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Detection of carbohydrates by electrospray ionization–ion mobility spectrometry following microbore high-performance liquid chromatography

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Abstract

This paper reports the first example of electrospray ion mobility spectrometry as a detection method for HPLC separation, demonstrating its potential for the quantitative and selective detection of non-volatile and non-chromophoric organic compounds. Reduced mobility constants (K_0) for 21 carbohydrates, including simple sugars, sugar alcohols and amino sugars, were determined to range from 0.68 to 1.37 $cm^2\ V^{-1}\ s^{-1}$. Minimum detectable quantities were measured from as low as $5.8 \cdot 10^{-14}$ mol for D(+)-cellobiose to as high as $8.2 \cdot 10^{-11}$ mol for L-iditol. A plot of carbohydrate mass with respect to inverse ion mobility indicated that, to a first approximation, mobility was inversely proportional to mass for most carbohydrates. However, in several cases, isomeric separation could be achieved by ion mobility spectrometry, demonstrating that ion mobility separation is based on size and shape of the molecule rather than molecular mass. Other notable exceptions to the mobility–mass relation were β-D-maltose and D(+)-cellobiose, which exhibited significantly lower mobilities than expected, and maltoheptaose, which exhibited a significantly higher mobility than expected. Dimerization and multiple charges may have caused these deviations from the simple mass–mobility correlation. Parametric optimization coupled with further basic investigations of this ionization and detection method should lead to a novel approach for the analytical determination of carbohydrates.

Keywords: Ion mobility, spectrometry; Detection, LC; Carbohydrates

1. Introduction

Carbohydrates are some of the most difficult biomolecules to separate and measure at low levels. Traditional methods of analysis include separation and detection by gas and liquid chromatography. For gas chromatographic techniques the low volatility of most carbohydrates requires derivatization of the hydroxyl, amino and carboxyl groups associated with this class of compounds. Unfortunately derivatization of these multi-functional molecules often result in incomplete derivatization, leading to a complex mixture of derivatives to separate and detect. Thus, liquid chromatography (LC) is the common method of choice for most carbohydrate analyses. However, the lack of UV absorption makes detection of carbohydrates after LC difficult. The insensitive and non-selective method of monitoring the effluent's

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refractive index (RI) is the most common detection method used after LC separation. Reviews of current chromatographic methods used for the separation and detection of carbohydrates can be found in Refs. [1–3].

A detection method is required, which can sensitively and selectively respond to carbohydrates dissolved in the mobile phase of a liquid chromatograph. Recently, we have developed an ion mobility spectrometric (IMS) method which can introduce non-volatile compounds into an ion mobility spectrometer for separation and detection [4,5]. Based on a unique cooled electrospray source design, polar and high-molecular-mass compounds can be efficiently nebulized, ionized and desolvated into gas phase ions at atmospheric pressure. These gas phase ions can be separated and detected according to their mobility differences in the drift gas. Electrospray ionization (ESI) has been used as an introduction and ionization method for carbohydrates into a mass spectrometer under low-pressure conditions [6], but not into ion mobility spectrometers under atmospheric pressure conditions. Because of the simplicity of ion mobility spectrometers, their application as a reliable and routine detection method for carbohydrates after LC seemed promising.

To test this hypothesis, the ESI and ion mobility separation behavior of a wide variety of carbohydrates were evaluated. Do these compounds electrospray efficiently under atmospheric pressure conditions? Can the solvents used in their liquid chromatographic separation be desolvated effectively, leaving behind an unclustered gas phase molecular analyte ion? How then do these ions behave in an ion mobility spectrometer? Very little information is available on the behavior of large molecular gas-phase ions at atmospheric pressures.

