Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Roberto Bauza[a]; Freddy Ysambertt[a]; Nelson Marquez[a]; Graciela O. De Rodriguez[a]; José L. López[b]
[a] DEPARTAMENTO DE QUIMICA FACULTAD EXPERIMENTAL DE CIENCIAS, UNIVERSIDAD DEL ZULIA, MARACAIBO, VENEZUELA [b] INVESTIGACION Y DESARROLLO C.A. COMPLEJO PETROQUIMICO ZULIA EL TABLAZO, MARACAIBO, VENEZUELA

To cite this Article Bauza, Roberto , Ysambertt, Freddy , Marquez, Nelson , De Rodriguez, Graciela O. and López, José L.(1992) 'Separation of Mono-, Di-, and Tristearin from an Industrial Mixture of Glycerides by Normal- and Reverse-Phase HPLC', Separation Science and Technology, 27: 5, 645 — 661
To link to this Article: DOI: 10.1080/01496399208018908
URL: http://dx.doi.org/10.1080/01496399208018908

SEPARATION SCIENCE AND TECHNOLOGY, 27(5), pp. 645–661, 1992

Separation of Mono-, Di-, and Tristearin from an Industrial Mixture of Glycerides by Normal- and Reverse-Phase HPLC

ROBERTO BAUZA, FREDDY YSAMBERTT,*
NELSON MARQUEZ, and GRACIELA O. DE RODRIGUEZ
DEPARTAMENTO DE QUIMICA
FACULTAD EXPERIMENTAL DE CIENCIAS
UNIVERSIDAD DEL ZULIA
APARTADO 526, MARACAIBO 4002, VENEZUELA

JOSÉ L. LÓPEZ
INVESTIGACION Y DESARROLLO C.A.
COMPLEJO PETROQUIMICO ZULIA
EL TABLAZO, EDO. ZULIA
APARTADO 10319, MARACAIBO 4002, VENEZUELA

Abstract

This work reports on a quantitative and qualitative study of reverse and normal-phase HPLC to separate mono-, di-, and tristearin from an industrial mixture of glycerides. For normal-phase chromatography a CN-bonded packed column was used, while C8 and C18 columns were used for reverse-phase chromatography. Both types of chromatography were studied with UV and RI detection. The influence of some chromatographic factors, such as the bonded phase, mobile-phase composition, and flow rate, on the retention times, selectiveness, and effectiveness were studied.

INTRODUCTION

Some applications in the manufacture of certain polymers (*1*, *2*) have predominantly used derivatives of stearic acid (such as the mono-, di-, and tristearic acids of glycerin) and salts from the stearates of zinc, magnesium,

*To whom correspondence should be addressed.

sodium, and calcium in order to guarantee the properties of the product during processing and finishing. Most of these compounds initially come from the sterification of an excess of glycerol with stearic acid in the presence of a catalyst (*3*).

Commercial mixtures of glycerides from stearic acid can be found in the marketplace and are named GMS (glycerol monostearate). These mixtures consist of different percentages of mono-, di-, and triglycerides which can exist in different isomeric forms.

It was once believed that all monoglycerides exist only in the alpha form. However, modern tests indicate that most commercial monoglycerides contain between 2 and 5% beta isomers (*4*). Likewise, the distearin can exist as alpha,beta and alpha,alpha isomers. Electronic diffraction studies (*5*) showed that both isomers can be interconverted in the solid phase by thermal conditions or catalyzed by acids or bases (*6*). They are more stable in the alpha,alpha form.

Methods for the quantification of glycerides employ wet as well as spectroscopic and chromatographic techniques. The proper application of these procedures depends upon the type of compound and the efficiency of the standardized equipment required for each technique (*3*, *4*, *7*, *8*). A chromatographic method using capillary gas chromatography (*6*, *9*) which allows the separation of the isomers alpha,beta and alpha,alpha diglycerides of the respective triglycerides has recently been highlighted. The glycerides have been quantified by size exclusion chromatography (*10*, *11*). CDF Chimie has developed a method using this type of technique which is capable of separating the mono- and triglycerides with good results.

A few studies have used interactive chromatography for triglycerides. Among these studies are those of Kathleen and coauthors (*12*) who used Partisil columns with systems of gradients as the mobile phase with refractive index detection for high resolution of mono-, di-, and triglycerides. Besides, Yukhido and coauthors (*13*) reported improving this separation by using postderivatization and ODS columns.

The first study of triglyceride separation was reported by Pei and coauthors (*1*). They used two types of columns: ODS and OS, with a methanol:water mixture as the mobile phase. Their analysis showed poor resolution and a loss of samples in the column. Some other authors (*14*, *15*) reported the same results.

Some authors (*14–17*) believe that reverse-phase chromatography is the best method to separate triglycerides. Riisom and coauthors (*18*) reported separations of the mono-, di-, and triglycerides by this mode for the separation of triglycerides.

