Journal of Chromatography, 209 (1981) 61–66
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,612

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATION OF FREE ACIDS, MONO-, DI- AND TRIGLYCERIDES USING AN INFRARED DETECTOR

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(Received December 24th, 1980)

SUMMARY

Methyl ester, mono-, di- and triglyceride classes were separated and quantitated by high-performance liquid chromatography. Baseline separations were achieved on an amino-, cyano-bonded Partisil column with a gradient from hexane–chloroform (60:65) to hexane–chloroform–acetonitrile (25:65:35). The eluate was monitored by an infrared detector set at 5.72 μm, and peak areas were determined by computer integration. Methyl 9,10-dihydroxystearate was used as an internal standard. The total run time and recycling time was 35 min. A precision of 10% or better was obtained for all components, even at the 1% (w/w) level.

INTRODUCTION

Separation and quantitation of mixtures of fatty acids and mono-, di- and triglycerides are often desired in natural products, emulsifier additives and food chemistry. Thin-layer (TLC)[1–4] and column[5,6] chromatographic methods separate lipid classes, but are time consuming and require additional techniques for quantitation of the fractions. Chromarods[7,8] and gas chromatography (GC)[9–11] have been used to quantitate certain components, but they do not permit isolation of material for further study. High-performance liquid chromatography (HPLC) with ultraviolet detection at 213 nm is not quantitative for mixtures containing a variety of unsaturated components[12].

To date, a dual-gradient HPLC system utilizing the transport flame ionization or "moving wire" detector has shown the most success in quantitation of lipid classes[13]. We have now developed a system of single-gradient HPLC utilizing infrared (IR) detection that separates and quantitates acyl-containing lipid classes.

0021-9673/81/0000–0000/$02.50

TABLE I
COMPOSITION AND ANALYSIS OF STANDARD MIXTURES

Mixture No.	*No. of trials*	*Standard**		*Methyl palmitate*				*Tripalmitin*			
		mmoles/ml	*mg/ml*	*mmoles/ml*	*mg/ml*	M_y/M_s**	$\bar{x}(A_y/A_s)$***	*mmoles/ml*	*mg/ml*	M_y/M_s	$\bar{x}(A_y/A_s)$
1	5	0.0303	10.01	0.0298	8.04	0.984	1.096	0.0125	10.06	0.413	1.303
2	6	0.0243	8.01	0.0112	3.01	0.461	0.494	0.0075	6.03	0.309	1.130
3	4	0.0152	5.01	0.0223	6.02	1.467	1.460	0.0037	3.02	0.243	0.840
4	5	0.0091	3.00	0.0372	10.03	4.088	3.919	0.0121	9.76	1.330	4.043
5	5	0.0303	10.01	0.186	5.02	0.614	0.608	0.0061	4.88	0.201	0.628

Mixture No.	*No. of trials*	*Standard**		*1,3-Dipalmitin*				*Monopalmitin*			
		mmoles/ml	*mg/ml*	*mmoles/ml*	*mg/ml*	M_y/M_s	$\bar{x}(A_y/A_s)$	*mmoles/ml*	*mg/ml*	M_y/M_s	$\bar{x}(A_y/A_s)$
1	5	0.0303	10.01	0.0177	10.06	0.584	1.203	0.0305	10.05	1.007	0.965
2	6	0.0243	8.01	0.0177	10.06	0.728	1.504	0.0152	5.03	0.626	0.574
3	4	0.0152	5.01	0.0142	8.05	0.934	1.820	0.0304	10.05	2.000	1.685
4	5	0.0091	3.00	0.0089	5.03	0.978	1.851	0.0292	9.64	3.209	2.545
5	5	0.0303	10.01	0.0025	1.41	0.083	0.145	0.0146	4.82	0.482	0.383

* Methyl 9,10-dihydroxystearate.

