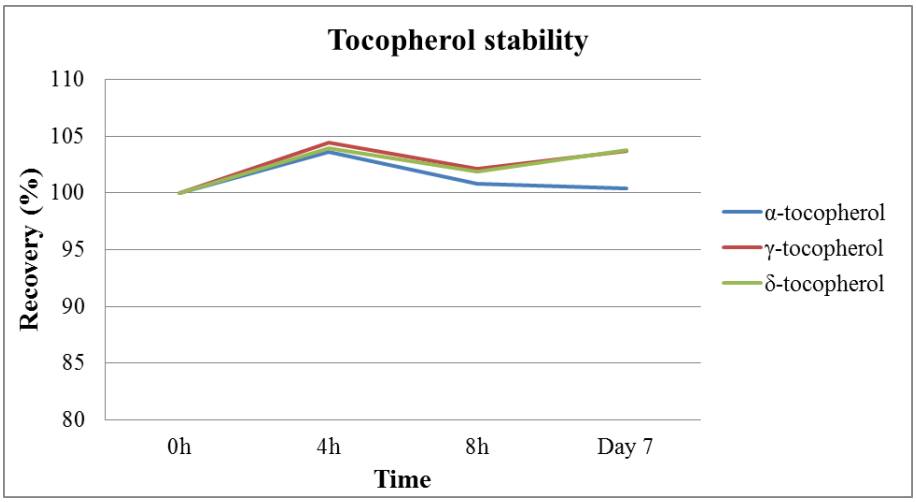

Figure 42: Stability of standard solution of tocopherols.

7.1.4.3 Selectivity

To investigate method selectivity for tocopherols, blank, individual solutions of tocopherols, mixture of tocopherols as well as mixture of tocopherols and phytosterols, were analysed. Obtained chromatograms are presented from Figure 43 to Figure 49.

Individual tocopherols were well separated in both mixtures and no interference with blank was observed. For each tocopherol was determined UV maximum and relative retention time, relative to ergosterol (Table 68). Absorption maxima varied from 199 nm to 201 nm, depending from chemical structure of each isomer. Retention time corresponded to substitutions on phenyl ring, with the shortest retention in δ-tocopherol (RRT = 1.29) and the longest in α-tocopherol (RRT = 1.70).

Method was proven to be selective for tocopherols and proper for their quantification.

Table 68: UV maxima and relative retention times of tocopherols.

Tocopherol	UV maximum (nm)	Relative retention time
α-tocopherol	201	1.70
γ-tocopherol	199	1.50
δ-tocopherol	200	1.29

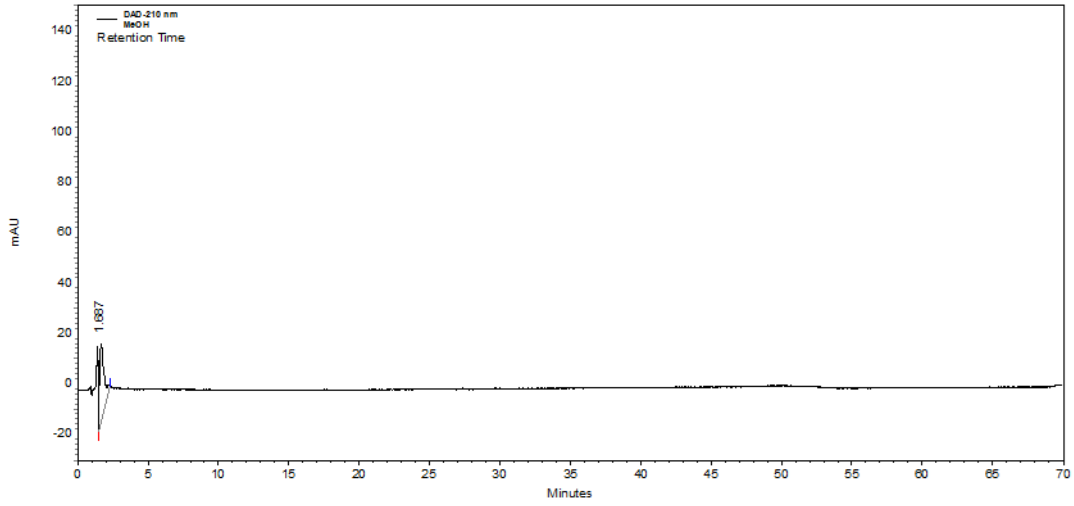

Figure 43: Chromatogram of blank (MeOH).

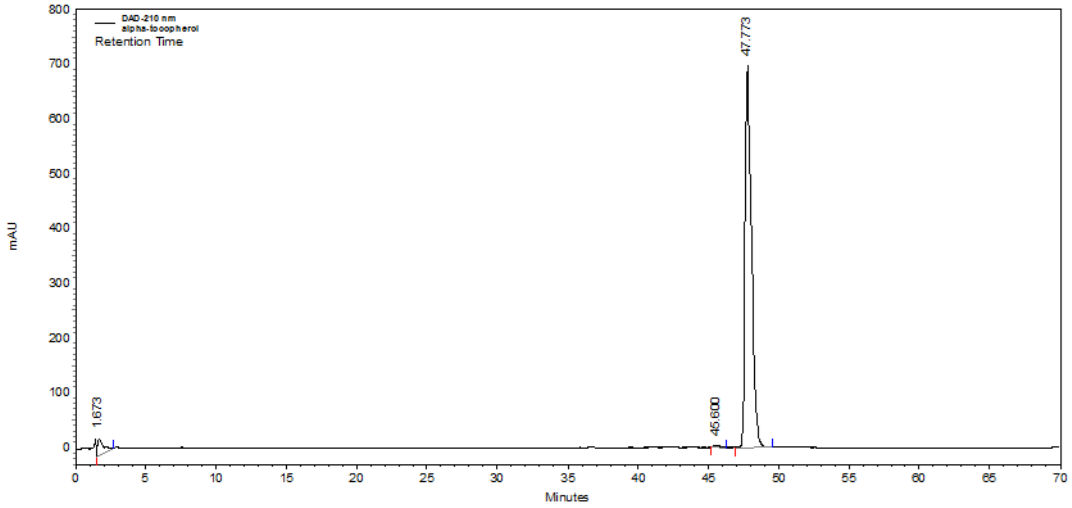

Figure 44: Chromatogram of α-tocopherol.

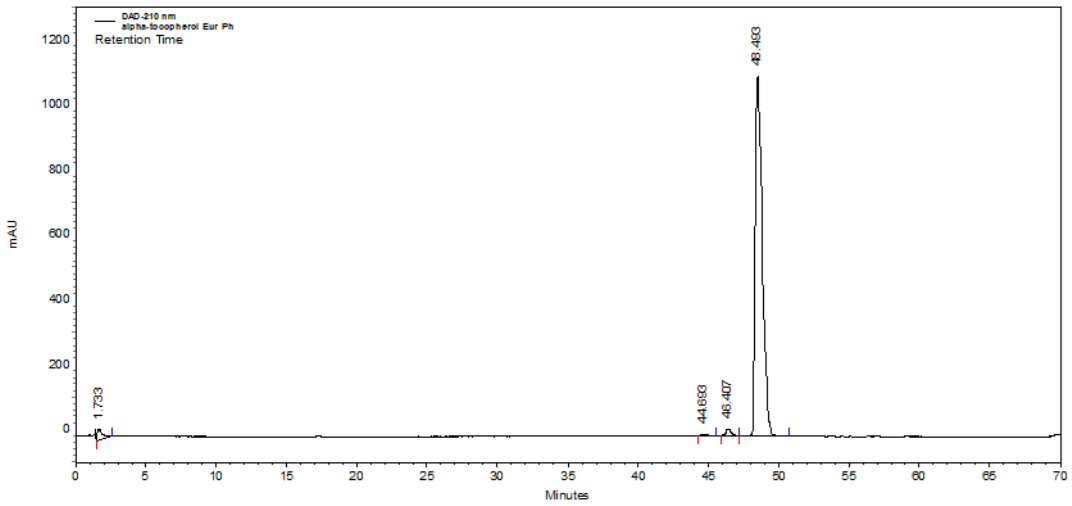

Figure 45: Chromatogram of α-tocopherol Eur. Ph. standard.

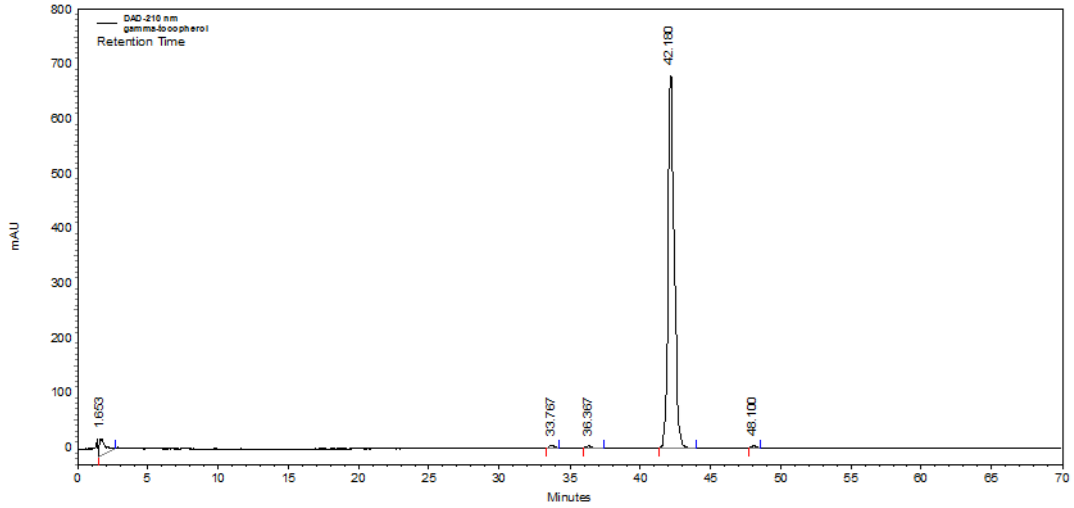

Figure 46: Chromatogram of γ-tocopherol.

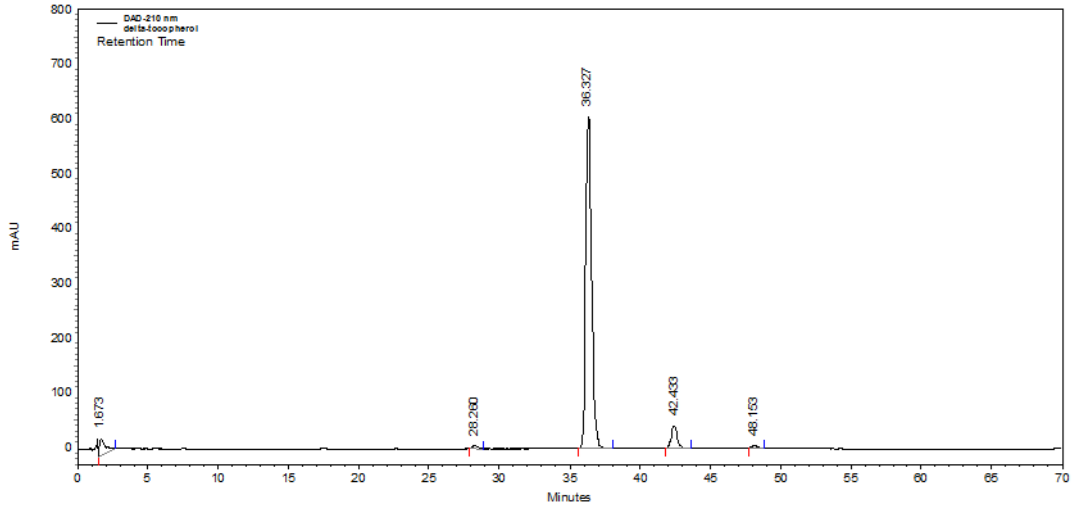

Figure 47: Chromatogram of δ-tocopherol.

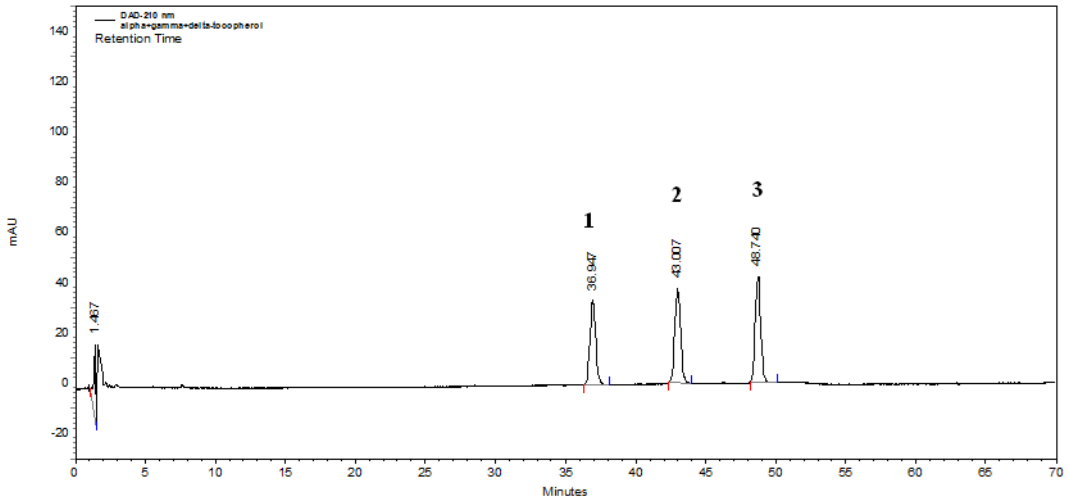

Figure 48: Chromatogram of the mixture of tocopherols.

Identified peas are: 1 – δ-tocopherol, 2 – γ-tocopherol, 3 – α-tocopherol.

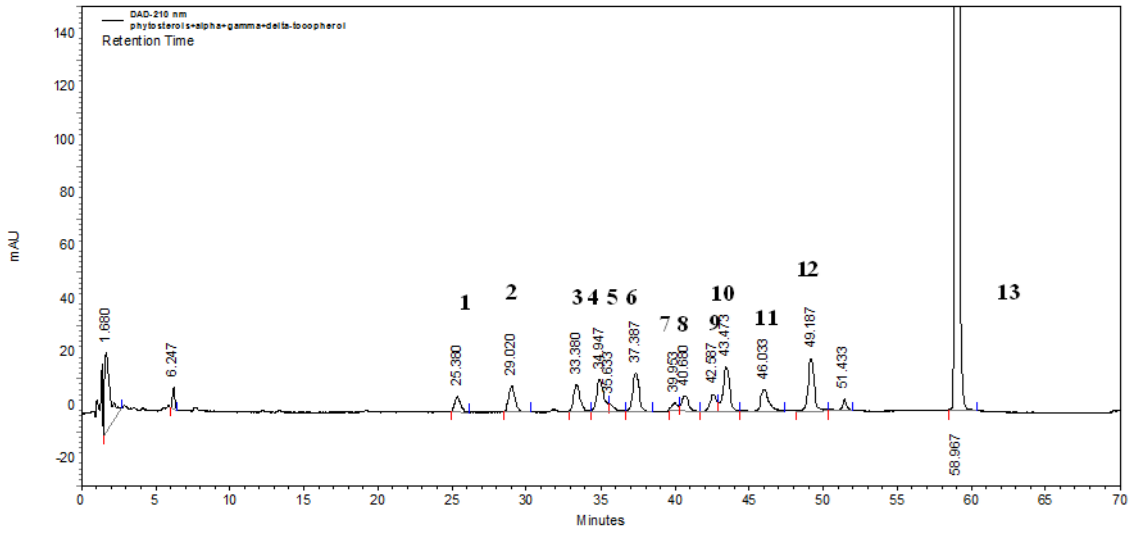

Figure 49: Chromatogram of the mixture of phytosterols and tocopherols.

Identified peaks are: 1 – desmosterol, 2 – ergosterol, 3 – lathosterol, 4 –cholesterol, 5 – brassicasterol, 6 – δ-tocopherol, 7 – campesterol, 8 – lanosterol, 9 – stigmasterol, 10 – γ-tocopherol, 11 – β-sitosterol, 12 –α-tocopherol, 13 – squalene.

7.1.4.4 Linearity

Tocopherol standard solution of 500 µg/mL (with dilutions 1/1, 1/2, 1/5, and 1/25) and 300 µg/mL mL (with dilutions 1/1, 1/2, 1/5, 1/10 and 1/20), were prepared in triplicate to establish calibration curves with minimum five points for each tocopherol. Mean linearities and corresponding statistical evaluations are presented from Table 69 to Table 74 and from Figure 50 to Figure 52.

All tocopherols demonstrated a good correlation ($R > 0.999$ and $R^2 > 0.990$) between concentration and mean peak area. Good fitness of data was confirmed with confidence intervals of intercept, passing the zero, as well as slope confidence interval, different from zero, in all tocopherols.

Isomers α- and δ- tocopherol showed good correlation between mean peak areas at different concentrations. Deviation of response factors complied established acceptance criteria (response factor RSD < 2%). Higher variation of response factors in γ-tocopherol was attributed to preparation errors and detection at low concentrations.

Method was proved linear for determination of tocopherols.

Table 69: Data of α-tocopherol linearity.

	Concentration (µg/mL)	**Mean area (mAU*min)**	**Response factor**
α-tocopherol	15.26	4860431	3.18E+05
	20.35	6556370	3.22E+05
	30.53	10046430	3.29E+05
	61.06	20387941	3.34E+05
	101.76	33344621	3.28E+05
	152.65	49917594	3.27E+05
	254.41	82928072	3.26E+05
	305.29	100015567	3.28E+05
	508.82	162641333	3.20E+05
R	0.9999	**Average RF**	3.26E+05
R^2	0.9998	**SD**	4.88E+03
Intercept	569536	**RSD**	1.50%
Slope	321052		

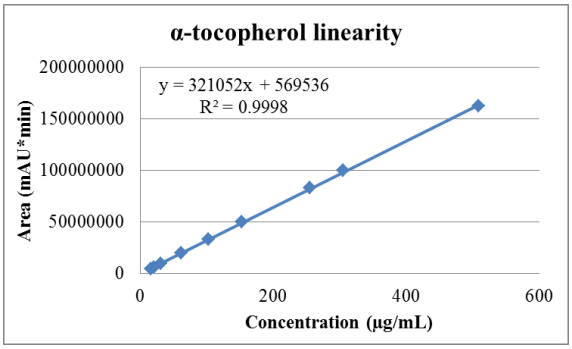

Figure 50: Graph of α-tocopherol linearity.

Table 70: Statistical data of α-tocopherol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999888328
R Square	0.999776668
Adjusted R Square	0.999744764
Standard Error	854792.362
Observations	9

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2.28967E+16	2.28967E+16	31336.53763	4.84501E-14
Residual	7	5.11469E+12	7.3067E+11		
Total	8	2.29018E+16			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	569535.8949	408144.3124	1.395427739	0.205556299	-395572.0444	1534643.834	-395572.0444	1534643.834
X Variable 1	321051.6498	1813.632966	177.0212915	4.84501E-14	316763.0893	325340.2103	316763.0893	325340.2103

Table 71: Data of γ-tocopherol linearity.

	Concentration (µg/mL)	**Mean area (mAU*min)**	**Response factor**
γ-tocopherol	1.23	548084	4.47E+05
	2.45	1223960	5.00E+05
	3.92	1486620	3.79E+05
	4.9	2552182	5.21E+05
	19.6	7737793	3.95E+05
	49	19284579	3.94E+05
	98	38887667	3.97E+05
R	0.9999	**Average RF**	4.33E+05
R^2	0.9997	**SD**	5.71E+04
intercept	164742	**RSD**	13.19%
slope	394024		

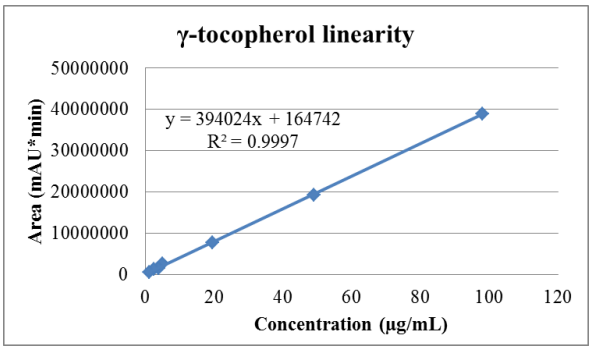

Figure 51: Graph of γ-tocopherol linearity.

Table 72: Statistical data of γ-tocopherol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999858071
R Square	0.999716163
Adjusted R Square	0.999659395
Standard Error	263133.6297
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.21935E+15	1.21935E+15	17610.72322	4.60889E-10
Residual	5	3.46197E+11	69239307089		
Total	6	1.2197E+15			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	164742.0934	125148.5629	1.316372235	0.245160713	-156962.5292	486446.716	-156962.5292	486446.716
X Variable 1	394023.7826	2969.161634	132.7054001	4.60889E-10	386391.3096	401656.2556	386391.3096	401656.2556

Table 73: Data of δ-tocopherol linearity.