2. Experimental

A schematic diagram of the ESI–IMS system coupled with microbore HPLC is shown in Fig. 1. The mobile phase used throughout this study was a mixture of HPLC-grade methanol–water–0.1 *M* acetic acid (48.75:48.75:2.5, v/v) (pH=4.17) (J.T. Baker, Phillipsburg, NJ, USA). Solvents were sparged with helium. A dual piston syringe pump

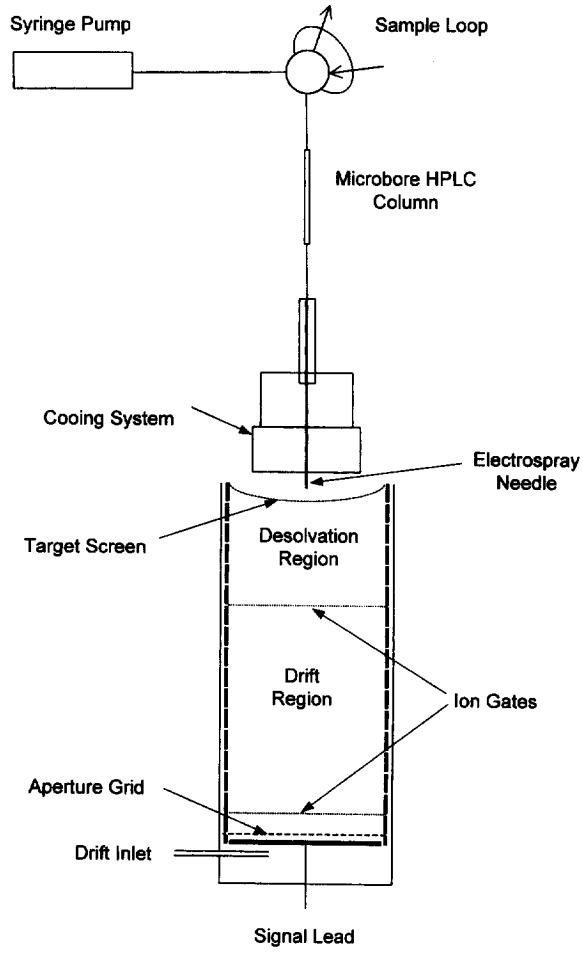

Fig. 1. Schematic diagram of electrospray ionization ion mobility spectrometry.

(Microgradient system, BrownLee Labs., Applied Biosystems, Santa Clara, CA, USA) was used to transport the mobile phase to the 27-gauge stainless steel (SS) electrospray needle. A six-port injector (C6W, Valco Instruments, Houston, TX, USA or Rheodyne 7125, Rheodyne, Cotati, CA, USA) was coupled to a microbore packed column (Hypersil 5 C_{18}, 250×1 mm, 5 μm, Phenomenx, Torrance, CA, USA) for the separation experiments. A 0.1 mm I.D. and 0.2 mm O.D. deactivated vitreous silica tubing (J&W Scientific, Folsom, CA, USA) of approximately 1 m in length was used as a transfer line. One end of the transfer line was connected to a low dead volume Valco split tee through a 1.6–0.8 mm zero

dead volume internal reducer (1ZR1.5T, Valco Instruments). The other end of the transfer line was inserted through the 27-gauge electrospray needle. Transfer line tubing was also connected between the split tee and a waste bottle, splitting the effluent 1:10, with one part going to the IMS system and 10 parts going to waste.

The electrospray needle was insulated with 0.4 mm I.D. PTFE tubing with a wall thickness of 0.2 mm. The spray needle was externally cooled with nitrogen gas and water via an external jacket surrounding air chamber which housed the insulated spray needle in order to avoid volatilization of the liquid stream in the needle. The electrospray needle was operated at approximately 40°C. Unless cooled, the spectra became unstable when the liquid stream vaporized before it reached the tip of the needle.

A target screen was added to the first guard ring of the ion mobility spectrometer, directing the sprayed ions into the desolvation region of the drift tube. A series of SS rings alternating with a series of Pyrex insulating rings were assembled to produce the atmospheric pressure ion drift tube. The SS rings were connected by a series of 1 MΩ resistors. The total resistance was about 16 MΩ. The guard rings had an I.D. of 4.22 cm, an O.D. of 5.71 cm, and were 0.8 cm in height. Pyrex insulators, fitted around each ring, served to isolate the guard rings from the aluminum housing as well as from each other. By applying drift voltage to the first ring of the tube, a uniform electric field directed electrosprayed ions through the drift tube to a terminal Faraday plate where the ion current was collected and amplified. Inside the drift tube, a preheated countercurrent dry nitrogen gas was employed as both drift gas and desolvation gas for the electrospray process.

