Technical

Quantitative Determination of Medium Chain Triglycerides in Infant Formula by Reverse Phase HPLC

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Two methods were developed for the separation of medium chain triglycerides (MCT) using reverse phase HPLC. Both methods employed a C18 microbond HPLC column as the stationary phase and an isocratic solvent system. The first method described consists of acetonitrile/acetone as the mobile phase with a differential refractometer as the detector. In the second method, acetonitrile/water was used as the mobile phase and a UV detector at 210 nm. Trinonanoin was used as the internal standard for quantitative determination. This method is suitable for milk-, whey- and soy protein-based matrices. With minor modification, it is applicable to MCT levels ranging from 10 to 50% of total fat.

Medium chain triglycerides (MCTs) were introduced in 1950 for the treatment of disorders in lipid absorption. A large fraction of MCTs can be absorbed as triglycerids, whereas long chain triglycerides (LCTs), such as corn oil, soy oil or coconut oil, cannot (1). Infant formulas containing MCTs were used for infants with low birth weight to circumvent the developmental delay in the fat digestion mechanism (2,3).

The fatty acid composition of MCT is predominantly C8:0 and C10:0 (98–99%) with a trace of C6:0 and C12:0 (1-2%). The relative percentages of C8:0 and C10:0 can vary from 60/40 to 75/25, resulting in different molecular species of triglycerides with the common name MCT oil. The shortest possible equivalent chain length (ECL) is TG18 (all C6) and the longest ECL is TG36 (all C12). In practice, more than 97% of the triglycerides in MCT oil are less than TG30, allowing the separation of the MCT from corn and soy oil which consist of no triglycerides less than TG30.

Analysis of triglycerides by high performance liquid chromatography (HPLC) using a reverse phase column can separate triglycerides according to their molecular weights as well as their degree of unsaturation. Earlier work in the HPLC analysis of seed oil triglycerides has been reported by several laboratories (4,5,6). Refractive index (RI) is the most commonly used quantitative detector. Low wavelength UV detections are limited in quantitative use due to the absorption of double bonds, which resulted in distinctly different response factors for saturated and unsaturated fatty acids. Detection at low wavelengths also prohibited the use of some solvent systems such as acetone. In this study two HPLC systems were investigated for the separation and quantitation of MCTs from LCTs in different infant formula matrices, using both RI and UV detectors.

MATERIALS AND METHODS

Materials. Infant formula of milk, casein/whey and soy protein matrices were products of Ross Laboratories (Ross Laboratories, Columbus, Ohio). All lipid standards were purchased from Nu Chek-Prep (Elysian, Minnesota). All organic solvents used in the HPLC system were glass distilled, purchased from Burdick & Jackson. Organic solvents used in extractions were analytical grade. Water was purified by Barnstead Nanopore II System (Barnstead Co.). All other chemicals were ACS analytical grade.

Methods. Extraction: Total fat was extracted from 10 gm of infant formula according to the Mojonnier Method (7,8). The extracts were dissolved in methylene chloride before HPLC analysis.

HPLC analysis: The HPLC unit consisted of an HP1090 (Hewlett Packard) main frame equipped with auto sampler and Filter Photometric Detector (FPD). The detector was operated at 210nm. A differential refractometer (R401 Waters Associates, RID) also was used in the study. The HPLC column used was C-18 reverse phase, 5 mcm particle size, 4.6 mm × 25 cm (Munhall OD5250). The conditions used with the RI detector were: mobile phase acetonitrile/acetone 93/7, flow rate: 2 ml/min: the sample size of MCT oil was 2 mg in a 20 mcl injection. For the UV 210 nm detector, the conditions used were: mobile phase acetonitrile/water 97/3; the sample size was 300-400 mcg per 4 mcl injection.

GLC analysis: An HP 5840 GLC unit equipped with auto sampler, flame ionization detector and digital integrator was used in the study. The methyl esters of the MCT oil were analyzed using a 6 ft × 1/4 in. glass column packed with 10% SP2340 on 100/120 mesh chromosorb WAW (Supelco, Inc., Bellefonte, Pennsylvania). The temperature program was held at the initial column temperature of 100 C for 4 min, then programmed up at 8 C/min to 148 C and held one min, then programmed up at 6 C/min to 200 C and held for 10 min.

Methyl esters were prepared from MCT oil by sulfuric acid catalyzed esterification (9).

