Journal of Chromatography, 522 (1990) 245–253
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 745

Chromatographic separations of sucrose monostearate structural isomers

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(First received April 26th, 1990; revised manuscript received July 26th, 1990)

ABSTRACT

High-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) methods are described for separating sucrose monostearate isomers. The HPLC procedure provides baseline separation of purified monoester isomers into three main peaks at room temperature, and completely separates monoesters with different acyl chain lengths (C_{14}, C_{16}, C_{18}). The TLC method separates up to six of the eight possible positional monostearate isomers, which can be further differentiated by specific color development with a visualizing agent. The GLC technique used resolves monoesters with different acyl chain lengths, and partially separates monostearate isomers. Isomers collected from HPLC were subjected to treatment with invertase, then resolved and detected by TLC to determine fatty acid substitution patterns on the sucrose molecule.

INTRODUCTION

Since sucrose esters of fatty acids were recognized as effective emulsifiers, they have been used in foods, cosmetics and pharmaceuticals [1,2]. The preparation of these compounds by procedures using the transesterification reaction [3] yields a complex mixture of monoesters, diesters and higher esters with fatty acyl groups of various chain lengths. Acylation of sucrose with a single fatty acid can yield 255 different esters, from mono- to octaesters [2]. Several thin-layer (TLC), gas–liquid (GLC) and high-performance liquid chromatographic (HPLC) methods have been developed to separate and identify the different fractions (*e.g.* monoesters, diesters, esters of higher degree of substitution) [4–6], but good separation of the different positional isomers in each fraction has been difficult and an efficient chromatographic system has not been reported. The emulsifying properties of different sucrose monoester preparations depend upon the number and kind of the different isomers [2]. Thus, an effective method for the separation and analysis of sucrose monoesters during synthesis, purification and structural studies is essential. We have developed methods to separate up to six positional isomers of sucrose monostearate. These protocols serve to characterize synthesis of monostearates and other sucrose monoesters and thus meet all requirements of a qualitative analytical procedure.

Invertase catalyses the hydrolysis of sucrose to D-glucose and D-fructose. By combining the separation techniques of HPLC and TLC with the specificity of

0021-9673/90/$03.50

hydrolytic enzymes, we were able to determine whether the fatty acyl residue was located on the glucose or fructose residue of sucrose monoester isomers.

EXPERIMENTAL

Materials

Sucrose monoesters were obtained from Dai-Ichi Kogyo Seiyaku (DKS) (Tokyo, Japan). The sample used (DKS F-160) contained sucrose monomyristates, sucrose monopalmitates and sucrose monostearates [2]. Sucrose monostearates were synthesized in dimethyl sulfoxide (DMSO) using the procedure by Osipow *et al.* [3]. Both monoester samples were purified by chromatography on silica gel 60 (Merck 7734). Samples were dissolved in a minimal volume of chloroform–ethanol (1:1) and monoesters were eluted from the column with hexane–diethyl ether–ethanol (3:1:1).

High-performance liquid chromatography

The Waters Assoc. HPLC system used consisted of a M6000 pump, a U6K injector and a R401 refractive index detector. A Maxima 820 chromatography workstation equipped with an I-200 system interface module (SIM) was used to acquire, save and process data (Waters Assoc.). The column system consisted of a 15 cm × 3.9 mm μBondapak C_{18} (10 μm irregular particle size) stainless-steel column connected to a 15 cm × 3.9 mm Nova-Pak C_{18} (4 μm spherical particle size) stainless-steel column, both of Waters Assoc. Columns were equilibrated and developed isocratically with acetone–water (7:3) at a flow-rate of 0.5 ml/min. HPLC-grade acetone (Aldrich) and water were filtered through a 0.45-μm nylon filter (Millipore) and degassed prior to chromatography. Monoester solutions (2%) in acetone–water (3:1) were passed through a 0.45-μm filter and 10-μl aliquots were used for analysis.

Sucrose monostearate fractions collected from the HPLC effluent were lyophilized after evaporation of the acetone, dissolved in a small amount of chloroform–ethanol (1:1), and subjected to TLC analysis.