	Concentration (µg/mL)	**Mean area (mAU*min)**	**Response factor**
δ-tocopherol	13.89	3629739	2.61E+05
	18.52	4876415	2.63E+05
	27.78	7502504	2.70E+05
	55.57	15249470	2.74E+05
	92.61	24845278	2.68E+05
	138.92	37206697	2.68E+05
	231.53	61693856	2.66E+05
	277.84	74630989	2.69E+05
	463.07	120392794	2.60E+05
R	0.9998	**Average RF**	2.67E+05
R^2	0.9996	**SD**	4.53E+03
intercept	548253	**RSD**	1.70%
slope	261486		

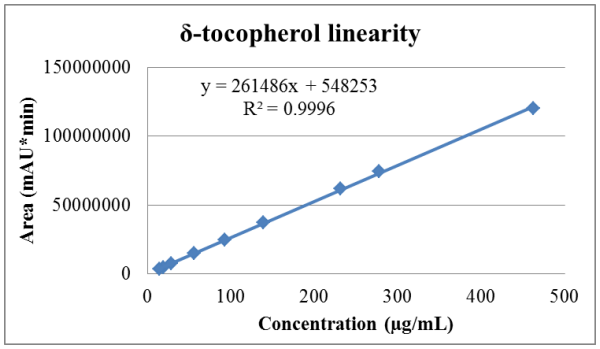

Figure 52: Graph of δ-tocopherol linearity.

Table 74: Statistical data of δ-tocopherol linearity.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999810677
R Square	0.99962139
Adjusted R Square	0.999567303
Standard Error	825020.4421
Observations	9

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.25797E+16	1.25797E+16	18481.68768	3.07375E-13
Residual	7	4.76461E+12	6.80659E+11		
Total	8	1.25845E+16			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	548252.5477	393928.8839	1.391755137	0.206618214	-383241.2445	1479746.34	-383241.2445	1479746.34
X Variable 1	261485.9503	1923.435138	135.947371	3.07375E-13	256937.7489	266034.1517	256937.7489	266034.1517

Obtained data from linearity were used to establish concentration range for tocopherols, used in validated analytical method (Table 75). Limits were set according to their potential assay in lipid emulsions.

Table 75: Concentration range of tocopherols.

Tocopherols	**Concentration interval (µg/mL)**
α-tocopherol	15-500
γ-tocopherol	1.25-100
δ-tocopherol	15-500

7.1.4.5 Precision

Repeatability of the instrumental system

To investigate the repeatability of the instrumental system, 10 consecutive injections of the standard solutions at different concentrations: 500 µg/mL, 100 µg/mL and 15 µg/mL, were analysed on two different days. The results are presented in Table 76.

Variation of instrumental system repeatability was higher than established acceptance criteria for day precision (RSD < 1%) and for interday precision (RSD < 2%). Deviations, attributed to the transfer of analytical method, were low enough to consider instrumental system as repeatable and precise for validation of tocopherols.

Table 76: Instrumental system repeatability data for tocopherols.

Tocopherol	Day precision RSD (%)			Interday precision RSD (%)		
	15 µg/mL	**100 µg/mL**	**500 µg/mL**	**15 µg/mL**	**100 µg/mL**	**500 µg/mL**
α-tocopherol	1.9%	1.4%	1.2%	3.0%	2.2%	2.0%
γ-tocopherol	1.3%	1.3%	1.3%	3.4%	3.1%	3.2%
δ-tocopherol	5.4%	1.3%	1.4%	8.3%	2.9%	3.1%

Repeatability of the method

Method repeatability included repeatability of tocopherol standard solution at three different concentration levels and repeatability of the test solution on two different days.

Repeatability of the standard solution was investigated by analysing tocopherol standard solution, corresponding to the concentrations 15 µg/mL, 100 µg/mL and 500 µg/mL, and calculating response factors. The results are presented from Table 77 to Table 79 for each tocopherol separately.

Variation of response factors was lower than 11% for α- and δ-tocopherol, which complied acceptance criteria, whereas deviation of γ-tocopherol was slightly higher, which was attributed to the lower concentrations.

Method was proven repeatable and precise for determination of tocopherols.

Table 77: Standard solution repeatability for α-tocopherol.

α-tocopherol	Concentration (µg/mL)	Area (mAU*min)	Response factor
15 µg/mL	15.25	4842748	3.17E+05
	15.27	4939746	3.23E+05
	15.27	4798801	3.14E+05
100 µg/mL	101.70	33363229	3.28E+05
	101.80	32911090	3.23E+05
	101.80	33759545	3.32E+05
500 µg/mL	508.49	165135844	3.25E+05
	508.99	161773768	3.18E+05
	508.99	161014386	3.16E+05
		Average RF	3.22E+05
		SD	5.82E+03
		RSD	1.81%

Table 78: Standard solution repeatability for γ-tocopherol.

γ-tocopherol	Concentration (µg/mL)	Area (mAU*min)	Response factor
1.25 µg/mL	1.23	432847	3.53E+05
	1.23	653917	5.34E+05
	1.23	557487	4.55E+05
20 µg/mL	19.60	7818178	3.99E+05
	19.60	7911697	4.04E+05
	19.60	7483504	3.82E+05
100 µg/mL	98.00	38580868	3.94E+05
	98.00	37641283	3.84E+05
	98.00	40440850	4.13E+05
		Average RF	4.13E+05
		SD	5.29E+04
		RSD	12.81%

Table 79: Standard solution repeatability for δ-tocopherol.

δ-tocopherol	Concentration (µg/mL)	Area (mAU*min)	Response factor
15 µg/mL	13.74	3605249	2.62E+05
	13.91	3665753	2.64E+05
	14.02	3618217	2.58E+05
100 µg/mL	91.63	24853722	2.71E+05
	92.74	24531583	2.65E+05
	93.47	25150530	2.69E+05
500 µg/mL	458.16	123382775	2.69E+05
	463.68	120624402	2.60E+05
	467.36	120087884	2.57E+05
		Average RF	2.64E+05
		SD	5.11E+03
		RSD	1.94%

To investigate the repeatability of the test solution, there were prepared six samples of lipid emulsion, according to the sample preparation, on two different days. Results are presented in Table 80.

From the analysed lipid emulsion, only α-tocopherol was extracted and quantified. Test solution repeatability showed low variability in sample preparation between two different days and complied acceptance criteria (RSD < 11%).

The method was proven repeatable and precise for determination of tocopherols.

Table 80: Test solution repeatability for α-tocopherol.

α-tocopherol	Concentration (µg/mL)	Area (mAU*min)	Response factor
Day 1	162.6	24489235	1.51E+05
	166.8	24210574	1.45E+05
	165.9	25890136	1.56E+05
	165.3	24693887	1.49E+05
	165.8	24648263	1.49E+05
	162.3	23966751	1.48E+05
Day 2	148.7	23113711	1.55E+05
	149.6	22603096	1.51E+05
	147.7	24224515	1.64E+05
	153.7	24054466	1.56E+05
	150.6	23257716	1.54E+05
	152.1	23434388	1.54E+05
		Average RF	1.53E+05
		SD	5.06E+03
		RSD	3.3%

7.1.4.6 Accuracy

Tocopherol standard solution was prepared at concentrations 15 µg/mL, 100 µg/mL and 500 µg/mL, analysed and mean percentage of recovery was calculated. The results are presented from Table 81 to Table 83 for each tocopherol separately.

Recoveries of α- and δ-tocopherol were within established limits (80-110%), meanwhile γ-tocopherol showed higher deviation in percentage of recovery, which was attributed to integration at lower concentrations. Statistical study of concentration influence on accuracy, with t-student test and Cochran's Q test, confirmed no statistically significant difference between the recoveries ($t_{exp} < 2.306$ and $G_{exp} < 0.871$; $p < 0.05$) for α- and δ-tocopherol for both tests. In γ-tocopherol, t-student test showed no significant difference. However, more sensitive Cochran's Q test detected the difference in recoveries at different concentration levels.

Analytical method was confirmed accurate for determination of tocopherols.

Table 81: Accuracy for α-tocopherol.

α-tocopherol	**Added concentration (µg/mL)**	**Recovered concentration (µg/mL)**	**Recovery (%)**
15 µg/mL	15.25	15.04	98.62
	15.27	15.35	100.50
	15.27	14.91	97.63
100 µg/mL	101.70	103.64	101.91
	101.80	102.24	100.43
	101.80	104.87	103.02
500 µg/mL	508.49	512.99	100.89
	508.99	502.55	98.73
	508.99	500.19	98.27
		Average recovery (%)	100.00
		SD	1.81
		RSD	1.81%
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.368$	$G_{crit} = 0.871$	$p < 0.05$

Table 82: Accuracy for γ-tocopherol.

γ-tocopherol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
1.25 µg/mL	1.23	0.95	77.65
	1.23	1.44	117.31
	1.23	1.23	100.01
5 µg/mL	4.90	5.65	115.21
	4.90	5.84	119.16
	4.90	5.34	109.03
100 µg/mL	98.00	84.79	86.52
	98.00	82.72	84.41
	98.00	88.88	90.69
		Average recovery (%)	100.00
		SD	15.77
		RSD	15.77
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.916$	$G_{crit} = 0.871$	$p < 0.05$

Table 83: Accuracy for δ-tocopherol.

δ-tocopherol	Added concentration (µg/mL)	Recovered concentration (µg/mL)	Recovery (%)
15 µg/mL	13.74	13.66	99.39
	13.91	13.89	99.86
	14.02	13.71	97.79
100 µg/mL	91.63	94.18	102.78
	92.74	92.96	100.24
	93.47	95.30	101.96
500 µg/mL	458.16	467.53	102.05
	463.68	457.08	98.58
	467.36	455.05	97.37
		Average recovery (%)	100.00
		SD	1.94
		RSD	1.94
t-student test	$t_{exp} = 0$	$t_{crit} = 2.306$	$p < 0.05$
Cochran's test	$G_{exp} = 0.674$	$G_{crit} = 0.871$	$p < 0.05$

7.1.4.7 Robustness

Method robustness for tocopherols was based on previously studied parameters, used in validation of phytosterols. Tocopherol standard solution was analysed using small variations of detector wavelength, temperature of the column, injection volume and different HPLC instruments.

Optimal conditions were fixed at wavelength of 210 nm, column temperature of 30°C, injection volume of 30 μL and the use of chromatograph Dionex UltiMate 3000. The obtained data were evaluated for statistically significant influence.

Variation of the wavelength

The detection wavelength was modified from 207 nm to 213 nm, with reference at 210 nm. Results are presented in Table 84 and statistical evaluation, using ANOVA test, showed no significant differences between the obtained recoveries (F_{exp} = 0.622, F_{crit} = 3.403; $p < 0.05$), when minor variations in the detector wave length were applied.

Method is robust within the interval 210 ± 3 nm in detector wavelength for determination of tocopherols.

Table 84: Wavelength robustness data for tocopherols.

Tocopherols	**Recovery (%)**		
	207 nm	**210 nm**	**213 nm**
α-tocopherol	100.52	100.45	99.52
	100.44	100.43	99.74
	99.04	100.46	100.75
γ-tocopherol	99.94	100.00	99.43
	100.36	100.25	99.67
	99.71	100.72	100.90
δ-tocopherol	99.49	99.44	99.38
	99.87	99.68	99.68
	100.64	100.88	100.94
Average recovery (%)	100.00	100.26	100.00
SD	0.53	0.47	0.66
RSD	0.53%	0.47%	0.66%
ANOVA	F_{exp} = 0.622	F_{crit} = 3.403	$p < 0.05$

Variation of the column temperature

The column temperature was modified from 27°C to 33°C, with reference at 30°C. The obtained results are presented in Table 85 and statistical evaluation, using ANOVA test, showed no significant differences between the obtained recoveries (F_{exp} = 1.284, F_{crit} = 3.403; $p < 0.05$), when minor variations to temperature were applied.

Method is robust within the interval 30 ± 3ºC in the temperature of the column for determination of tocopherols.

Table 85: Column temperature robustness data for tocopherols.

Tocopherols	**Recovery (%)**		
	27°C	**30°C**	**33°C**
α-tocopherol	98.17	100.45	100.25
	100.11	100.43	101.32
	99.25	100.46	99.56
γ-tocopherol	98.87	100.00	99.51
	100.90	100.25	100.70
	99.88	100.72	99.18
δ-tocopherol	99.06	99.44	99.60
	101.04	99.68	101.06
	99.90	100.88	99.34
Average recovery (%)	99.69	100.26	100.06
SD	0.94	0.47	0.80
RSD	0.95%	0.47%	0.80%
ANOVA	F_{exp} = 1.284	F_{crit} = 3.403	$p < 0.05$

Variation of the injection volume

The volume varied from 25 µL to 35 µL, with reference at 30 µL. Results are presented in Table 86 and statistical evaluation, using ANOVA test, showed no significant differences between the obtained recoveries (F_{exp} = 0.055, F_{crit}=3.403; $p < 0.05$), when minor variations in the volume of injection of the samples were applied.

Method is robust within the interval 30 ± 5 μL in the volume of injection of the samples for determination of tocopherols.

Table 86: Injection volume robustness data for tocopherols.

Tocopherols	**Recovery (%)**		
	25 μL	**30 μL**	**35 μL**
α-tocopherol	100.45	100.45	98.71
	101.83	100.43	103.52
	97.72	100.46	97.77
γ-tocopherol	100.60	100.00	98.81
	101.49	100.25	103.44
	97.91	100.72	97.75
δ-tocopherol	100.71	99.44	99.07
	101.27	99.68	103.91
	98.02	100.88	97.02
Average recovery (%)	100.00	100.26	100.00
SD	1.65	0.47	2.79
RSD	1.65%	0.47%	2.79%
ANOVA	$F_{exp} = 0.055$	$F_{crit} = 3.403$	$p < 0.05$

Variation of the HPLC

Variation of HPLC instrument was performed on Hitachi LaChrom (HPLC 14) and Dionex UltiMate 3000 (CQ 52). Results are presented in Table 87 and statistical evaluation, using t-student test, showed no significant differences between the obtained recoveries ($t_{exp} = 1.608$, $t_{crit} = 1.746$; $p < 0.05$), when analysis was performed at two different HPLC (Dionex UltiMate 3000 and Hitachi LaChrom).

Method is robust to different HPLC equipment for determination of tocopherols.

Table 87: HPLC instrument robustness data for tocopherols.

Tocopherols	Recovery (%)			
	Hitachi LaChrom (HPLC 14)		Dionex UltiMate 3000 (CQ 52)	
α-tocopherol	100.45		99.91	
	100.43		99.95	
	100.46		100.14	
γ-tocopherol	100.00		99.95	
	100.25		100.01	
	100.72		100.04	
δ-tocopherol	99.44		99.95	
	99.68		100.03	
	100.88		100.01	
Average recovery (%)	100.26		100.00	
SD	0.47		0.07	
RSD	0.47%		0.07%	
t-student test	$t_{exp} = 1.608$	$t_{crit} = 1.746$	$p < 0.05$	

7.1.5 Determination of phytosterols and tocopherols

Lipid emulsions for PN based on vegetable oils and fish oil demonstrated beneficial clinical effects apart from caloric intake. Naturally occurring oils were found to be an important source of vitamins, fatty acids, and other compounds that contribute to anti-inflammatory effect and reduced lipid peroxidation (15,150). However, in long-term administration of lipid emulsions, there was observed deterioration of hepatic function, which was attributed to phytosterols (52). On the other hand, recent studies showed that tocopherols, especially α-tocopherol, with antioxidant function, have possible hepatoprotective effects. Therefore, analyses of commercially available lipid emulsions help to evaluate the influence of phytosterols on hepatotoxic effects as well as protective function of tocopherols.

Phytosterol, cholesterol and squalene content was determined in lipid emulsions commercially available on Spanish pharmaceutical market. Content was investigated on six commercially available parenteral lipid emulsions from various providers, each in three different batches, collected from December 2015 until December 2016. Analyses were broadened to determination of tocopherols, as possible prevention for PNALD. The obtained results were studied for statistically relevant influences.

7.1.5.1 Determination of phytosterols, cholesterol and squalene

Phytosterols in parenteral lipid emulsions were identified and quantified with developed and validated analytical method. Each sample was analysed in triplicate and the average concentrations of phytosterols, cholesterol, and squalene are presented in Table 88.

Obtained results are comparable to the previously published ones (40,41,64,151), considering the variability of phytosterols' assay in vegetable oils in different batches and sensitivity of applied analyses.

Phytosterols were present in lipid emulsions, based on vegetable oils, whereas in fish oil based emulsion Omegaven 10%, no phytosterols were determined. Therefore, the exclusive vegetable origin of phytosterols was confirmed.

Table 88: Content of phytosterols in lipid emulsions.

Batch	Phytosterol content (μg/mL ± SD)						
	β-sitosterol	**campesterol**	**cholesterol**	**lanosterol**	**stigmasterol**	**squalene**	**Total phytos**
ClinOleic 20% (Baxter)							
14H29N30	173 ± 10	18 ± 2	51 ± 4	14 ± 2	27 ± 3	633 ± 22	232 ± 16
15F15N31	167 ± 7	11 ± 2	66 ± 3	19 ± 1	11 ± 1	1060 ± 36	209 ± 8
15F15N31 (bottle 2)	176 ± 2	8 ± 1	69 ± 1	28 ± 1	34 ± 1	656 ± 2	246 ± 2
16F22N30	122 ± 2	7 ± 1	46 ± 1	12 ± 1	7 ± 1	788 ± 11	149 ± 4
Intralipid 20% (Fresenius Kabi)							
10HB3671	277 ± 2	33 ± 4	361 ± 7	13 ± 1	129 ± 18	14 ± 1	451 ± 22
10IK7012	283 ± 17	100 ± 7	369 ± 24	13 ± 1	158 ± 12	18 ± 1	554 ± 36
10KC3584	163 ± 9	33 ± 1	212 ± 11	7 ± 1	59 ± 3	23 ± 1	262 ± 13
Lipofundina MCT 20% (Braun)							
143638082	120 ± 2	18 ± 1	64 ± 2	3 ± 1	39 ± 1	5 ± 1	179 ± 4
144718082	126 ± 5	20 ± 2	83 ± 4	2 ± 1	42 ± 4	9 ± 1	190 ± 9
154818081	134 ± 2	18 ± 1	76 ± 1	1 ± 1	42 ± 1	7 ± 1	195 ± 4
Lipoplus 20% (Braun)							
144538082	102 ± 5	17 ± 1	182 ± 9	ND*	26 ± 1	6 ± 1	146 ± 6
153938083	108 ± 1	19 ± 1	176 ± 4	ND*	33 ± 1	8 ± 1	161 ± 2
160128082	73 ± 1	13 ± 1	113 ± 1	ND*	27 ± 1	11 ± 1	114 ± 2
SMOFlipid 20% (Fresenius Kabi)							
16IF1650	100 ± 1	13 ± 1	421 ± 5	7 ± 1	17 ± 1	104 ± 1	138 ± 3
16HI0273	100 ± 1	13 ± 2	399 ± 3	10 ± 2	16 ± 3	108 ± 1	139 ± 8
16IG1719	94 ± 1	6 ± 1	576 ± 4	2 ± 1	10 ± 1	109 ± 1	113 ± 1
16IG1719 (bottle 2)	97 ± 1	8 ± 1	582 ± 7	3 ± 1	21 ± 1	114 ± 1	129 ± 3
16K65043	74 ± 2	8 ± 1	301 ± 13	5 ± 1	15 ± 2	143 ± 5	102 ± 3
Omegaven 10% (Fresenius Kabi)							
16H60131	ND*	ND*	401 ± 3	ND*	ND*	31 ± 1	ND*
16IE1319	ND*	ND*	510 ± 4	ND*	ND*	33 ± 1	ND*
16IE1319 (bottle 2)	ND*	ND*	404 ± 9	ND*	ND*	39 ± 1	ND*
16KF4628	ND*	ND*	349 ± 3	ND*	ND*	46 ± 1	ND*

(*ND - not detected)

Concentrations of total phytosterols varied according to differences in composition of lipid emulsions. The highest concentration, 550 μg/mL, was determined in Intralipid 20%, based only on soya bean oil and the lowest concentration, 100 μg/mL, was in SMOFlipid 20%, containing the mixture of vegetable oils and fish oil. Assay of total phytosterols between analysed lipid emulsions of different providers was proved statistically different ($F = 42.97$; $p = 0.00$), as expected.

Higher concentrations of total phytosterols were detected in lipid emulsions containing higher percentage of soya bean oil, being the highest in Intralipid 20%, with 100% of soya bean oil, and the lowest in SMOFlipid 20%, with 30% of soya bean oil. Correlation was previously described (40,41,64,151) and mixtures of vegetable oils in lipid emulsions were used to decrease the concentration of total phytosterols, while maintaining the calorie intake.

Comparing non-consecutive batches, statistically significant differences, were observed for all providers. The highest variation was observed in Lipoplus 20% ($F = 123.53$; $p = 0.00$) and the lowest was in Lipofundina MCT 20%, ($F = 5.43$; $p = 0.045$). Differences may be attributed to the use of vegetable oils, naturally occurring compounds, with expected heterogeneous composition.

