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Chloride attachment negative-ion mass spectra of sugars by combined liquid chromatography and atmospheric pressure chemical ionization mass spectrometry

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ABSTRACT

A method has been developed for the rapid determination of sugars, including molecular weight measurements, using high-performance liquid chromatography coupled with negative-ion, atmospheric-pressure chemical-ionization mass spectrometry. The chromatography was carried out on a 250 × 4 mm I.D. column packed with 7 μm NH_2-silica. The mobile phase consisted of a high percentage of methanol or acetonitrile with a small amount of chloroform. During the mass spectrometry, a strong base is formed from the polar solvent molecules by corona discharge, followed by ion–molecule reactions in the chemical ionization ion source (*e.g.* the methoxy anion is formed from methanol). This strong base reacts with the chloroform, generating chloride ions, which in turn react with the neutral sugar molecules as they elute from the chromatograph. The chloride ion and sugar interactions yield chloride-attachment ions, which are further stabilized by successive collisions. In this method, authentic monosaccharides and some oligosaccharides show dominant quasi-molecular ions, $[M + Cl]^-$, with little fragmentation, and it is particularly useful for the molecular weight determination of sugars.

INTRODUCTION

Sugars and other important carbohydrates present in tissues and organs of plants and animals play important biological roles. Further studies of these biological roles will require less laborious and more sensitive and informative analytical means for the separation, quantitation, and structural determination of saccharides. For this purpose new analytical procedures, such as gas chromatography (GC) [1], GC–mass spectrometry (MS) [2], high-performance liquid chromatography (HPLC) [3], fast atom bombardment (FAB) MS [4] and HPLC–MS [5] have been developed during the past 20 years. These compounds are thermally labile, and not amenable to separation by GC and GC–MS without derivatization, which itself is time-consuming and laborious. On the other hand, saccharides can be separated by HPLC without prior derivatization. However, saccharides have no characteristic absorption in either the UV or the visible region, and although they can be detected at near-UV wavelengths, photometry in this region is subject to strong interference from buffer salts. Consequently they have usually been analysed by pre- or post-column labelling methods.

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Honda *et al.* [3] reported a new separation and identification method using anion-exchange chromatography and post-column labelling with borate complexes and spectrophotometric detection. However, HPLC is able to provide only separation (*e.g.* retention time), not structural and molecular information.

In MS, saccharides have been investigated using soft ionization techniques, such as FAB or liquid secondary-ion MS (LSI-MS) [5], field desorption [6] and laser desorption (LD) [7]. Soft ionization allows the detection of molecular ions from underivatized saccharides. Usually saccharides are present in the complex mixture obtained from biological sources, so strict isolation and purification are required prior to implementing the above MS analyses.

The HPLC–MS has emerged as a powerful tool for the analysis of biological samples since it permits the separation and ionization of non-volatile and/or thermolabile compounds without derivatization. Analysis of some saccharides has been also achieved by HPLC–thermospray (TSP) MS [8]. Saccharide mass spectra from TSP are characterized by some fragment ions and quasi-molecular ions, such as $(M+H)^+$ or $(M+NH_4)^+$. However, HPLC-TSP-MS has not proved amenable for all saccharides, since tri- or higher saccharides have not been analysed successfully. These compounds suffer attack by the reagent ions at the glycosidic linkage, resulting in severe fragmentation and little or no molecular ion intensity. In addition, many ions are not formed by simple chemical ionization (CI) processes but by reactions (*e.g.* thermally catalysed acetylysis) making the TSP mass spectrum very complex [8].

Sakairi and Kambara [9] reported a new HPLC–MS technique that is able to provide molecular weights of saccharides. At very high nebulizer temperatures (*ca.* 500°C) in an atmospheric pressure ionization source without corona discharge, dominant alkali-metal cationized molecular ions appeared in the mass spectra for many saccharides. The authors called this an atmospheric pressure spray (ASP) method as a new ion-evaporation ionization. However, the choice of mobile phase might be limited in this ASP method, since high-intensity quasi-molecular ions were detected with aqueous solutions but poor results were obtained with high concentrations of organic solvents.

Our objective was to develop a new HPLC–MS method that will provide separation and molecular information for the many molecular species of intact saccharides that are present in complex mixtures. The present study describes the determination of saccharides as their chloride attachment ions by HPLC combined with negative-ion atmospheric pressure chemical ionization (APCI) MS.

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile, methanol and chloroform were obtained from Wako (Osaka, Japan). Water was distilled, deionized and stored in high-density polypropylene plastic bottles. Mobile phases were filtered (0.45 nm) and degassed

before use. Saccharides used in this experiment were purchased from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification.

Liquid chromatograph

HPLC separations were performed with a Hitachi Model L-6200 chromatographic pump with a controller (Hitachi, Tokyo, Japan). A LiChrosorb NH_2 column (250 × 4 mm I.D., 7 μm particle size) (E. Merck, Darmstadt, F.R.G.) was used for to separate mixtures of some saccharides.

Samples were dissolved in methanol–water (50:50, v/v), or acetonitrile–water (50:50, v/v) and introduced through a Rheodyne Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.). The chromatograph was connected to the nebulizer in the interface by a PTFE tube (1 m × 1.6 mm O.D. × 0.25 mm I.D.).

Interface, APCI source and mass spectrometer

The chromatograph, mass spectrometer equipment, and operating procedures have been previously, reported [10] and are described only briefly here. A schematic diagram of the HPLC–MS interface is shown in Fig. 1. The interface consisted of a nebulizer and a vaporizer unit. The nebulizer consisted of a stainless-steel capillary pipe (1.6 mm O.D., 0.1 mm I.D.) brazed to a stainless-steel block (40 × 40 × 50 mm), which could be heated to 400°C with two cartridge heaters. The vaporizer consisted of a bored (60 mm × 5 mm I.D.) stainless-steel block, which could be also heated to 400°C by two cartridge heaters. The temperature of each unit was controlled independently, thereby enabling the optimal temperature conditions for each unit to be obtained by carefully investigating quasi-molecular ion intensities for several saccharides. Column effluents were nebulized and vaporized, and introduced into the APCI ion source. Here a corona discharge generated ions, which then underwent a variety of ion–molecule reactions.

