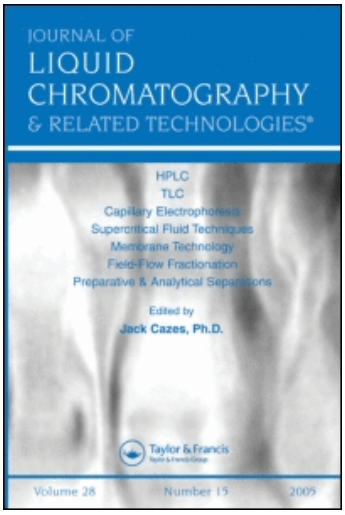

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Separation and Quantitation of Sucrose Esters Using HPLC with Evaporative Light Scattering Detection

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Abstract: A high performance liquid chromatographic method with evaporative light scattering detection (ELSD) for the separation and quantitation of sucrose esters is described. Samples are analyzed by means of a reversed-phase column using methanol and water under gradient conditions as the mobile phase. This procedure provides a complete separation and determination of mono- to octa-esters, with different positional isomers in each fraction on a single run, in combination with the electrospray ionization mass spectrum (ESI-MS) technology. With this method, it is possible to determine the approximate compositions of mono- to octa-esters in one analysis and the precise quantitation of pure positional isomers using an external standard method. The described method is found to be straight forward, rapid, inexpensive, and reproducible, and can be readily applied for the analysis of sucrose esters during synthesis, purification, and structure studies.

Keywords: Sucrose esters, HPLC, ELSD, ESI-MS, Analysis, Positional isomers

INTRODUCTION

Since amphiphilic sucrose fatty acid esters, also known as sucrose esters, have interesting physicochemical properties, in particular as emulsifiers, they have been used in foods, cosmetics, and pharmaceuticals.[1–8] In addition, several types of synthetic sucrose have shown potent insecticidal activity against

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soft-bodied arthropods,[7–10] and, thus, give sucrose esters a potential for future use. The preparation of sucrose esters by procedures using the esterification or transesterification reaction yields a complex mixture of mono-, di-, and higher esters with different positional isomers as shown in Figure 1. Even the acylation of sucrose with a single fatty acid can, theoretically, yield 255 different possible isomers in from mono- to octa-esters.[11] In order to separate, identify, and quantitate the different fractions (e.g., monoesters, diesters, and esters of higher degree of substitution), several thin-layer (TLC), gas (GC) and high performance liquid chromatographic (HPLC) methods have been developed.[11–16] TLC gives qualitative and quantitative ideas about the ratio of mono-, di-, and higher esters, while GC and GC-mass spectrometric methods were used for quantitative and qualitative results after derivatization. As a fast and more reproducible method of analysis, HPLC with UV detection or refractive index detection (RI) has been used for the analysis of sucrose esters. However, both UV and RI detection have disadvantages. The absorbance of sucrose esters arises primarily from double bonds in the fatty acid moieties, which accounts for the extremely poor response when using UV detection. RI detection only permits the isocratic elution whereas gradient elution is essential to obtain a good separation of sucrose esters. None of these methods enables a complete separation of sucrose esters. Quantitative analysis is, therefore, extremely difficult.

Some of these problems could be overcome with the evaporative light scattering detector (ELSD). ELSD is a mass detection method, which is based on LC column effluent nebulization into droplets by a nebulizing gas, the resulting vapor enters a temperature controlled evaporator tube, which causes the evaporation of mobile phase.[17–19] The resulting non-volatile analyte particles are then directed towards a narrow light beam. Light is scattered by residual particles and measured using a photomultiplier or photodiode. The signal intensity is related to the mass of the analyte in the scattering chamber, and the signal is indicative of molecular size and shape, but not the chemical identity of the residual particles passing through the light beam. The ELSD can provide a stable baseline even with gradient elution, and has been recognized as a universal detector compatible with gradient analysis.

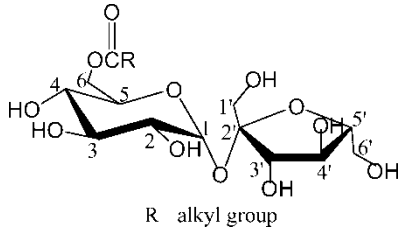

Figure 1. Structure of 6-mono-sucrose ester.

