❧Separation and Quantitative Determination of Monoacylglycerol Mixtures by Reversed Phase HPLC

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The separation and quantitative determination of underivatized saturated and unsaturated monoacylglycerol were examined by reversed phase high performance liquid chromatography using acetonitrile/water as the mobile phase. Monoacylglycerol (MG) mixtures containing saturated MG (C_6-MG–C_{18}-MG), monooleoylglycerol and monolinoleoylglycerol were sufficiently resolved, but mixtures of 1-MG and 2-MG with the same acyl group could not be separated under the conditions given below: column, C-8 chemical bonded column (4.6 × 250 mm); sample solvent, diethyl ether (UV detector at 210–215 nm) or chloroform; mobile phase, acetonitrile/water (7:3, v/v). Quantitative determinations were made for mixtures of three components (representative standard MGs) on a reversed phase column (Unisil Q, C-8) using a UV detector and the internal standard method. Standard deviation and coefficient of variation gave accurate, reproducible results.

The periodate oxidation method (1) has been commonly used for quantitative determination of 1-monoacylglycerol mixtures. But the analytical results by this method show total amounts of MGs and, therefore, values of individual component MGs in the samples are not obtained. Other analytical methods such as column chromatography (2), high performance liquid chromatography (HPLC) (3,4), thin layer chromatography (TLC) (5,6) and gas liquid chromatography (GLC) (7) have been reported for separation and quantitative determination of mixtures of mono-, di- and triacylglycerols. R.D. Wood et al. (8) reported the GLC analysis of MGs as their trimethylsilylether derivatives. This paper describes the separation and quantitative determination of mixtures of various MGs without derivatization by reversed phase HPLC with isocratic elution.

EXPERIMENTAL

Materials. Saturated fatty acids (C_6–C_{18}), oleic acid and linoleic acid were obtained from Yamakei Sangyo Co. Ltd., Osaka, Japan; Nippon Yushi Co. Ltd., Tokyo, Japan, and Yashiro Yushi Co. Ltd., Osaka, Japan, respectively. Purities of each compound were found by GLC to exceed 99%, except stearic acid and hexanoic acid, which were both 97%.

Monoacylglycerols were prepared from the corresponding acid and glycerol by direct esterification and were purified by recrystallization and column chromatography (purity, 99% or better as determined by GLC and TLC).

HPLC. HPLC was carried out using a Gasukuro Kogyo liquid chromatograph (model 570B) equipped with a variable wavelength UV detector and a differential refractometer (RI) detector (Showa Denko, Model SE-11). Separations were achieved on a stainless steel column (4.6 × 250 mm) packed with reversed phase particles such as LiChrosorb (RP-2, RP-8, RP-18, 7μm; Merck, Darmstadt, Federal Republic of Germany) and Unisil Q (C-8, C-18, 5 μm; Gasukuro Kogyo Co. Ltd, Tokyo, Japan.) The samples were eluted isocratically with acetonitrile/water. Other details are given in the table and figure legends. Pure 1-monooctanoylglycerol (C_8-MG) was employed as the internal standard in the quantitative evaluations. The peak areas were determined by triangulation (half width × height).

Solubility of saturated MG. Solubilities of the saturated MGs were determined by placing 2 g of each monoacylglycerol into an Erlenmeyer flask (30 ml) and adding 10 ml of each solvent with a pipette. The flask was fitted with a glass stopper and placed in a thermostated water bath at 26.8 C for 30 min. The flask was periodically swirled during the warming period. At the end of this treatment, 2 ml of the sample solution was transferred to a tared round-bottomed flask with a pipette. The solvent was removed under reduced pressure, and the remaining MG was weighed.

RESULTS AND DISCUSSION

Selection of HPLC conditions. To analyze MGs, HPLC conditions such as stationary phase, mobile phase, detector response and sample solvent were examined.

(i) Stationary phase. Three types of reversed phase columns, LiChrosorb RP-2, RP-8 and RP-18, were examined to select a suitable phase for separation of saturated MGs. The influence of stationary phases on retention times was evaluated using the following parameters: acetonitrile/water (8:2, v/v), 0.5 ml/min, and a UV wavelength at 220 nm. The RP-18 column showed good resolution for saturated MGs (C_6-MG–C_{18}-MG). The RP-8 column also showed good resolution between C_{10}-MG and C_{18}-MG. The RP-2 column, however, did not resolve the MGs as well as the RP-18 and RP-8. Retention times (min) of each MG were as follows for RP-18 and RP-8 columns, respectively: C_6-MG, 5.1 and —; C_8-MG, 5.7 and 5.9; C_{10}-MG, 7.2 and 6.6; C_{12}-MG, 9.8 and 8.0; C_{14}-MG, 15.7 and 10.1; C_{16}-MG, 27.7 and 13.6; C_{18}-MG, 36.0 and 19.4.