Differences in composition of lipid emulsions reflected in profile of identified analytes. All analysed emulsions contained cholesterol and squalene, whereas the phytosterols varied according to the mixture of vegetable oils.

Concentrations of cholesterol were higher in emulsions with higher percentage of fish oil, as was expected due to its animal origin. The highest concentration was found in SMOFlipid 20% and Omegaven 10%, approximately 450 μg/mL, and the lowest, 46 μg/mL, was in ClinOleic 20%, mainly based on olive oil and without fish oil.

Squalene is abundantly present in olive oil, therefore, the highest concentration, almost 800 μg/mL, was found in ClinOleic 20%. On the other hand, Lipofundina MCT 20% and Lipoplus 20%, mainly based on soya bean oil and MCT, showed the lowest concentration (10 μg/mL).

Determined concentrations of cholesterol and squalene varied considerably according to the composition of lipid emulsions. Previously published clinical results show that high concentrations of cholesterol and squalene did not reflect in higher plasmatic levels. There was even observed a slight decrease in concentration of cholesterol after long-term administration of lipid emulsion with fish oil (43).

Majorly present β-sitosterol and stigmasterol were correlated with the content of soya bean oil. Accordingly, Intralipid 20% demonstrated the highest concentration, 240 μg/mL, whereas, Lipoplus 20% and SMOFlipid 20% had 90 μg/mL of β-sitosterol.
Stigmaterol was present at the highest concentration, 120 μg/mL, in Intralipid 20% and the lowest, at 20 μg/mL, in SMOFlipid 20% and ClinOleic 20%.
Some studies suggest that phytosterols β-sitosterol and stigmasterol are responsible for hepatoxic effects in PNALD due to their abundance in vegetable oils (65,97).

Campesterol and lanosterol were present in lipid emulsions at relatively low concentrations. In Intralipid 20% was determined 50 μg/mL of campesterol, whereas in SMOFlipid 20%, 10 μg/mL, was found. Content of campesterol was higher in lipid emulsions richer with soya bean oil.

Lanosterol was present in ClinOleic 20%, at 20 μg/mL, as the highest concentration and in Lipofundina MCT 20%, at approximately 2 μg/mL, demonstrated the lowest concentration. In Lipoplus 20%, lanosterol, was not detected. There was observed that lipid emulsions, based on MCT, contained lower concentrations of lanosterol.

Variation in composition among commercially available lipid emulsion reflects in significantly different content of phytosterols, cholesterol and squalene. Moreover, the use of vegetable oils results in high variability between batches of the same provider. Therefore, analyses of phytosterols in lipid emulsions would contribute to better quality and safety of PN.

7.1.5.2 Determination of tocopherols

Lipid emulsions, analysed for phytosterol content, were investigated also for tocopherol content. Isomers, α-, γ- and δ-tocopherol, were studied due to their abundance in vegetable and fish oils as well as their demonstrated antioxidant activity.

Some commercially available lipid emulsions already contained α-tocopherol to ensure stability of lipid excipients. Declared concentrations of added α-tocopherol were 200 μg/mL in Lipofundina MCT and 150-296 μg/mL in Omegaven 10%. Manufacturers did not specify the content of α-tocopherol in Lipoplus 20% and SMOFlipid 20%, whereas ClinOleic 20% and Intralipid 20% did not contain α-tocopherol as an extra added excipient.

Each sample was analysed in triplicate and average concentrations of tocopherols are presented in Table 89.

Results confirmed differences in tocopherol content between various manufacturers. Declared concentrations of α-tocopherol, in Lipofundina MCT 20% and Omegaven 10% corresponded to the determined concentrations.

Concentrations of total tocopherols depended mainly on concentrations of α-tocopherol and varied from 40 μg/mL to 250 μg/mL, which was attributed to composition of analysed lipid emulsions. The highest concentration of total tocopherols, 250 μg/mL, was found in Lipoplus 20%, which was based on mixture of vegetable and fish oils and contained α-tocopherol as antioxidant. On the other hand, the lowest concentrations of total tocopherols were found in Intralipid 20% (40 μg/mL) and ClinOleic 20% (60 μg/mL), two lipid emulsions without added tocopherols. Comparing the lipid emulsions with the lowest concentration, a lower content is observed in soya bean oil based emulsion Inatralipid 20%, whereas, mainly olive oil based emulsion ClinOleic 20% demonstrates higher content of tocopherols.

According to the published studies, α-tocopherol present in lipid emulsions demonstrated an improved antioxidant status of patients (22,152–157). However, supplementation was required to prevent vitamin E deficiency, common during long-term PN, and hepatoprotection (69,118,158–161). Moreover, addition of α-tocopherol to the composition

of lipid emulsions contributed to better stability of emulsions, as prevented peroxidation of lipid compounds (121,161).

Table 89: Content of tocopherols in lipid emulsions.

	Tocopherol content (μg/mL ± SD)			
Batch	**α-tocopherol**	**γ-tocopherol**	**δ-tocopherol**	**Total tocopherols**
ClinOleic 20% (Baxter)				
14H29N30	38 ± 1	ND*	ND*	38 ± 1
15F15N31	45 ± 1	ND*	ND*	45 ± 1
16F22N30	40 ± 1	ND*	ND*	40 ± 1
Intralipid 20% (Fresenius Kabi)				
10HB3671	30 ± 1	40 ± 1	< 1.25	70 ± 1
10IK7012	19 ± 1	37 ± 1	< 1.25	56 ± 1
10KC3584	25 ± 2	36 ± 3	< 1.25	61 ± 3
Lipofundina MCT 20% (Braun)				
143638082	169 ± 4	27 ± 1	ND*	181 ± 4
144718082	169 ± 10	25 ± 3	ND*	194 ± 10
154818081	171 ± 2	26 ± 1	ND*	197 ± 2
Lipoplus 20% (Braun)				
144538082	228 ± 10	25 ± 1	< 1.25	253 ± 10
153938083	223 ± 10	21 ± 1	< 1.25	244 ± 10
160128082	196 ± 3	13 ± 1	< 1.25	209 ± 3
SMOFlipid 20% (Fresenius Kabi)				
16HI0273	169 ± 17	7 ± 1	ND*	176 ± 17
16IG1719	191 ± 2	9 ± 1	ND*	200 ± 2
16K65043	197 ± 3	9 ± 1	ND*	206 ± 3
Omegaven 10% (Fresenius Kabi)				
16H60131	209 ± 3	ND*	ND*	209 ± 3
16IE1319	215 ± 2	ND*	ND*	215 ± 2
16KF4628	199 ± 2	ND*	ND*	199 ± 2

(*ND - not detected)

Isomer γ-tocopherol was found in concentrations from 10 to 40 μg/mL, which is considerably lower compared to α-tocopherol and for clinical antioxidant activity, higher concentrations of γ-tocopherol would be needed (116,118,161).

There has been observed a correlation between the concentration of γ-tocopherol and the soya bean oil content. The highest concentration, 40 μg/mL, was found in Intralipid 20%, based only on soya bean oil, meanwhile, in ClinOleic 20%, with lowest content of soya bean oil, and Omegaven 10%, based only on fish oil, the levels of γ-tocopherol were below the validated quantification range (1.25 μg/mL).

The isomer δ-tocopherol was identified only in Intralipid 20% and Lipoplus 20%, at concentrations below the validated quantification range. In other lipid emulsions, no peaks for δ-tocopherol were observed. Difficulties in quantification of δ-tocopherol were expected due to its low content in vegetable oils and method sensitivity. Moreover, the antioxidant activity contribution of δ-tocopherol is minor compared to α-tocopherol and γ-tocopherol, as both are present at higher concentrations and are stronger hydrogen donors (121).

Analysis of three non-consecutive batches of each lipid emulsion confirmed expected differences in tocopherol content, similar as in phytosterols. Variation in the concentration is mainly attributed to the use of natural constituents. There were observed higher deviations in tocopherol content (176-206 μg/mL) in lipid emulsions composed of mixture of various vegetable oils in combination with fish oil, such as SMOFlipid and Lipoplus 20%. On the other hand, Omegaven 10% and Lipofundina 20%, both with added known concentration of α-tocopherol, resulted in relatively low variation (199-215 μg/mL and 181-197 μg/mL, respectively).

Use of vegetable and fish oils in PN provides intake of naturally occurring compounds, among others antioxidants tocopherols, and contributes to beneficial clinical effects. However, heterogeneity of natural excipients requires their control and specification of the content of tocopherols for each released batch.

7.2 Clinical study

7.2.1 Survey on use of lipid emulsions in Catalan hospitals

During the years, many national health organisations prepared guidelines for PN to facilitate the decisions about proper administration and to prevent malpractice. ASPEN and ESPEN started with and unification of different PN protocols and with years established guidelines for each specific patient status. There are many similarities between American and European recommendations. However, harmonisation of both will be required in the future (27,28,162).

In practice, it was observed that each hospital still has established its own protocol for PN. The majority of them base their protocols on ESPEN guidelines and on recommendations from manufacturers.

In order to evaluate the PN tendencies in Catalonia, a survey was performed among various hospitals in the region. The main objective was to determine the extent of use of lipid emulsions and compare protocols for PN. There were investigated the number of commercially available brands of lipid emulsions used in individual hospital, the preference of presentation and source of lipids in the composition of emulsions according to the size of hospital. Observed presentations/hospital were statistically evaluated by ANOVA and presented in Table 90.

In the survey participated 22 hospitals of different sizes and from various cities to ensure sufficiently heterogeneous population. Hospitals were divided into groups I to III, according to the number of beds, for statistical evaluation. The majority of surveyed hospitals were medium sized, corresponding to the group II (200-499 beds), which prevail in the cities of the region. The number of large ($\geq$ 500 beds) and small ($<$ 200 beds) participating hospitals was sufficient for adequate evaluation of trends in PN in Catalonia.

The use of lipid emulsions was categorised according to the number of brands, presentation and source of lipids. In evaluation of brands of lipid emulsions, there was observed an average of three different brands per hospital. Larger hospitals had more varied selection of commercially available emulsions, compared to smaller ones. The variability was

statistically significant (p = 0.019), which may be attributed to the specific requirements of different types of patients, which are more common in larger hospitals.

Table 90: Results of the survey on use of lipid emulsions (presentation/hospital ± SD).

	Group I	**Group II**	**Group III**	**p**
Hospitals				
size of hospital	≥ 500 beds	200-499 beds	< 200 beds	-
number of hospitals	4	12	6	-
Lipid emulsions				
Number of brands	3.00 ± 0.82	2.33 ± 0.98	1.33 ± 0.52	0.019
Presentations				
one-chamber bag	2.75 ± 0.96	2.17 ± 1.53	0.33 ± 0.82	0.014
multi-chamber bag	3.25 ± 2.50	2.58 ± 2.06	2.83 ± 1.60	0.849
Source of lipids				
first generation	0.00 ± 0.00	0.08 ± 0.29	0.17 ±0.41	0.199
second generation	2.50 ± 0.58	2.17 ± 1.64	0.83 ± 1.60	0.432
third generation	3.50 ± 1.73	2.50 ± 1.51	2.17 ± 1.17	0.550

Presentations of lipid emulsions in form of multi-chamber infusion bags prevailed in all participating hospitals, regardless of the size. On the other hand, larger hospitals used more frequently one-chamber bags compared to small ones, which was statistically confirmed (p = 0.014). The use of multi-chamber bags, with separated lipid, glucose and amino acid compartments, enables more complex PN and is more stable at room temperature compared to one-chamber bags (13). However, according to ESPEN guidelines, administration of one-chamber bag is preferred in order to avoid additional manipulations and complications during the administration (35).

The survey revealed predominate administration of lipid emulsions of third generation, based on the mixture of vegetable and fish oil. The preference of use may be attributed to their wide availability on the market and safety in long-term use, as recommended by guidelines. Among them, the most frequently used brands were SMOFlipid 20% and ClinOleic 20%. Lipid emulsions of second generation, based on MCT and olive oil, were more common in larger hospitals, whereas the emulsions of first generation, based exclusively on soya bean oil, were rare. The use of lipid emulsions of first generation is generally unadvised, especially due to higher incidence PNALD (27,28).

The comparison of protocols for PN in surveyed hospitals in Catalonia has demonstrated variability in administration of lipid emulsions. Each hospital had its own protocol, based on instructions from manufacturers and type of patient. The highest dose, 3 g lipids/kg/day, was used in Lipofundina MCT/LCT and SMOFlipid 20%, for paediatric patients. The lowest dose of 0.7 g lipids/kg/day was observed in Lipoplus 20%. The average dose was of 0.96 ± 0.08 g lipids/kg/day, which corresponds to a half of the maximum daily dose recommended by manufacturers. Clinical studies have demonstrated that administration of lipid emulsions in doses up to 1 g lipids/kg/day is safe and appropriate.

Observed dosing of lipid emulsions for PN is suitable according to recommendations and guidelines of ESPEN and manufacturers. According to manufacturers, the maximum dose for adults is 2 g lipids/kg/day, for ClinOleic 20% even up to 2.5 g lipids/kg/day, whereas for paediatric patients, the maximum doses are 3-4 g lipids/kg/day. ESPEN guidelines for PN recommend administration of lipid emulsions in doses 0.7-1.5 g lipids/kg/day for adult (29,33,35) and 2-4 g lipids/kg/day for paediatric patients (39). The dosing depends on clinical status of patients. Administration of higher doses (1.5 g lipids/kg/day) is recommended for critically ill patients to ensure adequate caloric intake (163). Higher doses of lipid emulsions in paediatric patients are due to underdeveloped metabolism (39).

Protocols of PN in surveyed hospitals are adequate and generally follow the ESPEN guidelines. Administration of third generation lipid emulsions in dosage below 1 g lipids/kg/day is according to the recommendations. However, more uniform administration of lipid emulsions would enable easier and safer nutrition for patient, without compromising specific needs of individuals.

7.2.2 Clinical trial

The performed clinical trial was first one, carried out on hospitalised adult patients, to demonstrate the effects of phytosterols on hepatic function in short time and significant improvement, when fish oil based lipid emulsion is administered.

7.2.2.1 Selection of patients and randomisation

Selection of patients, suitable to enter in the clinical study, lasted from March 2015 until March 2017. During that period, 19 patients, majority had diagnosed neoplasia in gastro-intestinal tract, were included and finalised the study, which was approved by Spanish Agency of Medicine and Medical Devices (AEMPS), and represented sufficiently large population for further statistical evaluation.

Adult patients on PN received vegetable based lipid emulsion ClinOleic 20% at dose 0.8 g lipid/kg/day for seven days in the phase of selection. Plasmatic levels of GGT were monitored and patients, who demonstrated alteration of hepatic function, entered into the following phase of clinical study, randomisation.

In the phase of randomisation, patients were randomly allocated into two groups (Table 91). Ten patients were included into group A, receiving vegetable oil based lipid emulsion ClinOleic 20%, and nine patients were in group B, receiving fish oil based Omegaven 10%. Both groups were equally distributed regarding demographic parameters and plasmatic levels of cholesterol and total phytosterols ($p > 0.05$).

Table 91: Randomisation of patients (mean ± SD).

	Group A (ClinOleic 20%)	**Group B** (Omegaven 10%)	**p**
Total patients	10	9	-
Men (%)	90%	56%	0.089
Age (years)	65.7 ± 13.5	67.9 ± 8.3	0.681
Weight (kg)	80.5 ± 8.9	68.7 ± 18.2	0.085
Cholesterol (μg/mL)	1023.0 ± 261.3	954.8 ± 389.8	0.657
Total phytosterols (μg/mL)	22.2 ± 6.4	19.7 ± 6.6	0.420

Group A majorly consisted of men (90%), averagely 65.7 years old and weighing 80.5 kg. In Group B also prevailed male patients, with average age of 67.9 years and 68.7 kg. Plasmatic concentration of cholesterol was 1023.0 μg/mL in group A and 954.8 μg/mL in group B. Total phytosterols were 22.2 μg/mL in group A and 19.7 in group B.

7.2.2.2 Administered treatment and sampling

Patients received lipid emulsions, according to randomisation, at dose of 0.4 g lipid/kg/day for seven days. The dose was reduced in order to avoid accumulation of phytosterols and prevent alterations of hepatic function, while maintaining the sufficient caloric intake. Plasma samples were collected on Day 0 of randomisation and on Day 7 of post-randomisation. Apart from investigated clinical parameters, the nutritional parameters, inflammatory parameters and renal function were monitored to ensure safe investigation.

7.2.2.3 Analysis of sterols in plasma

Collected plasmatic samples were analysed in Clinical Laboratory at Bellvitge University Hospital.

Obtained results in Table 92 show plasmatic concentrations (average ± SD) of sterols and statistical significance before administration of lipid emulsions. Levels of sterols on Day 0 of randomisation correspond to the accumulation of phytosterols after seven-day administration of ClinOleic 20% in the selection phase. Comparison of both groups demonstrates that the patients of both groups had similar levels of sterols before entering to the clinical trial ($p > 0.05$). Phytosterol content was related to the administration of lipid emulsion, while the concentration of cholesterol was not proportional to PN intake. The evaluation of influence of PN to plasmatic concentrations of cholesterol is conditioned to previous basal concentration, individual to each patient.

The highest concentration on Day 0 was found in β-sitosterol, between 11.5 μg/mL and 13.1 μg/mL, while the lowest concentration was of stigmasterol 0.7 μg/mL. Total phyosterols were from 19.7 μg/mL to 22.2 μg/mL.

Table 92: Plasmatic concentrations of sterols in patients on Day 0 (mean ± SD).

Sterols	Group A (ClinOleic 20%)	Group B (Omegaven 10%)	p
cholesterol (μg/mL)	1023.0 ± 261.2	954.8 ± 389.8	0.657
β-sitosterol (μg/mL)	13.1 ± 4.1	11.5 ± 3.0	0.342
campesterol (μg/mL)	2.2 ± 0.7	1.9 ± 1.0	0.428
lanosterol (μg/mL)	1.2 ± 0.7	1.0 ± 0.5	0.607
stigmasterol (μg/mL)	0.7 ± 0.3	0.7 ± 0.5	0.915
total phytosterols (μg/mL)	22.2 ± 6.4	19.7 ± 6.6	0.420

Results obtained on Day 7 of clinical trial are presented in Table 93. There was observed a significant difference of total phytosterols in plasma ($p < 0.05$) between the two groups. Major decrease of total phytosterols was in Group B, from initial 19.7 ± 6.6 μg/mL to 13.5 ± 5.2 μg/mL, whereas in Group A total phytosterols in plasma were maintained, from 22.2 ± 6.4 μg/mL to 23.3 ± 6.9 μg/mL, despite the reduced dose.

Concentrations of β-sitosterol. campesterol and lanosterol were significantly lower in Group B compared to Group A. Stigmasterol in Group B showed tendency of decrease, which was not yet significant after seven days. Cholesterol levels were similar in both groups.

Table 93: Plasmatic concentrations of sterols in patients on Day 7 (mean ± SD).

Sterols	Group A (ClinOleic 20%)	Group B (Omegaven 10%)	p
cholesterol (μg/mL)	1145.7 ± 212.8	996.2 ± 355.2	0.478
β-sitosterol (μg/mL)	13.8 ± 4.7	5.4 ± 2.05	0.001
campesterol (μg/mL)	2.3 ± 0.7	1.5 ± 0.7	0.033
lanosterol (μg/mL)	1.1 ± 0.4	0.3 ± 0.2	0.000
stigmasterol (μg/mL)	0.7 ± 0.4	0.4 ± 0.3	0.108
total phytosterols (μg/mL)	23.3 ± 6.9	13.5 ± 5.2	0.003

The difference in plasmatic concentrations was attributed to rate of elimination of each phytosterol fraction. The fastest elimination was observed in β-sitosterol, from initial 11.5 ± 3.0 μg/mL to 5.4 ± 2.05 μg/mL, whereas campesterol was eliminated the slowest, and maintained the plasmatic concentration from 1.9 ± 1.0 μg/mL to 1.5 ± 0.7 μg/mL. Higher tendency of campesterol for accumulation was already reported (164).

From the comparison of results of two groups, it was concluded that changing the lipid source from vegetable oil to fish oil efficiently reduces the concentration of plasmatic phytosterols. The intake of phytosterols is stopped and accumulated phytosterols can be efficiently eliminated, without new accumulation. However, there was observed an increase of plasmatic cholesterol. Therefore, use of fish oil based lipid emulsions can be a good option for PNALD preventive, while monitoring the plasmatic levels of cholesterol.

On the other hand, reduction of dosage of vegetable based lipid emulsion was not efficient in reducing the plasmatic levels of phytosterols. The accumulated phytosterols could not eliminate efficiently due to constant intake of phytosterols. It was concluded that dose reduction is not effective PNALD prevention.

7.2.2.4 Analysis of hepatic function

To investigate influence of PN on hepatic function, clinical parameters were determined on Day 0 and Day 7. Levels of GGT, AP, ALT and BIL were compared between group of patients receiving ClinOleic 20% and Omegaven 10%, in order to confirm alterations in hepatic function after administration of lipid emulsion based on vegetable oil.

