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ENHANCEMENT OF PROTONATED MOLECULAR IONS AND AMMONIUM·MOLECULAR ION COMPLEXES IN DIRECT-LIQUID-INTRODUCTION–MASS SPECTROMETRY OF CARBOHYDRATES*

JAMES M. LAU[†], MICHAEL MCNEIL[‡], ALAN G. DARVILL**, AND PETER ALBERSHEIM**,[§]

Department of Chemistry, University of Colorado, Boulder, Colorado (U.S.A.) and Complex Carbohydrate Research Center and School of Chemical Sciences, University of Georgia, Athens, Georgia 30613 (U.S.A.)

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ABSTRACT

Addition of ammonium acetate to the mobile phase in direct-liquid-introduction mass spectrometry enhances the abundance of the protonated molecular ion or ammonium·molecular ion complex for compounds of biological interest. The efficacy of the method was investigated by comparing mass spectra obtained, with and without ammonium acetate, for a variety of underivatized, per-*O*-acetylated, and per-*O*-alkylated carbohydrates, and for several underivatized peptides. The mass spectra of the per-*O*-alkylated carbohydrates obtained by direct-liquid-introduction mass spectrometry with ammonium acetate were also compared to those obtained by thermospray mass spectrometry.

INTRODUCTION

Analysis by direct-liquid-introduction mass spectrometry (d.l.i.-m.s.) involves the introduction of ~3–5% (20–30 μL/min) of the effluent of a liquid chromatography (l.c.) column directly into the chemical-ionization source of the mass spectrometer; the rest (~96%) of the effluent is collected in fractions for other analyses. This procedure has proved to be an effective method for analyzing mixtures of complex carbohydrates and mixtures of peptides[1–4].

D.l.i.-m.s. offers several advantages over refractive index detection of carbohydrates in l.c. column effluents. The benefits include the ability to (*1*) use concentration gradients of acetonitrile or methanol in water for the elution of per-*O*-

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[†] Current address: Hewlett–Packard Company, West 120 Century Road, Paramus, NJ 07652, U.S.A.

[‡] Current address: Department of Microbiology, Colorado State University, Fort Collins, CO 80523, U.S.A.

**Current address: Complex Carbohydrate Research Center, Russell Labs, P.O. Box 5677, Athens, GA 30613, U.S.A.

[§] To whom correspondence should be addressed.

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alkylated oligoglycosyl-alditols; (*2*) obtain chemical-ionization mass spectra (c.i.-m.s.) of the compounds as they are eluted from the l.c. column, which permits a simple assessment of the purity of collected l.c. fractions; and (*3*) detect the presence or absence of a particular per-*O*-alkylated oligoglycosyl-alditol in the l.c. effluent *via* extracted-ion chromatograms[1]. The analysis of peptides by d.l.i.-m.s.[3] has proved to have advantages similar to those just described for carbohydrates, compared to detection by refractive index or ultraviolet absorption.

D.l.i.-m.s. is limited as an analysis technique by its poor performance for compounds of low volatility. In order to increase d.l.i.-m.s. sensitivity and prevent compounds of low volatility from condensing on the chemical-ionization source, the mass spectrometer must be operated with a high source temperature (>200° for carbohydrates). However, c.i. mass spectra of carbohydrates recorded at high source temperatures are characterized by a high degree of fragmentation and a low abundance of protonated molecular ions [p.m.i.; $(M + H)^+$]. Such spectra are of less value in identifying components in l.c. effluents than are spectra that contain abundant p.m.i.

Standard mobile phases for d.l.i.-m.s. are methanol, acetonitrile, or water, and mixtures thereof. The low proton-affinity of each of these solvents leads to increased fragmentation in the chemical ionization process. Thus, the use of the l.c. solvent as the reagent gas for the chemical ionization process lessens the abundance of the p.m.i. in the d.l.i.-mass spectra obtained for carbohydrates.

