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Pressure-assisted capillary electrophoresis–electrospray ion trap mass spectrometry for the analysis of heparin depolymerised disaccharides

V. Ruiz-Calero, E. Moyano, L. Puignou, M.T. Galceran*

Departament de Química Analítica, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1–11, 08028 Barcelona, Spain

Abstract

A pressure-assisted capillary electrophoresis–ion trap mass spectrometry method was developed for the analysis of eight heparin-derived disaccharides. A 30 m*M* formic acid buffer at pH 3.20 was selected as running electrolyte, and the separation was performed by the simultaneous application of a CE voltage of −30 kV and an overimposed pressure of 0.5 p.s.i. (3.45 kPa). The application of pressure assistance was needed to provide stable electrospray conditions for successful coupling. The linearity of the CE–MS and CE–MS–MS methods was checked under these conditions. Quality parameters such as run-to-run precision and limits of detection were established in both CE–MS and CE–MS–MS modes. Finally, enzymatically depolymerised bovine and porcine mucosal heparins were analysed in this CE–MS system and the characteristic relative molar percentages of major and minor disaccharides were calculated. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Capillary electrophoresis–mass spectrometry; Heparin; Disaccharides

1. Introduction

Heparin, a widely used anticoagulant, is a glycosaminoglycan with molecular mass ranging from 3000 to 40 000 composed of disaccharide units consisting of hexuronic acids (L-IdoA or D-GlcA) linked to glucosamine units (which can be either *N*-sulfated or *N*-acetylated) by means of $\alpha(1\rightarrow4)$ bonds. Both units can also be variously sulfated at C-2 of the hexuronic acid and at C-6 of the glucosamine [1–3]. Heparin is commonly obtained from either porcine or bovine intestinal mucosa and lung after proteolytic digestion followed by precipitation as quaternary ammonium complexes or barium salts [4,5]. It is known that the biological activity is related to specific oligosaccharide and disaccharide sequences, which occur differently in heparins from different sources [6,7]. The commonest strategy for the characterisation of heparins from different origins relies on the determination of the fragments released after enzymatic depolymerisation with heparin lyases I, II and III. Using a suitable mixture of the three lyases, heparin is depolymerised giving compositional Δ-disaccharides with unsaturated uronic acid residues, whose determination provides characteristic compositional patterns [8–10]. High-performance liquid chromatography (HPLC) [8,11,12] and capillary electrophoresis (CE) [13–18] have been applied to the determination of oligosaccharides and disaccharides mostly using direct UV detection. Owing to

*Corresponding author. Tel.: +34-93-4021-275; fax: +34-93-4021-233.

E-mail address: galceran@apolo.qui.ub.es (M.T. Galceran).

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the low volumes which are required for the depolymerisation procedure, CE is a more advantageous technique for these determinations. LC and CE with fluorescence and UV detection of derivatised disaccharides have also been performed improving the sensitivity but at the expense of an additional derivatisation step [19,20]. LC–mass spectrometry (MS) with an atmospheric pressure interface has been used for the selective detection of the most abundant disaccharide in depolymerised heparin [21,22].

In recent years, advances in the electrospray interface systems has extended the applicability of coupling of CE with MS, especially in the biological and pharmaceutical fields [23–31]. However, at present, CE–MS seems to be less robust than LC–MS, and it has been mostly used for qualitative purposes [24]. In fact, most electrophoretic separations are commonly performed under conditions which are not suitable for coupling to a mass spectrometer. Therefore, coupling conditions must be carefully optimised to enhance the robustness of the CE–MS system and obtain reproducible results for quantitative applications.

In this work, a pressure-assisted CE–MS method was optimised for the analysis of eight heparin-derived disaccharides using a Finnigan LCQ ion-trap mass spectrometer. Electrolyte composition and other separation conditions were studied to obtain stable electrospray conditions. A volatile electrolyte consisting of 30 m*M* formic acid buffer adjusted to pH 3.20 with ammonium hydroxide was used as running electrolyte at reversed polarity. Moreover, the utility of pressure assisting to stabilise the coupling conditions and provide a stable electrospray was studied [32,33]. Recently, the ability of ion trap mass spectrometers to perform MS^n experiments has been applied to the characterisation of the oligosaccharide fragments of complex glycoforms [34–36]. Here, this feature has been advantageously used to obtain the MS and MS–MS spectra of the disaccharides, showing the potentiality of this technique for the analysis of positional monosulfated and disulfated isomers. Finally, the optimised CE–MS method was applied to the analysis of porcine mucosa and bovine mucosa heparin depolymerisation disaccharides.

2. Experimental

2.1. Chemicals

Eight heparin standard disaccharides (sodium salts) were obtained from Sigma (St. Louis, MO, USA): ΔUA2S(1→4)α-D-GlcNS6S (IS), ΔUA(1→4)α-D-GlcNS6S (IIS), ΔUA2S(1→4)α-D-GlcNS (IIIS), ΔUA(1→4)α-D-GlcNS (IVS), ΔUA2S(1→4)α-D-GlcNAc6S (IA), ΔUA(1→4)α-D-GlcNAc6S (IIA), ΔUA2S(1→4)α-D-GlcNAc (IIIA), ΔUA(1→4)α-D-GlcNAc (IVA). Their structures are shown in Fig. 1. Stock solutions of each disaccharide were prepared at concentrations ranging from 400 to 1000 mg l^{-1} on the basis of their absorbance at 232 nm [14]. Porcine mucosa heparin was kindly provided by Bioibérica (Palafolls, Barcelona, Spain) and bovine mucosa heparin was obtained from Sigma.

Heparin lyase I (50 Sigma units), heparin lyase II (10 Sigma units) and heparin lyase III (5 Sigma units) were from Sigma. One unit produces 0.1 μmol of unsaturated uronic acid per hour at pH 7.5 at 25°C. One international unit (IU) corresponds to ca. 600 Sigma units.

Formic acid 98%, ammonium hydroxide solution 25% (w/v), tris(hydroxymethyl)aminomethane, calcium chloride, sodium chloride and sodium hydroxide were obtained from Merck (Darmstadt, Germany). 2-Propanol was obtained from Fluka (Ronkonkoma, NY, USA). Water purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used for all solutions.

