Comparison of HPLC and GLC Techniques for the Determination of the Triglyceride Profile of Cocoa Butter

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Current methods for the authentication of cocoa butter (CB) are mainly based on a knowledge of its triglyceride (TG) composition. The performances of capillary GLC and nonaqueous HPLC with an evaporative light-scattering detector (ELSD) for the quantification of TG of CB of different geographical origins were compared. Use of capillary columns coated with a polarizable stationary phase or two reversed-phase HPLC columns coupled in series efficiently separated the major TG species contained in CB. The velocity of the GLC carrier gas influenced the FID response factors of TG standard compounds, which were linearly related to the retention times of the analytes studied. Within a certain mass range the ELSD response of standard TG solutions did not deviate from unity to a greater extent, independent of the molecular structure of the TG species. The quantities of individual TG as obtained by both methods were in close agreement, and the precisions of the methods were also of comparable magnitude, so that either method can be applied to assess the purity of CB. Capillary GLC has the advantage of higher sample throughput due to a shorter run time and because the consumption of chemicals is negligible.

Keywords: *Cocoa butter; triglycerides; capillary GLC; HPLC; evaporative light-scattering detector*

INTRODUCTION

Cocoa butter (CB) consists predominantly of symmetrical triglyceride (TG) species (for a review on compositional data see Lipp and Anklam (1998)). This unique TG composition dictates to a large extent the textural characteristics of chocolate (Chaiseri and Dimick, 1989). Other vegetable fats which could be used in chocolate manufacturing have to resemble the TG composition of CB closely; otherwise, they will interfere with the formation of a stable crystal network of the finished product (Shukla, 1997). These specialty fats are commonly termed cocoa butter equivalents (CBE). In some countries the addition of up to 5 g of vegetable fats other than CB per 100 g of chocolate is permitted, provided that its use and the correct amount added are mentioned on the product label. Detection and quantification of CBE in chocolate is a very demanding analytical task (reviewed by Lipp and Anklam, 1998). The most promising approaches make use of the TG profile either in combination with graphical data evaluation (Padley and Timms, 1980; Fincke, 1980; Young, 1984; Simoneau et al., 1999a) or by multivariate statistical methods (Anklam et al., 1996; Simoneau et al., 1999).

In principle, either GLC or HPLC may be used to determine the TG profile of fats and oils. GLC on apolar stationary phases allows only a separation according to the acyl-C number of the TG even in capillary columns (Ruizgutierrez and Barron, 1995), while use of a so-called "polarizable" medium-polarity phase facilitates not only a separation according to acyl-C numbers but also according to the number of double bonds in the molecule (Geeraert and Sandra, 1987). Although cold on-column injection improves the recovery of high molecular weight substances, unsaturated triglycerides are lost to varying degrees during high-temperature GLC (Mareš and Hušek, 1985; Mayer and Lorbeer, 1997). Reversed-phase HPLC, though a powerful separation technique for TG, has the disadvantage that the commonly employed UV detectors can only be used with limitations, since TGs possess only weak chromophores. To overcome this problem, the refractive index (RI) detector may be used for isocratic separation of lipids, but the more recently introduced evaporative light-scattering detector (ELSD) opened up new possibilities for using gradients to facilitate sophisticated separations (Lipp and Anklam, 1997). Nonetheless, quantitation may represent a problem, since the response to different components may vary and the response function is not rectilinear at very low or high concentrations (Christie, 1992).

As most procedures to quantitate CBE in chocolate rely on an accurate knowledge of its TG profile, the dependable determination of the TG spectrum is an essential prerequisite. Only a few authors (Řezanka and Mareš, 1991; Carelli and Cert, 1993) have compared directly the technical merits of GLC and HPLC for TG profiling of fats, including CB. However, in one of these comparative studies a split injector was used for GLC, which might have biased some results via discriminative sampling, and in the other the HPLC effluent was monitored by a UV detector, whose response is to a large extent influenced by the degree of unsaturation of separated components. Therefore, we felt the need to re-address the analytical aspects of TG profiling of CB as the basis for detection and quantification of a potential CBE admixture to chocolate. In this paper we

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Table 1. Design Matrix of a 2^3-Factorial Design (Independent Variables: Final Column Temperature, Carrier Gas Flow Rate, Temperature Gradient) to Study Recovery of Major Triglyceride Fractions of Cocoa Butter

POP FID response factor	0.894	0.887	0.960	0.966	0.905	0.896	0.965	0.969	0.944
POS FID response factor	1.009	1.010	1.014	1.009	1.005	1.006	1.011	1.011	1.011
SOS FID response factors	1.128	1.140	1.031	1.029	1.115	1.127	1.028	1.023	1.054
final temp (°C)	340	370	340	370	340	370	340	370	355
flow rate (mL/min)	1	1	4	4	1	1	4	4	2.5
gradient (°C/min)	5	5	5	5	30	30	30	30	17.5

detail the results obtained by analyzing a set of CB samples by high-temperature GLC using a polarizable capillary column as well as by reversed-phase HPLC in combination with an ELSD.

