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Non-aqueous capillary electrophoresis of the positional isomers of a sulfated monosaccharide

Stéphanie Descroix[a], Anne Varenne[a,*], Nicole Goasdoue[b], Joaquin Abian[c], Montse Carrascal[c], Régis Daniel[d], Pierre Gareil[a]

[a]*Laboratoire d'Electrochimie et Chimie Analytique, UMR CNRS 7575, ENSCP, 11 Rue Pierre et Marie Curie, 75231 Paris cedex 05, France*
[b]*Laboratoire de Structure et Fonction de Molécules Bioactives, UMR 7613, Université Paris 6, 4 Place Jussieu, 75251 Paris Cedex 05, France*
[c]*Unidad de Espectrometria de Masas Estructural y Biologica, IDIBAPS, IIBB-CSIC, E-08036 Barcelona, Spain*
[d]*Laboratoire de Recherches sur les Macromolécules, UMR CNRS 7540, Université Paris 13, Avenue Jean-Baptiste Clément, 93430 Villetaneuse, France*

Abstract

A non-aqueous capillary electrophoresis (NACE) method coupled to indirect absorbance detection has been developed for the separation of the three positional isomers of monosulfated fucose. The optimized electrolyte was composed of 12 m*M* ethanolamine, 2 m*M* trimesic acid buffer in a methanol–ethanol (1:1, v/v) mixture. As the retained electrolyte entails no separating agent other than the pH buffer, the NACE separation of the positional isomers has been ascribed mainly to selective ion-pairing with the electrolyte counter-ion and the possibility of a selective solvation effect in the alcohol mixture. In the absence of pure isomeric standards, peak identification was completed by MS and NMR spectroscopy and selective enzymatic desulfation. This method should be of interest for the structure elucidation of monosulfated fucose-based polysaccharides and for the screening of sulfoesterase of unknown activity.

Keywords: Non-aqueous capillary electrophoresis; Positional isomers; Sulfated fucose; Fucoidan; Carbohydrates

1. Introduction

The separation of small molecules presenting an identical charge-to-mass ratio is commonly considered a difficult and hazardous issue in capillary electrophoresis (CE), as this ratio is usually regarded as the major electrophoretic discriminating factor. Among such molecules are positional isomers, which are often preferably separated by chromatographic techniques. Alternatively, aqueous separation of charged positional isomers can be contemplated by CE whenever the compounds of interest present different weak acidic–basic behavior or can be selectively complexed by, for example, a neutral ligand. In addition, minute differences in electrophoretic migration velocities can be enhanced in the presence of an electroosmotic flow, dragging the analytes in the opposite direction to their migration direction (counter-electroosmotic mode), but this is at the expense of the analysis time [1].

Recently, non-aqueous capillary electrophoresis (NACE) has proven to be a very promising approach

*Corresponding author. Tel.: +33-1-5542-6372; fax: +33-1-4427-6750.
E-mail address: varenne@ext.jussieu.fr (A. Varenne).

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[2–4] for the separation of a large range of compounds, encompassing both ionic and neutral hydrophobic compounds, which are difficult to separate in aqueous electrolytes [5–9]. The use of organic solvents as separation medium opens up new ways to manipulate the separation selectivity, involving discriminating solvation effects, ion-pairing or modification of analyte ionization states due to shifts in acidity or complex formation constants. Recently, interest in NACE for the separation of molecules having similar structures or identical charge-to-mass ratios has been demonstrated [10,11]. According to the particular case, the other operational advantages of NACE are: (i) increased solubility of analytes sparingly soluble in purely aqueous electrolytes, (ii) reduction of Joule heating due to lower conductivities, implying the possible use of larger electric fields and thus improving the efficiency and reducing the analysis time, and (iii) modification of the electroosmotic flow without adding a surfactant.