EXPERIMENTS AND RESULTS

Separation of medium chain triglycerides according to their molecular weights. Samples were prepared and analyzed as described in the methods section. Chromatograms of MCT oil and total fat extracted from infant formula are shown in Figures 1 and 2.

Both HPLC systems are capable of separating triglycerides according to their molecular weights, which are determined by the chain length of the fatty acids esterified to the glycerol molecule. Medium chain triglycerides are completely separated from the triglycerides of corn and soy oil which consist of no detectable molecular species less than TG30. For coconut oil, however, a peak was eluted slightly in front of TG30 as detected by UV at 210 nm.

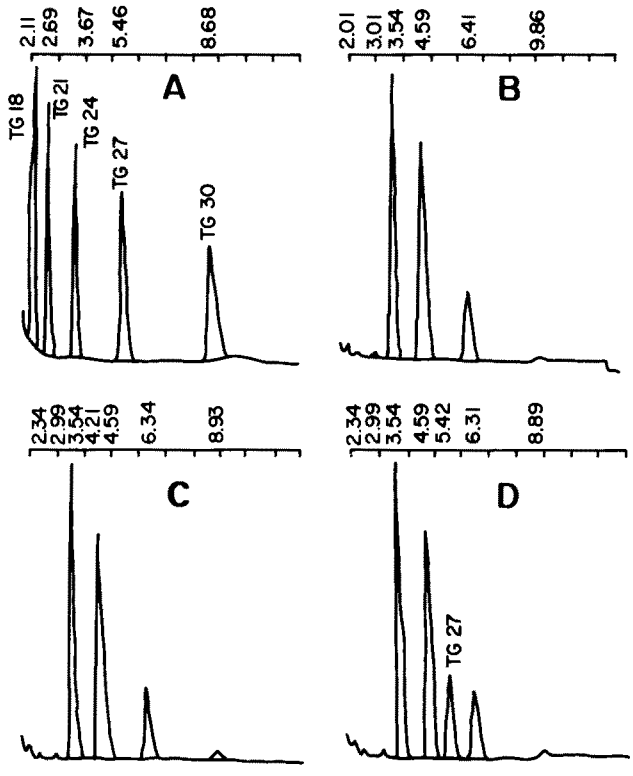

FIG. 1. Triglycerides profile of MCTs in standard, MCT oil and product extracts as monitored by differential refractometer. Mobile phase acetonitrile/acetone 93/7. The horizontal scale is retention time in minutes. A, triglyceride standard mix; B, MCT oil; C, oil extract from milk base infant formula, and D, oil extract from spiked milk base infant formula.

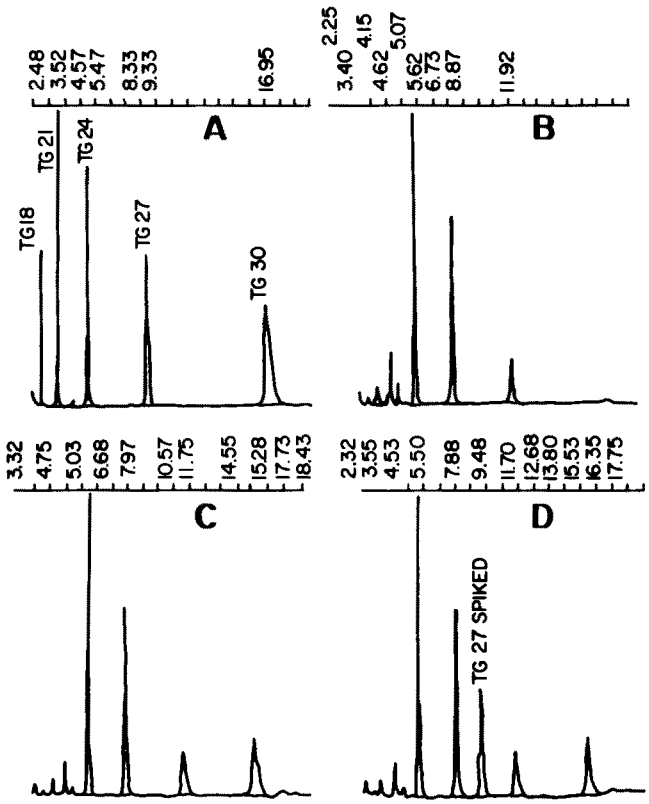

FIG. 2. Triglycerides profile of MCTs in standard, MCT oil and product extracts as monitored by UV 210 nm. Mobile phase acetonitrile/water 97/3. The horizontal scale is retention time in minutes. A, triglyceride standard mix; B, MCT oil; C, oil extract from milk base infant formula, and D, oil extract from spiked milk base infant formula.

