Quantitative Analysis of Synthetic Mixtures of Triacylglycerols with Fatty Acids from Caprylic to Stearic

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Separation and quantitation of triacylglycerols tricaprylin to tristearin was achieved by high-performance liquid chromatography with refractive index detection and an eluent composed of propionitrile and butyronitrile (80:20, vol/vol). Peak identification was based on the logarithms of retention times, and quantitation was achieved by way of theoretical relative response factors. The validity of the response factor calculations was tested by analysis of primary standard mixtures of saturated triacylglycerols and of triacylglycerols obtained by interesterification of binary mixtures of monoacid triacylglycerols.

KEY WORDS: HPLC, propionitrile/butyronitrile, refractive index detection, theoretical relative response factors, triacylglycerols.

Nonaqueous reversed-phase (NARP) high-performance liquid chromatography (HPLC) has been extensively employed for the separation of complex triacylglycerol (TG) mixtures. Such separations, with alkyl-bonded silica columns and various detection systems, have been reviewed previously (1). Factors that complicate HPLC analysis of TG mixtures include the limited solubility of long-chainlength saturated TG in common elution solvents (2), the identification of peaks in complex mixtures and their quantitation in cases of varying detector response.

The main problems associated with the refractive index (RI) detector are its low sensitivity and the fact that it is not possible to employ gradient elution, though it is otherwise well established as a lipid detector (3). Ultraviolet (UV) detectors are of limited application because TG absorb only in the far UV range, thus limiting the choice of mobile phases. In addition, the UV absorption by TG varies markedly with unsaturation, leading to variable response factors. The recently developed light-scattering detector has been applied to TG analysis (4); however, it does not respond equally for all TG (5). Kondoh and Takano (6) described a glyceride-selective post-column reaction detector, which gave quantitative molar response. Both of these detectors are sample-destructive and are not as yet commonly available to the analyst.

Schulte (7) established the effectiveness of propionitrile as a mobile phase for resolution of cocoa butter TG. Geeraert and De Schepper (8) used propionitrile as the mobile phase in HPLC analysis of a number of unsaturated oils. However, they were unable to reproduce the results of Schulte (7). Deffense (2) was able to reproduce Schulte's (7) analysis but found propionitrile unsuitable as an eluent for the analysis of more saturated animal fats, presumably because of solubility problems. Podlaha and Toregard (9) could not elute tristearin with propionitrile at room temperature but managed to do so at 30°C with a relatively long elution time of 105 min. Thus, the analysis of fats and oils high in saturated TG is complicated by either (or both) solubility and long retention times.

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The identification of peaks in NARP HPLC is complicated by the large number of TG species encountered in natural fats and oils. A number of papers have dealt with the problem (9–15). The quantitation of peaks, particularly in the case of RI detection, has been investigated (16), and the necessity for knowledge of response factors for this purpose has been pointed out (17,18).

In this paper these problems have been addressed and a description is given of quantitative HPLC analysis of TG, up to and including tristearin, by means of RI detection and a propionitrile/butyronitrile mixture as eluent. Identification of TG was based on retention times and their quantitation on relative theoretical response factors.

EXPERIMENTAL PROCEDURES

Solvents and reagents. Propionitrile and butyronitrile (99 and 98%, Aldrich Chemical Co. Milwaukee, WI) were both distilled over phosphorus pentoxide prior to use. The propionitrile/butyronitrile eluent was recycled through the HPLC system for approximately 50 injections. At this point the recycled eluent was redistilled over phosphorus pentoxide, and its composition was adjusted to that required. This was achieved by addition of butyronitrile to the extent indicated by RI measurement, to compensate for changes in solvent composition as a result of redistillation. Other solvents were of analytical grade. All lipid materials were 99% pure (Sigma Chemical Co., St. Louis, MO).

HPLC. TG mixtures (0.5% in butyronitrile) were heated to 60°C and 10 μL was injected (Valco injector, Valco Instruments, Houston, TX; fitted with a 10-μL loop) into a system consisting of an ISCO pump (model 2350, ISCO Inc., Lincoln, NE) and three columns in series (one Phenomenex Ultremex C18, 3 μm 150 × 4.6 mm, and two Novapak C18 4 μm 150 × 3.9 mm columns, Waters Associates, Milford, MA). The columns were maintained at 40°C in a Biorad column heater. The RI detector (model 7512, Erma Inc., Tokyo, Japan) was set at 40°C at sensitivity 1 (10^{-5} RIU range). The solvent was recycled, and the reservoir contents were stirred continuously. Data were analyzed with a DAPA (DAPA Scientific, Kalamunda, W.A., Australia) acquisition and software system.