Among the bioactive macromolecules, carbohydrates offer the greatest potential for information due to their incomparable variety of combinations and regioselective modification of their constitutive monomers. This huge structural diversity has given rise to the concept of an isomer barrier, which represents a major challenge in glycosciences [12]. Carbohydrate analysis [13] is recognized as one of the most challenging fields for the analytical chemist, considering the molecular sample complexity in terms of monomer composition, isomeric forms, degree of branching and polymerization, and difficulty in detection. Determination of the monosaccharide composition of complex carbohydrates is generally the first analytical request for carbohydrate characterization. Monosaccharide analysis has conventionally been performed by gas chromatography (GC) of volatile derivatives [14], normal-phase partition liquid chromatography and cation-exchange chromatography coupled to fluorescence detection or anion-exchange chromatography with pulsed amperometric detection [15] and polyacrylamide gel electrophoresis (PAGE) [16]. These methods usually require a relatively large amount of sample and, for some of them, time-consuming derivatization procedures, which, in addition, may further add to sample heterogeneity. Among the separation techniques, capillary electrophoresis offers powerful features that have led to exponential development and numerous applications, especially in the field of carbohydrates [17–19]. It allows on-line detection and requires a small amount of sample, keeping the consumption of carbohydrates to a minimum.

In the present paper, we describe the development of an effective method for the separation of the positional isomers of monosulfated fucose by NACE, employing no electrolyte additive (such as surfactant or complexing agent) other than a plain buffer. Fucose (6-deoxygalactose) is one of the most abundant deoxyhexoses occurring in biological structures (milk, antigens, glycolipids, glycoproteins), which suggests diverse biological functions. It has thus aroused interest because of its biochemical roles in various diseases. In addition to the neutral form, fucose also occurs in modified, mostly sulfated, forms, as in some bacterial and vegetal walls. Among these is fucoidan, a polysaccharide mainly constituted of α-linked sulfated L-fucopyranosyl units that can be extracted from the cell wall of marine brown algae. It is endowed with important properties and acts especially as a potent inhibitor in the coagulation cascade and the complement system [20]. However, its molecular structure, and hence the structural basis of its biological properties, still remain to be established [21]. Data have been reported in the literature concerning the position of the sulfate groups in algal fucoidan, the fucose residues being proposed to be sulfated at positions 2 and/or 4 and/or 3 [22]. New insights into the structure of fucoidan and the structure–activity relationships are expected from chemical and enzymatic depolymerization of the polysaccharide, leading to sulfated oligo- and monosaccharides [23]. Therefore, an analytical method able to quantitatively discriminate between the different sulfated fucose isomers appears to be of paramount biological importance.

2. Materials and methods

2.1. Materials

Monosulfated fucoses were kindly provided as a mixture of the three isomers (2-, 3- and 4-sulfated-L-fucose) by Dr. M. McLean (Grampian Enzymes,

Orkney, UK). The sulfoesterase from *Pecten maximus* was isolated and purified as previously described [24].

Other chemicals and reagents (ethanolamine and trimesic acid) were obtained from current commercial sources at the highest level of purity available. All aqueous solutions were prepared with ultrapure water produced by an Alpha Q laboratory water purification system (Millipore, Milford, MA, USA). The organic solvents used (methanol, ethanol, isopropanol, acetonitrile), of HPLC Ultra Gradient Grade, were obtained from Aldrich (Steinheim, Germany) and Prolabo (Paris, France). They were used without further purification.

2.2. Capillary electrophoresis

CE experiments were carried out with an HP^{3D} CE apparatus (Agilent, Waldbronn, Germany) equipped with an on-column diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. Data were handled by HP Chemstation software. Bare fused-silica capillaries, 40 cm (31.5 cm from inlet to detector)×50 μm I.D.×360 μm O.D., were from Beckman (Gagny, France). Samples were introduced in the hydrodynamic mode (30 mbar, 3 s). Under optimal non-aqueous separation conditions, separations were performed under a negative voltage (−20 kV) and the temperature in the capillary cartridge was set at 12 °C. Indirect absorbance detection was performed at 200 nm using trimesic acid as background chromophore. The acquisition rate was 10 points/s.

The final non-aqueous separation electrolyte was a 12 m*M* ethanolamine, 2 m*M* trimesic acid mixture (6 m*M* ionic strength) in a methanol–ethanol (50:50, v/v) solvent mixture. All electrolytes were filtered through 0.2 μm filter units before use. The monosulfated fucose mixture was 10^{-5} *M* in water and injected as described previously. New capillaries were conditioned by successive flushes with 1 and 0.1 *M* NaOH and then with water for 10, 5 and 10 min, respectively. Prior to each sample injection, the capillary was rinsed with the separation electrolyte for 5 min. Capillaries were rinsed with water and dried by air when not in use. Replicate injections were performed to check the short-term repeatability.

