High-Throughput Analysis of Sucrose Fatty Acid Esters by Supercritical Fluid Chromatography/Tandem Mass Spectrometry

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INTRODUCTION

Sucrose fatty acid esters (SEs) are nonionic surfactants consisting of sucrose as the hydrophilic group and a fatty acid such as palmitic acid or stearic acid as the lipophilic group. In SEs, a wide range of hydrophilic-lipophilic balance (HLB) values can be attained, and this balance can be modulated by varying the alkyl chain length of the acyl groups and the number of ester groups per molecule. Therefore, SEs are widely used as emulsifiers in foods, cosmetics, pharmaceuticals, and agrochemicals.1–5)

Supercritical fluid chromatography (SFC) coupled with triple quadrupole mass spectrometry was applied to the profiling of sucrose fatty acid esters (SEs). The SFC conditions (column and modifier gradient) were optimized for the effective separation of SEs. In the column test, a silica gel reversed-phase column was selected. Then, the method was used for the detailed characterization of commercial SEs and the successful analysis of SEs containing different fatty acids. The present method allowed for fast and high-resolution separation of monoesters to tetra-esters within a shorter time (15 min) as compared to the conventional high-performance liquid chromatography. The applicability of our method for the analysis of SEs was thus demonstrated.

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sensitivity and selectivity by multiple reaction monitoring (MRM) as compared to ultraviolet detection and ELSD. Commercial SE preparations were also analyzed for their diverse fatty acid ester contents.

**EXPERIMENTAL**

**Chemicals**

Sucrose monopalmitate (SE (C16:0), purity: 95%) and sucrose distearate (SE (C18:0/C18:0), purity: 95%) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Sucrose monostearate (SE (C18:0), purity: 25–33%) was obtained from Carbosynth Limited (Compton, UK). Commercial SEs (Ryoto sugar ester P-170 and S-170) were obtained from Mitsubishi-kagaku Foods Corporation (Tokyo, Japan). Carbon dioxide (99.99% purity) was purchased from Neriki Gas (Osaka, Japan). Methanol (HPLC grade) was purchased from Kishida Chemical (Osaka, Japan). Ammonium formate was used as an additive to the modifier (concentration of ammonium formate was 0.1% in the modifier). Ammonium formate was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Chloroform and acetone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Supercritical fluid chromatography**

An analytical Method Station SFC system was used (Waters, MA, USA) for SFC. The columns used were YMC-Pack CN (YMC Co., Ltd., Kyoto, Japan), YMC-Pack Diol (YMC Co., Ltd., Kyoto, Japan), Inertsil NH 2 (GL Science, Tokyo, Japan), Inkertsil C8 (GL Science, Tokyo, Japan), Inkertsil ODS-SP (GL Science, Tokyo, Japan), Inertsil ODS-EP (GL Science, Tokyo, Japan), Inertsil ODS-4 (GL Science, Tokyo, Japan), and Inertsil ODS-P (GL Science, Tokyo, Japan). The size of Inertsil NH 2, Inertsil ODS-SP, Inertsil ODS-EP, Inertsil ODS-4 and Inertsil ODS-P columns was 4.6 mm i.d.×250 mm, 5 µm, 100 Å. The size of YMC-Pack CN and YMC-Pack Diol columns was 4.6 mm i.d.×250 mm, 5 µm, 120 Å. The size of Inertsil C8 column was 4.6 mm i.d.×250 mm, 5 µm, 150 Å. The column temperature was set at 35°C. The back pressure was set at 10 MPa. The mobile phase was carbon dioxide, and the modifier was methanol. Ammonium formate was used as an additive to the modifier (concentration of ammonium formate was 0.1% in the modifier). The flow rate was adjusted to 3 mL/min. Separations were performed using a gradient of increasing modifier concentration: 17% from 0 to 3 min, 17–40% from 3 to 4 min, 40% from 4 to 17 min, 40–17% from 17 to 18 min, and 17% from 18 to 20 min. A sample volume of 5 µL was injected.