Gas-liquid chromatography (GLC). GLC of methyl esters was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a 60 m × 0.25 μm fused-silica capillary column coated with 0.25 mm DB23 (J&W Scientific, Folsom, CA). A Hewlett-Packard integrator (model 3396A) was used for analyzing results. Two to three μL of an approximately 3% solution was injected under the following conditions: injector temperature, 350°C; column temperature, 175°C isothermal; detector temperature, 250°C; hydrogen (carrier gas) flow velocity approximately 30 cm/s; and a split ratio of 1/100. In admixture with the carrier gas stream, hydrogen (30 mL/min) and nitrogen (24 mL/min) were also fed to the detector as fuel and make-up gases, respectively. Air flow was 400 mL/min.

Transesterification. Fatty acid methyl esters were prepared from TG according to Bannon *et al.* (19).

Interesterification. The sample (0.5 g) was dried at 70 mm Hg/90°C for 2 h. After this time, sodium methoxide (12.4% in methanol, 5 mg) was added, and the reaction was allowed to proceed at 70 mm Hg/90°C for 1 h. Distilled water (10 mL) and glacial acetic acid (2 drops) were added, followed by chloroform (5 mL), and the mixture was added to sodium chloride solution (10%, wt/vol) in a separating funnel. The chloroform layer was recovered and a second chloroform extraction was performed. The combined extracts were dried with anhydrous sodium sulphate.

TG synthesis. Mixed TG were synthesized from partial glycerides. The synthetic procedures recommended by Jensen and Pitas (20) were followed.

RESULTS AND DISCUSSION

Ideally, in HPLC the injection solvent should be identical to the eluent (21). In early work, propionitrile was used both as elution and injection solvent, by heating the injection sample to 60°C prior to injection and maintaining the columns and the detector at 40°C. Under these conditions, recovery of tristearin was only approximately 50%. Accordingly, butyronitrile was incorporated to increase solubility of the more saturated species. An eluent composed of 80% propionitrile and 20% butyronitrile by volume allowed quantitative recovery of tristearin, along with satisfactory TG resolution, and hence was adopted for routine use.

The use of temperatures above ambient was found to be beneficial for increased solubility of the TG and speed of analysis. It also eliminated the problem of baseline wandering caused by temperature fluctuations when operating at or near ambient conditions. With incorporation of butyronitrile into the eluent and operation at 40°C, recovery of tristearin was quantitative, resolution and analysis times were acceptable and no base line drift or fluctuations were noticed.

A flow rate of 0.3mL/min was chosen as a compromise between resolution, analysis time, pump noise and baseline drift. Injection quantities of 0.05 mg in 10 μL provided adequate peak sizes to allow identification and quantitation. This may be compared with 0.025 mg, the lowest amount found in the literature for RI detection (13).

The algorithm of Goiffon *et al.* (10,11) was used for peak identification. The partial selectivities contributed by the most common fatty acids were calculated from TG standards. Trimyristin was chosen as internal standard rather than triolein, which is frequently used for this purpose, because a saturated TG is much easier to maintain in a pure state. Goiffon *et al.* (10,11) have demonstrated that the additivity of logarithms of selectivities allows calculation of retention properties of mixed-acid TG from those of mono-acid TG. However, it must be borne in mind that retention behavior also depends on column temperature, packing material and exact composition of the eluent. A small FORTRAN program was written for the calculation of the logarithms of retention times and is available from the authors upon request.

The validity of the algorithm for identification of mixed-acid TG was checked by way of standards produced by interesterification of binary mixtures of mono-acid TG. By this means, all the TG containing the two selected fatty acids could be prepared in predictable amounts, bearing in mind that positional isomers, such as MMO and MOM, (M, myristyl; O, oleoyl) are not resolved by NARP HPLC. Two mixtures of TG were interesterified; namely MMM/OOO and PPP/OOO (P, palmitoyl). Results (Table 1) show good agreement between measured and calculated values. Logarithms of retention times have been applied successfully as a method for peak identification, although fresh data should be generated for individual chromatographic systems and operating procedures.

