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# Analysis of mixtures containing free fatty acids and mono-, di- and triglycerides by high-performance liquid chromatography coupled with evaporative light-scattering detection

Bruno Marcato\*, Giuliano Cecchin

*MONTELL Polyolefins, Centro Ricerche G. Natta, 44100 Ferrara, Italy*

## Abstract

Commercial monostearates of glycerol, generally used as antistatic agents for thermoplastic polymers, consist of a complex mixture of mono-, di- and trisubstituted glycerides and the corresponding fatty acids. Thus far, the glycerides and the fatty acids have been analyzed separately by high-performance liquid chromatography (HPLC). In fact, the simultaneous analysis of glycerides and fatty acids requires esterification of the acids in order to avoid overlapping of the chromatographic peaks. This paper describes an HPLC method which allows the direct separation and identification, simultaneously and in a single run, of the variously substituted glycerides, and also the corresponding saturated fatty acids that are found as by-products in commercial glycerol monostearates. The procedure is based on the use of a ternary gradient HPLC instrument equipped with an evaporative light-scattering detector; the stationary phase was a reversed-phase RP-8 end-capped (Merck) column; the mobile phase was two consecutive binary gradients consisting of acetonitrile–water plus acetic acid (0.1%, v/v) and acetonitrile–methylene chloride. The method is fast and shows high sensitivity and selectivity, being able to separate also the positional isomers of mono- and diglycerides in addition to the mixed di- and triglycerides.

*Keywords:* Evaporative light-scattering detection; Fatty acids; Glycerides

## 1. Introduction

It has long been known that mono- and disubstituted glycerides of fatty acids are particularly effective in providing static electric control in a large variety of thermoplastic polymers such as poly(vinyl chloride) (PVC), polypropylene (PP) and polyethylene (PE). Since the antistatic effect is related to the number of hydroxyl groups (the hydrocarbon moiety of the fatty acid provides compatibility with the polymer chain) mono-

glycerides are far more effective than the corresponding diglycerides.

Commercial antistatic products, although being sold nominally as monoglycerides and, more particularly, monostearates (GMS), can consist of a complex mixture of monoglycerides (main component), diglycerides and their positional isomers, triglycerides and by-products resulting from the incomplete conversion of the starting components (free fatty acids and glycerol). In addition, since the fatty acid component is a mixture of different fatty acids, such as stearic (main component), palmitic, myristic and

\* Corresponding author.

lauric acids, it leads, upon esterification with glycerol, to a variety of variously substituted esters, including di- and trisubstituted mixed esters. Therefore, commercial monostearates are complex mixtures of components having a considerable overlap of physical/chemical and thus chromatographic properties.

In the past, the identification and quantification of glycerides were generally performed using gas-liquid chromatography (GLC) with flame ionization detection (FID) [1–7]. However, while triglycerides were analysed as such, although with difficulty for those bearing long aliphatic chains [8], the mono- and diglycerides required prior derivatization, generally to methyl or propyl esters, in order to enhance volatility and avoid isomerization or decomposition of heat-labile compounds [9–11]. This procedure, besides being time consuming, was not able to provide a complete identification of the components in complex mixtures. Better results were subsequently obtained using capillary gas chromatography (cGC) [11]. More recently, the problem was approached by both normal- and reversed-phase HPLC. On the other hand, also in this case, previous workers had to resort to derivatization reactions, pre- or postcolumn, generally consisting of esterification or introduction of other suitable chromophores, in order to overcome separation and/or detectability problems [12]. However, also with these techniques, separation of complex glyceride mixtures was unsatisfactory. This stems from the fact that the formerly used detectors, either UV, RI or IR, are not able to provide sufficient sensitivities for the generality of underivatized lipids and, in addition, they can be limited by the incompatibility with absorbing eluents and with gradient elution [12].

