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Use of lipolysis in the isolation of sterol esters

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The isolation of sterol esters from edible oils is hindered by the presence of relatively large amounts of triacylglycerols that are similar in polarity to the sterol esters. This paper describes the use of lipolysis with porcine pancreatic lipase to selectively hydrolyse the triacylglycerols. The sterol esters can then be readily separated from the resulting free fatty acids, monoacylglycerols and any remaining diacylglycerols by flash chromatography. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) can then be used to analyse and partially identify the intact sterol esters. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

The analysis of intact sterol esters is of interest as a method of characterizing edible oils. Previous methods of isolating the sterol esters prior to analysis have been well documented. The most common method for this isolation is preparative thin layer chromatography (TLC), often with a preliminary purification step. Johansson (1979) combined low-temperature crystallization to remove the triacylglycerols with preparative TLC, using hexane–diethyl ether–acetic acid as the solvent mixture, to separate the sterol esters from other lipids. However, the recovery of sterol esters from the low-temperature recrystallization was only 45% after one run and residual triacylglycerols remained in the sterol ester fraction after TLC. Kiousseoglou & Boskou (1987) combined column chromatography, with a silicic acid column, with preparative TLC to achieve the isolation of sterol esters. Evershed *et al.* (1987) used column chromatography on alumina to obtain a fraction containing the sterol esters followed by either preparative TLC or Sep-Pak cartridges to isolate the sterol esters from the mixture. A major problem with the use of preparative TLC in the isolation of sterol esters is that the preponderance and similar polarity of triacylglycerols in edible oils tend to give rise to a slight contamination of the sterol ester fraction.

A more efficient method for the isolation of sterol esters is preparative high-performance liquid chromatography (HPLC). Use of a non-destructive detector to track the eluting compounds allows collection of the separated fractions. Worthington & Hitchcock (1984) used a preparative polyethylene column packed with silica gel and a refractive index detector to isolate sterol esters and sterols from seed oils. By increasing the polarity of the solvent mixture during the run, they were able to separate sterol esters, hydrocarbons, triacylglycerols and sterols from seed oils. An advantage of this method over preparative TLC is that a larger amount of oil can be used. Trost (1989) also used preparative HPLC to separate non-polar material from oils prior to analysis. More than one separation was needed to collect sufficient material for the subsequent analysis, and it was necessary to re-run the collected fractions to totally remove the triacylglycerols. An improved method of preparative HPLC for oil analysis is on-line coupled liquid chromatography–gas chromatography (LC–GC) proposed by Grob *et al.* (1990). However, their method involved the use of a destructive detector (flame ionization) to analyse the eluting compounds after GC. Thus, further analysis of the separated compounds was not possible.

The use of lipases to catalyse the hydrolysis of triacylglycerols is also well documented. The most common reaction conditions used for this hydrolysis with porcine pancreatic lipase are those of Luddy *et al.* (1964), in which triacylglycerol and lipase, plus tris(hydroxymethyl)methylamine, bile salts and calcium chloride solution, are stirred together at 40°C. Thorough mixing of the sample is required.

This paper discusses the development of a small-scale method based on lipolysis for the isolation of sterol esters from edible oils. The proposed method uses porcine pancreatic lipase to hydrolyse triacylglycerols to acylglycerols and free fatty acids. Suitable reaction conditions have been devised such that the sterol esters remain intact throughout the reaction. The free fatty acids, monoacylglycerols and any diacylglycerols remaining after lipolysis are then separated from the sterol ester fraction, which includes monomethyl and

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dimethyl sterol esters, by means of flash chromatography. Gas chromatographic (GC) or other analyses of the intact sterol esters can then be performed. Partial identification of the sterol esters present in oils can be carried out by electron impact (EI) gas chromatography–mass spectrometry (GC–MS) and comparison with relative retention time data.

MATERIALS AND METHODS

Rapeseed oil and sunflower oil were purchased from a UK retail outlet. Porcine pancreatic lipase (No. L3126, EC 3.1.1.3), *Candida cylindracea* lipase (No. L1754, EC 3.1.1.3), and samples of pure cholesterol esters and triacylglycerols were purchased from Sigma Chemical Company Ltd (Poole, UK). Lipozyme (a *Mucor miehei* lipase, EC 3.1.1.3) was donated by Novo Enzyme Products Ltd (Farnham, UK). Bakerbond octadecyl (C_{18}) packing was purchased from J. T. Baker (Swallowfield, UK).