TABLE 1

Logarithms of Experimental and Calculated Selectivities for Interesterified MMM/OOO and PPP/OOO Standards[a]

TG	log S_e	log S_c
MOO	0.0519	0.0508
MMO	0.1035	0.1016
POO	0.188	0.1873
PPO	0.222	0.2222

[a]TG, triacylglycerols; MMM, trimyristin; OOO, triolein; PPP, tripalmitin; MOO, myristyldiolein; MMO, oleyldimyristin; POO, palmityldiolein; and PPO, oleyldipalmitin. Log S_e and S_c are logarithms of experimental and calculated selectivities, respectively.

Quantitation of TG based on their RI detector responses was addressed next. The RI of TG increases with chain-length and unsaturation, and response factors are necessary for quantitation with this type of detector, as previously pointed out (17,18). The method of Perrin and Naudet (16) was used for calculation of theoretical relative response factors (TRF). This method involves first calculating the refractive indices of mixed TG from the partial values of individual fatty acids with the relation:

$$N_{xyz} = (N_{xxx} + N_{yyy} + N_{zzz})/3 \quad [1]$$

where N_{xyz} is the refractive index of TG_{xyz} at the chosen temperature, and $N_{xxx,yyy,zzz}$ are the refractive indices of the mono-acid $TG_{xxx,yyy}$ and $_{zzz}$ at the same temperature. The TRF is then calculated for mixed TG by the equation:

$$k_i = (N_{std} - N_0)/(N_i - N_0) \quad [2]$$

where k_i is the response factor for TG_i, N_i is the RI of TG_i, N_0 is the RI of the solvent and N_{std} is the RI of the internal standard.

Table 2 shows the good agreement between experimental and calculated values of RI for a number of mixed TG. The experimental values of mixed and mono-acid TG were all measured at 60°C (22) because not all TG are liquid at 40°C. This avoids discrepancies between measured values due to different measuring temperatures. The algorithm can thus be used for calculating the RI of mixed TG from those of mono-acid TG. Although the RI of TG varies with temperature, this variation is relatively small in the 20–60°C range. The solvent RI was measured at 20°C. These different temperatures must introduce some error, which should, however, be minimal and constant for all values calculated. With trimyristin as standard and a 80:20 propionitrile/butyronitrile solvent mixture, the equation for the calculation of the relative response factor for a TG_i becomes apparent in Equation 3.

TABLE 2

Measured and Calculated RI of Triacylglycerols at 60°C[a]

TG	RI_m	RI_c
PPS	1.4467	1.4458
PSS	1.4463	1.4465
PPO	1.4480	1.4484
SSO	1.4494	1.4497
MOO	1.4505	1.4508
POO	1.4511	1.4516
SOO	1.4524	1.4522

[a]TG, triacylglycerols; PPS, stearyldipalmitin; PSS, palmityldistearin; PPO, oleyldipalmitin; SSO, oleyldistearin; MOO, myristyldiolein; POO, palmityldiolein; and SOO, stearyldiolein. RI_m and RI_c are measured and calculated refractive indices, respectively.

TABLE 3

High-Performance Liquid Chromatography (HPLC) Measurement of Triacylglycerols in Standard Mixtures of Known Composition[a]

TG	TRF	HPLC wt%	Known wt%
Mixture 1 (n = 3)			
CyCyCy	1.126	7.7 ± 0.2	8.6
CCC	1.085	6.6 ± 0.3	6.4
LaLaLa	1.034	47.2 ± 0.3	46.9
MMM	1.000	18.9 ± 0.3	18.7
PPP	0.968	8.9 ± 0.4	9.3
SSS	0.942	10.2 ± 0.4	10.1
Mixture 2 (n = 5)			
LLL	0.769	33.4 ± 0.8	33.8
OOO	0.859	20.0 ± 0.6	19.5
POO	0.893	10.4 ± 0.3	9.9
PPO	0.929	12.0 ± 0.4	12.3
PPP	0.968	14.6 ± 0.4	14.4
PPS	0.959	9.7 ± 0.4	10.0

Grade mixture 1 = 97.7%, Grade mixture 2 = 97.8%.