The analysis of underivatized triglycerides was reported by Barron et al. [13], Gilkison [14] (using a UV-compatible reversed-phase gradient) and Andrikopolous et al. [15] (analysis was performed in the presence of phenolic antioxidants). Identification of untreated monoglyceride mixtures was described by Takano and Kondoh [16]; however, the use of a glyceride-selective postcolumn reactor detector was necessary. Un-

derivatized mixtures of monoglycerides were identified by Semporé and Bézard [17] using an octadecylsilyl column and a differential refractometer. Bauza et al. [18] were able to separate mono-, di- and triglyceride mixtures using both normal- and reversed-phase HPLC and UV or RI detection; however, the analysis was confined to the most common GMS components and separation was limited. Mono- and diglycerides could also be analysed without derivation by supercritical fluid chromatography coupled with FID [19]. A high-performance size-exclusion method (HPSEC), coupled with RI detection, was described by Christopoulou and Perkins [20] for the separation and quantification of fatty acids and mono-, di and triglyceride mixtures. However, esterification of any free acid present in the sample was necessary owing to incomplete separation from the monoglyceride peak; moreover, separation of the various molecular species within each lipid class was not possible. In general, mixtures of underivatized fatty acids were found difficult to analyse, especially when mixed with glycerides, since the peaks tend to tail and overlap with those of diglycerides [21]. Direct HPLC analysis of underivatized fatty acid mixtures is limited to few examples concerning the identification of components by IR or UV detection and using eluents containing suitable counter ions [22–24].

Substantial progress was made in this area with the introduction of evaporative light-scattering detection (ELSD), which, being sensitive just to the mass of the vaporized analyte, is not limited by the absorption characteristics of the individual components and/or the nature of the eluent. In particular, the full exploitation of gradient elution techniques, coupled with the most appropriate eluent mixtures, possibly in the presence of suitable counter ions, was made possible. Since then, several groups have dealt with the analysis of underivatized lipids. For instance, Bruns [25] analysed mixtures of mono-, di- and triglycerides and methyl esters of fatty acids using a cyano-modified silica phase; identification of triglyceride mixtures was reported by Dreux and Lafosse [26], Hierro et al. [27] and Letter [28].

Recently, a method has been described by Liu et al. [29] concerning the separation of neutral lipid classes by normal-phase HPLC–ELSD, using a hexane–2-propanol–ethyl acetate–formic acid eluent gradient. However, the analysis was limited to mono- and diglycerides; in addition, for monoglycerides, the separation was optimized only between positional isomers with an identical fatty acyl moiety.

In conclusion, at least to our knowledge, no direct, complete and simultaneous identification of mixtures containing both free acids and glycerides has been reported. This paper describes a method which allows the separation and identification, directly, simultaneously and in a single run, not only of a series of saturated glycerides but also of glycerol and the saturated fatty acids that can be found in GMS. The procedure is based on the use of ternary-gradient HPLC with ELSD and a reversed-phase RP-8 end-capped column.

## 2. Experimental

### 2.1. Chemicals

Acetonitrile and water for HPLC were obtained from Merck (Darmstadt, Germany). Methylene chloride for HPLC, ethyl acetate and acetic acid were obtained from Carlo Erba (Milan, Italy).

The following reference standards were purchased from Sigma (St. Louis, MO, USA): palmitic acid (code P-0500); stearic acid (S-4751); 1-monostearoyl-*rac*-glycerol (M-2015); 1-monomyristoyl-*rac*-glycerol (M-1890); 1-monopalmitoyl-*rac*-glycerol (M-1640); 1-monoerucin (M-1155); 2-monopalmitoylglycerol (M-8385); 1,3-distearin (D-8269); 1,2-distearoyl-*rac*-glycerol (D-8519); 1,2-dipalmitoyl-*rac*-glycerol (D-2135); dipalmitin, mixed isomers (D-2636); 1,2-dimyristoyl-*rac*-glycerol (D-8769); 1,3-dimyristin (D-9144); dilaurin, mixed isomers (D-9758); diarachidin, mixed isomers (D-1402); 1,3-dierucin (D-4284); 1-palmitoyl-3-stearoyl-*rac*-glycerol (P-3544); tristearin (T-5016); tripalmitin (T-5888); trimyristin (T-5141); trilaurin (T-4891); and 1,2-

