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Determination of regioisomeric distribution in carbohydrate fatty acid monoesters by LC–ESI-MS

Ignacio Pérez-Victoria,[a,b] Alberto Zafra[a] and Juan Carlos Morales[a,*]

[a]*Departamento de Química, Puleva Biotech, SA, 18004 Granada, Spain*
[b]*Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain*

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Abstract—A new LC–ESI-MS method for characterizing the regioisomeric distribution in carbohydrate monoesters with long-chain fatty acids is described. Sucrose monolaurate mixtures were used for development of the method. The surfactant nature and high polarity of these compounds make them appropriate analytes for being detected by electrospray-ionization mass spectrometry (ESI-MS). Despite the structural similarity of regioisomers, an excellent resolution of all regioisomers present in the different samples studied (sucrose monodecanoates, sucrose monolaurates, sucrose monopalmitates and melezitose monolaurates) is achieved. Reversed-phase liquid chromatography with isocratic acetonitrile–water mixtures was used for a proper separation of the analytes. Finally, the superiority of this chromatographic method for determining the regioselectivity in enzymatic carbohydrate acylation reactions, with respect to the typical methodology based on routine ^{13}C NMR spectroscopy, is also discussed.

Keywords: Oligosaccharide; Oligosaccharide fatty acid monoesters; Regioisomeric separation; Transesterification; Sucrose ester; LC–ESI-MS

1. Introduction

Sugar fatty acid esters are nonionic surfactants with broad applications in food, cosmetic and pharmaceutical industries.[1] Carbohydrate fatty acid monoesters are the most important group of this type of compounds because of their better water solubility compared to di-, tri- and higher ester derivatives. It is important to note that some of their properties depend on the acylation position. For example, regioisomeric sucrose fatty acid monoesters have different surfactant properties since they possess different CMC values.[2,3] Biological properties may also be different between regioisomers. In fact, better antimicrobial activity has been observed for 6-*O*-palmitoylraffinose than for 1″-*O*-palmitoylraffinose when tested against *Bacillus subtilis*.[4] On the other hand, when the monoacylation of a carbohydrate is a protective step within a synthetic sequence, the achievement of the maximum regioselectivity is a fundamental goal.[5] Consequently, it is important to have precise and sensitive analytical methodologies for the determination of the regioisomeric distribution in samples of carbohydrate fatty acid monoesters prepared by chemical or enzymatic synthesis.[6,7] The information about the regioselectivity of the acylation reaction is obtained from such type of analysis.

Although an example has been published of sucrose fatty acid ester analysis by high-temperature gas chromatography,[8] HPLC is a more convenient technique since it does not require previous derivatization of the sample. As this type of compound cannot be detected by UV absorption, refractive index or evaporative light-scattering detection is typically employed. Curiously, the majority of analytical methods that have appeared in the literature describing the synthesis of these types of compounds are focused on the monitoring of the starting carbohydrate and on the analysis of the degree of substitution rather than on the analysis of

* Corresponding author at present address: Instituto de Investigaciones Químicas, CSIC, 49 Americo Vespucio, 41092 Seville, Spain. Tel.: +34 954 489 568; fax: +34 954 460 565; e-mail: jcmorales@iiq.csic.es

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the regioisomeric compounds that are obtained.[9] Queneau and co-workers have described with certain detail the HPLC analysis of regioisomeric sucrose fatty acid monoesters in a study about acyl group migrations in basic media.[10] Nevertheless, the information on the enzymatic regioselectivity in acylation reactions of carbohydrates reported by majority of authors is usually based exclusively on routine ^{13}C NMR spectra.[11]

In this work, a new method for the analysis of regioisomeric distribution in carbohydrate long-chain fatty acid monoesters based on HPLC with ESI mass spectrometry detection is described. At the same time, the superiority of this method in the determination of the regioselectivity of an enzymatic carbohydrate acylation with respect to the classic methodology based on routine ^{13}C NMR spectra is also discussed.