Plasmatic levels of GGT, AP, ALT, and total BIL, determined before the randomisation, in patients of both groups are presented in Table 94.

On Day 0 (randomisation day), patients had levels of GGT at 3.8 μg/mL, AP at approximately 2.5 μg/mL, ALT at approximately 0.4 μg/mL and total BIL at approximately 8 μg/mL in both groups. Statistical evaluation did not demonstrate significant difference between Group A and B ($p > 0.05$), which implies the patients were equally randomised and suitable for further trial.

Table 94: Hepatic function clinical parameters of patients on Day 0 (mean ± SD).

Clinical parameters	Group A (ClinOleic 20%)	Group B (Omegaven 10%)	p
GGT (μg/mL)	3.85 ± 1.54	3.87 ± 2.89	0.983
AP (μg/mL)	2.58 ± 1.25	2.35 ± 1.65	0.727
ALT (μg/mL)	0.35 ± 0.22	0.38 ± 0.32	0.814
total BIL (μg/mL)	8.30 ± 3.65	7.11 ± 4.20	0.518

After seven days of administration of lipid emulsions, hepatic parameters were measured again and the results are presented in Table 95.

Table 95: Hepatic function clinical parameters of patients on Day 7 (mean ± SD).

Clinical parameters	Group A (ClinOleic 20%)	Group B (Omegaven 10%)	p
GGT (μg/mL)	4.94 ± 2.43	2.43 ± 0.86	0.010
AP (μg/mL)	3.03 ± 1.36	1.82 ± 1.22	0.059
ALT (μg/mL)	0.48 ± 0.04	0.42 ± 0.38	0.767
total BIL (μg/mL)	6.40 ± 2.59	8.11 ± 6.37	0.445

Comparison of clinical parameters in patients from Group A, between Day 0 and Day 7, showed increase of GGT (approximately 4.9 μg/mL), AP (approximately 3.0 μg/mL) ALT (approximately 0.5 μg/mL) and decrease of total BIL to 6.4 μg/mL.

Administration of vegetable oil based lipid emulsion during seven days altered hepatic function, which was reflected in increased activity of hepatic enzymes and elimination of BIL. The observed hepatic alterations coincide with the similar clinical studies (66).

On the other hand, patients in Group B had lower levels of GGT (approximately 2.4 μg/mL), AP (approximately 1.8 μg/mL) and ALT (approximately 0.4 μg/mL), whereas total BIL increased to 8 μg/mL, compared to Day 0. Reduced intake of phytosterols, due to

administration of fish oil based lipid emulsion, reduced activation of hepatic enzymes for elimination of accumulated phytosterols. Therefore, particularly, GGT and AP, which are first indicators of changes in hepatic function, were significantly reduced. Enzyme ALT remained at initial concentration, whereas total BIL increased, which was attributed to lower elimination due to normalisation of hepatic function.

On Day 7, the liver function test demonstrated a difference in levels of GGT and AP between group A and B. Levels of GGT were significantly lower in group B ($p = 0.010$), whereas levels of AP, although lower, only indicated the tendency of significant difference ($p = 0.059$).

Administration of vegetable oil based lipid emulsion during seven days at reduced dose 0.4 g lipids/kg/day (50% of initial dose) in comparison to fish oil based lipid emulsion did not result in significant decrease of none of the studied parameters of liver function test.

Contrary to early markers of liver function, such as GGT and AP, there was no significant alteration of the parameters of liver function test ALT and BIL in neither of the two arms under the conditions of the study. In hospitalised adult patients treated with PN at dose of 0.4 g/kg/day was observed that 7-day period is too short for alterations of indicators of hepatocellular damage, such as ALT, and cholestasis, such as BIL. However, this does not imply that in multifactorial liver damage, associated with sepsis and a systemic inflammatory response, vegetable oil based lipid emulsions may not contribute to the early appearance of PNALD.

Therefore, it was concluded that the administration of lipid emulsions based on fish oil at dose 0.4 g lipids/kg/day during seven days significantly improved decrease of GGT in comparison to emulsion based on vegetable oil at the same dose. Moreover, a significant tendency of AP decrease was observed.

GLOBAL DISCUSSION

Lipid emulsions are an important preparation for PN, which ensure adequate caloric intake without alterations of glucose metabolism. Vegetable oils were used as lipid component of emulsions, as they represent an adequate source of energy and naturally occurring lipophilic macronutrients, such as vitamins and essential fatty acids.

First lipid emulsions were based on soya bean oil, which resulted in higher incidence of PNALD during the long-term use. Several studies have correlated high concentrations of phytosterols in soya bean oil to alterations of hepatic functions (40,43,49). Exact aetiology is still not clear, although the lack of an efficient elimination pathway for phytosterols is probably the cause. Phytosterols are exogenous compounds, structurally similar to cholesterol, present abundantly in plants and consequently in vegetable oils. Enteral administration of phytosterols results in low absorption due to the lack of transporters. However, intravenous administration results in complete administration of phytosterols into the organism. Lack of proper metabolism and elimination pathways result in accumulation in long-term administration of lipid emulsions (21,67,164,165).

Formulations were designed using MCT and olive oil, which improved the clinical effects. With further studies, fish oil was introduced in order to prevent PNALD (68). Currently, the most widely used lipid emulsions for PN are based on a mixture of vegetable and fish oil. The mixture contributes to diverse macronutrients and reduces the concentration of oil constituents that have demonstrated harmful effects (21,48,150).

The use of natural excipients, such as vegetable and fish oil, in formulations has also disadvantages. The excipients have heterogeneous composition, which depends on production conditions, from growth, harvesting to extraction and isolation. Consequently, the control of quality of used excipients during the manufacturing is essential. In PN, manufacturers report the concentrations of α-tocopherol, which is added as antioxidant. On the other hand, quality control of oils is not reported. Clinical studies have demonstrated the importance of concentration of phytosterols in lipid emulsions for PN. Therefore, their quantification and reporting should be established in the future.

This way, the intake of phytosterols would be controlled and PNALD complications may be prevented.

Phytosterols have been analysed mainly in biology as constituents of various plants (73–78) and in food sciences as additives to reduce absorption of cholesterol (83–89). Different analytical methods have been developed, but, all for specific determinations. Samples with different matrix have been saponified to reduce the interference of lipid constituents and to enable extraction of phytosterols. Methods of analysis of phytosterols were developed for liquid and gas chromatography. In RP-HPLC, the main problem in simultaneous determination is low selectivity between structurally similar phytosterols, mainly stigmasterol and campesterol, the most abundant ones.

On the other hand, analytical methods requiring specific detectors, based on NP-HPLC or gas chromatography were successful in separation of phytosterols. However, their use in routine is limited due to more complex analysis and availability of instrumentation. Therefore, a novel method was developed for simultaneous analysis of phytosterols, cholesterol, squalene and tocopherols in lipid emulsions by RP-HPLC. The sample preparation conditions were adapted for adequate saponification and efficient extraction of analytes from lipid matrix. The chromatographic conditions were optimised and the use of common solvents, such as ACN, MeOH and H_2O, under gradient conditions, on the column C_8 and UV detection was established. The developed method is the first RP-HPLC method, which successfully separates phytosterols, cholesterol, squalene and tocopherols. Furthermore, the method was validated for all analytes and was proven suitable and transferable for routine use.

The analyses of commercially available lipid emulsions on Spanish pharmaceutical market have been performed with the established validated analytical method. Comparison between six lipid emulsions from different providers, each one in three non-consecutive batches, was performed. The obtained results confirmed that the variation of concentration of phytosterols and tocopherols between manufacturers and between batches are statistically significant (40,41,64,126,151). The observed variability, due to the use of natural excipients, should be monitored and controlled by manufacturers in order to ensure quality in PN.

The highest concentrations of phytosterols were found in lipid emulsions based on soya bean oil, which endorse previous studies (40,41,64,151). Furthermore, β-sitosterol was abundant in soya bean oil, high concentrations of cholesterol were correlated with higher percentage of fish oil, whereas squalene was high in olive oil lipid emulsions. The highest concentrations of tocopherols were detected in emulsions, where α-tocopherol was added as antioxidant, particularly in fish oil based emulsions. Analyses of sterol fractions in lipid emulsions confirmed that the mixtures of various vegetable and fish oil reduce the total concentration of phytosterols. It was also concluded that addition of α-tocopherol to lipid emulsions is suitable for potential clinical antioxidant effects.

In order to evaluate the use of lipid emulsions in practice, a survey was carried out among various hospitals in Catalonia. It was observed that lipid emulsions of third generation are widely used, due to their availability on the market and according to the ESPEN guidelines. Larger hospitals have wider selection of emulsions in order to cover specific requirements of different pathologies. The dosage is mainly based on recommendations of manufacturers and guidelines. However, more uniform administration would improve the quality of PN (3).

The effects of phytosterols in lipid emulsions for PN were evaluated in a clinical trial, conducted at Bellvitge University Hospital. Adult hospitalized patients treated with PN longer than seven days were administered lipid emulsion, based on vegetable oil, and changes in hepatic function were evaluated. Patients who demonstrated significant increase in GGT, one of the most sensitive enzymes, were selected for the trial. Participants were randomised into group with lipid emulsion containing phytosterols and group with lipid emulsion without phytosterols. The dose of lipid emulsion with phytosterols was reduced and clinical parameters were monitored, particularly GGT, ALT, AP and total BIL, in order to evaluate liver function. Results have demonstrated statistically significant alterations of hepatic function after seven day administration of lipid emulsion with phytosterols.

Replacing vegetable oil based lipid emulsion with fish oil resulted in effective elimination of plasmatic phytosterols and improved hepatic parameters after one week administration.

Therefore, it was confirmed that the use of fish oil based lipid emulsion is suitable for PNALD prevention.

Dose reduction of vegetable oil based lipid emulsion after one week administration maintained the initial levels of liver function parameters and plasmatic concentration of phytosterols. It may be concluded that dose reduction strategy prevents increase of liver function alteration, although it is not effective in its decrease.

Plasmatic concentrations of cholesterol were not significantly different in both groups, regardless of the difference in intake with lipid emulsions. The fish oil based emulsion contained approximately 400 μg/mL of cholesterol, whereas in vegetable oil based emulsion only approximately 60 μg/mL of cholesterol was found. It may be concluded that elimination pathways of cholesterol were not impaired and functioned properly in both groups.

In the obtained results, accumulation of plasmatic phytosterols was observed and attributed to their slower elimination compared to the administration rate of lipid emulsion. In the group where dosage was reduced from 0.8 g to 0.4 g of vegetable oil based lipid emulsion, no reduction in administered plasmatic sterols was observed. On the other hand, in patients treated with fish oil based lipid emulsion, a significant reduction of plasmatic phytosterols was observed after seven days. However, not all fractions showed significance, among them campesterol and stigmaterol, which suggests that these fractions are excreted at a slower rate and may contribute to hepatoxicity more than other fractions.

Comparison of liver function test after a week of PN treatment has demonstrated a statistically significant decrease of initially elevated GGT and a tendency of significant decrease of AP in fish oil based lipid emulsion at dose 0.4 g lipids/kg/day. On the other hand, plasmatic levels of GGT and AP were maintained or increased slightly with the administration of lipid emulsion, based on vegetable oils, which was previously expected (46,57,58). Concentrations of ALT and total BIL were not significantly different between the two groups and would require monitoring of hepatic function for a longer period for more conclusive results.

Clinical trial has demonstrated that differences in commercially available lipid emulsion should be considered, particularly in long-term PN. The exclusive use of lipid emulsions, based on vegetable oils, leads to alterations of hepatic function and consequently to PNALD. Regular monitoring of clinical parameters for liver function and replacement of vegetable oil to fish oil based lipid emulsion would prevent liver damage.

CONCLUSIONS

The research work, presented in this thesis, can be resumed with the following conclusions:

1. An innovative and simple analytical method for simultaneous determination of phytosterols, cholesterol and squalene, using RP-HPLC-DAD was successfully developed. Furthermore, the sample preparation was optimised to successfully extract sterols and squalene from lipid emulsion matrix.

2. The proposed method was validated according to ICH, USP and AOAC International guidelines and has demonstrated an adequate selectivity, linearity, precision, accuracy and robustness for routine use in analyses of parenteral lipid emulsions.

3. Further investigations demonstrated the method has a good selectivity for tocopherols, so that the validation was extended. Moreover, the inter-laboratory precision and transferability of the analytical method was proved with the collaboration of Faculty of Pharmaceutical sciences at KU Leuven.

4. Analyses of lipid emulsions for PN, available on Spanish pharmaceutical market, confirmed the differences in concentrations of phytosterols, cholesterol, tocopherols and squalene, according to the oil composition. Moreover, observed variability in determined analytes, among various providers and between different non-consecutive batches of the same provider, is significant and would require quality control and specification from manufacturers. On the other hand, addition of α-tocopherol to lipid emulsions as antioxidant is suitable and may have clinical effects.

5. Evaluating the use of lipid emulsions for PN among diverse hospitals in Catalonia revealed the plurality of protocols for administration, which could be improved and unified in the future in order to ensure better quality. Surveyed hospitals mainly use lipid emulsions of third generation, based on mixture of vegetable oils and fish oil, with dosing based on ESPEN guidelines and recommendations by manufacturers.

6. Performed clinical study among patients at Bellvitge University Hospital confirmed the correlation between administration of lipid emulsions, based on vegetable oils, and alteration of hepatic function. An increase of liver function test was observed after seven days, which can lead to serious liver damage on long-term. On the other hand, administration of fish oil based lipid emulsion resulted in effective elimination of plasmatic levels of phytosterols and improved hepatic parameters.

7. The obtained data confirm that PN with lipid emulsions of third generation is suitable and safe in doses lower than 1 g lipids/kg/day. The use of fish oil based lipid emulsion is recommended and effective in prevention of PNALD.

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ANNEX 1

Summary of the procedure used for the measurement of mass concentration of "total" sterols (esterified + non-esterified) in serum by UHPLC-APCI-MS/MS

1. Reagents

- β-Sitostanol (Sigma-Aldrich-Merck; Ref: S462330-250MG)
- β-Sitostanol-D7 (Toronto Research Chemicals; Ref: S495002)
- β-Sitosterol (Toronto Research Chemicals; Ref: S497050)
- β-Sitosterol-D7 (Toronto Research Chemicals; Ref: S497052)
- 2,6-Di-tert-butyl-4-methylphenol (BHT) (Sigma-Aldrich-Merck; Ref: B1378-100G)
- 2-Propanol (isopropanol) LC-MS (Sigma-Aldrich-Merck; Ref: 1.02781.1000)
- Absolute ethanol (Merck; Ref: 1.07017)
- Acetonitrile UHPLC-MS (Sigma-Aldrich; Ref: 34967-1L)
- Brasicasterol (Toronto Research Chemicals; Ref: B676850)
- Campesterol (Toronto Research Chemicals; Ref: C155360)
- Campesterol-D3 (Toronto Research Chemicals; Ref: C155362)
- Desmosterol (Toronto Research Chemicals; Ref: D296860)
- Desmosterol-D6 (Toronto Research Chemicals; Ref: D296862)
- Dihydrolanosterol (Toronto Research Chemicals; Ref: D449855)
- Ergosterol (Toronto Research Chemicals; Ref: E599240)
- Hexane HPLC (Sigma-Aldrich; Ref: 34859-1L)
- Lanosterol (Toronto Research Chemicals; Ref: L174580)
- Methanol LC-MS (Sigma-Aldrich; Ref: 14262-1L)

- Potassium hydroxide (Sigma-Aldrich-Merck; Ref: 000000001050125000)
- Stigmasterol (Toronto Research Chemicals; Ref: S686750)
- Stigmasterol-D3 (Toronto Research Chemicals; Ref: S686753)
- Water UHPLC-MS (Sigma-Aldrich; Ref: 14263-1L)

2. Materials and equipment

- Analytical balance ADA-120/L (Adam Equipment)
- Centrifuge Biofuge® 13 (Heraeus Holding GmbH)
- Repetitive dispenser Nichimate® Stepper (Nichiryo Co Ltd)
- Nitrogen evaporator/concentrator MD200-2 (Xian Toption Instrument Co., Ltd.)
- Volumetric flasks of 10 mL, 50 mL and 100 mL BLAUBRAND® (BRAND GMBH + CO KG)
- Adjustable volume mechanical pipette 100-1000 μL Acura® 825 (Socorex)
- Adjustable volume mechanical pipette 20-100 μL Nichipet® EX II (Nichiryo Co Ltd)
- Adjustable volume mechanical pipette 2-10 μL Acura® 825 (Socorex)
- Sonicator Branson® 3510 MTH Ultrasonic (Branson)
- Beakers of 50 mL and 100 mL BLAUBRAND® (BRAND GMBH + CO KG)

3. Preparation of calibration and control materials

1. Two primary solutions of 1000 mg/L are prepared for each of the sterols in 2-propanol.

2. From the different primary solutions, two secondary solutions of 100 mg/L in 2-propanol containing all sterols are prepared. One of the solutions will be used to prepare the calibration materials and another one for the control materials.

3. From the secondary solutions, 9 calibration materials (0.10, 0.10, 0.25, 0.50, 1.00, 5.00, 10.0, 30.0 and 50.0 mg/L) and 3 control materials (0.30, 20.0 and 40.0 mg/L) are prepared in 2-propanol.

All solutions and materials are stored at -80ºC.

4. Preparation of working solution of internal standards

Relationship between internal standard and sterol:

Sterol	Internal standard
Brassicasterol	Colesterol-D_6
Campesterol	Campesterol-D_3
Desmosterol	Desmosterol-D_6
Ergosterol	Colesterol-D_6
Lanosterol	Dihydrolanosterol
β-Sitostanol	β-Sitostanol-D_7
β-Sitosterol	β-Sitosterol-D_7
Stigmasterol	Stigmasterol-D_3

Preparation of working solution of internal standard:

1. Primary solutions of 500 mg/L are prepared for each internal standard. Solutions are aliquoted in Eppendorf tubes and stored at -80ºC.

2. At the time of the analysis, and from the different primary solutions, a working solution of 20 mg/L in 2-propanol is prepared, which contains all the internal standards.

5. Preparation and treatment of serum samples, calibration materials (CAL) and control materials (CTRL)

The preparation and treatment consists of an alkaline hydrolysis, followed by a liquid-liquid extraction, an evaporation of the extract with nitrogen and a subsequent reconstitution with methanol:

250 µL serum or CAL or CTRL

+

750 µL KOH 0,7 M in EtOH/H_2O (96/4 *v/v*)

+

50 µL working sol. IS (20 mg/L in 2-propanol)

+

10 µL BHT 1000 mg/L in MeOH

↓ **Alcaline hydrolisis: 68°C, 30 min in sonicator**

500 µL H_2O

+

1.5 mL Hexane

↓ **1) Vortex 5 s**
2) Centrifuge 2500 *g* 20 min

Collect supernatant (1200 µL) in glass tube

↓ **Evaporate with N_2 until dryness (approx. 10 min)**

Reconstitute with 600 µL MeOH and inject onto UHPLC-APCI-MS/MS

6. Chromatographic and mass spectrometry equipment

An ACQUITY®-UPLC® measuring system coupled to a triple quadrupole mass spectrometer ACQUITY®-TQD®, both from Waters SA Chromatography, is used.