distearoyl-3-palmitoyl-*rac*-glycerol (D-3782). The following reference standards were supplied by Tokyo Kasei (Tokyo, Japan): 1-monoolein (code G 0082); 1-monolaurin (G 0081); erucic acid (D 0965); and behenic acid (B 1248). The following reference standards were supplied by Merck: myristic acid (code 800399) and lauric acid (805333). Glycerol was supplied by Carlo Erba. Atmer 129, GMS 40 and Witconol-MST were commercial “monostearates” obtained from ICI (Middlesbrough, UK), SOGIS (Sospino, Italy) and Witco (Houston, TX, USA), respectively.

### 2.2. Equipment

The HPLC instrument was a Varian (Palo Alto, CA, USA) Model 5560, equipped with a Rheodyne (Cotati, CA, USA) Model 7125 injection valve and a 10- $\mu$ l loop, a Sedex 45 evaporative light-scattering detector (SEDERE, Alfortville, France) and a Spectra-Physics (San Jose, CA, USA) SP 4270 integrator. The analytical column was a LiChroCART 250-4 packed with LiChrospher 100 RP-8, end-capped (5  $\mu$ m) (25  $\times$  0.4 cm I.D.), Code 50837 (Merck).

### 2.3. Preparation of the standard and sample solutions

Standard mixtures of aliphatic acids, mono-glycerides, diglycerides and triglycerides were prepared by dissolving 100–200 ppm (w/v) of each compound in ethyl acetate–chloroform (9:1, v/v). The sample solutions were prepared by dissolving the commercial monostearates in ethyl acetate–chloroform (9:1, v/v), resulting in a final concentration of 2500 ppm (w/v) [the presence of chloroform is necessary to favour the solution of compounds having long aliphatic chains, e.g., tristearin; on the other hand, its concentration must be less than 15% (v/v) in order to avoid the occurrence of split and tailed peaks].

### 2.4. HPLC analysis

The standard and the sample solutions were injected into the chromatograph by means of a 10- $\mu$ l sample loop. The chromatographic con-

Table 1  
Binary gradient elution conditions

Binary gradient (step)	Time (min)	Water–acetic acid (99.9:0.1) (% v/v)	Acetonitrile (% v/v)	Methylene chloride (% v/v)
I	0	16	84	0
	4	0	100	0
II	12	0	70	30
	18	0	70	30
	19	16	84	0
	25	16	84	0

ditions were as follows: flow-rate, 1.3 ml/min; column temperature, 40°C; eluents of the mobile phase, acetonitrile, methylene chloride and water–acetic acid (99.9:0.1, v/v) (the eluent gradients are reported in Table 1); detector temperature, 27°C; gain, 6; and pressure of nebulizing gas, 2.0 bar.

The identification of each compound present in the sample solution was performed by comparing its retention time with that of the corre-

sponding peak in the standard mixture; quantification of monoglycerides was performed by the external standard method.

### 3. Results and discussion

Under the adopted chromatographic conditions, the peaks of the various analytes (peak identification in the standard mixture was per-

