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

Ana Novak[a], Mercè Gutiérrez-Zamora[b], Lluís Domenech[b], Josep M. Suñé-Negre[a,c], Montserrat Miñarro[a,c], Encarna García-Montoya[a,c*], Josep M. Llop[a,c], Josep R. Ticó[a,c], Pilar Pérez-Lozano[a,c]

[a] Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, Av. Joan XXIII, 27-31, 08028 Barcelona
[b] Service of Development of Medicines (SDM), Faculty of Pharmacy and Food Sciences, University of Barcelona, Av. Joan XXIII, 27-31, 08028 Barcelona
[c] Pharmacotherapy, Pharmacogenetics and Pharmaceutical Technology research group. Bellvitge Biomedical Research Institute (IDIBELL), Av. Granvia de l'Hospitalet, 199-203, 08090 L'Hospitalet de Llobregat

* Corresponding author. *E-mail:* encarnagarcia@ub.edu; *phone number*: +34934034712

Abstract

A simple analytical method for simultaneous determination of phytosterols, cholesterol, and squalene in lipid emulsions was developed due 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 x 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: phytosterol, cholesterol, squalene, high-performance liquid chromatography, validation

Abbreviations: ACN – acetonitrile, MeOH – methanol

1. Introduction

Phytosterols are substances of plant origin, considered as cholesterol equivalents, owing to similar sterol structure (Figure 1) and analogous functions in cell membrane regulation. Recently, their clinical importance has increased due to their beneficial effects in reducing cholesterol when administered perorally (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 they are used in the 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. Their 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 favourable 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).

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, ELSD or coupled mass spectrometry, mobile phases with n-hexane, 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 is 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 ºC.

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 ºC.

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 is adapted to HPLC according to previously described protocols (Duelund, 2012; Xu et al., 2012). 1 mL 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). Mixture was vortexed for 10 s and heated for 20 min at 60 °C. After saponification mixture had cooled down, 5 mL of water for HPLC was added and vortexed for 10 s. Extraction was performed by adding 2 x 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 0.45 µm PVDF filter and prepared for HPLC analysis.

2.4. HPLC conditions

HPLC analysis was performed on Dionex UltiMate 3000, equipped with pump (LPG–3400 M), autosampler (WPS3000), thermostated column compartment (TCC-3100, 6P), and DAD (PDA-3000). Robustness was performed on 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 x 4,6 mm, 5 µm; Waters), Zorbax SB-Phenyl (150 x 4,6 mm, 5 µm; Agilent) and Zorbax Eclipse XDB C_8 (150 x 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 10-30 µL, whereas column temperature was maintained at 30 ºC 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 ºC 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. 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 retention time of ergosterol, set as internal standard, and t_{r1} 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 obtained calibration curves, mean linearity and regression statistics were calculated.

2.5.4. Precision

Repeatability of the instrumental system was performed 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 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. Percentage of recovery was calculated and statistically evaluated.

2.5.6. Robustness

The standard solution was subjected to minor chromatographic variations, presented in Table 1. Recovery was calculated and statistically significant differences were investigated by ANOVA and t Student test.

2.6. Data analysis

Chromatographic data were obtained and analyzed with 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 hydrophobicity and selectivity were used under

isocratic conditions. Figure 2 shows chromatograms with optimal mobile phase composition, at flow 1 mL/min, injection volume of 30 μL, detection at 210 nm and temperature was maintained at 30 ºC.

The most hydrophobic column used in our method development was C_{18} and with mobile phase ACN/MeOH = 98:2 (v/v) resulted in relatively good separation of sterols in less than 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 mentioned 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 lathosterol (Figure 2, peak 6) as well as peak overlapping 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 column hydrophobicity and selectivity (Slavin & Yu, 2012). 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 more than 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 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 longer than 120 min, due 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.

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

Phytosterol standard solution was stable during a period of 12 days at room temperature (25 ± 2 ºC) 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 are attributed to physicochemical properties of each standard. Squalene, as the only analyzed compound which lacks sterol structure, differs in absorption maximum and in validated concentration range demonstrates high positive intercept. On the other hand, brassicasterol has slightly positive intercept possibly due to interactions with cholesterol. All standards demonstrated good correlation between concentrations and response factors, coefficients are 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.

