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# Determination of non-ionic surfactants with ester groups by high-performance liquid chromatography with post-column derivatization

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## ABSTRACT

A high-performance liquid chromatographic (HPLC) method with a post-column reaction detector was developed for the determination of non-ionic surfactants with ester groups (NSEG) such as sorbitan fatty acid esters and sucrose fatty acid esters. NSEG from the mono- to pentaester are separated by reversed-phase HPLC with gradient elution. NSEG eluted from a chromatographic column are hydrolysed with potassium hydroxide, and the resulting fatty acids are reacted with 2-nitrophenylhydrazine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to form 2-nitrophenylhydrazides. The reaction mixture is then made strongly alkaline in order to reduce the blank absorbance and develop a violet colour of the hydrazide, which is detected at 550 nm. The post-column reaction detector, for example, can detect 1 μg of monomyristin. With the proposed method, NSEG are well separated and determined with high sensitivity and selectivity.

## INTRODUCTION

Non-ionic surfactants with ester groups (NSEG) such as sorbitan fatty acid esters, sucrose fatty acid esters and polyoxyethylene sorbitan fatty acid esters are widely used as emulsifiers in the food and cosmetics industries. They are usually a complicated mixture, because they have distributions of the alkyl chain length of the fatty acids, the number of ester groups and the number of oxyethylene chain units. NSEG have been determined by means of thin-layer chromatography (TLC) [1–3], gas chromatography (GC) [4,5] and high-performance liquid chromatography (HPLC) [6–9]. However, TLC has poor separation and quantitativity and NSEG of high molecular weight cannot be measured by GC because they are non-volatile. On the other hand, many studies have been reported on the application of HPLC to NSEG, as HPLC is very suitable for the analysis of non-volatile compounds.

Reversed-phase and gel permeation chromatography are mainly used for the separation of NSEG. However, no satisfactory separation of NSEG from the mono- to pentaester has been achieved by either method. Refractive index (RI) and UV detection are frequently used, but both methods have poor sensitivity and selectivity. Hence there is no satisfactory method for the analysis of NSEG in terms of resolution, selectivity, sensitivity and quantitativity.

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We tried to separate NSEG from the mono- to pentaester by reversed-phase chromatography using an ODS column and a gradient elution method, and also tried to detect them by using a new post-column reaction detector with high sensitivity and selectivity. The basis of the post-column reaction was that NSEG are hydrolysed and the resulting fatty acids are derivatized and detected selectively and sensitively. For the colour development of fatty acids, the 2-nitrophenylhydrazine (ONPH) method [10], which has been used for the post-column reaction detector of water-soluble carboxylic acids [11], was chosen, because it was found that it is also applicable to fatty acids.

This paper describes the optimization of the chromatographic separation and the post-column reaction and also the determination of NSEG with high resolution and sensitivity.

## EXPERIMENTAL

### *Apparatus*

A schematic diagram of the liquid chromatograph equipped with a post-column reaction detector is shown in Fig. 1. The liquid chromatograph consisted of a reciprocating piston pump (Model 655; Hitachi, Tokyo, Japan), a gradient controller (Model L-5000; Hitachi), a variable-wavelength UV–VIS detector (Uvidec 100-VI; Japan Spectroscopic, Tokyo, Japan), an injector with a 100-$\mu$l loop (Model 7125; Rheodyne, Berkeley, CA, U.S.A.) and a circulator (Model FE; Haake, Karlsruhe, Germany) for column temperature control. The injector was heated at 60°C with a specially designed apparatus [12]. The post-column reaction detector consisted of an acid-resistant pump (NP-S-253; Nihon Seimitsu, Tokyo, Japan), a three-channel reaction pump (655A-13; Hitachi), a constant-temperature circulating bath (Model D1-L; Haake) for condensation reaction and two constant-temperature reaction baths (NRB-15; Nihon Seimitsu) for hydrolysis and the colour development reaction. The post-column reaction coil was made of Teflon tubing (0.5 mm I.D.). Chromatograms, peak areas and retention times were obtained by using a data processor (D-2000; Hitachi).