Plattner (*16*) made a series of comparisons of reverse-phase chromatography with different ODS columns. Among the columns considered, the

Zorbax ODS showed the greatest retention and selectivity for the glycerides, obtaining high values of K for the analysis.

Recently Barron and coauthors (*17*) reported similar studies using Novapak ODS and Ultrasphere ODS columns in which separations made with homogeneous saturated triglycerides (with equal substituted acid groups) were performed in an isocratic system with tetrahydrofuran/acetonitrile in different proportions. The results indicated high retention of saturated triglycerides of hydrocarbonated chains, showing that such triglycerides as palmitin, stearin, araquidin, etc. are not conveniently separated in C-18 columns.

Many studies on the mobile phase to optimize the separation of triglycerides have been reported. Some authors (*15*, *17*) have pointed out that for the separation of saturated triglycerides with large hydrocarbonated chains in the reverse phase, it is necessary to decrease the polarity of the mobile phase in order to obtain short analysis times. Parris (*19*) reported obtaining adequate mobile phases by using solvents of low polarity such as methylene chloride and tetrahydrofuran to get fast elutions of nonpolar compounds. Paul (*20*) suggested binary mixtures of acetonitrile with such organic solvent of low polarity as dichloromethane, chloroform, and tetrahydrofuran. Parris (*21*) used 40% dichloromethane and 60% acetonitrile to improve the resolution of such critical mixtures as SLLn (Ln = linolenic, L = linoleic, S = stearic). On the other hand, El Handy (*22*) reported separations of triglycerides such as LaLaLa, LLL, MMM, and PPP (La = lauric, M = miristic, P = palmitic) by using ternary mixtures of acetonitrile, acetone, and tetrahydrofuran.

The application of gradient elution in complex mixtures of triglycerides has been used during the last few years. Parris (*21*) reported gradients of tetrahydrofuran and acetonitrile with refractive index detection. Robinson and Macrae (*23*) proposed an ethanol gradient in acetonitrile using a UV detector. A flow gradient for the analysis of saturated triglycerides with RI detection was recently reported (*24*); the author found an improvement in separation.

Detection is one of the factors that has limited the development of a chromatographic method for triglycerides. The property most commonly sought in this kind of analysis is the refractive index (*15*, *16*, *25*). Some authors (*15*, *25*) have recommended refractive index detection for triglycerides due to their low molar absorption coefficient (*3*) in UV-visible detection. Nevertheless, other workers (*14*, *23*) have indicated that the sensitivity depends on the type of glyceride to be studied.

This work shows the separation and quantification achieved in mixtures of mono-, di-, and tristearins in the normal phase by using CN columns, and in the reverse phase by using C-8 and C-18 columns with UV and RI

detection. The results obtained by the two different types of phases and detectors are compared.

EXPERIMENTAL

Materials

High purity standards (approximately 99%) of tristearin (Matry, USA); 1,3-distearin, 1,2-distearin, 1-monosteryl glycerol, and stearic acid (Sigma Chemical Co., USA); and glycerol (Merck, USA) were used.

An industrial monoglycerol (GMS) mixture containing approximately 40% di-, 45% mono-, and 15% tristearin (GMS1), and a monostearin mixture of approximately 90% purity (GMS2) were supplied by Estireno del Zulia, El Tablazo, Venezuela.

Dichloromethane, acetonitrile, heptane, hexane, acetone, chloroform, and tetrahydrofuran were spectrophotometric or HPLC grade (J. T. Baker Chemical Co. and Fisher Scientific Co.). The solvents were degasified by using an ultrasonic apparatus (Brandson).

Three types of columns were used to perform these studies. The first column was a Zorbax-CN (DuPont, USA) of 25 cm × 4.6 mm i.d. with 10 μm particle size. The second column was a Lichrosorb RP-18 (Hibar-Merck) of 25 cm × 4.5 mm i.d. with 10 μm particle size. The third column was a Lichrosorb RP-8 (Hibar-Merck) of 25 cm × 4.5 mm i.d. with 10 μm particle size.

Solubility Studies

The solubilities of the samples were studied in tetrahydrofuran, acetone, dichloromethane, chloroform, hexane, heptane, and acetonitrile. Approximately 10 mg of an industrial mixture (GMS), of monostearin (GMS2), and of mono-, di-, and tristearin (GMS1) were dissolved in 25 mL solvent. All analyses were performed at room temperature.

Apparatus

The separations were carried out in two Waters Associates High Performance Liquid Chromatographs. Wavelength selection was achieved by using the following system: a pump (model 600 E) with a flow rate of 1.0 mL/min and a universal injector (model 991) coupled to a NEC computer. The other analyses were carried out in the following system: a pump (model 510) with an optimized flow rate of 0.7 mL/min (650 psi pressure), a UV-visible variable wavelength detector (model 484), and a refractive index (RI) model 401.