MATERIALS AND METHODS

Materials. Genuine CB samples were donated by ADM Cocoa (Koog aan de Zaan, The Netherlands). TG reference substances (1,3-dipalmitoyl-2-oleoylglycerol (POP), 1,2-dioleoyl-3-palmitoylglycerol (POO), 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS), 1,3-distearoyl-2-oleoylglycerol (SOS), 1,2-dioleoyl-3-stearoyl-glycerol (SOO)) with a purity of at least 99% were obtained from Sigma-Aldrich (Milan, Italy). TG standard solutions were prepared by dissolving each in isooctane (1 mg/mL). Dichloromethane and acetonitrile for HPLC were from Ultrafine Ltd. (London, U.K.), and AR grade isooctane and chloroform were from Carlo Erba (Milan, Italy).

GLC. Triglycerides were separated on a HP 6890 Plus GC equipped with an HP 7683 autoinjector in combination with the ChemStation software Ver. A.06.01 (Hewlett-Packard, Cernusco, Italy). Fused-silica capillary columns used were either from J&W Scientific (0.25 mm × 30 m, 0.15 μm DB-17-HT) or from Chrompack (0.25 mm × 25 m, 0.1 μm CP-TAP) and were obtained through CPS Analytica srl (Milan, Italy). For TG separations 0.5 μL of a solution containing 0.5 mg/mL isooctane was on-column-injected at a column temperature of 100 °C. The oven was programmed at 50 °C/min to 300 °C and at programming rates varying from 5 to 30 °C/min to a final temperature of 340−370 °C depending on the upper temperature limit of the capillary column used. Hydrogen was used as carrier gas, and the flow rate was held constant by the electronic pressure control system of the HP 6890. The temperature of the on-column injector was set to the "oven-track" mode; i.e., it was maintained 3 °C above the oven temperature during the temperature program. The FID temperature was 360 °C.

Peaks were identified by retention time matching and by reference to retention data published by Geeraert and Sandra (1987) and Řezanka and Mareš (1991). Quantitation was by area normalization.

HPLC. Triglycerides were separated by either (i) a 150 × 4.6 mm Chromsep column packed with 3 μm Spherisorb ODS-2 (Chrompack) or (ii) one or two 250 × 4.6 mm, 5 μm Hypersil ODS columns (Hypersil, Runcorn, U.K.) operated in series at 30 °C. A Waters 625 LC system controlled by the Millennium 2010 Chromatography Manager (Waters SpA, Milan, Italy) was used. Samples of 10 μL (5 mg/mL chloroform) were injected by a Gilson 231 autosampling injector (Gilson Italia srl, Milan, Italy). The effluent was monitored by an ELSD-MKIIA (Varex, Burtonsville, MA). The temperature of the drift tube was set to 100 °C, and a stream of N_2 was regulated so as to read 40 mm on the flow meter.

Acetonitrile/dichloromethane (70:30) was the mobile phase for isocratic TG separations; the same solvents were also used to generate a linear gradient of acetonitrile/dichloromethane (from 80:20 to 46:54) over 60 min.

Peaks were identified by retention time matching and by reference to retention data published by Podlaha et al. (1984) and Řezanka and Mareš (1991). Quantitation was by area normalization.

Experimental Design. A 2^3-factorial design was generated and statistically evaluated by STATISTICA for Windows (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Detection and quantification of foreign fats in chocolate relies on an accurate and precise determination of certain characteristics of the extracted fat. The determination of the TG profile in combination with data analysis by linear regression is a widely used technique for approving the authenticity of CB (Padley and Timms, 1980). Since the publication of that method, GLC column technology has made considerable progress and capillary columns have superseded packed columns. Moreover, nonaqueous RP-HPLC has found wide application for the analysis of fats and oils, so that at present the TG spectrum can be analyzed by several optional routines. However, for authenticity assessment of expensive edible fats and oil samples, it is of prime importance that results obtained by different technological platforms are comparable.