The electronic components of the ESI-IMS system included a high-voltage supply for the spray needle (Model 602B-200P Bertan Assoc., Hicksville, NY, USA), a high-voltage supply for the drift field supply (Model PMT 50A, Bertan Assoc.), a temperature controller for the IMS housing oven (Watlow, St. Louis, MO, USA), gate drivers under software control (WSU Technical Service), and a current amplifier (Model 427, Keithley Instruments, Cleveland, OH, USA). In most of the experiments, 8800 V was applied to the electrospray needle and 4800 V to the target screen. Thus, electrospray ionization was generated with a potential difference of 4000 V. The gate drivers were controlled by software developed at WSU based on the Burr-Brown PCI-20000 data acquisition and control cards (Intelligent Instrumentation, Tucson, AZ, USA) installed in an IBM AT compatible computer (Cactus Computer, Moscow, ID, USA). The detector output was amplified and sent to the data acquisition cards on the computer. Chromatograms were recorded by a HP3392A integrator (Hewlett-Packard, Wilmington, DE, USA).

In these experiments, the ion mobility spectrometer was operated at 250°C. Nitrogen gas, preheated to the same temperature, was used as drift gas at a flow-rate of 900 ml min^{-1}. The electric field strength in both desolvation and drift region was controlled at 300 V cm^{-1}. Ion mobility spectra were obtained by continuously infusing sample solution into the ion mobility spectrometer using a 24-μl sample loop. To obtain spectra, the ion gate located between the desolvation region and the drift region was used to generate ion pulses (~0.2 ms in length) and the other ion gate was left open. When the ion mobility spectrometer was used as a chromatographic detector, the first ion gate was operated in a similar manner, but the second ion gate was opened for only a short period of time after a defined time delay from the first ion gate pulse. This method of operation only allowed ions with a selected drift time to be detected.

ESI-IMS is suitable for interfacing to HPLC with lower volumetric flow-rates. In this study, a microbore C_{18} column was used for the separation of glucose and sorbitol as model compounds. A mixture of methanol–water–0.1 *M* acetic acid (48.75:48.75:2.5, v/v, pH=4.17) was used as mobile phase. ESI-ion mobility spectra in the positive ion detection mode of carbohydrate standards were obtained by dissolving 1 mg of each carbohydrate in a 1 ml of methanol–water–0.1 *M* acetic acid (48.75:48.75:2.5, v/v) solution and continuously injecting individual standards into the IMS system using the 24-μl injection loop.

From measured drift times, reduced mobilities (K_0) were calculated from Eq. (1) [12].

$$K_0 = (L^2/Vt_d)(273/T)(P/760) \quad (1)$$

where L is the drift length in cm, V is the drift

voltage, t_d is measured drift time in seconds, T is the drift tube temperature in Kelvin and P is the drift gas pressure in Torr (1 Torr = 133.322 Pa).

3. Results and discussion

3.1. Ion mobility spectrometric characterization of carbohydrates

Twenty-one carbohydrate standards with molecular masses ranging from 180 to 1153 were characterized by ESI-IMS. The compounds investigated fell into three subgroups: simple sugars including monosaccharides and polysacharides, sugar alcohols and amino sugars. Acetic acid was added to the carbohydrate solutions to change the acidity of the sample solution in order to enhance the sensitivity of the electrospray process. Fig. 2 shows ion mobility spectra of simple sugars. In the figure, the peaks at around 10 ms were solvent-related ions. In each spectrum, a single molecular ion peak was commonly observed, presumably in the singly protonated form. Dimerization, decomposition, or different ion conformations may have caused multiple peaks in a spectrum. It is difficult to identify each individual peak without mass analysis. The drift times of every peak and reduced mobility for the most dominant peaks are listed in Table 1. Similarly, ion mobility spectra of sugar alcohols and amino sugars are illustrated in Figs. 3 and 4. Their mass, drift times and reduced mobilities are given in Table 1.