Lipolysis

The lipolysis reaction was first tested on a model system consisting of a mixture of triacylglycerols containing 1% cholesterol palmitate. In later experiments, pure oils were used. Triacylglycerol–cholesterol palmitate mixture or oil (200 mg) and porcine pancreatic lipase (100 mg) were mixed together in a screw-top vial. Tris(hydroxymethyl)methylamine buffer (4.0 ml, 1 M, pH 8.0), calcium chloride (0.2 ml, 22% aq.) and bile salts solution (1.0 ml, 1% aq.) were added to the vial. The mixture was placed in a water bath at 45°C and thoroughly stirred with a magnetic follower. An initial reaction time of 30 min was used, after which time the reaction mixture was cooled. This aqueous mixture was then extracted with diethyl ether (3×10 ml). Solid residue present in the diethyl ether after separation was removed from the combined ether extract by vacuum filtration. Following extraction and filtration, the diethyl ether solution was evaporated down to *ca* 2ml. The experiment was then repeated using reaction times of 45, 60, 90 and 120 min. The entire experiment was then repeated replacing the pancreatic lipase with *Candida cylindracea* lipase and with Lipozyme.

Flash chromatography

A column (2 cm i.d.) containing Bakerbond octadecyl (C_{18}) packing (to a depth of 15 cm) was washed with methanol. The ether extract from lipolysis (*ca* 2 ml) was added to the top of the column and allowed to evaporate. The sample was eluted with methanol (50 ml) followed by hexane (50 ml) to yield two factions. The hexane fraction was evaporated to dryness and the residue taken up in fresh hexane (5 ml). GC analysis of the residue with and without cholesterol palmitate as internal standard was then carried out.

GC analysis

GC analysis was performed on a capillary gas chromatograph fitted with a cooled on-column injector and a flame ionization detector. The carrier gas was hydrogen at a pressure of 8.0 bar. The column was a wall coated open tubular column of fused silica, i.d. 0.25 mm, coated with TAP CB [from Chrompack (UK) Ltd, London, UK]. Sterol ester samples with and without cholesteryl palmitate as internal standard were analysed by on-column capillary injection with the temperature programme: 50°C, 0.5 min, ramp rate 30°C min^{-1}; 255°C (5 min), ramp rate 5°C min^{-1}; 350°C (15 min). The detector temperature was 405°C.

Samples were analysed six times and the mean concentration for each sterol ester calculated by comparison with the internal standard peak.

Identification of sterol esters in oils

Identification of intact sterol esters isolated from crude rapeseed and crude sunflower oils was carried out both by comparison of the GC relative retention times (R.R.T.) and by elution sequences of the isolated sterol esters with those in the literature (Gordon & Griffith, 1992) and with authenticated standard sterol esters (Table 1) and by EI GC–MS.

The relative retention times quoted in Table 1 were calculated by performing GC on samples of authentic standard sterol esters on the same TAP-CB column used for analysis of the intact sterol esters. The retention times of the individual esters were then calculated relative to a cholesteryl palmitate internal standard as before.

GC–MS of the intact sterol esters in crude rapeseed and sunflower oils was carried out at Bristol University on a Finnigan 4500 quadrupole mass spectrometer directly coupled to a Carlo Erba 5160 gas chromatograph fitted with a Restek 30 m×0.25 mm i.d. Rtx-

Table 1. Calculated relative retention times for standard sterol esters

Fatty acid/sterol	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:0
Cholesterol	0.71	0.78	0.85	0.92	1.00	1.06	1.07	1.08	1.10	1.13
Brassicasterol	0.72	0.80	0.88	0.94	1.02	1.07	1.09	1.10	1.12	1.13
Campesterol	0.73	0.82	0.91	0.96	1.03	1.08	1.10	1.12	1.13	1.14
Stigmasterol	0.77	0.84	0.91	0.97	1.03	1.09	1.12	1.13	1.14	1.14
β-Sitosterol	0.77	0.85	0.93	1.01	1.06	1.11	1.13	1.14	1.16	1.17
Δ^5-Avenasterol	0.82	0.90	0.96	1.02	1.08	1.13	1.15	1.16	1.17	1.18

All relative retention times have been calculated relative to cholesteryl palmitate.