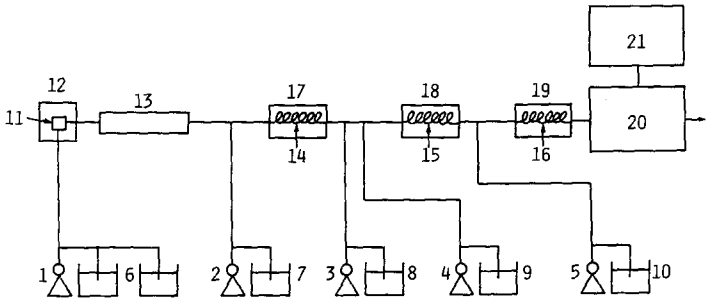


Fig. 1. Schematic diagram of the liquid chromatograph 1–5 = Pumps; 6 = eluent reservoirs; 7 = reservoir for potassium hydroxide reagent; 8 = reservoir for ONPH reagent; 9 = reservoir for EDC reagent; 10 = reservoir for sodium hydroxide reagent; 11 = sample injector; 12 = injector heater; 13 = analytical column; 14 = reaction coil (10 m × 0.5 mm I.D.); 15 = reaction coil (20 m × 0.5 mm I.D.); 16 = reaction coil (10 m × 0.5 mm I.D.); 17 = reaction bath (120°C); 18 = water-bath (70°C); 19 = reaction bath (100°C); 20 = UV–VIS detector; 21 = data processor.

*Reagents.*

Hitachi gel 3057, which is an ODS packing of average particle diameter 3 $\mu$m, was used as the stationary phase. Authentic fatty acids, monoacylglycerols and triacylglycerols were purchased from Sigma (St. Louis, MO, U.S.A.), methanol and ethanol of HPLC grade from Kanto Chemical (Tokyo, Japan), 2-nitrophenylhydrazine hydrochloride from Tokyo Chemical Industry (Tokyo, Japan), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Wako (Osaka, Japan), sorbitan fatty acid esters (Wako practical grade) from Wako and sucrose fatty acid esters from Ryoto (Tokyo, Japan) and Dai-ichi Kogyo Seiyaku (Kyoto, Japan). Other reagents were of analytical-reagent grade.

In the post-column reactor, the following reagents were used: for the hydrolysis of NSEG, 0.45 *M* potassium hydroxide in ethanol–water (50:50); for the condensation of ONPH and fatty acids, 0.035 *M* ONPH in 0.25 *M* hydrochloric acid and 0.15 *M* EDC in 6% pyridine; and for the colour development reaction, 1.5 *M* sodium hydroxide. These reagents were renewed each day.

*Procedure for the manual ONPH method*

To 4 ml of 100 ppm myristic acid in methanol–water (85:15) containing 5.7 m*M* triethylamine (TEA) are added 1 ml of 0.45 *M* potassium hydroxide in ethanol–water (50:50) and 2 ml of ONPH and EDC solution and the mixture is heated at 60°C for 30 min. After cooling, 2 ml of sodium hydroxide solution are added and the mixture is heated at 80°C for 10 min. After cooling, the absorbance of the resulting solution is measured at 550 nm.

*Post-column reactor*

As shown in Fig. 1, potassium hydroxide solution (reservoir 7) is added at a flow-rate of 0.2 ml/min to the eluate from the chromatographic column and passed through PTFE tubing (reaction coil 14; 10 m × 0.5 mm I.D.) into a reaction bath (bath 17) kept at 120°C, where NSEG are hydrolysed to form fatty acids. Then ONPH and EDC solution (reservoirs 8 and 9) are added at a rate of 0.4 ml/min and passed through PTFE tubing (reaction coil 15; 20 m × 0.5 mm I.D.) in a circulating bath (bath 18) kept at 70°C, where fatty acids are condensed with ONPH. Sodium hydroxide solution (reservoir 10) is added to the flow at a rate of 0.4 ml/min, and the mixture is passed through PTFE tubing (reaction coil 16; 10 m × 0.5 mm I.D.) in a reaction bath (bath 19) kept at 100°C, where the blank absorbance is reduced and a violet colour of the hydrazide is developed. Finally, the flow is passed through the UV–VIS detector set at 550 nm.