(ii) Mobil phase. Acetonitrile/water has generally been used as the mobile phase for reversed phase analysis of acylglycerols with a UV detector. The influence of water on retention times for separation of MGs was examined using the RP-8 column under the same conditions as for stationary phase, except the flow rate was 2.0 ml/min. Retention times of each MG were naturally prolonged with increasing water content in acetonitrile. Water content of 30% gave sufficient separation of saturated MGs from C_6-MG to C_{18}-MG. The retention times were 1.6, 1.8, 2.2, 2.8, 4.0, 6.1 and 9.5 from C_6-MG to C_{18}-MG,

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respectively.

(iii) Detector response. As MGs have absorption only at low UV wavelength, the influence of the wavelengths from 205–225 nm on the relative responses was examined.The response for each saturated MG at 210–215 nm was ca. 1.5 times greater than that at 220 nm. Therefore, it is better for saturated MG determinations to operate a UV detector in this range. The responses of monolinoleoylglycerol (MLi) and monooleoylglycerol (MO) were far more sensitive than those of saturated MGs at 210–215 nm. On the other hand, the response of an RI detector was lower than that of a UV detector at 210 nm. Therefore, it is necessary to inject concentrated samples for analysis with an RI detector.

(iv) Sample solvent. Since the RI detector showed low responses for long chain saturated MGs such as C_{16}-MG and C_{18}-MG which have low solubility, solubilities of representative saturated MGs were determined (Table 1). The MGs were more soluble in chloroform than in other solvents. Sample solvent also affects resolution and detectability when chromatography of MGs is carried out on reversed phase columns. Chromatography of a mixture of short chain MGs in several solvents was carried out on the C-18 column, using both detectors, the mobile phase (8:2, v/v) and flow rate of 1.0 ml/min. In the case of the RI detector, chloroform, carbon tetrachloride, benzene and diethyl ether overlapped with C_{10}-MG, and ethanol overlapped with C_6-MG. Methanol, however, was well separated. The retention times (min) were as follows: CH_3OH, 2.8; C_6-MG, 3.8; C_8-MG, 4.7; C_{10}-MG, 6.0; C_{12}-MG, 9.7.

In the case of the UV detector, methanol and ethanol have no absorption and peaks for the MGs were shown on the chromatogram. The other solvents were essentially the same as in the case of RI detector, except for sensitivity. Therefore, methanol is a good solvent for analysis of short chain MGs under the above conditions, but it does not dissolve long chain MGs.

Resolution on monooleoyl-, monolinoleoylglycerol and saturated MGs. Resolutions of isomers such as 1- and 2-lauroylglycerol (prepared from 1,3-benzyliden glycerol) and 1- and 2-palmitoylglycerol (prepared from direct esterification) were achieved on the reversed phase columns, but sets of isomers could not be separated into two peaks.

A mixture of MO and MLi was adequately separated

TABLE 1

Solubilities of Saturated Monoacylglycerols (g/100 ml)[a]

Solvent	C_6-MG	C_{12}-MG	C_{16}-MG	C_{18}-MG[b]
n-Hexane	0.6	0.6	1.6	0.4
Petroleum ether	0.4	0.7	2.0	0.9
Diethyl ether	>40[c]	4.8	2.8	1.7
Benzene	>40	5.5	6.5	1.5
Acetone	>40	18.5	6.0	4.3
Chloroform	>40	43.5	31.5	19.5
Methanol	>40	>40	4.8	4.2
Ethanol	>40	>40	5.1	4.4

[a]Determined at 26.8 C. Data were converted from g/2 ml to g/100 ml.
[b]Component: C_{18}-MG, 59%; C_{16}-MG, 40%; C_{14}-MG, 1%.
[c]>40 Shows that 2.0 g of the sample material was soluble enough in 5 ml of the solvent after standing for 30 min at 26.8 C.