2. Results and discussion

2.1. HPLC–ESI-MS of carbohydrate fatty acid mono esters

HPLC chromatography coupled to mass spectrometry detection is a very useful analytical technique due to its high sensitivity and the structural information that can be obtained about the analytes.[12] The electrospray-ionization (ESI) interface is very appropriate for the analysis of polar nonvolatile compounds. For example, underivatized oligosaccharides have been separated and characterized by LC–ESI-MS.[13] Due to the ESI mechanism, compounds of surfactant nature present a high response in this class of mass spectrometry detectors, and very low detection limits can be achieved.[14] For example, nonionic surfactants of the family of polyethers have been analyzed by HPLC–ESI-MS.[15] Carbohydrate monoesters with long-chain fatty acids are another type of nonionic surfactant, and, consequently, their HPLC analysis with ESI-MS detection is very appropriate and presents obvious advantages in terms of operability and sensitivity with respect to the refractive index detection.

The development of an HPLC–ESI-MS method for characterizing the regioisomeric distribution in sugar fatty acid monoesters prepared by chemical or enzymatic synthesis involves, on one hand, an optimization of the ionization conditions for the analytes in the ESI source, and, on the other hand, an optimization of the chromatographic separation of the different regioisomers.

For development of the method we employed two commercial sucrose monolaurates, each one comprising a different regioisomeric mixture, and sucrose monolaurates prepared in our laboratory using chemical or enzymatic catalysis following previously described procedures (see Experimental).

2.2. Ionization conditions

In order to optimize the ionization conditions, a diluted aqueous sample (ca. 0.1 mg L^{-1}) of commercial sucrose monolaurate (Fluka) was injected directly in the mass spectrometer with the ESI probe operating in the positive-ionization mode. Under these conditions, polar analytes without basic functional groups are usually detected as adducts with sodium ions, even though sodium salts have not been added to the sample (low concentration of sodium can be derived from glassware and storage bottles, or can be present as impurities even in analytical grade solvents).[14] Consequently, it is expected that the observable molecular ion peak for sucrose monolaurate has m/z 547 ($[M+Na]^+$).

Figure 1 confirms that, without adding sodium salts to the sample, sucrose monolaurate forms stable sodium adducts. Pseudomolecular ions and very few fragments are produced when working in ESI conditions since this is a very soft ionization method. Although we are working with a single quadrupole mass spectrometer in which tandem MS is not possible, in-source fragmentation may be induced by increasing the voltage on the sampling cone. The low-energy collisions in the CID region (the intermediate vacuum region between the sampling and extraction cones) often involve breaking of the weakest bonds in the original ion.[16] In the case of sucrose

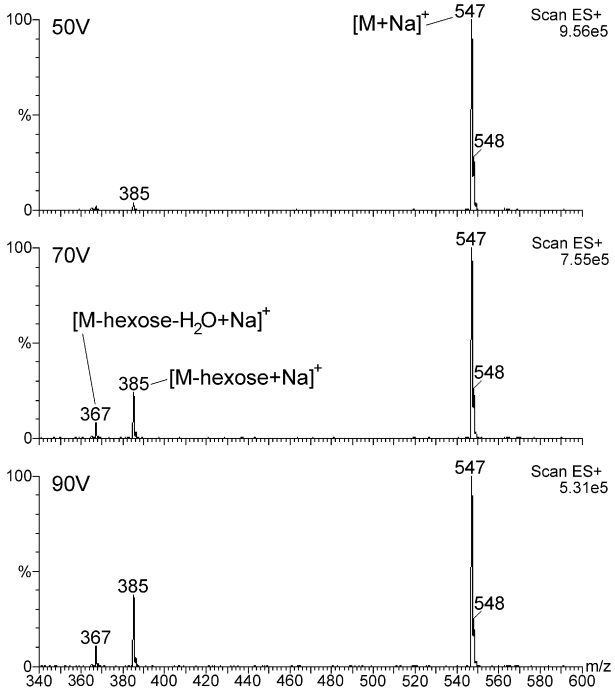


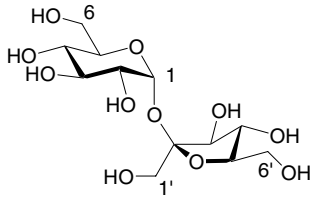
Figure 1. Mass spectra in the ESI positive-ion mode showing the in-source cone voltage induced fragmentation of commercial sucrose monolaurate (Fluka). Numbers in the upper right corner reflect the total ion counts.

monolaurate, the in-source cone voltage induced fragmentation is shown in Figure 1.