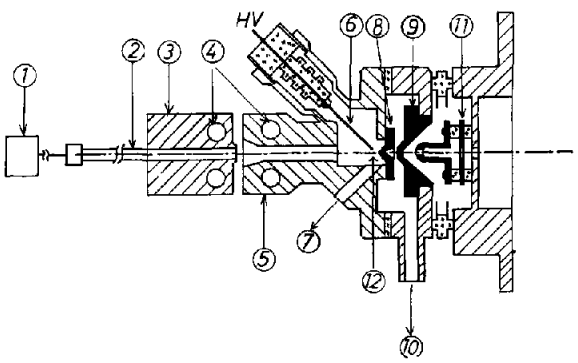

Fig. 1. Schematic diagram of the API ion source: 1 = liquid chromatograph; 2 = stainless-steel capillary pipe; 3 = nebulizer; 4 = cartridge heaters; 5 = vaporizer; 6 = needle electrode; 7 = exhaust; 8 = first electrode/aperture; 9 = second electrode/aperture; 10 = mechanical pump; 11 = lens; 12 = API source.

The corona discharge current was *ca.* $5 \cdot 10^{-6}$ A throughout all the experiments.

The ion source, including the first and second electrodes, was heated to 150°C with two ceramic molded heaters in order to reduce the contamination by solvents and samples. These ions, produced at atmospheric pressure, were introduced into the mass spectrometer through the intermediate pressure region located between two conical electrodes, the first with a 250-μm aperture and the second with a 400-μm aperture.

A "drift voltage" was applied between these two electrodes in order to increase the transmission efficiency of formed ions into the mass spectrometer, and to dissociate cluster ions produced by adiabatic expansion into quasi-molecular ions. This intermediate region was evacuated by a 1500 l/min mechanical booster pump and a 500 l/min rotary pump to *ca.* 60 Pa. The resultant ions were mass-analysed with a Hitachi Model M-2000A double-focusing mass spectrometer. The ion-source housing and the mass-analysing region were evacuated with two 900 l/s and 700 l/s diffusion pumps to *ca.* $1 \cdot 10^{-2}$ and 10^{-4} Pa, respectively. The acceleration energy was determined to be 4 kV by the second electrode potential.

All mass spectral data were gathered under full-scan operation mode, scanning the range m/z 1–800 in 4 s in a negative-ion detection mode. Investigations of the optimal conditions (*e.g.* nebulizer temperature, drift voltage, etc.), and sensitivity measurements were carried out with selected-ion monitoring (SIM) by flow-injection analysis without use of a column.

RESULTS AND DISCUSSION

Principle of method

Gas-phase ionization processes have been reported to play a role in "filament-on" HPLC–TSP-MS [11] and HPLC–APCI-MS [12,13]. It is suggested that these ionization mechanisms are very similar to CI. Negative-ion chemical ionization (NICI) has been investigated for many years.

There are many processes leading to the formation of negative ions: (1) proton transfer; (2) anion attachment; (3) nucleophilic attack; (4) charge transfer. Proton-transfer and anion-attachment mechanisms are especially important in practical negative HPLC–TSP-MS and HPLC–APCI-MS because they can provide molecular ions. Systematic studies have demonstrated that fluoride (F^-) and chloride (Cl^-) anions can be used as reagent ions to generate NICI mass spectra [12,14]. When halide ions (*e.g.* Cl^-) are associated with a neutral molecule that has some polar functional group, such as hydroxy, carboxylic, etc., then unstable intermediates with excess kinetic energy are generated. Normally, these intermediates dissociate to neutral molecules and chloride ions. However, under atmospheric pressure conditions, these intermediates become stabilized by removal of excess internal energy by successive collisions, resulting in stable ions with hydrogen bonding.

The conjugate acids (*e.g.* HCl) of halide ions (*e.g.* Cl^-) are very strong, so

proton transfer from a neutral molecule to the halide ion is difficult. Because of the frequent collisions that occur in the APCI source, adduct ions and cluster ions are formed more effectively than with ionization mechanisms that operate in vacuum or at low pressures.

The reactions mentioned above are generalized as follows:

(1) RO generation

$$ROH + e^- \xrightarrow{\text{Discharge}} RO^- + H \ (R = H, CH_3, \text{etc.})$$

(2) Cl generation [15,16]

$$CCl_3H + RO^- \longrightarrow Cl_3C:^- + ROH$$

$$Cl_3C:^- \longrightarrow :CCl_2 + Cl^-$$

$$:CCl_2 \xrightarrow{H_2O} CO + 2Cl^-, \quad HCOOH + 2Cl^-$$

(3) Cl attachment

$$M\text{–}OH + Cl^- \longrightarrow (M\text{–}OH \cdots Cl)^* \xrightarrow{\text{[Collisional stabilization]}} (M\text{–}OH \cdots Cl)^-$$

Hydrogen bonding

Formation of chloride ions

Fig. 2 shows the HPLC–APCI-MS mass spectrum obtained with methanol (negative-ion detection mode). Methanol was introduced continuously at flow-rate of 1 ml/min through the APCI source. The characteristic ions include:

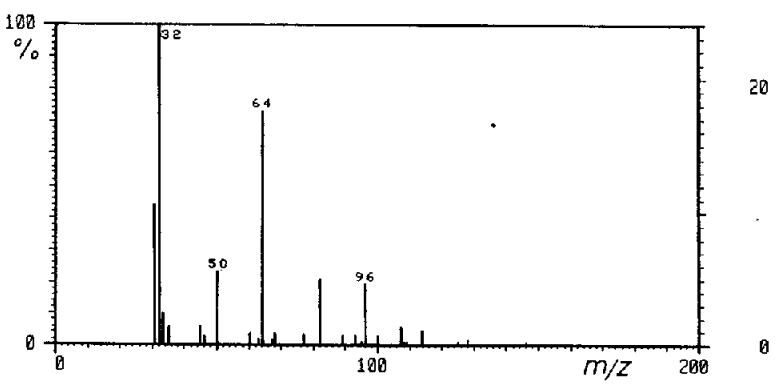

Fig. 2. HPLC–NI-APCI-MS mass spectrum of methanol: nebulizer temperature, 250°C; vaporizer temperature, 400°C; drift voltage, 150 V; flow-rate, 1 ml/min.

