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ORIGINAL PAPER

Stability of Minor Lipid Components with Emphasis on Phytosterols During Chemical Interesterification of a Blend of Refined Olive Oil and Palm Stearin

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Abstract Minor compounds such as tocopherols and phytosterols in vegetable oils play an important role in their stability and nutritional value. This study monitored the effects of chemical interesterification on the levels of tocopherols, tocotrienols, phytosterols and phytosterol oxidation products (POPs) in an olive oil and palm stearin blend (50/50 w/w). Tocopherols and tocotrienols were dominated by α-tocopherol (192 ppm) and γ-tocotrienol (70 ppm) and decreased during interesterification. Among the tocopherols, δ-tocotrienol had the highest decrease (35%) at 120 °C. During interesterification at 90 and 120 °C, total sterol content in the oil blend (509 ppm) declined slightly, by 3 and 5%, respectively. Phytosterols were esterified at a higher level at 120 °C (7%) than at 90 °C (4%) during this process. Distribution of fatty acids in the esterified sterols followed the fatty acid composition of the oil blend. Total POP content was 4.3 ppm, and remained generally unchanged during interesterification. Among the nine POPs tentatively identified by their mass spectra, 6-hydroxysitostanol and 6-hydroxycampestanol dominated in the oil blend and in the interesterified product. The formation pathways of these saturated di-hydroxyphytosterols have yet to be identified. Although the interesterification process comprised several treatments, there were only minor losses of tocopherols and phytosterols and virtually no increases in the POPs.

Keywords Interesterification · Minor lipid components · Olive oil · Palm stearin · Phytosterols · Phytosterol oxidation products · POPs · Sterol esters · Tocopherols · Tocotrienols

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Introduction

Most of the native vegetable oils have limited applications in their original form due to their specific chemical composition. Vegetable oils can be modified to widen their commercial use, either physically by fractionation or blending, or chemically by hydrogenation or interesterification [1]. Blending does not result in chemical modification of the triacylglycerol composition. Furthermore, the blended oils can result in phase separation during storage. During partial hydrogenation, some *cis* double bonds are isomerised into their *trans* form. Nutritional studies have suggested a direct relationship between *trans* fatty acids and increased risk of coronary heart disease [2]. Chemical interesterification has been used as an alternative to partial hydrogenation to obtain zero *trans* oil products. Both chemical interesterification and enzymatic interesterification are common in use to improve plasticity, crystallisation behaviour and functional properties of fats and oils [1, 3].

There are many reports on the effects of chemical interesterification on physical and chemical properties of the end-product. Melting properties, oxidative stability, triacylglycerol (TAG) modification (positional distribution of fatty acid in TAG), crystallisation and nutritional properties of chemically interesterified fats/oils blend have been

extensively studied [1]. In addition, studies on the fate of different tocopherols during this process show these compounds to be decreased to some extent depending on the interesterifications and type of tocopherols [4]. However, to our knowledge there are no reports on the fate of other minor compounds such as phytosterols in interesterified fats and oils.

Phytosterols (plant sterols) are minor components of vegetable oils and form a major proportion of the unsaponifiables [5]. Phytosterols in vegetable oils are important from a nutritional point of view because they contribute to lowering serum cholesterol levels in humans [6] and to oxidative and thermal stability and shelf-life [7]. It has been shown that during chemical interesterification there is a minor increase in the formation of esterified sterols of vegetable oils. However, different results have been observed in the formation of sterol esters when using lipase from *R. meihei* and *C. cylindracea* [8].

Phytosterols can also oxidise in the same way as other unsaturated lipids and produce phytosterol oxidation products (POPs) when exposed to air, heat, light or catalysts [9]. Recently, the POP content of oils and foods has gained interest due to its possible negative biological effects [9]. The effects of vegetable oil refining processes, particularly bleaching and deodorisation, on the formation of POPs have been studied in detail [10, 11]. However, to our knowledge, no literature is available on the effects of chemical interesterification on POPs.

This paper reports the first study on the effects of chemical interesterification of a blend of refined olive oil and palm stearin conducted at different temperatures on phytosterols and their oxidation products (POPs). In addition, changes in fatty acid composition of the esterified sterols and in levels of tocopherols and tocotrienols during chemical interesterification were investigated.