The ions at *m*/*z* 385 and 367 originate from the molecular ion $[M+Na]^+$ by loss of a hexose and a [hexose$-H_2O$] neutral fragment due to glycosidic bond cleavage. This fragmentation pattern had been already observed in the FAB MS/MS collision-induced spectrum of the same commercial compound.[17] It is important to note that spectra in Figure 1 really correspond to a mixture of regioisomers, and this must be taken into account for the development of the chromatographic separation method since fragment abundance could be different among regioisomers.

2.3. Chromatographic separation and development of the LC–ESI-MS method

The nonreducing disaccharide sucrose may theoretically yield eight different regioisomeric monoesters with a given fatty acid. Queneau and co-workers have tackled the separation of regioisomeric sucrose fatty acid monoesters by HPLC using an amino-bonded stationary phase with refractometric detection.[10] Unfortunately, neither chromatograms nor any comments regarding the chromatographic resolution obtained appear in this publication. This type of stationary phase is typically employed for the separation of oligosaccharides. In this work, we have approached the separation using a reversed-phase column taking advantage of the lipophilicity of the acyl fatty acid chain in the analytes (see Experimental).



Sucrose

The achievement of a satisfactory chromatographic resolution is a significant challenge since all the different regioisomeric carbohydrate fatty acid monoesters have the same hydrophilic–lipophilic balance (HLB) and are structurally very similar. For the optimization of the separation conditions, the same commercial sample of sucrose monolaurate (Fluka) was used again. The establishment of the relative abundance of each regioisomer in the sample requires that all of them have the same response in the detector, that is, all of them ionize equally. This fundamental requirement obliges one to use isocratic conditions in the mobile phase and a constant flow rate to avoid any possible ionization differences among regioisomers due to a solvent effect. As the analytes easily form stable sodium adducts that can be detected in ESI positive-ion mode in the absence of sodium salts, there is no need to use any additive in the mobile phase. On the other hand, the use of mild basic buffers could promote intramolecular acyl-group migrations during the elution, masking the actual regioisomeric composition of the starting sample.[10] The flow rate was set to 0.3 mL/min, low enough to get a satisfactory sensitivity. After different trials using methanol–water and acetonitrile–water mixtures, we found that the optimum mobile phase for separating the different regioisomers present in the commercial sucrose monolaurate (Fluka) was 48:52 acetonitrile–water. The resolution achieved under these conditions is very satisfactory (Fig. 2).

Chromatograms in Figure 2 show eight different peaks corresponding to the eight possible regioisomers. The signal-to-noise ratio is much larger in SIR (selected-ion recording) chromatograms than in the scan-mode chromatograms, and thus they are more appropri-

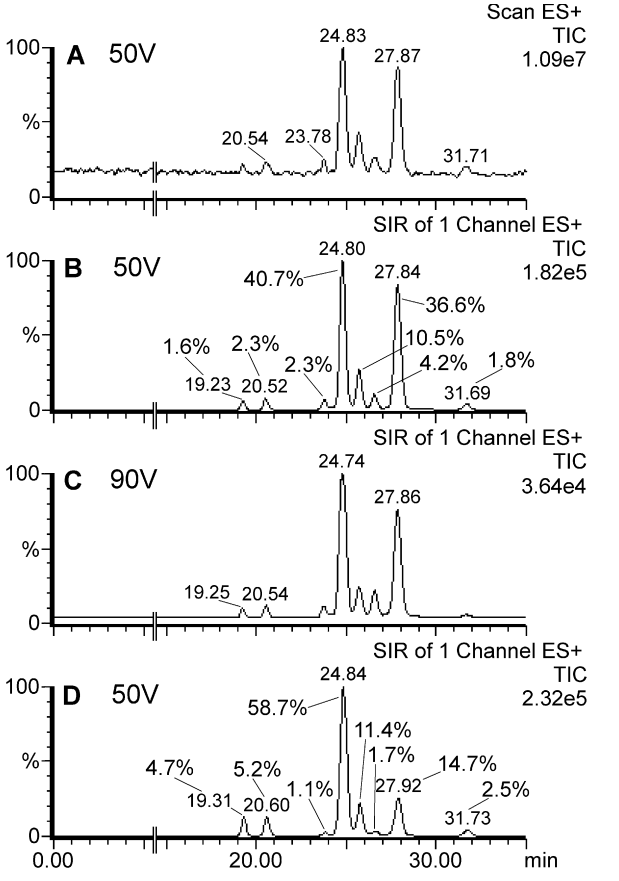


Figure 2. HPLC–ESI-MS chromatograms of commercial sucrose monolaurate (Fluka) (A, B and C) and sucrose monolaurate purified from the commercial L-1695 (Mitsubishi Kagaku Foods Corp.) (D). Detection was done in the scan mode, *m*/*z* from 350 to 570 (A) and the SIR mode selecting the following ions: *m*/*z* 547 (B and D) and *m*/*z* 385 (C). Cone voltages are indicated in each chromatogram. Column: Spherisorb ODS2 (250 mm × 4.6 mm, 3 μm). Mobile phase: 48:52 acetonitrile–water; flow rate at 0.3 mL/min.

