High Performance Liquid Chromatography Analyses of Emulsifiers: I. Quantitative Determinations of Mono- and Diacylglycerols of Saturated Fatty Acids

T. RIISOM and **L. HOFFMEYER**, Grindsted Products Division, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark

ABSTRACT

A high performance liquid chromatography (HPLC) method is described for the separation of mono-, di-, and triacylglycerols of fatty acids on a 25-cm column packed with 10 μm LiChrosorb DIOL. The acylglycerols are eluted isocratically with isooctane-isopropanol (95:5) within 10 min, and the components monitored by UV-absorption at 213 nm. The applicability of the method for the quantitative determination of the mono- and diacylglycerol content of fully hardened monoglycerides using an internal standard method has been demonstrated. The method shows excellent reproducibility and accuracy with standard deviations of a distilled monoglyceride containing 92.1% monoacylglycerols and 6.5% diacylglycerols of 0.70% and 0.42%, respectively. The method is applicable to other types of emulsifiers, for instance, acetylated monoglyceride emulsifiers and propylene glycol esters of fatty acids.

INTRODUCTION

In the quality control of monoglyceride emulsifiers, determination of the monoacylglycerol content is important. Also, in other respects, quantitative determination of the amounts of mono- and diacylglycerols is important, e.g., to see if an emulsifier complies with its specifications, to compare emulsifiers from different suppliers, and to examine certain foodstuffs for emulsifier content. The method commonly used is the quantitative oxidation of 1-monoacylglycerols by excess periodic acid to formaldehyde and an aldehydoester, and back-titration of the excess periodic acid (addition of potassium iodide and titration with sodium thiosulfate) (1). This procedure shows good reproducibility and accuracy, but it also has several disadvantages: (a) the method is tedious and time consuming, (b) free glycerol interferes unless it is extracted by water or a salt solution, and (c) the method determines merely the 1-monoacylglycerol content, and equilibrium between the 1-mono- and 2-monoacylglycerols is assumed. Moreover, this equilibrium is dependent on temperature, and thus a number of factors has to be ascertained before an analysis can be performed.

Several other methods based on thin layer chromatography (TLC), (2,3), liquid chromatography (LC) (4,5), and notably gas chromatography (GC) (6-8) have been proposed as substitutes for the periodic acid oxidation, but none of these methods has yet proved accurate enough to be an alternative to the periodic acid titration.

Only recently has high performance liquid chromatography (HPLC) been applied to the separation of surfactants. Aitzetmüller (9,10) presented fingerprint chromatograms of nonionic emulsifiers intended for human consumption. The emulsifiers were separated on silica gel under gradient elution conditions, and the effluent monitored by a moving-wire detector. The procedure yielded semi-quantitative evaluations, and it might prove a useful technique in the screening of, for example, instant foods, toppings, ice creams, and snacks for emulsifier content.

Brüschweiler (11) published results on the separations of emulsifiers and detergents on 10 μm silica gel. The samples were eluted under gradient elution conditions and the components detected by means of their UV-absorbance, either as derivatives containing a UV-chromophore or without derivation at 220 nm.

In this study we report the separation of saturated mono-, di-, and triacylglycerols on 10 μm LiChrosorb DIOL eluting isocratically with isooctane 95%-isopropanol 5% (v/v). The components were monitored by UV-absorption at 213 nm. This method is shown to give reproducible results and quantitative evaluations with standard deviations comparable to or better than the periodic acid oxidation method. The procedure is fast and easy to perform, and both the mono- and diacylglycerol content are determined. With commercial monoglyceride emulsifiers though, the HPLC diacylglycerol values are high due mainly to the elution of free fatty acids with the diacylglycerols. Provisional experiments indicate the method to be applicable with other types of emulsifiers, notably acetylated monoglyceride emulsifiers and propylene glycol esters of fatty acids.

EXPERIMENTAL PROCEDURES

Materials

Glycerol tristearate (puriss. ≥99% by GC), glycerol 1,3-dipalmitate (puriss.), and glycerol 1-monostearate (puriss.) were obtained from Fluka AG, Buchs, Switzerland, while glycerol 1,2-dipalmitate (synth. pure) was from Koch-Light Laboratories, Colnbrook, England. Glycerol 1-monopalmitate and glycerol 2-monopalmitate were synthesized in our laboratory (purity >99% as determined by GLC). Isooctane (Uvasol) and isopropanol (Uvasol) were purchased from E. Merck, Darmstadt, West Germany.