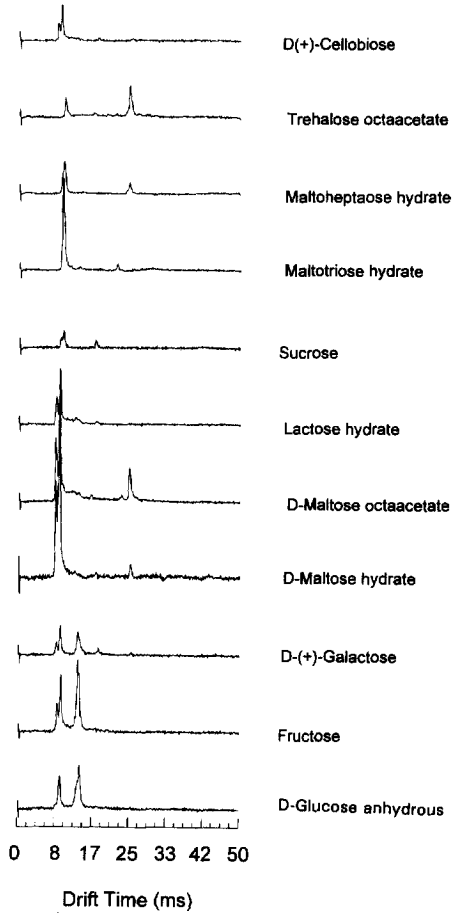

Fig. 2. Ion mobility spectra of simple sugars. Analytes at 1 mg/ml in mobile phase was continuously introduced with a flow-rate of 5 μl/min. The y-axes show relative response; the scale is the same for each spectrum.

One important observation from this data is that isomers of various carbohydrates produced different mobility constants. For example, D-glucose, fructose and D(+)-galactose all have a molecular mass of 180.16 but have different K_0 values, which were measured to be 1.25, 1.31 and 1.28, respectively. Because each of these mobilities defer by more than 0.02 K_0 units, these isomers can be separated by IMS, demonstrating that the shape of these three molecules are different from one another. Other examples of isomer separation are β-D-maltose and lactose. Both have a molecular mass of 360.32 but mobilities of 0.70 and 1.37, respectively. Sucrose and D(+)-cellobiose with a molecular mass of 342.3 had mobilities of 1.00 and 0.68. Finally, an interesting set of isomers were found in the amino sugars. D-Mannosamine, D-galactosamine and D(+)-glucosamine have a molecular mass of 215.64. D-Mannosamine ($K_0 = 1.29$) can hardly be separated from D-galactosamine ($K_0 = 1.27$) but D(+)-glucosamine ($K_0 = 1.37$) can be completely separated from both of the other isomers.

In general, however, the differences in mobility with respect to shape is small and the primary difference in the mobility of these compounds was due to the effect that mass had on the size of the ion. Fig. 5 demonstrates the relation of inverse reduced mobilities and molecular mass of the 21 sugars. With only three exceptions, reduced mobilities demonstrated a narrow distribution around a linear regres-

Table 1
Molecular mass (M_r), K_0 values and drift times (t_d)[a] of carbohydrates