Since the physical and chemical properties of sucrose esters depend upon the composition of positional isomers, as well as each fraction,[20,21] an effective method for the separation and analysis of sucrose esters during synthesis, purification, and structural studies is essential. This paper describes a procedure which separates the sucrose ester into their esters from mono- to octa-esters, with different isomers being resolved simultaneously in each fraction on a single run by HPLC/ELSD using gradient elution. The proposed method also aimed at developing an easy and rapid method for analysis of sucrose esters with different positional isomers in each fraction, to meet all requirements of qualitative and quantitative analytical procedures.

EXPERIMENTAL

Reagents and Chemicals

All solvents were of either analytical or HPLC grade. Water was obtained by distillation of demineralized water. Sucrose and other chemicals were all analytical grade and were purchased from Shenyang Chemical Co. (Shenyang, China).

Synthesis and Purification of Sucrose Esters

Synthesis of sucrose esters were as the procedure by Chortyk et al.[9] For example, sucrose was dissolved in dimethylformamide at a concentration of 27.4 g (0.08 mol) of sucrose/100 mL of DMF, with gentle heating (up to 100°C) and stirring until the sucrose dissolved. Then, 20 mL of pyridine was added, and the solution was cooled to 65°C. Octanoyl chloride (0.18 mol) was dissolved in 60 mL of acetonitrile and poured into a separatory funnel, and this solution was added at a fast drop rate (over a 45 min period) to the sucrose solution, while stirring vigorously. The reaction temperature was maintained at 65°C. After addition of the acid chloride, the reaction mixture was stirred for 1 h at 65°C, cooled to about 40°C, and poured into 100 mL of acetone. About 17 g of sodium bicarbonate (0.2 mol) was mixed with 2 mL of water, and the paste was added slowly into the reaction mixture. After the evolution of CO_2 ceased, anhydrous, crystalline sodium sulfate (100 g) was added. The reaction mixture was then filtered and evaporated to dryness using a vacuum pump. The crude residue was then partitioned between a phosphate buffer (pH 7) and ethyl acetate. The aqueous phase was extracted twice with ethyl acetate and the combined organic layers were washed three times with water and concentrated. Octanoyl sucrose esters (C_8SE) were thus obtained. Five C_8SE samples (OS-1, OS-2, OS-3, OS-4, OS-5) were prepared with the varying molar ratio (1, 2, 3, 4, 5) of

octanoyl chloride to sucrose. Sample OS-t was a mixture of OS-2 and OS-5 and the mass ratio was 1:1.

Electrospray Ionization Mass Spectrum (ESI-MS)

Identification of the components of C_8SE was carried out with a quadrupole HP-1100 MS system in the electrospray positive mode. The mass ions (m/z) were recorded in a full scan mode with mass ranges of 100–1600. A sample solution (20 mg mL^{-1}) used for ES-MS analysis was prepared by dissolving sucrose esters in HPLC grade methanol.

HPLC Apparatus and Chromatographic Conditions

The HPLC system consisted of the following components: a Hewlett-Packard Model 1050 series, equipped with an autosampler, an Alltech 2000 evaporative light scattering detector (ELSD), and Agilent chemstation.

The chromatographic separation was performed using gradient elution. The mobile phase components were methanol:water, and delivered at a flow rate of 1.0 mL/min. The separation was carried out at 40°C, on a reversed-phase C18-ODSA column (150 × 4.6 mm, 5 μm particle size) purchased from Elite Analytical Instruments Co., Ltd (Dalian, China). All injections were 10 μL in volume. Solvent gradient conditions are reported in Table 1. The column effluent was directed to ELSD. Nebulization of the eluent in the ELSD was provided by a stream of dried air using an air compressor at a flow rate of 2.4 L/min. The nebulization was performed at room temperature, and the nebulized effluents were evaporated at 90°C.

The mobile phase was prepared by filtering HPLC grade methanol or water through a 0.2 μm nylon filter using a vacuum filtration assembly. Filtered solvents were degassed using a water vacuum assembly with gentle stirring using a magnetic stirrer for about 5 min.

