ANALYSIS OF ACYLGLYCEROLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN DERIVATIZATION

IV. SIMULTANEOUS ANALYSIS OF MONO-, DI- AND TRIACYLGLYCEROLS

YUKIHIRO KONDOH and SATOSHI TAKANO*

Tochigi Research Laboratories, Kao Corporation, 2606, Akabane, Ichikai-machi, Tochigi 321–34 (Japan)

(First received November 26th, 1986; revised manuscript received January 20th, 1987)

SUMMARY

A simple and convenient method for the simultaneous determination of mono-, di- and triacylglycerols has been developed. Acylglycerols from monolaurin to tristearin were separated by reversed-phase high-performance liquid chromatography and detected with the acylglycerol-selective post-column reaction detector. The detector indicated the molar response of all acylglycerols from monolaurin to tristearin. It can, for example, detect 0.07 nmol of 1-monopalmitin and give a linear calibration graph between 0.2 and 121 nmol of 1-monopalmitin. Furthermore, acylglycerols in commercial products could be selectively detected and quantitated without any pretreatment. Therefore, the proposed method should offer a versatile method for the analysis of acylglycerols in natural and commercial products.

INTRODUCTION

In a previous paper[1], a new post-column reaction detector for the analysis of triacylglycerols with high sensitivity, high selectivity and molar response was described. By using this acylglycerol-selective post-column reaction detection (GS-PCRD) system, triacylglycerols of natural fats and oils were sufficiently separated and quantitatively analyzed in non-aqueous reversed-phase chromatography on an octadecyl chemically bonded silica (ODS) column. Furthermore, the combination of argentation high-performance liquid chromatography (HPLC) using an infrared detector with non-aqueous reversed-phase chromatography using GS-PCRD was investigated for the full analysis of triacylglycerols of natural fats and oils[2]. The analysis of monoacylglycerols in commercial products was also demonstrated using reversed-phase HPLC and GS-PCRD[3].

For the simultaneous determination of mono-, di- and triacylglycerols, gas chromatographic analysis has been employed after the derivatization of mono- and diacylglycerols with chlorotrimethylsilane[4]. Recently, direct analysis of these acylglycerols was carried out using capillary super critical fluid chromatography[5]. How-

ever, acylglycerols that contain the same number of acyl carbon atoms, but differ in the number or position of double bonds, are difficult to separate by both techniques. Thus, reversed-phase HPLC seems to be most suitable for this purpose. Since GS-PCRD is compatible with gradient elution, we have developed a simple and convenient method for the simultaneous determination of mono-, di- and triacylglycerols in natural and commercial products by reversed-phase HPLC and GS-PCRD.

EXPERIMENTAL

Apparatus

The apparatus was similar to that previously described[1,2]. A sample injector able to maintain an ambient temperature from 30 to 100°C was specially designed and used.

Reagents

Hitachi gel 3057 (Hitachi, Tokyo, Japan) was used as a stationary phase, comprising ODS packings of average diameter 3 μm. Authentic mono-, di- and triacylglycerols were obtained from Sigma (St. Louis, MO, U.S.A.) and P-L Biochemicals (Milwaukee, WI, U.S.A.). Acetonitrile and ethanol of HPLC analysis grade were obtained from Kanto Chemical Co. (Tokyo, Japan). Other reagents were of analytical reagent grade.

Procedure for analysis of acylglycerols

A stainless-steel column (250 mm × 4.0 mm I.D.) packed with Hitachi gel 3057 (3 μm) was used and kept at 30°C. The mobile phases were ethanol–acetonitrile–water (40:48:12) (solvent A) and (65:35:0) (solvent B) at a flow-rate of 0.8 ml/min. The procedure was as follows. Starting with 100% solvent A, during 15 min the amount of solvent B was increased at 4.67%/min to 70% and then to 100% at 1%/min during 30 min. This mobile phase was maintained for 25 min, and then was changed to the starting condition for reconditioning (20 min). The effluent was monitored by GS-PCRD.

A sample containing 0.2–200 μg of mono-, di- and triacylglycerols, dissolved in 5–40 μl of acetone or less than 5 μl of tetrahydrofuran (THF) was injected for HPLC.