Identification of MCT Peaks. Two mg of MCT oil in 20 mcl of methylene chloride were injected on the column. Fractions were collected from the outlet of the column as the peaks were eluted. Figure 3 shows the span of each fraction collected. Three sample runs were collected, and corresponding fractions were pooled. The solvents were then evaporated under nitrogen and methyl esters were prepared and analyzed by GLC as described in the method. Results are shown in Table 1. Oil extracted from milk base formula was analyzed by the same method; the results are shown in Table 2. The equivalent chain length was projected from the retention time of reference standard. The fatty acid composition agreed very well with the calculated chain length, and the ratios of triglyceride molecular species are also in good agreement as predicted by the ratio of fatty acid composition (10).

Quantitative determination of MCTs in infant formula. Trinonanoin (TG27) was used as an internal standard because it does not exist in natural oils and its molecular weight is between those of other major components of the MCT oil. TG-27 was accurately weighed and dissolved in hexane. Aliquots of known concentration were spiked in samples before extraction. They were analyzed by the HPLC system described, and the peak area was integrated. The total peak areas of MCTs were combined and the calculation was based on the known amount of TG27 and its peak area. The area of the peak which was contributed by the coconut oil, as detected by UV, was excluded from the calculation. Results are shown in Table 3.

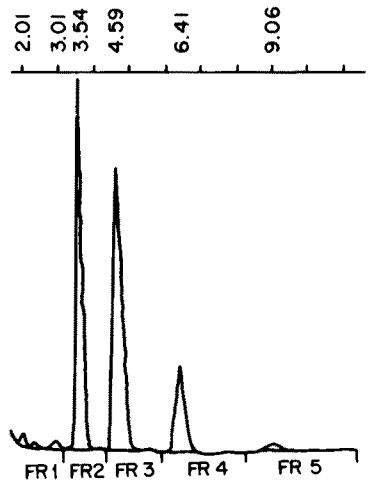

FIG. 3. Span of the fractions 1-5 collected from HPLC and analyzed by GLC for fatty acid compositions. The horizontal scale is retention time in minutes.

Precision study of determination of MCT in milk base infant formula. Two technicians performed the analysis of a milk base sample on two different days.

TABLE 1

Fatty Acids composition of MCT Oil Fractions Collected from HPLC

	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Percent of Total	0.5	34.4	42.9	19.2	3.0
Equivalent chain length as projected from retention time calculations	<24	24	25.5	28.5	31
Fatty Acid Composition, %					
C8:0	—	93.9	50.0	24.4	—
C10:0	—	1.6	43.4	63.2	56.0
C12:0	—	—	—	1.6	—
C14:0	—	—	—	1.6	—
C16:0	—	1.3	—	2.8	—
C18:0	—	—	1.9	1.4	10.9
C18:1	—	1.9	1.8	2.2	23.0
C18:2	—	1.3	2.9	2.8	10.1

TABLE 2

Fatty Acid Composition of MCT Fractions Collected from HPLC of Milk Base Matrix Infant Formula Oil

	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Percent of Total	3.70	26.7	40.6	22.2	6.8
Equivalent chain length as projected from retention time calculations	<24	24	25.5	28.5	31
Fatty Acid Composition, %					
C8:0	17.8	95.2	58.9	24.0	—
C10:0	12.3	—	37.1	62.3	48.0
C12:0	5.5	1	—	—	14.2
C14:0	—	—	—	—	—
C16:0	8.2	—	—	2.0	4.6
C18:0	—	—	0.8	1.1	2.5
C18:1	35.6	2.4	2.0	6.8	16.8
C18:2	20.6	2.4	1.2	3.8	13.9

TABLE 3

Quantitative Determination of MCT in Infant Formula

Sample		W/W % of MCT Theoretical %	Analytical Results
Milk base formula	A	1.79	1.78 ± 0.05[a]
	B	2.20	2.20 ± 0.13[b]
Soy protein base formula	A	1.43	1.35 ± 0.08[b]
	B	1.11	1.04 ± 0.05[b]
	C	0.76	0.76 ± 0.01[b]
	D	0.37	0.36 ± 0.02[b]
Casein/whey base formula	A	1.08	1.13 ± 0.04[b]
	B	0.73	0.74 ± 0.03[b]
	C	0.36	0.37 ± 0.01[b]

[a]Average of eight replicates.