Chromatographic conditions:

- Column: Acquity UPLC® BEH™ 2.1 x 100 mm; 1.7 μm (Waters)
- Pre-column: Acquity® UPLC® BEH™ C_{18} VanGuard Pre-column (5 mm x 2.1 mm; 130 Å, 1.7 μm)
- Filter: 0.2 μm ACQUITY UPLC® Col. In-Line Filter Kit (Waters)
- Column temperature: 30ºC
- Sampling temperature: 15ºC
- Injection volume: 10 μL
- Mobile phase A: water
- Mobile phase B: methanol
- Flow: 0.5 mL/min
- Elution: gradient

Time (min)	**Flow (mL/min)**	**Mobile phase A (%)**	**Mobile phase B (%)**	**Type of elution in gradient**
0.0	0.5	15	85	-
0.2	0.5	15	85	Linear
0.5	0.5	0	100	Linear
3.3	0.5	0	100	non-linear
4.0	0.5	15	85	non-linear

- Total time of chromatography: 5.5 min

Mass spectrometry conditions:

- Ionisation: Atmospheric pressure chemical ionisation (APCI)
- Triple quadrupole mode: Multiple reaction monitoring (MRM)
- Nebulizer gas: nitrogen
- Collision gas: argon

- Intensity of corona: 10 μA
- Temperature of ionisation source: 130ºC
- Temperature of desolvatisation: 600ºC
- Flow of desolvatisation gas: 600 L/h
- Flow of nitrogen in cone: 60 L/h
- Flow of collision gas: 0.20 mL/min
- Dwell time: 0.04 s
- Other parameters:

Sterol	***m/z*** **Ion precursor**	***m/z*** **Ion product**	**Cone potential (V)**	**Collision energy (eV)**
Brassicasterol	381.5	161.3	30	30
Campesterol	383.5	161.3	30	20
Campesterol-D_3	386.5	164.3	30	20
Colesterol-D_6	375.5	167.5	30	20
Desmosterol	367.5	161.3	30	20
Desmosterol-D6	373.5	167.3	30	20
Ergosterol	379.5	161.3	30	20
Lanosterol	409.5	149.3	30	25
Dihydrolanosterol	411.5	205.5	30	25
β-Sitostanol	399.5	81.4	30	30
β-Sitostanol-D_7	406.5	81.4	30	30
β-Sitosterol	397.5	161.3	30	20
β-Sitosterol-D_7	404.5	168.3	30	20
Stigmasterol	395.5	161.3	30	20
Stigmasterol-D_3	398.5	164.3	30	20

ANNEX 2

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WILEY Biomedical Chromatography

RESEARCH ARTICLE

Development and validation of a simple high-performance liquid chromatography analytical method for simultaneous determination of phytosterols, cholesterol and squalene in parenteral lipid emulsions

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Abstract

A simple analytical method for simultaneous determination of phytosterols, cholesterol and squalene in lipid emulsions was developed owing to increased interest in their clinical effects. Method development was based on commonly used stationary (C_{18}, C_8 and phenyl) and mobile phases (mixtures of acetonitrile, methanol and water) under isocratic conditions. Differences in stationary phases resulted in peak overlapping or coelution of different peaks. The best separation of all analyzed compounds was achieved on Zorbax Eclipse XDB C_8 (150 × 4.6 mm, 5 μm; Agilent) and ACN–H_2O–MeOH, 80:19.5:0.5 (v/v/v). In order to achieve a shorter time of analysis, the method was further optimized and gradient separation was established. The optimized analytical method was validated and tested for routine use in lipid emulsion analyses.

KEYWORDS

cholesterol, high-performance liquid chromatography, phytosterol, squalene, validation

1 | INTRODUCTION

Phytosterols are substances of plant origin, considered as cholesterol equivalents, owing to their similar sterol structure (Figure 1) and analogous functions in cell membrane regulation. Recently, their clinical importance has increased owing to their beneficial effects in reducing cholesterol when administered per orally (de Jong, Plat, & Mensink, 2003; Fernandes & Cabral, 2007; Jones, MacDougall, Ntanios, & Vanstone, 1997). However, in parenteral nutrition, lipid emulsions containing vegetable oils rich with phytosterols resulted in a higher incidence of liver dysfunction when used long term (Harvey et al., 2014; Meisel et al., 2011; Savini et al., 2013; Xu et al., 2012). Commercially available lipid emulsions for parenteral nutrition have different compositions of vegetable oils and, consequently, concentrations of phytosterols vary. The detailed determination of fractions will help to identify potentially harmful phytosterols and correlate them with observed clinical effects. Furthermore, quantification of unwanted fractions of phytosterols will enable establishment of dosage protocols in hospitals to prevent clinical damage and provide the basis for complete elimination of the fractions during the manufacturing process.

Squalene is the triterpene precursor of cholesterol and phytosterols (Bavisetty & Narayan, 2015; Maguire, O'Sullivan, Galvin, O'Connor, & O'Brien, 2004), with important antioxidant activity, drug carrying functions and favorable clinical effects (Reddy & Couvreur, 2009). It is present in parenteral lipid emulsions as a constituent of vegetable oils, especially in olive oil (Xu et al., 2012).

Abbreviations: ACN, acetonitrile; AOAC, Association of Official Analytical Chemists; MeOH, methanol; RRT, relative retention time.

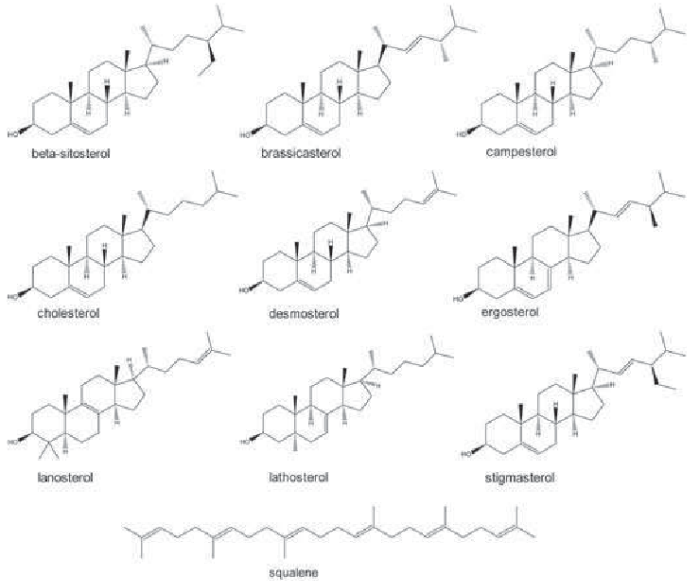

FIGURE 1 Chemical formulas of phytosterols, cholesterol, and squalene

Several gas chromatography (GC) and high-performance liquid chromatography (HPLC) analytical methods have been developed for qualitative and quantitative determination of phytosterols, cholesterol and squalene, especially for food and plant extract analyses (Abidi, 2001; Lagarda, García-Llatas, & Farré, 2006; Moreau, Whitaker, & Hicks, 2002). Simultaneous determination is described only with GC, whereas available HPLC methods enable identification of only a few majorly occurring phytosterols, such as *β*-sitosterol, campesterol and stigmasterol. Existing HPLC methods use C_{18}, C_8 and phenyl columns in combination with specific chromatographic conditions, such as fluorescence detectors, Evaporative Light Scattering Detector (ELSD) or coupled mass spectrometry, mobile phases with *n*-hexane or dicholoromethane or high-speed counter-current chromatography (Carretero et al., 2008; Duong et al., 2016; Feng, Liu, Luo, & Tang, 2015; Maguire et al., 2004; Mitei, Ngila, Yeboah, Wessjohann, & Schmidt, 2009; Sánchez-Machado, López-Hernández, Paseiro-Losada, & López-Cervantes, 2004; Schröder & Vetter, 2012; Sheng, 2009; Slavin & Yu, 2012; Warner & Mounts, 1990; Yuan, Ju, Jin, Ren, & Liu, 2014). However, no available HPLC analytical method allows simultaneous separation of all available phytosterols, cholesterol and squalene under simple chromatographic conditions.

The purpose of our investigation was the development and validation of a simple RP-HPLC-DAD analytical method for qualitative and quantitative determination of phytosterols, cholesterol and squalene for routine use in parenteral lipid emulsions analyses.

2 | EXPERIMENTAL

2.1 | Materials and reagents

Brassicasterol (≥98% purity), campesterol (≥65% purity), desmosterol (≥84% purity), ergosterol (≥95% purity), lanosterol (≥93% purity), lathosterol (≥99% purity), *β*-sitosterol (≥85% purity), stigmasterol (≥95% purity) and squalene (≥98% purity) were purchased from Sigma Aldrich (St Louis, MO, USA). Cholesterol (≥97% purity) was obtained from Fagron (Barcelona, Spain). Acetonitrile and methanol, UHPLC grade, were acquired from Panreac (Darmstadt, Germany). Potassium hydroxide (KOH) from Fagron (Barcelona, Spain), 96% ethanol from Panreac (Darmstadt, Germany), pyrogallol (≥99% purity) from Sigma Aldrich (St Louis, MO, USA), and heptane from Panreac (Darmstadt, Germany) were used for sample preparation. Three commercially available parenteral lipid emulsions were analyzed.

2.2 | Stock and standard solution

Stock solutions of phytosterol standards, cholesterol and squalene were prepared in methanol and stored at 4–6°C. The standard solution was prepared as a mixture of stock solutions to obtain final concentrations of squalene (500 μg/mL), cholesterol (250 μg/mL), *β*-sitosterol (250 μg/mL), ergosterol (125 μg/mL), stigmasterol (125 μg/mL), campesterol (50 μg/mL), desmosterol (50 μg/mL), lanosterol (50 μg/mL) and lathosterol (50 μg/mL) and stored at 4–6°C.

2.3 | Sample preparation

Lipid emulsions require saponification and extraction before the analysis of sterols and squalene to remove the matrix effect of the other lipid constituents. The preparation protocol was adapted to HPLC according to previously described protocols (Duelund, 2012; Xu et al., 2012). A 1 mL aliquot of lipid emulsion, 10 mL of 7% KOH and 3 mL of 1% of pyrogallol were added to Pyrex test tubes with screw caps. To spiked samples, an internal standard was added, which was 1 mL of ergosterol standard solution (100 μg/mL). The mixture was vortexed for 10 s and heated for 20 min at 60°C. After the saponification mixture had cooled down, 5 mL of water for HPLC was added and

vortexed for 10 s. Extraction was performed by adding 2 × 5 mL of heptane. The upper heptane layer was collected into an evaporative flask and evaporated with a rotary evaporator at room temperature in order to obtain a dry layer, which was afterwards dissolved in 2 mL of methanol, filtered through a 0.45 µm PVDF filter and prepared for HPLC analysis.

2.4 | HPLC conditions

HPLC analysis was performed on a Dionex UltiMate 3000, equipped with a pump (LPG–3400 M), an autosampler (WPS3000), a thermostated column compartment (TCC-3100, 6P), and DAD (PDA-3000). Robustness was tested on an Agilent 1100 with pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A) and DAD (G1315A).

To achieve optimal separation, various HPLC conditions were investigated. Different columns were used: Symmetry C_{18} (150 × 4.6 mm, 5 µm; Waters); Zorbax SB-Phenyl (150 × 4,6 mm, 5 µm; Agilent); and Zorbax Eclipse XDB C_8 (150 × 4.6 mm, 5 µm; Agilent). The mobile phase consisted of acetonitrile (ACN), methanol (MeOH) and water (H_2O) in various proportions. Flow varied from 1 to 2 mL/min, injection volume was 10–30µL, column temperature was maintained at 30°C and UV detection was set at 210 nm.

2.5 | Method validation

2.5.1 | Standard solution stability

The stability of prepared standard solutions was analyzed from 0 to 12 days. The solution was maintained at room temperature at 25 ± 2°C in order to investigate the stability during analysis.

2.5.2 | Specificity

Standard solutions were characterised to obtain UV absorption maximums and relative retention times (RRT) for each phytosterol, cholesterol and squalene. The RRT was calculated according to USP 39-NF 34 (The United States Pharmacopeial Convention, 2016), as RRT = t_{r0}/t_{r1}, where t_{r0} is the retention time of ergosterol, set as the internal standard and t_{r1} is the retention time of other sterols and squalene.

2.5.3 | Linearity

To establish the linearity of analytes, a standard solution was prepared in triplicate, according to the following dilutions: 1:1, 1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200 and 1:500. From the obtained calibration curves, mean linearity and regression statistics were calculated.

2.5.4 | Precision

Repeatability of the instrumental system was determined at different concentrations of standard solution, corresponding to the dilutions 1:1, 1:5 and 1:20, prepared for linearity. Ten consecutive injections were performed at each concentration and the statistics of the obtained response factors were determined. The procedure was repeated on different days to investigate interday precision.

2.5.5 | Accuracy

Accuracy was determined from the data, obtained from the linearity, corresponding to the dilutions 1:1, 1:20 and 1:500. The percentage of recovery was calculated and statistically evaluated.

2.5.6 | Robustness

The standard solution was analyzed under minor variations in chromatographic conditions, such as detection wavelength, column temperature, injection volume and the use of different HPLC equipment, presented in Table 1. The investigated variations can be critical in simultaneous separation of several analytes. The use of two different chromatographs ensures the method's transferability between different HPLC equipment. Recovery was calculated and statistically significant differences were investigated by ANOVA and Student *t*-test.

2.6 | Data analysis

Chromatographic data were obtained and analyzed with the Chromeleon datasystem (version 6.80 SR15, Dionex) and ChemStation (version A.08.03, Agilent Technologies). Statistical studies were performed with MS Excel 2007.

3 | RESULTS AND DISCUSSION

3.1 | Method development

In the study of optimal chromatographic conditions for separation of phytosterols, cholesterol, and squalene, three columns with different hydrophobicities and selectivities were used under isocratic conditions. Figure 2 shows chromatograms with optimal mobile phase composition, at flow rate 1 mL/min, injection volume 30 µL, detection 210 nm and temperature maintained at 30°C.

The most hydrophobic column used in our method development was C_{18} and with mobile phase ACN–MeOH 98:2 (v/v), which resulted in relatively good separation of sterols in <60 min. However, in spite of method optimization, it was unable to separate stigmasterol and campesterol (Figure 2, peak 7), two phytosterols of major interest for their clinical effects. Coelution was previously reported in the literature under different chromatographic conditions (Breinhölder, Mosca, & Lindner, 2002; Lagarda et al., 2006; Sánchez-Machado et al., 2004). There was also noted coelution between cholesterol and

TABLE 1 Robustness conditions

Chromatographic condition	Variation
Detection wavelength	207 nm 210 nm 213 nm
Column temperature	27°C 30°C 33°C
Injection volume	25 µL 30 µL 35 µL
Different HPLC equipment	Dionex UltiMate 3000 Agilent 1100

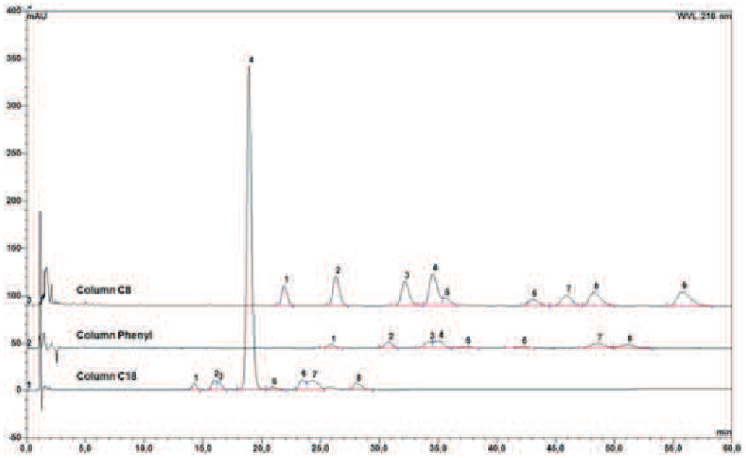

FIGURE 2 Method development chromatograms with column C_{18} (upper), phenyl (middle) and C_8 (lower), and isocratic conditions

lathosterol (Figure 2, peak 6) as well as peak overlap between ergosterol (Figure 2, peak 2) and lanosterol (Figure 2, peak 3).

In order to improve sterol separation, a column with phenyl end-capping was used to change the column hydrophobicity and selectivity (Slavin & Yu, 2012). The Zorbax SB-Phenyl column and mobile phase ACN–H_2O–MeOH, 48:29.5:22.5 (v/v/v) showed stronger column–analyte interaction and time of analysis was prolonged to >60 min. Furthermore, there was observed coelution of β-sitosterol, stigmasterol and lanosterol (Figure 2, peaks 7 and 8) as well as peak overlapping of cholesterol and lathosterol (Figure 2, peaks 3 and 4). Squalene eluted after 100 min as a wide peak. Changes of chromatographic conditions did not improve the separation of the analyzed compounds.

The selection of Zorbax Eclipse XDB C_8, which is less hydrophobic column than C_{18} and without added functional groups, resulted in weaker column–analyte interaction (Warner & Mounts, 1990) and enabled the simultaneous identification of all analytes. Mobile phase composition was ACN–H_2O–MeOH, 80:19.5:0.5 (v/v/v) and the time of analysis was >120 min, owing to longer retention of squalene. Peak overlapping between cholesterol and brassicasterol (Figure 2, peaks 4 and 5) was observed; however, the repetitive identification of both standards was proved. The method was selected for further optimization with gradient elution in order to shorten the time of analysis, and especially to accelerate squalene elution.

Optimal gradient conditions are presented in Table 2, the mobile phase was ACN (component A) and H_2O–MeOH (component B), 95:5 (v/v) and the rest of the chromatographic conditions remained the same as in isocratic elution.

TABLE 2 Gradient conditions

Time	Component A	Component B
0 min	75%	25%
45 min	90%	10%
50 min	100%	0%
65 min	100%	0%

Figure 3 shows the chromatogram obtained under optimized chromatographic conditions. Time of analysis was 65 min, which allows good separation of all analyzed compounds.

3.2 | Validation study

System suitability was evaluated according to the USP 39-NF 34 (The United States Pharmacopeial Convention, 2016) and it was concluded that the proposed analytical method is within the specifications and appropriate for routine work.

3.2.1 | Stability of the solution

The phytosterol standard solution was stable during a period of 12 days at room temperature (25 ± 2°C) as the percentage of recovery remained within the limits of 80–110%, specified by the AOAC (Association of Official Analytical Chemists).

3.2.2 | Selectivity

Standards were analyzed separately, according to the described method and UV absorption maximum was attributed to each one of them, as presented in Table 3. To facilitate the identification, relative retention times were also calculated, relative to internal ergosterol as internal standard.

3.2.3 | Linearity

Linearity was determined for each standard separately. Mean linearity values of slope, intercept and determinant coefficient are presented in Table 4. Differences in linearity curves were attributed to physicochemical properties of each standard. Squalene, as the only analyzed compound to lack a sterol structure, differed in absorption maximum and in the validated concentration range showed a high positive intercept. On the other hand, brassicasterol had a slightly positive intercept possibly owing to interactions with cholesterol. All standards demonstrated good correlation between

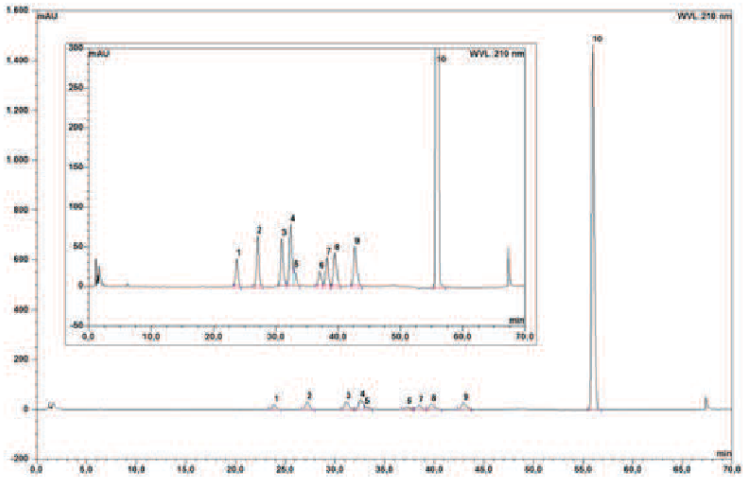

FIGURE 3 Chromatogram of optimal gradient method. 1, Desmosterol; 2, ergosterol; 3, lathosterol; 4, cholesterol; 5, brassicasterol; 6, campesterol; 7, lanosterol; 8, stigmasterol; 9, β-sitosterol; 10, squalene)

concentrations and response factors; coefficients were all $r^2 > 0.9900$, as specified according to AOAC.

The concentration interval was also established, where linearity, precision and accuracy were proven. The lower limit represents the limit of quantification (LOQ) of each phytosterol and the upper limit was set according to their potential assay in lipid emulsions.

TABLE 3 Selectivity data

Standard	UV maximum (nm)	Relative retention time (min)
β-Sitosterol	193.4	0.64
Brassicasterol	190.4	0.82
Campesterol	192.6	0.73
Cholesterol	193.5	0.83
Desmosterol	193.9	1.14
Ergosterol	281.2	1.00
Lanosterol	194.3	0.71
Lathosterol	190.6	0.87
Squalene	199.4	0.49
Stigmasterol	193.0	0.69

TABLE 4 Linearity data

Standard	Slope	Intercept	Determinant coefficient (r^2)	Concentration interval (μg/mL)
β-Sitosterol	0.1714	−0.0240	0.9998	9–179
Brassicasterol	0.1672	+0.0138	0.9974	3–49
Campesterol	0.3292	−0.0989	1.0000	2–32
Cholesterol	0.1674	−0.2430	0.9997	12–249
Desmosterol	0.4037	−0.0593	0.9998	2–42
Ergosterol	0.2418	−0.0429	1.0000	6–121
Lanosterol	0.4237	−0.1614	0.9999	2–46
Lathosterol	0.5943	−0.1516	0.9999	2–49
Squalene	1.6678	+8.8607	0.9980	25–498
Stigmasterol	0.2121	−0.1509	0.9997	6–122

3.2.4 | Precision

Precision was studied at different concentrations and on different days. The obtained data (Table 5) are within the working limits.

3.2.5 | Accuracy

Accuracy was determined as mean percentage of recovery of standards at different concentrations (Table 6). Recovery specifications, according to AOAC, were established at 80–110% and all standards are within the interval. Variations in recoveries were attributed to small area integration. Student's *t*-test ($t_{exp} < 2.306$; $p < 0.05$) and Cochran's *Q* test ($G_{exp} < 0.8709$; $p < 0.05$) were applied to evaluate the influence of concentration on accuracy and with both tests calculated statistics showed no statistically significant influence.