2.2. Electrophoretic conditions

The experiments were carried out on a P/ACE System 5500 (Beckman Instruments, Fullerton, CA, USA). The electrophoretic separations were performed using uncoated fused-silica capillaries of 77 cm (70 cm separation length)×75 μm I.D. from Supelco (Bellefonte, PA, USA) for the studies using a CE–UV system, and with a total length of 80 cm when the CE system was coupled to the mass spectrometer. For the CE–UV system, a running electrolyte of 30 m*M* formic acid adjusted to pH 3.2 with ammonium hydroxide (4%, w/v), and a voltage of −25 kV were used. An overimposed pressure of

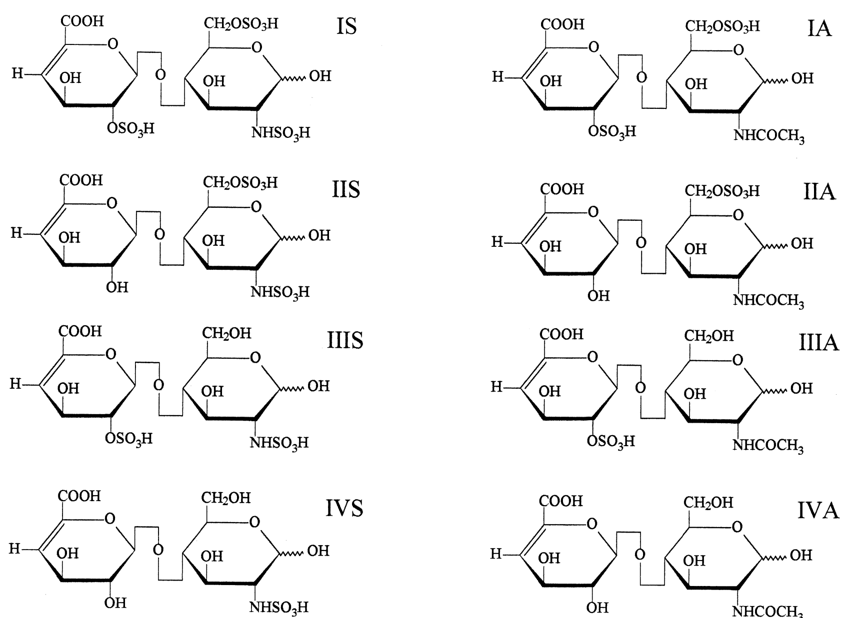

Fig. 1. Chemical structures of the eight heparin disaccharides obtained by enzymatic depolymerisation.

3.45 kPa (0.5 p.s.i.) for 10 min starting at minute 22, was applied from the inlet vial to the outlet vial. For CE–MS, the pressure-assisted electrophoretic separations were performed by applying simultaneously a voltage of −30 kV and an overimposed pressure of 0.5 p.s.i. from the inlet vial to the mass spectrometer during the whole run. Capillaries were daily pretreated with 100 m*M* sodium hydroxide for 15 min, then rinsed with water for 15 min and finally conditioned by rinsing with the running buffer for 30 min. During these treatments the electrospray source was retracted from the mass spectrometer. Samples were loaded by pressure injection at 0.5 p.s.i. for 7 s. Running electrolytes were filtered through a 0.45-μm membrane, and degassed by sonication before use. The temperature was held at 25°C.

2.3. CE–electrospray ionisation MS conditions

A Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a tricoaxial electrospray ionisation (ESI) source (Finnigan MAT, San Jose, CA, USA) was used for coupling to the electrophoresis system. A sheath liquid consisting of 2-propanol–5 m*M* HCOOH, pH 3.20 (adjusted with ammonium hydroxide, 4%, w/v) was delivered at a flow-rate of 2 μl min^{-1} by a 500-μl Unimetrics (Shorewood, IL, USA) syringe pump. The sheath gas flow (N_2) was set to 6.7 l h^{-1} and the heated capillary was maintained at 225°C. The ESI source operated in the negative mode and voltages ranging from −3.5 to −5 kV were applied to the electrospray needle.

The MS and MS–MS spectra were acquired by infusing the individual disaccharide solutions of 100 μg ml^{-1} in running electrolyte. The infusion was performed by the simultaneous application of the separation voltage (−30 kV) and pressure (0.5 p.s.i.) from the inlet vial containing the standard solution. The m/z was generally scanned from 220 to 600. MS–MS spectra were obtained using activation amplitudes of 14–24% (0 to 100% corresponded to 0 to 5 kV). The mass selection window was set to 1.5 units and Q value was 0.250. In general, the maximum ion injection time was 500 ms and three microscans were accumulated per scan in the full scan mass range. Data were processed using the Xcalibur software 1.0.

2.4. Enzymatic depolymerisation conditions

Extensive depolymerisation was carried out as previously described [16] to release the heparin disaccharides. Briefly, 100 μg of heparin was depolymerised with heparin lyase I, heparin lyase II and heparin lyase III (7.8, 1.0 and 0.9 units, respectively) in 80 μl buffer consisting of 20 m*M* Tris, 50 m*M* NaCl and 3 m*M* $CaCl_2$ adjusted to pH 7.2 with 1 *M* HCl. The depolymerisations were performed in a water bath with agitation at 37°C for 24 h. The reactions were stopped by boiling at 100°C for 1 min.

3. Results and discussion

3.1. Preliminary studies

In order to implement the electrophoretic conditions of the CE–UV method previously published [16] to the MS coupling, some modifications were needed. As MS requires volatile and low-ionic-strength buffers, the concentration of formic acid was decreased from 60 to 30 m*M* and ammonium hydroxide (4%, w/v) was used to adjust the pH instead of NaOH (1 *M*). Moreover, the coupling required the enlargement of the capillary length to a minimum of 80 cm. Thus, the initial conditions tested were: 30 m*M* formic acid, pH 3.2 adjusted with ammonium hydroxide (4%, w/v) at −15 kV (reversed polarity) and an overimposed pressure of 0.5 p.s.i. at 40 min. Under these conditions, the sulfated disaccharides migrated in 35 min and analysis time was 45 min. In order to reduce analysis time a high negative voltage of −25 kV was applied and an overimposed pressure of 0.5 p.s.i. was applied. Satisfactory resolution was obtained for the sulfated disaccharides, as can be seen in Fig. 2. Migration of sulfated disaccharides occurred in 21 min, and 5 min of simultaneous voltage and pressure was needed for the migration of disaccharide IVA.