Thin-layer chromatography

TLC was performed according to a modified procedure of Lee *et al.* [7]. Separation was achieved on Whatman K5 silica gel plates which had been dipped in 0.2 *M* potassium phosphate buffer and dried at 85°C for 1 h.

The plates were developed for 30 min using ethyl acetate–pyridine–water (80:20:5), then air dried for 15 min; the same procedure was used to redevelop the plates two additional times, for a total of three times. Visualization of sucrose monoesters on the TLC plates was achieved by saturating the dry plates with a solution of 4 g diphenylamine, 4 ml aniline, 30 ml 85% H_3PO_4 and 200 ml acetone [8], and drying them in the hood for 20 min prior to heating at 110°C for 4 min.

Invertase hydrolysis experiments

Approximately 1 mg yeast invertase (β-fructofuranosidase, Sigma) was added to lyophilized isomer fractions collected from HPLC which had been hydrated with 20 m*M* acetate buffer, pH 4.5. The solutions were stirred and heated at 45°C for 24 h and then tested for hydrolysis by TLC analysis.

Gas–liquid chromatography

GLC separations were performed using a 3700 Varian gas chromatograph with a flame ionization detector. The column was a 6 ft. × 1/8 in. O.D. stainless-steel column filled with 3% OV-17 on Chromosorb W-HP, 80–100 mesh. The column temperature was set at 280°C for 12 min, then programmed to increase at 2°C/min to a final temperature of 330°C. The temperature of the injection port was 300°C and the detector temperature was 400°C. The flow-rate of the carrier gas (nitrogen) was 30 ml/min. Samples (2 mg) were silylated with 1 ml TRISIL (Pierce) and heated at 70°C for 30 min prior to analysis. The injection volume was 2 μl.

RESULTS AND CONCLUSIONS

Fig. 1 shows the HPLC separation of the purified DKS F-160 monoester fraction, and standard monostearates. In Fig. 1A, three sets of peaks are completely resolved from each other. The first corresponds to myristoyl monoesters, the second to palmitoyl monoesters and the third to steroyl monoesters. Monostearate peaks were identified based on the chromatographic mobilities of authentic, pure monostearates prepared in our laboratory (Fig. 1B). The assignments of M-I, M-II, P-I, P-II, and P-III (Fig. 1A) are based on their mobilities and their reported composition in DKS F-160 [2]. The three peaks corresponding to the groups of monopalmitates and monostearates, are also well resolved. Others have attempted to separate the monostearate isomers by HPLC, but the limited solubility of sucrose monoesters in the solvents they chose required elevated temperatures and did not provide optimal resolution of positional isomers [9–11]. Cormier *et al.* [9] first reported HPLC analysis of sucrose fatty acid esters, but it is not clear whether they separated positional isomers of both monostearates and distearates, or if they only separated monoesters from diesters and triesters present in their sample. Gupta *et al.* [6] have found HPLC

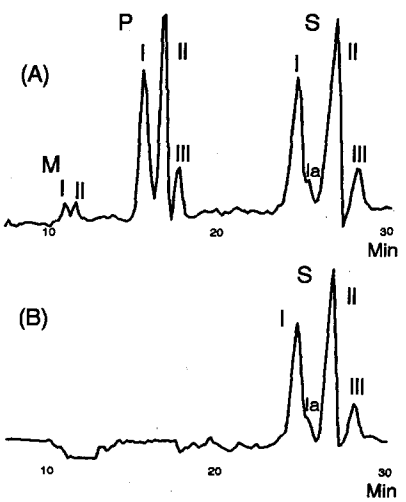

Fig. 1. Reversed-phase HPLC separation of highly purified sucrose monoesters. (A) DKS F-160 monoesters: M-I, M-II = monomyristates; P-I, P-II, P-III = monopalmitates; S-I, S-Ia, S-II, S-III = monostearates. (B) Standard monostearates: S-I, S-Ia, S-II, S-III.

separation of sucrose esters unsatisfactory due to the deleterious effect of potassium soaps and residual sucrose on the separation process. Moreover, they also report that sucrose esters tend to be absorbed strongly on silica gel [6]. More recently Jaspers *et al.* [11] developed a procedure which separated monoesters with different acyl chain lengths. However, their method required an elevated temperature (65°C) and did not separate positional isomers.