Materials and Methods

Reagents and Solvents

The standard fatty acid methyl ester (FAME) mixture FO7 was obtained from Larodan Fine Chemicals AB (Malmo, Sweden); reference samples of tocopherols from Merck KGaA (Darmstadt, Germany); Tri-Sil reagent from Pierce Chemical Co. (Rockford, IL, USA); thin-layer chromatography (TLC) (Silica gel 60. 10 × 20 cm, 0.25 mm thickness) from Merck, Darmstadt, Germany; and SPE cartridge (1 g silica) from IST (Mid Glamorgan, UK). All other chemicals and solvents used in this study were of analytical grade and were purchased from VWR International AB (Stockholm, Sweden) unless otherwise stated.

Samples

Refined olive oil and palm stearin were gifts from AarhusKarlshamns Sweden AB, Karlshamn, Sweden.

Blend Preparation

Palm stearin was melted at 60 °C in a water bath prior to use. The liquefied palm stearin and refined olive oil were mixed in proportions of 50/50 (w/w) for the interesterification process.

Chemical Interesterification

Interesterification of the oil blend was carried out according to methods described previously with slight modifications [12]. In brief, 100 g of moisture-free olive oil and palm stearin blend were mixed and charged into a 500-mL round-bottom flask. Sodium methoxide powder [0.5% (w/w)] was added as a catalyst to the starting oil blend. The oil blend was interesterified at two different temperatures, 90 and 120 °C, for 60 min under vacuum. After cooling of the interesterified product to approx. 60 °C to deactivate the residual catalyst, 4 mL of citric acid solution (20% in water) were added and mixed for 15 min. Thereafter, the mixture was transferred into a separating funnel and washed with warm water several times to ensure that it was cleansed of citric acid and residual catalyst. The oil phase was then passed through a Watman 4 filter paper to remove possible residual catalyst and interesterified product was stored at −20 °C until further analysis.

Triacylglycerol Profiles

Triacylglycerol profiles were determined by GC according to the method described by Farmani et al. [13]. The GC analysis was performed with a Chrompack CP 9001 gas chromatograph (Chrompack, Middelburg, The Netherlands).

Separation of Sterol Esters by Preparative Thin-Layer Chromatography (TLC)

To determine the fatty composition of sterol esters, the oil sample (approx. 50 mg × 3) in 0.5 mL hexane was applied onto a TLC plate. To correctly identify the esterified sterol bands, a reference sample of sterol esters was also loaded onto the same TLC plate. The TLC plate was then developed twice in *n*-hexane/diethyl ether/acetic acid (85:15:1, v:v:v) solvent mixture. After elution, the reference band on

the TLC plate was exposed to iodine vapour, while the sample area was covered with a glass plate. The esterified sterols band was marked, scraped off and extracted three times with 2 mL hexane. The extracted sterol ester fraction was methylated and fatty acid methyl esters (FAMEs) were analysed by GC as described below.

Preparation of FAMEs

FAMEs of oil samples and separated esterified sterols from the previous step were prepared according to the method described by Dutta et al. [14].

FAME Analysis by GC

GC analyses were performed with a Chrompack CP 9001 gas chromatograph (Chrompack, Middelburg, The Netherlands). The GC was equipped with a flame ionisation detector and split/splitless injector. A 50 m × 0.22 mm, 0.25 µm film thickness fused-silica capillary column BPX70 (SGE, Austin, TX, USA) was used for analysis. Injector and detector temperatures were 230 and 250 °C, respectively. Oven conditions were 158 °C increased to 220 °C at a rate of 2 °C/min and maintained for 5 min. Helium was used as a carrier gas and nitrogen as a make-up gas at a flow rate of 30 mL/min. FAMEs were identified by comparison of their retention time with standard FAMEs. The peak areas were integrated by Maestro version 2.4 (Chrompack, Middelburg, The Netherlands) and reported as a percentage of total fatty acids.

Analysis of Tocopherols and Tocotrienols by High-Pressure Liquid Chromatography

Tocopherols and Tocotrienols were analysed by high-pressure liquid chromatography (HPLC) according to the method described by Dutta et al. [14] with slight modification. A 7725 Rheodyne Injector fitted with a 20 µL loop and connected to a 510 HPLC pump (Waters, Milford, CT, USA) was used. The column used was LiChroCART 250-4 packed with LiChrosphere 100 NH_2, 5-µm particle size and coupled to a guard column LiChroCART 4-4 (Merck KGaA, Darmstadt, Germany). Approximately 10 mg fats were dissolved in 1 mL *n*-heptane and 10 µL was directly injected into the HPLC column. Tocopherols and tocotrienols were detected by fluorescence detector Varian 9070 (Walnut Creek, CA, USA) at a wavelength of 294 and 320 nm for excitation and emission, respectively. The isocratic mobile phase was a mixture of *n*-heptane/*tert*-butylmethylether/tetrahydrofuran/methanol (79:20:0.98:0.02, v:v:v:v) at a flow rate of 1 mL/min. Each tocopherol and tocotrienol was quantified using an external standard method with reference samples of tocopherols, and a Hewlett Packard integrator HP 3396A was used to calculate the peak areas. All samples were analysed in triplicate and the results reported are the mean of these.