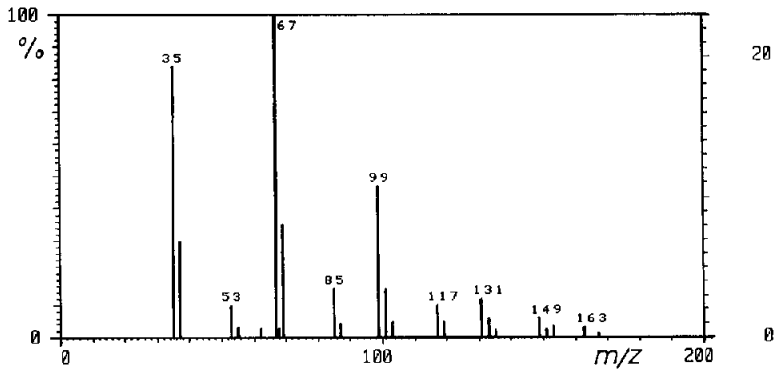

Fig. 3. HPLC–NI–APCI-MS mass spectrum of chloroform–methanol (5:95): nebulizer temperature, 250°C; vaporizer temperature, 400°C; drift voltage, 150 V; flow-rate, 1 m/min.

$[CH_3O\ (CH_3OH)\ n]^-$	$n=0$ and 1 at m/z 31 and 63
$[O_2\ (H_2O)\ n]^-$	$n=0$, 1 and 2 at m/z 32, 50 and 68
$[O_2\ (CH_3OH)\ n]^-$	$n=0$, 1, 2 and 3 at m/z 32, 64, 96 and 128

It is thought that atmospheric O_2 is the source of the oxygen atoms ionized by the corona discharge in the APCI.

Fig. 3 shows the HPLC–NI-APCI-MS mass spectrum obtained with methanol–chloroform (95:5, v/v). In this mass spectrum, the two groups of mass peaks are attributable to:

$[Cl\ (CH_3OH)\ n]^-$	$n=0$, 1 and 2, at m/z 35, 67 and 99
$[Cl\ H_2O(CH_3OH)\ n]^-$	$n=0$, 1, 2 and 3 at m/z 53, 85, 117 and 149

Alkyl halides, especially chloroform, are well known to be reactive toward strong bases, such as hydroxy and methoxy anions, etc. [15,16]. Thus, it is evident that in negative-ion APCI, chloroform is attacked by strong base, such as OH^- or CH_3O^-, to give the chloride anion Cl^-. The chloride ions formed in the APCI source will then form adduct ions with the analyte molecules with polar hydroxy functional groups. The chloride ion reacts with analyte molecules exactly analogously to chloride CI using alkyl halide–methane as reagent gas [12,14]. Another advantage of using chloride anions as a reagent is that chloride adducts show the characteristic 3:1 isotopic pattern of chlorine. This makes it easy to identify the quasi-molecular ion in the mass spectrum.

Fig. 4 is the mass spectrum from 1 μg of underivatized sucrose, which was dissolved in methanol–water (50:50, v/v) and introduced through the HPLC–APCI interface into the mass spectrometer using methanol–chloroform (95:5, v/v) as a mobile phase. This spectrum has a dominant chloride adduct ion at m/z 377 in the molecular-ion region and at very low intensity, other adduct ions,

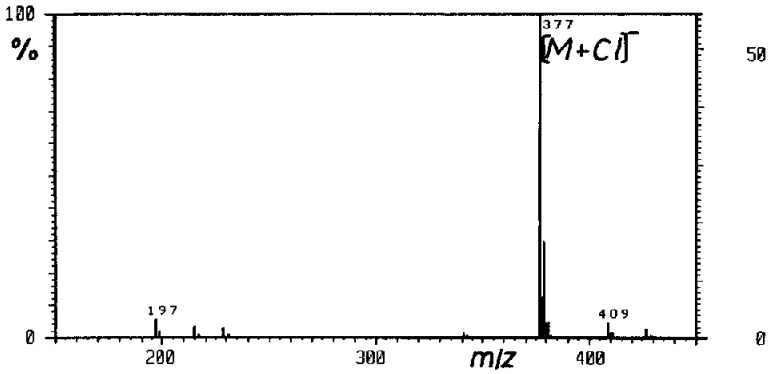

Fig. 4. LC–NI-APCI-MS mass spectrum of underivatized sucrose: mobile phase, chloroform–methanol (5:95); nebulizer temperature, 250°C; vaporizer temperature, 400°C; drift voltage, 150 V; flow-rate, 1 ml/min.

which are assigned to $[M+Cl+CH_3OH]^-$ at *m/z* 409, $[M+Cl+H_2O+CH_3OH]^-$ at *m/z* 427. A few fragment ions with low intensities in the low-mass region from *m/z* 150 to *m/z* 300 are attributable to $[162+Cl]^-$ at *m/z* 197, $[162+Cl+H_2O]^-$ at *m/z* 215, and $[162+Cl+CH_3OH]^-$ at *m/z* 229. The ion with a mass of 162 is the fragment ion resulting from the 1,2-cleavage of the glycosidic linkage [17].