ate for peak integration. The aforementioned possible differences in fragment intensities among different regioisomers can be observed when comparing the peaks at 25.5 and 27 min in chromatograms B (m/z 547, 50 V) and C (m/z 385, 90 V). For this reason, it is more convenient to perform the integration in the SIR chromatogram obtained selecting the molecular ion $[M+Na]^+$ at a cone voltage of 50 V, ensuring a maximum intensity for the molecular ion and minimum fragmentation, thus avoiding differences in response among regioisomers.

Meeting the requirement of equal response, the relative abundance of each regioisomer in the sample can be obtained by integration according to the following equation:

$$\%\ \text{regioisomer X} = \left[(\text{Area peak regioisomer X})/\left(\sum \text{Area all peaks}\right)\right] \times 100$$

The regioisomeric distribution in the commercial sucrose monolaurate (Fluka) was thus determined (Fig. 2, chromatogram B). Likewise, chromatogram D in Figure 2 shows the regioisomeric distribution in the purified sucrose monolaurate obtained from the commercial L-1695 (Mitsubishi Kagaku Food Corp.). In all cases the automatic integration algorithm of MassLynx 3.5 software was used.

Differentiation of regioisomeric sucrose monoesters by ionspray tandem mass spectrometry has been reported.[18] Such an approach could be used for a tentative identification of the different peaks in the chromatograms if the mass spectrometer coupled to the chromatograph were able to perform MS/MS analyses. When working with a single quadrupole mass spectrometer, such analysis is not possible. In order to identify the identity of the regioisomers corresponding to some peaks of the chromatograms, we compared the retention times in the previous chromatograms with the retention times observed in the chromatograms of different samples of sucrose monolaurate that contain a main known regioisomer. Each of these samples was prepared in our laboratory according to previously described procedures. The main regioisomer obtained in each case was: 6-*O*-lauroylsucrose with catalysis by *Thermomyces lanuginosus* lipase (Lipozyme TL IM),[19] 1′-*O*-lauroylsucrose with catalysis by the crude protease of *B. subtilis* (Proteinase N),[20–22] 6-*O*-lauroylsucrose and 6′-*O*-lauroylsucrose in almost equimolar proportion with catalysis by *Candida antarctica* lipase B (Novozym 435),[23] 2-*O*-lauroylsucrose with basic catalysis by Na_2HPO_4.[6] Figure 3 shows the comparison between the chromatogram obtained for the commercial sucrose monolaurate (Fluka) and the different samples prepared in our laboratory. Reproducibility of retention times is excellent. This allows the identification of 6-*O*-lauroylsucrose