Total, Free and Esterified Phytosterols

Total, free and esterified phytosterols of oil samples were determined according to the method described by Azadmard-Damirchi and Dutta [15].

Transesterification for Determination of Phytosterol Oxidation Products (POPs)

The method described by Schmarr et al. [16] was used after slight modification. In brief, for transesterification of the oil sample (approx. 250 mg), 2.5 mL of 10% sodium methylate in methanol, diluted with *tert*-butyl methyl ether (4:6, v/v), were added and mixed vigorously for 1 min. The mixture was kept for 1 h at room temperature with additional mixing after 30 min. After adding 2 mL of water and 5 mL of chloroform, the sample was shaken and then centrifuged at 2,500 rpm for 3 min to facilitate phase separation, after which the aqueous phase was removed. To neutralise excessive alkali, 2 mL of citric acid solution (1% in water) were added, and after shaking and centrifuging, the aqueous phase was again removed. The remaining chloroform extract was then evaporated under a stream of nitrogen and the residue dissolved in 1 mL *n*-hexane/diethyl ether (9:1, v:v) for further analysis.

Enrichment of POPs by Solid Phase Extraction (SPE)

For this purpose a new SPE method was used. The transesterified samples were dissolved in 1 mL of *n*-hexane/diethyl ether (9:1; v:v) and loaded onto the SPE cartridge (1 g silica) previously conditioned with 5 mL *n*-hexane. The non-POP compounds were eluted with 15 and 10 mL of *n*-hexane/diethyl ether (9:1, v:v) and (1:1, v:v), respectively. The POPs were then eluted with 10 mL of acetone (unpublished data). A known amount of 5α-cholestane (5 µg dissolved in hexane was added to the eluted POP fraction as internal standard for quantification by GC. The acetone fraction containing POPs was stored at −20 °C until further analysis. The efficiency of the SPE method for separation and enrichment of POPs from the transesterified oil samples was checked by analytical TLC according to the method described by Dutta and Appelqvist

[17]. The non-POP and POP fractions were applied on TLC and developed in the solvent system; diethyl ether/cyclohexane (9:1, v:v). The TLC plate was dried briefly in air and then sprayed with the reagent prepared by dissolving 1 g each of phosphomolybdic acid and cerium sulphate in 5.4 mL of concentrated sulphuric acid and diluted up to 100 mL with water. The plate was placed in an oven at 120 °C for 10 min for colour development and then evaluated visually for the completeness of POPs from other compounds.

Preparation of Trimethylsilyl (TMS) Ether Derivatives of Phytosterols and POPs

TMS ether derivatives of POPs were prepared prior to GC and GC–MS analyses according to the method described by Azadmard-Damirchi and Dutta [15].

Analysis of POPs by GC

Analyses were performed with a GC model 6890 N (Agilent Technologies, Wilmington, DE, USA) equipped with an autosampler GC PAL (CTC Analytics AG, Zwingen, Switzerland) and a flame-ionisation detector. A combination of two columns, DB-5MS (15 × 0.18 mm, 0.18 μm) and DB-35MS (10 m × 0.2 mm, 0.33 μm) (J&W Scientific, Folsom, CA, USA), which were joined together by a universal press-fit connector (NTK Kemi, Sweden) was used for POP analysis. Injector and detector temperatures were 260 and 310 °C, respectively. Oven conditions were 60 °C for 1 min, then increased to 290 °C at a rate of 50 °C/min and maintained for 5 min, and then raised to the final temperature of 305 °C at rate of 1 °C/min and maintained for 15 min. The TMS ether derivatives of POPs were injected at splitless mode of injection. Helium was used as a carrier gas and nitrogen as a make-up gas at a flow rate of 0.7 and 30 mL/min, respectively. The peak areas were computed with Agilent ChemStation Rev. B.02.01 (Agilent Technologies) and quantification was carried out relative to the 5α-cholestane as an internal standard. All samples were analysed in triplicate, and the results are reported as the mean of these.