**Mass spectrometry**

MS analyses were carried out using a Xevo mass spectrometer (Waters, MA, USA), with electrospray ionization (ESI). ESI-MS analysis was performed in the positive ion mode. The MS parameters are as follows: capillary voltage, 3.0 kV; cone voltage, 30 V; desolvation temperature, 600°C; desolvation gas flow, 800 L/h; cone gas flow, 60 L/h. All data were analyzed using Mass Lynx (Waters, MA, USA).

**Sample preparation**

The sample preparation method for the standard SE is as follows. Approximately 10 mg of the standard SE sample was weighed into a screw-capped glass tube, to which 1 mL of chloroform was added. The mixture was shaken until the oil completely dissolved. The sample was first diluted 10-fold with chloroform and further diluted 10-fold using acetone. Finally, this sample was diluted 100-fold using methanol, yielding a standard solution with a concentration of 1 µg/mL.

The sample preparation for the commercial SE is as follows. Approximately 10 mg of the commercial SE sample was weighed into a screw-capped glass tube, to which 1 mL of chloroform was added. The mixture was shaken until the oil completely dissolved, and the solution was diluted 10-fold with chloroform. The resulting solution was then diluted 10-fold using acetone, yielding an SE solution with a concentration of 100 µg/mL.

**RESULTS AND DISCUSSION**

**Mass spectrometry conditions for sucrose ester analysis**

To establish accurate and sensitive MS conditions, the mass spectrum of each SE (SE (C16:0), SE (C18:0), and SE (C18:0/C18:0)) was acquired by infusion analysis. Dominant peaks showed that all compounds were detected as sodium adducts, which are easily formed.20 Next, to obtain the fragment spectra of SE, collision-induced dissociation (CID) was performed using authentic standards of SE. The precursor ion was set as the sodium adduct of each SE.

In the case of SE (C18:0), two product ions (m/z=469 and m/z=451) were detected from the precursor ion m/z=631.2 (Table 1, Fig. 1). The product ion (m/z=469) was generated by the elimination of hexose from the precursor ion, and the other product ion (m/z=451) was generated by the elimination of H2O from the first product ion. This fragmentation result was coincident with that observed in a previous study.21

In the case of SE (C18:0/C18:0), four product ions (m/z=735, m/z=717, m/z=469, and m/z=451) were detected from the precursor ion m/z=897.5 (Table 1). The product ion (m/z=735) was generated by the elimination of hexose from the precursor ion, and the other product ion (m/z=717) was generated by the elimination of H2O from the product ion (m/z=735). On the other hand, the product ion (m/z=469) was generated by the elimination of a hexose unit bearing a fatty acid from the precursor ion, and the remaining product ion...
As a result of this study, the product ion was generated by the elimination of hexose from the precursor ion in all authentic standards (SE (C16:0), SE (C18:0), and SE (C18:0/C18:0)). These results indicated that SEs were easily cleaved at the glycosidic bond by CID. In many studies, the molecular species of lipid was determined by CID. By using CID, authentic standards were not needed to determine the molecular species. This was because, in the case of lipid, the cleavage site by CID was almost the same. As mentioned above, CID was very useful for the determination of the molecular species of lipid. The result in this study indicated that the cleavage site of SE by CID was the same. Thus, it was shown that the molecular species of SE could be determined by CID. Moreover, the result in this study indicated that the attachment sites of fatty acid could be determined by CID.

SFC separation conditions for sucrose ester analysis

The separation conditions were investigated using normal-phase columns, YMC-Pack CN (250×4.6 mm i.d., 5 µm, 120 Å), YMC-Pack Diol (250×4.6 mm i.d., 5 µm, 120 Å), and Inertsil NH2 (250×4.6 mm i.d., 5 µm, 100 Å). A broad peak for SE (C16:0) and SE (C18:0) was observed when using YMC-Pack CN and YMC-Pack Diol, which show affinity for high-polarity compounds. It was thought that the highly polar SE (C16:0) and SE (C18:0) were retained firmly on these columns. In the case of Inertsil NH2, which shows affinity for compounds containing hydroxyl groups, no peak was detected because the SEs contain many hydroxyl groups and hence are strongly retained on the column.