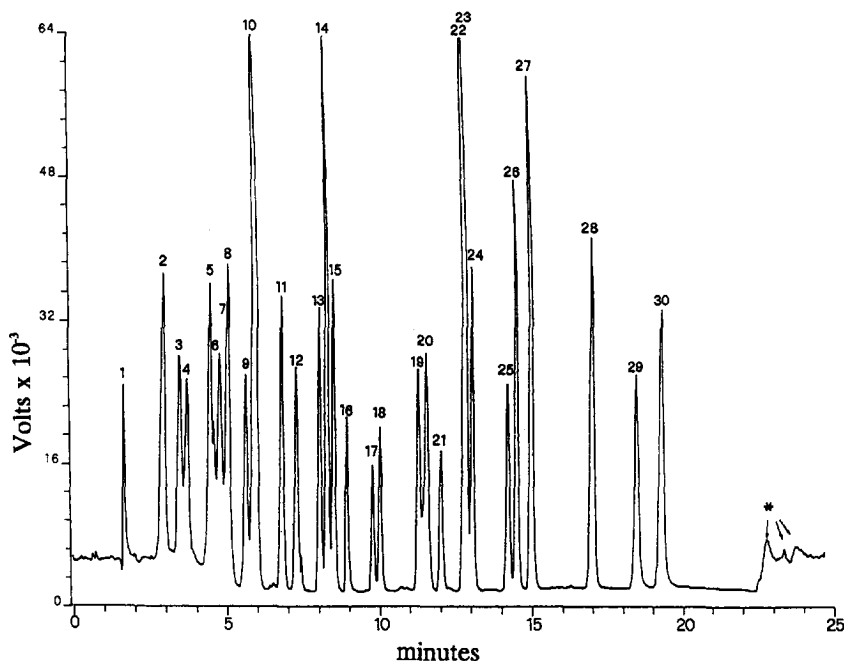


Fig. 1. HPLC of a standard mixture of glycerol, free fatty acids, monoglycerides, diglycerides and triglycerides. Amount injected of each component, 1–2  $\mu$ g. See text for HPLC conditions and Table 2 for peak identification.

formed by matching the corresponding retention times with those of the pure standards) are symmetric and well resolved, and their retention times show excellent reproducibility. Fig. 1 shows the chromatogram of the "global mixture" of standard reference compounds (glycerol, aliphatic acids and mono-, di- and triglycerides). These compounds are listed in Table 2 together with their retention times under the conditions given in Section 2.4. The presence of the counter ion ( $H^+$ ), provided by acetic acid, was found to be particularly important, since it makes possible the shift of the acid–base equilibrium of the acid analytes towards their associated form and thus avoids the occurrence of broadened, superimposed and tailed peaks. This can be clearly seen

Table 2  
Retention times of standard compounds (see text for HPLC conditions)

Peak No.	Standard compound	Retention time (min)
1	Glycerol	1.67
2	1-Monolaurin	2.95
3	Lauric acid	3.55
4	1-Monomyristin	3.70
5	2-Monopalmitin	4.50
6	Myristic acid	4.55
7	1-Monopalmitin	4.85
8	1-Monoolein	4.95
9	Palmitic acid	5.70
10	1-Monostearin	5.95
11	Stearic acid	6.90
12	1-Monoerucin	7.35
13	Erucic acid	8.10
14	1,3-Dilaurin	8.30
15	1,2-Dilaurin	8.50
16	Behenic acid	8.95
17	1,3-Dimyristin	9.80
18	1,2-Dimyristin	10.05
19	1,3-Dipalmitin	11.30
20	1,2-Dipalmitin	11.55
21	1-Palmitin-3-stearin	12.00
22	1,3-Distearin	12.75
23	Trilaurin	12.80
24	1,2-Distearin	13.00
25	1,3-Diarachidin	14.15
26	1,2-Diarachidin	14.40
27	Trimyristin	14.90
28	Tripalmitin	16.80
29	1,2-Distearin-3-palmitin	18.25
30	Tristearin	19.10

in Fig. 2, where the chromatograms are depicted of the same standard mixture of free fatty acids, eluted (A) without and (B) with acetic acid. It is likely that peak broadening and tailing, observed in the absence of acetic acid, are due to the simultaneous occurrence of both associated and dissociated forms of fatty acids; in contrast, in the presence of acetic acid, well resolved and symmetrical peaks appear, probably corresponding to the associated fatty acid species, which allows not only easy separation and identification, but also accurate quantification of the various analytes. It was observed that the pres-