3.2. MS and MS–MS spectra of heparin disaccharides

3.2.1. MS spectra

The full-scan spectra (Table 1) of heparin disaccharides showed abundant deprotonated molecular ions, and some fragments resulting from the loss of SO_3 were observed. Trisulfated and disulfated disaccharides (IS, IIS, IIIS and IA) spectra exhibited doubly charged ions $[M-2H]^{2-}$ due to the deprotonation of the molecule. The confirmation of these double charge ions was accomplished by acquisition in ZoomScan mode (i.e., a scan performed at a 1/20th slower scan rate in a range of 10 *m/z* units to obtain high resolution [37]). The ZoomScan spectra showed the isotopic peaks $[M-2H+1]^{2-}$ and $[M-2H+2]^{2-}$ at 0.5 and 1.0 *m/z* units, respectively, from the doubly deprotonated molecular ion, while the isotopic peaks of the singly charged ions appeared at 1.0 *m/z* and 2.0 units from the $[M-H]^{-}$ ion. An example of doubly charged ion confirmation is given for disaccharide IS in Fig. 3. Moreover, the MS–MS spectra of these doubly charged ions gave product ions at higher *m/z* values (Fig. 3d).

The loss of SO_3 was observed for the trisulfated (IS), and disulfated disaccharides (IIS, IIIS, IA), although the intensities were lower than 40%. MS spectra of positional isomers of disulfated IIS and IIIS and monosulfated IIA and IIIA showed no significant differences, as can be seen in Table 1. Thus, the acquisition of the MS–MS spectra was required to identify these disaccharides.

3.2.2. MS–MS spectra

MS–MS spectra were obtained by infusing the standard solutions of disaccharides. The precursor and main product ions as well as the values of activation amplitude (AA) are given in Table 2. Three general fragmentation events occurred: the loss of H_2O and SO_3, the glycosidic bond fragmentation and the cross-ring cleavage. The most abundant MS–MS fragments of the $[M-H]^{-}$ trisulfated and disulfated disaccharides were produced by the loss of SO_3 appearing at the *m/z* values corresponding to less sulfated disaccharides. In contrast, loss of H_2O and cross-ring fragmentation were predominant in the MS–MS spectra of monosulfated and non-sulfated disaccharides. Different relative abundances of product ions for positional isomer disaccharides were found, as observed in the MS–MS spectra of disaccharides IIA and IIIA, which are shown in Fig. 4. These differences could be used to identify these disaccharides.

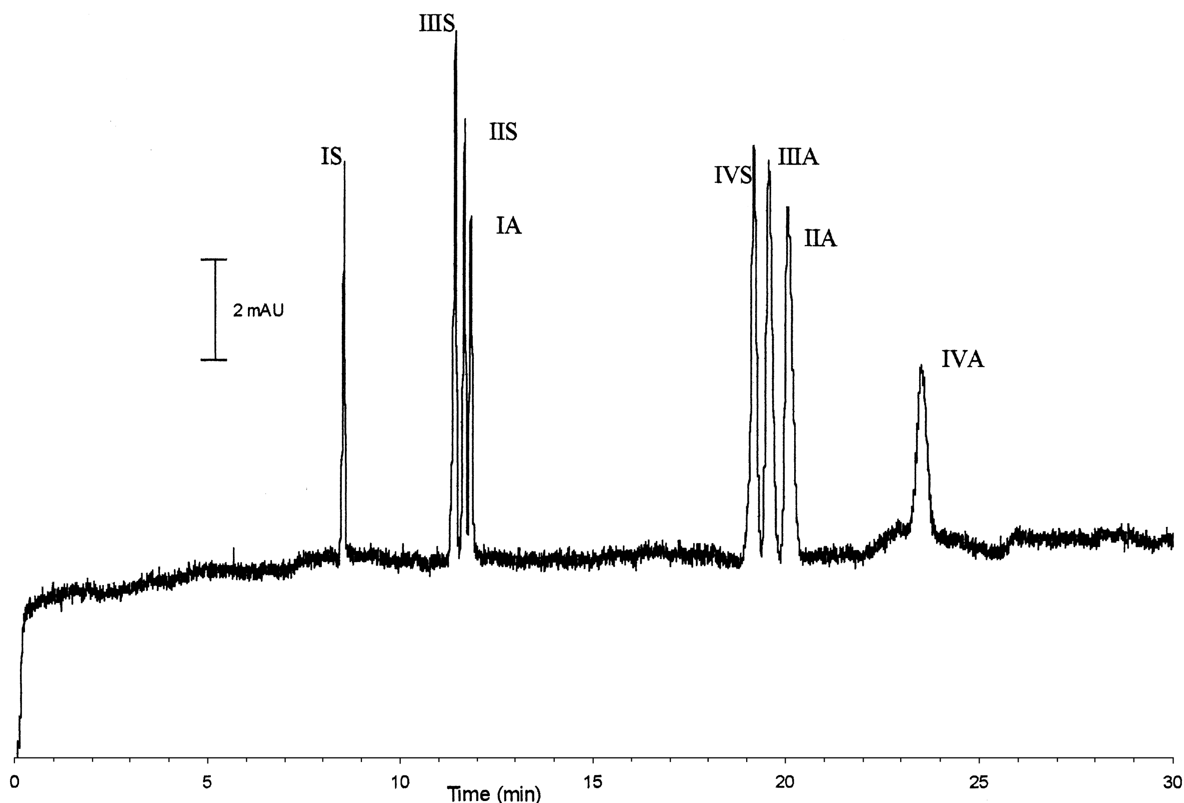

Fig. 2. CE–UV electropherogram of sulfated disaccharides (approx. 60 μg ml^{-1}). Experimental conditions: 30 m*M* formic buffer, pH 3.20, voltage applied: −25 kV, injection time (0.5 p.s.i.): 7 s. An overimposed pressure (0.5 p.s.i.) was applied at 22 min.

3.3. Optimisation of CE–MS coupling

Several attempts were made to obtain CE–MS electropherograms using the modified CE conditions. However, the application of the separation voltage (−25 kV) when CE was directly coupled to MS gave unstable electrospray currents with values dropping rapidly from the initial 13–12 to ca. 3 μA. A drop in the CE current to values similar to the electrospray current also occurred and when ca. 3 μA was reached, the CE voltage was automatically interrupted. This may be due to deficient contact between the electrophoretic buffer and the sheath liquid.

In general, when reversed polarity conditions and acidic running electrolytes are used, only the anionic analytes with high electrophoretic mobility, such as the sulfated disaccharides, migrate towards the anode. Under these conditions the contribution of the anionic migration is insufficient to maintain the contact with the sheath liquid and close the electrical circuit. To solve this problem some modifications of the coupling system were tested, such as the flow-rate of sheath liquid (2–10 μl min^{-1}) and sheath gas (6.7–11.9 l h^{-1}), and the position of the capillary in the tricoaxial interface (0.10–0.30 mm), but no better results were obtained. The effect of a hydrostatic pressure between the capillary ends generated by raising the CE system was also examined. However, the additional bulk flow generated towards the anode did not improve the electrospray stability.