No.	Sample name	M_r	K_0 ($cm^2 V^{-1} s^{-1}$)	t_d (ms)
(A) Simple sugars				
1-1	D-Glucose	180.16	1.25	**13.84**
1-2	Fructose	180.16	1.31	**13.45**
1-3	D(+)-Galactose	180.16	1.28	**13.45**, 18.00
1-4	β-D-Maltose	360.32	0.70	**25.06**
1-5	Lactose	360.32	0.99	12.77, **17.61**
1-6	Sucrose	342.31	1.00	**17.12**
1-7	Maltotriose	504.44	0.80	13.54, **21.96**
1-8	Maltoheptaose	1153.02	0.70	**24.67**
1-9	Trehalose	678.60	0.70	16.74, 19.74, 21.77, **24.86**
1-10	D(+)-Cellobiose	342.30	0.68	17.80, **25.25**
(B) Sugar alcohols				
2-1	D-Sorbitol	434.40	0.89	**19.54**
2-2	D-Mannitol	182.17	1.27	**13.84**
2-3	Maltitol	344.32	0.99	**17.61**, 24.96
2-4	L-Iditol	182.17	1.29	**13.64**
2-5	Inositol	180.16	1.21	**14.61**
2-6	Lactitol	362.33	1.00	12.87, **17.51**, 25.06
(C) Amino sugars				
3-1	D-Mannosamine	215.64	1.29	**13.54**
3-2	D-Galactosamine	215.64	1.27	**13.84**
3-3	D(+)-Glucosamine	215.64	1.37	**12.87**
3-4	Glucuronamide	193.16	1.27	12.67, **13.84**, 19.25
3-5	*N*-Methyl-D-glucamine	195.22	1.34	**13.06**

[a] In cases of multiple peaks detected, the drift times of base peaks were bolded. Note that the drift time may vary with ambient pressure change from day-to-day experiments. The variation was corrected when K_0 values were calculated according to Eq. (1).

sion line. The three exceptions were D-maltose, D-cellobiose and maltoheptaose. D-Maltose and D-cellobiose had smaller ion mobility compared with other sugars of similar molecular mass. In their ion mobility spectra (Fig. 3), another peak having a shorter drift time can be observed. However, the most intense peak was used to determine the reduced mobility plotted in Fig. 5. The formation of a dimer ion may have caused the outlier data points for these compounds.

Maltoheptaose demonstrated the opposite effect; a shorter drift time than that expected from Fig. 5. This effect may have resulted from multiple protonation. Because of its polysacharide nature, formation of doubly or triple charged ions are possible in an electrospray ionization source. A more interesting explanation of the phenomenon would be the effect of gas phase ion conformation. If the protonated polysacharide stayed in a folded form in the gas phase, it could result in a higher mobility measurement than expected. The existence of varied gas phase conformations of large molecules has been observed in ESI-IMS experiments [7]. Nevertheless, mass identification of electrosprayed ions will provide a definite answer for these observations.

The behavior of the electrospray ionization source for ion mobility spectrometry was investigated by observing the change in response of glucose and background ions as glucose was introduced into the electrospray. Ion mobility spectra were collected every 30 s for a single injection. The injection loop was 24 μl. At a sample flow-rate of 5 μl min^{-1}, the sample, 5.6 m*M* glucose, could be continuously sprayed for about 4.7 min. The response intensities of solvent ion peaks and the glucose ion peak were measured as a function of time after injection. As shown in Fig. 6, intensities of solvent ion peaks decreased in a range from 2 to 7 min when the