Ammonia has traditionally been used as the reagent gas for chemical ionization in d.l.i.-, d.c.i.- (desorption chemical ionization), and g.l.c.–m.s. when less fragmentation is desired[5-7]. Introduction of ammonia gas directly into the chemical ionization source in conjunction with introduction of the l.c. effluent gave improved abundance of p.m.i. and ammonium·molecular ion complexes [a.m.i.c.; $(M + NH_4)^+$] during d.l.i.-m.s. analysis[6]. The "soft" chemical ionization characteristics (low degree of fragmentation) resulting from the use of ammonia as the reagent gas were evident in the mass spectra of carbohydrates obtained by this method. The introduction of ammonia gas into the source of the mass spectrometer during d.l.i.-m.s. is cumbersome, however, and can make source pressures difficult to regulate.

Recently, ammonium acetate has been introduced as an ionizing buffer in the mobile phase of thermospray mass spectrometry[8]. The success of use of ammonium acetate in this application, and the desire to employ ammonia c.i., albeit with a need to regulate source pressure easily, led us to add ammonium acetate to the l.c. effluent during d.l.i.-m.s. Here we report on the use of ammonium acetate in the mobile phase of d.l.i.-m.s. of carbohydrates and peptides, and on the improved quality of the spectra obtained.

EXPERIMENTAL

Liquid chromatography–mass spectrometry conditions. — D.l.i.-m.s. was performed on either a Hewlett–Packard (HP) 5985 l.c.-m.s. data system with a HP

1084B l.c. and the HP option 004 l.c.-m.s. interface, or a HP 5987 l.c.-m.s.-data system with a HP 1090 l.c. and HP option 004 l.c.-m.s. interface. Thermospray l.c.-m.s. was conducted on a HP 5987 l.c.-m.s. data system with a HP 1090 l.c. and Vestec thermospray probe, source, and control unit. Mass spectra were recorded at a scan rate of 550 amu/s using an integration time of 50 μs. The mass spectra are reported in the form of raw data from the scan that contained the maximum total-ion abundance.

Liquid chromatograph conditions. — The l.c. was operated during d.l.i.-m.s. in the following way, in order to provide a mobile phase consisting of 0.0–0.5M ammonium acetate in acetonitrile–water at a constant flow-rate of 1 mL/min. Acetonitrile–water (1:1) was introduced through port A of the gradient mixer at a flow rate set between 0.5 and 1.0 mL/min, while the same solvent containing M ammonium acetate was introduced through port B at a flow rate set between 0.0 and 0.5 mL/min. Hence, a flow rate of 0.9 mL of acetonitrile–water/min through port A, and a flow rate of 0.1 mL of M ammonium acetate in acetonitrile–water/min through port B yielded a total flow rate of 1 mL of 0.1M ammonium acetate in acetonitrile–water per min. The samples of pure model compounds were analyzed without an analytical column by injection into a guard column (4.5 mm × 5 cm), which served as an in-line filter, and broadened the chromatographic peak so that 5–6 mass-spectral scans of the compound could be obtained. A 0.5-μm filter was placed directly before the probe handle.

During thermospray l.c.-m.s., a solvent flow-rate of 0.5 mL of 1:1 acetonitrile–or methanol water/min was maintained by introducing acetonitrile (100%) or methanol (100%) through port A of the gradient mixer at 0.25 mL/min and water through port B at 0.25 mL/min. When desired, 0.14M ammonium acetate in water was added at 0.7 mL/min to the solvent by an external pump *via* a mixing tee. A 0.5-μm in-line filter was also used prior to the thermospray probe handle; however, it is no longer believed to be necessary.