Preparation of Internal Standard

Di-n-propyltartrate was employed as internal standard in the quantitative evaluations of the HPLC chromatograms, and it was synthesized according to the following procedure: 35 g tartaric acid is refluxed for 1 hr with 315 g n-propanol (May & Baker) and 7 ml concentrated H_2SO_4 (May & Baker). The di-n-propyltartrate is extracted from the reaction mixture with diethyl ether, and the ether phase washed several times with water to remove unreacted n-propanol and possibly monoester. The ether phase is dried with anhydrous sodium sulfate and the solvent evaporated under vacuum (60-80 C and 10^{-2} mm Hg). TLC and GLC showed the synthesized di-n-propyltartrate to be >99% pure (n_D^{20} = 1.4472, acid value <1).

UV-Spectroscopy

UV-spectra were obtained on a Pye-Unicam SP1800 spectrophotometer at 190-300 nm. The samples were dissolved in 95% issooctane-5% isopropanol and were run with pure solvent in the reference cell.

TABLE I

Molar Extinction Coefficients of Fatty Acid Esters, and Internal Standard

Compound	ε at 213 nm[a,b]
Glycerol 1-monopalmitate	66
Glycerol 2-monopalmitate	63
Glycerol 1-monostearate	64
Glycerol 1,2-dipalmitate	118
Glycerol 1,3-dipalmitate	117
Glycerol tristearate	180
Di-n-propyltartrate	295

[a]Molar extinction coefficient.
[b]Accurate to ± 5%.

HPLC

The analyses were performed on a Pye-Unicam LC-20 chromatograph equipped with a Perkin-Elmer LC-55 variable wavelength UV-detector. The separations were achieved on 25 cm x 4.6 mm ID steel columns prepacked with 10 μm LiChrosorb DIOL purchased from Chrompack, Middelburgh, The Netherlands. The samples were eluted isocratically with 95% isooctane-5% isopropanol (v/v) at a flow rate of 2 ml/min and a pressure of about 600 psi, and the chromatograms were printed by a Perkin-Elmer model 159 chart recorder. The detector response was treated by a Perkin-Elmer PEP-2 electronic integrator to achieve peak areas and retention times. The samples were dissolved in the eluent (0.5-1.0%, w/v) and 25 μl or 100 μl of solution were injected via loop injector (Pye-Unicam). The internal standard was dissolved in eluent (250 mg in 100 ml eluent) and 1 ml aliquots added to the samples prior to quantitative evaluations.

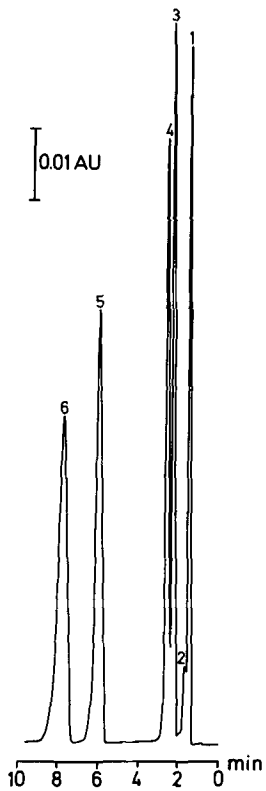

FIG. 1. HPLC chromatogram of a test mixture. Peak identification: 1) glycerol tristearate; 2) artefact peak due to loop injection; 3) glycerol 1,3-dipalmitate; 4) glycerol 1,2-palmitate; 5) di-n-propyltartrate (internal standard); and 6) glycerol 1-monostearate.