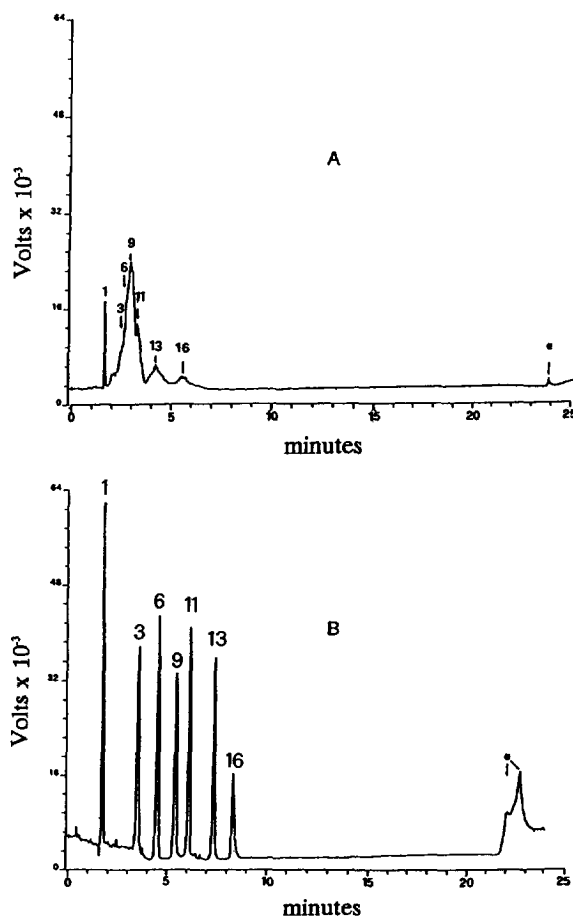


Fig. 2. HPLC of a mixture of glycerol and free fatty acids (A) without acetic acid in the water eluent and (B) with acetic acid in the water eluent. Amount injected of each component, 1–2  $\mu\text{g}$ . See text for HPLC conditions and Table 2 for peak identification. Asterisks indicate peaks present in the elution gradient.

ence of acetic acid in the mobile phase, besides not impairing the intrinsic sensitivity of ELSD since it is completely vaporized at the working

temperature, does not bring about any hydrolytic decomposition of the variously substituted glycerides that are contained in the sample.

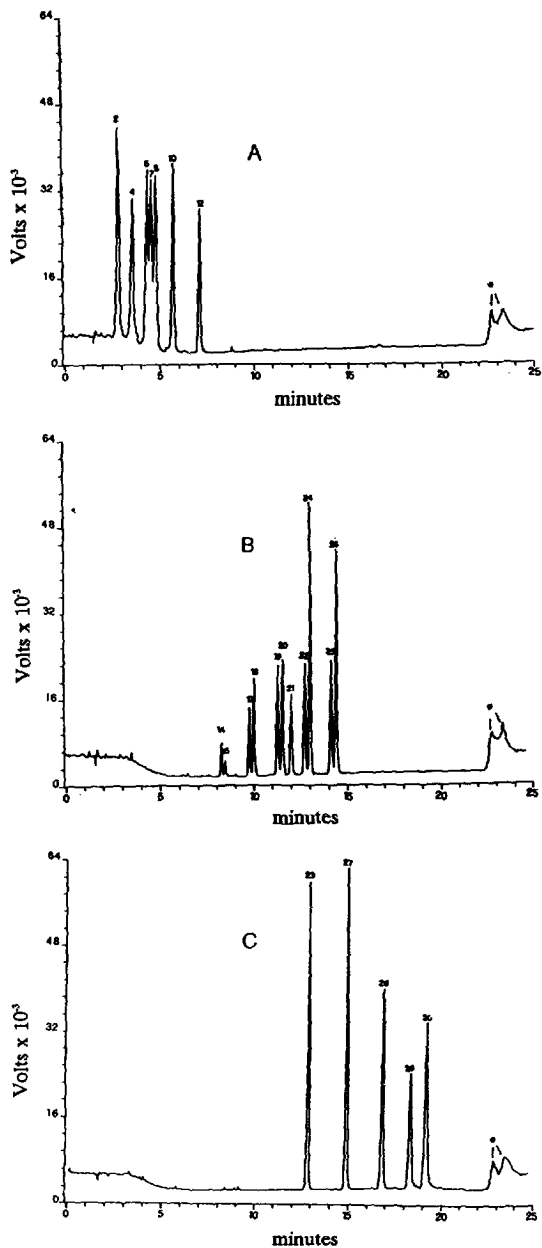


Fig. 3. HPLC of a standard mixture of (A) monoglycerides, (B) diglycerides and (C) triglycerides. Amount injected of each compound, 0.2–2  $\mu\text{g}$ . See text for HPLC conditions and Table 2 for peak identification. Asterisks indicate peaks present in the elution gradient.