Materials. — Cellobiose, maltotriose, stachyose, glycylglycylglycylglycine (gly$_4$), glycylglycylglycylglycylglycine (gly$_5$), and prolylglycylglycine (progly$_2$) were obtained from Sigma Chemical Co. Cellobiose octaacetate was obtained from Eastman Organic Chemicals. Per-*O*-methylated maltotriitol was prepared as follows. Maltotriose (5 mg) was reduced with sodium borohydride (10 mg) in M ammonium hydroxide (1 mL) for 1 h. The di-D-glucosyl-D-glucitol (maltotriitol) was recovered by quenching the reaction with acetic acid, and evaporating the solvent under a stream of filtered air. The borate remaining after the reduction was removed by three evaporations with acidic methanol under a stream of filtered air, and the sodium ions were removed by passing a water solution of the mixture through a column (5 mL) of Dowex-AG50W-X12 resin. The eluate was lyophilized. The resulting maltotriitol was per-*O*-methylated by the method of Harris and coworkers[9]. The per-*O*-methylated carbohydrate was purified from the reaction mixture by chromatography on Sep-Pak C_{18} cartridges as described[10]. Per-*O*-methylated stachyose was obtained by methylation of stachyose (5 mg), and the products isolated as for per-*O*-methylated maltotriitol.

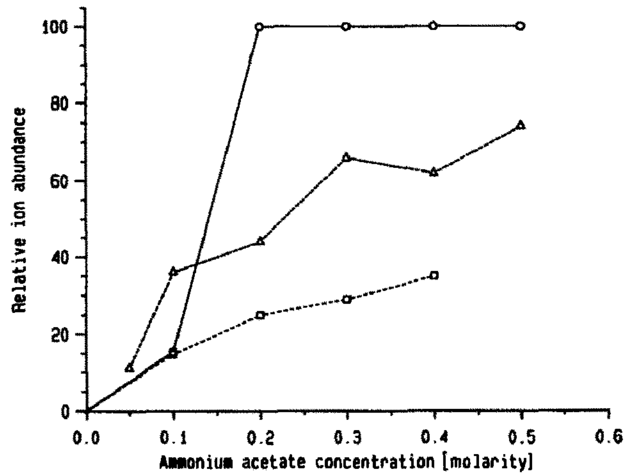

Fig. 1. Concentration of ammonium acetate in the mobile phase of d.l.i.-m.s. *versus* the relative abundance of the a.m.i.c. (*m/z* 692) of per-*O*-methyl maltotriitol (■), the a.m.i.c. (*m/z* 696) of per-*O*-acetylated cellobiose (▲), and the a.m.i.c. (*m/z* 360) of cellobiose (●). [In all cases, the mass spectrometer was operated with a source temperature of 200° and at a d.l.i. flow-rate of 1 mL/min. The solvent was 1:1 acetonitrile–water.]

RESULTS AND DISCUSSION

The optimum concentration of ammonium acetate for d.l.i.-m.s. — Initial d.l.i.-mass spectra of per-*O*-methylated maltotriitol, per-*O*-acetylated cellobiose, and underivatized cellobiose were recorded at concentrations of ammonium acetate ranging from 0.05 to 0.5M in 0.05 or 0.1M increments. The spectra were examined for total ion current (sensitivity), which was not appreciably affected by the concentration of ammonium acetate (data not presented), and for abundance of the a.m.i.c. (the most diagnostic ion), which was dependent on the concentration of ammonium acetate. The maximum or near-maximum abundance of a.m.i.c. was observed at ~0.3M ammonium acetate (see Fig. 1). All subsequent d.l.i.-m.s. experiments employed 0.3M ammonium acetate unless stated otherwise.

The optimum source temperature for ammonium acetate-enhanced d.l.i.-m.s. — D.l.i.-mass spectra (obtained without ammonium acetate) of identical samples recorded at different source temperatures are often different[11]. In order to examine the effect of the source temperature on d.l.i.-m.s. with 0.3M ammonium acetate present, mass spectra of per-*O*-methylated maltotriitol were recorded at source temperatures ranging from 160° to 300° in 25° increments, and the total ion current and a.m.i.c. abundance were determined (see Fig. 2). A source temperature of 200° gave maximum relative abundance of the a.m.i.c. and high total ion current (high sensitivity). A source temperature of 200° was also found to yield excellent d.l.i.-mass spectra for cellobiose and per-*O*-acetylcellobiose in the presence of am-