Analysis of POPs by GC–MS

For identification of POPs, GC–MS analysis was performed on a GC8000 Top Series gas chromatograph (CE Instruments, Milan, Italy) coupled to Voyager mass spectra with MassLab data system version 1.4V (Finnigan, Manchester, UK). The column combination and conditions for the analysis were the same as used for GC analysis. The full scan mass spectra were recorded at EI^+ mode at electron energy of 70 eV and ion source temperature of 200 °C. The spectra were scanned in the range of *m*/*z*: 40–700. POPs were identified on the basis of the mass spectral data reported in the literature [11, 18, 19].

Results and Discussion

In this study, we used only one olive oil and palm stearin blend as a reaction matrix to monitor the effects of chemical interesterification mainly on phytosterols, POPs, tocopherols and tocotrienols. However, a few observations were made on the TAG and fatty acids. Interesterification altered the TAG profiles of the starting oil blend, as observed in GC analysis (results not shown). However, no efforts were made to determine the detailed quantitative and qualitative distribution of TAG species in the final products. Oleic acid (C18:1) was the predominant fatty acid (48%) in both the starting blend and the interesterified fats, followed by palmitic acid (C16:0) (33%) and linoleic acid (C18:2) (10%). Fatty acid composition of starting blend remained unchanged during chemical interesterification (Table 1). It is well known that chemical interesterification does not change the fatty acid profile of the oils or oil blends and only alters the distribution of the fatty acids on the TAG molecules [20].

Table 2 shows the results obtained from tocopherol and tocotrienol analysis of the blend and interesterified blends by HPLC. Among the tocopherols, α-tocopherol dominated (91%), followed by γ-tocopherol (9%). Among the tocotrienols, γ-tocotrienol dominated (52.4%), followed by α-tocotrienol (27%), δ-tocotrienol (14%) and β-tocotrienol (7%). Interesterification caused a minor reduction in

Table 1 Fatty acid composition (%) of an olive oil and palm stearin blend and in the final product interesterified at different temperatures

Fatty acid	Starting blend (%)	Interesterified product (%)	
		90 °C	120 °C
Myristic acid (C14:0)	0.6	0.6	0.6
Palmitic acid (C16:0)	33.2	33.2	33.7
Stearic acid (C18:0)	4.3	4.3	4.2
Oleic acid (C18:1)	47.7	47.6	47.5
Linoleic acid (C18:2)	9.8	9.7	9.7
Linolenic acid (C18:3)	0.6	0.6	0.6
Eicosanoic acid (C20:0)	0.5	0.5	0.5
Gadoleic acid (C20:1)	0.2	0.2	0.2
Behenic acid (C22:0)	0.1	0.1	0.1
Lignoceric acid (C24:0)	0.1	0.1	0.1

Each value is the mean of triplicate analyses (CV generally <3%)

Table 2 Changes in tocopherol and tocotrienol contents of an olive oil and palm stearin blend interesterified at different temperatures

POPs	Starting blend µg/g oil (%)	Interesterified product (µg/g oil) (%)	
		90 °C	120 °C
α-Tocopherol	191.9 (91.1)	191.1 (91.1)	186.9 (91.2)
γ-Tocopherol	18.8 (8.9)	18.6 (8.9)	18.0 (8.8)
Total tocopherol	210.7	209.7	204.9
α-Tocotrienol	35.5 (26.7)	35.2 (27.1)	34.4 (28.1)
β-Tocotrienol	8.8 (6.6)	7.6 (5.9)	7.1 (5.8)
γ-Tocotrienol	69.7 (52.4)	68.9 (53.1)	68.8 (56.2)
δ-Tocotrienol	18.9 (14.2)	18.1 (13.9)	12.2 (9.9)
Total tocotrienol	132.9	129.8	122.5

Each value is the mean of triplicate analyses (CV generally <3%)

tocopherols and tocotrienols content. The total loss of tocopherols was 0.5 and 3% at 90 and 120 °C, respectively, compared with their content in the starting oil blend. Loss of tocotrienols was higher than that of the tocopherols, 2 and 8% at 90 and 120 °C, respectively, compared with their content in the starting oil blend. Among these compounds, the loss of δ-tocotrienol during the interesterification at 120 °C was the highest (35%) (Table 2). It is known that tocopherols, the natural antioxidants in vegetable oils are decreased at various levels during chemical interesterification [4].