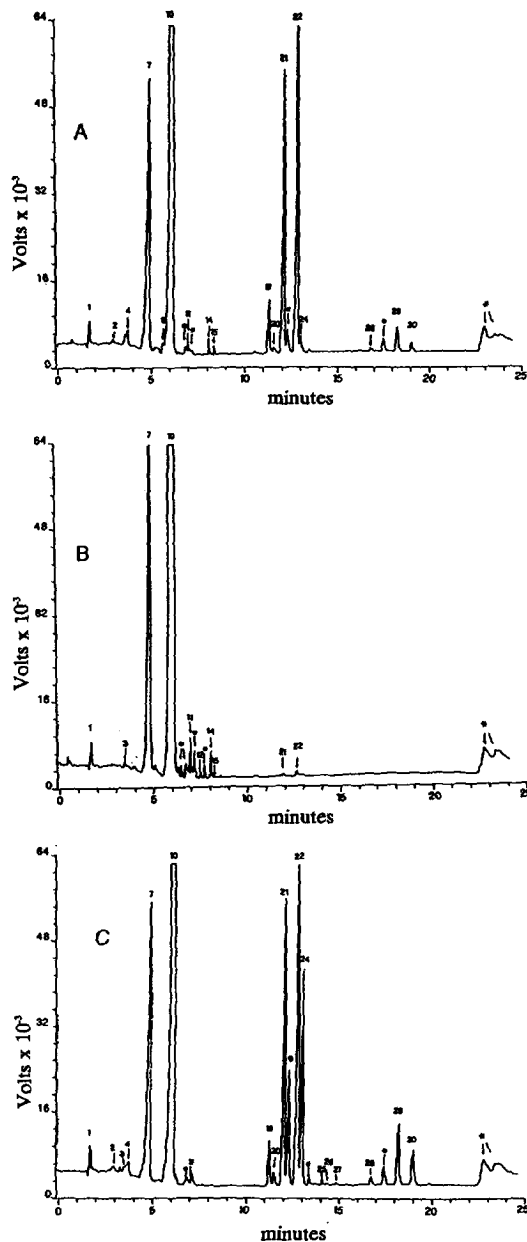


Fig. 4. HPLC of (A) GMS 40, (B) Atmer 129 and (C) Witconol-MST. Amount injected of each sample, ca. 25  $\mu\text{g}$ . See text for HPLC conditions and Table 2 for peak identification. Asterisks indicate peaks present in the elution gradient; ● = unidentified peaks.

This procedure thus allows both qualitative and quantitative analyses of complex mixtures containing glycerol, free carboxylic acids bearing long aliphatic chains, monoglycerides, diglycerides and triglycerides. This not only can be accomplished directly, simultaneously and in a single run but also, because of the separation level achieved, even allows the identification and quantification of positional isomers such as 1-monoglycerides and 2-monoglycerides (Fig. 3A) or 1,2-diglycerides and 1,3-diglycerides (Fig. 3B). In addition, mixed triglycerides can also be separated and identified (Fig. 3C).

Regarding the composition of commercial GMSs and, more particularly, their content in monoglycerides, which constitute the most effective antistatic component, seventeen samples were analyzed according to the present procedure. Considerable differences were found among the samples in terms of type and content of monoglycerides, diglycerides, triglycerides and free fatty acids.