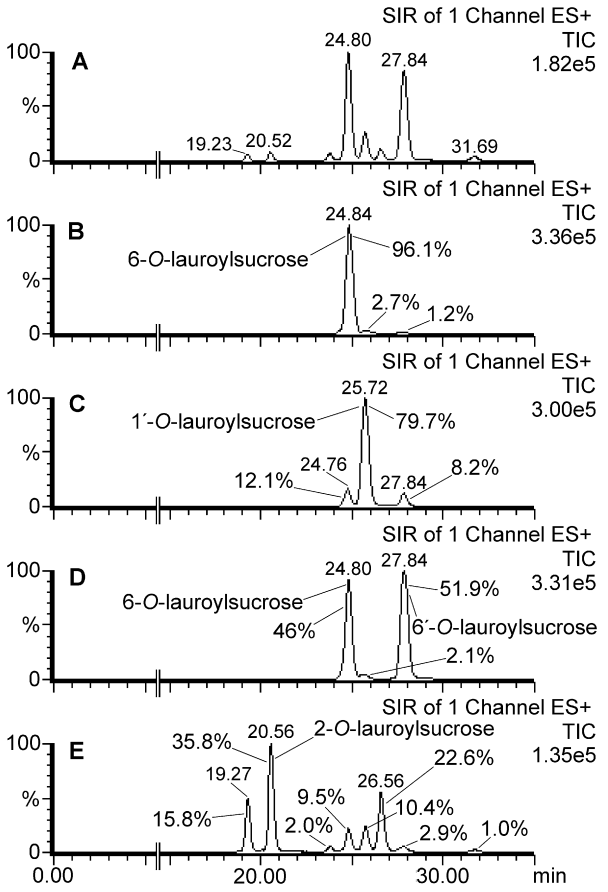


Figure 3. Comparison of the HPLC–ESI-MS chromatogram of the commercial sucrose monolaurate (Fluka) (A) with that obtained for the sample synthesized using as biocatalysts *T. lanuginosus* lipase (B), *B. subtilis* protease (C), *C. antarctica* lipase B (D) and Na_2HPO_4 as basic catalyst (E). Main regioisomers in each chromatogram and relative abundance are indicated. Detection was done in the SIR mode selecting ion m/z 547 and using a cone voltage of 50 V. Column: Spherisorb ODS2 (250 mm × 4.6 mm, 3 μm). Mobile phase: 48:52 acetonitrile–water; flow rate at 0.3 mL/min.

and 6′-*O*-lauroylsucrose as the main regioisomers present in the commercial sucrose monolaurate (Fluka). In the corresponding commercial sample from Mitsubishi Kagaku Food Corp., 6-*O*-lauroylsucrose is the main component. Chromatograms B–D in Figure 3 show that this HPLC–MS method is a very powerful tool for determining the enzymatic regioselectivity in biocatalyzed sucrose acylation reactions.

Next, we analyzed the regioisomeric distribution in a commercial sample of sucrose monodecanoate (Fluka), and a sample of purified sucrose monopalmitate from the commercial D-1615 (Mitsubishi Kagaku Food Corp.), in order to show how this LC–ESI-MS method can be adapted to other sucrose fatty acid monoesters with different HLB. The only required modifications in the method are variations in the mobile phase composition (higher acetonitrile proportions are used for the longer acyl chains of the monoester), selecting the corresponding molecular ion $[M+Na]^+$ in each case. Figure 4

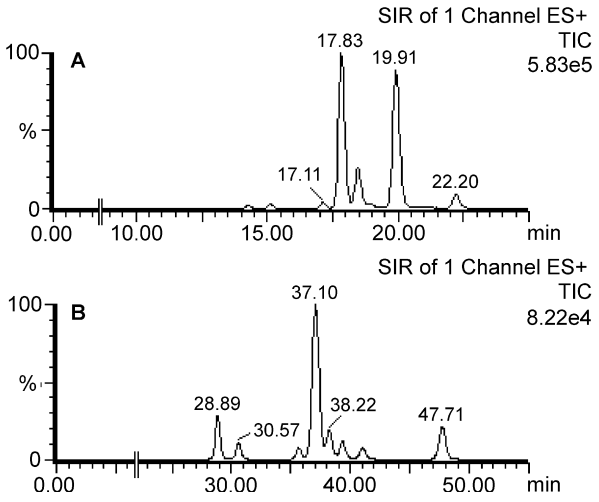
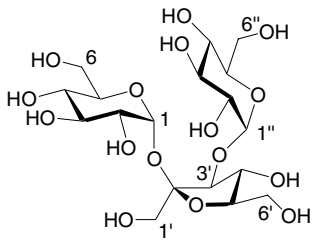


Figure 4. HPLC–ESI-MS chromatograms of the commercial sucrose monodecanoate (Fluka) (A) and sucrose monopalmitate purified from the commercial D-1615 (Mitsubishi Kagaku Foods Corp.) (B). Detection was done in the SIR mode selecting ion *m*/*z* 519 (A) or *m*/*z* 603 (B) using a cone voltage of 50 V. Column: Spherisorb ODS2 (250 mm × 4.6 mm, 3 μm). Mobile phase: 43:57 acetonitrile–water (A) or 62:38 acetonitrile–water (B); flow rate at 0.3 mL/min.

shows the separation of the eight regioisomers present in both samples. The similar chromatographic profile compared with the corresponding monolaurates of the same commercial brands clearly proves that the same synthetic procedure for the acylation of sucrose has been employed in both cases.