Prior to each run, the HPLC-ELSD system was allowed to warm up for 20–30 min, and the pumps were primed using the protocol suggested by the

Table 1. Gradient elution program

Time (min)	Methanol (%)	Water (%)
0	60	40
10	60	40
11	70	30
30	75	25
80	100	0

manufacturer. Using freshly prepared mobile phase, the baseline was monitored until stable and the samples run.

Optimization of ELSD Parameters

In order to obtain minimum noise and maximum detection signal in ELSD, three basic parameters, nebulizer gas flow rate (pressure), evaporating temperature, and gain were varied to optimize the detection of samples.[17–19] The performance of the detector was periodically evaluated by executing the electronic noise, solvent noise, column noise, and signal stability tests, using the procedures recommended by the manufacturer. Dried air was used as the driving gas for nebulization and nitrogen was used as the carrier gas for analyte transport. The carrier gas was passed through filter frits prior to entering the detector to assure the absence of stray particles introduced by the gas.

Preparation of Sample Solutions

Individual stock solutions of C_8SE samples (OS-1, OS-2, OS-3, OS-4, OS-5, and OS-t) were prepared in HPLC grade methanol. The sample was dissolved and transferred with methanol to a 10 mL volumetric flask and made up to volume with methanol. The concentrations of stock solutions were all 10 mg mL^{-1}. Also, three controls (1.0, 5.0, and 10.0 mg mL^{-1}) of OS-t were prepared by diluting the stock solution with methanol in appropriate quantities. All working solutions were stored at $-20^{\circ}C$ and brought to room temperature before use. The sample solution was filtered through an 0.2 μm hydrophilic membrane filter into a HPLC sample vial just before HPLC/ELSD analysis.

Calibration Curve and Standards

Sucrose (Su) was used as the calibration standard. In a clean, dry 100 mL volumetric flask, 100 mg sucrose was accurately weighed and dissolved in a mixture containing HPLC grade methanol and water (60/40, v/v) to make a stock solution. It should be noted that this stock solution was found to be stable at $-20^{\circ}C$. Calibration standards were prepared by diluting the stock solution with the same mixture in appropriate quantities. Twelve calibration standards were made at concentrations between the detection limit of sucrose and the concentration at which saturation of the detector occurred. Three controls were also prepared so as to lie in the lowest, middle, and highest regions of the calibration curve, i.e., 12.8, 64, and 128 μg mL^{-1}.

Reproducibility

The precision and accuracy of the method were assessed by within and between run validations. The variation was evaluated by injecting three sets of sucrose controls (12.8, 64, and 128 μg mL^{-1}, n = 3) on three separate days. By substituting the peak area into the calibration curve equation from the same run, the measured concentrations were obtained. The relative standard deviations were calculated to check the precision of the method. The accuracy was expressed as percentage bias.[22,23]

$$\text{Bias} = \frac{\text{Nominal concentration} - \text{Measured concentration}}{\text{Nominal concentration}} \times 100\%$$

RESULTS AND DISCUSSION

In ELSD, a constant nebulization process is important for satisfactory repeatability. Several factors influence the average diameter of the droplets and their distribution, which include density, viscosity, and liquid surface tension.[17–19] Among these factors, the nebulizer gas flow rate affects the signal responses most significantly. When the gas flow rate is too low, large droplets are formed, resulting in spikes and random noise. But, when the gas flow rate is too high, the droplets decrease in size, which results in a decreased signal response. The evaporating temperature is also an important parameter affecting the signal response. At low temperature solvent evaporation is not complete, and at high temperature the detector response is decreased, owing to the decrease in particle size by improper vaporization of the nebulized analytes in the drift tube. In this work, the optimum nebulizer gas flow rate was determined to be 2.4 L min^{-1} and the evaporating temperature was set to 90°C.