2.3. Micro-preparative CE

For the recovery of the monosulfated fucose fraction for MS–MS analysis, the sample tray of the CE instrument was used as a fraction collection system. The developed procedure was as follows: (1) the sample mixture was separated under optimal conditions; (2) the voltage was cut when the peak zone of interest appeared at the detection window; (3) a 50 mbar pressure was applied to the capillary inlet to elute the zone from the capillary; and (4) after a specific elution time, the sample zone was collected in a collection vial for a specific collection time. Both of these times were calculated from the velocity of the sample zone under the selected elution pressure and the geometrical parameters of the capillary. This procedure was repeated automatically about 100 times so as to collect sufficient material for MS–MS analysis.

2.4. MS–MS

Experiments were performed with a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, Finnigan MAT, San Jose, CA, USA) equipped with a nanospray interface (Protana, Odense, Denmark). Acquisition was carried out in the negative ion mode. The spray voltage applied was 0.8 kV and the capillary temperature was 115 °C. For MS–MS experiments, the width of the isolation window was 3 m.a.u. and the relative collision energy was 25–30%. The sample was dissolved in 5 μL methanol–water (1:1, v/v).

2.5. NMR spectroscopy

NMR spectroscopy was performed with a Bruker DMX 500 spectrometer, operating at the proton Larmor frequency of 500.11 MHz. The experiments were carried out with a 5-mm probe, equipped with self-shielded Z-gradients. Spectra were recorded at 25 °C with suppression of the residual solvent signal ($H_2O/^2H_2O$) by presaturation. Chemical shifts are reported in ppm using sodium 3-trimethylsilylpropanoate as the internal reference. The sample of monosulfated fucopyranoses was exchanged twice with 99.8% ^2H_2O (Sigma, La Verpillière, France)

with intermediate lyophilization and dissolved in 0.5 mL of 100% $^{2}H_2O$ for NMR analysis.

2.6. Enzymatic desulfation of the sulfated-fucose isomers

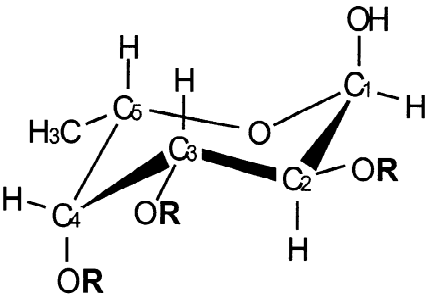
Sulfoesterase activity was routinely assayed with *p*-nitrocatechol sulfate as substrate using the spectrophotometric determination of *p*-nitrocatechol at 515 nm ($\varepsilon_{515} = 12\,200\ M^{-1}\ cm^{-1}$). The enzyme specific activity was defined as the amount of μmoles of *p*-nitrocatechol released per minute per milligram of protein, as previously reported [24]. The enzymatic reaction was performed on 6 mg of the sulfated fucopyranose mixture in 130 μL of 25 m*M* acetate buffer, 0.1 *M* NaCl, pH 5.25, containing 0.2 units of sulfoesterase. The reaction was carried out at 30 °C for 24 h, and then stopped by freezing. The samples were then submitted to NMR and CE analysis.

3. Results and discussion

Before this work was completed, samples of monosulfated fucoses were mainly checked for purity by 500 MHz ^{1}H-NMR, which also provides a rough evaluation of their positional isomer content. To the best of our knowledge, no analytical method is effective for a separate determination of these isomers. As a separation method is clearly needed to this end, CE and, especially, NACE were evaluated in this work. Furthermore, it can be anticipated that this latter technique would be of special relevance for analysis of this isomer mixture.

3.1. Optimization of non-aqueous CE separation

The positional isomers of monosulfated fucose (Fig. 1) can be viewed as UV-transparent, anionic compounds having exactly identical nominal charge-to-mass ratios. Considering the properties of the sulfate group, there is no possibility of playing with the acid–base equilibria to modulate the charge of the three isomers, except, perhaps, at very high pH, through partial dissociation of the hydroxyl groups [25,26]. As is common, aqueous media were investigated as a starting point for the CE separation of the three isomers. Experiments were conducted in three



R = H or SO_3^-

Fig. 1. Structures of the positional isomers of monosulfated fucose.

directions: (i) variation of the pH to vary the electroosmotic-to-electrophoretic mobility ratio; (ii) in-situ complexation with borate-based electrolytes to alter the charge rate; and (iii) the addition of an organic modifier to modify the solvation shell did not provide sufficient separation. But improvement of the selectivity for the latter prompted us to further investigate the separation of monosulfated fucose isomers in purely non-aqueous electrolytes.