2.4. LC–ESI-MS versus routine ^{13}C NMR spectroscopy as a method for determining the regioselectivity of enzyme-catalyzed carbohydrate acylations

The use of routine ^{13}C NMR spectroscopy is absolutely necessary for the elucidation of the substitution position in the main regioisomer obtained in the synthesis of carbohydrate monoesters.[24] At the same time, ^{13}C NMR spectroscopy has been currently used to obtain the regioselectivity data of carbohydrate acylation reactions catalyzed by enzymes.[11] A comparison of this commonly used technique for determining the acylation regioselectivity with the LC–ESI-MS methodology herein described was carried out. A recently reported example by our laboratory was used: the acylation of



Melezitose

the nonreducing trisaccharide melezitose with vinyl laurate catalyzed by *C. antarctica* lipase B, Novozym 435.[25] There are eleven different acylation positions for melezitose; nevertheless, the four primary hydroxyl groups are the more reactive in the acylation reaction catalyzed by the lipase.

For the analysis of the isolated melezitose monolaurate fraction, we used slight modifications in the LC–ESI-MS method. Flow rate was increased to 1.1 mL/min, using a postcolumn splitter so that 0.2 mL/min entered the detector. The proportion of water in the mobile phase was increased to a final ratio of 35:65 acetonitrile–water. The chromatogram was acquired in the SIR mode, selecting the ion at *m*/*z* 709 working with a cone voltage of 40 V (50 V produces more fragmentation than in the previous cases). Figure 5A shows the regioisomeric distribution in the sample. There are three regioisomers present; however, only two appear in the routine ^{13}C NMR spectrum (Fig. 5B). The methylene region shows the signals of the carbons corresponding to the four primary hydroxyl groups in the trisaccharide (those more reactive with the lipase). Two groups of signals are clearly observed in this region: the four more intense signals corresponding to the main regioisomer and the four of low intensity corresponding to the next regioisomer present in abundance in the sample. Interestingly, the signals of the minor regioisomer present in the sample do not appear, probably because they are hidden in the background noise of the spectrum. This example clearly proves that the regioselectivity determined exclusively from routine ^{13}C NMR spectroscopy is less precise than that obtained from our LC–ESI-MS method.

Indeed, the high sensitivity achieved with the SIR detection mode allows the detection of regioisomers present in the sample in very small proportion. On the other hand, the intensities observed in a routine ^{13}C NMR spectrum are highly dependent on the number of acquisition scans, and regioisomers present in small proportion in the sample may not even appear in the spectrum. In addition, a given carbon of the carbohydrate ester may present different relaxation times depending on the particular regioisomer. Most likely, these aspects make the regioselectivity data obtained by ^{13}C NMR more approximate and less accurate than the data that can be obtained with the LC–ESI-MS methodology.

In summary, we have developed a robust LC–ESI-MS method for the analysis of regioisomeric mixtures of carbohydrate fatty acid monoesters. The chromatographic resolution achieved using a reversed stationary phase is excellent, and the acquisition in the SIR mode allows an accurate integration. Although the use of routine ^{13}C NMR spectroscopy is necessary to establish the substitution position in the main regioisomer obtained in the synthesis of carbohydrate monoesters,[24] it is more convenient to use the chromatographic method