As sucrose has eight free hydroxyl groups,[9,20,21] esterification with octanoyl chloride could result in the formation of a mixture containing eight groups of C_8SE, from mono- to octa-. Figure 2 shows the total electrospray mass spectrum of OS-t. Mono- to octa-C_8SE can be identified by the presence of their quasi-molecular ions $[M + Na]^+$ (positive mode). The ions at m/z 491.2, 617.3, 743.5, 869.5, 995.7, 1121.7, 1247.9, and 1373.9 are attributed to the $[M + Na]^+$ ions of mono-, di-, tri-, tetra-, penta-, hexa-, hepta-, and octa-C_8SE, respectively. The ions at m/z 1337.7 are assigned as [mono- + tetra-C_8SE + Na]$^+$ or [di- + tri-C_8SE + Na]$^+$. Similarly, the ions at m/z 1464.8 correspond to [mono- + penta-C_8SE + Na]$^+$, [di- + tetra-C_8SE + Na]$^+$ or [tri- + tri-C_8SE + Na]$^+$, and the ions at m/z 1590.9 are assigned as [mono- + hexa-C_8SE + Na]$^+$, [di- + penta-C_8SE + Na]$^+$ or [tri- + tetra-C_8SE + Na]$^+$.

In order to obtain a good resolution of the different fractions and the different positional isomers in each fraction on a single run, which has been

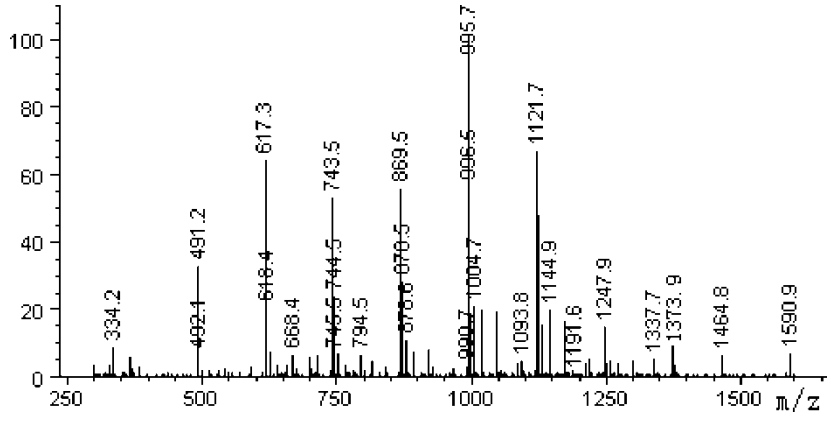

Figure 2. Mass spectrum of OS-t.

difficult to obtain, several combinations of methanol and water were evaluated for use as the mobile phase. After trying several types of gradients and varying duration, an optimum solvent system was found as described in Table 1.

Figure 3 shows a typical HPLC/ELSD chromatogram of OS-t. Nine groups of peaks are completely resolved from each other based on their difference in polarity. Higher esters are less polar due to a greater substitution of hydroxyl groups of the sucrose molecule. The first single peak, Su, corresponds to sucrose based on a comparison with reference compounds. The assignments of G1, G2, G3, G4, G5, G6, G7, and G8 are based on their mobility in reversed-phase HPLC, which are opposite to their polarity. G1 corresponds to mono-C_8SE, G2 to di-C_8SE, G3 to tri-C_8SE, and G4 to tetra-C_8SE, etc., all the way up to octa-C_8SE. Confirmation of their identities could be established by LC-MS. Also, these assignments can be verified by the HPLC/ELSD chromatograms and mass spectrums of the other five C_8SE samples. In the synthesis of C_8SE, varying the molar ratio of octanoyl chloride to sucrose results in the variety of final products. Figure 4 shows a comparison of HPLC/ELSD chromatograms of the five C_8SE samples with

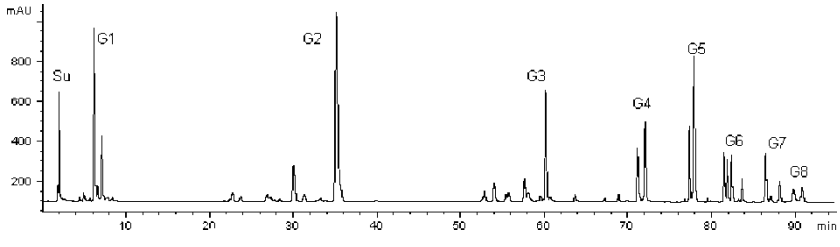

Figure 3. Reversed-phase HPLC separation of OS-t with ELSD detection.