The content and composition of sterols were determined in the starting blend and in the interesterified product (Table 3). Sitosterol was the predominant sterol (425 ppm), followed by campesterol (65 ppm) and stigmasterol (20 ppm). A few other sterols including Δ^5-avenasterol were present at insignificant concentrations as the amounts of these sterols were very low in refined olive oil and palm stearin. The content of total sterols were reduced to 3.2 and 5.5% during interesterification at 90 and 120 °C, respectively (Table 3). However, the distribution

Table 4 Changes in the fatty acid composition (%) of the esterified sterols in an olive oil and palm stearin blend interesterified at different temperatures

Fatty acid	Starting blend (%)	Interesterified product (%)	
		90 °C	120 °C
Palmitic acid (C16:0)	19.1	20.6	20.6
Stearic acid (C18:0)	8.0	8.5	7.1
Oleic acid (C18:1)	31.9	36.2	34.9
Linoleic acid (C18:2)	7.8	11.6	11.9
Eicosanoic acid (C20:0)	12.0	9.2	10.3
Behenic acid (C22:0)	11.4	7.6	8.7
Lignoceric acid (C24:0)	8.9	6.6	6.7

Each value is the mean of triplicate analyses (CV generally <5%)

of sterols remained unchanged in the esterified product. Free sterol content (335 ppm) was higher than esterified sterol content (140 ppm) in the starting blend (Table 3). Interesterification caused an increase in esterified sterol content of the oil blend of 4.0 and 6.6% at 90 and 120 °C, respectively, compared with stating blend (Table 3). Ferrari et al. [8] reported that during chemical interesterification at 110 °C for 30 min, 10% of free β-sitosterol was esterified with fatty acids. Free and esterified sterol contents in vegetable oils are important because they have different physical properties and free and esterified sterols may also have different physiological effects [21]. It has also been reported that esterified phytosterols are oxidised to a greater extent than free phytosterols during prolonged heating at 100 °C. In contrast, free phytosterols are oxidised to a greater extent than phytosteryl esters at 180 °C [22].

In the esterified sterols of the starting oil blend, oleic acid was the dominant fatty acid (32%), followed by palmitic acid (19%) and eicosanoic acid (C20:0) (12%) (Table 4). Myristic acid (C14:0), linolenic acid (C18:3) and gadoleic acid (C20:1) were not present in the esterified

Table 3 Changes in the content and distribution of sterols and esterified sterols in an olive oil and palm stearin blend interesterified at different temperatures

Sterol	Starting blend			Interesterified product							
				90 °C				120 °C			
	Free esterified µg/g oil (%)		Total µg/g oil (%)	Free esterified µg/g oil (%)		ES (%)[a]	Total µg/g oil (%)	Free esterified µg/g oil (%)		ES (%)	Total µg/g oil
Campesterol	38.2 (62.1)	23.3 (37.8)	65.3	34.3 (57.1)	25.8 (42.9)	5.1	63.1	32.8 (54.6)	27.4 (45.4)	7.6	62.0
Stigmasterol	13.6 (78.1)	3.8 (21.9)	19.6	12.5 (74.6)	4.2 (25.4)	3.5	18.8	12.0 (71.2)	4.8 (28.8)	6.9	17.5
Sitosterol	283.4 (71.4)	113.2 (28.5)	424.5	263.2 (67.6)	126.3 (34.4)	5.9	411.0	251.2 (65.1)	134.8 (34.9)	6.4	401.3
Total	335.2 (70.5)	140.3 (29.5)	509.4	310.0 (66.5)	156.3 (33.5)	4.0	492.9	296.1 (63.9)	167.0 (36.1)	6.6	480.8

Each value is the mean of triplicate analyses (CV generally <5%)

[a] ES, esterified sterols, % increase of esterified sterols in the interesterified product

sterols. During interesterification, palmitic, oleic and linoleic acid contents increased slightly in the esterified sterols whereas eicosanoic, behenic (C22:0) and lignoceric (C24:0) acid contents decreased to some extent. These results show that the distribution of fatty acids in esterified sterols is altered to reach equilibrium with fatty acid composition of the reaction matrix during chemical interesterification (Tables 1, 4). Fatty acid moieties in phytosteryl ester molecules play an important role in the oxidation of phytosterols [22]. It has been shown that unsaturated fatty acid moieties can accelerate the rate of phytosterol oxidation in the saturated lipid matrix comparable with that in the unsaturated lipid matrix [22].