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 are attributed to small area integration. t-Student 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 various factors on different levels. Statistical significance was evaluated for each influence.

Minor changes in detection 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 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 had no statistically significant influence on analysis (F_{exp}=1.207, F_{crit}=2.456; $p<0.05$). Lower injection volume does not affect the identification of peaks and at the same time increased injection volume does not result in peak saturation, due to low standard concentrations.
Statistical study with t-Student test shows no statistically significant differences between the obtained recoveries (t_{exp}= 0.147, t_{crit}=1.734; $p<0.05$), when analysis is completed at two different HPLC. 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 of 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 internal standard, due 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 non-spiked samples were prepared.
The identification of peaks was based on retention times and UV maximums 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 composition. Preparation protocol successfully removes the effect of matrix, which enables identification and quantification of sterols and at the same time does not produce modification of analytes. The obtained results are comparable to the previously published ones (Xu et al., 2012), considering the 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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References

Abidi, S. L. (2001). Chromatographic analysis of plant sterols in foods and vegetable oils. *J. Chromatogr. A*, *935*(1–2), 173–201.

Bavisetty, S. C. B., & Narayan, B. (2015). An improved RP-HPLC method for simultaneous analyses of squalene and cholesterol especially in aquatic foods. *Journal of Food Science and Technology*, *52*(9), 6083–6089. https://doi.org/10.1007/s13197-015-1793-4

Breinhölder, P., Mosca, L., & Lindner, W. (2002). Concept of sequential analysis of free and conjugated phytosterols in different plant matrices. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *777*(1–2), 67–82. https://doi.org/10.1016/S1570-0232(02)00093-4

Carretero, A. S., Carrasco-Pancorbo, A., Cortacero, S., Gori, A., Cerretani, L., & Fernández-Gutiérrez, A. (2008). A simplified method for HPLC-MS analysis of sterols in vegetable oil. *European Journal of Lipid Science and Technology*, *110*(12), 1142–1149. https://doi.org/10.1002/ejlt.200700237

de Jong, A., Plat, J., & Mensink, R. P. (2003). Metabolic effects of plant sterols and stanols (Review). *The Journal of Nutritional Biochemistry*, *14*(7), 362–369. https://doi.org/S0955286303000020 [pii]

Duelund, L. (2012). Determination of sterols by HPLC with UV-Vis detection Saponification. Retrieved from http://memphys.dk/sites/default/files/files/basic_page/%3Cem%3EEdit Basic page%3C/em%3E Lab procedures/Sterol_UV_121225.pdf

Duong, S., Strobel, N., Buddhadasa, S., Stockham, K., Auldist, M., Wales, B., … Cran, M. (2016). Rapid measurement of phytosterols in fortified food using gas chromatography with flame ionization detection. *Food Chemistry*, *211*, 570–576. https://doi.org/10.1016/j.foodchem.2016.05.104

Feng, S., Liu, S., Luo, Z., & Tang, K. (2015). Direct saponification preparation and analysis of free and conjugated phytosterols in sugarcane (Saccharum officinarum L.) by reversed-phase high-performance liquid chromatography. *Food Chemistry*, *181*, 9–14. https://doi.org/10.1016/j.foodchem.2015.02.073

Fernandes, P., & Cabral, J. M. S. (2007). Phytosterols: Applications and recovery methods. *Bioresource Technology*, *98*(12), 2335–2350. https://doi.org/10.1016/j.biortech.2006.10.006

Harvey, K., Xu, Z., Walker, C., Pavlina, T., McGrath, S., Zaloga, G., & Siddiqui, R. (2014). Parenteral lipid emulsions in guinea pigs differentially influence plasma and tissue levels of fatty acids, squalene, cholesterol, and phytosterols. *Lipids*, *49*(8), 777–793. https://doi.org/10.1007/s11745-014-3927-2

Jones, P. J., MacDougall, D. E., Ntanios, F., & Vanstone, C. a. (1997). Dietary phytosterols as cholesterol-lowering agents in humans. *Canadian Journal of Physiology and Pharmacology*, *75*, 217–227. https://doi.org/10.1139/y97-011