Wavelength Selection

A mixture of 0.5% (w/v) of industrial mono-, di-, and tristearin was prepared in THF. The HPLC instrument was conditioned by passing dichloromethane through a CN column for 15 h. Injection of 100 μL samples was done with a 0.36 AUFS sensitivity. The chromatograms were recorded in three-dimensional form by using a diode array detector.

RESULTS AND DISCUSSION

Through a solubility study it is possible to predict the behavior of these glycerides in a chromatographic system. Some of these organic molecules have a polar group (OH groups) and nonpolar groups (C_{18} saturated hydrocarbons). The tristearin is the less polar of these compounds because its three positions are substituted by hydrocarbonated groups. It is expected that the solubility will depend on the balance of these substituted groups. The results of this study are not sufficient to establish a scale of polarity for these compounds. Mono-, di-, and triglycerides are soluble in such low polarity solvents as CH_2Cl_2, $CHCl_3$, and THF, and nonsoluble in such high polarity solvents as acetonitrile and methanol. Singleton (*26*) studied the low polarity and the hydrophobic character of these triglycerides in these types of solvents.

Figure 1 shows the three-dimensional chromatogram of a mono-, di-, and tristearin mixture as the result of wavelength studies at 200 to 250 nm. The absorption maximum was found at 230 nm, showing the possibility of detection of all glycerides at this wavelength, which is in accordance with studies done by Wojtusik (*14*) for some saturated and nonsaturated glycerides. In view of the great solubility of these glycerides in methylene chloride, this solvent was used as the mobile phase. In spite of the poor resolution, this solvent showed good selectivity for these compounds. To improve the resolution, a modifier of low polarity was added to the mixture, and this increased the retention time of these glycerides in the column. Different proportions of hexane were tested (up to 40%), but the separation of di- and tristearin remained poor. A polar modifier (ACN) was added to change the selectivity and to improve the resolution. The best resolution was found at 6% ACN. Amounts of ACN up to 6% increased the strength of the mobile phase. Figure 2A shows the chromatogram of mono-, di-, and tristearin in this final mobile phase. In addition, different flow rates were studied, from 0.5 to 1.3 mL/min, to find the lower equivalent theoretical plate (H). The optimum flow rate was 0.7 mL/min for this CN column and this mobile phase.

Figures 2A and 3A show chromatograms of the GMS(1) and GMS(2) mixture, respectively. These compounds were identified by comparison