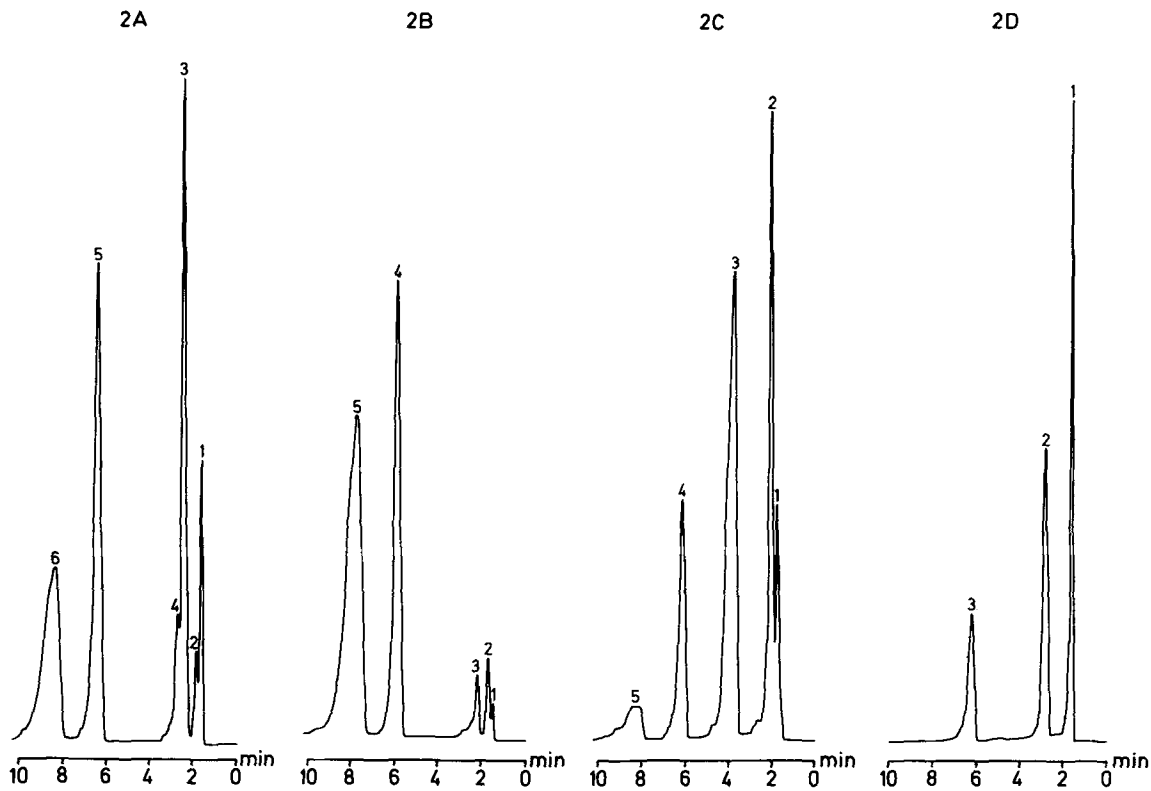

FIG. 2. HPLC chromatograms of nonionic emulsifiers. 2A. Mono-diglyceride: 1) triacylglycerols, 2) artefact peak, 3) and 4) diacylglycerols, 5) internal standard, and 6) monoacylglycerols. 2B. Distilled monoglyceride: 1) triacylglycerols, 2) artefact peak, 3) diacylglycerols, 4) internal standard, and 5) monoacylglycerols. 2C. Acetic acid esters of distilled monoglycerides: 1) triacylglycerol, 2) diacetic acid esters of monoacylglycerols, 3) monoacetic acid esters of monoacylglycerols, 4) internal standard, and 5) monoacylglycerol. 2D. Propylene glycol esters of fatty acids: 1) diacylpropyleneglycols, 2) monoacylpropyleneglycols, and 3) internal standard.

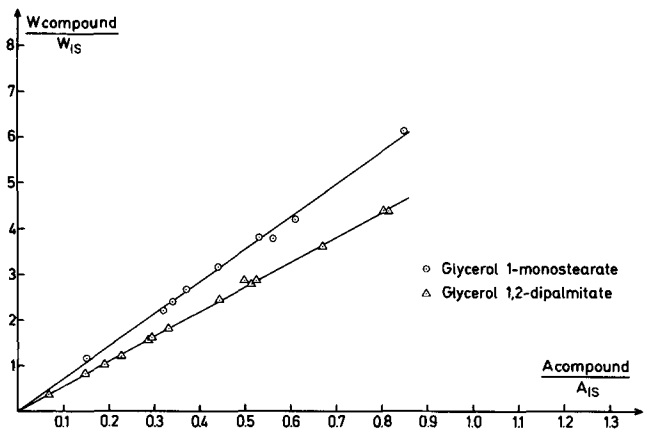

FIG. 3. Internal standard calibration for glycerol-1-monostearate and glycerol 1,2-dipalmitate.