Optimization of analytical conditions

Fig. 5 shows the influence of 5% each of the three polyhalogenated solvents mixed with methanol, on the chloride attachment NI APCI mass spectra of un-

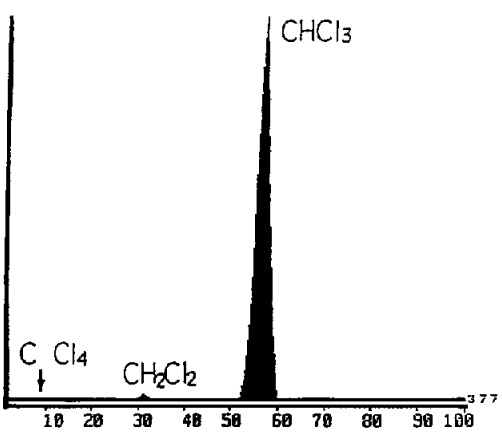

Fig. 5. Influence of the polyhalogen solvents mixed with methanol on the chloride-attachment HPLC–NI-APCI-MS mass spectra of underivatized sucrose. Mobile phase, carbon tetrachloride–methanol (5:95), dichloromethane–methanol (5:95) or chloroform–methanol (5:95); nebulizer temperature, 250°C; vaporizer temperature, 400°C; drift voltage, 150 V.

derivatized sucrose. A 10-μl volume of the methanol–water solution, containing 100 ng/ml of sucrose, was injected and the intensity of the $[M+Cl]^-$ ion was monitored by SIM (*m/z* 377) in three different mobile phases. The mixture of 5% chloroform and methanol generally produced the highest $[M+Cl]^-$ ion response. The $[M+Cl]^-$ ion intensity for sucrose using chloroform–methanol was *ca.* 8-fold higher than that using dichloromethane–methanol, and three orders of magnitude higher than that using carbon tetrachloride–methanol. These results are in fair agreement with the tendency of the hydrolysis of the polychloromethanes in strong base solutions : $CHCl_3 > CH_2Cl_2 > CCl_4$. From this experiment, it is evident that chloroform is the most suitable of the three solvents for generating chloride ions in the APCI source.

The calibration curves for glucose at different concentrations of chloroform in acetonitrile are shown in Fig. 6A. No major differences were observed with chlo-

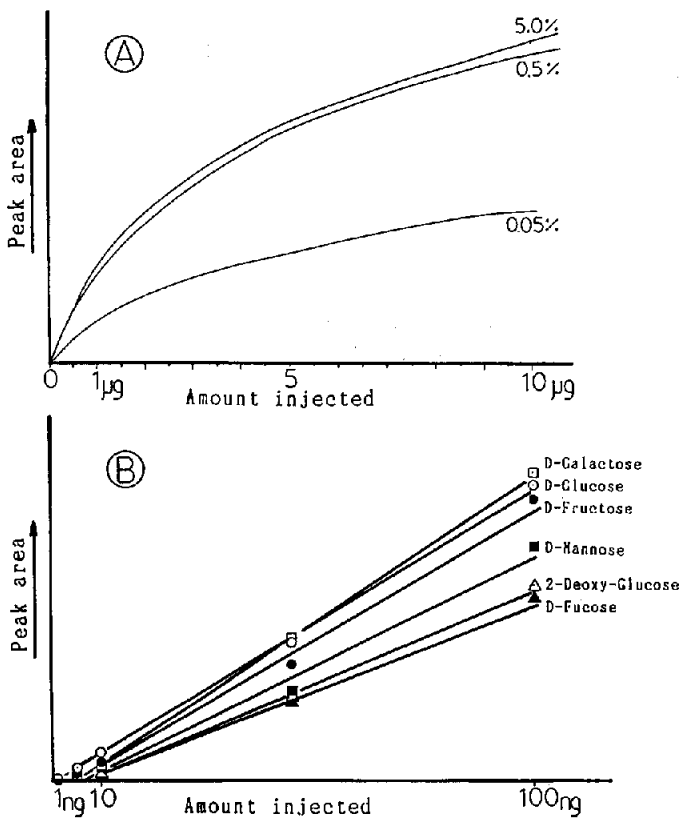

Fig. 6. Quantitative analysis by chloride-attachment HPLC–NI-ACPI-MS. Peak area as a function of amount of saccharide injected: (A) for different chloroform content in the mobile phase (for glucose); (B) for several saccharides [chloroform–acetonitrile (5:95) solution]. Analytical conditions: column, LiChrosorb NH_2 (250 × 4.0 mm I.D.); mobile phase, 95% acetonitrile–water (80:20) and 5% chloroform; flow-rate, 0.8 ml/min; nebulizer temperature, 170°C; drift voltage, 150 V.

roform content ranging from 0.5% to 5% in acetonitrile, as seen from Fig. 6A. However, with 0.05% chloroform in acetonitrile as a mobile phase, the $[M+Cl]^-$ response was *ca.* 50% of that using $\geq$ 0.5% chloroform solution. This indicates that a chloroform concentration of more than 0.5% in acetonitrile is adequate for quantitative analyses of sample amounts ranging from 1 ng to 10 μg. The calibration for glucose was found to be linear in the range from 1 ng to 100 ng when eluted with more than 0.5% $CHCl_3$ in acetonitrile; *i.e.* the $[M+Cl]^-$ ion intensity increased proportionally to the amount of sample injected. Saturation of the response was observed for samples larger than 1 μg.