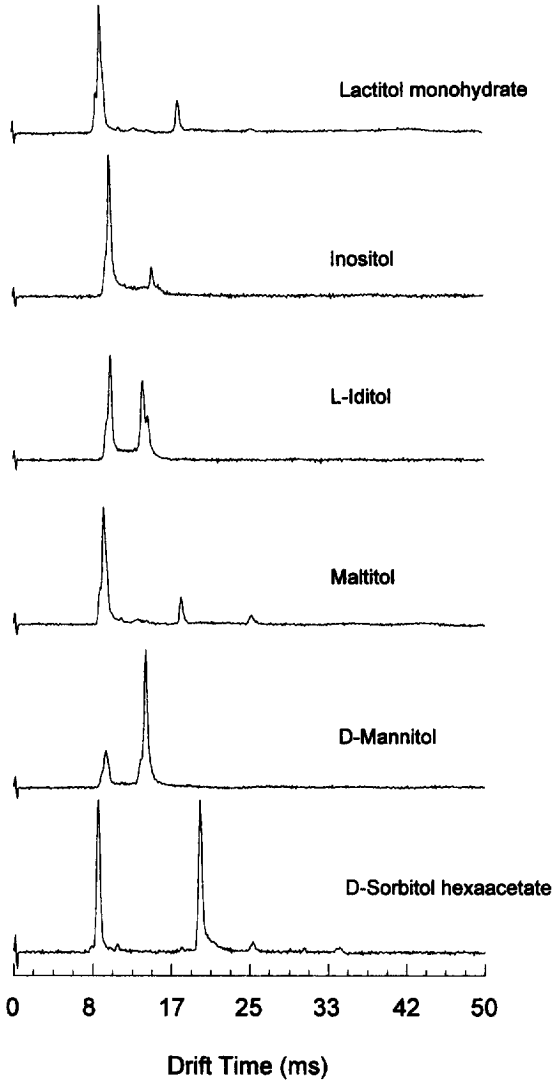

Fig. 3. Ion mobility spectra of sugar alcohols. Analytes at 1 mg/ml in mobile phase was continuously introduced with a flow-rate of 5 μl/min. The *y*-axes show relative response; the scale is the same for each spectrum.

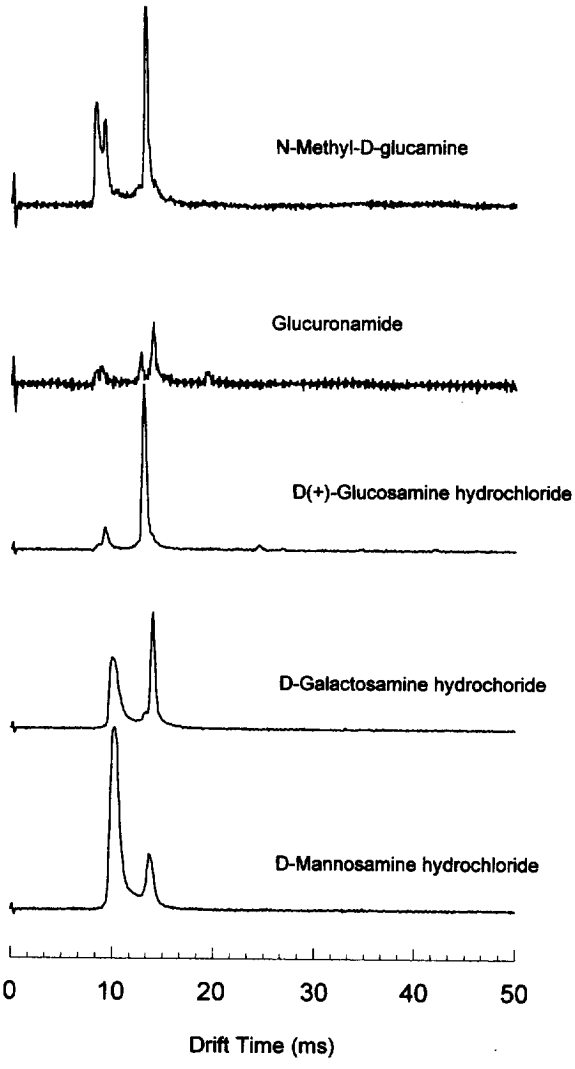

Fig. 4. Ion mobility spectra of amino sugars. Analytes at 1 mg/ml in mobile phase was continuously introduced with a flow-rate of 5 μl/min. The *y*-axes show relative response; the scale is the same for each spectrum.

glucose ion was present. This result demonstrated the charged competition process among the analyte and background electrolytes. A high concentration of glucose suppressed the formation of background ions. When the sample was exhausted, the background electrolytes carried most of the charge and the solvent peaks reappeared. Note that the solvent peaks continued to increase gradually after 10 min indicating glucose residue was slowly washed out of the liquid supply system. The concentration of 5.6 m*M* was too high for this sensitive device. Overloading the detector was evident because ions from the background electrolytes were completely eliminated from the spectrum during the elution of glucose.