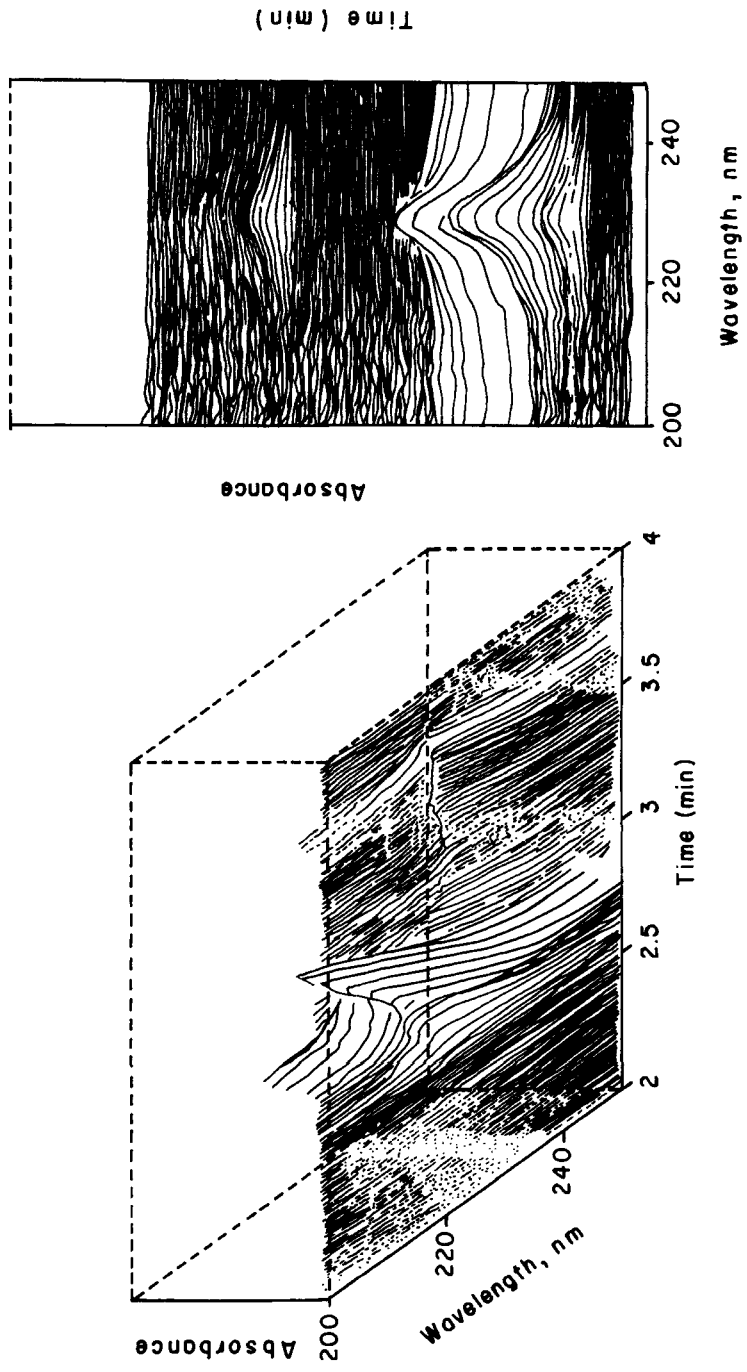

FIG. 1. Chromatographic profile of GMS(1) for wavelength analysis.

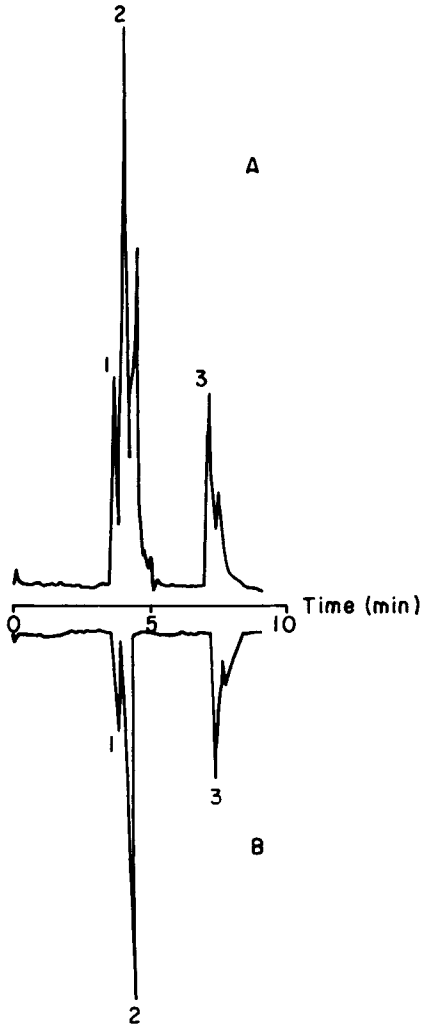

FIG. 2. (A) Normal phase HPLC separation of GMS(1) components with UV (230 nm) detection. Conditions: 10 μm Zorbax CN column (25 cm × 4.6 mm i.d.); mobile phase, CH_3CN/CH_2C_{12}/heptane (6:54:40); flow rate, 0.7 mL/min. Peaks: (1) tristearin, (2) distearin, and (3) monostearin. (B) Normal-phase HPLC separation of GMS(1) components with RI detection. Chromatographic conditions: As in Fig. 2(A). Peaks: (1) tristearin, (2) distearin, (3) solvent, and (4) monostearin.

with the retention times of mono-, di-, and tristearin standards. As expected, the retention times of these glycerides increase as the polarities increase in normal-phase chromatography. Moreover, the peak contribution of the mobile-phase components was studied by injecting equal vol-