3.2.6 | Robustness

Robustness of the analytical method (Table 7) was studied with variation of chromatographic conditions and the statistical significance was evaluated for each influence. Optimal conditions

TABLE 5 Precision data

	Precision – instrumental RSD (%)			Precision – interday RSD (%)		
Standard	1:20	1:5	1:1	1:20	1:5	1:1
β-Sitosterol	1.86	2.16	0.97	2.12	2.69	1.28
Brassicasterol	16.90	6.96	2.79	15.51	13.10	6.79
Campesterol	11.26	7.68	3.55	11.99	6.98	3.97
Cholesterol	6.19	1.91	0.82	5.53	2.37	1.31
Desmosterol	1.71	2.64	0.65	5.42	3.66	0.69
Ergosterol	1.95	1.43	0.67	6.08	1.98	0.99
Lanosterol	3.55	3.53	1.57	4.62	5.57	1.62
Lathosterol	1.76	2.43	0.63	4.18	3.70	0.78
Squalene	0.60	0.47	0.43	2.35	0.37	0.71
Stigmasterol	4.50	2.98	1.22	6.50	3.32	1.19

TABLE 6 Accuracy data

	Accuracy – recovery (%) ± SD		
Standard	1:20	1:5	1:1
β-Sitosterol	99.56 ± 3.27	99.93 ± 2.06	100.51 ± 0.65
Brassicasterol	94.75 ± 2.76	100.96 ± 7.67	104.30 ± 2.06
Campesterol	93.72 ± 5.46	100.79 ± 4.99	105.48 ± 2.70
Cholesterol	96.29 ± 3.53	98.77 ± 3.06	104.94 ± 0.34
Desmosterol	93.44 ± 5.54	102.39 ± 3.46	104.17 ± 1.67
Ergosterol	99.69 ± 5.12	99.66 ± 3.68	100.65 ± 2.66
Lanosterol	94.50 ± 7.85	101.34 ± 10.1	104.16 ± 2.45
Lathosterol	94.26 ± 1.81	100.54 ± 3.59	105.20 ± 3.66
Squalene	106.89 ± 0.28	100.00 ± 8.11	100.00 ± 8.11
Stigmasterol	97.47 ± 4.36	100.00 ± 5.66	100.00 ± 5.66

were fixed at wavelength of 210 nm, column temperature of 30°C, injection volume of 30μL and the use of chromatograph Dionex UltiMate 3000.

Minor changes in detection wavelength (210 ± 3 nm) showed no statistically significant difference (F_{exp} = 1.427, F_{crit} = 2.456; p < 0.05), as the studied wavelengths were close to UV absorption maximums of standards.

It was demonstrated that applied minor variations of column temperature (30± 3°C) had no statistically significant influence on the recovery (F_{exp} = 1.423, F_{crit} = 2.456; p < 0.05) as it did not significantly change the fluidics of standard solution.

Minor modifications of injection volume (30 ± 5 μL) had no statistically significant influence on analysis (F_{exp} = 1.207, F_{crit} = 2.456; p < 0.05). Lower injection volume did not affect the identification of peaks and at the same time increased injection volume did not result in peak saturation, owing to low standard concentrations.

The statistical study with Student t-test showed no statistically significant differences between the obtained recoveries (t_{exp} = 0.147, t_{crit} = 1.734; p < 0.05), when analysis was completed in two different HPLC systems (Dionex UltiMate 3000 and Agilent 1100). Therefore, the proposed analytical method is sufficiently robust in terms of using different HPLC equipment.

3.3 | Analyses of commercial lipid emulsions for parenteral nutrition

The procedure for sample treatment of different commercially available lipid emulsions is an adapted version of already published ones (Duelund, 2012; Xu et al., 2012), considering the properties of sample and analytical method requirements. The volume of samples was larger in order to ensure proper detection and MeOH was used as a final solvent to avoid incompatibilities with the mobile phase. Saponification time was reduced to 20 min, in order to prevent the sterol oxidation and shorten the total preparation time, while maintaining the effectiveness of medium chain triglyceride removal. 5α-Cholestane, which was normally used as internal standard in GC, lacks chromophores for UV detection and ergosterol was defined as the internal standard, owing to its different UV detection maximum, and its possible assay in parenteral lipid emulsions was previously discarded. To confirm the absence of ergosterol in each analyzed sample, spiked and nonspiked samples were prepared.

The identification of peaks was based on retention times and UV maxima of standards. Three commercially available parenteral lipid emulsions with various composition were analyzed to investigate the possibility of routine analysis. Chromatograms are presented in Figure 4. It was demonstrated that the proposed analytical method is suitable for samples with different lipid compositions. The preparation protocol successfully removes the matrix effect, which enables identification and quantification of sterols and at the same time does not produce modification of analytes. The obtained results are comparable with the previously published ones (Xu et al., 2012), considering the

TABLE 7 Robustness data

	Robustness – recovery (%)									
Variation	β-Sitosterol	Brassicasterol	Campesterol	Cholesterol	Desmosterol	Ergosterol	Lanosterol	Lathosterol	Squalene	Stigmasterol
Detection wavelength										
207 nm	99.79	99.12	99.64	98.64	99.32	100.01	99.37	99.74	99.85	99.54
210 nm	99.69	100.40	100.38	100.50	99.00	99.40	99.72	99.26	99.86	99.91
213 nm	99.71	100.01	101.17	100.33	98.37	99.39	100.42	100.51	99.86	100.63
Column temperature										
27°C	99.86	98.87	100.70	101.49	101.45	100.02	99.91	100.19	100.24	99.08
30°C	99.69	100.40	100.38	100.50	99.00	99.40	99.72	99.26	99.86	99.91
33°C	99.87	99.59	100.57	100.38	100.07	99.94	100.22	99.98	100.00	99.88
Injection volume										
25 μL	100.26	99.77	99.22	97.92	99.51	99.01	98.81	96.55	100.01	99.82
30 μL	99.69	100.40	100.38	100.50	99.00	99.40	99.72	99.26	99.86	99.91
35 μL	99.47	98.66	99.72	99.65	100.32	99.69	99.59	99.19	100.30	99.45
Different HPLC equipment										
Dionex UltiMate 3000	99.69	100.40	100.38	100.50	99.00	99.40	99.72	99.26	99.86	99.91
Agilent 1100	99.99	99.34	99.91	99.11	99.92	99.71	99.81	100.67	100.02	99.95

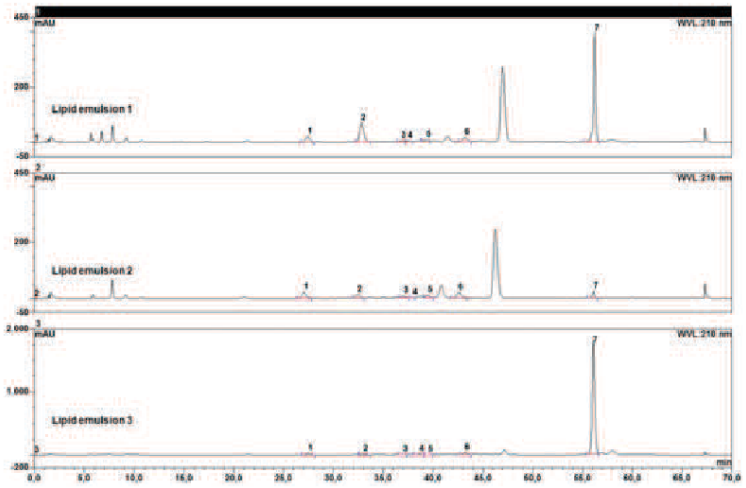

FIGURE 4 Chromatogram of three different lipid emulsions. Identified peaks are: 1, ergosterol; 2, cholesterol; 3, campesterol; 4, lanosterol; 5, stigmasterol; 6, β-sitosterol; and 7, squalene

variability of phytosterols assay in vegetable oils in different batches and characteristics of applied analyses.

4 | CONCLUSION

The proposed analytical method consists of a simplified sample preparation and a single analysis, which successfully separates eight phytosterols, cholesterol and squalene. Validation demonstrated that the method is suitable for routine analysis.

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Nutrición Hospitalaria

Trabajo Original

Nutrición artificial

Selección de emulsiones lipídicas en nutrición parenteral: parámetros bioquímicos y hematológicos

Lipid emulsion selection in parenteral nutrition. Biochemical and hematological paramaters

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Resumen

Introducción: el patrón de ácidos grasos (AG) de las emulsiones lipídicas (EL) utilizadas en nutrición parenteral (NP) condiciona diferentes respuestas fisiológicas.

Objetivos: estudiar si los criterios clínicos de prescripción de EL en NP establecidos en nuestro protocolo abierto y basados en recomendaciones se correlacionan con marcadores bioquímicos y hematológicos iniciales.

Métodos: estudio observacional retrospectivo de cuatro años. Se recogieron variables demográficas, clínicas, nutricionales y analíticas al inicio de la NP. Se realizó un análisis uni y multivariante para estudiar la asociación entre los valores iniciales de los parámetros bioquímicos y hematológicos (PBHE) y el tipo de emulsión lipídica empleada.

Resultados: de los 1.558 pacientes, 460 pacientes (29,5%) tenían PBHE al inicio de la NP y utilizaron mayoritariamente las combinaciones soja (AS) + triglicéridos de cadena media (MCT) + oliva (AO) + pescado (AP) (37,4%) y AS+MCT+AO (35,6%). Se encontraron diferencias estadísticamente significativas en el patrón EL utilizado entre los pacientes con y sin PBHE: patrón de AG con AP 44,8% *vs.* 39,5%, respectivamente.

Las situaciones clínicas con proteína C-reactiva (PCR) elevada se asociaron con mayor uso de EL con AP: AS+AO+AP (OR: 4,52 [IC 95%: 1,43-13,91] y AS+MCT+AO+AP (OR: 3,34 [IC 95%: 2,10-5,33]). En situaciones clínicas complejas asociadas con paciente crítico se utilizó EL con MCT: afectación hepática (AS+MCT OR: 2,42 [IC 95%: 1,03-5,68]) y afectación renal (AS+MCT+AP OR: 3,34 [IC 95%: 1,12-9,99]).

Conclusiones: la inclusión protocolizada de PBHE al inicio de la NP permite complementar criterios clínicos y metabólicos en la elección de la EL.

Palabras clave:

Nutrición parenteral. Emulsiones lipídicas intravenosas. Ácidos grasos omega-3. Parámetros biológicos. Paciente crítico. Paciente quirúrgico.

Abstract

Introduction: Lipid emulsions (LE) are a component of parenteral nutrition (PN) and its fatty acid (FA) profile determines various physiological responses.

Objectives: To assess the adequacy of a clinical not restricted protocol in the choice of LE by studying complementary biochemical and hematological parameters (BHP) at the beginning of the PN.

Methods: A 4-year retrospective observational study of LE administered to patients with PN. Demographic, clinical, nutritional and analytical variables at the beginning of the PN were collected. Univariate and multivariate analyses were performed to study the correlation between the initial clinical and biochemical parameters and the LE profile used.

Results: Four hundred and sixty patients (29.5%) out of 1,558 had BHP at the beginning of PN and used mainly the LE combinations soybean (SO) + medium-chain triglycerides (MCT) + olive (OO) + fish (FO) (37.4%) and SO + MCT + OO (35.6%). Statistically significant differences on the LE pattern were observed between patients with and those without initial BHP (44.8% *vs* 39.5% received FO, respectively). Conditions regularly associated with elevated C-reactive protein (CRP) were associated with increased use of FO LE: SO+OO+FO (OR: 4.52 [95% CI: 1.43-13.91]) and SO+MCT+OO+FO (OR: 3.34 [95% CI: 2.10-5.33]). In those complex conditions related with the critical patient MCT were used: hepatic failure (SO+MCT OR: 2.42 [95% CI: 1.03-5.68]) and renal failure (SO+MCT+FO OR: 3.34 [95% CI: 1.12-9.99]).

Conclusions: The use of BHP at the beginning of PN treatment allows complementing the clinical and metabolic criteria in LE selection.

Key words:

Parenteral nutrition. Intravenous fat emulsions. Fatty acids omega-3. Biomarkers. Critical illness. Postoperative period.

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INTRODUCCIÓN

Desde mediados de los años 70, cuando las emulsiones lipídicas (EL) se introdujeron como un componente básico de la nutrición parenteral (NP), las ventajas y los riesgos que conlleva su utilización han sido fuente constante de debate.

Las EL son una fuente calórica eficiente y aportan ácidos grasos (AG) esenciales. Con su introducción en la NP se consiguió reducir las complicaciones asociadas a la administración de altas cargas de glucosa (1). Posteriormente, con el desarrollo y la comercialización de diferentes patrones lipídicos, su espectro de actividad ha variado y se ha introducido el concepto de farmaconutrición ligado a su administración. En función del patrón de AG utilizado se observan diferentes respuestas inmunológicas e inflamatorias, así como la modificación de rutas metabólicas y de la transducción de señales de ellas derivadas (1-3).

Las EL actuales pueden contener soja (AS), AG de cadena media (MCT), oliva (AO) o pescado (AP). Las EL con AS son las primeras que se utilizaron con éxito y se consideran EL de primera generación. Son ricas en el AG esencial linoleico, que es un AG poliinsaturado (AGPI) de la serie omega-6. Los AG de la serie omega-6 promueven la producción de prostanoides, leucotrienos, factor de necrosis tumoral (TNF-α) e inerleucina (IL)-6, que aumentan la inflamación sistémica y el estrés oxidativo (1,4,5), y se correlacionan con acciones inmunosupresoras como la afectación del sistema reticuloendotelial y la inhibición de funciones de los linfocitos, macrófagos y neutrófilos (6). Las EL con MCT tienen un metabolismo energético más eficiente porque entran más fácilmente en la mitocondria (no dependen de carnitina), presentan un aclaramiento más rápido y no se almacenan como triglicéridos (TG) en tejido adiposo. Además, son resistentes a la peroxidación y no son precursores de mediadores inflamatorios (4,6). Estas EL con MCT se consideran de segunda generación y suelen formularse conjuntamente con AS en proporción 50:50. También de segunda generación son las EL que incorporan un patrón de AO y se formulan junto a AS en proporción 80:20. Esta formulación permite reducir el contenido de ácidos grasos omega-6 manteniendo el aporte de AG esenciales. El componente mayoritario del patrón AO es el ácido oleico, un AG monoinsaturado (AGMI) de la familia omega-9 que se caracteriza por ser resistente a la peroxidación lipídica y no ser precursor de medidores inflamatorios. Esta EL se comporta como inmunoneutra o incluso levemente antiinflamatoria (5). La última generación de EL está constituida por el patrón de AP que se formula solo o en combinación con alguna o todas las emulsiones anteriores. La EL con AP contiene cantidades significativas de AGPI omega-3: ácido linolénico, ácido eicosapentaenoico (EPA) y ácido docosahexaenoico (DHA). Esos AGPI inhiben la producción de citoquinas proinflamatorias (TNFα, IL-6 y IL-1β) y, por tanto, mejoran la inflamación y la capacidad antioxidante (6,7), ya que pueden modular la vía de producción de eicosanoides (8) y de citoquinas antiinflamatorias (IL-10) inhibiendo la expresión nuclear del factor-κ B (1,3). Por otro lado, los AGPI omega-3 producen resolvinas y protectinas, que también tienen un papel importante en la resolución de la inflamación (3) y en la modulación de la producción de citoquinas (4,6). Estas EL suponen un cambio importante por su actividad farmaconutriente, que trasciende al mero aporte energético. También se postula que la administración intravenosa de AGPI omega-3 beneficia el perfil cardiovascular porque modifica el metabolismo lipídico, las concentraciones lipídicas plasmáticas y los parámetros de la coagulación (9-11).

El mejor conocimiento de las ventajas y limitaciones del soporte nutricional endovenoso ha llevado a estandarizar cada vez más la prescripción de estos preparados. No obstante, dada la coexistencia de diferentes EL y la posibilidad de elección, es importante establecer criterios adicionales de utilización que trasciendan los criterios exclusivamente económicos y logísticos.

A partir de la hipótesis de que no todas las EL tienen la misma indicación porque presentan actividades fisiológicas diferentes según el tipo de AG que las componen, pretendemos establecer qué parámetros bioquímicos y hematológicos, obtenidos al inicio de la terapia con NP, complementan los criterios clínicos-metabólicos establecidos en nuestro protocolo para la elección del tipo de EL. Estos criterios de inclusión basados en su actividad farmaconutriente no siempre están recogidos; así, en una reciente revisión en 22 hospitales catalanes (12) se observa que la media de utilización de patrones lipídicos por hospital es de 2.

Cuando en los protocolos se utilizan más de dos emulsiones lipídicas habría que proceder a su evaluación para ver si la utilización es correcta, especialmente cuando, como en el caso de nuestro hospital, la sistematización de la prescripción puede llevar a la desviación de los criterios de prescripción.

El presente trabajo es un estudio observacional de utilización de EL en pacientes hospitalizados tratados con NP. Su objetivo es estudiar si los criterios clínicos de prescripción de EL en NP establecidos en nuestro protocolo abierto y basado en recomendaciones (no restringido ni por especialidad médica ni por tipo de lípido) se correlacionan con marcadores bioquímicos y hematológicos iniciales.

MATERIAL Y MÉTODOS

Estudio observacional retrospectivo de todos los pacientes mayores de 18 años tratados con NP en un hospital de tercer nivel durante cuatro años (2005-2008). Las EL administradas en la NP durante este periodo se muestran en la tabla I.

Se recogieron las siguientes variables: demográficas (edad y peso), clínicas (diagnóstico, unidad de hospitalización, sepsis, afectación hepática inicial o afectación renal en el momento de inicio de la NP y mortalidad), nutricionales (tipo de EL administrada durante todo el tratamiento con NP) y analíticas al inicio de la NP (proteína C reactiva, PCR; prealbúmina; TG; glucemia; leucocitos; pruebas de función hepática [gamma-glutamiltransferasa, GGT; fosfatasa alcalina, FA; alanina-aminotransferasa, ALT; bilirrubina total, BIL]; y creatinina).

La variable sepsis se definió por la presencia de infección y, como mínimo, dos de los siguientes parámetros: temperatura > 38 °C o < 36 °C, taquicardia (> 90 latidos/min), leucocitosis (> 12 x 10^9) o leucopenia (< 4 x 10^9), y taquipnea de

Tabla I. Marcas de emulsiones lipídicas administradas durante el estudio

Marca comercial	Laboratorio	Composición			
		Aceite de oliva	Aceite de soja	Triglicéridos de cadena media	Aceite de pescado
Structolipid®/Structokabiven®[1]	Fresenius Kabi Bad Homburg, Germany	-	36%	64%	-
Clinoleic®/Oliclinomel®[1]	Baxter Lessines, Belgium	80%	20%	-	-
SMOFlipid®	Fresenius Kabi Bad Homburg, Germany	25%	30%	30%	15%
Omegaven®	Fresenius Kabi Bad Homburg, Germany	-	-	-	100%

[1]*Bolsas tricompartimentales de nutrición parenteral con aminoácidos, glucosa y lípidos.*

> 20 respiraciones/min o presión parcial de CO_2 < 4,2 kPa (13). La afectación hepática se definió como la elevación de bilirrubina > 34 µmol/l o > 2 mg/dl, a partir de los criterios establecidos por la escala Child-Pugh (14). La afectación renal se definió como filtración glomerular < 60 ml/min/1,73 m^2, utilizando la fórmula CKD-EPI (15).

Las variables analíticas PCR, prealbúmina, TG y glucosa se categorizaron con los criterios siguientes:

- La PCR es una proteína plasmática que participa en la respuesta inmunológica y sus niveles pueden elevarse rápidamente después de una inflamación aguda (16). Se consideran valores normales concentraciones entre 5 y 10 mg/l. En infecciones leves y víricas los niveles pueden alcanzar los 10-40 mg/l; en inflamaciones graves e infecciones bacterianas, 40-200 mg/l; en infecciones bacterianas graves y en quemados los niveles superan los 200 mg/l (17). En el estudio se consideró PCR ≥ 200 mg/l como nivel de corte.
- La prealbúmina es un marcador de desnutrición, de inflamación aguda y de estrés metabólico (18,19). El rango normal oscila entre 170-420 mg/l (18). Dado que valores inferiores a 100 mg/l se asocian con desnutrición grave (20), fue este punto de corte el que se utilizó en el estudio, teniendo en cuenta que, al tratarse de una proteína inversa de fase aguda, estos valores se ven enmascarados en estados inflamatorios.
- La administración de lípidos en la NP debe equilibrarse con la utilización del sustrato y mantener una concentración plasmática de TG < 3 mmol/l (21). Con niveles > 4,5-5 mmol/l se recomienda parar el aporte lipídico (22,23). En el estudio, se estableció el corte a partir de niveles por encima de 3 mmol/l.
- La administración de glucosa en la NP tiene las mismas consideraciones metabólicas que la administración de lípidos. El objetivo es equilibrar el aporte con la utilización del sustrato para mantener niveles plasmáticos de 5-8 mmol/l (21). Cuando se superan 10 mmol/l, se consideran valores de hiperglucemia asociados a pronóstico negativo con riesgo incrementado de complicaciones infecciosas (24). En el estudio se consideró hiperglucemia a partir de niveles > 10 mmol/l.