As already pointed out, NACE has emerged as a complementary approach for the analysis of compounds that are difficult to separate in aqueous electrolytes. Compared with water, organic solvents afford a variety of physico-chemical properties that can be exploited to improve separation selectivity and efficiency. For the analytes of interest, the main issue is to benefit from different solvation and ion-pairing effects. Solvation phenomena are closely related to the nature of the solvent, but may also depend on the structural arrangement of the analytes. This results from a particular balance between hydrogen bonding, dipolar or dispersive interactions. Depending on the solvent dielectric constant, additional ion–ion interactions may also take place between oppositely charged species to form a neutral species. The effective charge of the species to be separated can be differently affected by this phenomenon according to the steric arrangement in the vicinity of the charge involved.

In the case of the positional isomers of monosulfated fucose, different solvation and ion-pair formations may arise because of the nature and

position of the substituents on the fucose ring. We thus investigated CE separations in various non-aqueous media, keeping the same electrolyte buffer and varying the solvent composition and proportion.

The electrolyte was prepared from ethanolamine and trimesic acid in proportions such that it was buffered at a pH close to the pK_a of ethanolamine in each solvent, and that it provided indirect UV absorbance detection. The selection of the organic solvents to be studied was mainly dictated by differences in solvation ability, high UV transparency and a low value of the relative dielectric constant ε (Table 1). It is commonly reported that ε should not exceed 30–40 to allow significant ion-pair formation to take place. Another relevant parameter is the ratio of the relative dielectric constant to the viscosity, which is proportional to the electroosmotic mobility [27]. As stated above, for a given pair of effective electrophoretic mobilities, the electroosmotic mobility can be tuned to enhance electrophoretic resolution. According to previous reports [28–31] and our own experience, the best candidate solvents are alcohols and acetonitrile. With respect to the choice of the alcohol, the length of the chain affects the dielectric constant and may also alter the solvation properties [31].

For the solvents and solvent mixtures tested (Table 2), the electroosmotic mobilities were always inferior, in absolute value, to the electrophoretic mobilities of the monosulfated fucoses. Therefore, the voltage polarity was negative. Table 2 indicates the resolution and migration times obtained. The most effective separation was achieved in a methanol–ethanol (1:1, v/v) mixture. This improved separation can be explained by an increased contribution of selective ion-pairing between the sulfate group of the analytes and the electrolyte counter-ion (ethanolamine), resulting from the lower dielectric constant (Table 1). The stability of these ion-pairs may be influenced by the position of the sulfate group on the fucose ring. If this assumption holds true, fucose-4-sulfate will form the most stable ion-pair with ethanolamine, as it is detected last. Apart from ion-pairing, the differences in alkyl chain length of methanol and ethanol may also induce more selective solvation of the fucose isomers in the alcohol mixture, according to the position of the sulfate group.

With the preceding optimal non-aqueous separation conditions, the influence of temperature was examined between 12 and 40 °C. It has been reported that the temperature may considerably affect resolution and analysis time, especially for carbohydrates due to the presence of anomeric forms [32]. The evolution of the electropherograms, while varying the temperature between 12 and 40 °C, is shown in Fig. 2. It can be seen that the migration times decrease with increasing temperature. This migration shift can first be explained by the decrease in electrolyte viscosity. Reduced ion-pairing and solvated radii, however, may also account for the increase in analyte electrophoretic mobility, as this latter phenomenon is also accompanied by a

Table 1
Relevant physico-chemical parameters of the organic solvents used for the NACE separation of the positional isomers of monosulfated fucose at 25 °C [27]

Solvent	d[a]	ε	η[a] (cP)	$\lambda_{cut\text{-}off}$[a] (nm)
Water	1	78.3	0.89	190
Methanol (MeOH)	0.79	32.7	0.54	205
Ethanol (EtOH)	0.78	24.5	1.09	210
Isopropanol (i-PrOH)	0.8	19.4	2.08	210
Acetonitrile (ACN)	0.77	35.9	0.34	190

d, density; ε, relative dielectric constant; η, viscosity; $\lambda_{cut\text{-}off}$, cut-off wavelength.
[a] At 25 °C.