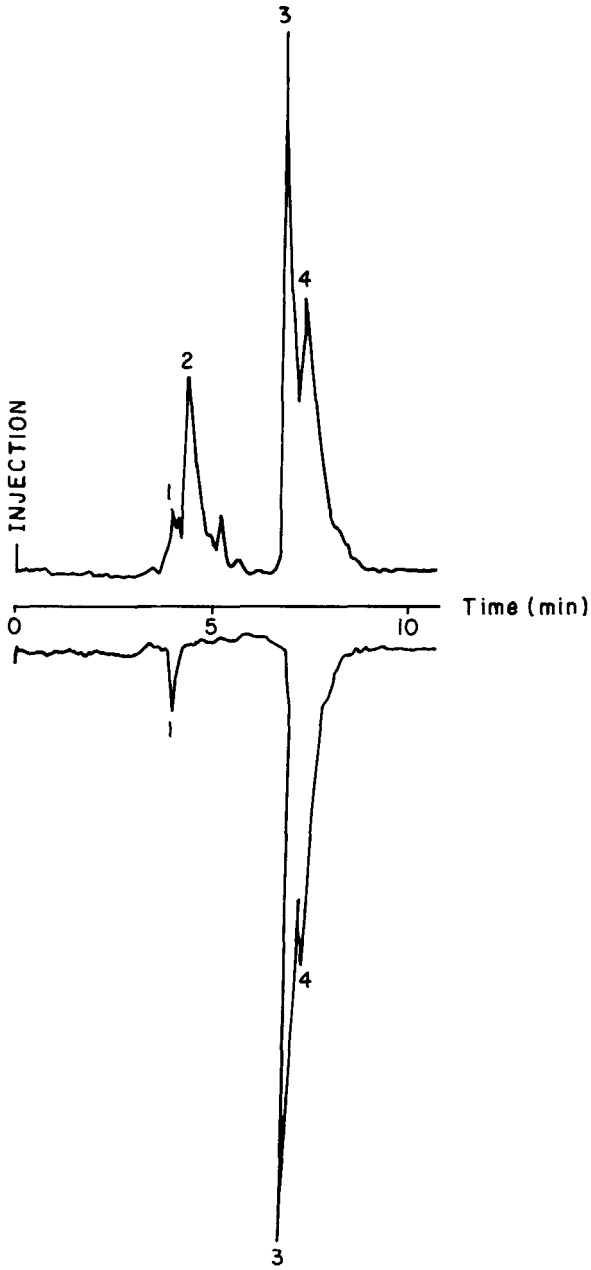

FIG. 3. (Top) Normal-phase HPLC separation of GMS(2) components with UV detection (230 nm). Conditions: As in Fig. 2. Peaks: (1) distearin, (2) solvent, (3) monostearin, and (4) impurity. (Bottom) Normal-phase HPLC separation of GMS(2) components with IR detection. Conditions: As in Fig. 2. Peaks: (1) distearin, (3) monostearin, and (4) impurities.

umes to the sample. In both chromatograms a "shoulder," located in the extreme of the monostearin peak, is present. These peaks could be hydrolysis products of stearic acid and glycerol. To investigate this, standards of stearic acid and glycerol were added to the GMS(1) mixture, but not one of these compounds corresponded to stearic acid or glycerol. We believed that these peaks are contaminant products. The 1,2- and 1,3-distearin isomers could not be resolved under these chromatographic conditions. Little is known about the isocratic separation of these isomers in bonded-phase HPLC. Kathleen (*12*) reported the separation of dipalmitin isomers with a hexanal/chloroform/acetonitrile gradient.

To determine the composition of the GMS mixture, a calibration curve was constructed. Figure 4 shows an increase of the slope related to an increase in the ester group in the molecule. The correlation between the structure and sensitivity can be explained by the additivity principle (*3*).

Table 1 shows the concentration of the GMS mixture in normal-phase chromatography. The composition of GMS mixture was calculated based on the calibration curve. Marginal values for these samples were reported by the SEC technique (*27*, *28*).

To evaluate the chromatographic conditions, a mixture of mono-, di-, and tristearin standards was used. Figure 5 shows the separation of mono-, di-, and tristearin standards in the normal phase with UV detection, and Table 2 shows the quantitative analysis of this mixture under the same

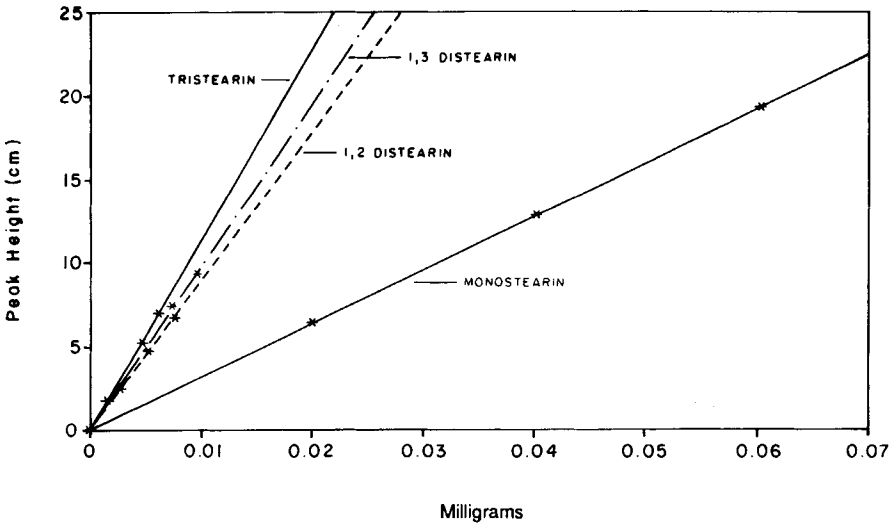

FIG. 4. Calibration curve with CN column. Conditions: As in Fig. 2 with UV detection.