Lagarda, M. J., García-Llatas, G., & Farré, R. (2006). Analysis of phytosterols in foods. *Journal of Pharmaceutical and Biomedical Analysis*, *41*(5), 1486–1496. https://doi.org/10.1016/j.jpba.2006.02.052

Maguire, L. S., O'Sullivan, S. M., Galvin, K., O'Connor, T. P., & O'Brien, N. M. (2004). Fatty acid profile, tocopherol, squalene and phytosterol content of walnuts, almonds, peanuts, hazelnuts and the macadamia nut. *International Journal of Food Sciences and Nutrition*, *55*(3), 171–178. https://doi.org/10.1080/09637480410001725175

Meisel, J. A., Le, H. D., De Meijer, V. E., Nose, V., Gura, K. M., Mulkern, R. V., … Puder, M. (2011). Comparison of 5 intravenous lipid emulsions and their effects on hepatic steatosis in a murine model. *Journal of Pediatric Surgery*, *46*(4), 666–673. https://doi.org/10.1016/j.jpedsurg.2010.08.018

Mitei, Y. C., Ngila, J. C., Yeboah, S. O., Wessjohann, L., & Schmidt, J. (2009). Profiling of

phytosterols, tocopherols and tocotrienols in selected seed oils from botswana by GC-MS and HPLC. *JAOCS, Journal of the American Oil Chemists' Society*, *86*(7), 617–625. https://doi.org/10.1007/s11746-009-1384-5

Moreau, R. A., Whitaker, B. D., & Hicks, K. B. (2002). Phytosterols, phytostanols, and their conjugates in foods: Structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research*, *41*(6), 457–500. https://doi.org/10.1016/S0163-7827(02)00006-1

Reddy, L. H., & Couvreur, P. (2009). Squalene: A natural triterpene for use in disease management and therapy. *Advanced Drug Delivery Reviews*, *61*(15), 1412–1426. https://doi.org/10.1016/j.addr.2009.09.005

Sánchez-Machado, D. I., López-Hernández, J., Paseiro-Losada, P., & López-Cervantes, J. (2004). An HPLC method for the quantification of sterols in edible seaweeds. *Biomedical Chromatography*, *18*(3), 183–190. https://doi.org/10.1002/bmc.316

Savini, S., D'Ascenzo, R., Biagetti, C., Serpentini, G., Pompilio, A., Bartoli, A., … Carnielli, V. P. (2013). The effect of 5 intravenous lipid emulsions on plasma phytosterols in preterm infants receiving parenteral nutrition: A randomized clinical trial. *American Journal of Clinical Nutrition*, *98*(2), 312–318. https://doi.org/10.3945/ajcn.112.056556

Schröder, M., & Vetter, W. (2012). Investigation of unsaponifiable matter of plant oils and isolation of eight phytosterols by means of high-speed counter-current chromatography. *Journal of Chromatography A*, *1237*, 96–105. https://doi.org/10.1016/j.chroma.2012.03.033

Sheng, Y. (2009). Isolation and identification of an isomer of β-sitosterol by HPLC and GC-MS. *Health*, *1*(3), 203–206. https://doi.org/10.4236/health.2009.13034

Slavin, M., & Yu, L. (2012). A single extraction and HPLC procedure for simultaneous analysis of phytosterols, tocopherols and lutein in soybeans. *Food Chemistry*, *135*(4), 2789–2795. https://doi.org/10.1016/j.foodchem.2012.06.043

The United States Pharmacopeial Convention. (2016). *<621> Chromatography* (USP 39-NF). Rockville, MD.

Warner, K., & Mounts, T. L. (1990). Analysis of tocopherols and phytosterols in vegetable oils by HPLC with evaporative light-scattering detection. *Journal of the American Oil Chemists' Society*, *67*(11), 827–831. https://doi.org/10.1007/BF02540500

Xu, Z., Harvey, K. A., Pavlina, T., Dutot, G., Hise, M., Zaloga, G. P., & Siddiqui, R. A. (2012). Steroidal compounds in commercial parenteral lipid emulsions. *Nutrients*, *4*(8), 904–921. https://doi.org/10.3390/nu4080904

Yuan, C., Ju, Y., Jin, R., Ren, L. L., & Liu, X. (2014). Simultaneous HPLC-DAD Analysis of Tocopherols, Phytosterols, and Squalene in Vegetable Oil Deodorizer Distillates. *Chromatographia*, *78*(3–4), 273–278. https://doi.org/10.1007/s10337-014-2826-2

Figure 1: Chemical formulas of phytosterols, cholesterol, and squalene.