Los criterios de utilización para las diferentes EL vienen establecidos por diferentes situaciones clínicas agrupadas en función del estrés metabólico y fracaso orgánico (Tabla II).

Las EL utilizadas se agruparon en seis categorías, teniendo en cuenta todas las combinaciones utilizadas:

- Patrón AS+MCT (Structolipid® o Structokabiven®)
- Patrón AS+MCT+AO Clinomel® y Structokabiven® secuencialmente
- Patrón AS+AO (Clinoleic® o Clinomel®)
- Patrón AS+AO+AP (Clinoleic® o Clinomel®) +Omegaven®
- Patrón AS+MCT+AP (Structolipid® o Structokabiven®) +Omegaven®
- Patrón AS+MCT+AO+AP (SMOFlipid®)

El protocolo establece la administración de 25 a 30 kcal/kg/d si el IMC es inferior a 25. Se administran lípidos entre 0,8-1 g/kg/d, glucosa entre 2-3 g/kg/d y proteínas entre 1-1,2 g/kg/d en paciente no crítico y 1,5 g/kg/día en paciente crítico. En insuficiencia renal y técnicas de depuración renal se ajustan las cantidades de proteínas administradas. Todas las nutriciones preparadas llevan vitaminas y elementos traza diariamente. En cuanto a electrolitos, en críticos solo se aditiva fosfato y magnesio y en el resto de los pacientes, sodio, potasio, fosfato, magnesio y calcio según necesidades y estabilidad de la elaboración.

En nuestro hospital, con un largo recorrido en la utilización de NP, se ha simplificado su prescripción. En el periodo estudiado se procedía a su petición mediante una receta de inicio (hoy en día informatizada) en la que el clínico tenía que especificar una de las diferentes opciones detalladas en la tabla II y que se basan en la elección de diferentes patrones de lípidos en función de la bibliografía existente sobre el tema, así como recogiendo los criterios de uso de los diferentes hospitales de la red pública de Cataluña (10). Nuestro laboratorio de bioquímica configuró un perfil nutricional específico que se utiliza principalmente para el seguimiento clínico más que para la elección de las fórmulas de inicio.

Tabla II. Protocolo de utilización de emulsiones lipídicas para la nutrición parenteral durante el periodo de estudio

Fórmula	Criterios	Emulsión lipídica recomendada	Patrón lipídico
Mantenimiento	Intervención quirúrgica no compleja sin complicaciones infecciosas	Clinoleic® o Clinomel®	AS+AO
Estrés moderado	Postcirugía compleja[1], infecciones, fístula	Structolipid® o Structokabiven®	AS+MCT
Estrés grave	Paciente crítico[2]	SMOFlipid®	AS+MCT+AO+AP
Estrés grave + fracaso orgánico	Paciente crítico + shock séptico o fracaso orgánico (renal, distrés respiratorio,etc.)	(Clinoleic® o Clinomel®) + Omegaven® o (Structolipid® o Structokabiven®) + Omegaven®	AS+AO+AP o AS+MCT+AP[3]

AS: aceite de soja; AO: aceite de oliva; MCT: triglicéridos de cadena media; AP: aceite de pescado. [1]Trasplante hepático, bricker, *esofagectomía, gastrectomía total, duodenopancreatectomía, resección intestino delgado, etc. [2]Ingreso en unidades críticos, ventilación mecánica, infecciones, politraumatismo. [3]Patrones suplementados con aceite de pescado a dosis mayores a SMOFlipid®.*

ANÁLISIS ESTADÍSTICO

Las variables descriptivas categóricas se describieron como porcentajes y las continuas, como medias con su desviación estándar.

Se hizo un análisis univariante mediante análisis de la varianza de un factor (*one-way* ANOVA) para estudiar la asociación entre los valores iniciales de los parámetros clínicos y bioquímicos y el tipo de patrón lipídico utilizado. Se utilizó el test de Chi-cuadrado para determinar la asociación entre las variables clínicas categorizadas y el tipo de patrón lipídico. En esta aproximación univariante la variable patrón lipídico se categorizó en seis en función de las combinaciones de EL utilizadas. Se estableció la significación estadística para $p < 0,05$.

Se hizo un análisis multivariante construyendo seis modelos de regresión logística múltiple por pasos *(stepwise).* La variable dependiente de cada modelo fue el tipo de patrón lipídico y como variables independientes se incluyeron las variables clínicas y bioquímicas categorizadas. El criterio de inclusión fue $p < 0,2$, y la significación estadística se estableció mediante los intervalos de confianza del 95%.

Se utilizó el programa estadístico SPSS versión 19.0.

En este estudio se contempló la exención del consentimiento informado de los pacientes, dado que se trataba de un análisis retrospectivo de nuestra práctica clínica asistencial. Los datos de los pacientes se anonimizaron para los fines de este estudio. La información confidencial de los pacientes se trató de acuerdo con lo establecido por la normativa legal vigente en nuestro país en materia de protección de datos. Este manuscrito ha sido aprobado para su publicación por el Comité Ético de Investigación Clínica del Hospital Universitario de Bellvitge.

RESULTADOS

Durante los cuatro años de estudio, recibieron NP 1.558 pacientes, de los cuales solo 460 (29,5%) presentaban parámetros bioquímicos y hematológicos (PBHE) de inicio. De estos últimos, 212 estuvieron ingresados en una unidad de críticos. El 65% eran hombres, la edad media era de 63 ± 15 años y la media del peso fue 72 ± 14 kg. La mortalidad fue del 13,5% (62 pacientes). El diagnóstico más frecuente fue neoplasia digestiva (207 pacientes, 45%) (Tabla III). Los valores iniciales de las variables clínicas y analíticas se muestran en la tabla IV.

Las combinaciones más utilizadas fueron AS+MCT+AO+AP en 172 (37,4%) pacientes y AS+MCT+AO en 164 (35,6%) pacientes (Tabla V). Se encontraron diferencias estadísticamente significativas en el patrón EL entre los pacientes que tenían PBHE iniciales y los que no. Un 44,8% de los pacientes con PBHE iniciales recibieron AP, en comparación con el 39,5% de pacientes que no tenían PBHE. El patrón exclusivamente con AO se administró en un 9,8% de pacientes con PBHE iniciales frente al 13,1% en los que no las tenían. Entre ambos grupos de pacientes no existían diferencias estadísticamente significativas en cuanto a días de tratamiento con NP, estancia en unidades de cuidados intensivos y *exitus.*

En el análisis univariante se encontraron diferencias estadísticamente significativas entre los valores iniciales de los parámetros bioquímicos y la utilización de los seis patrones lipídicos excepto en el caso de los TG, que solo mostraron tendencia a la significación (Tabla VI). Asimismo, en el análisis univariante de las variables

Tabla III. Diagnósticos

Diagnóstico	n (%)
Neoplasia digestiva	207 (45)
Patología digestiva no tumoral	162 (35,2)
Neoplasia no digestiva	25 (5,4)
Traumatismo	17 (3,7)
Patología cardiovascular	17 (3,7)
Infecciones	15 (3,3)
Otros	11 (2,4)
Trasplante	6 (1,3)
Total	*460 (100)*

Tabla IV. Valores iniciales de las variables clínicas y analíticas

Parámetros clínicos y bioquímicos iniciales	
Datos iniciales	**Media ± desviación estándar**
Proteína C reactiva (mg/l)	135,9 ± 108,9
Prealbúmina (mg/l)	129,7 ± 73,4
Triglicéridos (mmol/l)	2,2 ± 1,2
Glucosa (mmol/l)	8,3 ± 3,1
Filtración glomerular (ml/min)	89,0 ± 31,4
Bilirrubina (µmol/l)	33,6 ± 75,9
Variables clínicas categorizadas	
Datos iniciales	**n (%)**
Proteína C reactiva ≥ 200 mg/l	126 (27,4%)
Prealbúmina ≤ 100 mg/l	195 (42,4%)
Triglicéridos ≥ 3 mmol/l	87 (18,9%)
Glucosa ≥ 10 mmol/l	95 (20,7%)
Sepsis	61 (13,3%)
Afectación hepática	42 (9,1%)
Afectación renal	50 (10,9%)

clínicas categorizadas se encontraron diferencias estadísticamente significativas entre los patrones lipídicos para las variables PCR ≥ 200 mg/l, prealbúmina ≤ 100 mg/l, afectación renal y hepática. Las variables glucosa ≥ 10 mmol/l y sepsis mostraron una tendencia a la significación, mientras que la variable triglicéridos ≥ 3 mmol/l no mostró significación estadística (Tabla VI).

Tabla V. Patrones utilizados

Patrón	PBHE iniciales n (%)		p
	Sí (n = 460)	No (n = 1.098)	
AS+MCT+AO+AP	172 (37,4%)	393 (35,8%)	0,01
AS+MCT+AP	21 (4,6%)	24 (2,2%)	
AS+AO+AP	13 (2,8%)	17 (1,5%)	
AS+MCT+AO	164 (35,6%)	436 (39,7%)	
AS+MCT	45 (9,8%)	84 (7,7%)	
AS+AO	45 (9,8%)	144 (13,1%)	

PBHE: parámetros bioquímicos y hematológicos; AS: aceite de soja; AO: aceite de oliva; MCT: triglicéridos de cadena media; AP: aceite de pescado.

En el análisis multivariante con seis modelos de regresión logística (Tabla VII), la variable PCR ≥ 200 mg/l entró en los seis patrones con una asociación estadísticamente significativa en tres de ellos; dos con AP: AS+AO+AP (OR: 4,52 [IC 95%: 1,43-13,91] y AS+MCT+AO+AP (OR: 3,34 [IC 95%: 2,10-5,33] y uno sin AP: AS+MCT+AO (OR: 0,26 [IC 95%: 0.15-0.46]). Por lo tanto, los pacientes con PCR ≥ 200 mg/l tenían mayor probabilidad de recibir EL con AP, mientras que los pacientes con PCR < 200 mg/l tenían mayor probabilidad de recibir EL sin AP.

Tabla VI. Análisis univariante de los patrones lipídicos y las variables continuas y categóricas

	AS+AO (n = 45)	AS+MCT (n = 45)	AS+MCT+AO (n = 164)	AS+AO+AP (n = 13)	AS+MCT+AP (n = 21)	AS+MCT+AO+AP (n = 172)	p
Variables continuas*							
Datos iniciales	**Media ± DE**	**Media ± DE**	**Media ± DE**	**Media ± DE**	**Media ± DE**	**Media ± DE**	
PCR (mg/l)	94,2 ± 96,8	119.6 ± 92,8	92,1 ± 81,7	208,5 ± 97,4	204,5 ± 137,9	179,3 ± 112,9	0,00
Prealbúmina (mg/l)	158,2 ± 65,4	127,69 ± 66,9	143,3 ± 67,9	108,2 ± 70,9	112,6 ± 71.1	113,7 ± 78,3	0,00
Triglicéridos (mmol/l)	1,98 ± 0,9	1,94 ± 1,5	2,3 ± 1,1	1,8 ± 0,9	2,4 ± 1.6	2,4 ± 1,20	0,06
Glucosa (mmol/l)	8,3 ± 3,4	7,34 ± 2,2	7,9 ± 2,9	8,8 ± 3,2	8,5 ± 3,1	8,8 ± 3,3	0,02
FG (ml/min)	102,7 ± 31,0	85,25 ± 31,8	89,7 ± 26,3	8,7 ± 36,9	6,1 ± 42,8	88,7 ± 32,4	0,00
Bilirrubina (µmol/l)	15,9 ± 4,3	52,20 ± 138,9	25,3 ± 56,7	13,2 ± 12,2	64,3 ± 105,2	39,0 ± 74,3	0,03
Variables categóricas**							
Datos iniciales	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	
PCR ≥ 200 mg/l	6 (13,3%)	7 (15,6%)	19 (11,6%)	8 (61,5%)	10 (47,6%)	76 (44,2%)	0,00
Prealbúmina ≤ 100 mg/l	11 (24,4%)	20 (44,4%)	52 (31,7%)	9 (69,2%)	10 (47,6%)	93 (37,4%)	0,00
Triglicéridos ≥ 3 mmol/l	6 (13,3%)	6 (13,3%)	29 (33,3%)	1 (7,7%)	4 (19%)	41 (23,8%)	0,33
Glucosa ≥ 10 mmol/l	9 (20%)	4 (8,9%)	28 (17,1%)	5 (38,5%)	5 (23,8%)	44 (25,6%)	0,07
Sepsis	6 (13,3%)	6 (13,3%)	13 (7,9%)	2 (15,4%)	8 (38,1%)	26 (15,1%)	0,07
Afectación hepática	1 (2,2%)	8 (17,8%)	12 (7,3%)	0 (-)	4 (19,0%)	17 (9,9%)	0,04
Afectación renal	3 (6,7%)	7 (15,6%)	12 (7,3%)	3 (23,1%)	8 (38,1%)	17 (9,9%)	0,00

**Análisis de la varianza de un factor, estadístico F de Snedecor. **Tablas de contingencia, estadístico Chi-cuadrado.*

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Tabla VII. Análisis multivariante de cada patrón lipídico

	Patrones lipídicos											
	AS+AO (n = 45)		AS+MCT (n = 45)		AS+MCT+AO (n = 164)		AS+AO+AP (n = 13)		AS+MCT+AP (n = 21)		AS+MCT+AO+AP (n = 172)	
Datos iniciales	**OR (IC 95%)**	**p**	**OR (IC 95%)**	**p**	**OR (IC 95%)**	**p**	**OR (IC 95%)**	**p**	**OR (IC 95%)**	**p**	**OR (IC 95%)**	**p**
PCR ≥ 200 mg/l	0,47 (0,18-1,20)	0,11	0,48 (0,21-1,11)	0,08	0,26 (0,15-0,46)	0,00	4,52 (1,43-13,91)	0,01	2,08 (0,83-5,19)	0,11	3,34 (2,10-5,33)	0.00
Prealbúmina ≤ 100 mg/l	0,54 (0,25-1,14)	0,10	------	--	0,74 (0,48-1,14)	0,17	------	--	----	--	1,46 (0,95-2,23)	0,08
Afectación hepática	0,23 (0,03-1,71)	0,15	2,42 (1,03-5,68)	0,04	------	--	------	--	----	--	----	--
Sepsis	-----	--	-----	--	0,48 (0,25-0,94)	0,03	----	--	2,52 (0,85-7,43)	0,09	----	--
Glucosa ≥ 10 mmol/l	-----	--	0,35 (0,12-1,00)	0,05	-----	--	2,33 (0,72-7,52)	0,16	----	--	1,54 (0,95-2,49)	0,08
Afectación renal	-----	--	-----	--	-----	--	----	--	3,34 (1,12-9,99)	0,03	0,60 (0,31-1,17)	0,136
Triglicéridos ≥ 3 mmol/l	-----	--	-----	--	-----	--	-----	--	----	--	1,53 (0,93-2,51)	0,09

[Nutr Hosp 2017;34(4):767-775]

La variable prealbúmina ≤ 100 mg/l entró en tres patrones, dos de ellos sin AP. En ninguno de los tres casos, a pesar de mostrar tendencia, las diferencias fueron estadísticamente significativas (Tabla VII). Valores de prealbúmina ≤ 100 mg/l reducían la probabilidad de recibir EL sin AP y valores de prealbúmina > 100 mg/l aumentaban la probabilidad de recibir el patrón AS+MCT+AO+AP.

La variable afectación hepática entró en dos de los tres patrones lipídicos sin AP. Los pacientes con afectación hepática tenían mayor probabilidad estadísticamente significativa de recibir la combinación AS+MCT (OR: 2,42 [IC 95%: 1,03-5,68]).

La sepsis entró en dos patrones, pero únicamente en el patrón AS+MCT+AO con significación estadística, de tal forma que los pacientes sépticos tenían menos probabilidad (OR: 0,48 [IC 95%: 0,25-0,94]) de recibir esta combinación. Sin significación estadística, los pacientes sépticos presentaban una tendencia a recibir uno de los patrones con AP (AS+MCT+AP).

La variable glucosa ≥ 10 mmol/l entró en tres patrones, pero la relación únicamente fue estadísticamente significativa en el patrón AS+MCT (OR: 0,35 [IC 95%: 0,12-1,00]). En los otros dos modelos, aunque la relación no era estadísticamente significativa, los pacientes con glucosa ≥ 10 mmol/l tenían una mayor probabilidad de recibir combinaciones con AP (AS+AO+AP o AS+MCT+AO+AP).

La variable afectación renal entró en dos patrones lipídicos con AP, con significación estadística en uno de ellos. Los pacientes con afectación renal tenían mayor probabilidad de recibir la combinación AS+MCT+AP (OR: 3,34 [IC 95%: 1,12-9,99]).

La variable triglicéridos ≥ 3 mmol/l solo entró y se asoció con el patrón AS+MCT+AO+AP sin relación estadísticamente significativa.

DISCUSIÓN

Actualmente, las EL en la NP, además de ser fuente calórica, se utilizan por sus propiedades como farmaconutrientes. Es bien conocido el papel de las diferentes EL en la respuesta inflamatoria, en la afectación del sistema inmune y de las rutas metabólicas y en la transducción de señales (1-3). Las EL con AP han demostrado su papel antiinflamatorio al inhibir la síntesis de citoquinas proinflamatorias, modular la producción de eicosanoides e inhibir la expresión de factores de transcripción nucleares (1,3,6-8).

Diversos ensayos clínicos han demostrado los efectos beneficiosos de la suplementación de la NP con AP en el paciente quirúrgico, tales como la modulación de los marcadores inflamatorios, la reducción de la estancia hospitalaria y la reducción de morbilidad infecciosa (3). En el paciente crítico los resultados son más controvertidos (3). En una revisión, Stapleton y cols. (2) analizaron el papel del AP administrado vía enteral o parenteral en los procesos inflamatorios en paciente críticos, especialmente en sépticos y en pacientes con insuficiencia respiratoria aguda, sin llegar a conclusiones firmes. En un metaanálisis de Pradelli y cols. (25) concluyeron que, en pacientes críticos y quirúrgicos, la administración de EL con AP se asocia con una reducción significativa de infecciones y de días de estancia hospitalaria. En otro metaanálisis en paciente crítico, Palmer y cols. (26) no encontraron una reducción significativa de mortalidad, infecciones ni días de estancia en UCI, mientras que Manzanares y cols. (27) en su metaanálisis encuentran diferencias significativas en la reducción de infecciones y una cierta tendencia a la significación en la reducción de necesidad de ventilación mecánica y de los días de estancia hospitalaria.

Nuestros resultados evidencian que los pacientes con PBHE iniciales presentaban una mayor utilización de AP, a diferencia de aquellos en los que no se obtuvieron PBHE. Estos últimos recibieron más emulsiones no adaptadas a la fase de estrés. No obstante, hay que considerar un posible sesgo de selección, dado que a los pacientes más críticos se les solicitan más pruebas. También hay diferencias en los PBHE iniciales de los pacientes según la EL administrada. Las diferencias más destacables se encuentran en los valores de PCR, que se utiliza tanto como indicador de respuesta inflamatoria en paciente crítico o séptico, como en el seguimiento de la respuesta nutricional. En nuestro estudio, a los pacientes con una PCR inicial más elevada se les administró EL con AP, coincidiendo tanto con Gultekin y cols. (28) y Hall y cols. (29), que encontraban una disminución de la PCR en los pacientes sépticos tratados con EL con AP, como con Grau-Carmona y cols. (3), que en pacientes críticos observaron una disminución de la PCR al administrar EL con AP. En nuestro estudio con pacientes quirúrgicos (30) la disminución de la PCR no fue significativa, pero la administración de NP con EL que contienen AP se limitó a cinco días. Los resultados obtenidos y la bibliografía apuntan a que administrar EL con AP puede mejorar el perfil inflamatorio del paciente, pero faltan más estudios para confirmar estos resultados.

La prealbúmina es una proteína de vida media corta que refleja bien los cambios agudos en el estado nutricional del paciente, pero es también una proteína de fase aguda que disminuye en los procesos inflamatorios (18,19). En nuestro análisis univariante, los pacientes con los valores más bajos de prealbúmina recibieron EL con AP con diferencias estadísticamente significativas entre los diferentes patrones lipídicos. En el análisis multivariante, la prealbúmina se relacionó con tres patrones de EL, pero en ninguno de ellos con significación estadística. Pocos estudios analizan la relación entre EL y prealbúmina y los que lo hacen no encuentran asociaciones significativas (28,30). Por lo tanto, el parámetro prealbúmina no parece ser crítico para seleccionar la EL a administrar, aunque harían falta más estudios que analizaran específicamente la prealbúmina para confirmar los resultados.