[a]TG, triacylglycerols; CyCyCy, tricaprylin; CCC, tricaprin; LaLaLa, trilaurin; MMM, trimyristin; PPP, tripalmitin; SSS, tristearin; LLL, trilinolein; OOO, triolein; POO, palmityldiolein; PPO, oleyldipalmitin; PPP, triplamitin; and PPS, stearyldipalmitin. TRF are theoretical relative response factors. HPLC wt% and Known wt% are levels of individual TG measured by HPLC and in the standard mixtures, respectively.

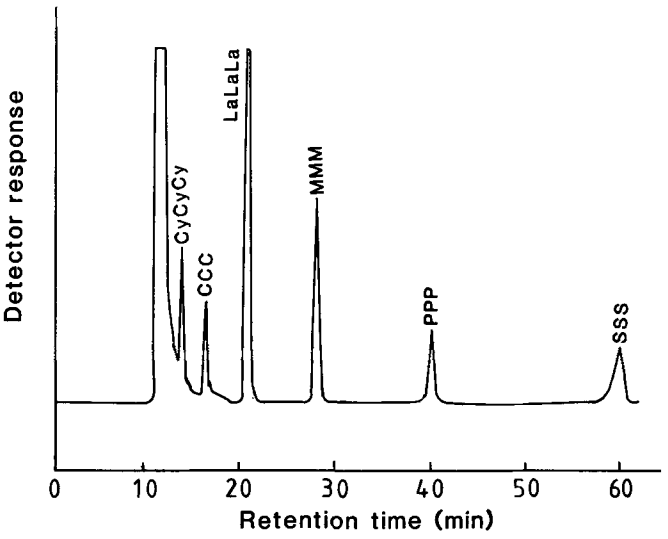

FIG. 1. Nonaqueous reversed-phase high-performance liquid chromatography of trisaturated monoacid triacylglycerols with three C18 columns in series at 40°C, Refractive index detection and propionitrile/butyronitrile (0.3 mL/min) as eluting solvent. Triacylglycerols were CyCyCy (tricaprylin), CCC (tricaprin), LaLaLa (trilaurin), MMM (trimyristin), PPP (tripalmitin) and SSS (tristearin).

TABLE 4

Mole% of Fatty Acids from High-Performance Liquid Chromatography (HPLC) and Gas-Liquid Chromatography (GLC) Analyses (grade = 97.2%)[a]

FA	FAME TRF wt%	Mole% GLC	Mole% HPLC
L	0.9865	32.2	32.5
O	0.9932	29.3	30.4
P	1.0193	34.8	33.8
S	1.0000	3.7	3.3

[a]FA, fatty acids; L, linoleic; O, oleic; P, palmitic; and S, stearic. FAME TRF are fatty acid methyl ester theoretical relative response factors by GLC. Mole% GLC and mole% HPLC are values determined by the respective techniques.

$$k_i = 0.0731/(Ni-1.3696) \quad [3]$$

The validity of relative response factors obtained was tested with a mixture of trisaturated TG of known composition (mixture 1), and one of saturated, and mono-, di- and triunsaturated TG (mixture 2). Table 3 shows the TRF and the measured weight percentages for both mixtures. Figure 1 shows the chromatogram obtained for this mixture. Grade analysis (23) was applied as a measure of agreement between percentages in the mixtures measured by HPLC and that originally weighed. Grades of 97.7 and 97.8% were obtained for mixtures 1 and 2, respectively. This confirms the quantitative elution of trisaturated TG tricaprylin to tristearin. The TRF range from 0.769 (LLL, trilimolein) to 0.968 (PPP), and the widely varying nature of TG ensures that the accuracy of the TRF was rigorously tested. The mixed TP PPO, POO and PPS (S, stearoyl) were obtained with yields of 77, 69 and 89%, respectively, with HPLC purities above 98%.

A second test of the accuracy of the HPLC method was to perform grade analysis between the molar percentages of fatty acids deduced from the HPLC analysis and those deduced from GLC fatty acid methyl ester analysis. GLC TRF in this case must be known, and the GLC analysis must be optimized (23). Table 4 shows these results, and the grade of 97.2% confirmed the accuracy of the HPLC method.

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