Next, to find a suitable column for good separation, five kinds of reverse-phase columns were examined: Inertsil C8 (250×4.6 mm i.d., 5 µm, 150 Å), Inertsil ODS-SP (250×4.6 mm i.d., 5 µm, 100 Å), Inertsil ODS-EP (250×4.6 mm i.d., 5 µm, 100 Å), Inertsil ODS-4 (250×4.6 mm i.d., 5 µm, 100 Å), and Inertsil ODS-P (250×4.6 mm i.d., 5 µm, 100 Å).

Three monomeric columns (Inertsil C8, Inertsil ODS-SP, Inertsil ODS-EP, and Inertsil ODS-4) were compared.

SE (C18:0) and SE (C18:0/C18:0) were successfully separated on all columns. With the use of Inertsil ODS-4, not only SE (C18:0) and SE (C18:0/C18:0) but also SE (C16:0) and SE (C18:0) could be separated satisfactorily (data not shown). The differing density of carbon in Inertsil ODS-4 (11%), Inertsil ODS-EP (9%), Inertsil ODS-SP (8.5%), and Inertsil C8 (8%) could be the cause of this. Hydrophobic interaction is depended on the carbon content of column. By carbon content of column is increased, hydrophobic interaction is increased.

A broad peak for SE (C18:0/C18:0) was observed when using Inertsil ODS-P, which is a polymeric-type column. Because the carbon content in Inertsil ODS-P (29%) is higher than that in Inertsil ODS-4, the low-polarity SE (C18:0/C18:0) was thought to be retained more firmly on the former. Finally, Inertsil ODS-4 was selected as the column of choice for this analytical method, since it demonstrated...
good separation of SEs.
Subsequently, the separation conditions were investigated using methanol containing 0.1% ammonium formate as the modifier solvent. SE (C16:0), SE (C18:0), and SE (C18:0/C18:0) were successfully separated within 5 min (Table 1, Fig. 2).

**Analysis of commercial products of sucrose esters by SFC/MS**

SE (C16:0), SE (C18:0), and SE (C18:0/C18:0) were analyzed in commercial sucrose ester preparations by using the newly developed analytical method. The reproducibility of the retention time and peak area was analyzed repeatedly (n=3) using P-170 (SE containing mainly palmitic acid side chains) and S-170 (SE containing mainly stearic acid side chains). From a preliminary investigation using the single ion resolution (SIR) method, SE (C16:0) was detected in P-170, while SE (C18:0) and SE (C18:0/C18:0) were detected in S-170. However, SE (C18:0) and SE (C18:0/C18:0) were not detected in P-170, and SE (C16:0) was not detected in S-170 (data not shown).

Therefore, MRM was used to detect these compounds. MRM is able to be performed high sensitivity and selectivity analysis. Thus, MRM is suitable for high sensitivity analysis compared to SIR. The MRM conditions (precursor ion, product ion, cone voltage, and collision energy) were set using standard SEs (Table 2). Under the optimized MRM conditions, SE (C18:0) and SE (C18:0/C18:0) were detected

| Compound         | MRM transition | Cone voltage (V) | Collision energy (V) |
|------------------|----------------|------------------|----------------------|
| SE (C16:0)       | 603.2>441.0    | 38               | 26                   |
| SE (C18:0)       | 631.2>469.0    | 40               | 28                   |
| SE (C18:0/C18:0) | 897.5>469.0    | 40               | 28                   |

Fig. 3. MRM chromatograms obtained from P-170 analysis (100 µg/mL). The chromatogram of 897.5>469.0 is SE (C16:0), 631.2>469.0 is SE (C18:0), and 603.2>441.0 is SE (C16:0).
in P-170, and SE (C16:0) was detected in S-170. As a result of this analysis, SEs containing different fatty acids could be detected simultaneously (Fig. 3).