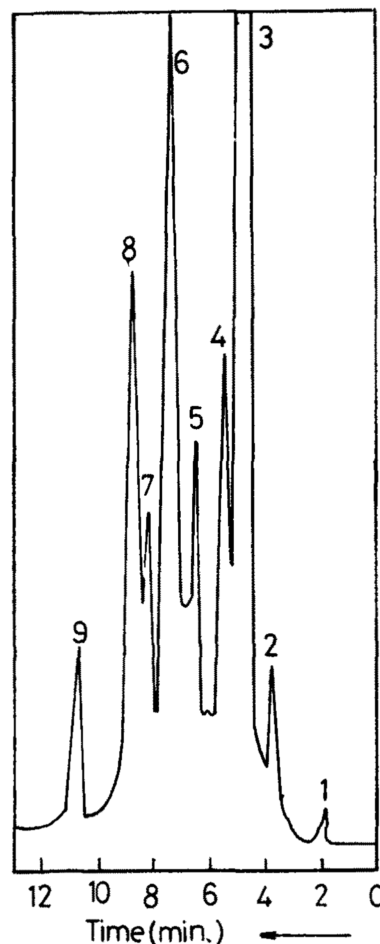

FIG. 1. Reversed phase HPLC of saturated and unsaturated monoacylglycerols. Conditions: column—Unisil Q, C-8, CH_3CN/H_2O (7:3, v/v), 1.0 ml/min, UV 210 nm. Peak number: 1 and 2, impurity from diethyl ether; 3, chloroform + diethyl ether; 4, C_{12}-MG; 5, C_{14}-MG; 6, $C_{18:2}$-MG; 7, C_{16}-MG; 8, C18:1-MG; 9, $C_{18:0}$-MG.

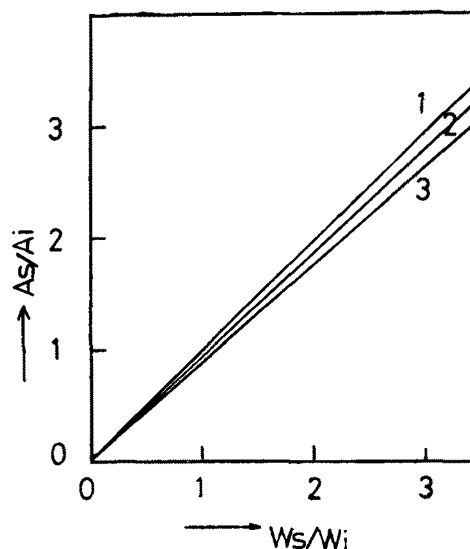

FIG. 2. Calibration curves for monolauroyl-, monomyristoyl- and monopalmitoylglycerols. Ws, weight of sample; Wi, weight of internal standard (C_8-MG); As, area of sample; Ai, area of internal standard (C_8-MG). 1, C_{12}-MG; 2, C_{14}-MG; 3, C_{16}-MG. Conditions: Unisil Q, C-18, CH_3CN/H_2O (8:2, v/v), 1.0 ml/min; RI detector.

TABLE 2

Analysis of Saturated Monoacylglycerol Mixtures with an RI Detector[a]

Sample no.	Component	Known (%)	Found (%)[b]	Standard deviation	Coefficient of variation (%)	Error
1	C_{12}-MG	60.4	60.8	1.09	1.8	+0.4
	C_{14}-MG	20.7	18.8	1.14	6.1	−1.9
	C_{16}-MG	18.9	20.5	0.86	4.2	+1.6
2	C_{12}-MG	35.2	35.9	1.29	3.6	+0.7
	C_{14}-MG	33.1	31.8	1.46	4.6	−1.3
	C_{16}-MG	31.7	32.3	1.42	4.4	+0.6
3	C_{12}-MG	23.3	25.0	0.50	2.0	+1.7
	C_{14}-MG	55.7	52.4	0.94	1.8	−3.4
	C_{16}-MG	21.0	22.5	1.17	5.2	+1.5
4	C_{12}-MG	23.9	24.1	0.40	1.7	+0.2
	C_{14}-MG	19.3	15.8	0.68	4.4	−3.5
	C_{16}-MG	56.9	60.0	0.60	1.0	+3.1

[a]HPLC conditions: Unisil Q, C-18, CH_3CN/H_2O (8:2, v/v), 1.0 ml/min.
[b]Average, n = 9 or 10.

TABLE 3

Analysis of Saturated Monoacylglycerol Mixtures with an UV Detector at 210 nm[a]

Sample no.	Component	Known (wt %)	Found (%)[b]	Standard deviation	Coefficient of variation (%)	Error
1	C_{12}-MG	27.5	29.7	0.55	1.9	+2.2
	C_{14}-MG	22.5	22.0	0.51	2.3	−0.5
	C_{16}-MG	50.0	48.4	0.67	1.4	−1.6
2	C_{12}-MG	30.8	33.3	0.35	1.0	+2.5
	C_{14}-MG	43.3	41.4	0.24	1.3	−1.9
	C_{16}-MG	25.9	25.3	0.47	1.9	−0.6
3	C_{12}-MG	58.9	62.4	0.61	1.0	+3.5
	C_{14}-MG	11.0	10.7	0.39	2.8	−0.3
	C_{16}-MG	30.1	26.9	0.50	1.9	−3.3

[a]HPLC conditions: Unisil Q, C-8, CH_3CN/H_2O (7:3, v/v), 1.0 ml/min.
[b]Average, n = 9 or 10.

on a C-8 or C-18 bonded column using acetonitrile/water (former column, 7:3; later, 8:2, v/v, respectively). A mixed sample of saturated MGs, MO and MLi was almost completely separated using the C-8 column (Unisil Q, C-8) with the same mobile phase (7:3, v/v) (Fig. 1). Their K′ values were as follows: C_{12}-MG, 0.14; C_{14}-MG, 0.28; $C_{18:2}$-MG, 0.53; C_{16}-MG, 0.74; $C_{18:1}$-MG, 0.91; $C_{18:0}$-MG, 1.26. The peaks of MO and MLi appeared between C_{16}-MG and $C_{18:0}$-MG, and C_{14}-MG and C_{16}-MG, respectively. In the case of the C-18 bonded column (Unisil Q, C-18), only the $C_{18:1}$-MG and C_{16}-MG peaks were partially overlapped; the others were separated from each other.