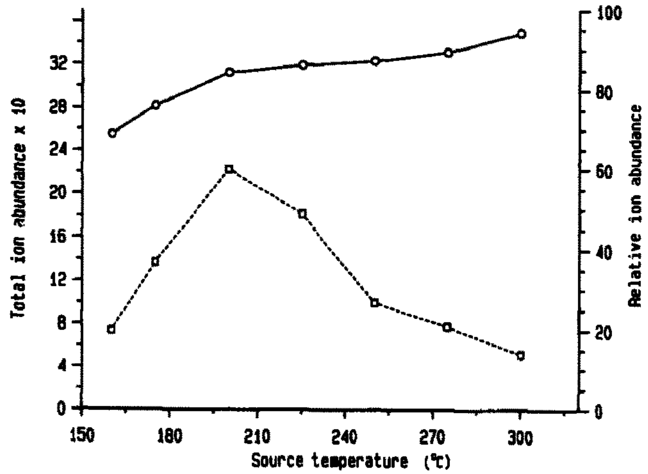

Fig. 2. Variation of source temperature *versus* a.m.i.c. abundance of per-*O*-methylmaltotriitol [(M + NH$_4$)$^+$ at *m/z* 692] (□) and total ion current of per-*O*-methylmaltotriitol (0.75 μg delivered to the source) (○). [The mass spectrometer was operated at a d.l.i. flow-rate of 1 mL/min. and an ammonium acetate concentration of 0.3M in a mobile phase consisting of 1:1 acetonitrile–water.]

monium acetate. Based on these results, a source temperature of 200° was used for all subsequent experiments unless stated otherwise.

The effect of ammonium acetate on d.l.i.-m.s. analysis of carbohydrates and peptides. — The d.l.i.-mass spectra of a number of derivatized and underivatized carbohydrates and underivatized peptides were obtained with and without 0.3M ammonium acetate in the mobile phase. In each case, the relative abundance of the a.m.i.c. or p.m.i. was enhanced by 0.3M ammonium acetate.

The d.l.i.-mass spectra of per-*O*-methylated maltotriitol recorded with and without ammonium acetate are shown in Figs. 3A and 3B. The mass spectrum recorded in the presence of ammonium acetate (see Fig. 3B) had a ten-fold increase in the relative abundance of the a.m.i.c. (at *m/z* 692) over that of the p.m.i. (at *m/z* 675) in the absence of ammonium acetate. The ammonium acetate-enhanced spectrum (see Fig. 3B) also contained a number of useful fragment ions, including a reducing-end fragment-ion at *m/z* 253, which provided information about the structure of the reducing end of the molecule, and several nonreducing-end fragment-ions (*m/z* 254 and 458), which provided information about the structure of the nonreducing end of the molecule[7]. The ammonium acetate-enhanced mass spectrum of per-*O*-methylated maltotriitol did not contain an ammonium adduct of the often-misleading c.i. rearrangement ion that would have been at *m/z* 488. This ion results from elimination of internal glycosyl residues[11]. The rearrangement ion was present in the d.l.i.-mass spectrum of per-*O*-methylated maltotriitol obtained in the absence of ammonium acetate (at *m/z* 471), and it has the same *m/z* value as the p.m.i. for a mono-*O*-glycosylalditol (see Fig. 3A).