Optimization of GLC Parameters. To find out how the FID response of the three major TGs of CB, i.e., POP, POS, and SOS, was influenced by instrumental parameters, a factorial design at two factor levels was set up. The variables included (a) the carrier gas flow rate (1 and 4 mL/min), (b) the gradient of the second temperature ramp (5 and 30 °C/min), and (c) the final column temperature (340 and 370 °C). As injection was done by the cold on-column technique, discrimination due to erroneous sample introduction was ruled out (Grob, 1979). The DB-17HT column was employed for these experiments, and the oven temperature after sample injection was in all cases increased at a rate of 50 °C/min (first ramp) up to 300 °C. Detector response factors (RFs) of POP, POS, and SOS served as response variables and are listed along with the experimental conditions in Table 1.

The FID response of POS remained fairly constant within the experimental domain. The effect exerted by changing the carrier gas flow rate was statistically significant ($p < 0.05$) for the FID response of both POP and POS, while the other variables tested did not influence the detector response. A high carrier gas flow rate (4 mL/min) reduced discrimination effects as evidenced by RF approaching unity, but analysis of a CB sample revealed that it affected resolution of critical pairs, e.g., POS/POO. Therefore, the following parameters were selected for GLC of CB TG: 1 mL of H_2/min as carrier gas and a temperature ramp of 30 °C/min to 340 °C. The relevant section of a chromatogram of a South American CB obtained with these conditions is shown in Figure 1. The major TG fractions were clearly separated, except the coelution of SLS and OOO, known to be inseparable on medium-polarity columns (Geeraert and Sandra, 1987; Řezanka and Mareš, 1991).

The dominating effect of the carrier gas velocity on the recovery of TG has also been noticed by Mareš and Hušek (1985), although they studied only trisaturated TG. In an earlier study, one of the authors found that N_2 was superior to H_2 as carrier gas for the quantitation of TG using a short metal capillary column (Ulberth and

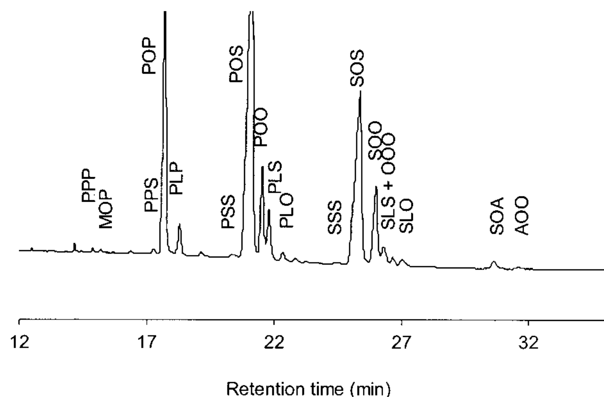

Figure 1. Separation of the triglyceride fraction of cocoa butter by a 0.25 mm × 30 m DB-17HT capillary column.

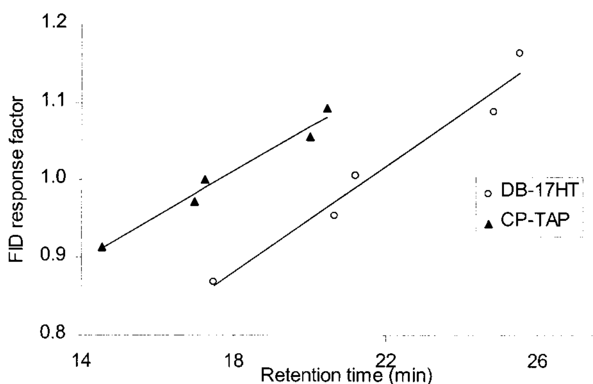

Figure 2. Relationship between the retention times of reference triglycerides and the FID response.

Gabernig, 1997). Contrary to that, H_2 proved to be better suited for long, narrow-bore capillary columns in the present investigation. With the short column the carrier gas head pressure was held constant, which lead to a dynamic alteration of the carrier gas flow rate during temperature programming. This in turn changed the composition of the FID fuel gases. It was shown that a variation of the total N_2 flow reaching the FID altered its response behavior to a much lesser extent compared to that of H_2. These effects were overcome by using an electronic pressure control system which held the gas flow rates constant in the present experiments.

The performance of the CP-TAP column for GLC of CB TG was equivalent to that of the DB-17HT column. Again, only the carrier gas flow rate exhibited a statistically significant effect on the RFs of the calibration standards. For further experiments the same conditions as selected for the DB-17HT column were applied, except that the flow rate of the carrier gas was set to 0.8 mL/min to take into account the lower film thickness of the stationary phase (0.1 μm CP-TAP vs 0.15 μm DB-17HT).