*Procedure for HPLC*

A stainless-steel column (150 mm × 4.6 mm I.D.) packed with Hitachi gel 3057 was used and kept at 50°C. The mobile phases used were methanol–water (85:15) containing 5.7 m*M* TEA (solvent A) and ethanol–methanol (75:25) containing 5.7 m*M* TEA (solvent B) at a flow-rate of 0.8 ml/min. The analysis was done according to the gradient programme shown in Table I. A sample containing 10–500 $\mu$g of NSEG dissolved in 5–50 $\mu$l of ethanol or isopropanol was injected into the HPLC system.

TABLE I

GRADIENT ELUTION PROGRAMME

| Time (min) | Solvent A (%) | Solvent B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 5 | 60 | 40 |
| 10 | 28 | 72 |
| 15 | 12 | 88 |
| 20 | 4 | 96 |
| 25 | 0 | 100 |
| 45 | 0 | 100 |
| 45.1 | 100 | 0 |
| 70 | 100 | 0 |

## RESULTS AND DISCUSSION

### *Optimization of separation*

The determination of NSEG by HPLC has been investigated in the reversed-phase mode using an ODS column and mixtures of isopropanol, methanol and water as the mobile phase [6,7]. In this study, Hitachi gel 3057 was chosen as the stationary phase because of the high resolution obtained. Mixtures of acetonitrile, methanol, ethaol, isopropanol and water were investigated as mobile phases. Among these solvents, acetonitrile was not suitable as it was hydrolysed to acetic acid, which gave a high blank absorbance. Poor resolutions were obtained by using isopropanol–water and ethanol–water mobile phases. Methanol–water could not elute esters higher than the triester. Finally, methanol–ethanol–water was adopted as the mobile phase because of the high resolution and good compatibility with the post-column reaction.

Gradient elution was necessary to separate NSEG from the mono- to pentaester simultaneously. The mobile phase composition and the gradient programme were determined as described under Experimental, taking the resolution and baseline drift into account. Under these conditions, sorbitan fatty acid esters from the mono- to tetraester and sucrose fatty acid esters from the mono- to pentaester could be simultaneously determined. A small amount of triethylamine was added to the mobile phase in order to eliminate the interferences from free fatty acids, as will be described later.

### *Optimization of post-column reaction*

In order to investigate the post-column reaction, each reaction was optimized in the order of hydrolysis, condensation and colour development reaction.

The condensation and colour development reaction were investigated by the manual ONPH method with myristic acid instead of NSEG as a sample.

### *Optimization of hydrolysis reaction*

Optimization of the hydrolysis reaction was investigated with triacylglyerol (tristearin) as a standard sample, which is difficult to hydrolyse [13] as a pure standard of the tetraester of sorbitan fatty acid esters and the pentaester of sucrose fatty acid

esters could not be obtained. If hydrolysis of tristearin is performed perfectly, one mole tristearin produces three moles of stearic acid. Therefore, the yield of hydrolysis was expressed as the relative peak area of tristearin to stearic acid of three times mole amount. With the tube length fixed at 10 m, the effects of the concentration of potassium hydroxide and reaction temperature on the relative peak area were investigated. The relative peak area increased with increase in concentration of potassium hydroxide and reached nearly 100% above 0.40 *M* potassium hydroxide. Therefore, 0.45 *M* potassium hydroxide was used for the hydrolysis reaction. As the relative peak area decreased at ethanol contents below 40% in the potassium hydoxide solution, the ethanol content was fixed at 50%. The temperature selected for the hydrolysis reaction was 120°C, as the relative peak area reached nearly 100% above 110°C.

Under these conditions, good linearities of concentration *versus* peak area were observed for NSEG, as will be described later. Further, the ester compositions of NSEG obtained by using these conditions were the same as those obtained by using a higher hydrolysis temperature and were in good agreement with the literature data and supplier's data, as will be described later. These results indicate that these conditions are suitable for NSEG, all of which are hydrolysed at the same rate. On the basis of these results, the conditions adopted for the hydrolysis reaction were those given under Experimental.

*Optimization of condensation reaction*

Fig. 2 shows the effect of the concentration of ONPH on the absorbance at 550 nm. The absorbance increased with increase in the concentration of ONPH and reached a nearly constant value above 0.02 *M*. Therefore, the concentration of ONPH was fixed at 0.035 *M*. The concentration of EDC adopted was 0.15 *M*, as the absorbance reached nearly constant value above 0.08 *M*.