The mass spectra of per-*O*-methylated stachyose (d.p. 4) obtained with and without ammonium acetate are illustrated in Figs. 4A and 4B. For this larger carbo-

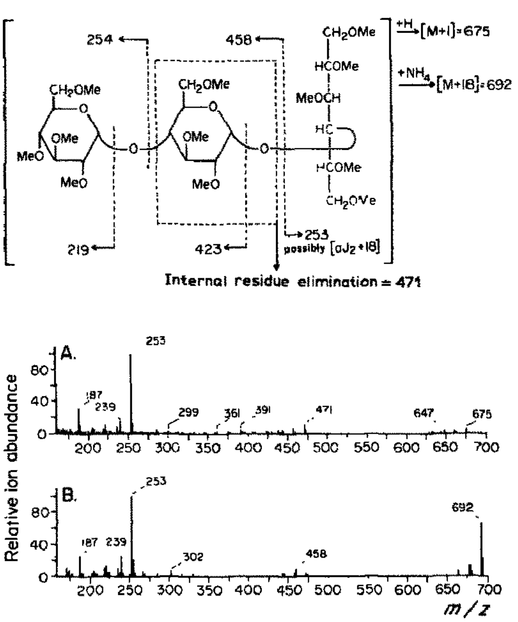

Fig. 3. Chemical ionization mass spectra of per-*O*-methylated maltotriitol obtained by d.l.i.-m.s. (A) Mobile phase entering the mass spectrometer consisted of 1:1 acetonitrile–water (1 mL/min) without ammonium acetate; (B) mobile phase entering the mass spectrometer consisted of 1:1 acetonitrile–water containing 0.3M ammonium acetate (1 mL/min). [The origins of the fragment ions are shown in the top half of the Figure.]

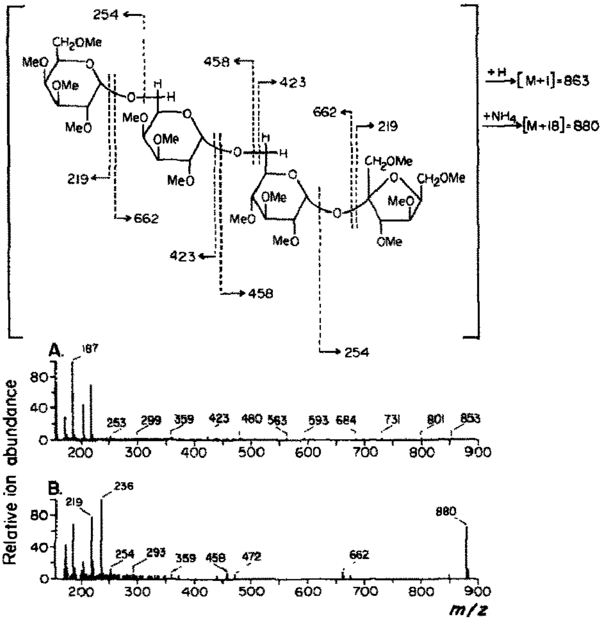

Fig. 4. Chemical ionization mass spectrum of per-*O*-methylated stachyose obtained by d.l.i.-m.s. (For additional information, see legend to Fig. 3.)

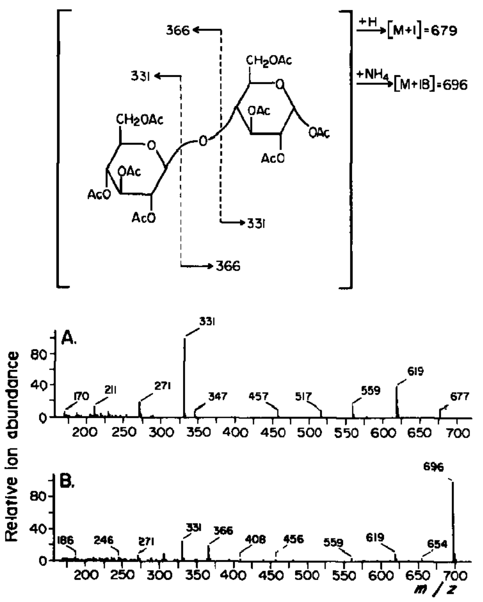

Fig. 5. Chemical ionization mass spectrum of per-*O*-acetylated cellobiose, obtained by d.l.i.-m.s. (See legend to Fig. 3.)

hydrate, the p.m.i. was not detected in the absence of ammonium acetate (see Fig. 4A). Use of ammonium acetate (see Fig. 4B) enhanced the a.m.i.c. (at *m/z* 880). The p.m.i. at *m/z* 863 was not even detected. Informative, nonreducing-end fragment-ions, which provided the sequence of glycosyl residues, were present in the ammonium acetate-enhanced d.l.i.-mass spectrum at *m/z* 254, 458, and 662.