The use of an hydrodynamic overimposed pressure during the whole electrophoretic run in CE–MS systems to enhance the robustness of the method and decrease run times has recently been reported [36,37]. To test the utility of this procedure for the stabilisation of our CE–MS system, a pressure of 0.5 p.s.i. and voltages ranging from −15 to −30 kV were applied during the whole electrophoretic run.

Table 1
Mass spectral data of heparin disaccharides

Disaccharide	Fragment	*m/z*	Relative abundance (%)
IS	$[M-H]^-$	575.8	29
	$[M-H-SO_3]^-$	495.9	36
	$[M-2H]^{2-}$	287.5	100
	$[M-2H-SO_3]^{2-}$	247.5	17
IIS	$[M-H]^-$	495.9	100
	$[M-H-SO_3]^-$	416.1	31
	$[M-2H]^{2-}$	247.5	71
IIIS	$[M-H]^-$	495.9	100
	$[M-H-SO_3]^-$	416.1	20
	$[M-2H]^{2-}$	247.5	50
IVS	$[M-H]^-$	416.1	100
IA	$[M-H]^-$	537.8	100
	$[M-H-SO_3]^-$	458.1	33
	$[M-2H]^{2-}$	268.5	70
IIA	$[M-H]^-$	458.1	100
IIIA	$[M-H]^-$	458.1	100
IVA	$[M-H]^-$	378.0	100

Under these conditions both ESI (12–14 μA) and CE currents (17.5–18.0 μA) were stable during the whole run. However, the electrophoretic separation was negatively affected. A CE–MS electropherogram obtained using the pressure-assisted system is shown in Fig. 5. A significant loss of resolution between the disulfated and monosulfated disaccharides can be seen. The disulfated disaccharide IA and monosulfated IVS were identified and quantified by their individual $[M-H]^-$ traces, *m/z* 537.8 and 416.0, respectively, whereas the positional isomers IIS–IIIS and IIA–IIIA could not be individually identified and determined. It must be mentioned that the variation of the CE voltage did not affect the efficiency of the separation in the pressure assisted system probably due to the relatively high pressure used (0.5 p.s.i.). Therefore, the highest voltage, −30 kV, was selected as it shortened the run time to 11 min.

Owing to the co-migration, identification and quantitation of disaccharides IIS–IIIS and IIA–IIIA had to be performed with MS–MS. For the *N*-sulfated disaccharides (IIIS–IIS), product ions at *m/z* 300.1 and 337.9 appeared with relative high abundances in the MS–MS spectrum of the precursor ion 247.5, which corresponded to the $[M-2H]^{2-}$ ion of disaccharide IIS. Therefore, from the traces of these product ions selective determination of disaccharide IIS could be performed, but at the expense of a loss in sensitivity. MS–MS fragmentation of the $[M-H]^-$ ion 458.1 could also be used to identify and quantify disaccharides IIA and IIIA. The MS–MS spectra of disaccharide IIA showed intense product ions at *m/z* 357.0 and 300.1, which enable its determination.

3.4. Quality parameters

Linearity, day-to-day precision and limits of detection (LODs) were determined for CE–MS and CE–MS–MS using two sets of disaccharide standards with concentration ranging from 3 to 124 μg ml^{-1}. The highest limit was extended to 440 and 310 μg ml^{-1} for disaccharides IS and IIIS, respectively. Set A contained disaccharides IIIS, IA, IVS, and IIA, while set B consisted of disaccharides IS, IIS, IIIA