To analyse POPs, a single-step new SPE method was used to enrich these compounds, which were subsequently analysed by GC and GC–MS. The efficiency of this new SPE method to separate POPs from unoxidised sterols and FAMEs was checked by analytical TLC and GC–MS. The TLC analysis showed that the SPE method efficiently separated POPs from other compounds, such as unoxidised phytosterols and FAMEs. The GC–MS analysis also showed that there were no unoxidised phytosterols eluting with POPs fraction. It should be mentioned that complete separation of phytosterols from POPs is important because during analysis by GC, unoxidised phytosterols can overlap with some POPs owing to similar retention times [18]. In this study we used a combination of two GC capillary columns, DB-5MS and DB-35MS, to analyse POPs. Both GC and GC–MS analysis showed that by using this column combination system, POPs can be separated efficiently without any overlapping and with a considerably reduced analysis time of 50 min compared with more than 70 min in a previous study [23].

This is the first report showing the possible effect of chemical interesterification on POP content. Generally, chemical interesterification had no effects on the POP content of the starting oil blend (Table 5). These results shows that processing of vegetable oils at the temperature used for interesterification (90–120 °C), along with catalysis and other steps to produce interesterified fats, does not generate POPs. The total amounts of POPs in the starting blend and in the interesterified products were rather low (4–5 ppm), possibly due to the low phytosterol content in the starting oil blend.

Although phytosterols can generate a large number of oxidation products, generally epimers of 7-hydroxy-, epimers of 5,6-epoxy-, triols, 7-keto-, and 25-hydroxy derivatives are reported [9]. We could not find any published report with which to compare our results on POPs during interesterification. A few recent reports on the levels of POPs in crude vegetable oils and their fate during refining processes are of interest [10, 11]. These authors report a POP content in crude peanut, sunflower, corn, lampante olive and palm oils ranging from 2 to 68 ppm. They further show that during bleaching with different types of bleaching earth and deodorisation at 180 °C, the levels of POPs in the crude oils decrease to some extent. In another study, Lambelet et al. [11] showed that semi-refined (no deodorization) low erucic acid rapeseed oil contained 70 ppm of total POP and that the dominant POP was 7-ketobrassicasterol, while the total levels of POPs did not change considerably during deodorisation at 200, 225 and 250 °C. Those authors also reported for the first time that 6β-hydroxybrassicastanol, 6β-hydroxycampestanol and 6β-hydroxysitostanol levels dominated in the semi-refined and deodorised rapeseed oil samples. They did not find any epoxyphytosterols or triols in the samples.

We were able to identify the following POPs in the starting blend and in the interesterified product by comparing their mass spectra with those of published data [11, 18, 19]: 24-hydroxycampesterol; 7α-hydroxysitosterol; 3, 6-hydroxycampestanol; 24-hydroxysitosterol; 6-hydroxysitostanol; 24-methylcholest-4-ene-6 α-ol-3-one; 25-hydroxystigmasterol; 25-hydroxysitosterol; 7-ketositosterol (Fig. 1). However, we were unable to identify a few peaks, a matter that requires further study (Fig. 1). Among the

Table 5 Changes in levels of phytosterol oxidation products (POPs) in an olive oil and palm stearin blend interesterified at different temperatures

POPs	Starting blend µg/g oil (%)	Interesterified product (µg/g oil) (%)	
		90 °C	120 °C
24-Hydroxycampesterol	0.16 (3.70)	0.28 (6.09)	0.30 (6.49)
7α-Hydroxysitosterol	0.12 (2.78)	0.10 (2.17)	0.10 (2.16)
6-Hydroxycampestanol	0.50 (11.57)	0.52 (11.30)	0.50 (10.82)
24-Hydroxysitosterol	0.10 (2.31)	0.10 (2.17)	0.10 (2.16)
6-Hydroxysitostanol	2.86 (65.28)	3.10 (67.39)	3.14 (67.69)
24-Methylcholest-4-ene-6 α-ol-3-one	0.10 (2.31)	0.10 (2.17)	0.10 (2.16)
25-Hydroxystigmasterol	0.18 (4.17)	0.16 (3.48)	0.10 (2.16)
25-Hydroxysitosterol	0.10 (2.31)	0.10 (2.17)	0.10 (2.16)
7-Ketositosterol	0.2 (4.63)	0.14 (3.04)	0.18 (3.90)
Total	4.32	4.60	4.62

Each value is the mean of triplicate analyses (CV generally <5%)