The effect of ammonium acetate on the d.l.i.-m.s. analysis of a per-*O*-acetylated carbohydrate was examined by using per-*O*-acetylcellobiose (see Fig. 5A and 5B). Without ammonium acetate, the base peak in the mass spectrum was the aA_1 ion at *m/z* 331, and there was no detectable p.m.i. at *m/z* 679 (see Fig. 5A). With ammonium acetate, the a.m.i.c. at *m/z* 696 was the base peak. Ammonium acetate also increased the total ion abundance, which demonstrated the increased sensitivity of the analysis.

Underivatized cellobiose and maltotriose, and the peptides gly_4, gly_5 and $progly_2$ were also examined by d.l.i.-m.s. with and without ammonium acetate. The spectra obtained for cellobiose are presented in Figs. 6A and 6B. The ammonium acetate-enhanced d.l.i.-mass spectrum of cellobiose (see Fig. 6B) contained the a.m.i.c. (at *m/z* 360) in good abundance, as well as a series of ions that result from the consecutive losses of H_2O and ketene from the a.m.i.c. In contrast, the d.l.i.-mass spectrum of cellobiose obtained in the absence of ammonium acetate lacked all of the high-mass ions. Ammonium acetate also enhanced the d.l.i.-mass spectrum of maltotriose, although not to the same extent as for cellobiose. The ammonium acetate-enhanced mass spectrum, like the mass spectrum obtained for

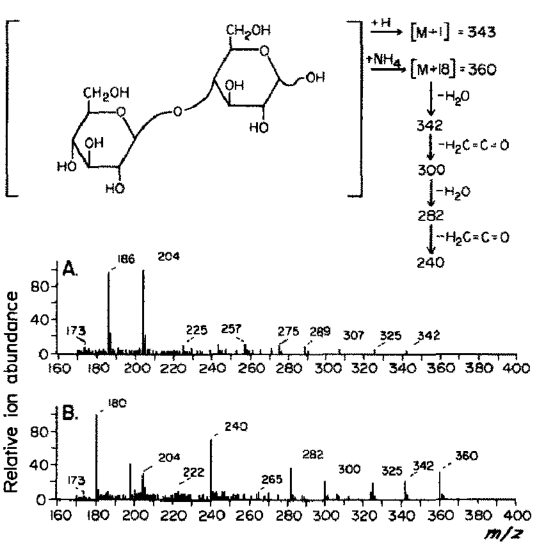

Fig. 6. Chemical ionization mass spectrum of cellobiose obtained by d.l.i.-m.s. (See legend to Fig. 3.)

TABLE I

LIQUID CHROMATOGRAPHIC-MASS SPECTROMETRIC ANALYSIS OF MODEL COMPOUNDS BY NORMAL DIRECT LIQUID INTRODUCTION, AMMONIUM ACETATE DIRECT LIQUID INTRODUCTION, AND THERMOSPRAY

Compound analyzed	*Ammonium acetate*[a]	*Base peak*	*P.m.i./a.m.i.c.*[b]
Per-*O*-methylmaltotriitol	−	253	675 (16)[c]
	−	253	675 (1)
	+	253	692 (66)
	+ Tsp	253	692(49)
Per-*O*-methylstachyose	−	187	863 (0)
	+	236	880 (64)
Cellobiose octaacetate	−	331	679 (0)
	+	696	695 (100)
Cellobiose	−	204	343 (0)
	+	240	360 (52)
Maltotriose	−	204	505 (0)
	+	240	522 (0)
$(Gly)_5$	−	286	304 (7)
	+	286	304 (92)
$(Gly)_4$	−	247	247 (100)
	+	247	247 (100)
$Pro(Gly)_2$	−	230	230 (100)
	+	230	(230 (100)