TABLE III

Correlation between the HPLC Method and the Periodic Acid Oxidation[a]

HPLC % Monoacylglycerol	Periodate oxidation % 1-Monoacylglycerol
93.7	90.2
96.7	91.2
96.2	91.0
96.3	92.8
94.7	92.3
95.3	91.1
95.4	91.8
97.4	95.8
93.3	90.9
95.1	92.1
66.7	63.7
63.2	60.6
69.0	64.7
70.5	66.3

[a]Ten commercial distilled monoglycerides and four commercial mono-diglyceride emulsifiers.

RESULTS AND DISCUSSION

UV-Spectroscopy

UV-spectra of glycerol 1-monostearate, glycerol 1- and 2-monopalmitate, glycerol 1,2- and 1,3-dipalmitate, glycerol tristearate, and di-n-propyltartrate were obtained at 190-300 nm. The spectra show the mono-, di-, and triacylglycerols to have absorption maxima at 213-215 nm. The molar extinction coefficients were calculated for each compound at 213 nm, and the results are given in Table I.

The results demonstrate that glycerol 1- and 2-monopalmitate have identical molar extinction coefficients, as is the case for glycerol 1,2- and 1,3-dipalmitate. Comparison of the observed extinction coefficients for glycerol 1-monostearate and 1-monopalmitate indicates the coefficients to be independant of the chain length of the fatty acid moiety supporting the assumption that the UV-absorption is basically due to the ester function of these molecules.

HPLC

Figure 1 shows the separation of a standard solution mixed from glycerol 1-monostearate, glycerol 1,2- and 1,3-dipalmitate, glycerol tristearate, and di-n-propyltartrate. The chromatogram demonstrates that it is possible to achieve good separations between mono-, di-, and triacylglycerols, even between 1,2- and 1,3-diacylglycerols, and still retain peak symmetry for the polar monoacylglycerols. Di-n-propyltartrate elutes with a retention time which makes it suitable as an internal standard in quantitative evaluations. The system is also capable of separating 1-monoacylglycerols from 2-monoacylglycerols (not shown), the latter eluting ca. 1 min later than the 1-monoacylglycerols. However, due to the low content of 2-monoacylglycerols in commercial monoglycerides and mono- and diglycerides, the 2-monoacylglycerol peak usually disappears in the tailing of the much larger 1-monoacylglycerol peak.

The loop injection gives rise to two small artefact peaks interfering with the triacylglycerol peak and thus makes accurate quantitative evaluations of the triacylglycerol content difficult to obtain, especially in samples low in triacylglycerol content. The artefact peaks, usually two, appear even when injecting pure eluent, and it has not yet been possible to find a reasonable explanation for the occurrence of these artefact peaks.

The separation is achieved within 10 min, and as the system needs no equilibration time (isocratic elution), it is immediately ready for another injection. Figure 2 shows the separations of a few nonionic emulsifiers, and it is readily seen that good separations are achieved in each case within 10 min.

Internal Standard Calibration

Mixtures of di-n-propyltartrate (internal standard) and glycerol 1-monostearate were chromatographed, and the area ratios obtained (area of 1-monostearate/are of di-n-propyltartrate) were plotted against the corresponding weight ratios to establish whether the detector response is linear over the entire range considered. The di-n-propyltartrate concentration was varied between 0-0.5% (w/v) for constant glycerol 1-monostearate concentration (0.5%), and also the 1-monostearate concentration was varied between 0-1% (w/v) while the internal standard concentration was kept constant (0.15%). A similar procedure was followed for mixtures of glycerol 1,2-dipalmitate and internal standard. The plots are presented in Figure 3, and they reveal that the detector response is linear over the range considered.

Detector response factors were calculated from these plots (the slope) using least square analysis giving f = 7.02 for glycerol 1-monostearate and f = 5.46 for glycerol 1,2-dipalmitate. Before applying these response factors to quantitative evaluations of monoacyl- and diacylglycerol con-

TABLE II

HPLC Analyses of Standard Mixtures

	I		II		III	
	Amount taken (%)	HPLC	Amount taken (%)	HPLC	Amount taken (%)	HPLC
1-Monostearate	81.78	81.8	84.93	90.6	94.95	94.9
2-Monopalmitate			4.93			
1,2-Dipalmitate	11.41	11.4	5.02	5.4	5.05	4.9
Tristearate	6.80	8.1	5.12	5.7		

tents of monoglyceride and mono-diglyceride emulsifiers due consideration has to be given to the fatty acid composition of the emulsifiers considered. The following equations may be applied for samples, where the fatty acid composition is known.