RESULTS AND DISCUSSION

Separation of mono-, di- and triacylglycerols

The HPLC analysis of triacylglycerols has previously been investigated using a reversed-phase ODS column[1]. Only a few solvents could be used as the mobile phase because of interference with the post-column reaction. Among these solvents, ethanol and acetonitrile were used as the mobile phase for GS-PCRD. Using ethanol–acetonitrile (60:40), triacylglycerols from tricaprylin to tristearin could be sufficiently separated with Hitachi gel 3057 (3 μm) as a stationary phase[1]. The separation of monoacylglycerols from monolaurin to monolinolein was achieved with acetonitrile–water (58:42) as a mobile phase on Hypersil MOS (3 μm)[3].

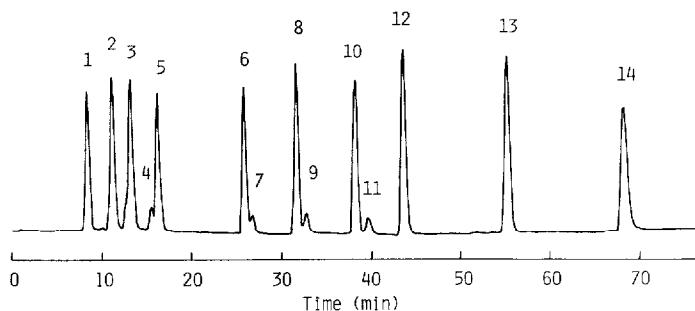On the basis of these results, the simultaneous separation of mono-, di- and

Fig. 1. Typical chromatogram of a standard mixture of mono-, di- and triacylglycerols using GS-PCRD. Peaks: 1 = THF; 2 = monomyristin; 3 = monopalmitin; 4 = 2-monostearin; 5 = 1-monostearin; 6 = 1,3-dimyristin; 7 = 1,2-dimyristin; 8 = 1,3-dipalmitin; 9 = 1,2-dipalmitin; 10 = 1,3-distearin; 11 = 1,2-distearin; 12 = trimyristin; 13 = tripalmitin; 14 = tristearin.

triacylglycerols was investigated using mixtures of ethanol–acetonitrile–water as a mobile phase. Hitachi gel 3057 (3 μm) was chosen as a stationary phase because of the high resolution. Excellent resolution of acylglycerols from monolaurin to tristearin was obtained under the conditions described in the Experimental section, and a typical chromatogram is shown in Fig. 1. A long chromatographic column (250 mm) was used since the chromatographic peaks underwent a two-fold broadening caused by diffusion of eluted substances in the detector. The column temperature was set at 30°C in order to avoid the isomerization of 2-monoacylglycerol to 1-monoacylglycerol[3].

Response and reproducibility of the detector

It is most important to establish the molar response of the detector. Glycerol was repeatedly injected with an interval of 5 min under the gradient conditions. Fig. 2 shows the peak area of glycerol *versus* the analysis time. This result indicates that GS-PCRD affords a constant response to glycerol regardless of the mobile phase composition. However, in the determination of triacylglycerols from trilaurin to tri-
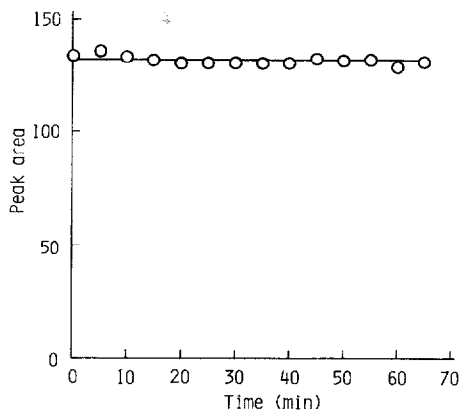

Fig. 2. Response of the detector to glycerol for the gradient analysis. Glycerol (2 μg) was repeatedly injected with an interval of 5 min under the gradient conditions.

TABLE I

EFFECTS OF CHROMATOGRAPHIC CONDITIONS ON THE RESPONSE OF THE ACYLGLY-
CEROL-SELECTIVE POST-COLUMN REACTION DETECTOR

Mobile phase*						
Solvent A	25:65:10	25:65:10	20:68:12	20:72:8	20:72:8	40:48:12
Solvent B	65:35:0	65:35:0	65:35:0	65:35:0	65:35:0	65:35:0
Injection solvent	THF	THF	THF	Acetone	THF	THF
Injection temp. (°C)	25	50	60	60	60	60
Relative molar response to trilauarin						
tricaprylin						1.00
tricaprin						1.02
trilaurin	1.00	1.00	1.00	1.00	1.00	1.00
trimyristin	1.03	1.02	1.00	1.03	1.00	1.02
tripalmitin	1.04	0.98	0.96	0.98	0.97	0.98
tristearin	0.47	0.88	0.86	0.93	0.96	0.98
triolein						0.98