Fig. 3 shows the effect of heating time and reaction temperature on the absorbance at 550 nm. As a high reaction rate is required in the post-column reaction, the reaction temperature was set at 70°C. The pH in the condensation reaction was

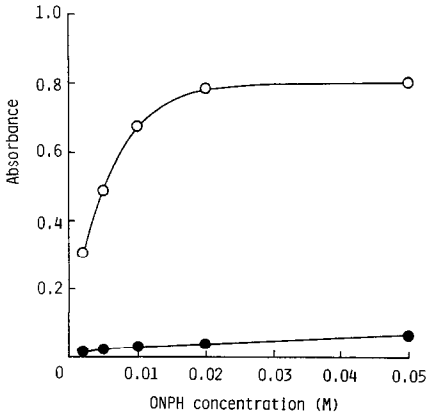


Fig. 2. Effect of concentration of 2-nitrophenylhydrazine on the absorbance at 550 nm. ○ = 100 ppm of myristic acid; ● = reagent blank.

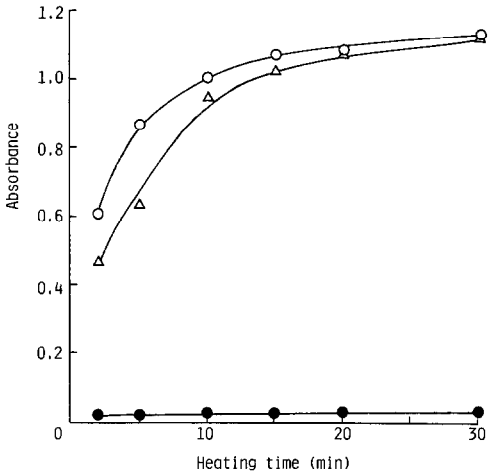


Fig. 3. Effect of heating time and reaction temperature on the absorbance at 550 nm. △ = 100 ppm of myristic acid, reaction temperature 40°C; ○ = 100 ppm of myristic acid, reaction temperature 60°C; ● = reagent blank, reaction temperature 60°C.

adjusted with pyridine–hydrochloric acid buffer, according to Horikawa and Tamimura [10]. It was found that the response of this post-column reaction detector varied under the gradient conditions because of the variation of the pH in the condensation reaction. Therefore, the concentrations of pyridine and hydrochloric acid were

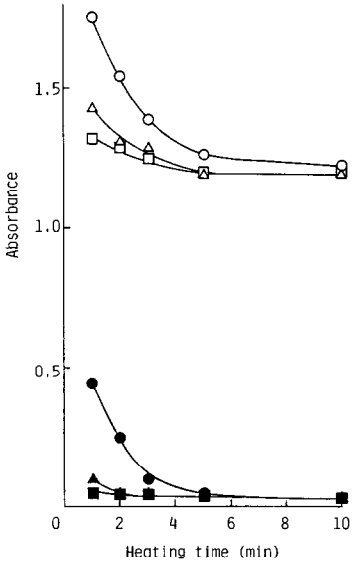


Fig. 4. Effect of concentration of sodium hydroxide and heating time on the absorbance at 550 nm (reaction bath temperature 80°C). ○ = 100 ppm of myristic acid, NaOH concentration 0.5 *M*; △ = 100 ppm of myristic acid, NaOH conentration 1.0 *M*; □ = 100 ppm of myristic acid, NaOH concentration 1.5 *M*; ● = reagent blank, NaOH concentration 0.5 *M*; ▲ = reagent blank, NaOH concentration 1.0 *M*; ■ = reagent blank, NaOH concentration 1.5 *M*.

investigated so as to minimize the variation of the response as will be described later. Finally, the effect of the length of Teflon tubing on the condensation reaction (peak area) was investigated and the optimum was determined to be 20 m. On the basis of these results, the conditions adopted for condensation reaction were those given under Experimental.

*Optimization of colour development reaction*

After the condensation reaction, the reaction mixture is made strongly alkaline and heated to develop a violet colour of the hydrazide and reduce the blank absorbance. Fig. 4 shows the effect of the concentration of sodium hydroxide and heating time on the absorbance at 550 nm. The blank absorbance decreased with increase in concentration of sodium hydroxide and reaction temperature. Therefore the concentration of sodium hydroxide adopted was determined 1.5 *M* and the reaction bath temperature was set at 100°C [11]. On the basis of these results, the conditions adopted for the colour development reaction were those given under Experimental.