$$C_{mono} = 7.02 \times \overline{MW}_{mono}/MW_{monostearate} \times A_{mono}/A_{is} \times C_{is} \quad (I)$$

and

$$C_{di} = 5.46 \times \overline{MW}_{di}/MW_{dipalmitate} \times A_{di}/A_{is} \times C_{is} \quad (II)$$

C_{mono}, C_{di}, and C_{is} are the concentrations of monoacylglycerol, diacylglycerol, and internal standard, respectively, and A_{mono}, A_{di}, and A_{is} the corresponding peak areas. $\overline{MW}_{mono}$ and $\overline{MW}_{di}$ are the mean molecular weights of the monoacylglycerols and diacylglycerols, respectively, calculated according to the following equations

$$\overline{MW}_{mono} = (M + 92.09) - 18.02 \quad (III)$$

and

$$\overline{MW}_{di} = (2M + 92.09) - 2 \times 18.02 \quad (IV)$$

where M is the average molecular weight of the fatty acids determined as described in AOCS Official Method Cd 11-57 (12).

These response factors are applicable to saturated or fully hardened emulsifiers only, as the double bonds of unsaturated fatty acids possibly contribute to the UV-absorption at 213 nm and thus alter the detector response factors.

Accuracy of the Method

A method that is used in quality control has to be fast, accurate, easy to operate, and it must not show day-to-day or operator-to-operator variations. To test the accuracy of the method developed and whether any variations occur, a solution of a distilled monoglyceride made from fully hardened lard (monoacylglycerol content ca. 90%) was chromatographed on ten consecutive working days by two operators, each carrying out a double determination each day. The order in which the operators carried out their analyses was determined at random.

The results, expressed as weight percents of monoacylglycerol and "diacylglycerol" in the distilled monoglyceride, where the latter contains contributions from free fatty acids, showed a mean composition of 92.1% monoacylglycerols and 6.51% "diacylglycerols."

A two-tailed variance test revealed that the monoacylglycerol determinations showed neither day-to-day nor operator-to-operator variations (95% confidence level), while the "diacylglycerol" evaluations showed weak significance for day-to-day variations ($p = 0.05$) but no operator-to-operator variations.

The standard deviations, 0.70% for the monoacylglycerol determination and 0.42% for the "diacylglycerol" determination, demonstrate that the HPLC method gives reproducible results with an accuracy for the monoacylglycerol evaluation (0.8% relative standard deviation) comparable to or better than the periodic acid oxidation method.

In Table II the results of the analyses of three mixtures of glycerol 1-monostearate, 2-monopalmitate, 1,2-dipalmitate, and tristearate are given. The glycerol tristearate concentration is calculated using an estimated response factor of 5.9, and therefore these results are not very accurate. Furthermore, the artefact peaks interfere with the triacylglycerol peak, and the results consequently are high. The table demonstrates that the method gives reliable results for such standard mixtures, and although the glycerol 1-monostearate and 2-monopalmitate peaks are not separated enough to allow quantitation of the individual peaks, the total monoacylglycerol content calculated by this method gives a value in accordance with the amount actually weighed in.

To establish the correlation between the two methods, ten commercial distilled monoglyceride emulsifiers and four commercial mono-diglycerides were chromatographed, and the weight percents obtained were compared to the results obtained from the periodic acid oxidation. The results are tabulated in Table III.

It readily appears from the table that the correlation between the results obtained by the HPLC method and the periodic acid oxidation method is good. The HPLC-derived values for the content of monoacylglycerols are on average 3.5% higher than the values for the 1-monoacylglycerol content determined by the periodate oxidation method. This is probably caused by the presence of 3-4% 2-monoacylglycerols in the samples which is measured as 1-monoacylglycerol in the HPLC method. This value is in the lower part of the range of 2-monoacylglycerols considered normal (4-12% of total monoacylglycerols), but the value is in accordance with the results given by Krog and Lauridsen (13) employing a column chromatographic procedure.

The diacylglycerol contents were not determined, as it has been revealed that the free fatty acids, which are present in small amounts in distilled monoglycerides and mono-diglycerides (<1%), elute together with the 1,2-diaclyglycerols. Thus, they make a contribution to the diacylglycerol determination, which is not negligible for distilled monoglycerides (diacylglycerol content usually ca. 3.5%). If the response factors are determined for, for example, stearic acid, the erroneous values for the diacylglycerol contents may be corrected, if the acid value is known and a fatty acid distribution of the free fatty acids similar to the overall fatty acid distribution is assumed.

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