* Ethanol–acetonitrile–water.

stearin the detector did not indicate the molar response as shown in Table I. The relative responses of tristearin to trilaurin are a little lower than those of other triacylglycerols; however use of THF as an injection solvent increases the relative molar response. Furthermore, it was found that the relative response of tristearin also depended on the initial mobile phase composition (solvent A). These results suggested that low solubility of tristearin in the mobile phase caused these problems. Therefore, the effect of the temperature of the injection port was investigated along with the effect of the composition of solvent A. An increase in the temperature of the injection port drastically increased the relative molar response of tristearin, as shown in Table I. Thus, the injection port was maintained at 60°C with a specially designed apparatus as described in the Experimental section. The molar responses from trilaurin to tristearin were finally established under the conditions shown in the right-hand column of Table I. Under these conditions, glycerol, mono-, di- and tripalmitin gave the molar responses shown in Table II.

Calibration graphs and reproducibilities
        The linear range of the calibration graphs for mono-, di- and tripalmitin under

TABLE II

RESPONSIBILITY OF THE ACYLGLYCEROL-SELECTIVE POST-COLUMN REACTION DE-
TECTOR TO GLYCEROL AND MONO-, DI- AND TRIACYLGLYCEROLS AND LINEAR
RANGE OF CALIBRATION GRAPHS

Acylglycerol	Relative molar response to glycerol	Range of calibration graph (nmol)	C.V. (%)*
Glycerol	1.00		
Monopalmitin	1.04	0.2–121	0.62
Dipalmitin	1.03	0.3– 70	1.12
Tripalmitin	1.01	0.3– 50	2.33

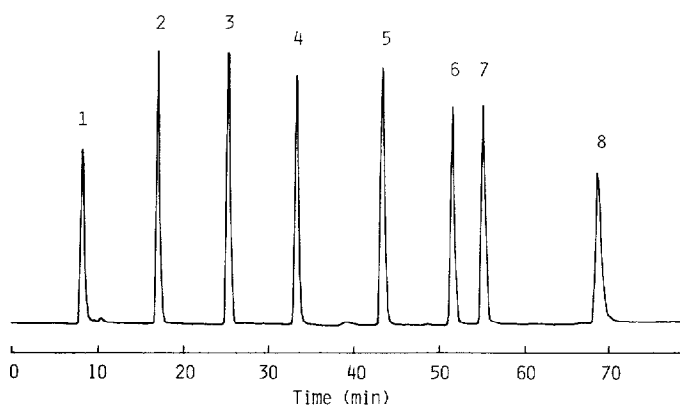* Each sample (5.0 μg) was injected and analyzed five times.

Fig. 3. Typical chromatogram of a standard mixture of triacylglycerols using GS-PCRD. Peaks: 1 = THF; 2 = tricaprylin; 3 = tricaprin; 4 = trilaurin; 5 = trimyristin; 6 = triolein; 7 = tripalmitin; 8 = tristearin.
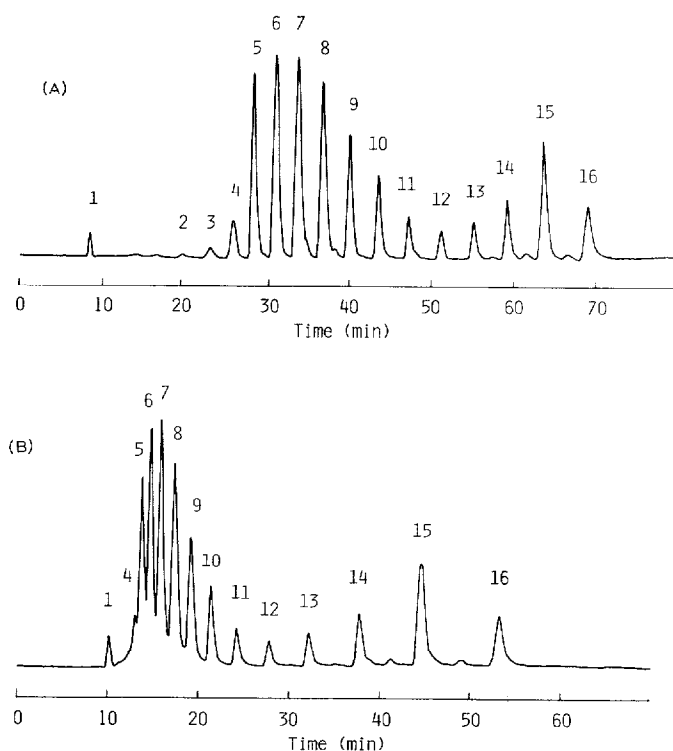