Table 2
Influence of the nature and composition of the solvent on the resolution of the positional isomers of monosulfated fucose and on the migration time of the latest-detected isomer

Solvent (%, v/v)	Resolution	Migration time (min)
MeOH	No effective separation	25.0
MeOH–water (80:20)	No effective separation	28.5
MeOH–ACN (80:20)	No effective separation	30.0
MeOH–EtOH (80:20)	No effective separation	23.0
MeOH–EtOH (60:40)	$R_{1/2} = 0.5$, $R_{2/3} = 1.0$	19.5
MeOH–EtOH (50:50)	$R_{1/2} = 1.1$, $R_{2/3} = 1.7$	14.0
MeOH–EtOH–i-PrOH (40:40:20)	$R_{1/2} = 1.3$, $R_{2/3} = 2$	21.0

Separation electrolyte, 12 m*M* ethanolamine, 2 m*M* trimesic acid in the specified solvent medium; fused-silica capillary, 50 cm×50 μm I.D.; applied voltage, −20 kV; temperature, 12 °C. Other conditions: see Materials and methods.
$R_{1/2}$, resolution between the first and second peak; $R_{2/3}$, resolution between the second and third peak.

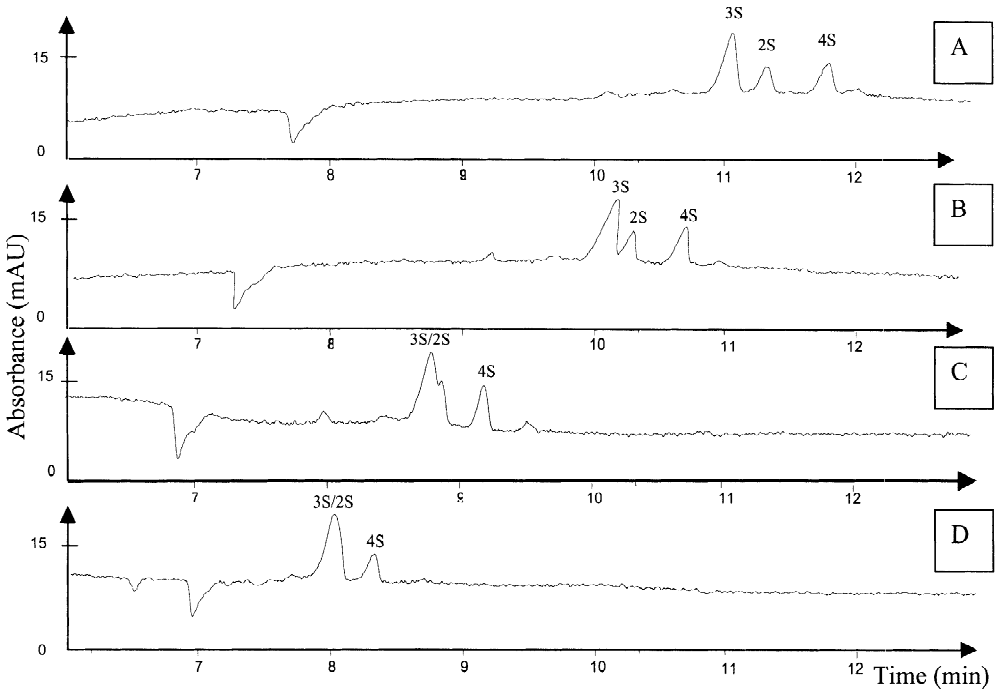


Fig. 2. Effect of temperature on the separation of the three positional isomers of monosulfated fucose in NACE: (A) 12 °C, (B) 25 °C, (C) 30 °C, (D) 40 °C. Fused-silica capillary, 40 cm (detection, 31.5 cm)×50 μm I.D.; applied voltage, −20 kV; separation electrolyte, 2 m*M* trimesic acid, 12 m*M* ethanolamine in MeOH–EtOH (50:50, v/v); indirect absorbance detection at 200 nm; sample mixture at a total concentration of 1.9 m*M* in water. Peaks: 3S = fucose-3-sulfate, 2S = fucose-2-sulfate, 4S = fucose-4-sulfate.

dramatic decrease in selectivity, especially for the pair fucose-2-sulfate and fucose-3-sulfate. Finally, the best isomeric separation was obtained at 12 °C. It should also be noted that no superimposed anomeric separation appeared at this low temperature, but the apparent peak efficiency was not very high.