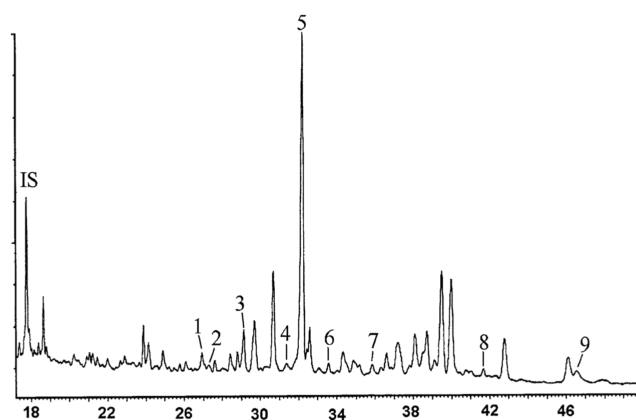

Fig. 1 GC–MS total ion chromatogram of phytosterol oxidation products (POPs) from starting oil blend. *IS* internal standard (5α-colestane), *1* 24-hydroxycampesterol, *2* 7α-hydroxysitosterol, *3* 6-hydroxycampestanol, *4* 24-hydroxysitosterol, *5* 6-hydroxysitostanol, *6* 24-methylcholest-4-ene-6 α-ol-3-one, *7* 25-hydroxystigmasterol, *8* 25-hydroxysitosterol, *9* 7-ketositosterol

POPs tentatively identified, 6-hydroxysitostanol dominated, followed by 6-hydroxycampestanol (Table 5). These dihydroxyphytostanols were identified in this study by comparing the mass spectral data with data in the literature [11, 24]. However, further studies are needed to confirm the spatial configuration of these compounds because their mass spectral data in our study (Fig. 2a, b) did not conform completely with the published data.

Vegetable oils such as rapeseed oil, refined olive oil and palm stearin contain only minor amounts of campestanol and sitostanol. Therefore, 6-hydroxycampestanol and 6-hydroxysitostanol cannot be generated solely from oxidation of campestanol and sitostanol. The possible pathway for formation these saturated dihydroxyphytosterols, 6-hydroxycampestanol and 6-hydroxysitostanol, maybe a reductive cleavage of 5,6-epoxyphytosterols during refining of oils. This proposed reductive cleavage of 5,6-epoxyphytosterols to saturated 6-hydroxyphytostanols during the refining process needs further study. It should be noted here that absence of 5,6-epoxyphytosterols was reported by Lambelet et al. [11]. It has been previously shown that type of bleaching earth and processing conditions can affect the formation of 5β,(β-epoxycholesterol in tallow during the bleaching process [25].

The full scan mass spectra of the tentatively identified 6-hydroxycampestanol and 6-hydroxysitostanol are shown in Fig. 2a, b, respectively. The major fragmentation patterns of these two compounds are also given in Table 6. Lambelet et al. [11] report some of the mass spectra data of

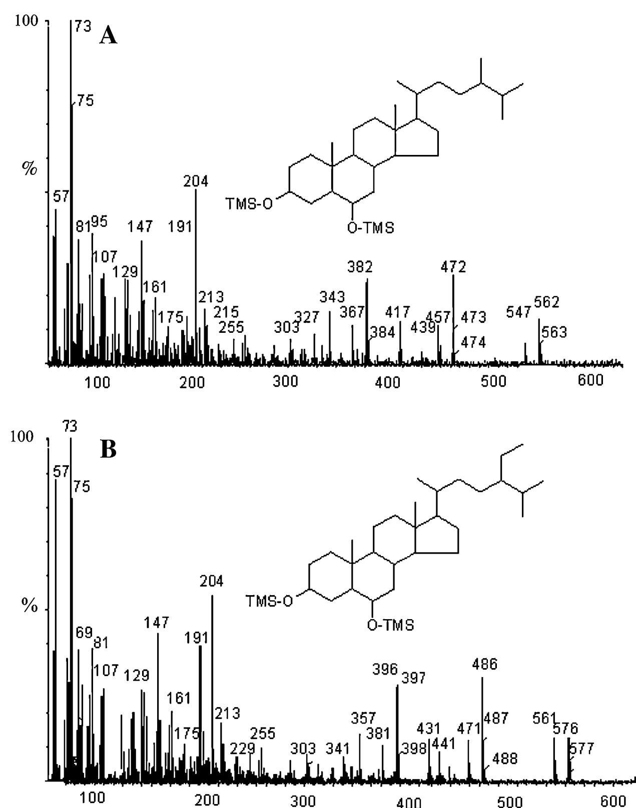

Fig. 2 **a** Mass spectrum of trimethylsilyl (TMS)-ether derivative of 6-hydroxycampestanol showing molecular ion at *m/z* 562, **b** Mass spectrum of TMS-ether derivative of 6-hydroxysitostanol showing molecular ion at *m/z* 576