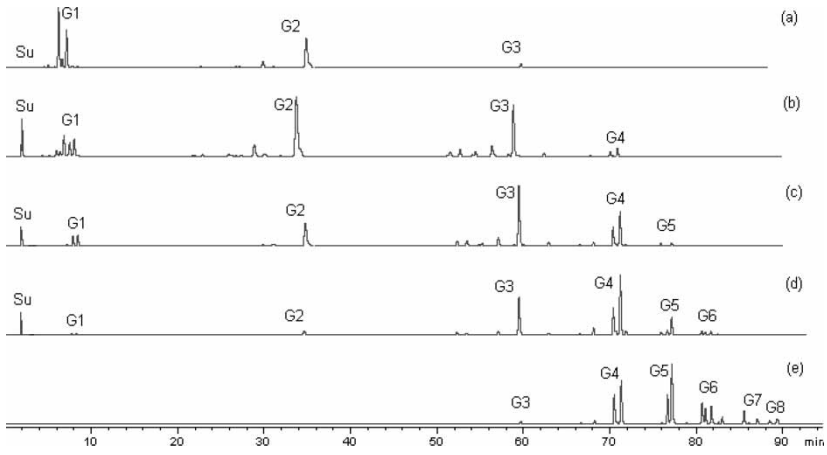

Figure 4. A comparison of HPLC/ELSD chromatograms of five C_8SE samples: (a) OS-1, (b) OS-2, (c) OS-3, (d) OS-4, (e) OS-5.

molar ratio of octanoyl chloride to sucrose from 1 to 5 in their synthesis. Su, G1, G2, and G3 in OS-1 (Figure 4a), Su, G1, G2, G3, and G4 in OS-2 (Figure 4b), Su, G1, G2, G3, G4, and G5 in OS-3 (Figure 4c), Su, G1, G2, G3, G4, G5, and G6 in OS-4 (Figure 4d), and G3, G4, G5, G6, G7, and G8 in OS-5 can be observed in the chromatograms. In combination with the mass spectrums (not shown in this paper) of these C_8SE samples, assignments of G1 to G8 are easily justified.

Good resolutions of positional isomers in each fraction are also obtained by the developed method. Separation of isomers in G1, G2, G3, and G8 is illustrated in Figure 5. In view of the fact that sucrose has three primary hydroxyl groups on the 6, 6′, and 1′ carbons, acylation at these positions is much more likely to occur than that on the more hindered, secondary hydroxyls on the 2, 3, 4, 3′, and 4′ carbons (Figure 1). This was confirmed by Chortyk et al. with NMR experiments, which showed the main structures of mono-C_8SE to be 6-, 6′-, and 1′-monoacyl sucrose, di-C_8SE to be 6, 6′-diacyl sucrose, and tri-C_8SE to be 6, 1′, 6′-triacyl sucrose according to the reactivity of 6- > 6′- > 1′- > secondary hydroxyl.[9] In the group of G1 (Figure 5a), five peaks at least are observable, whereas M3, M4, and M5 are assigned to 6-, 1′-, and 6′-mono-C_8SE, respectively. Thus, it can be inferred that mobility of monoacyl sucrose is as follows: 6- > 1′- > 6′-mono-C_8SE. Therefore, mobility of diacyl sucrose should be such: 6, 1′- > 6, 6′- > 1′, 6′-di-C_8SE. In G2, peak D2 corresponds to 6, 6′-di-C_8SE, whereas D1 should be 6, 1′-di-C_8SE for 1′, 6′-di-C_8SE would have a longer retention time than D2. Peak T1 in G3 can be undoubtedly assigned to 6, 1′ 6′-triC_8SE. It is interesting that there are two commensal peaks in G8, whereas there should be only one single peak theoretically. All these,