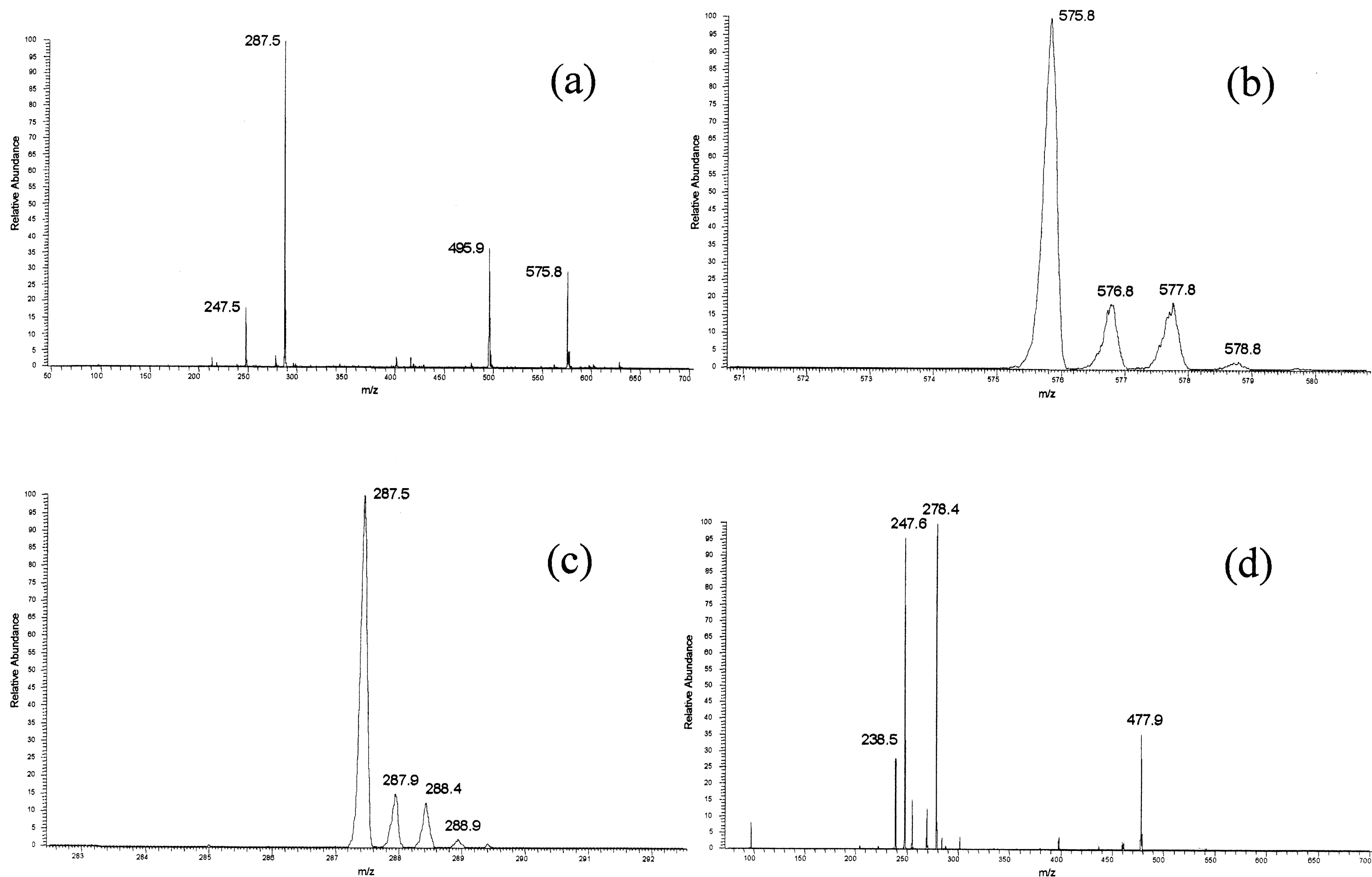

Fig. 3. MS spectra of disaccharide IS. (a) MS spectrum, high-resolution spectra acquired in the ZoomScan mode of (b) $[M-H]^{-}$, and (c) $[M-2H]^{2-}$ ions, and (d) MS–MS of m/z 287.5. MS conditions given in the Experimental section.

Table 2
MS–MS data of heparin disaccharides

Disaccharide	Precursor ion	Activation amplitude (%)	Fragment ions (% relative abundance)[a]
IS	575.8	14.0	495.8 (100)
	287.5	14.5	278.4 (100), 247.6 (95)
IIS	495.9	17.5	416.1 (100)
	247.5	17.0	357.0 (100), 138.0 (42), 300.1 (49), 258.0 (35), 238.5 (20), 337.9 (20)
IIIS	495.9	17.5	416.1 (100)
	247.5	17.0	357.0 (100), 138.0 (84), 238.5 (55), 258.1 (41), 97.0 (20)
IVS	416.1	22.0	138.0 (100)
IA	537.8	15.5	458.1 (100)
	268.5	17.0	259.5 (100)
IIA	458.1	24.0	440.0 (100), 357.0 (82), 300.1 (86)
IIIA	458.1	24.0	440.0 (100), 236.9 (48), 360.0 (32), 156.9 (32), 377.8 (20)
IVA	378.0	17.0	276.8 (100)

[a] Only fragment ions with relative abundance higher than 75% are given, except for positional isomers IIS–IIIS and IIA–IIIA (higher than 20%).

and IVA. In CE–MS, quantitation was performed by external calibration using the traces of the most abundant molecular ion, singly or doubly charged. Linearity, migration time precision, concentration precision and bias are given in Table 3. Relative standard deviations (RSDs) were calculated at the 35–60 μg ml^{-1} level on both migration time ($n=6$) and concentration ($n=3$). Migration times were satisfactorily reproducible (RSD lower than 2.8%). RSD values (between 2.0 and 18.2%) for concentration were similar to those obtained with CE–UV [16]. The accuracy of the results was similar to those published for other CE–MS methods, which are, in general, worse than those of LC–MS [26,28,30].

Linearity in CE–MS–MS was studied using the individual traces of the most abundant product ions obtained from the fragmentation of the most abundant precursor ions, $[M-H]^{-}$ or $[M-2H]^{2-}$. In the case of disaccharides IIS and IIA, linearity of the signals of characteristic ions (300.1 and 337.9 for IIS, and 300.1 and 357.0 for IIA) was also checked. In general, run-to-run precision for concentration, RSDs between 5.3 to 9.9%, were similar in both CE–MS and CE–MS–MS modes, but more accurate results (5.2 to 17.4%), were obtained using CE–MS–MS (Table 4).

LODs for this coupling were established as the amount injected that gave a signal-to-noise ratio higher than 3 and/or a signal higher than 10^3 in both CE–MS and CE–MS–MS modes. As expected, LODs in the CE–MS–MS mode were lower, sometimes by one order of magnitude, than those of CE–MS due to the increase of selectivity. LODs are given in Table 5, along with those corresponding to the CE–UV method previously published [16]. The LODs corresponding to the characteristic ions of disaccharides IIS and IIA are also given. LODs of the CE–UV method were in general lower than those of CE–MS. However, for most of the disaccharide the CE–MS–MS values were of the same order as the corresponding CE–UV values. Moreover, for *N*-acetylated disaccharides a significant improvement in sensitivity, from 2 to 25 times, was obtained.