TABLE 1
Quantitative Analysis of the GMS Mixture in Normal-Phase HPLC with UV Detection

Mixture	Mono (%)	1,3-Di (%)	Tri (%)
GMS(1)	30.8	28.3	9.3
GMS(2)	64.2	3.0	—

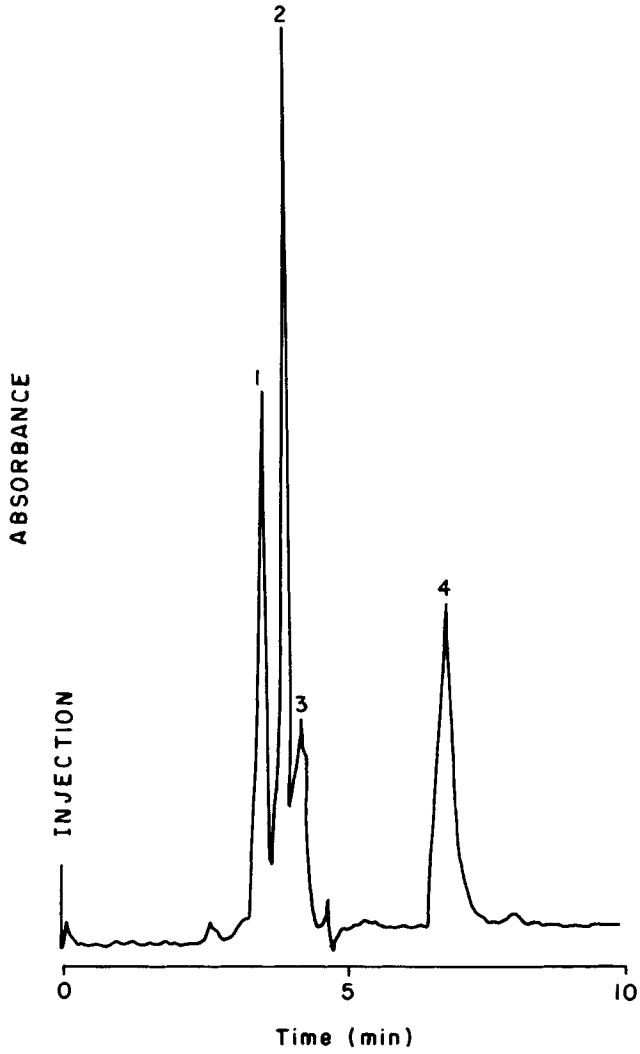

FIG. 5. Normal-phase HPLC separation of mono-, di-, and tristearin in a standard mixture with UV detection. Peaks: (1) tristearin, (2) distearin, (3) solvent, and (4) monostearin.

TABLE 2
Quantitative Analysis of Mono-, Di-, and Tristearin Mixture in Normal-Phase HPLC with UV Detection

	Composition (% w/w)		
Standard	Expected	Experimental	Error (%)
Tristearin	19.32	19.53	1.10
1,3-Distearin	40.33	41.20	2.20
Monostearin	40.33	39.70	−1.60

chromatographic conditions. Good resolution and quantification of the mixture were found by using these chromatographic conditions.

Figures 2B and 3B represent the separations obtained with the RI detection of mixtures of GMS(1) and GMS(2), respectively. The good reproducibility of the retention times can be observed. The chromatogram corresponding to GMS(2) has a small peak preceding the solvent, assigned to the distearin due to the retention time. This peak was observed with difficulty in the UV detection because of a partial overlapping of the solvent. All of this indicates that the mixtures of GMS(2) consist of a small amount of distearin. In regard to the first observation, the same volume of the mobile phase was injected as in the analyzed sample, and the same retention time and a behavior identical to the preceding chromatograms was obtained. Consequently, these peaks were assigned to the solvent. These observations are critical for analysis of the stearic acid in the mixture because the solvent eluted at the same retention time as the acid, resulting in a complete overlapping of the peak for the compound, which eliminates stearic acid as a possible hydrolysis product (if it is present in the mixture).

The quantitative aspect related to the sensitivity of the triglycerides in the detection systems (RI and UV) has not been studied in much detail. Some authors (*14*, *15*) have reported better detection in separations with RI, whereas others have indicated considerable losses in sensitivity with this kind of detection. Some studies relate detection to the type of glyceride being analyzed (*16*). In any case, the results obtained showed that the sensitivity of the analysis decreases when the detection is changed from UV to RI; this can be seen in Fig. 2. Comparison of the two chromatograms obtained from GMS(1) with both detection systems in this figure indicate that RI detection is not recommended for the quantification of glycerides of stearic acid.