Figure 2: Method development chromatograms with column C_{18} (upper), Phenyl (middle) and C_8 (lower), and isocratic contitions.

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).

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.

Variable	Level		
	-1	0	+1
Wavelength	207 nm	210 nm	213 nm
Column temperature	27 ºC	30 ºC	33 ºC
Injection volume	25 µL	30 µL	35 µL
Different HPLC	Dionex UltiMate 3000		Agilent 1100

Table 1: Robustness conditions.

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

Table 2: Gradient conditions.

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 3: Selectivity 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

Table 4: Linearity data.

Standard	Precision – instrumental RSD (%)			Precision – interday 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

Table 5: Precision data.

Standard	Accuracy – recovery (%) ± SD		
	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

Table 6: Accuracy data.

Standard	Level	Robustness – recovery (%)			
		Wavelength	Column temperature	Injection volume	Different HPLC equipment
β-sitosterol	-1	99.79	99.86	100.26	99.69
	0	99.69	99.69	99.69	99.99
	+1	99.71	99.87	99.47	
Brassicasterol	-1	99.12	98.87	99.77	100.40
	0	100.40	100.40	100.40	99.34
	+1	100.01	99.59	98.66	
Campesterol	-1	99.64	100.70	99.22	100.38
	0	100.38	100.38	100.38	99.91
	+1	101.17	100.57	99.72	
Cholesterol	-1	98.64	101.49	97.92	100.50
	0	100.50	100.50	100.50	99.11
	+1	100.33	100.38	99.65	
Desmosterol	-1	99.32	101.45	99.51	99.00
	0	99.00	99.00	99.00	99.92
	+1	98.37	100.07	100.32	
Ergosterol	-1	100.01	100.02	99.01	99.40
	0	99.40	99.40	99.40	99.71
	+1	99.39	99.94	99.69	
Lanosterol	-1	99.37	99.91	98.81	99.72
	0	99.72	99.72	99.72	99.81
	+1	100.42	100.22	99.59	
Lathosterol	-1	99.74	100.19	96.55	99.26
	0	99.26	99.26	99.26	100.67
	+1	100.51	99.98	99.19	
Squalene	-1	99.85	100.24	100.01	99.86
	0	99.86	99.86	99.86	100.02
	+1	99.86	100.00	100.30	
Stigmasterol	-1	99.54	99.08	99.82	99.91
	0	99.91	99.91	99.91	99.95
	+1	100.63	99.88	99.45	

Table 7: Robustness data.