Table 6 Fragmentation pattern and abundance EI^+ mass spectral data for the 6-hydroxycampestanol and 6-hydroxysitostanol as trimethylsilyl-ether derivatives

Ion fragment	6-Hydroxysitoestanol	6-Hydroxycampestanol
M^{+a}	562 (13)[b]	576 (13)
M^+-15[c]	547 (12)	561 (12)
M^+-90[d]	472 (26)	486 (30)
M^+-90–15	457 (11)	471 (12)
M^+-Sc[e]	421 (1)	435 (1)
M^+-145[f]	417 (12)	431 (12)
M^+-227[f]	335 (5)	349 (4)
M^+-Sc-90	331 (2)	345 (2)
M^+-90 × 2	382 (24)	396 (27)
M^+-(90 × 2)–15	367 (11)	381 (10)
M^+- (90 × 2)–Sc	241 (3)	255 (10)
M^+-358[g]	204 (51)	–
M^+-372[g]	–	204 (54)
M^+-371[g]	191 (36)	–
M^+-385[g]	–	191 (40)

[a] Molecular ion

[b] Relative abundance is reported in brackets

[c] CH_3

[d] $(CH_3)_3SiO$

[e] Side chain

[f] Fragment originating from side chain

[g] Fragment originating possibly from ring structure

two compounds, identified as 6β-hydroxycampestanol and 6β-hydroxysitostanol by comparing their mass fragmentation pattern with published data on authentic samples of these compounds [24]. However, Aringer and Nordström [24] report that typical ion fragments of 3,6-dihydroxystanols at *m/z* 403 and 321 (for 3,6-dihydroxycholestanol) originate from the side-chain. Because the corresponding ion fragments at *m/z* 431 (403 + 28) and 349 (321 + 28) were abundant in the mass spectrum of 3α,6β-hydroxysitostanol and that the intensity of these ion fragments varies considerably among the saturated 3,6-dihydroxystanols but form the base peak of 5β-cholestane-3α,6β-diol. In addition, they report that two fragments at *m/z* 204 and 191 are present in high intensities in the di-TMS ether derivatives of 5α-cholestane-3β,6α-diol and 5α-cholestane-3α,6α-diol but are scarce in the mass spectra of 5β-cholestane-3α,6β-diol and 5β-cholestane-3β,6β-diol. These two fragments at *m/z* 204 and 191 were also present at significantly high intensities in both 6-hydroxycampestanol and 6-hydroxysitostanol in this study (Table 6 and Fig. 2a, b). Since these two fragments are present in the derivatives of both campesterol and sitosterol they probably originate from the ring structure but further study is needed to confirm the position of fragmentation in the sterol ring structure. By comparing the mass spectrometric data of these compounds, in our study of the 6-hydroxyphytostanols, which contain the ion fragments from different epimers of 6-hydroxystanols [24], we can conclude that the capillary column system used in this study presumably could not separate different possible epimers present in the sample. It should be mentioned here that an unknown compound of molecular ion at *m/z* 574 was eluted between 6-hydroxycampestanol and 6-hydroxysitostanol. However, there was no assertive evidence to conclude that the unknown was 3,6-dihydroxystigmasterol as no ion fragments other than molecular ion and the fragment *m/z* 559 (M^+-15) could not be matched with corresponding fragments from 3,6-dihydroxysitostanol or 3,6-dihydroxycampestanol.

To our knowledge, this is the first study monitoring the effects of chemical interesterification on phytosterols, POPs and their changes during this process. The data presented here indicated that the various several steps used to produce interesterified product (treatment of the starting oil blend with catalyst and citric acid, washing with water and filtration) seemed to be too mild to cause significant increases in the oxidation of phytosterols. Further studies should focus on the stability of interesterified fats and oils having a wide range of unsaturated fatty acids and various levels of phytosterols, tocopherols and other minor lipid components. In addition, the formation pathways of the saturated dihydroxyphytostanols in fats and oils during refining processes need to be identified under controlled experimental conditions.

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