For the study of sensitivity, known amounts of mono-, di-, and tristearin standards were injected. A plot of these data supplies an estimate of the quantitative aspect of the sensitivity; this is shown in Fig. 6. The slopes in

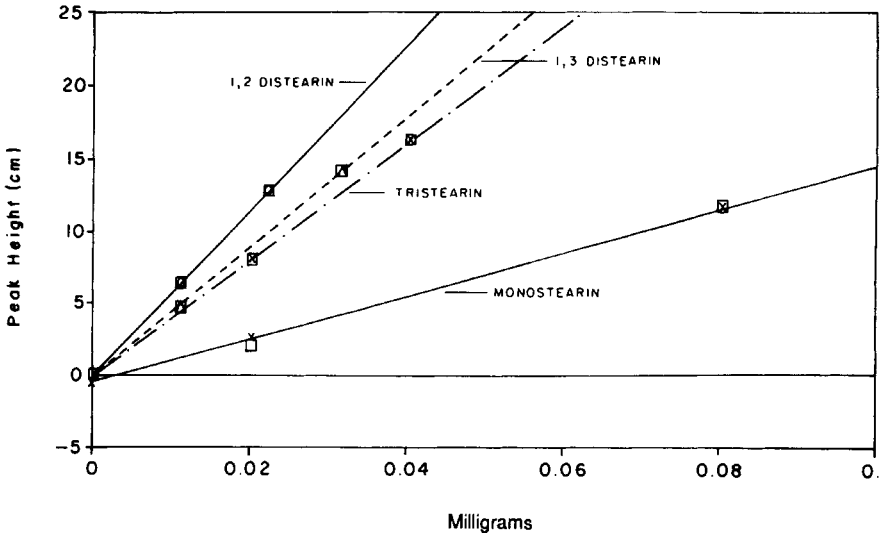

FIG. 6. Calibration curve with CN columns and RI detection. Conditions as in Fig. 2.

this figure do not ascend in the same order as in the case of UV; their values were lower. In this type of detection the order of the sensitivity is due to some factors resulting from a change of refraction in the cell: the greater the change, the greater the sensitivity. The compositions of the mixtures being studied were determined by using the calibration curves obtained with the standards. These values are shown in Table 3. The percentages decrease in comparison with those of UV detection. These results were expected, and they agree with some previous studies of the sensitivity with this type of detection.

The influence of two types of stationary nonpolar phases was studied. These phases consist of saturated hydrocarbonated groups: Octylxylane (C_8) and octadecylxylane (C_{18}). Both studies employed UV detection.

In view of the good selectivity shown by the CN column in normal-phase HPLC, it was decided to include acetonitrile in the mobile phase as the

TABLE 3
Quantitative Analysis of the GMS Mixtures in Normal-Phase HPLC with RI Detection

Mixture	Mono (%)	1,3-Di (%)	Tri (%)
GMS(1)	24.9	17.9	6.0
GMS(2)	50.0	1.8	—

polar modifier together with a low polarity solvent like dichloromethane in order to avoid insolubility of the compound. The separation of the GMS(1) components with a 60/40 acetonitrile/dichloromethane mixture at a flow rate of 1.0 mL/min was initially studied. We observed that the compounds elute simultaneously with the solvent. Ways of achieving high retention are by making the mobile phase more polar or by increasing the difference in the column; in this case the proportion of acetonitrile was increased. An important change in selectivity was attained, although adequate resolution has not been achieved. The best way to get good separation seems to be by increasing the polarity of the mobile phase by adding more acetonitrile. This method would not be appropriate because these glycerides are totally insoluble in this solvent and because precipitation could arise inside the column (*25*).

The results obtained indicate that 18-carbon saturated chains present in the glycerides do not give considerable retention in the small C-8 group