** M_y = moles of component y; M_s = moles of standard.

*** A_y = peak area of component y; A_s = peak area of standard.

EXPERIMENTAL*

Methyl palmitate, tripalmitin, dipalmitin and monopalmitin of purity greater than 99% were obtained from Nu-Chek-Prep (Elysian, MN, U.S.A.). A product sold as "methyl 10,11-dihydroxystearate" was obtained from K & K Labs. (Plainview, NY, U.S.A.). Four grams of this product were placed on a dry column (21 cm × 2 cm I.D.) of Hi-Flosil and eluted with a litre of chloroform. The eluate was evaporated to dryness, and the residue was recrystallized from 600 ml of cold (−10°C) diethyl ether. Mass spectrometry proved that the purified ester was actually methyl 9,10-dihydroxystearate.

Five standard mixtures (Table I) were prepared by dissolving known amounts of each of the following in chloroform: methyl palmitate, tripalmitin, dipalmitin, methyl 9,10-dihydroxystearate and monopalmitin. The concentration of each component in each solution is given in Table I. In addition, the ratio of the moles of each component (M_y) to moles internal standard (M_s) is given for comparison with the analytical findings. The ratio of the peak area (A_y) of each component to the area of the internal standard (A_s) was calculated for each trial, and the averages of the trials, $\bar{x}$ (A_y/A_s), are given in Table I.

Two other standards were also prepared. Crude soybean oil was lipolyzed and recovered[14], and the resulting mixture was treated with diazomethane[15] to convert the free acids into methyl esters. Methyl 9,10-dihydroxystearate (65.2 mg) was added to 28.2 mg of this mixture in 0.7 ml of chloroform. In a second sample, 282.8 mg of commercial shortening and 2.07 mg of methyl 9,10-dihydroxystearate were dissolved in 0.25 ml of chloroform.

HPLC was accomplished on a column (25 cm × 4.6 mm) of Partisil PXS 10/25 PAC (Whatman, Clifton, NJ, U.S.A.), using gradient elution. Solvent A was hexane–chloroform (60:65) and solvent B was acetonitrile–hexane–chloroform (35:25:65). A linear gradient of these two solvents went from 2 to 95% B in 20 min at 2 ml/min. The chromatographic system included two M-6000A pumps, a Model 660 solvent programmer (all from Waters Assoc., Milford, MA, U.S.A.) and an IR detector for liquid chromatography (DuPont, Wilmington, DE, U.S.A.) set at 5.72 μm and 0.1 A attenuation.

Typically, samples of 0.5 to 1.5 mg were injected, although 55 mg of the commercial shortening were injected because the components of interest represented only 1 to 4% (w/w) of the total mixture. A typical run time was 35 min, with 6–7 min needed to recycle before the next injection.

A laboratory-wide computer system[16] was used to determine peak areas. Each HPLC run was stored in the computer and then displayed on an interactive graphics terminal. The cross-hairs of the terminal were used to mark the beginning, end and baseline location for each peak. The area above the baseline was calculated by the computer, and an area report was printed. This rapid interactive method was required because a solvent front and the triglyceride peak eluted simultaneously. In laboratories not equipped with a manually controlled computer integration system, similar

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

control over beginning, end and baseline of each peak can be obtained by using the "cut and weigh" method.

RESULTS AND DISCUSSION

In the early stages of work, it was found that free acids tailed and overlapped the diglycerides. Esterification of the free acids in the sample before analysis eliminated these difficulties and allowed quantitation of the free acids (as methyl esters). Inclusion of an internal standard permitted independent quantitation of individual components. Methyl 9,10-dihydroxystearate was selected as internal standard because it is readily available, reasonably inexpensive, and gives a peak that is well resolved from others in the chromatogram.

Most available solvents (both Spectro and ACS grades) contain traces of materials that absorb at 5.72 μm. Hexane and acetonitrile had absorbances of similar intensities at 5.72 μm, but chloroform was one of the few solvents that had virtually no absorbance in that region. Careful balancing of the amounts of hexane, acetonitrile and chloroform in solvents A and B was necessary to produce a baseline that had relatively little drift as the program progressed. A typical baseline (Fig. 1a) was generated from a trial in which only chloroform, the solvent used in sample preparation, was injected. The chloroform produced a negative peak *ca.* 1.4 min after injection.