This saturation is attributed to the depletion of reagent ions in the APCI source. Fig. 7 shows the mass chromatograms for *m/z* 35 Cl^-, *m/z* 67 $[CH_3OH+Cl]^-$, *m/z* 99 $[2CH_3OH+Cl]^-$, and *m/z* 377 the chloride adduct ion with sucrose. Volumes of 20 μl of methanol solution, containing 10 μg of sucrose, were injected. When sucrose was eluted with 0.5% chloroform–methanol solution, the intensity of the $[M+Cl]^-$ ion increased while the intensities of ions at *m/z* 35, *m/z* 67, and *m/z* 99 decreased. This indicates that ions at *m/z* 35 Cl^-, *m/z* 67 $[CH_3OH+Cl]^-$, and *m/z* 99 $[2CH_3OH+Cl]^-$ play important roles in the APCI as reagent ions. This explanation is also supported by the experimental results that the $[M+Cl]^-$ ions for 10 ng of D-glucose injected with 5 μg of inositol was 0.53 times as abundant as the $[M+Cl]^-$ ions for 10 ng of D-glucose injected alone.

Fig. 6B shows peak area as a function of amount injected for several saccharides (with 5% chloroform in acetonitrile as mobile phase). Good linearity (Fig. 6B) was observed between 1 and 100 ng injected for all monosaccharides tested. Intensities of $[M+Cl]^-$ ions for all monosaccharides tested increased proportion-

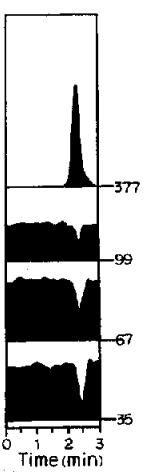

Fig. 7. Mass chromatograms of *m/z* 35 Cl^-, *m/z* 67 $[CH_3OH+Cl]^-$, *m/z* 99 $[2CH_3OH+Cl]^-$, and *m/z* 377 $[M+Cl]^-$, as sucrose eluted in 0.5:95.5 chloroform–methanol solution.

ally to sample amounts injected, but slight differences of sensitivity were observed between samples.

The effect of drift voltage and nebulizer temperature on ion intensity $[M+Cl]^-$ is shown in Fig. 8. A 1-μg mass of glucose dissolved in chloroform–acetonitrile (5:95) solution was injected, and the ion intensity at m/z 215 was measured in the SIM mode, with chloroform–acetonitrile (5:95) eluent. (In this experiment, the flow-rate and vaporizer temperature were maintained constant at 1 ml/min and 400°C, respectively.) The chloride adduct ions with glucose ($[M+Cl]^-$) peaked in the vicinity of 90 V drift voltage at a nebulizer temperature of 205°C, and at 150 V for a nebulizer temperature of 170°C. (The nebulizer temperature was monitored by a thermocouple located near the centre of the heating block of the nebulizer.) The optimum nebulizer temperature, giving abundant $[M+Cl]^-$ ions while not sacrificing overall sensitivity, was 170°C, and 150 V was the optimum drift voltage, using chloroform–acetonitrile (5:95) solution as mobile phase. At lower (135°C) and higher (240°C) nebulizer temperatures, the $[M+Cl]^-$ ion intensities were relatively low. At a lower temperature, nebulized droplet sizes are large, so a higher drift voltage is required to strip the solvating molecules from the droplets. At a higher drift voltage, the decreasing signal is attributed to a disruption of the chloride adduct ion complex resulting in the loss of the charge with the chlorine by a collision-induced dissociation (CID). At a higher nebulizer temperature, the decrease in signal can be caused by the neutralization occurring between formed ions and solvent molecules or positive ions due to the higher frequency of collisions or also due to the destabilization of

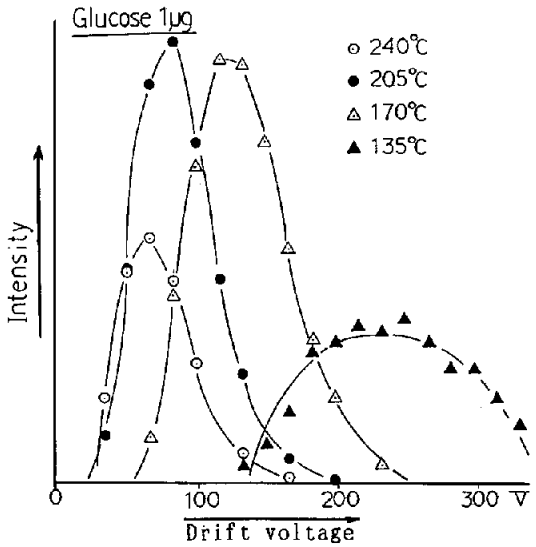

Fig. 8. The effect of drift voltage and nebulizer temperature on ion intensity $[M+Cl]^-$ for glucose. Mobile phase, chloroform–acetonitrile (5:95); flow-rate, 1 ml/min; vaporizer temperature, 400°C; injection, flow injection without a column.

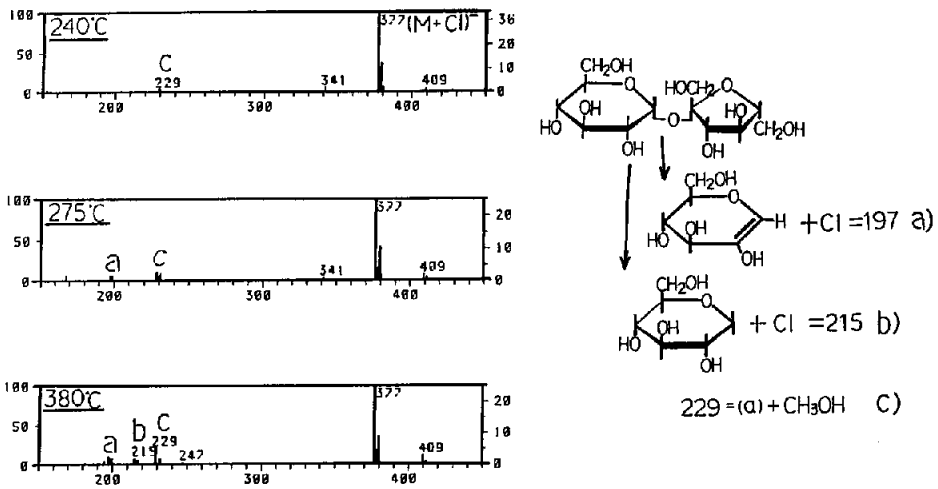

Fig. 9. Chloride-attachment HPLC–NI-APCI-MS mass spectra for sucrose taken at different nebulizer temperatures. Mobile phase, chloroform–methanol (5:95); drift voltage, 130 V; flow-rate, 1 ml/min; vaporizer temperature, 400°C; injection, flow injection without a column.

the chloride attachment complex. In Fig. 9 an increase in solvent molecules clustered with ions $[M + Cl + CH_3OH]^-$ does not appear to be the core as the temperature increases.