Subsequently, we focused on the analysis of other esters in P-170 and S-170 by scan mode analysis because authentic SE standards were obtained only for SE (C16:0), SE (C18:0), and SE (C18:0:18:0). In addition to peaks for the previous three compounds, peaks attributable to di-esters containing palmitic acid (SE C16:0/C16:0), tri-esters containing palmitic acid (SE (C16:0/C16:0/C16:0)), tri-esters containing stearic acid (SE (C18:0/C18:0/C18:0)), tetra-esters containing palmitic acid (SE (C16:0/C16:0/C16:0/C16:0)), and tetra-esters containing stearic acid (SE (C18:0/C18:0/C18:0/C18:0)) were detected (Figs. 4 and 5).

In order to confirm the fragmentation pattern of each peak, product ion scan was performed, and product ions due to eliminated hexose and/or hexose bearing one or more fatty acids were detected (Table 3). The MS/MS spectrum of SE (C16:0/C16:0/C16:0) is shown in Fig. 6. The fragmentation pattern for eliminated hexose was confirmed using the authentic SE (C16:0). Therefore, the peaks at m/z=841.4, m/z=1079.5, m/z=1317.5, m/z=1633.5, and m/z=1429.5 could be due to SE (C16:0/C16:0), SE (C16:0/C16:0/C16:0), SE (C16:0/C16:0/C16:0/C16:0), SE (C18:0/C18:0/C18:0), and SE (C18:0/C18:0/C18:0/C18:0), respectively. Thus, with the present method, separation of mono-esters to tetra-esters is achieved within a much shorter time (15 min) than that mentioned in previous reports.10

As a result of this study, it was found that SEs were easily cleaved at the glycosidic bond by CID. By using this property, the bonding-sites of fatty acid chains were determined.

Table 3. Details of detected SEs in P-170 and S-170.

| Peak No. | Compound | Selected ion | Precursor ion (m/z) | Product ion (m/z) | R.T. (min)a | Peak area | RSD (%)b |
|----------|----------|--------------|-------------------|------------------|-------------|-----------|----------|
| 1        | SE (C16:0) | [M+Na] +     | 603.2             | 423, 441         | 1.6         | 9.6E+04   | 0.0 1.8  |
| 2        | SE (C18:0) | [M+Na]+      | 631.2             | 451, 469         | 1.8         | 9.2E+04   | 0.7 7.4  |
| 3        | SE (C16:0) | [M+Na]+      | 841.4             | 423, 441, 661, 679 | 2.6        | 2.6E+05   | 0.0 3.7  |
| 4        | SE (C18:0) | [M+Na]+      | 897.5             | 451, 469, 717, 735 | 3.2        | 1.4E+05   | 0.6 4.4  |
| 5        | SE (C18:0) | [M+Na]+      | 897.5             | 469, 717, 735    | 3.5         | 1.0E+04   | 0.2 9.5  |
| 6        | SE (C16:0) | [M+Na]+      | 1079.5            | 423, 679         | 4.4         | 1.9E+04   | 0.0 18.3 |
| 7        | SE (C16:0) | [M+Na]+      | 1079.5            | 423, 441, 661, 679, 899, 917 | 4.7         | 1.9E+04   | 0.4 6.5  |
| 8        | SE (C16:0) | [M+Na]+      | 1079.5            | 441, 661, 679, 899 | 5.4        | 1.2E+05   | 0.0 19.2 |
| 9        | SE (C18:0) | [M+Na]+      | 1163.5            | 735              | 5.9         | 1.0E+04   | 0.3 28.3 |
| 10       | SE (C18:0) | [M+Na]+      | 1163.5            | 451, 469, 717    | 6.2         | 7.4E+04   | 0.2 11.8 |
| 11       | SE (C18:0) | [M+Na]+      | 1163.5            | 469, 717, 735    | 6.7         | 3.3E+04   | 0.2 8.7  |
| 12       | SE (C16:0) | [M+Na]+      | 1317.5            | —                | 6.8         | 1.5E+03   | 0.0 27.5 |
| 13       | SE (C16:0) | [M+Na]+      | 1317.5            | 661              | 7.1         | 2.4E+03   | 0.0 1.0  |
| 14       | SE (C16:0) | [M+Na]+      | 1317.5            | 661              | 7.4         | 1.3E+04   | 0.0 21.2 |
| 15       | SE (C16:0) | [M+Na]+      | 1317.5            | 661              | 7.5         | 3.2E+04   | 0.0 13.2 |
| 16       | SE (C16:0) | [M+Na]+      | 1317.5            | 661              | 7.6         | 2.6E+04   | 0.0 8.3  |
| 17       | SE (C16:0) | [M+Na]+      | 1317.5            | 661              | 8.0         | 1.4E+04   | 0.0 17.2 |
| 18       | SE (C16:0) | [M+Na]+      | 1317.5            | 441, 661, 917    | 8.2         | 3.7E+04   | 0.0 9.2  |
| 19       | SE (C16:0) | [M+Na]+      | 1317.5            | 661              | 8.5         | 1.5E+04   | 0.0 14.3 |
| 20       | SE (C16:0) | [M+Na]+      | 1317.5            | 899, 917         | 9.0         | 6.9E+04   | 0.0 8.1  |
| 21       | SE (C18:0) | [M+Na]+      | 1429.5            | —                | 11.0        | 3.6E+03   | 0.1 16.1 |
| 22       | SE (C18:0) | [M+Na]+      | 1429.5            | 11.3             | 9.6E+03     | 0.1 10.7 |
| 23       | SE (C18:0) | [M+Na]+      | 1429.5            | 11.6             | 6.3E+03     | 0.1 13.2 |
| 24       | SE (C18:0) | [M+Na]+      | 1429.5            | 12.3             | 4.3E+03     | 0.1 3.7  |
| 25       | SE (C18:0) | [M+Na]+      | 1429.5            | 12.9             | 1.1E+04     | 0.2 10.9 |
| 26       | SE (C18:0) | [M+Na]+      | 1429.5            | 13.5             | 6.0E+03     | 0.1 1.4  |
| 27       | SE (C18:0) | [M+Na]+      | 1429.5            | 14.4             | 1.7E+04     | 0.1 4.7  |