For the sake of comparison, the chromatograms of three typical commercial GMSs are depicted in Fig. 4, and the respective contents of monoglycerides are reported in Table 3. It can be observed that the samples clearly differ in both total content of monoglycerides, monostearate in particular, and type and amount of by-products, mainly constituted by diglycerides. In no case was a linear correlation found on plotting peak area vs. analyte concentration. In addition, different standards had to be used for different molecules because of the different response

coefficients. The calibration graphs for 1-monostearin and 1-monopalmitin are shown in Fig. 5. The parameters of the calibration graphs, obtained by fitting the experimental points according to an equation of the type  $y = ax + bx^2$ , where  $y$  represents the peak area expressed in counts  $\cdot 10^{-6}$  and  $x$  the analyte concentration expressed in mg/ml, are  $a = 3.60$ ,  $b = 1.92$  and  $a = 1.3$ ,  $b = 1.70$  for 1-monostearin and 1-monopalmitin, respectively.

The repeatability of the method was evaluated on the basis of six independent runs with the same commercial monostearate (SOGIS), in which the following monoglycerides were found: 1-monostearin, 1-monopalmitin, 1-monomyristin, 1-monoerucin and 1-monolaurin. The results obtained were as follows: 1-monostearin, average value  $\bar{X} = 27.3\%$  (w/w), SD = 0.71, confidence limits (95% probability level) =  $\pm 0.75$ ; 1-monopalmitin: average value  $\bar{X} = 13.5\%$  (w/w), SD = 0.54, confidence limits (95% probability level) =  $\pm 0.50$ . Based on the quoted values and confidence level, the maximum range expected between duplicates is  $\pm 3.7\%$  and  $\pm 5.8\%$  for 1-monostearin and 1-monopalmitin, respectively at the considered levels.

The data for monomyristin, monoerucin and monolaurin were not treated statistically owing to the very low values found for all three substances [ $\bar{X} = 1.4$ , 0.9 and  $< 0.5\%$  (w/w), respectively], under the experimental conditions tested. However, it is likely that satisfactory repeatability could also be obtained with these substances simply by increasing the sample size.

Table 3  
Concentrations of the main monoglycerides in commercial GMS samples

Sample	1-Monostearin (%, w/w)	1-Monopalmitin (%, w/w)	1-Monomyristin (%, w/w)	Total monoglycerides content (%, w/w)
GMS 40 (SOGIS)	25.0	12.0	1.5	40
Witconol-MST (Witco)	24.0	11.5	2.5	38
Atmer 129 (ICI)	75.0	15.0	1.0	93

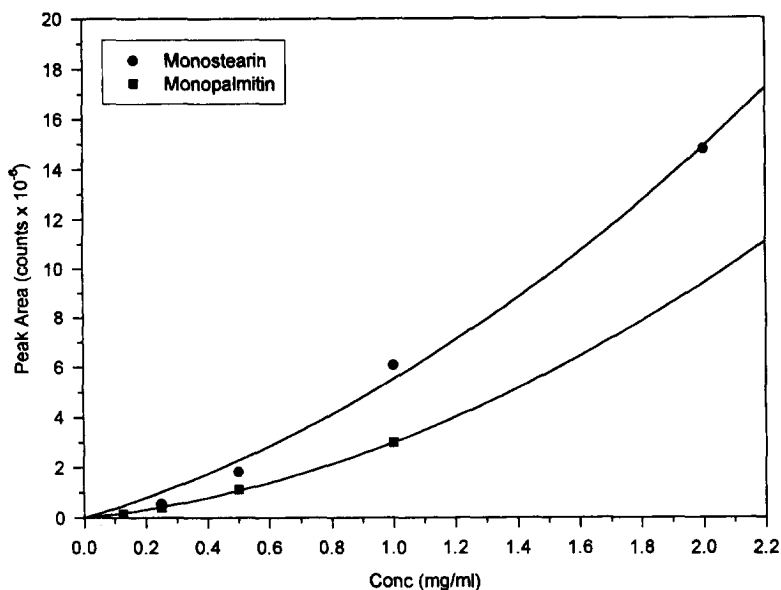


Fig. 5. Calibration graphs for 1-monostearin and 1-monopalmitin. See text for HPLC conditions.

The method allows the detection of concentration levels of monoglycerides down to 0.001 g/l in solution, which correspond to 0.4% (w/w) in the solid antistatic agent.

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