Our HPLC procedure has several advantages over previous methods including good sample solubility, chromatography at room temperature, and easy solvent removal from sucrose monoester fractions. In addition, we did not encounter difficulties with sucrose esters absorbing onto C_{18} reversed-phase silica gel. Inasmuch as the three main peaks are effectively resolved, we collected fractions of the different peaks peaks for preliminary structural analysis of the isomers, in combination with TLC techniques.

Although TLC separation of sucrose monostearate isomers has not been reported before, Soljic and Eskinja [12] separated six sucrose monopalmitates on silica gel G plates developed with chloroform–methanol–acetic acid (85:5:10). We were unable to obtain adequate separation of the monostearates by this method probably because silica gel does not give a good separation of sugar compounds unless it is impregnated with inorganic salts (*e.g.* boric acid, mono- and diphosphate, etc.) [13]. Thus, when plates were impregnated with phosphate, sucrose monoesters were effectively separated.

Fig. 2 illustrates the TLC separation of the DKS F-160 monoester fraction, and of pure monostearates. Although the monopalmitates and monostearates from the DKS F-160 sample did not separate from each other, separation of the pure monostearate isomers was good. At least five well resolved components can be seen, which exhibit different colors. The spot labeled 2–3 (Fig. 2) represents two unresolved isomers, which appear pinkish-brown, and are clearly resolved when the initial quantity of sample is lower. Additionally, component 1 appears as a grayish-blue spot, component 4 as a blue spot, and components 5, 6 and 7 as spots of different shades of purple. Component 6 was faint or absent except in heavily spotted samples. Under the conditions of these analyses, sucrose imparts a dark purple color on a TLC plate, fructose a pink color and glucose a blue color [13]. All three sugars are clearly resolved just above the origin. The colors of the different isomers appear to be produced mainly by reaction of the visualizing reagent with the part of the monoester sugar that is not substituted. Thus, sucrose with glucose esterified would most likely appear as a variation of the colors pink or purple and sucrose with fructose esterified would be seen as a variation of the color blue. These observations were confirmed by the experimental results obtained with invertase.

Twelve fractions of the sucrose monostearates were collected from the HPLC effluent (Fig. 3A) and were spotted on silica plates for TLC analysis (Fig. 3B, lanes 1–12). After reaction with the visualizing reagent, each isomer differed in color, intensity and position on the TLC plate. The first five fractions correspond to HPLC peak I and shoulder Ia. In lane 4 of Fig. 3B, the component just under the fastest migrating spot was faint and not always detected in all samples analyzed. Thus, this component may have been an artifact, or an isomer that exists in quantities too small to detect easily. Fractions 6–9 correspond to HPLC peak II (although fraction 6 was contaminated by components present in fraction 5), and fractions 10–12 correspond to

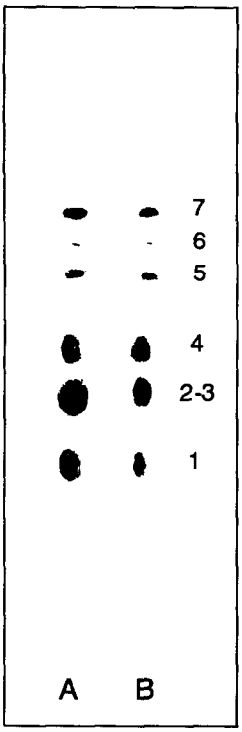

Fig. 2. TLC separation of DKS F-160 monoester samples (lane A) and pure sucrose monostearates (lane B).

HPLC peak III. The colors representing the four isomers in lanes 1–5 in Fig. 3 are predominantly pink or pinkish-purple. From this information we reasoned that the fatty acyl substitution for these fractions is on glucose. The isomers that predominate in lanes 6–12 in Fig. 3 appear as grayish-blue or blue. We interpreted this data to infer that for these fractions, the fructose moiety of sucrose has the fatty acyl substitution. While eight isomers are theoretically possible, only six well defined spots appear in the TLC analysis. There may be an additional isomer in fractions 10–12 that has a similar R_F value as the slowest moving component from fractions 6–9. This would explain the continued appearance of the slowest moving component in fractions 10–12. Another possibility is that the isomer(s) exist in quantities too small to detect.