Late-eluting TGs were degraded to a higher degree compared to TGs with a shorter retention time (Figure 2). Both capillaries behaved in a similar way, and retention times of individual reference substances were linearly related to the experimentally determined RFs (correlation coefficient 0.981 for the DB-17HT and 0.986 for the CP-TAP capillary columns). A similar relationship was reported for other capillary columns coated with a polarizable stationary phase, i.e., methyl−65% phenylsilicone (Carelli and Cert, 1993) and methyl−50% diphenylmethylsilicone (Mayer and Lorbeer, 1997).

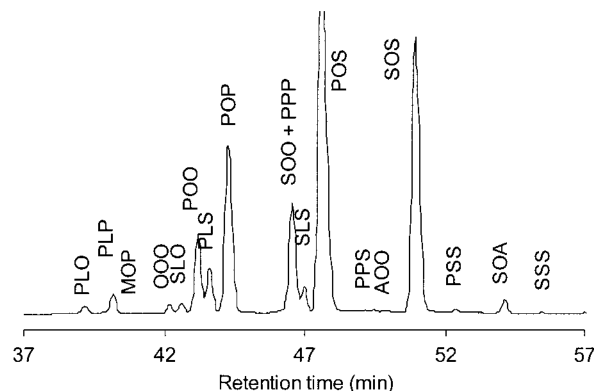

Figure 3. Separation of the triglyceride fraction of cocoa butter by two 4.6 × 250 mm Hypersil ODS HPLC columns coupled in series and a mobile phase gradient of acetonitrile/acetone (from 80:20 to 46:54) over 60 min.

Virtually no difference between the TG profile (expressed as grams of individual TG per 100 grams of total TG) was found by analyzing a soft South American, hard Malaysian, and one average West African CB with both capillaries (the maximum difference was 0.33% for SOS at a concentration of 24%).

Optimization of HPLC Parameters. For nonaqueous reversed-phase HPLC separations of TG, methanol or acetonitrile (ACN), modified by a more apolar solvent such as acetone, tetrahydrofuran, or dichloromethane (DCM), is mostly employed as the mobile phase. It has to be selected so as to ensure good solubility of TG, in particular trisaturated species. We found that ACN/DCM was more suitable in this respect than ACN/acetone.

Two Hypersil ODS columns in series and a linear gradient of 20% DCM to 54% DCM in ACN over 60 min separated most TG fractions of CB with a chromatographic resolution of >1.0 (Figure 3). However, the small amounts of PPP normally found in CB were not separated from SOO, which is in accordance with other reported HPLC systems for the analysis of CB TG (Podlaha et al., 1984; Hernandez et al., 1991). By using only one of the Hypersil ODS columns, SLS was poorly separated from POS. A 150 mm column packed with 3 μm Spherisorb ODS-2 and operated with the same ACN/DCM gradient failed also to resolve SLS from POS (data not shown), but allowed the duration of the analysis to be shortened considerably (run time 30 min).

The response function of the ELSD is known to be nonlinear (Christie, 1992). Within a mass range of 10−200 μg of an individual TG, the relationship between the mass of the analyte and ELDS response was best approximated by a power function (Figure 4). However, the curvature of the function was only evident at low concentrations (<20 μg), and the detector response was, in accordance with others (Christie, 1992; Hopia and Ollilainen, 1993), not influenced by the molecular structure of the TG tested. Consequently, ELSD response factors virtually did not deviate from unity when 10 μL of test solutions containing 10, 50, and 100 mg of total TG/mL were injected (Table 2).

GLC versus HPLC. The same number of identified peaks were obtained with both techniques. However, small qualitative differences existed due to incomplete separation of critical pairs (SLS/OOO was not separated by GLC, and PPP coeluted with SOO in HPLC). Therefore, these substances had to be summed up to enable a comparison of results. Provided optimized methods are

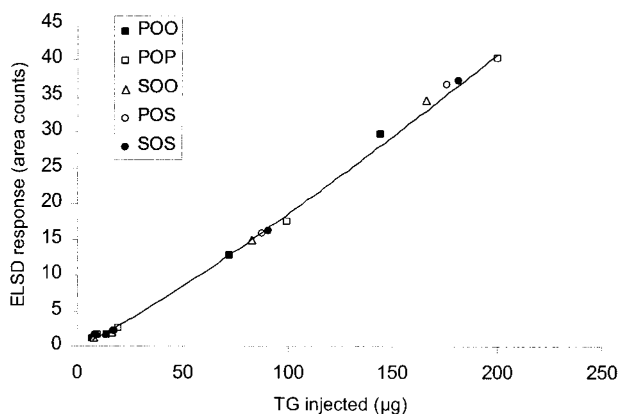

Figure 4. Power function describing the relationship between the mass of reference triglycerides and the ELSD response.