The effect of nebulizer temperature on the mass spectra was also investigated. Fig. 9 shows the mass spectra of sucrose obtained at different nebulizer temperatures, 240°C, 275°C and 380°C. All mass spectra were dominated by the $[M + Cl]^-$ ion at m/z 377 with a few low-intensity fragment ions. No significant differences were observed in these mass spectra, but too high a temperature ($\geqslant$ 400°C) caused partial decomposition of sucrose molecules. Good mass spectra were obtained at nebulizer temperatures ranging from 240°C to 350°C.

Fig. 10 shows the chloride attachment HPLC–NI-APCI mass spectra of sucrose taken at different drift voltages, using an eluent of chloroform–methanol (5:95) at a flow-rate of 1 ml/min without a column. All mass spectra show a dominant $[M + Cl]^-$ ion with a few cluster ions. The cluster ions appearing in the mass spectrum taken at a drift voltage of 70 V can be assigned to $[M + Cl + H_2O]^-$ at m/z 395, $[M + Cl + CH_3OH]^-$ at m/z 409, $[M + Cl + H_2O + CH_3OH]^-$ at m/z 427, $[M + Cl + 2CH_3OH]^-$ at m/z 441, $[M + Cl + H_2O + 2CH_3OH]^-$ at m/z 459, and $[M + Cl + 2H_2O + CH_3OH]^-$ at m/z 477. However, at a drift voltage of 100 V, all cluster ions except $[M + Cl + CH_3OH]^-$ at m/z 409 disappeared, and $[M - H]^-$ at m/z 341 appeared in the mass spectrum. At a drift voltage of 130 V, all cluster ions disappeared and only the dominant $[M + Cl]^-$ ion and $[M - H]^-$ ion were observed still with no fragment ions. The optimal analytical conditions are summarized in Table I.

TABLE I

OPTIMAL ANALYTICAL CONDITIONS

Solvent content	Nebulizer temperature (°C)	Desolvator temperature (°C)	Drift voltage (V)
Chloroform–methanol (5:95)	250	400	130
Chloroform–acetonitrile (5:95)	170	400	150

Mass spectra of various saccharides

Figs. 11–15 show the mass spectra for 1 μg of underivatized saccharides obtained using a 5% chloroform–methanol eluent at a flow-rate of 1 ml/min in the flow-injection mode (no column). All mass spectra were dominated by chloride adduct ions, showing the characteristic 3:1 isotopic abundance ratio, with some additional relatively low-intensity fragment ions. In the mass spectra of the monosaccharides, abundant chloride ions $[M+Cl]^-$ at *m/z* 215, and both chloride and solvent molecule attachment ions $[M+Cl+CH_3OH]^-$ at *m/z* 247 and $[M-H]^-$ at *m/z* 179, were observed with few fragment ions. The negative-ion mass spectra for disaccharides were also very simple, consisting primarily of $[M+Cl]^-$ with very low-intensity $[M+Cl+CH_3OH]^-$ and $[M-H]^-$ ions. A few fragment ions, $[M-90+Cl]^-$ at *m/z* 287, $[M-60+Cl]^-$ at *m/z* 317, and $[M-H_2O+Cl]^-$ at *m/z* 359, with intensities less than 20%, were detected in some mass spectra. These fragment ions $[M-90+Cl]^-$ and $[M-60+Cl]^-$ are attributed to sugar-ring fragmentation products, which were also observed with FAB

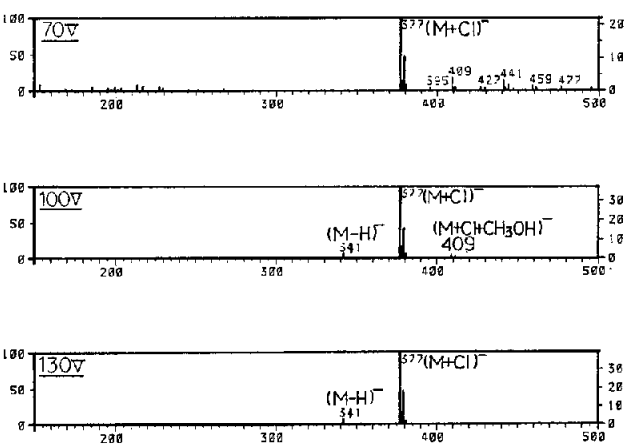

Fig. 10. Chloride-attachment HPLC–NI-APCI-MS mass spectra for sucrose taken at different drift voltages. Mobile phase, chloroform–methanol (5:95); nebulizer temperature, 250°C; vaporizer temperature, 400°C; flow-rate, 1 ml/min; injection, flow injection without a column.