Por lo que respecta a las tasas de infección, nuestro grupo (30), en un ensayo clínico en paciente quirúrgico, demostró que la incidencia de infecciones disminuye con significación estadística al suplementar la NP con AP. Aunque Palmer y cols. (26) en un metaanálisis de paciente crítico no encontraron una reducción significativa de mortalidad ni de infecciones, los últimos metaanálisis de Grau-Carmona y cols. (3) y de Manzanares y cols. (27) concluyen que se da una reducción de las infecciones en pacientes críticos tratados con EL con AP. En cuanto a la sepsis, que frecuentemente precede al desarrollo de fallo multiorgánico como consecuencia de una inflamación descontrolada, Hall y cols. (29)

encontraron que los pacientes tratados con AP presentaban una reducción significativa en la aparición de disfunción orgánica y en los valores máximos de PCR. En nuestro estudio univariante, la sepsis no fue un factor con relación estadísticamente significativa en la selección de un patrón lipídico frente a otro, pero sí que lo fueron la afectación hepática y la renal. Por otro lado, Gultekin y cols. (28) también observaron una disminución significativa de PCR, mientras que los resultados del resto de parámetros inflamatorios como prealbúmina, IL-6, TNFα y leucocitos no fueron significativos. Por lo tanto, estos estudios demuestran que en paciente quirúrgico y crítico se produce una disminución de parámetros inflamatorios y de infección administrando EL con AP.

En el análisis univariante, la asociación entre afectación hepática y el tipo de patrón lipídico fue significativa, y en el multivariante se confirmó que los pacientes con afectación hepática tenían más probabilidad de recibir el patrón lipídico MCT+AS que de recibir EL con AP. Las EL con MCT mejoran algunas de las características del metabolismo de las EL con AS. Entran más fácilmente en la mitocondria, se oxidan y aclaran más rápidamente, y no se almacenan como TG en el tejido adiposo (4,6). Gracias a las características de su metabolismo, MCT en el paciente con la función hepática alterada pueden ayudar a la nutrición sin sobrecargar el metabolismo hepático, preservando la función hepática (4,6,31). Las últimas guías de la European Society for Clinical Nutrition and Metabolism (ESPEN) en fracaso hepático recomiendan el uso de EL con AP en pacientes con alteraciones hepáticas (32).

El paciente crítico se asocia a un estrés catabólico y presenta una respuesta inflamatoria aumentada asociada a complicaciones como fracaso multiorgánico, hospitalización prolongada y elevada mortalidad (33). El fracaso renal es un elemento común en este fracaso multiorgánico del paciente crítico. En nuestro estudio, en el análisis univariante la relación entre la EL administrada y la afectación renal fue significativa, y en el multivariante se demostró que el paciente con afectación renal tenía más probabilidad de recibir EL con AP. En el estudio de Hall y cols. (29), la administración de EL con AP demostró atenuar los efectos de la inflamación exacerbada que se genera en el fracaso multiorgánico. Las guías ESPEN (34) describen que el hipercatabolismo presente en pacientes con fracaso renal agudo (por fracaso multiorgánico) podría estar generado por la respuesta inflamatoria y, por tanto, las EL con AP son una opción para tratar de controlar esta respuesta inflamatoria. Nuestros resultados coinciden con la propuesta de estas guías ESPEN ya que, según el análisis multivariante, los pacientes con afectación renal tienen más probabilidad de recibir EL con AP.

En nuestro estudio, los valores iniciales de TG no muestran diferencias significativas al comparar un patrón lipídico frente a otro y, aun cuando el aclaramiento de las EL con AP es *a priori* mejor (35), persiste una cierta controversia sobre los mecanismos de aclaramiento, por lo que las guías no proponen la utilización de EL con AP para contrarrestar la hipertrigliceridemia (22).

Una limitación del estudio es que no se evalúan la ventajas clínicas obtenidas con la elección de cada EL. No obstante, cabe destacar que el objetivo ha sido establecer el grado de cumplimiento de un protocolo basado en recomendaciones recogidas en la bibliografía y que nuestro interés es complementar los estudios efectuados por nuestro grupo y otros posteriores en cartera sobre los efectos de las EL con AP en situaciones clínicas que cursan con respuesta inflamatoria sistémica y en alteraciones de la función hepática.

CONCLUSIÓN

Los criterios clínicos establecidos en nuestro protocolo se correlacionan con los PBHE obtenidos al inicio de la terapia con NP. La utilización de parámetros de respuesta inflamatoria como la PCR, y los asociados a situaciones clínicas de estrés como glucosa, creatinina, bilirrubina y leucocitos son herramientas útiles en la modelización de fórmulas individualizadas.

Dado el reducido grupo de pacientes de los que se obtiene perfil nutricional inicial, nuestro estudio pone de manifiesto que la utilización protocolizada al inicio del tratamiento con NP de estos PBHE permitiría complementar los criterios clínicos y metabólicos en la elección de la EL a administrar. De todos los parámetros estudiados, la PCR es la que más se correlaciona con los diferentes escenarios de utilización de patrones de EL, mientras que la prealbúmina, parámetro utilizado de manera habitual como indicador de síntesis proteica, no ha resultado tan decisiva como criterio de elección.

Las situaciones que cursan con PCR elevadas se asocian con un mayor uso de EL con AP. En situaciones de complejidad clínica características del paciente crítico (fracaso hepático, fracaso renal, sepsis) se utilizan EL con MCT.

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Phytosterol determination in lipid emulsions for parenteral nutrition

Determinación de fitoesteroles en emulsiones lipídicas para nutrición parenteral

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Abstract

Objective: The presence of phytosterols in vegetal lipid emulsions has been associated with alterations of liver function tests. Determination of phytosterols content, currently undeclared, would allow the development of strategies to prevent or treat these alterations.

Method: 3-4 non-consecutive batches of 6 lipid emulsions from different providers (Clinoleic™, Intralipid™, Lipofundina™, Lipoplus™, Omegaven™ and Smoflipid™) were analyzed. Differences in total phytosterol assay between providers and batches were statistically studied by a one-way ANOVA and Kruskal-Wallis non-parametric approximation and post hoc Scheffé test ($p < 0.05$)

Results: The absence of phytosterols was confirmed in Omegaven™, emulsion based on fish oil. The highest assay of phytosterols (422.4 ± 130.5 µg/mL) has been related with the highest percentage of soya bean oil in Intralipid. In the remaining emulsions, concentrations were from 120 to 210 µg/mL related to the percentage of soya bean oil. Statistically significant differences of phytosterol content in lipid emulsions were observed among different providers ($F = 23.59$; $p = 0.000$) as well as among non-consecutive batches. Clinolenic™ ($F = 23.59$; $p = 0.000$), Intralipid™ ($F = 978.25$; $p = 0.000$), Lipofundina™ TCL/TCM ($F = 5.43$; $p = 0.045$), Lipoplus™ ($F = 123.53$; $p = 0.000$) and Smoflipid™ (16.78; $p = 0.000$). Except for Lipofundina™ TCL/TCM, the differences between batches were marked.

Conclusions: Lipid emulsions, registered on Spanish pharmaceutical market, contain variable quantities of phytosterols dependent on commercial brand and batch.

Resumen

Objetivo: La presencia de fitoesteroles en emulsiones lipídicas de origen vegetal se ha relacionado con la aparición de alteraciones de los parámetros de la función hepática. El objetivo es determinar la presencia de fitoesteroles en las emulsiones registradas en el mercado farmacéutico.

Método: Se analizaron tres-cuatro lotes no consecutivos de seis marcas distintas de emulsiones lipídicas (Clinoleic®, Intralipid®, Lipofundina®, Lipoplus®, Omegaven® y Smoflipid®) y las diferencias en contenido de fitoesteroles totales entre marcas y entre lotes se estudiaron estadísticamente (ANOVA de un factor, aproximación no paramétrica de Kruskal-Wallis y análisis *post hoc* Scheffé; $p < 0,05$).

Resultados: Se encontró ausencia de fitoesteroles en el preparado Omegaven® con aceite de pescado. El contenido más alto de fitoesteroles (422,4 ± 130,5 µg/mL) coincidió con el porcentaje más alto de aceite de soja (Intralipid®). En el resto de las emulsiones se detectaron concentraciones de fitoesteroles entre 120 y 210 µg/mL, relacionadas con el contenido de aceite de soja. Se observaron diferencias estadísticamente significativas entre todas las marcas de emulsiones lipídicas ($F = 42,97$; $p = 0,000$) y entre lotes no consecutivos. Clinolenic® ($F = 23,59$; $p = 0,000$); Intralipid® ($F = 978,25$; $p = 0,000$); Lipofundina® TCL/TCM ($F = 5,43$; $p = 0,045$); Lipoplus® ($F = 123,53$; $p = 0,000$),; y Smoflipid® (16,78; $p = 0,000$). Excepto en el caso de la Lipofundina® TCL/TCM las diferencias entre lotes fueron marcadas.

Conclusiones: Las emulsiones lipídicas registradas en el mercado farmacéutico español contienen cantidades variables de fitoesteroles en función de la marca comercial y el lote. La determinación del contenido de fitoesteroles, actualmente no declarados, permitiría desarrollar estrategias para prevenir o tratar la aparición de estas alteraciones.

KEYWORDS
Phytosterols; Lipid emulsions; Parenteral nutrition; Soybean oil; Liver function tests.

PALABRAS CLAVE
Fitoesteroles; Emulsiones lipídicas; Nutrición parenteral; Aceite de soja; Parámetros de función hepática.

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Introduction

Lipid emulsions (LEs) are routinely used in parenteral nutrition (PN). Prior to the inclusion of LEs in these formulas, PN required high amounts of glucose, which was associated with a range of problems[1]. The high energy efficiency of lipids led to a reduction in the use of glucose.

In Spain, the use of LEs in PN became routine practice in the 1980s. Initially, all LEs were based on soybeans, but since then a range of formulations has been developed. Currently, 5 LEs are registered for the Spanish pharmaceutical market. They are based on soybeans, olives, medium-chain triglycerides (MCTs), and fish oil in different concentrations and combinations.

Although LEs were initially used as an energy substrate, the anti-inflammatory effect of fish oil[2,3] and the lower lipid peroxidation effect of olive oil[4] has led to these lipids being proposed as pharmaconutrients.

Parenteral nutrition-associated liver disease is one of the most relevant complications of PN. Parenteral nutrition-associated liver disease has a multifactorial component[5,6,7], and the quantity and type of lipid[8,9] have clearly been established as among the factors associated with the disease. Therefore, it is relatively common in clinical practice to reduce doses or to even temporarily stop the administration of lipids altogether[10,11]. For several years, it was hypothesised that these complications were associated with the use of plant-based LEs. Since the time of the study by Clayton in the paediatric population[12], this possibility has been attributed to the presence of phytosterols, which hypothesis was subsequently confirmed in adult patients by our study group[13]. The phytosterol content of LEs is currently undeclared, and thus does not appear in the Summary of Product Characteristics or on the label. Currently, all emulsions available on the Spanish pharmaceutical market contain variable amounts of plant-based lipids and therefore contain phytosterols. This means that LE use entails their erratic administration.

Phytosterols occur in plants and are considered to be equivalent to cholesterol due to their having a similar sterol structure and similar functions in cell membrane regulation. There has been a recent increase in their clinical importance due to their demonstrated beneficial effects on cholesterol reduction when orally administered[14,15,16]. Due to their potential hepatotoxicity, the determination of phytosterol content in LEs would improve the management and prevention of hepatic complications in PN.

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) analytical methods, particularly for the analysis of food and plant extracts, are available for the qualitative and quantitative determination of phytosterols. Gas chromatography can simultaneously determine phytosterols, whereas the available HPLC methods can only identify a few phytosterols and only under particular conditions[17].

We developed a simple HPLC analytical method for the routine determination of phytosterol content in parenteral LEs. The objective of this study was to determine differences in the phytosterol content of LEs available on the Spanish pharmaceutical market according to their formulation, brand, and batch.

Methods

We prospectively analysed intravenous LEs with different compositions available on the Spanish pharmaceutical market (Table 1) to determine daily exposure to phytosterols in patients with PN.

To better simulate clinical practice in Spain, we established different scenarios according to the brand of LE and batch. Thus, we studied 3-4 batches of each of the 5 plant-based LEs available on the Spanish pharmaceutical market. Batches corresponded to non-consecutive shipments.

We included Omegaven™, which is an LE exclusively based on fish oil. This LE was imported because it is not registered in the Spanish pharmaceutical market.

We developed an HPLC analytical method for the routine quantification of phytosterols by establishing a sample preparation protocol. This method can simply and effectively separate phytosterols from the matrix. The aim was to obtain phytosterol samples with a high extraction percentage and good repeatability in a short period of time. Liquid chromatography was performed using a Dionex Ultimate 3000[18] chromatography system.

Differences in total phytosterol assay between the 5 brands and between batches were analysed using one-way ANOVA, post hoc multiple-comparison Scheffé test ($P<.05$), and nonparametric Kruskal-Wallis test.

Data were analysed using IBM SPSS 22.0 software. A P value of <.05 was used as a cutoff for statistical significance, using a two-tailed test.

Results

The proposed analytical method allowed us to simplify sample preparation and conduct a single analysis, which led to the successful separation of 8 phytosterols, cholesterol, and squalene. The validation process showed that the method is suitable for routine analysis.

The analysis of LE brands (Table 2) showed that the fish-oil-based LE Omegaven™ did not contain phytosterols. This finding was in line with previously published results[3,5], and therefore Omegaven™ was excluded from the statistical analysis. Intralipid is based completely on soybean oil. Its analysis showed that it contained the highest concentration of phytosterols (422.4 ± 130.5 µg/mL) and confirmed that soybean oil was the source of its high phytosterol content. The analysis showed that the other LE brands had variable phytosterol content ranging from 120 µg/mL to 210 µg/mL, depending on the percentage of soybean oil. Statistically significant differences were found between these brands ($F=42.97$; $p=0.00$). A weak correlation was found between phytosterol concentrations and greater plant-based lipid content, especially when the LE was based on soybeans.

The second part of the study analysed phytosterol content in various non-consecutive batches of LEs (Table 3). Statistically significant differences were also found between different batches: Clinoleic ($F=23.59$; $p=0.000$), Intralipid ($F=978.25$; $p=0.000$), Lipofundin LCT/MCT ($F=5.43$; $p<0.045$), Lipoplus ($F=123.53$; $p=0.000$), and Smoflipid (16.78; $p=0.000$). Except in the case of Lipofundin LCT/MCT, the differences between batches were substantial.

Discussion

We developed an HPLC analytical method to simplify and reduce the cost of determining phytosterol content in LEs[18]. The validation process demonstrated its selectivity, linearity, precision, accuracy, and robustness, all of which support its routine use[18]. The sample treatment protocol for the commercially available LEs is an adapted version of published protocols[19], and it took into account the properties of the samples and the requirements of the analytical method. We used this method to determine the phytoste-

Table 1. Intravenous Lipid Emulsions and Their Composition as Declared by the Manufacturer

Commercial name (pharmaceutical laboratory)	Composition
Clinoleic™ (Baxter)	80% olive oil and 20% soybean oil
Intralipid™ (Fresenius Kabi)	100% soybean oil
Lipofundin™ (LCT/MCT (Braun)	50% soybean oil and 50% MCT
Lipoplus™ (Braun)	50% MCT, 40% soybean oil, and 10% fish oil
Omegaven™ (Fresenius Kabi)	100% fish oil
Smoflipid™ (Fresenius Kabi)	30% soybean oil, 30% medium chain fatty acids, 20% olive oil, and 15% fish oil

MCT: medium chain triglycerides; LCT: long chain triglycerides.

Table 2. Differences in Total Phytosterol Content by Brand

ID	Lipid emulsion	Mean total phytosterol concentration (µg/mL)	Statistically significant differences by ID (*P*<0.05)*
1	Clinoleic™ 20% (n=12)	208,8±39,4	2 y 5
2	Intralipid™ 20% (n=9)	422,4±130,5	1,3,4 y 5
3	Lipofundin™ LCT/MCT (n=9)	187,9±9,1	2
4	Lipoplus™ 20% (n=9)	140,1±20,9	2
5	Smoflipid™ 20% (n=15)	124,2±15,3	1 y 2

F = 42.976; significance value = 0.000. Statistically significant difference using one-way ANOVA variance analysis and non-parametric Kruskal-Wallis test (Omegaven™ was excluded from the statistical analysis). *Post hoc Scheffé test: 1, Clinoleic™; 2, Intralipid™; 3, Lipofundin™ LCT/MCT; 4, Lipoplus™; 5, Smoflipid™.

rol content of all the LEs registered in the Spanish pharmaceutical market, and thus we were able to determine their impact on clinical practice in Spain.

A recent observational study on the use of LEs in 22 hospitals in Catalonia clearly showed the diversity of LEs used and differences in use criteria. These criteria were mainly based on economic management policies and, in some cases, on the level of stress of the candidate participants[20]. Apart from the established criteria for LE selection, our study introduces the new criterion of phytosterol content in order to prevent or correct the abnormalities in liver function parameters commonly seen in patients with PN.

Few studies have analysed different series of LEs to assess their phytosterol content and their impact on liver function. Meisel *et al.*[21] compared 5 LEs in a murine model and showed that liver function abnormalities depended on the formulation of the administered LE. In this murine model, fish oil prevented hepatic steatosis. Forchielli in 2010[22] found statistically significant differences in phytosterol content between different commercial preparations. In the clinical setting, Savini *et al.*[23] found an association between phytosterol intake and plasma phytosterol concentrations in uncomplicated preterm infants receiving routine PN. The latter two studies on different types of LEs showed that phytosterol content ranged from 50 µg/mL to 400 µg/mL. This range was also confirmed in our series.

In 2014, the American Society of Parenteral and Enteral Nutrition (ASPEN) published an updated position paper[24] that analysed several studies[25,26,27] on phytosterol concentrations in LEs in order to gain better knowledge of phytosterol content in LEs for clinical purposes. ASPEN consulted with the manufacturers to validate the accuracy of the information in the document.

The determination of phytosterols in LEs would enable the amount administered to be quantified, thus facilitating better control of one of the relevant factors that may lead to parenteral nutrition-associated liver disease. An alternative could be the administration of LEs with a low phytosterol content or of non-plant-based emulsions, such as fish oil. The promising results obtained by replacing plant-based LEs with fish oil-based LEs[28,29] suggest that the elimination of phytosterols could be associated with improvements in liver function parameters, although randomized studies are needed to determine if the absence of phytosterols is also compensated by other properties or components of fish oil-based LEs.

The present study is the first to determine the presence of phytosterols in all the lipid emulsions registered on the Spanish pharmaceutical market

Table 3. Differences in Total Phytosterol Content by Batch

Lipid emulsion* Snedecor´s F/ sig. (P value)	ID	Batch	Mean total phytosterol concentration (µg/mL)	Statistically significant differences between batches by ID (*P*<0.05)**
Clinoleic™ 20% F=23.59; *P*=0.000	1 (n=3)	14H29N30	231.9±15.7	3
	2 (n=6)	15F15N31	227.2±21.0	3
	3 (n=3)	16F22N30	149.0±3.9	1 and 2
Intralipid™ 20% F=97.26; *P*=0.000	1 (n=3)	10HB3671	451.3±23.2	2 and 3
	2 (n=3)	10IK7012	554.1±36.5	1 and 3
	3 (n=3)	10KC3584	261.6±12.8	1 and 2
Lipofundin™ LCT/MCT F=5.43; *P*=0.045	1 (n=3)	143638082	178.8±3.7	3
	2 (n=3)	144718082	189.7±9.3	-
	3 (n=3)	154818081	195.4±3.0	1
Lipoplus™ F=123.53; *P*=0.000	1 (n=3)	144538082	145.9±6.1	2 and 3
	2 (n=3)	153938083	160.5±1.5	1 and 3
	3 (n=3)	160128082	113.8±1.6	1 and 2
Smoflipid™ 20% F=16.79; *P*=0.000	1 (n=3)	16IF1650	137.6±2.9	3 and 4
	2 (n=3)	16HI0273	138.9±7.6	3 and 4
	3 (n=6)	16I61719	121.1±9.3	1, 2, and 4
	4 (n=3)	16K65043	102.3±1.9	1, 2, and 3

* Statistically significant differences with one-way ANOVA and non-parametric Kruskal-Wallis test.
** Post hoc Scheffé test: 1, Clinoleic™; 2, Intralipid™; 3, Lipofundin™ LCT/MCT; 4, Lipoplus™; 5, Smoflipid™.

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and, unlike the aforementioned studies, it confirms the great variability in phytosterol content by brand and batch with its consequent clinical implications. The results highlight the relevance of including the total phytosterol concentration of each preparation released onto the market in the Summary of Product Characteristics to facilitate better and safer use in clinical practice.

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Conflicts of interests

No conflict of interest.

Contribution to the scientific literature

Recent studies have shown that long-term PN leads to liver function abnormalities, which have been attributed to the phytosterol content of LEs. This study determined the total phytosterol content of the LEs registered on the Spanish pharmaceutical market. The results confirm that there is significant variability between different brands of LEs and between different batches. The results provide a basis on which to design strategies to prevent their hepatotoxic effects.

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