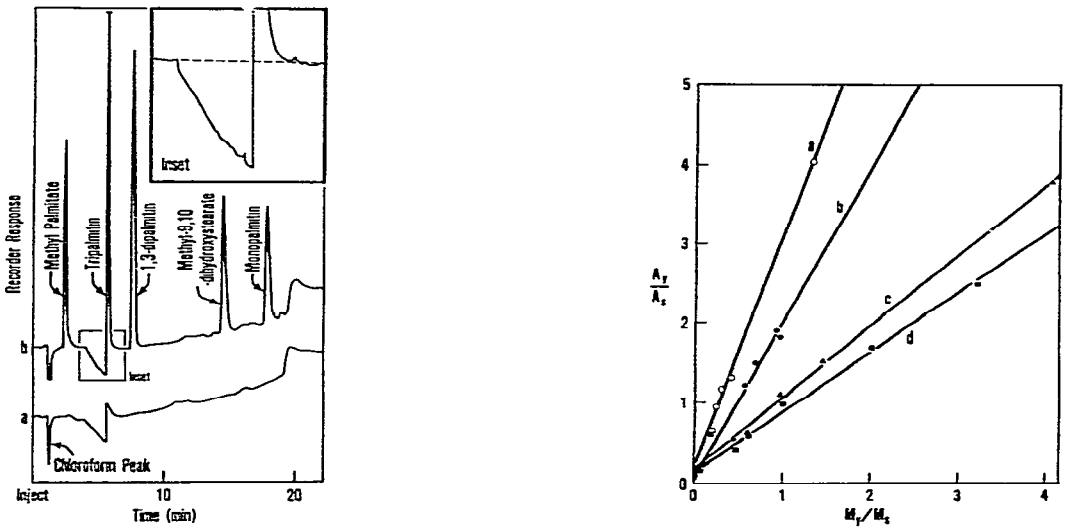


Fig. 1. (a) Typical baseline produced on a Partisil PXS 10/25 PAC column with a linear gradient of solvents A [hexane–chloroform (60:65)] and B [acetonitrile–hexane–chloroform (35:25:65)] from 2 to 95% in 20 min at 2 ml/min monitored at 5.72 μm; chloroform (5 μl) was injected at the start of the program. (b) HPLC chromatogram of standard mixture 1 (methyl palmitate, tripalmitin, dipalmitin, methyl 9,10-dihydroxystearate and monopalmitin) under the conditions in (a). Inset: Integration of triglyceride peak. Dotted line shows baseline used.

Fig. 2. Plots of area of component/area of standard methyl 9,10-dihydroxystearate (A_y/A_s) *versus* moles of component/moles of standard (M_y/M_s) for methyl palmitate, tripalmitin, dipalmitin, and monopalmitin based on the mixtures listed in Table I. (a), tripalmitin (slope, 2.9); (b), dipalmitin (slope, 1.9); (c), methyl palmitate (slope, 0.89); (d), monopalmitin (slope, 0.75).

Other irregularities in the baseline resulted from the gradient programming[17]. The baseline dipped from 4 to 6 min, then rose sharply. To integrate the triglyceride peak that eluted with this baseline shift, the baseline following the peak was extended horizontally through to the beginning of the peak (see inset in Fig. 1). This technique provided a consistent method of determining peak area and gave results in good agreement with the known amounts of sample. At *ca.* 20 min, a second baseline shift occurred. Interference between this shift and the monoglyceride peak was eliminated by running the system at 95% B for 10 min before recycling for the next run. Since re-equilibration of the column by switching directly back to initial conditions took only 6 to 7 min, a "reverse" gradient[17] was not necessary. When the system was idle for several hours or overnight, a "warm-up" run through the entire sequence optimized conditions for later chromatography.

Five standard mixtures of methyl palmitate, tripalmitin, dipalmitin, methyl 9,10-dihydroxystearate and monopalmitin (Table I and Fig. 1) were each analyzed four to six times. Since detection is based on absorbance due to carbonyl groups, molar response factors of 3 for triglycerides, 2 for diglycerides and 1 for monoglycerides and for methyl esters would be predicted. In Table I, the values of $\bar{x}(A_y/A_s)$ are approximately three times the values of M_y/M_s for tripalmitin, twice the values of M_y/M_s for dipalmitin and the same as the values of M_y/M_s for methyl palmitate and monopalmitin. To determine the actual response factors, values of $\bar{x}(A_y/A_s)$ for each component were plotted against (M_y/M_s) (Fig. 2). The slopes of the lines were: tripalmitin, 2.9 (σ 0.07); dipalmitin, 1.9 (σ 0.07); methyl palmitate, 0.89 (σ 0.05); and monopalmitin, 0.75 (σ 0.07). These values were used as response factors (f_y) in calculation of moles/g of subsequent samples using the equation