Fig. 4. Analysis of triacylglycerols in a mixture of hydrogenated coconut and tallow oil by gradient elution (A) and isocratic elution (B). Peaks: 1 = THF; 2 = $C_{26:0}$ (total carbon number of acyl groups = 26; number of unsaturation of acyl groups = 0); 3 = $C_{28:0}$; 4 = $C_{30:0}$; 5 = $C_{32:0}$; 6 = $C_{34:0}$; 7 = $C_{36:0}$; 8 = $C_{38:0}$; 9 = $C_{40:0}$; 10 = $C_{42:0}$; 11 = $C_{44:0}$; 12 = $C_{46:0}$; 13 = $C_{48:0}$; 14 = $C_{50:0}$; 15 = $C_{52:0}$; 16 = $C_{54:0}$.
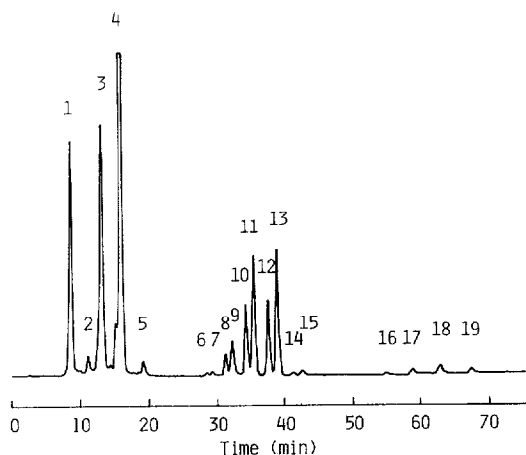
Fig. 5. Analysis of an emulsifier in a commercial household product. Peaks: 1 = glycerol; 2 = mono-myristin; 3 = monopalmitin; 4 = monostearin; 5 = monoarachidin; 6 = 1,3-diacylglycerol (DG) $C_{30:0}$; 7 = 1,2-DG $C_{30:0}$; 8 = 1,3-DG $C_{32:0}$; 9 = 1,2-DG $C_{32:0}$; 10 = 1,3-DG $C_{34:0}$; 11 = 1,2-DG $C_{34:0}$; 12 = 1,3-DG $C_{36:0}$; 13 = 1,2-DG $C_{36:0}$; 14 = 1,3-DG $C_{38:0}$; 15 = 1,2-DG $C_{38:0}$; 16 = triacylglycerol (TG) $C_{48:0}$; 17 = TG $C_{50:0}$; 18 = TG $C_{52:0}$; 19 = TG $C_{54:0}$.

the optimum conditions are shown in Table II, and their detection limits are 0.1–0.3 nmol. The reproducibilities of the analyses of 5 μg of each acylglycerol were within 2.3% relative standard deviation.

Application

Fig. 3 shows a chromatogram of a mixture of standard triacylglycerols from tricaprylin to tristearin, indicating that the present method is able to analyze a wide range of triacylglycerols. An example of an analysis of a mixture of hydrogenated coconut and tallow oil is shown in Fig. 4A, and compared with that obtained by a conventional isocratic method (Fig. 4B).

Commercial monoacylglycerols used as an emulsifier usually contain di- and triacylglycerols. Fig. 5 shows an example of an analysis of monoacylglycerols in a commercial household product. The contents of mono-, di- and triacylglycerols were found to be 0.48, 0.48 and 0.04%, respectively. No interfering peak was observed, although the sample was injected directly without any pretreatment. These results show the selectivity of the detector. Thus, the present method should offer a versatile method for the simultaneous analysis of mono-, di- and triacylglycerols in natural and commercial products.

REFERENCES

1 Y. Kondoh and S. Takano, *Anal. Chem.*, 58 (1986) 2380.
2 S. Takano and Y. Kondoh, *J. Am. Oil Chem. Soc.*, in press.
3 S. Takano and Y. Kondoh, *J. Am. Oil Chem. Soc.*, in press.
4 R. P. D'Alonzo, W. J. Kozarek and H. W. Wharton, *J. Am. Oil Chem. Soc.*, 59 (1981) 292.
5 T. L. Chester, *J. Chromatogr.*, 299 (1984) 424.