3.2. Identification of the positional isomers

In order to first ascertain that the three peaks resolved under optimal NACE conditions actually corresponded to monosulfated fucose material, a pool of these peaks was collected using the fraction collection device of the apparatus and subsequently analyzed by negative-ion electrospray ionization mass spectrometry (ESI-MS). The optimization of this micro-preparative procedure is detailed in Section 2.3. The recovery of each individual peak was not attempted because of their very close migration times, differing by less than 1 min. The ESI-MS spectra of the collected sample (Fig. 3A) shows a major peak at m/z 243 corresponding to the molecular ion [M^-] of monosulfated fucose. Other signals in the spectra correspond to the trimesic acid anion from the electrolyte (m/z 208.9) and to its dimer (m/z 418.7). Fragmentation of the molecular ion at m/z 243 produced the characteristic daughter spectra of monosulfated fucose, i.e. the hydrogenosulfate ion at m/z 97, fragments at m/z 139 and 183, corresponding to cleavages across the pyranose ring, and an ion at m/z 225 due to dehydration of monosulfated fucose (Fig. 3B). The signal observed at m/z 286 in Fig. 4A was identified as an artifact due to the formation of a Schiff adduct between ethanolamine from the electrolyte and the acyclic aldehyde form of the sulfated fucose. This assignment was supported by the daughter ion spectrum of this ion, which was in agreement with the proposed structure (not shown).

For the three separated peaks of monosulfated fucose obtained by NACE, the assignment of each peak to a specific positional isomer was conducted