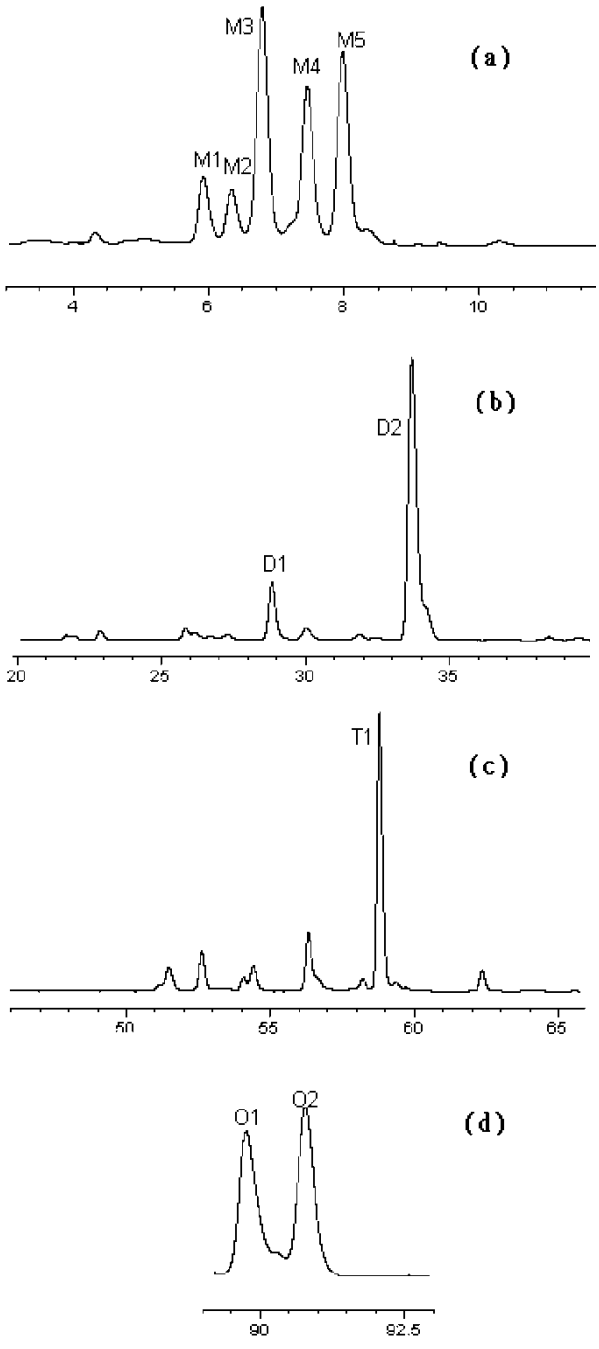

Figure 5. Separation of positional isomers in several fractions: (a) G1, (b) G2, (c) G3, (d) G8.

including assignment and determination of positional isomers in each fraction need further research, which would be considerable complicated work. The HPLC/ELSD techniques we developed also provide a feasibility to be scaled up to develop preparative methods, which can provide adequate quantities of the pure positional isomers in each fraction for structural studies.

Quantitative analysis of each fraction with different positional isomers is indispensable in quality control of synthesis, purification, and commercial products of sucrose esters, since the degree of substitution is one key parameter to their physicochemical properties[20,21] and bioactivities.[9] TLC and GC have been used extensively for the quantitative analysis of sucrose ester but only after preliminary treatment, which is complicated and time wasting.[11] Quantitative analysis using HPLC with UV and RI also have been promoted, however, high deviation were observed as well as TLC and GC when calculation was based on the area percent of each fraction. HPLC/ELSD overcomes some of these deficiencies, since ELSD response is primarily caused by the mass of the analyte.[17,18] It enables the area percent distributions more close to the actual results, especially in the quantitative analysis of a series of analogues such as sucrose esters. Table 2 shows a percent distribution of G1, G2, and G5 in OS-t. Analyses are carried out in three OS-t solutions with different concentrations and each repeated for another two times. The results from these analyses give a rough estimate of the content of each fraction in OS-t, whereas slight variations are observed (0.01–3.08%) for each in three concentrations.

On the other hand, many studies have aimed at the precise structure activity relationship of positional isomers in mixtures of sucrose esters.[20,21] Consequently, quantitative analyses of some positional isomers are needed. Similarly, the rough estimate content of each isomer can be obtained based on the area percent. In order to get a more precise content of a certain isomer, it is necessary to prepare pure sucrose ester of a defined

Table 2. Percent distribution of G1, G2 and G5 calculated in three concentrations of OS-t solutions

Concentration of OS-t solutions (mg mL^{-1})	Percent distribution (%)[a]					
	G1		G2		G5	
	Mean[b]	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
1.0	15.80	4.30	25.59	2.51	11.20	4.38
5.0	14.75	3.79	25.70	2.48	12.60	3.72
10.0	14.76	3.32	28.67	1.55	12.20	3.52

[a]Values of percent distribution are calculated from peak areas of HPLC-ELSD data for each fraction.