3.5. CE–MS of heparin depolymerised samples

The applicability of the pressurised CE–MS sys-

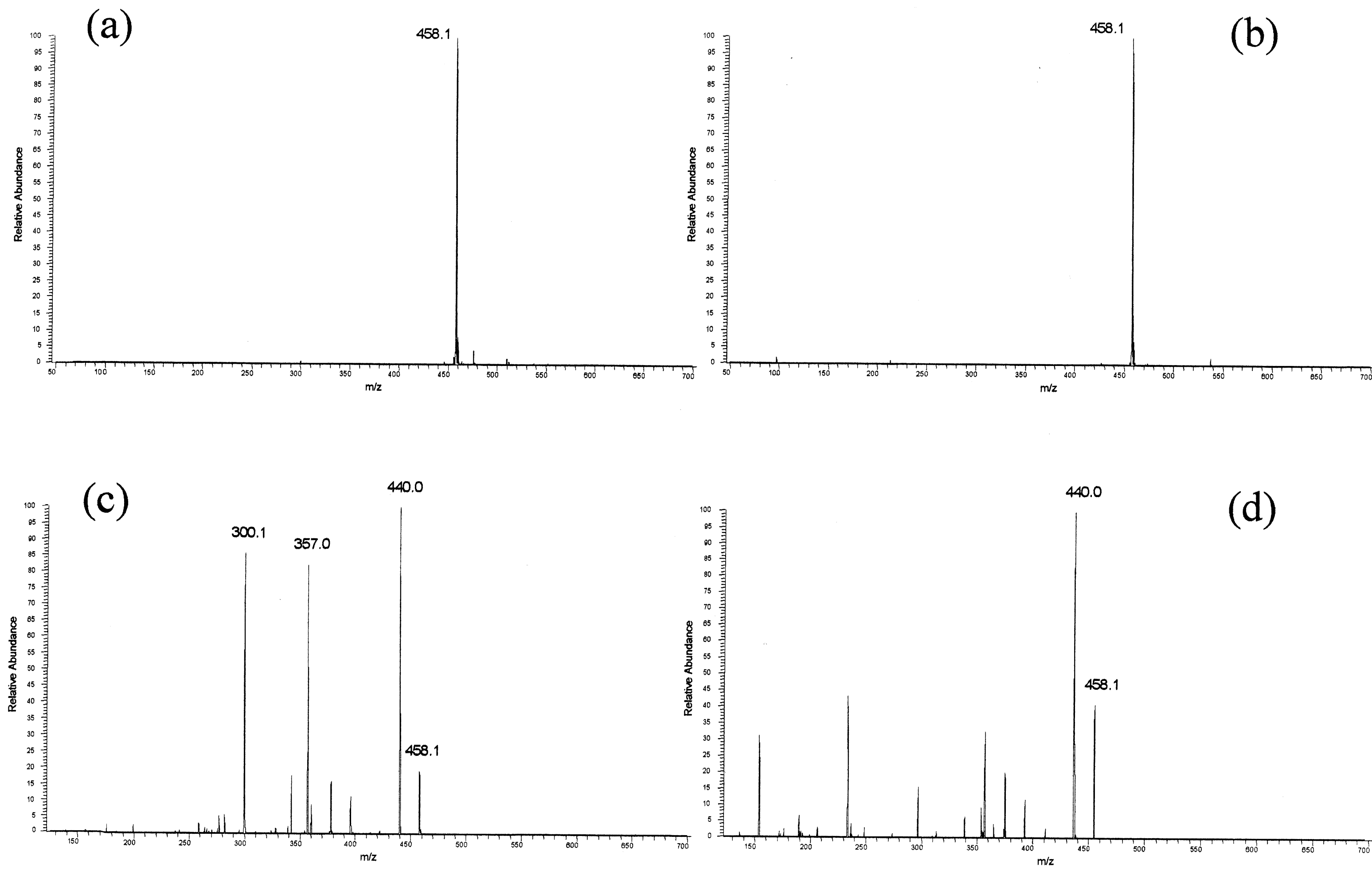

Fig. 4. MS–MS spectra of positional isomer disaccharides IIA and IIIA. (a) MS spectrum of disaccharide IIA, (b) MS spectrum of disaccharide IIIA, (c) MS–MS spectrum of m/z 458.1 of disaccharide IIA and (d) MS–MS spectrum of m/z 458.1 of disaccharide IIIA. An activation amplitude (AA) of 24% was applied. MS conditions given in the Experimental section.

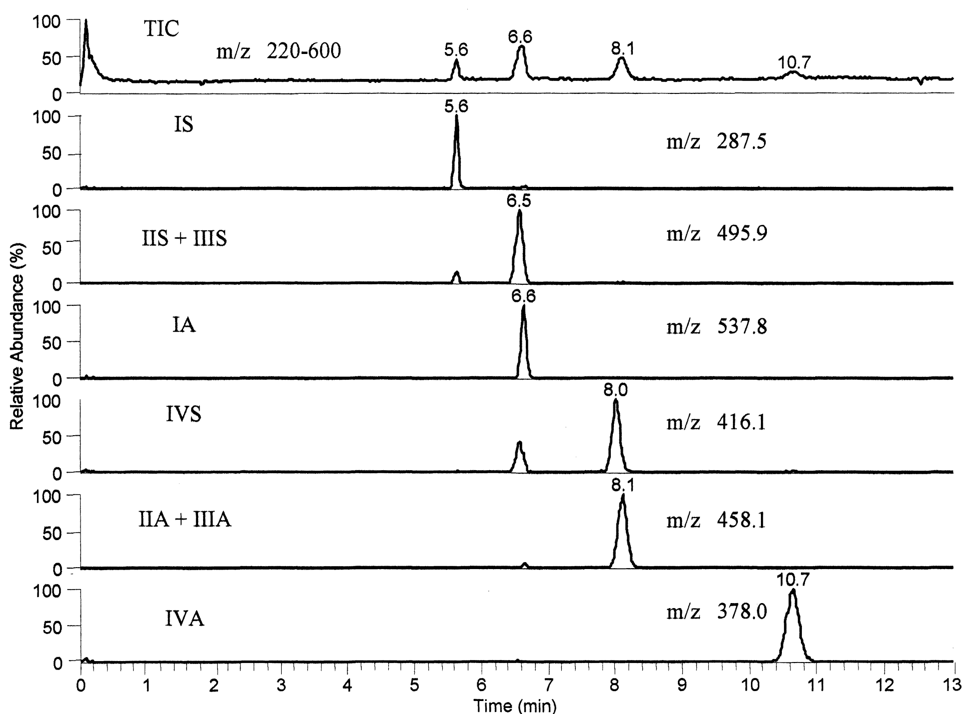

Fig. 5. Pressure-assisted CE–MS electropherogram of a disaccharide standard solution (approx. 60 μg ml^{-1}). Experimental conditions: 30 m*M* formic buffer, pH 3.20, voltage applied: −30 kV, injection time (0.5 p.s.i.): 7 s. An overimposed pressure gradient (0.5 p.s.i.) from inlet to mass spectrometer was applied. Full-scan CE–MS total ion current (TIC) and extracted ion current profiles for the base peaks of the disaccharides are shown.

tem was tested for the heparin depolymerised products in both MS and MS–MS modes, with the aim to obtain disaccharide characterisation. In Fig. 6, as an example, the electropherograms of depolymerised porcine intestinal mucosa heparin are given. The disaccharides were identified in their CE–MS traces.