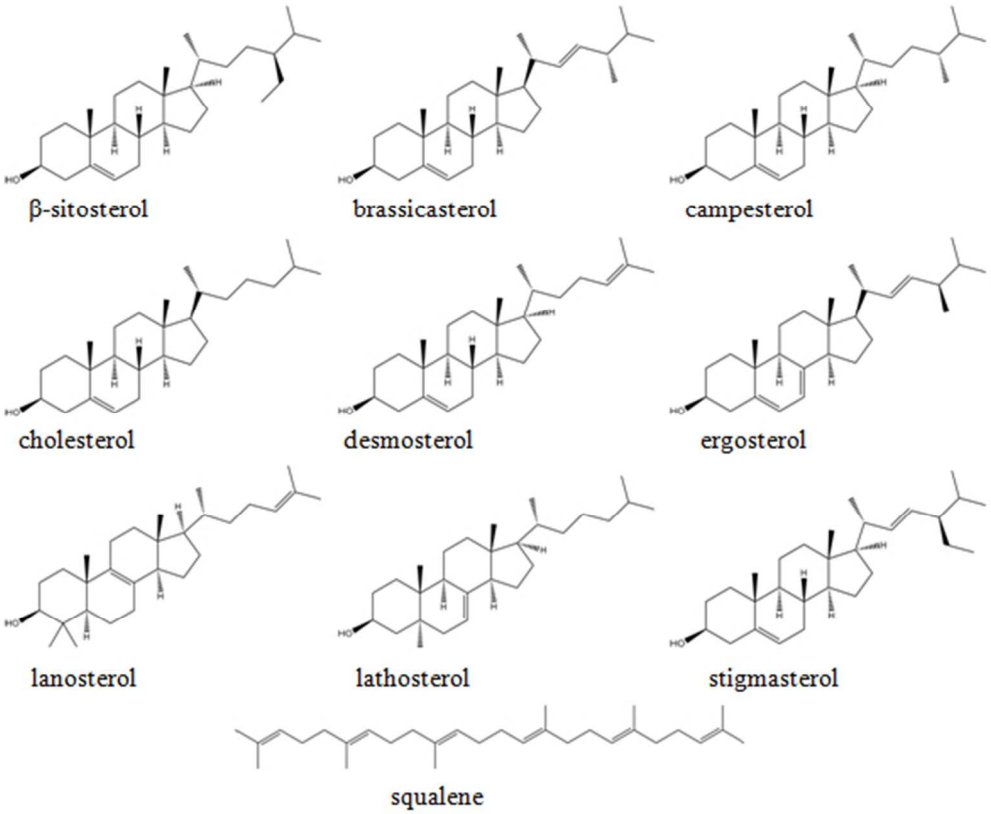

Figure 1: Chemical formulas of phytosterols, cholesterol, and squalene.

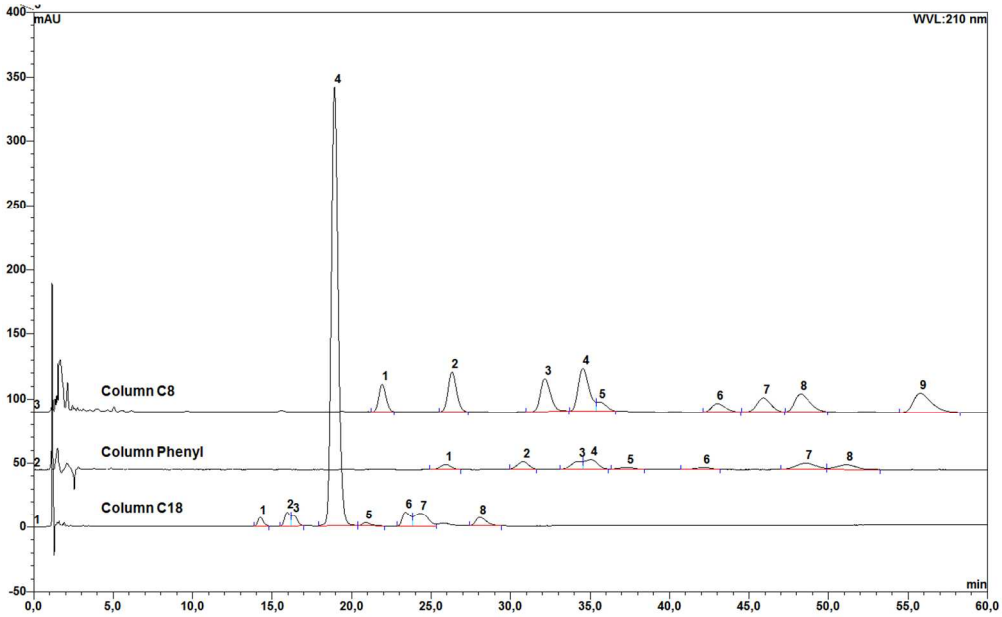

Figure 2: Method development chromatograms with column C18 (upper), Phenyl (middle), and C8 (lower) and isocratic contitions.