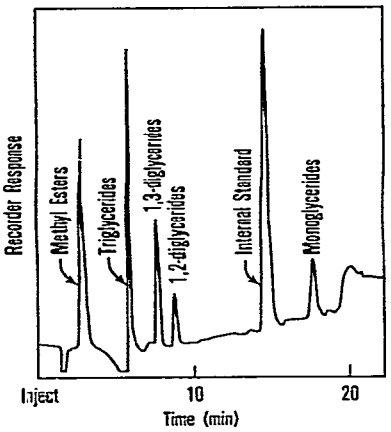
$$\frac{M_y}{\text{g sample}} = \frac{A_y M_s}{A_s f_y w}$$

where w is the weight of the sample.

However, the predicted response factors (number of carbonyl groups per molecule) are close enough to the experimental values that they could be used to obtain approximate values of M_y/g sample. The overall relative standard deviation for the method was 7.1%. By running each sample in duplicate, 95% confidence limits (C.L.) of $\pm 10.0\%$ were obtained ($2s/r$ = C.L.).

In the study of natural products, it is often desirable to quantitate classes of compounds such as glyceride fractions composed of species having several molecular weights. Results in molar units from IR detection of carbonyl functionalities are more convenient for evaluating these mixtures than the weight percentage obtained from flame ionization and "moving wire" detectors. For example, in the lipolysis procedure used to determine the glyceride structure of oils, the extent of hydrolysis is often calculated from molar ratios[9]. If results are originally in weight percent, an average molecular weight for each class must be determined before the results can be converted to moles.

To demonstrate an application of the HPLC–IR method to natural systems, a soybean oil lipolysis mixture[9,14] was prepared and analyzed (Fig. 3). Mean values (mmole/g) of four trials were: methyl esters, 4.49 (σ 0.07); triglycerides, 0.62 (σ 0.05); 1,3-diglycerides, 0.802 (σ 0.003); 1,2-diglycerides, 0.35 (σ 0.02); and monoglycerides 1.40 (σ 0.08). The overall relative standard deviation was 4.9%.



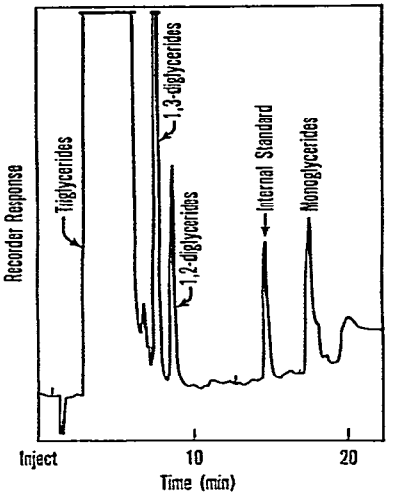


Fig. 3. HPLC chromatogram of soybean oil lipolysis mixture. Conditions as for Fig. 1.

Fig. 4. HPLC chromatogram of a commercial shortening. Conditions as for Fig. 1.

A further advantage of this method is that components present in small amounts (*ca.* 1%) can be measured to within $\pm 0.1\%$ by increasing the sample size. Mono- and diglycerides present at 1–4% (w/w) levels in a commercial shortening were quantitated by adding an appropriate amount of standard and injecting a large sample (55 mg) on to the column (Fig. 4). Mean values (mmole/g) in six trials were: 1,3-diglycerides, 0.0387 ($\sigma \pm 0.0025$); 1,2-diglycerides, 0.0195 ($\sigma \pm 0.0015$); and monoglycerides, 0.0527 ($\sigma \pm 0.0008$). The overall relative standard deviation was 5.9%. By adjusting the internal standard and sample sizes, even smaller amounts of monoglycerides could probably be quantitated with similar precision.

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