Invertase catalyses the hydrolysis of sucrose to D-glucose and D-fructose only if the fructose moiety of sucrose is unsubstituted [14]. The specificity of this enzyme provided a convenient test to categorize the monostearate isomers as glucose or fructose substituted. Fig. 4 shows TLC separations for three (fractions 3, 8 and 12) of the twelve isomer fractions collected from HPLC and tested with invertase. Fraction 3 (Fig. 4, lane A) is composed of three isomers represented by three differently colored spots: pinkish-brown (bottom), pinkish purple (middle), light purple (top). When fraction 3 was allowed to react with invertase, hydrolysis of the sucrose monoesters occurred. The three isomer spots were replaced by a glucose monostearate spot near the solvent front at the top of the plate, and a pink spot from fructose above the application point of the TLC plate (Fig. 4, lanes A and B). Fraction 8 appears to be

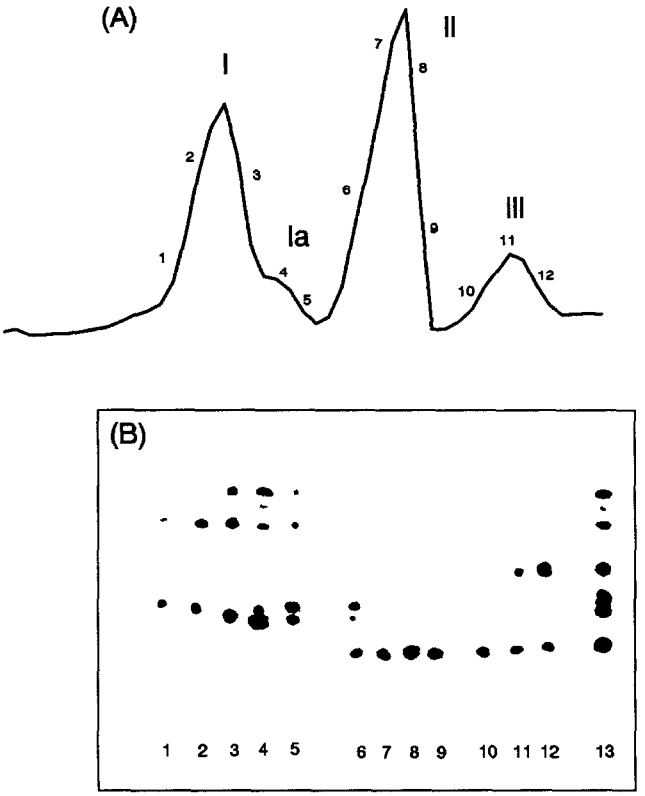

Fig. 3. Correlation of sucrose monostearate HPLC effluent fractions with the corresponding TLC analysis. (A) HPLC separation of monostearate isomers (collected fractions 1–12). (B) TLC of collected HPLC fractions (lanes 1–12) positioned beneath the corresponding HPLC tracing. Lane 13 = purified monostearate isomers sample.

composed of only one isomer (Fig. 4, lane C), represented by a bluish-gray spot, and fraction 12 (Fig. 4, lane E) is composed of two isomers represented by bluish-gray (bottom) and blue (top) spots. Hydrolysis of the sucrose monoester isomers was not detected in either fraction with the addition of invertase, as indicated on the TLC plate by an absence of change in their R_F values (Fig. 4, lanes C–F). A lack of invertase hydrolysis implies that the fatty acid substituent is located on the fructose moiety of the sucrose molecule.