[b]Each value is the mean of three experiments.

structure for use as external standard. Calibration curves of ELSD were non-linear.[17] Calibration functions were obtained by curve fitting using the equation:

$$a = Km^E$$

with peak area units a, mass of a component injected m, a constant K, and an exponent E. When the calibration functions are plotted on a logarithmic scale they are linear, following the equation:

$$\log a = E \log m + \log K$$

with E being the slope and log K the intercept. It would be interesting and significant to establish calibration functions of each isomer, in each fraction, so as to study the relationship between the exponents E or constant K, and the structure of isomers. However, there are so many difficulties to obtain appropriate amounts of pure isomers at present because of the instability of ester links on sucrose and the complicated purification process. As mentioned above, our HPLC/ELSD technology has provided a possibility to scale up to prepare pure isomers, and this will encourage us to continue further work on it. In an effort to show how this method run with sucrose was chosen as a standard example, not only because the sucrose molecule is the backbone of a pure sucrose ester compound, but also because quantitative analysis of residual sucrose is also needed in the quality control of sucrose ester products.

The detection limit ($S/N > 5$) of the described method was observed for sucrose at 64 ng on the column in the current assay. Figure 6 shows the fitted calibration curve for sucrose following the equation: $Y = aX + b$ with Y being the log value of the peak area, X the log value of sample mass, a the intercept, and b the slope with a regression coefficient of 0.9999. Values of E and K are thus obtained, which are 1.48 and 3.67×10^{-3}, respectively.

The reproducibility of the method was evaluated by analyzing a set of three controls (12.8, 64, and 128 $\mu g\ mL^{-1}$; $n = 3$) on three separate days ($n = 3$), and calculating the RSD% and the Bias%. As shown in Table 3, RSD% and Bias% of the controls on all three days were found to be within

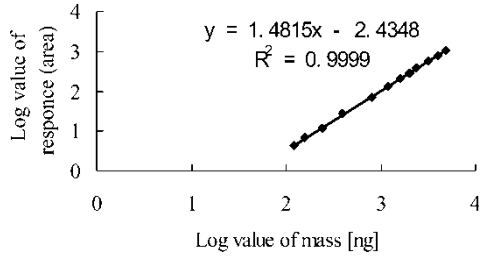

Figure 6. Log-transformed linear calibration curves of sucrose.

Table 3. Reproducibility of Su standards over three consecutive days

Theoretical concentration ($\mu g\ mL^{-1}$)	Days	Measured concentration ($\mu g\ mL^{-1}$)[a]		
		Mean	RSD (%)	Bias (%)
12.8	Day 1	13.31	0.98	3.98
	Day 2	13.09	1.68	2.27
	Day 3	12.97	2.31	1.33
64	Day 1	62.81	0.96	1.86
	Day 2	62.54	1.41	2.28
	Day 3	62.45	1.17	2.42
128	Day 1	126.85	0.66	0.90
	Day 2	125.71	0.76	1.79
	Day 3	125.08	0.97	2.28

[a]Mean values represent three different Su standards for each concentration.

2.31% and 3.98%, respectively. In general, the errors in quantitation were found to be the highest in the control with the lowest concentration. Because the response of ELSD is sigmoidal and the calibration curve is a log-log transformation of the date, there is a significant error in the quantitation of points at the lower and upper end of the curve. It can, therefore, be concluded that for optimum quantitation from the calibration curve, concentrations should be selected in such a way that they lie in approximately the mid region of the calibration curve when using ELSD.

CONCLUSION

A high performance liquid chromatography method has been developed for the separation and quantitation of sucrose esters using an evaporative light scattering detector. Mono- to octa-sucrose esters were successfully separated and determined on a single run in combination with ES-MS technology. Structural information of some positional isomers in several fractions was provided and more comprehensive structural studies are needed, which will be facilitated by the methods reported here. The present method also proposed a simple and reliable quantitative analysis of each fraction in sucrose esters based on the area percent of response in ELSD. Precise quantitation of a positional isomer using an external standard method was discussed with sucrose as an example. In principle, it can be used to quantitate any pure sucrose ester compound of interest. Thus, this procedure can be easily adopted for analysis of sucrose esters during synthesis, purification, and structure studies.

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