3.2. Detectability of carbohydrates

Minimum detectable quantities, defined as a sig-

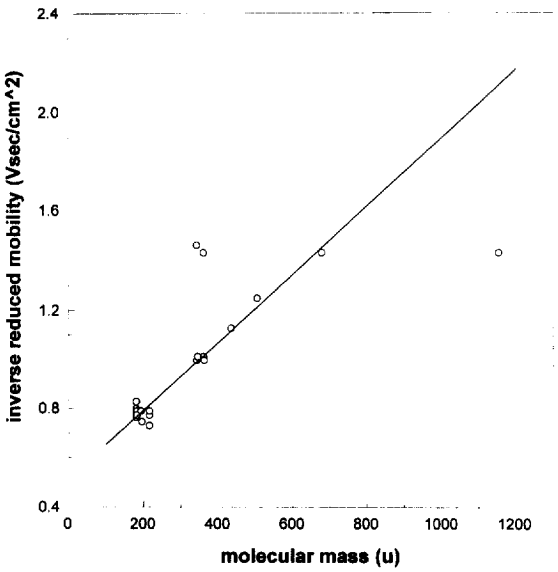

Fig. 5. Relationship of molecular mass of sugars and their inverse reduced mobilities derived from Table 2.

Table 2
Minimum detectable quantities of carbohydrates

Compound	Minimum detectable quantity (pmol)
D-Glucose	1.94
Fructose	0.166
D(+)-Galactose	0.422
β-D-Maltose	0.083
Lactose	0.105
Sucrose	0.120
Maltotriose	0.180
Maltoheptaose	4.86
Trehalose	0.928
D(+)-Cellobiose	0.058
D-Sorbitol	0.053
D-Mannitol	0.417
Maltitol	2.21
L-Iditol	82.3
Inositol	8.33
Lactitol	0.210
D-Mannosamine	24.6
D-Galactosamine	6.96
D(+)-Glucosamine	0.422
Glucuronamide	0.430
N-Methyl-D-glucamine	0.328

nal-to-noise ratio of 3, for each carbohydrate were determined in this study and are listed in Table 2. Most of the carbohydrates could be detected at the high-fmol level; however, detection limits for maltoheptaose, L-iditol, inositol, D-mannosamine and D-galactosamine were at the pmol level. These results represent an improvement in sensitivity of more than 10^6 to 10^3-times over those of RI detection. Although there was wide variation, the data indicated that detection limits for many compounds will be in an analytically useful range. Dynamic ranges of 10^3 to 10^4 were obtained in this investigation. In Table 3, our results were compared with other direct detection methods of HPLC for carbohydrates. Based on this comparison, ESI-IMS looks promising as a detection

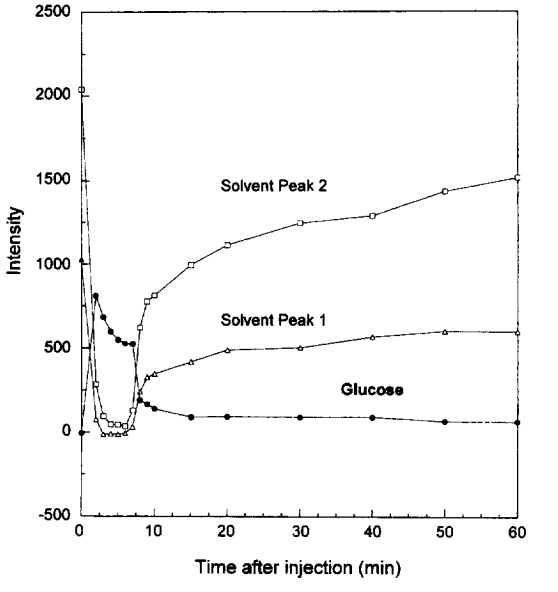

Fig. 6. Plots of response intensities of background ions or glucose ion as function of time after injection. Glucose solution at 1 mg/ml in mobile phase was continuously introduced with a flow-rate of 5 μl/min. The total infusion volume was 24 μl.