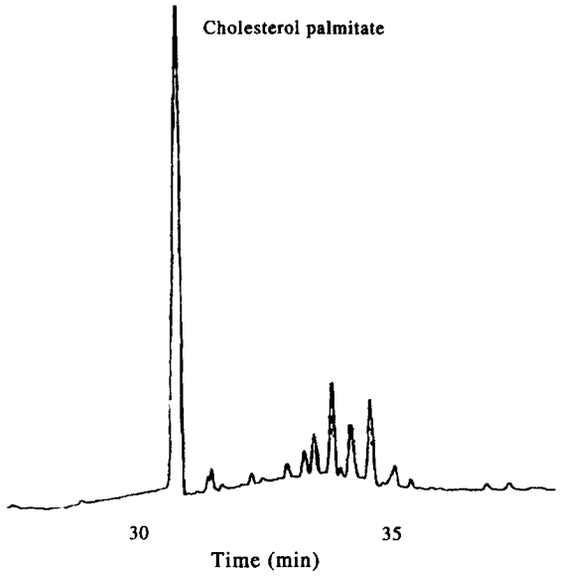

Fig. 1. Sterol ester region of the gas chromatogram of the hexane extract from lipolysis and flash chromatography of rapeseed oil (attenuation = 64).

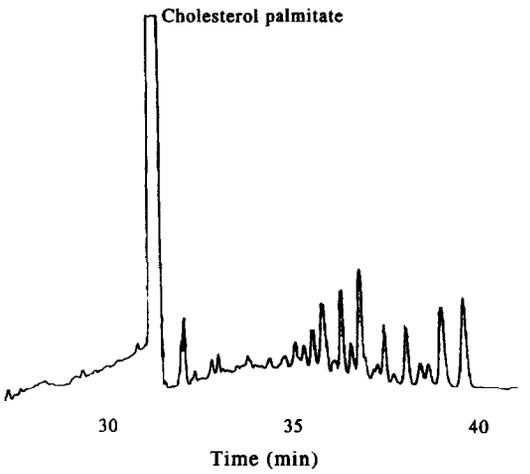

Fig. 2. Sterol ester region of the gas chromatogram of the hexane extract from lipolysis and flash chromatography of sunflower oil (attenuation = 16).

65TG (35% dimethyl, 65% diphenyl polysiloxane) capillary column and an on-column injector. The GC and MS conditions were as follows.

GC conditions

Temperature 1, 50°C; ramp rate 1, 20°C min^{-1}. Temperature 2, 270°C; ramp rate 2, 5°C min^{-1}. Temperature 3, 355°C; hold time, 20 min.

MS conditions

Source temperature, 170°C. Electron energy, 70 eV. Emission current, 350 μA. Data system, Finnigan INCOS.

RESULTS AND DISCUSSION

GC analysis of the mixtures after lipolysis and extraction with diethyl ether showed that the lipolysis with pancreatic lipase for a minimum time of 30 min had succeeded in hydrolysing nearly all of the triacylglycerols present to a mixture of diacylglycerols, monoacylglycerols and free fatty acids. Increasing the time to 1 h was sufficient to hydrolyse the triacylglycerols completely to free fatty acids and monoacylglycerols. However, even with a reaction time of 2 h, no free sterol was formed, indicating that the sterol esters had been left intact. This is consistent with the findings of Njar & Caspi (1987) that porcine pancreatic lipase does not catalyse the transesterification of sterol esters. Replacing the pancreatic lipase with both *Candida cylindracea* lipase and Lipozyme led to an increase in the amounts of unhydrolysed triacylglycerol at each of the reaction times in comparison with the pancreatic lipase. Figures 1 and 2 show the gas chromatograms of the hexane extracts collected after lipolysis and flash chromatography of rapeseed oil and sunflower oil, respectively, for 1 h with porcine pancreatic lipase.

Attempts were made to scale up the reaction such that 10 g of oil and 5 g of porcine pancreatic lipase were used. Because of the difficulties in ensuring thorough mixing of the sample, it was not possible to completely hydrolyse the triacylglycerols present in the oil, even when ultrasonic mixing was used. Even if small amounts of residual triacylglycerols remain after lipolysis, the large-scale procedure would be useful for isolating large quantities of sterol esters if followed by preparative HPLC or preparative TLC. However, further development of the method is necessary if large quantities of sterol esters are to be isolated without a final preparative chromatographic step.