*Response and reproducibility of the detector*

The ONPH method has been used for the analysis of water-soluble carboxylic acids, whereas fatty acids are not water soluble. Therefore, the linearity of concentration *versus* response for fatty acids was examined. Good linearities were observed from 0.5 to 250 μg for lauric, myristic, palmitic, stearic and oleic acid and the relative standard deviation of five measurements was found to be 0.80% for 20 μg of myristic acid. Moreover, the relative molar responses of lauric, palmitic stearic and oleic acid with respect to myristic acid were in the range 0.98–1.01, demonstrating equal molar responses.

It is most important to establish the molar response of the detector. Monomyristin was repeatedly injected at intervals of 5 min under the gradient conditions. Fig. 5 shows the relative peak area of monomyristin for each injection with respect to that of the first injection *versus* the analysis time. The response of the detector varies

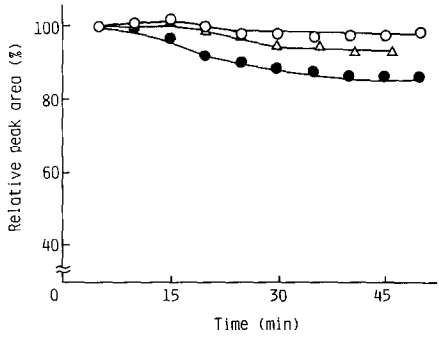


Fig. 5. Effect of concentration of hydrochloric acid in ONPH solution on the response of the detector to monomyristin for gradient analysis. Monomyristin (40 μg) was repeatedly injected at intervals of 5 min under the gradient conditions. Mobile phases: methanol–water (85:15) containing 5.7 m*M* TEA (solvent A) and ethanol–methanol (75:25) containing 5.7 m*M* TEA (solvent B). Pyridine concentration in EDC solution, 6%. HCl concentration in ONPH solution: (○) 0.25; (△) 0.30; (●) 0.40 *M*.

with the hydrochloric acid concentration in the ONPH solution and the degree of the variation is suppressed within 3% with 0.25 *M* hydrochloric acid. This result indicates that NSEG from the mono- to pentaester could be detected with a molar response regardless of the mobile phase composition. On the other hand, it is known that the low solubility of tri-, tetra- and pentaesters in the mobile phase decreases their molar response [12]. Therefore, the temperature of the injection port is maintained at 60°C with a specially designed apparatus [12]. The detection limit is 1 $\mu$g of monomyristin.

*Application*

NSEG usually contain small amount of free fatty acids, which overlap with the peaks of the monoesters and interfere in the analysis. This interference was eliminated by the addition of a small amount of triethylamine to the mobile phase and fatty acids were eluted at the void volume, as shown in Fig. 6. Fig. 7 shows chromatograms of commercial sorbitan fatty acid esters and sucrose fatty acid esters obtained by the proposed method. Each peak group was trapped immediately after the column and was identified by TLC [1,3]. The separated peaks in the peak groups for each ester will be due to the alkyl distribution of fatty acid moieties and positional isomers of NSEG [8]. However, their identification was difficult except for the monoester of sucrose fatty acid esters because no authentic standard sample was available. The identification of the monoester of sucrose fatty acid esters shown in Fig. 7 was done by comparing the chromatograms in Fig. 7C, D and E with their fatty acid compositions according to Jaspers *et al.* [8]. Good linearities of concentration *versus* peak area were observed from 10 to 500 $\mu$g for sorbitan fatty acid esters and sucrose fatty acid esters and the relative standard deviations for five measurements were in the range 1.1–2.0% for 200-$\mu$g amounts.