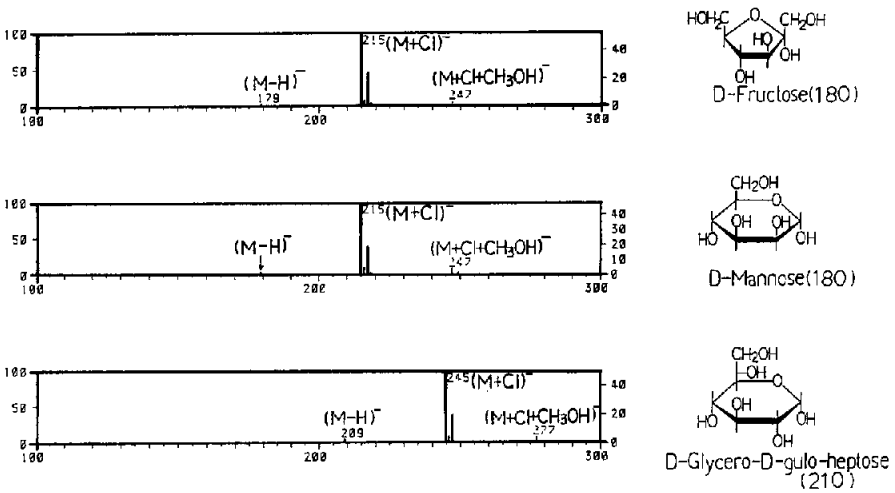

Fig. 11. Chloride-attachment HPLC–NI-APCI mass spectra for 1 μg each of underivatized fructose, mannose, and heptose. Mobile phase, chloroform–methanol (5:95); nebulizer temperature, 250°C; drift voltage, 150 V; flow-rate, 1 ml/min; injection, flow injection without a column.

reported by Garozzo *et al.* [4]. Mass spectra of tri- and tetrasaccharides also were simple, with the most aboundant ion being $[M+Cl]^-$. The same fragment ions $[M-162+Cl]^-$ or $[M-2\times162+Cl]^-$, due to cleavage of glucosidic linkages, were observed for raffinose and stachyose. Ions due to the cleavage of glycosidic linkage of carbohydrates have also been observed by FAB [4].

The chloride attachment sites could not be assigned from the mass spectra obtained by this method. However, investigation of the sensivities of some mono-

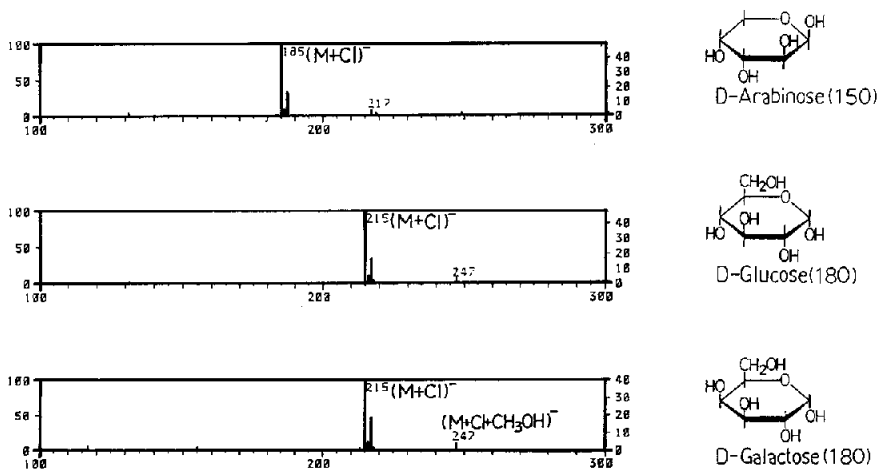

Fig. 12. Chloride-attachment HPLC–NI-APCI mass spectra for 1 μg each of underivatized arabinose, glucose, and galactose. Conditions as in Fig. 11.

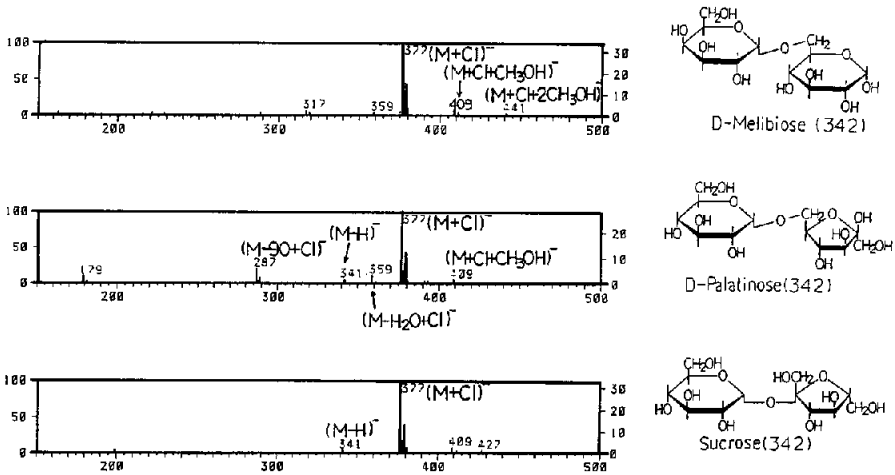

Fig. 13. Chloride-attachment HPLC–NI-APCI mass spectra for 1 μg each of underivatized melibiose, palatinose, and sucrose. Conditions as in Fig. 11.

saccharides reveals that chloride adduct ion formation is dependent on the structure of the sugars. $[M+Cl]^-$ ions for D-galactose and D-glucose are over 1.6 times as abundant as the $[M+Cl]^-$ ions of D-fucose (6-deoxy-D-galactose) and 2-deoxyglucose, respectively. This observation suggests that stereochemistry plays a role. The chloride anions bind to two or three adjacent hydroxy hydrogen atoms of sugar molecules with a greater affinity than to a single hydroxy hydrogen atom.