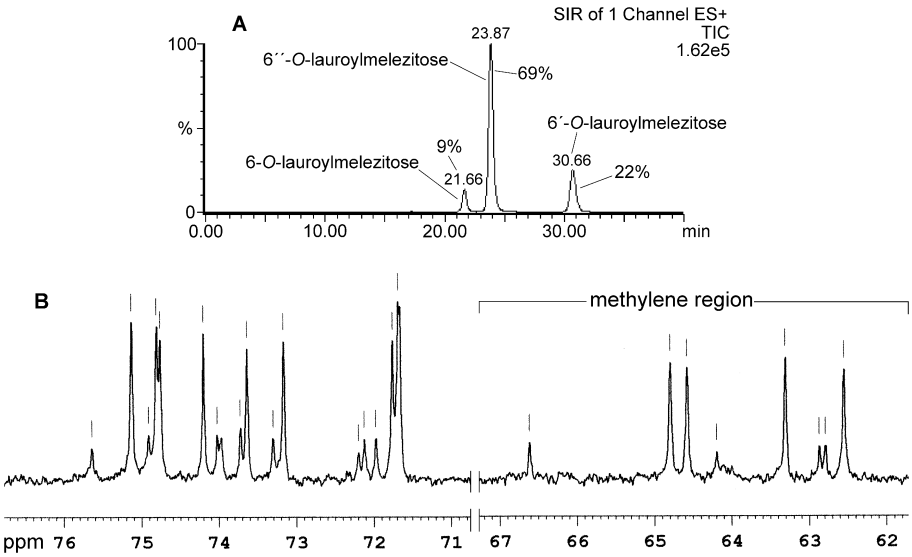


Figure 5. (A) HPLC–ESI-MS chromatogram showing the regioisomeric distribution in the isolated melezitose monolaurate prepared by catalysis with lipase Novozym 435. Detection was done in the SIR mode selecting ion *m/z* 709 using a cone voltage of 40 V. Column: Spherisorb ODS2 (250 mm × 4.6 mm, 3 μm). Mobile phase: 36:65 acetonitrile–water; flow rate at 1.1 mL/min (with postcolumn splitter). (B) Expansion of part of the routine ^{13}C NMR spectrum.

described in this work to determine the relative abundance of all regioisomers produced in the reaction, due to its higher precision and sensitivity.

3. Experimental

3.1. Chemicals and reagents

The following commercial samples were employed: sucrose monolaurate and sucrose monodecanoate from Fluka; sucrose monolaurate and sucrose monopalmitate purified as previously described from the sucroesters L-1695 and D-1615 from Mitsubishi Kagaku Foods Corporation.[26] The remainder of the sucrose monolaurates were prepared according to previously described procedures using different enzymatic or chemical catalysts: *T. lanuginosus* lipase,[19] *B. subtilis* crude protease,[20–22] *C. antarctica* lipase B,[23] and sodium dihydrogen phosphate.[6] The sample of melezitose monolaurate was prepared using catalysis by *C. antarctica* lipase B as described previously.[25]

All reagents used were of analytical grade unless specified otherwise. HPLC gradient-grade acetonitrile was purchased from Sharlab. Water was purified with a Milli-Q plus system (Millipore). Deuterated MeOH (99.9 atom %) for NMR experiments was purchased from Aldrich Chemical Co.

3.2. Liquid chromatography–mass spectrometry

LC–ESI-MS analyses were performed with a Waters Alliance 2695 separation module coupled to a Waters Micromass ZQ single quadrupole mass spectrometer. The electrospray-ionization (ESI) ion source was operated in the positive-ion mode. Chromatograms were acquired in the Scan mode (*m/z* range indicated in figures) and in the SIR mode (selecting ions indicated in figures). Nebulizing gas (N_2) flow was 60 L/h. Nitrogen drying gas was used at a flow rate of 300 L/h and 200 °C. MS parameters were optimized as follows: capillary voltage, 2.75 kV; extraction cone, 4 V; RF lens, 0.5 V; source temperature, 120 °C. Different sample cone voltages were used as indicated in the different figures.

HPLC separations were carried out on a Spherisorb ODS2 4.6 × 250 mm 3 μm column (Waters) operating at 40 °C. Isocratic acetonitrile–water mixtures were used as the mobile phase (ratio indicated in figures). The flow rate was set to 0.3 mL/min (for sucrose monoesters) or 1.1 mL/min with postcolumn splitting so that 0.2 mL/min entered the detector (for melezitose monolaurate). Samples for HPLC–ESI-MS analysis were prepared as diluted aqueous solution (ca. 1 mg L^{-1}) using ultrapure Milli-Q® water, filtered through 3-μm nylon filters by Millipore and analyzed immediately. Volume injection was 10 μL. MassLynx 3.5 software was used for data acquisition and processing.

3.3. Routine ^{13}C NMR spectroscopy

A routine ^{13}C NMR spectrum of melezitose monolaurate was acquired from a solution in CD_3OD (20 mg of product dissolved in 0.75 mL of solvent) prepared in a 5-mm tube. A Bruker ARX400 spectrometer was used, with the probe at room temperature (298 K). The spectrum was acquired using the standard pulse sequence and parameters of the instrument: 1200 scans of a sequence with

a 7 μs 90° excitation pulse, a delay of 1.5 s, and 2 s acquisition time (100 μs broadband proton decoupling).

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