The results of ester distribution analyses of commercial sucrose fatty acid esters by the proposed method are summarized in Table II. This method affords a molar response to fatty acids produced by the hydrolysis of NSEG as described above. Therefore, it is necessary to correct the peak areas with the corresponding ester value to obtain the molar composition of NSEG from the mono- to pentaester. In Table II,

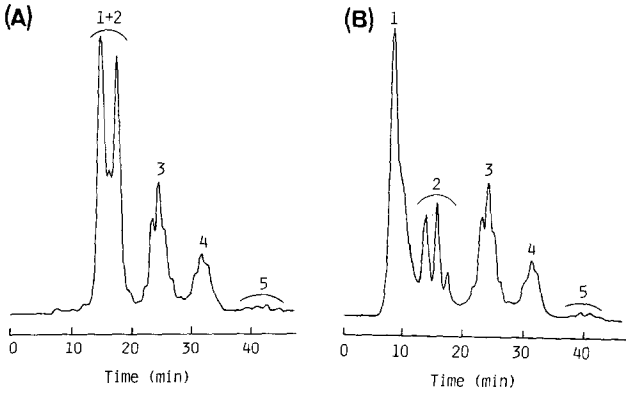


Fig. 6. Chromatogram of a mixture of sorbitan monostearate and fatty acids ($C_{16:0}$, $C_{18:0}$) using (A) eluents without triethylamine and (B) eluents with triethylamine. 1 = Fatty acid; 2 = monoester; 3 = diester; 4 = triester; 5 = tetraester.

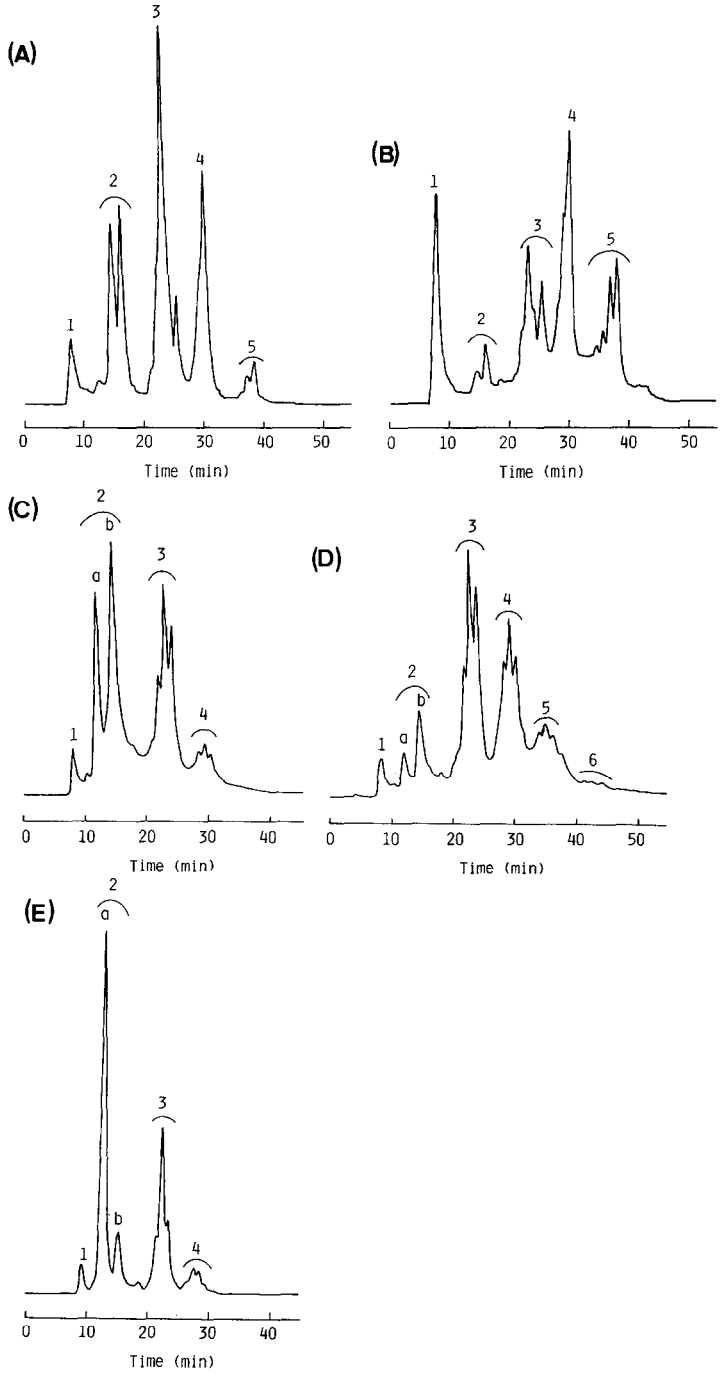


Fig. 7. Analysis of commercial sorbitan fatty acid esters and sucrose fatty acid esters. (A) Sorbitan monooleate; (B) sorbitan trioleate; (C) Ryoto sugar ester S-1570; (D) Ryoto sugar ester S-370; (E) Ryoto sugar ester P-1570. 1 = Fatty acid; 2 = monoester (a, $C_{16:0}$; b, $C_{18:0}$); 3 = diester; 4 = triester; 5 = tetraester; 6 = pentaester.