Quantitative determination of test samples. To analyze saturated MGs, calibration curves for C_{12}-MGs, C_{14}-MG and C_{16}-MG were prepared from the relationship between relative weight and relative response against an internal standard (C_8-MG) using a C-8 or C-18 bonded column with a UV detector for the former column and an RI detector for the latter. The plots of weight (MG/C_8-MG) against response (MG/C_8-MG) gave linear relationships on both columns. One of the calibration curves obtained from the C-18 column is shown in Figure 2.

Quantitative determinations were made to test the reproducibility and accuracy of the method. Test sample solutions containing known amounts of C_{12}-, C_{14}-, C_{16}- and C_8-MG (internal standard) were prepared and analyzed on both columns. In Tables 2 and 3, the results of the analysis for three or four samples composed of the

three MGs are given. Standard deviation and coefficient of variation (%) for C_{12}-, C_{14}- and C_{16}-MG on the C-8 column with a UV detector were 0.35-0.61 and 1.0-1.9%; 0.24-0.51 and 1.2-2.8%; and 0.47-0.67 and 1.4-1.9%, respectively. In the case of the C-18 column with an RI detector, the results were similar. Both HPLC methods gave reproducible results.

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Analysis for Trace Amounts of Geosmin in Water and Fish[1]

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Trace amounts of geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) were concentrated from relatively large volumes of water by vegetable oil extraction. After stirring the two phases for 30 min, the dispersed oil was allowed to separate. The oily layer was removed and centrifuged to break the emulsion and separate the two layers. The direct gas chromatographic technique was used to resolve the geosmin from other volatile components on a capillary gas chromatographic column. Volatiles were separated from the oil by securing an aliquot of the oil layer on volatile-free glass wool in the glass liner of the special gas chromatography inlet system. Geosmin was detected at the parts ber billion level with this simple and rapid technique. A technique also was developed for detecting geosmin in fish tissue; it involves steaming the fish to break up the tissue, centrifuging the residual oil phase, and detecting geosmin by capillary gas chromatography. The geosmin remains in the residual oil.

There have been many reports in the literature on the muddy or earthy odor of soil, and on the problem of muddy odor or flavor in surface waters and in fish taken from those waters (1). The major cause of this odor appears to be the production of geosmin (*trans*-1, 10-dimethyl-*trans*-9-decalol) by either Actinomycetes or blue-green algae or both, and methylisoborneol by Actinomycetes (2-9). These two compounds have been isolated and identified from surface water (7).

Yurkowski and Tabachek reported a study (1) that showed that all odor extracts from both muddy-flavored and non-muddy-flavored fish from the saline lakes of western Canada contained geosmin, suggesting that untainted fish (as determined by sensory panel) contained geosmin at levels below the taste threshold level. The results of their study also showed that, depending on the lake, the fish may be tainted throughout or for a short period of time during any portion of the growing season which lasts from early May to late October. Simple methods of analysis for geosmin content of fish, and of the water in which fish are raised, are therefore needed. Since geosmin has a very low organoleptic threshold level, estimated at 6 ppb in fish by Yurkowski and Tabachek (1), recovery of the odor-causing material from fish tissue or water is a primary consideration in any assay method. This problem is addressed in the present paper.

EXPERIMENTAL PROCEDURES

Materials. Catfish filets were obtained at local seafood markets. Geosmin (98% pure) was purchased from Givaudan Corp., Clifton, New Jersey.

Gas chromatography. The gas chromatograph (GC) used was a Hewlett Packard (HP) 5790 series equipped with flame ionization detector (FID). Flow rates for hydrogen and air were 30 and 240 ml/min, respectively. Nitrogen flow rates were 1.2 ml/min through the column and 30 ml/min for the auxiliary make-up gas. A HP ultra performance capillary column was used (50 m × 0.31 mm ID column coated with 0.52 micron film of crosslinked 5% phenyl methyl silicone). An external closed inlet device (Scientific Instrument Service, River Ridge, Louisiana) designed from the inlet system previously described (10) was interfaced at the carrier gas arm of the insert Weldment assembly of the GC to facilitate direct gas chromatography. The inlet temperature of this device was set at 140 C, and the temperature of the six-port rotary valve was set at 180

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