Table 3
CE–MS linearity, precision and accuracy

Disaccharide	Linearity range tested		Migration time		Concentration precision and accuracy		
	Concentration range (μg ml^{-1})	r^2 (*n*)	t_m (min)	RSD (%) (*n*=8)	Concentration (μg ml^{-1})	RSD (%) (*n*=3)	Bias (%)
IS	13.8–440.0	0.992 (6)	5.8	1.8	55.1	6.3	−11.3
IIIS	40.8–310.0	0.936 (5)	6.6	2.5	64.2	1.9	−5.4
IIS	18.7–124.6	0.997 (5)	6.6	2.5	52.6	8.6	−15.6
IA	17.5–133.7	0.999 (6)	6.8	2.4	40.5	8.7	−39.4
IVS	33.7–101.0	0.982 (6)	8.1	2.5	37.1	18.2	−26.5
IIIA	12.5–124.6	0.988 (5)	8.2	2.2	55.0	5.7	−11.7
IIA	13.9–139.2	0.971 (4)	8.3	2.2	55.3	9.4	−20.5
IVA	14.7–88.2	0.984 (4)	10.7	2.8	37.2	6.3	−15.7

Table 4
CE–MS–MS linearity, precision and accuracy

Disaccharide	Transition	Linearity range tested		Concentration precision and accuracy		
		Concentration range (μg ml^{-1})	r^2 (n)	Concentration (μg ml^{-1})	RSD (%) (n=3)	Bias (%)
IS	287.5→278.5	6.2–440.0	0.970 (5)	54.0	9.9	−13.6
IIIS	495.9→416.1	13.6–310.0	0.976 (5)	58.5	5.9	−13.9
	247.5→357.0	13.6–310.0	0.961 (6)	55.3	6.0	−10.1
IIS	495.9→416.1	13.8–124.6	0.974 (5)	53.0	7.1	−14.2
	247.5→357.0	13.8–124.6	0.998 (4)	59.1	8.0	−5.2
	247.5→300.1+337.9	18.5–124.6	0.998 (4)	56.4	8.6	−9.5
IA	537.8→458.1	6.7–133.7	0.935 (5)	55.2	5.3	−17.4
IVS	416.0→138.0	10.1–101.0	0.999 (6)	46.2	9.6	−8.6
IIIA	458.1→440.0	4.2–124.6	0.978 (5)	69.8	5.5	+12.3
IIA	458.1→440.0	7.0–139.2	0.976 (5)	65.4	5.6	−6.0
	458.1→300.1+357.0	46.4–139.2	0.951 (4)	58.6	6.9	−15.8
IVA	378.0→276.8	14.7–88.2	0.984 (4)	37.4	6.0	−15.3

The total ion current (TIC) electropherogram showed intense peaks at migration times coincident with those of the trisulfated and disulfated disaccharides. CE–MS was used for the quantitation of major disaccharides (IS, IIS and IIIS) while CE–MS–MS was used for minor disaccharides. Calculated concentrations of minor disaccharides were in the order of those obtained with CE–UV, but concentration

Table 5
Limits of detection in CE–MS, CE–MS–MS and CE–UV

Disaccharide	CE–MS		CE–MS–MS		CE–UV[a]	
	μg ml^{-1}	μM	μg ml^{-1}	μM	μg ml^{-1}	μM
IS	4.6	8.0	1.2	2.1	1.6	2.4
IIIS	13.6	27.3	3.1	6.2	1.6	2.9
IIS	6.2	12.5	3.1 (6.2)[b]	6.3 (12.5)[b]	1.4	2.5
IA	5.8	10.8	1.3	2.3	2.8	4.6
IVS	10.1	24.2	1.3	3.0	1.3	2.9
IIIA	3.5	7.6	0.6	1.3	2.2	4.4
IIA	3.9	8.5	1.3 (13.9)[c]	2.8 (30.3)[c]	2.0	4.0
IVA	4.4	11.6	0.2	0.5	5.0	12.5

[a] Ref. [16].
[b] Fragments 300.1+337.9.
[c] Fragments 300.1+357.0.

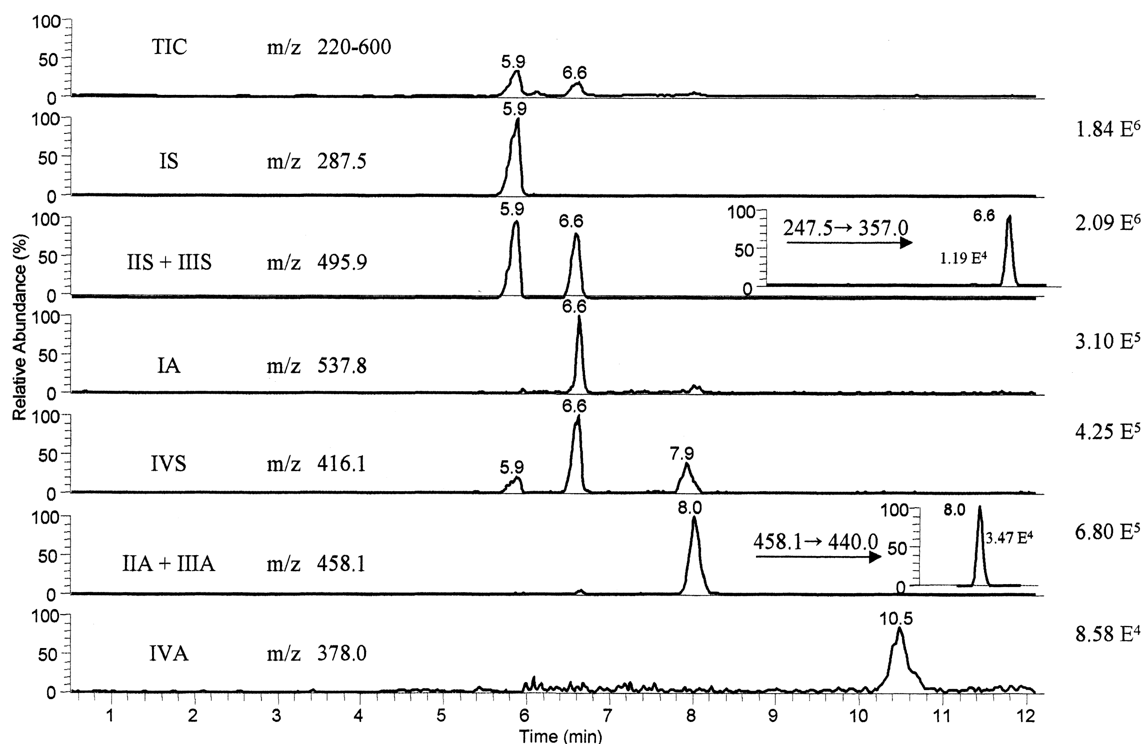

Fig. 6. Full-scan CE–MS total ion current and extracted ion current profiles for the base peaks of depolymerised porcine mucosal heparin. Insets show IIS+IIIS CE–MS–MS transition of m/z 247.5 to m/z 357.0 and IIA+IIIA CE–MS–MS transition of m/z 458.1 to m/z 440.0.

values of major disaccharides were lower than expected. This can be explained by the co-migration of an interference with the major compounds which can reduce the efficiency of ionic evaporation. A detailed MS study of peaks in the interval from 5.0 to 6.0 min (Fig. 7) gave MS spectra that were different from those obtained for disaccharide IS. Traces of the base peak revealed an interference almost one order of magnitude higher than that of the disaccharide trace. As a result, normalisation of molar concentrations of the eight disaccharides would lead to inaccurate percentage characterisations.