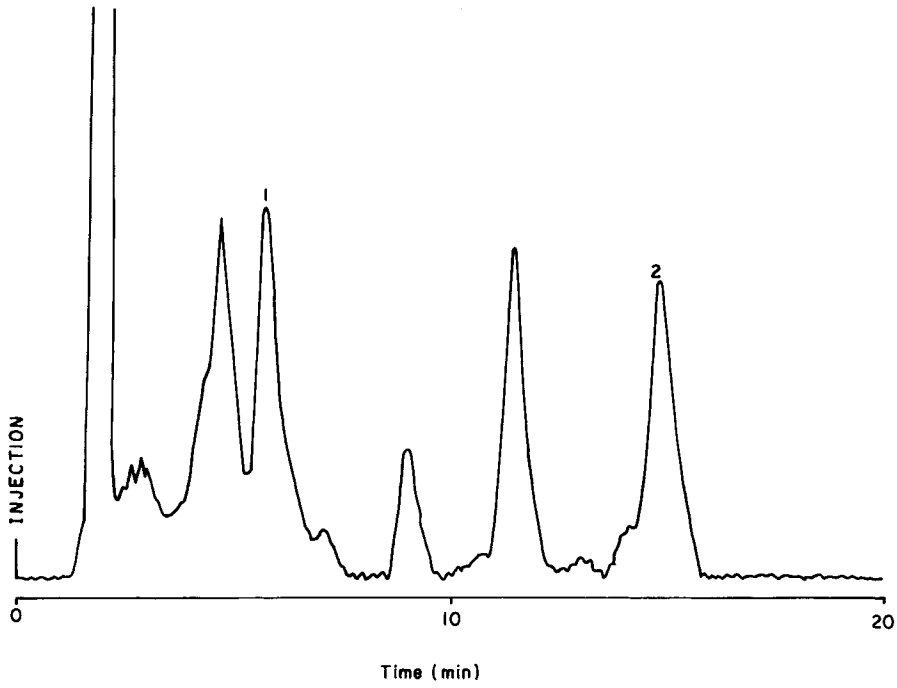

FIG. 7. Reverse-phase HPLC separation of GMS(1) components in octadesilsilane column with UV detection (230 nm). Chromatographic conditions: 10 μm Lichrosorb RP-18 column (25 cm × 4.5 mm i.d.); flow rate, 0.9 mL/min; mobile phase, (ACN/CH_2Cl_2/hexane 75:24:1). Peaks: (1) monostearin and (2) 1,2-distearin.

of the stationary phase. Similar results were reported by Barron (*15*) in separations of glycerides from palmitic acid (16 saturated carbon) in an octylsilane column.

It was previously indicated that one way of improving the selectivity and retention of compounds is by changing the stationary phase. This time a less polar stationary phase, an octadecylsilane column, with acetonitrile/dichloromethane (60/40) as the mobile phase was chosen. Consequently, a noticeable separation of some peaks was obtained. When the proportion of acetonitrile was increased to 75% in the mobile phase, an additional peak was separated. Finally, when a known polar modifier was added, greater competence for the stationary phase was achieved and better resolution was obtained, as seen in Fig. 7.

Identification of the peaks was done by injecting known amounts of a standard. The elution order was expected to depend on the polarities. The more polar compounds, like monostearin, elute first, and the less polar compounds, like distearin, elute last.

Retention of tristearin was excessively high; there was no elution in a 40-min run. This result is critical since the strength of the mobile phase can be increased by lowering the polarity, but with a loss of resolution of the initial peaks. For this reason, it is difficult to adjust to an adequate elution time for tristearin without losing the total resolution of the chromatogram.

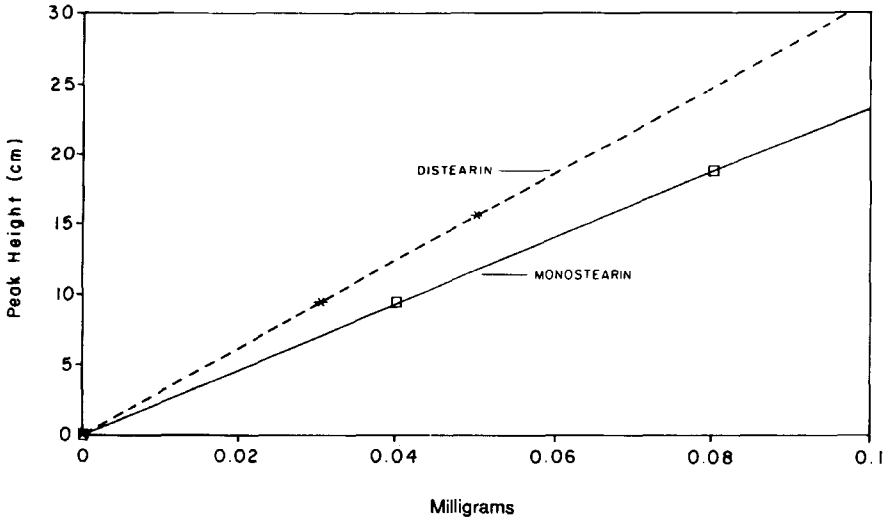

FIG. 8. Calibration curves of 1,3-distearin and monostearin in C-18 column with UV detection (230 nm).

TABLE 4
Quantitative Analysis of the GMS Mixtures in Reverse-Phase HPLC with UV Detection

Mixture	Mono (%)	1,3-Di (%)
GMS(1)	24.3	11.6
GMS(2)	50.3	—

An advantage of the reverse-phase system with C-18 column is the capacity for resolving the 1,2- and 1,3-distearin isomers. Quantification of the compounds in GMS(1) and GMS(2) was done by injecting known amounts of the mono- and 1,3-distearin standards. Figure 8 shows the calibration curve obtained. Table 4 shows the results of the analysis of GMS(1) and GMS(2) mixtures. It can be observed that the composition is less than expected in relation to the values found for the normal phases.

CONCLUSIONS

HPLC was very effective for separating the components of glycerides which have been used in the surface treatment of expansible polystyrenes.

The best separation was achieved by using a CN column with UV detection. Normal-phase HPLC using a CN column and the mixture CH_2Cl_2/ACN/hexane as the mobile phase were more effective than reverse-phase HPLC using either a C-8 or a C-18 column. UV detection was more sensitive than RI detection.

Acknowledgments

The authors are grateful to Universidad del Zulia and INDESCA for financial support.

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Received by editor July 2, 1991