Fractions 1, 2 and 5, like fraction 3, were also hydrolyzed after treatment with invertase. Fraction 4 seemed hydrolyzed; however it was difficult to determine whether the faint spot, appearing second from the top on the TLC plate (Fig. 3), had been hydrolyzed. Fractions 7 and 9, like fraction 8, appeared as a single major component which was not affected by treatment with invertase. Fraction 6 contained the same slow moving component which was not affected by invertase treatment, as well as trace amounts of two components hydrolyzed by invertase that had the same R_F values as the slowest moving components in fraction 5. The results of the hydrolysis experiments indicate that the monostearate isomers eluting in HPLC peaks I and Ia are glucose

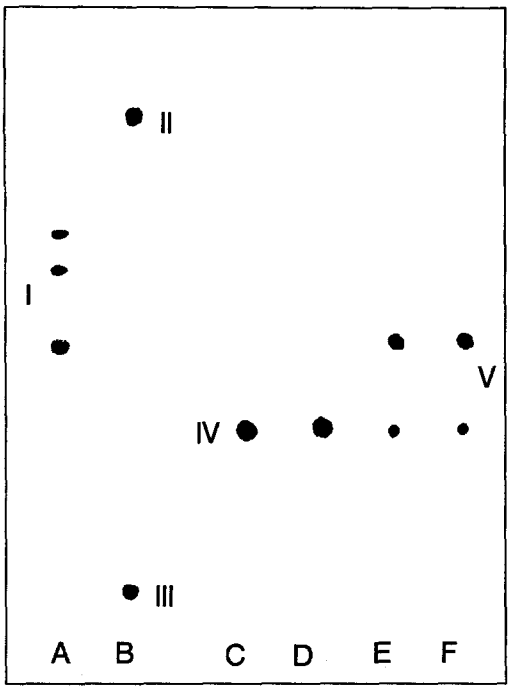

Fig. 4. TLC of invertase experiment samples. Isomer fractions 3, 8 and 12 (Fig. 3) before (lanes A, C and E) and after (lanes B, D and F) the addition of invertase. Lane A (fraction 3): I = monostearate isomers; Lane B (fraction 3, complete hydrolysis): II = glucose monostearates, III = fructose; Lane C (fraction 8): IV = monostearate isomers; Lane D (fraction 8, no hydrolysis detected): IV = monostearate isomers; Lane E (fraction 12): V = monostearate isomers; Lane F (fraction 12, no hydrolysis detected): V = monostearate isomers.

substituted (TLC spot colors are variations of pink and purple), while HPLC peaks II and III represent fructose substituted isomers (TLC spot colors are variations of blue). In every case, except in that of the faint spot in fraction 4, the preliminary visual evaluation of glucose or fructose fatty acyl substitution based on the TLC color given by the isomer was confirmed by enzymatic hydrolysis experiments. Definitive structural assignments will be made based on NMR experiments of the isomer fractions collected from HPLC.

GLC has been used extensively for the quantitative and qualitative analysis of sucrose monoesters and diesters [6]. GLC studies to determine the position of esterification has been done for sucrose monomyristates [15] and sucrose monostearates [16], but only after the isomeric mixtures were methylated, saponified and subjected to methanolysis to yield the methyl glycosides. In an effort to develop a simpler and faster procedure, we separated the monostearate trimethylsilyl derivatives on a 3% OV-17 column (Fig. 5). The DKS F-160 mixture separated into two main isomers and at least five minor ones (Fig. 5A), while the pure monostearate mixture separated into one main isomer and at least three minor ones (Fig. 5B). The individual peaks shown represent more than one isomer.

As an example, the main peak of the group labeled "stearates" in Fig. 5A and

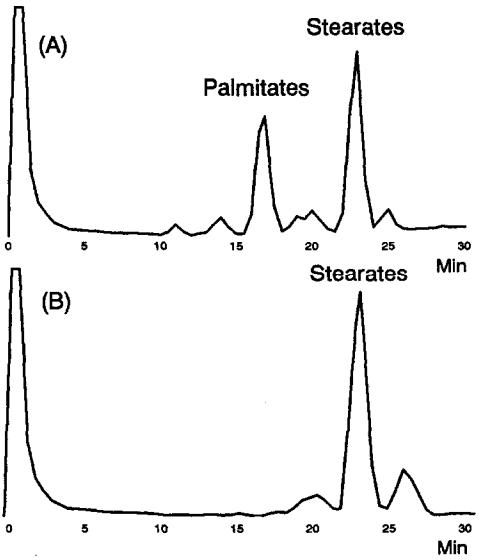

Fig. 5. Computer reconstructed GLC separation of highly purified sucrose monoesters. (A) DKS F-160 monoesters; (B) Standard monoesters.