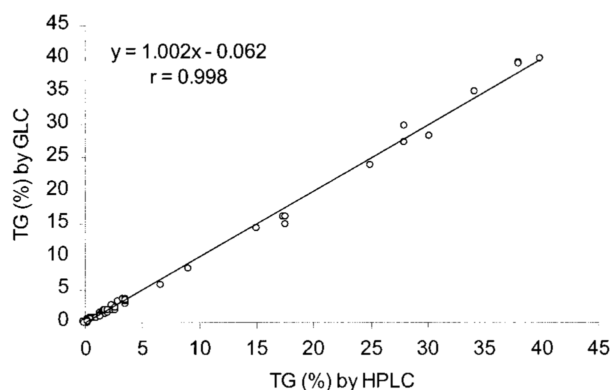

Figure 5. Comparison of the proportions of individual triglycerides of cocoa butter as obtained by HPLC vs capillary GLC.

Table 2. ELSD Response Factors of Standard Triglycerides

TG species	100 mg/mL	50 mg/mL	10 mg/mL	mean	RSD (%)
POO	1.00	1.01	1.07	1.02	3.61
POP	1.02	1.01	0.98	1.00	2.09
SOO	0.99	0.99	1.00	1.00	0.59
POS	0.99	0.99	0.98	0.98	0.59
SOS	1.00	1.00	1.00	1.00	0.24

applied, both chromatographic techniques produced nearly identical TG profiles for four CBs, differing widely in TG composition (soft South American, hard Asian, West African, and a commercial CB mix). Percentages for individual TGs as found by HPLC and GLC for the four CB samples are scattered around a line whose intercept was not statistically different from zero and whose slope did not differ from unity (Figure 5). In both methods the experimentally determined RF for the five major TG fractions (POP, POO, POS, SOS, and SOO) were used to convert area percent to mass percent, and for the remaining fractions RF values of 1.0 were used. Since the experimentally calibrated TG fractions made up around 90% of the total TG spectrum of CB, the error introduced by assuming a RF of 1.0 for the remaining, noncalibrated fractions was negligible. The maximum difference found amounted to 1.34% for the POS content of a commercial CB mix. For both methods the relative standard deviation as a measure of repeatability was <3% for TG fractions in excess of 1% of the TG spectrum, but became increasingly larger as the proportion of the TG fraction approached values around 0.1%. To illustrate the precision of the chromatographic methods used, the TG profile of a South American CB determined by HPLC as well as by GLC is listed in Table 3.

Table 3. Precision of the GLC and HPLC Methods Tested, Expressed as the Within-Series Relative Standard Deviation (RSD)

	GLC		HPLC	
	mean	RSD	mean	RSD
MOP	0.13	2.1	0.02	0.0
PPS	0.28	2.8	0.12	9.4
POP	14.44	0.1	14.98	0.3
PLP	1.97	0.5	1.64	1.8
PSS	0.39	3.4	0.19	5.3
POS	35.02	0.1	34.09	0.4
POO	5.80	0.2	6.57	0.6
PLS	3.70	0.6	3.31	0.9
PLO	0.73	2.1	0.69	4.7
SSS	0.20	4.4	0.07	15.8
SOS	23.98	0.1	24.98	0.4
SOO + PPP	8.27	0.2	9.02	0.7
SLS + OOO	1.89	0.8	2.28	1.3
SLO	0.77	2.4	0.75	2.7
SOA	0.83	1.0	0.95	0.6
AOO	0.17	11.2	0.12	8.3

The agreement of results obtained by the two chromatographic techniques was much better than reported previously. In one method comparison study (Řezanka and Mareš, 1991), where an UV detector at low wavelength was used to monitor the HPLC column effluent, differences of up to 5.0% (absolute) resulted for major TG fractions of CB. Deviations of similar magnitude were reported by Carelli and Cert (1993), who analyzed olive oil and sunflower oil by capillary GLC (split injection) and HPLC-ELSD.

In conclusion, capillary GLC as well as HPLC in combination with an ELSD are both equally suited to determine the TG spectrum of CB with high precision and accuracy, provided that the separation system has been properly optimized. Therefore, both techniques can be used for checking the authenticity of CB, on the basis of TG data. However, the shorter run time and the avoidance of toxic chemicals as mobile phase are strong arguments in favor of capillary GLC.

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