a Retention time (R.T.).
b Relative standard deviation (RSD).
(n=3)

Peak Nos. 4 and 5 were determined as SE (C18:0/C18:0) by the retention time and m/z. However, the product ion spectra of each peak were different (Fig. 7, precursor ion was set at m/z=8971). m/z=451 and 469 were major product ions of peak No. 4. These product ions were derived at hexose combined with one stearic acid. On the other hand, m/z=717 and 735 were major product ions in peak No. 5. These product ions were derived at hexose combined with two stearic acids. This was because, these SE (C18:0/C18:0) had different attachment sites for the fatty acid (Fig. 7).

Peak No. 7 was determined as SE (C16:0/C16:0/C16:0) by m/z and product ions. As a result of the product ion scan (precursor ion was set at m/z=1079), it was found that several forms of SEs (C16:0/C16:0/C16:0) were included in this peak (Fig. 6).

These results were new knowledge revealed by SE analysis. By analyzing the product ion spectra of each SE, the attachment sites of fatty acid could be determined in commercial SEs. These results indicate that the commercial SE preparation S-170 might contain at least two types of SEs (C18:0/C18:0) with different attachment sites.

Unfortunately, penta-esters and higher analogs were not detected by this MS method due to its insufficient sensitivity. In order to resolve this issue, optical detection using an evaporative light scattering detector (ELSD) system may be applied. It is suggested that an MS system in combination with an ELSD system might be applied for comprehensive SE analysis.
CONCLUSION

In this study, an SFC-QqQ MS system was applied for the analysis of SEs, and a high-throughput analytical method for mono-, di-, tri-, and tetra-esters was established. Moreover, SEs containing different fatty acids could be detected simultaneously. Moreover, by analyzing the fragment pattern of SEs, the bonding-site of fatty acid chains could be determined. Recent work in this area has focused on the emulsification, antibacterial activity, and bioactivity of SEs. If the present analytical method is used in this research area, more detailed information can be obtained easily and rapidly. Therefore, we expect this system to contribute greatly to the practical studies of SEs.

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