B contained three components as measured both by HPLC and TLC. Similarly, the leading and following minor peaks in Fig. 5 contained more than one sucrose ester derivative. Thus, we could not assign individual monoesters to GLC peaks. Perhaps the use of capillary GLC would facilitate a sufficient separation for this purpose.

Since we have found that the four isomers corresponding to HPLC peaks I and Ia (Fig. 3) are glucose substituted and the two (possibly three) isomers corresponding to HPLC peaks II and III are fructose substituted, we suspect that substitution of the steroyl group must be predominantly on the fructose moiety of the sucrose molecule. This is contrary to previous findings from studies on the competition of sucrose monostearate isomers [2,16] and sucrose monolaurate isomers [14]. However, the extensive work of Lemieux and McInnes [15] indicated that approximately 60% of the acyl substitution occurs on the fructose moiety of sucrose monomyristate. Clearly, more comprehensive structural studies are needed which will be facilitated by the methods reported here.

We conclude that monostearate isomers can be separated using a combination of HPLC and TLC techniques. Our protocols offer several advantages over previously reported procedures, such as simple and rapid screening of samples for the presence of individual isomers and improved resolution. These uncomplicated techniques can be scaled up to develop preparative methods which can provide adequate quantities of the pure positional monostearate isomers for structural studies.

ACKNOWLEDGEMENTS

The financial support of the Department of Economic Development, State of Nebraska is gratefully acknowledged. A special thanks to Virginia Stryker for her help in the purification of the sucrose monostearates.

This paper was published as Journal Series No. 9189, Agricultural Research Division, University of Nebraska.

REFERENCES

1 G. R. Ames, *Chem. Rev.*, 60 (1960) 541.
2 H. Chung, P. A. Seib, K. F. Finney and C. D. Magoffin, *Cereal Chem.*, 58 (1981) 164.
3 L. Osipow, F. D. Snell, W. C. York and A. Finchler, *Ind. Eng. Chem.*, 48 (1956) 1459.
4 H. Mima and N. Kitamori, *J. Am. Oil Chem. Soc.*, 39 (1962) 546.
5 J. Weiss, M. Brown, H. J. Zeringue and J. R. Feuge, *J. Am. Oil Chem. Soc.*, 48 (1971) 145.
6 R. K. Gupta, K. James and F. J. Smith, *J. Am. Oil Chem. Soc.*, 60 (1983) 1908.
7 K. Y. Lee, D. Nurok and A. Zlatkis, *J. Chromatogr.*, 174 (1979) 187.
8 S. A. Hansen, *J. Chromatogr.*, 105 (1975) 388.
9 R. Cormier, L. H. Mai and P. Pommez, *Proc. Tech. Sess. Can. Sugar Refin. Res.*, (1978) 37.
10 V. R. Kaufman and N. Garti, *J. Liq. Chromatogr.*, 4 (1981) 1195.
11 M. E. A. P. Jaspers, F. F. van Leeuven, H. J. W. Nieuwwenhuis and G. M. Vianen, *J. Am. Oil Chem. Soc.*, 64 (1987) 1020.
12 Z. Soljic and I. Eskinja, *J. Serb. Chem. Soc.*, 52 (1987) 105.
13 M. Ghebregzabher, S. Rufini, B. Monaldi and M. Lato, *J. Chromatogr.*, 127 (1976) 133.
14 W. C. York, A. Finchler, L. Osipow and F. D. Snell, *J. Am. Oil Chem. Co.*, 33 (1956) 424.
15 R. U. Lemieux and A. G. McInnes, *Can. J. Chem.*, 40 (1962) 2394.
16 M. Gee and H. G. Walker, Jr., *Chem. Ind. (London)*, 24 (1961) 829.