TABLE II

RESULTS OF ESTER DISTRIBUTION ANALYSES OF COMMERCIAL SUCROSE FATTY ACID ESTERS BY THE PROPOSED METHOD

| Sucrose fatty acid ester | Concentration | Proposed method | | | | | Literature data[c] | | | | | Supplier's data[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mono- | Di- | Tri- | Tetra- | Penta- | Mono- | Di- | Tri- | Tetra- | Penta- | Mono- | Di–penta- | Fatty acid composition |
| Ryoto sugar ester O-1570[a] | mol-% | 77.3 | 19.8 | 2.9 | – | – | | | | | | 70 | 30 | Oleic acid |
| | wt.-% | 69.7 | 25.4 | 4.9 | – | – | | | | | | | | (~70%) |
| Ryoto sugar ester P-1570[a] | mol-% | 74.9 | 21.4 | 3.7 | – | – | | | | | | | | Palmitic acid/ |
| | wt.-% | 66.9 | 27.0 | 6.1 | – | – | | | | | | 70 | 30 | Stearic acid |
| | | | | | | | | | | | | | | (~70:30) |
| Ryoto sugar ester S-1570[b] | mol-% | 74.8 | 21.5 | 3.7 | – | – | | | | | | | | Hydrogenated |
| | wt.-% | 66.5 | 27.4 | 6.1 | – | – | | | | | | 70 | 30 | tallow[f] |
| | | (0.3)[e] | (0.8)[e] | (4.0)[e] | – | – | | | | | | | | |
| Ryoto sugar ester S-370[b] | mol-% | 30.0 | 36.6 | 23.3 | 8.7 | 1.4 | | | | | | | | Hydrogenated |
| | wt.-% | 20.1 | 35.0 | 29.0 | 13.3 | 2.6 | | | | | | 20 | 80 | tallow[f] |
| | | (2.8)[e] | (1.1)[e] | (1.5)[e] | (3.0)[e] | (9.9)[e] | | | | | | | | |
| DK ester F-160[a] | mol-% | 76.8 | 20.9 | 2.3 | – | | | | | | | | | Hydrogenated |
| | wt.-% | 69.2 | 26.9 | 3.9 | – | – | 71.0 | 24.0 | 5.0 | – | | 70 | 30 | tallow[f] |
| DK ester F-110[a] | mol-% | 60.5 | 30.9 | 7.6 | 1.1 | – | | | | | | | | Hydrogenated |
| | wt.-% | 50.4 | 36.4 | 11.6 | 2.0 | – | 50.0 | 36.0 | 12.0 | 2.0 | – | 50 | 50 | tallow[f] |
| DK ester F-50[a] | mol-% | 47.2 | 40.9 | 10.5 | 1.4 | – | | | | | | | | Hydrogenated |
| | wt.-% | 36.8 | 45.5 | 15.2 | 2.5 | – | 33.0 | 49.0 | 16.0 | 2.0 | – | 30 | 70 | tallow[f] |

[a] Mean of two analyses.
[b] Mean of five analyses.
[c] Ref. 14.
[d] Catalogue data.
[e] Relative standard deviation (%).
[f] Stearic acid and palmitic acid.

ester compositions (mol-%) were calculated with the values obtained by division of the peak area by the corresponding ester value. Ester compositions (wt.-%) are also given in Table II; these were calculated with the values obtained by the product of ester composition (mol-%) and the corresponding avarage molecular weight, which was calculated with the fatty acid composition obtained by GC analysis of the methyl ester of fatty acids produced by alkaline hydrolysis of NSEG. The ester compositions (wt.-%) in Table II are in good agreement with the literature data [14] and supplier's data. The relative standard deviations for five measurements were within 4% for NSEG, except for the pentaester, which is a minor component.

By using the proposed method, NSEG can be determined with high sensitivity and high selectivity and the ester distributions of NSEG can also be determined.

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