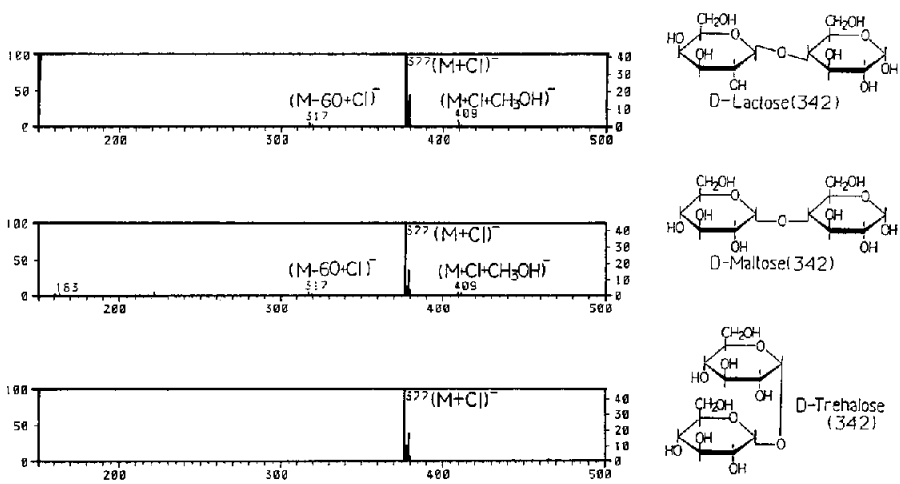

Fig. 14. Chloride-attachment HPLC–NI-APCI mass spectra for 1 μg each of underivatized lactose, maltose, and trehalose. Conditions as in Fig. 11.

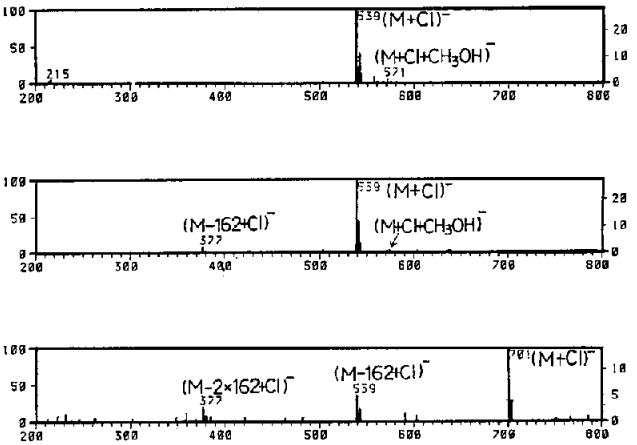

Fig. 15. Chloride-attachment HPLC–NI-APCI mass spectra for 1 μg each of underivatized melezitiose, raffinose, and stachyose. Conditions as in Fig. 11.

Separation and determination of saccharides

Fig. 16 shows the separation of two standard underivatized disaccharides by negative-ion HPLC–APCI-MS using an eluent of methanol–water–chloroform (81:9:10) at a flow-rate of 1 ml/min through a Merck LiChrosorb NH_2 250 × 4 mm I.D. column. Microgram levels of saccharides injected yielded satisfactory molecular-ion intensities. Two saccharides injected provided isobar chloride adduct molecular ions, but these compounds could be determined on the basis of their differing retention times.

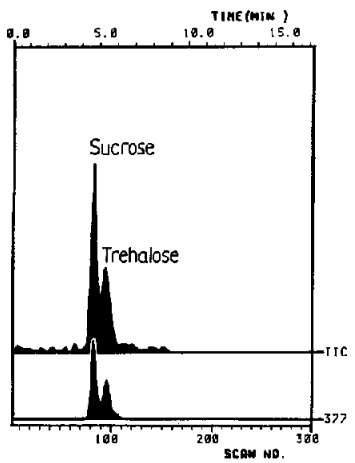

Fig. 16. Separation of two standard underivatized disaccharides by HPLC–APCI-MS using an eluent of methanol–water–chloroform (81:9:10) at a flow-rate of 1 ml/min through a Merck LiChrosorb NH_2 250 mm × 4 I.D. column. Nebulizer temperature, 260°C; drift voltage, 150 V.

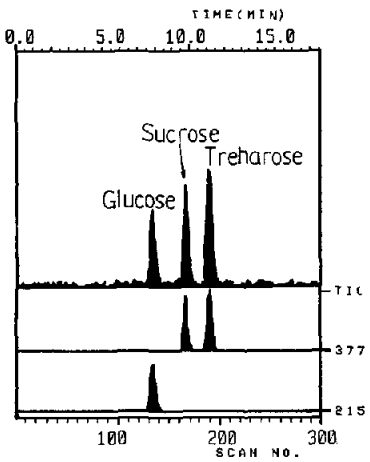

Fig. 17. Separation of underivatized saccharides by HPLC–NI-APCI-MS using an eluent of acetonitrile–water–chloroform (60:37:3) at a flow-rate of 1 ml/min through a Merck LiChrosorb NH_2 250 mm × 4 I.D. column. Nebulizer temperature, 170°c; drift voltage, 150 V.

Fig. 17 shows another example of the separation of underivatized saccharides using an eluent of acetonitrile–water–chloroform (60:37:3) at a flow-rate of 1 ml/min. (The column was the same as for Fig. 16). The temperature of the nebulizer was maintained at 170°C and the drift voltage was 150 V. The chromatograms show that glucose elutes first, followed by sucrose and then trehalose. Acetonitrile is a better mobile phase than methanol because it provides better separations of the saccharides.

CONCLUSION

In this study we have shown the determination of saccharides by HPLC–NI-APCI-MS using the chloride attachment mechanism. Although the applicability of this method to all types of saccharide has not yet been completely established, we have found it useful for the analysis of small amounts of saccharides. All samples investigated in this study have shown chloride-adduct molecular ions as the most abundant species, with a low intensity of fragment ions. The chloride-attachment technique is compatible with reversed-phase LC, requiring only the inclusion of a small amount of chloroform. The resulting negative-ion intensity is linear over more than two orders of magnitude of saccharide concentration. The detection limit is below 1 ng (glucose) by SIM detection. Thus, this technique is easily applied, requiring little modification of LC conditions or instrumentation. Not only does it provide a sensitive means of detecting saccharides, but it can also be used to detect alcohols, etc.

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