GC analysis of the compounds collected by flash chromatography of the diethyl ether extract, indicated that a very small amount of sterol ester, most of the free fatty acids and monoacylglycerols, and all of the unhydrolysed diacylglycerols were being removed from the column by methanol. However, more than 90% of the sterol esters remained on the column until it was washed with hexane. Since the free fatty acids elute well before the sterol ester peaks in the GC analysis, small amounts of free fatty acids left in the sterol ester fraction do not interfere with the analysis. Each sterol ester sample was analysed by GC and then reanalysed after the addition of 1 mg of cholesteryl palmitate. This allowed the calculation of relative retention times and accurate quantification of each sterol ester peak (Tables 2 and 3). GC–MS using electron impact allowed identification of the sterol moiety of the sterol esters. Comparison of retention time data with authentic standards and literature values allowed tentative identifications of the sterol esters corresponding to some of the peaks. Large differences in the composition of the sterol ester fraction isolated from rapeseed oil and sunflower oil were evident.

Table 2. Content and composition of sterol esters in crude rapeseed oil

R.R.T.	Average concentration (mg/g oil)	Principal fragment ions *m/z* (abundance)	Tentative identification
0.92	0.012	n.d.	n.d.
1.01		624 (20), 368 (74), 353 (15), 255 (100), 229 (32), 213 (52)	Δ^7-Cholesteryl palmitate from internal standard
1.02		624 (3), 366 (100), 351 (12), 235 (55), 211 (6)	$\Delta^{5,7}$-Cholesteryl palmitate from internal standard
1.03	0.015	n.d.	n.d.
1.04	0.010	n.d.	n.d.
1.05	0.099	380 (100), 365 (3), 337 (3), 255 (27), 228 (6)	β-Sitosteryl palmitate + brassicasteryl ester
1.08	0.144	n.d.	n.d.
1.09	0.274	380 (100), 365 (4), 337 (3), 255 (72), 228 (11),213 (20)	Brassicasteryl oleate
1.10	0.585	382 (100), 367 (70), 274 (16), 261 (12), 255 (10), 228 (6)	Campesteryl oleate
1.11	1.780	396 (12), 382 (100), 381 (3), 367 (12), 288 (2),274 (25), 261 (18), 255 (14), 213 (8)	Campesteryl linoleate + β-sitosteryl ester
1.13	0.525	396 (100), 382 (6), 381 (9), 288 (13), 275 (7),274 (3), 228 (3), 199 (7)	β-Sitosteryl oleate + campesteryl linolenate
1.14	1.520	396 (100), 381 (15), 288 (21), 275 (16), 255 (8), 199 (5)	β-Sitosteryl linoleate
1.15	0.363	n.d.	n.d.
1.16	0.021	396 (100), 394 (40), 381 (8), 296 (98), 288 (21), 281 (11), 275 (16), 255 (32)	β-Sitosteryl linolenate + Δ^5-avenasteryl linoleate
Sum	5.348		

Table 3. Content and composition of sterol esters in crude sunflower oil

R.R.T.	Average concentration (mg/g oil)	Principal fragment ions *m/z* (abundance)	Tentative identification
0.92	0.205	n.d.	n.d.
0.96	0.019	n.d.	n.d.
1.02		624 (3), 394 (4), 379 (6), 366 (100), 351 (12), 281 (11), 253 (55),228 (12), 213 (18), 211 (6)	$\Delta^{5,7}$-Cholesteryl palmitate from internal standard
1.04	0.103	394 (16), 351 (2), 255 (3), 213 (2)	Stigmasteryl palmitate
1.05	0.031	n.d.	n.d.
1.07	0.007	n.d.	n.d.
1.08	0.005	382 (100), 367 (5), 274 (18), 261 (12), 255 (11), 228 (6)	Campesteryl stearate
1.10	0.078	382 (100), 367 (9), 274 (21), 261 (18), 255 (19), 228 (12)	Campesteryl oleate
1.11	0.060	396 (100), 381 (10), 288 (7), 275 (4), 255 (56), 199 (21)	β-Sitosteryl stearate
1.12	0.094	394 (78), 382 (39), 367 (5), 282 (12), 279 (4), 274 (6), 261 (6),255 (22), 228 (27)	Stigmasteryl oleate + campesteryl linoleate
1.13	0.273	396 (28), 394 (5), 381 (10), 288 (8), 275 (3), 255 (56), 213 (12)	β-Sitosteryl oleate + stigmasteryl linoleate
1.14	0.534	396 (100), 381 (6), 288 (6), 275 (2), 255 (35)	β-Sitosteryl linoleate
1.15	0.144	394 (11), 379 (15), 296 (10), 281 (4), 228 (4), 213 (10)	Δ^5-Avenasteryl oleate
1.16	0.310	394 (44), 379 (12), 296 (75), 281 (9), 253 (48)	Δ^5-Avenasteryl linoleate + ?
1.18	0.121	n.d.	n.d.
Sum	1.984		

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