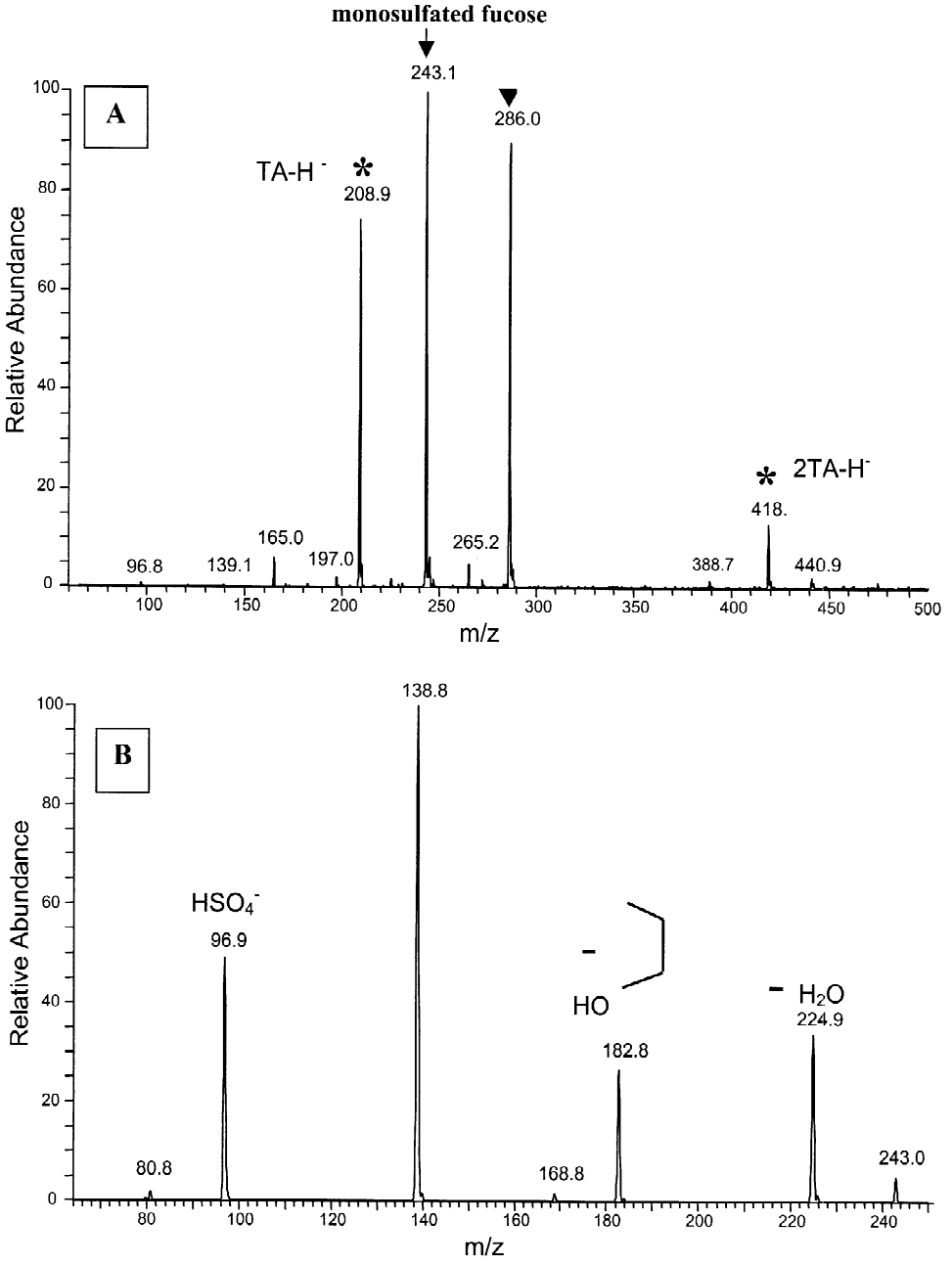


Fig. 3. Electrospray mass spectrometric analysis of the CE fraction containing the sulfated fucose isomers. (A) Electrospray spectrum of sulfated fucose (m/z 243) showing additional peaks corresponding to trimesic acid (TA) (*) from the electrolyte and to the Schiff adduct (▼) between ethanolamine and the acyclic aldehyde form of sulfated fucose. (B) Daughter ion mass spectrum obtained from the precursor ion at m/z 243.

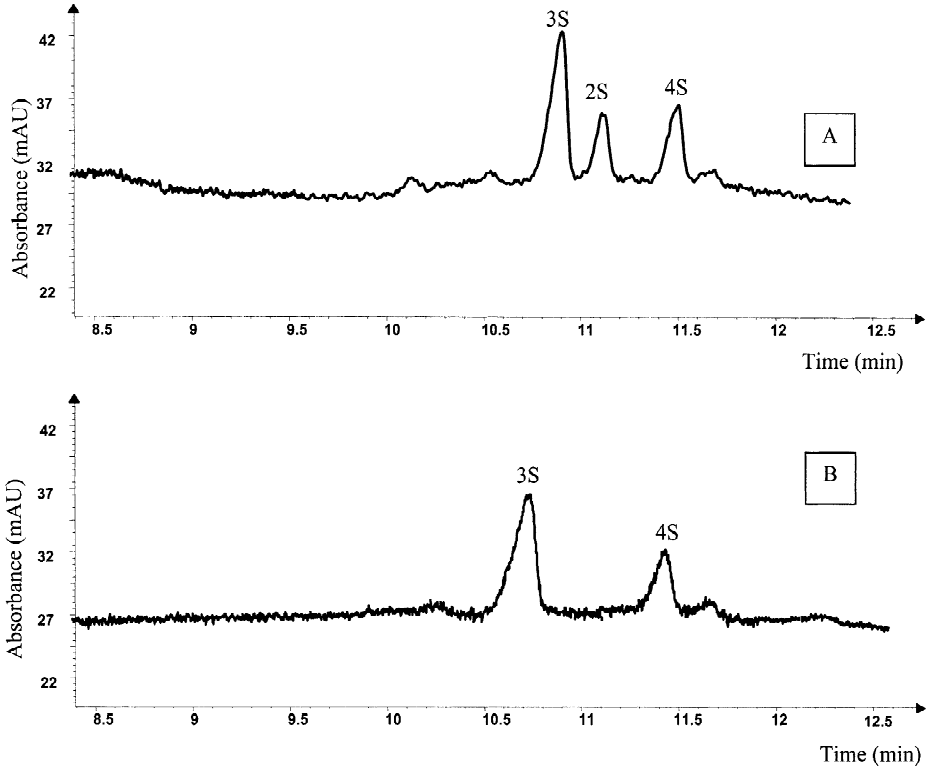


Fig. 4. NACE separation of a mixture of monosulfated fucose positional isomers before (A) and after (B) regioselective hydrolysis catalyzed by a fucose 2-*O*-sulfohydrolase. Separation conditions, see Fig. 3; temperature, 12 °C. Hydrolysis conditions, see Section 2.6. Peak identification as in Fig. 3.