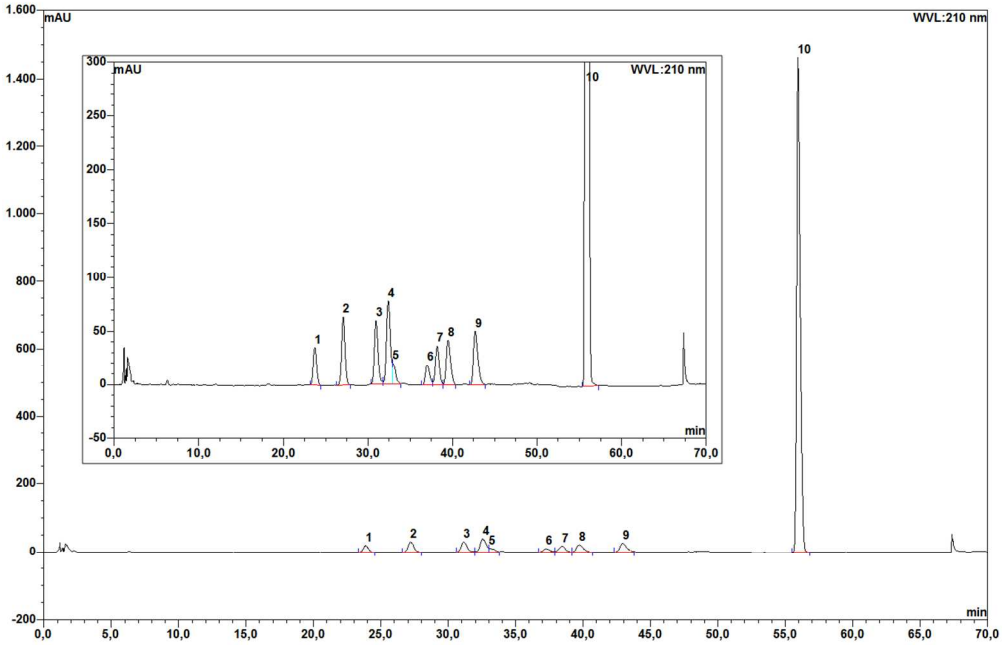

Figure 3: Chromatogram of optimal gradient method (1 – desmosterol, 2 – ergosterol, 3 – lathosterol, 4 – cholesterol, 5 – brassicasterol, 6 – campesterol, 7 – lanosterol, 8 – stigmasterol, 9 – β-sitosterol, 10 – squalene).

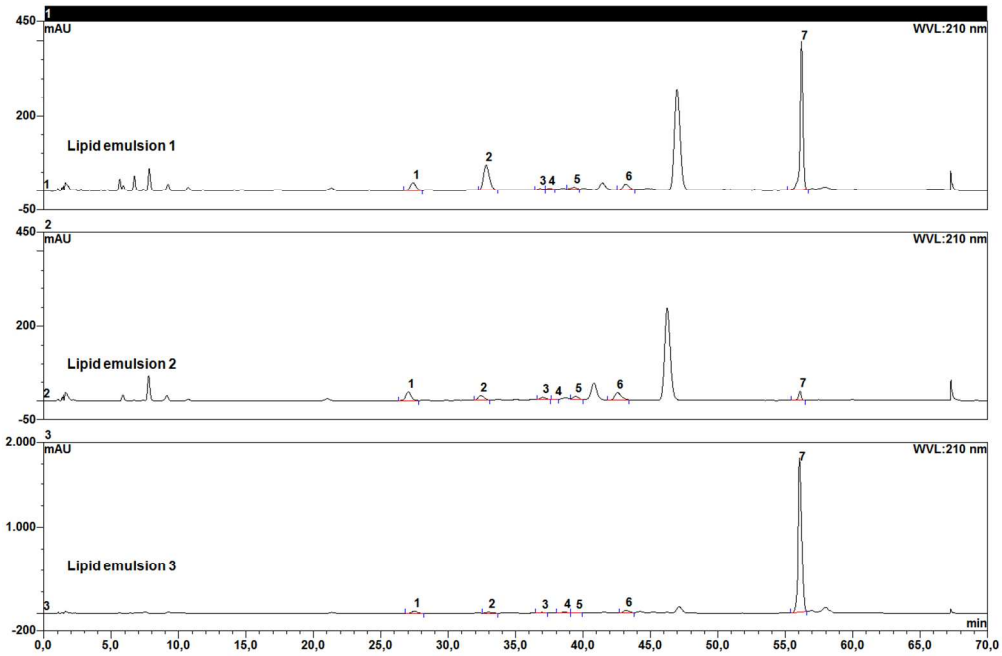

Figure 4: Chromatogram of three different lipid emulsions. Identified peaks are: 1 – ergosterol, 2 – cholesterol, 3 – campesterol, 4 – lanosterol, 5 – stigmasterol, 6 – β-sitosterol, and 7 – squalene.