To overcome the effect of the sample matrix on the disaccharide characterisation, ratios of the molar concentrations of major and minor disaccharides were separately calculated. The relative concentrations agree with those obtained by CE–UV (Table 6). Individual quantitation of positional isomers was not calculated in these samples as the signals obtained for the characteristic fragment ions of disaccharides IIS and IIA were below the detection limits.

4. Conclusions

The application of pressure-assistance in CE–MS coupling provided stable ESI conditions and enabled heparin-derived disaccharide determination in reversed polarity CE. However, the relatively high pressure used produced a significant loss of efficiency, which required the acquisition of CE–MS–MS electropherograms to enable identification of positional isomers. Lower LODs in concentration (0.5 to 6.3 μM) than those of CE–UV were obtained in the CE–MS–MS for most of the disaccharides.

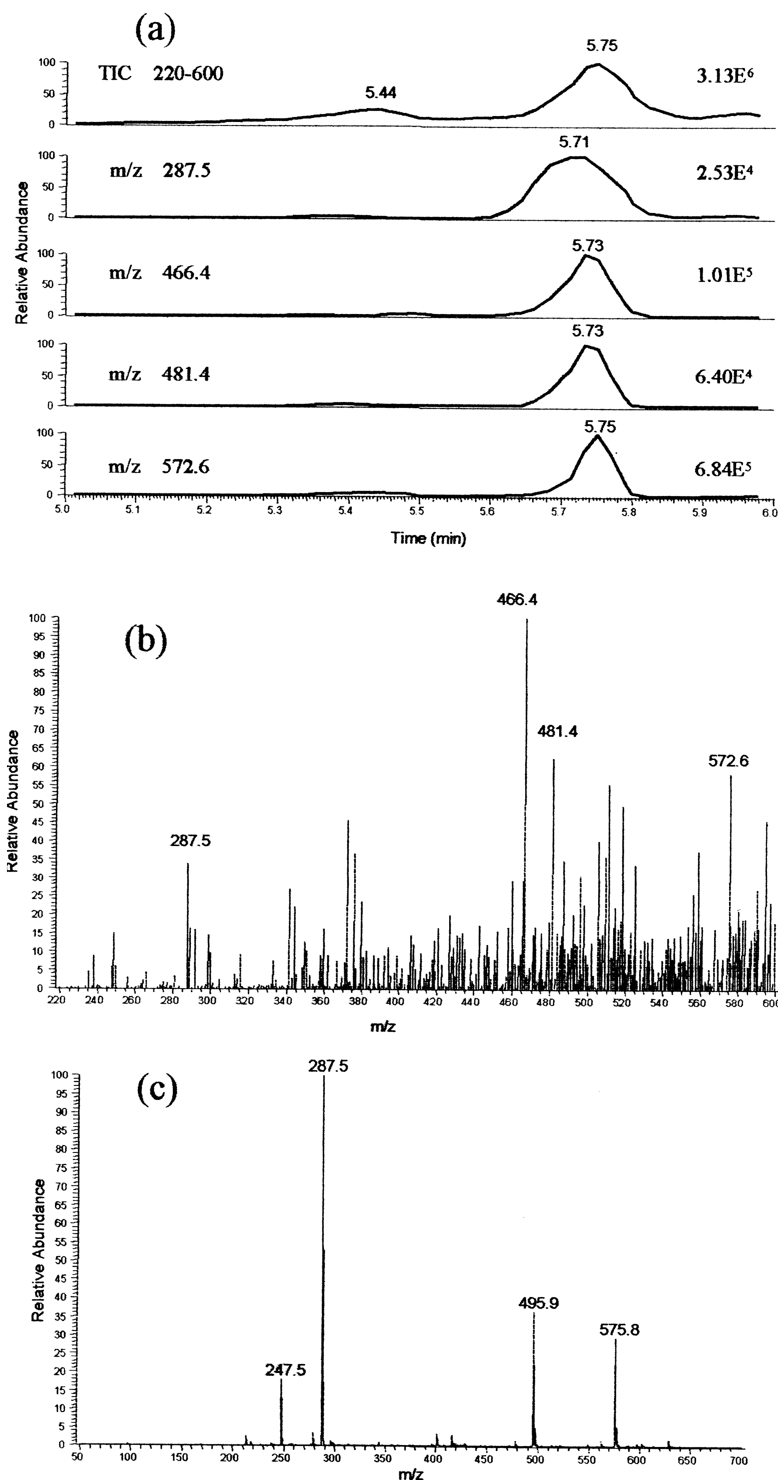

Fig. 7. (a) Full-scan CE–MS total ion current and extracted ion current profiles of the most abundant ions in the interval of 5.0 to 6.0 min of the depolymerised bovine mucosal heparin. (b) Average MS spectra of the peak migrating at 5.7 min and (c) of the disaccharide IS standard.

Table 6
Relative disaccharide percentage composition

	Bovine mucosa heparin		Porcine mucosa heparin	
	CE–UV	CE–MS	CE–UV	CE–MS
Major disaccharides				
IS/(IIS+IIIS)	1.4	1.9	3.1	3.3
Minor disaccharides				
IA/IVA	2.2	n.q.	2.1	3.0
IVS/IVA	11.9	8.0	1.6	1.6
(IIA+IIIA)/IVA	7.7	n.q.	3.2	2.7

n.q., Not quantified.

Good run-to-run precision values for migration times and concentration were obtained in both CE–MS and CE–MS–MS methods. The use of a CE system with pressure modulating capabilities would allow a better optimisation of the CE–MS separation conditions for this particular application and for other determinations where the resolution is compromised. Studies with variable pressure and voltage CE systems as well as modifications of the electroosmotic flow by using derivatised capillaries are currently in progress.

Despite the high selectivity of MS, the analysis of depolymerised heparins evidenced the influence of the sample matrix on the quantitative results, which could affect disaccharide compositional profile. This problem was avoided by the relative normalisation of major and minor disaccharides. In this way, characteristic disaccharide profiles, in agreement with those previously calculated by CE–UV, were obtained for bovine and porcine mucosal heparins.

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