using the following strategy. First, the electropherogram of the mixture of the three sulfated isomers was compared with that of a mixture containing only fucose-3-sulfate and fucose-4-sulfate isomers. The latter mixture (Fig. 4) was obtained by specific hydrolysis of fucose-2-sulfate catalyzed by a newly discovered, highly regioselective fucose 2-*O*-sulfohydrolase [24]. Accordingly, the NACE analysis of this mixture exhibited only two peaks, which should correspond to fucose-3-sulfate and fucose-4-sulfate, the missing peak then being assigned to fucose-2-sulfate. Identification of the electrophoretic peaks was completed by correlating the proportion of each isomer determined by ^{1}H-NMR measurements (Fig. 5) and by CE using the corrected peak areas (i.e. peak area/migration time). This approach is justified by the fact that the response coefficients in the indirect absorbance detection mode do not depend on any other analyte characteristics than its mobility and therefore analytes having close mobilities also have close response coefficients [33,34]. For a more precise quantitative analysis, response coefficients can be obtained, without the availability of isomerically pure standards, from the mobility values of each isomer and electrolyte co-ion and counter-ion. The major isomer molar fraction (57% from ^{1}H-NMR) was fucose-3-sulfate, which migrated first, followed by fucose-2-sulfate (24% from ^{1}H-NMR) and fucose-4-sulfate (19% from ^{1}H-NMR). This migration order may correspond to the increasing stability order of the ion-pairs, based on the accessibility of the sulfate groups to the electrolyte counter-ion.

4. Concluding remarks

This study emphasizes the usefulness of NACE for

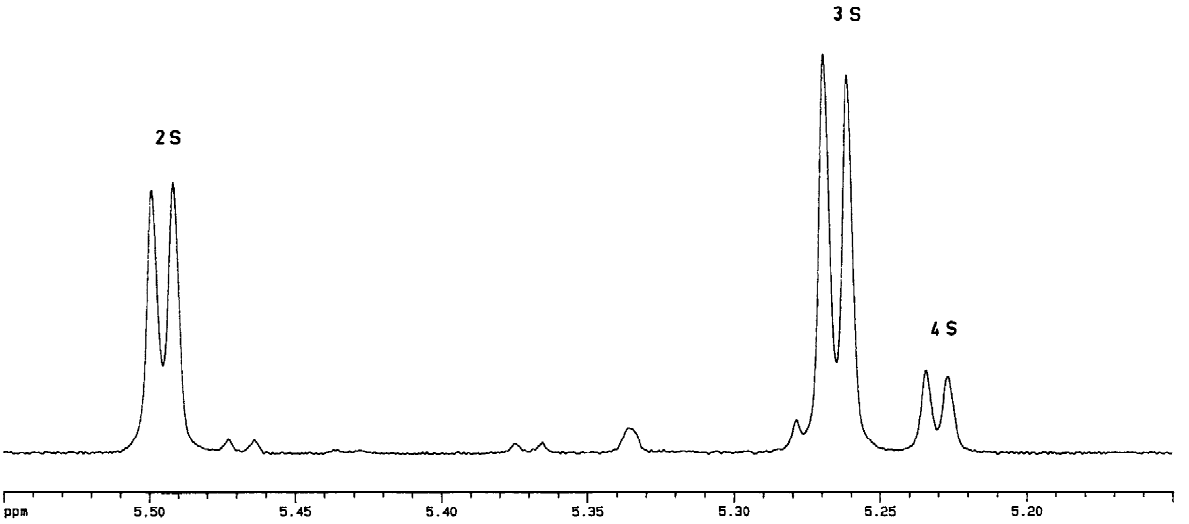


Fig. 5. Partial ^{1}H spectrum (500 MHz, 25 °C) of a mixture of monosulfated fucose positional isomers. Expansion of the H-1 signals of the α-anomers is shown from 5.55 to 5.15 ppm. Peak identification as in Fig. 3.

the discrimination of analytes with identical charge-to-mass ratios without the use of any additional separating agent than classical buffer pH through selective ion-pairing and solvation phenomena. For the first time a simple CE method for the separation of the positional isomers of monosulfated fucose has been described. This method can be applied to the screening of sulfoesterases of unknown activity and should constitute a new analytical tool for the determination of the structure of monosulfated fucose-based polysaccharides. In the course of a structure–activity study of fucoidan, the NACE method described here was used to determine and quantify each sulfated fucose isomer constituting this bioactive polysaccharide.

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