[b]Average of four replicates.

Four replicates were done on each day. Trinonanoin (TG27) was used as internal standard, and the percentage of MCT in sample was calculated as described above. The percentage recovery was calculated by the recovery of the spiked standard. Results of this experiment are shown in Table 4.

DISCUSSION

No previous report was found for the quantitation of MCT in the presence of coconut oil. The C8 and C10 fatty acids in the coconut oil prohibit the quantitative measurement of MCT after hydrolysis of the triglyceride molecule. Separation of triglycerides by their molecular weights can be achieved by both mobile phase systems reported in this study. Due to the absorption of short wavelength UV by the double bond of the unsaturated fatty acids and other oxidized materials, refractive index continues to be a very important detection method for quantitative analysis.

TABLE 4

Precision Study of Determination of MCT in A Milk Base Infant Formula

Recovery	W/W % of MCT		% Recovery	
	Day 1	Day 2	Day 1	Day 2
1	1.86	1.78	96	95
2	1.79	1.79	97	95
3	1.73	1.78	101	99
4	1.70	1.80	101	103
Average	1.77	1.79	98.8	98.0
Std. Dev.	±0.07	±0.01	±2.63	±3.83
R.S.D. %	3.99	0.6		
	2 Days Combined			
Average	1.78	(n = 8)		
Std. Dev.	±0.05			
R.S.D. %	2.67%			
Batch Record	1.79%			

The choices of mobile phase modifiers are abundant for an RID. However, the low sensitivity and the inability to use a gradient elution make the RI detector undesirable in some studies.

Water has been used as an acetonitrile mobile phase modifier in the separation of fatty acid methyl esters (11). It is found to be satisfactory for the separation of low molecular weight triglycerides as shown. The MCT oil containing predominantly triglycerides with ECL lower than TG30 and about all saturated fatty acids (96%) provide an unique opportunity to take advantage of using the acetonitrile/water mobile phase for separation and short wavelength UV detection for quantitation.

The precision of this method was tested between days, operators and replicates. The percentage recoveries were calculated to check the effeciency of the total process and were not used for calculation of the MCT content in the samples. The relative standard deviation (RSD) was higher on one day and lower on the other. Nevertheless, they were below 6.5% throughout the study, including the low level samples. Part of the deviations were contributed by the auto sampler which gave a 1.8% RSD when one sample was injected 10 times and the responses were measured by both peak heights on a chart recorder and peak area integrated by an integrator. The highest and lowest number deviated as much as 3.5%.

The application of this method in different matrices and different levels is shown in Table 3. The matrices tested included all the major matrices in infant formulas. The levels tested ranged from 0.36% to 2.2%, which represent 10% to 50% of the total fat content in the formulas. In all the tested conditions, this method appears to be satisfactory. The only modification between high and low level samples was that the low level samples were concentrated to keep the injection volume below 10 mcl. In this way, the interference of the injection solvent can be minimized.

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REFERENCES

1. Bach, A.C., and V.K. Babayan, *Am. J. Clin. Nutr. 36*:950 (1982).
2. Tantibhedhyangkul, P.,and S.A. Hashium, *Pediatrics 55*:359 (1975).
3. Roy, C.C., M. Ste-Marie, L. Chartrand, A. Weber, H. Bard and B. Doray, *J. Pediatr. 86*:446 (1975).
4. Plattner, R.D., G.F. Spencer and R. Kleiman, *J. Am. Oil Chem. Soc. 54*:51 (1977).
5. Singleton, J.A., and H.W. Pattee *J. Am. Oil Chem. Soc. 61*:761 (1984).
6. Phillips, F.C., W.L. Erdahl, J.A. Schmit and O.S. Privett, *Lipids 19*:880 (1984).
7. Instrument Manual for Mojonnier Milk Tester, Bulletin No. 101, Mojonnier Bros. Co.
8. *Official Methods of Analysis*, Association of Official Analytical Chemists, 12th edn, 1975, 16.020, 16.065, 51.011, 51.039, 51.073.
9. Wood, R., and J. Falch, *Lipids 9*:979 (1974).
10. Babayan, V.K., *J. Am. Oil Chem. Soc. 45*:23 (1967).
11. Wood, R., and T. Lee, *J. Chromatogr. 254*:237 (1983).

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