Table 3
Detection limits of various HPLC detection methods for carbohydrates

Detection method	Detection limit	Ref.
Refractive index	3–5 μg	[8,9]
UV absorption	1–10 μg	[8]
Evaporative light scattering	10–50 ppm	[10]
Pulsed amperometric	20 ng	[11]
Voltametric	100 μg	[8]
Conductivity	50–100 nmol	[8]
MS (thermospray)	20–100 pg	[8]
IMS (coronaspray)	0.2–6.9 pmol	[12]
IMS (electrospray)	20 pg–15 ng	This study

method for carbohydrates after liquid chromatographic separation.

3.3. Microbore HPLC detection

Fig. 7 shows chromatograms obtained using ESI–IMS after microbore HPLC separation. Two peaks (glucose and sorbitol) in Fig. 7 have been identified by using standards. The two model compounds shown here provided good resolution, response and reproducibility. The relative standard deviation was 0.06% ($n=6$) in the peak retention time, when known amounts of the standards were injected repeatedly. A noise level of 0.14 pA was usually obtained with a minimum detectable quantity of 23 pg for sorbitol. The linearity of response of the ESI process was confirmed by known amounts of standard from 10 ng to 10 μg. Significantly more stable baselines and signals were obtained in this study compared with chromatograms determined by coronaspray IMS [13].

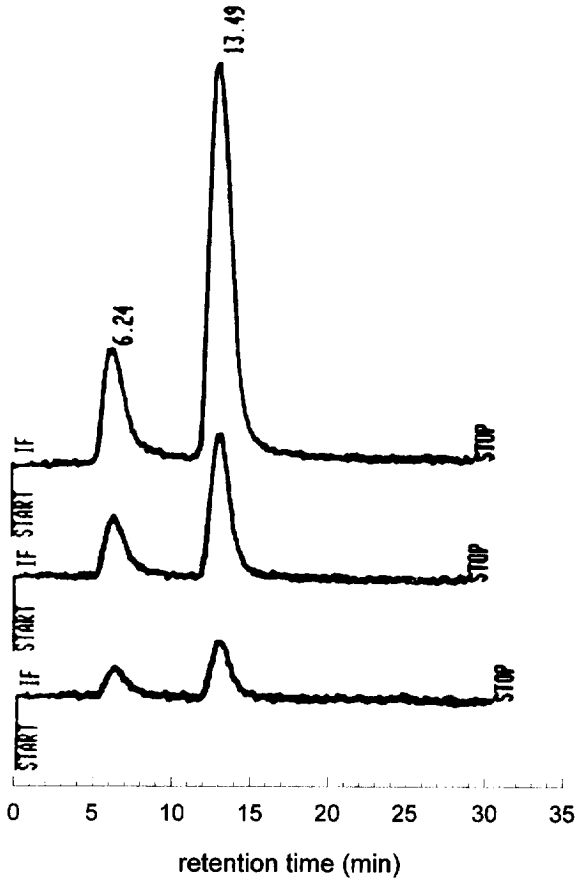

Fig. 7. Chromatographic profiles obtained by ESI-IMS after micro-HPLC separation. Samples were prepared in mobile phase and injected with a volume of 2 μl; mobile phase flow-rate was at 50 μl/min with a split ratio of 1:10. Peaks are glucose (6.24 min) and sorbitol (13.49 min), each in an amount of (top) 1 μg, (middle) 500 ng, (bottom) 100 ng.

4. Conclusions

ESI-ion mobility spectra of 21 different carbohydrates were measured and their reduced mobilities determined. With few exceptions, a linear relationship between inverse reduced mobilities and molecular masses was observed. The electrospray ionization source for IMS had a response behavior similar to that of ordinary electrospray ionization for mass spectrometry. As a detection method for liquid phase separation, ESI–IMS demonstrated fmol to pmol level detection limits for many carbohydrates. By successfully interfacing with a microbore HPLC column, this study demonstrated, for the first time, the promising potential of ESI–IMS as a HPLC detection method. This preliminary investigation demonstrated that ESI–IMS can be used for detecting non-volatile and non-chromophoric compounds after a HPLC separation, complementing other HPLC detection methods such as UV–visible and RI detectors.

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