[a] Normal d.l.i.-m.s. without ammonium acetate; + = d.l.i.-m.s. with 0.3M ammonium acetate present; Tsp = thermospray-m.s. The source temperature during the d.l.i.-m.s. analyses was 200°; the source temperature during the thermospray-m.s. analyses was 250°. [b] The *m/z* value is given first, followed by the relative abundance in parentheses. [c] The source temperature for this analysis was 165°.

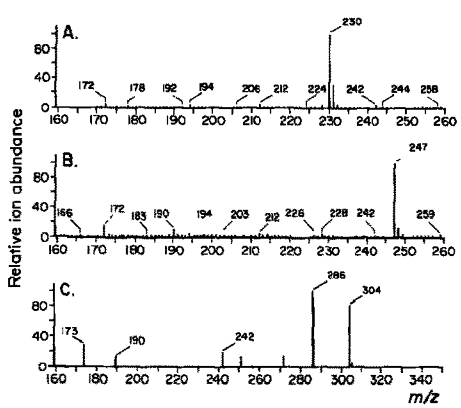

Fig. 7. Chemical ionization mass spectrum of (A) gly_4 (M + H at *m/z* 230), (B) gly_5 (M + H at *m/z* 247), and (C) $progly_2$ (M + H at *m/z* 304), obtained by d.l.i.-m.s. [Mobile phase entering the mass spectrometer consisted of 1:1 acetonitrile–water containing 0.3M ammonium acetate (1 mL/min).]

cellobiose, displayed consecutive losses of H_2O and ketene (see Table I). Furthermore, ammonium acetate enhanced the d.l.i.-mass spectrum of gly_5, the largest peptide analyzed (see Fig. 7), resulting in more-abundant p.m.i.; ammonium acetate did not lessen the effectiveness of d.l.i. to afford spectra of the smaller peptides gly_4 and $progly_2$.

The total ion current and the relative abundance of the p.m.i. or a.m.i.c. for each of the molecules examined by ammonium acetate-enhanced d.l.i.-m.s. are summarized in Table I. Ammonium acetate increased the relative abundance of the p.m.i. and a.m.i.c. for a number of the compounds analyzed. Indeed, for cellobiose, cellobiose octaacetate, and per-*O*-methylated stachyose, no p.m.i. was detected in the mass spectrum recorded in the absence of ammonium acetate.

Comparison of the use of ammonia acetate-enhanced d.l.i.-m.s. and thermospray l.c.–m.s. in the structural analysis of per-O-alkylated carbohydrates. — The mass spectrum of per-*O*-methylated maltotriitol obtained by thermospray l.c.–m.s. using ammonium acetate as the ionizing buffer (filament-on mode) is shown in Fig. 8. The spectrum obtained by thermospray m.s. is almost identical to that obtained by ammonium acetate-enhanced d.l.i.-m.s. (see Fig. 3B). Even the total ion abundances of the two spectra are equal (see Table I). However, the thermospray spectrum was obtained with 1.3 times as much material delivered to the source as was needed for the d.l.i.-mass spectrum.

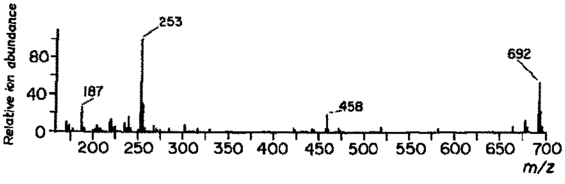

Fig. 8. Thermospray mass spectrum (filament on) of per-*O*-methylated maltotriitol obtained at a flow rate of 1.2 mL/min of acetonitrile–water containing 0.1M ammonium acetate. [The origins of the fragment ions are shown in the top half of Fig. 3.]

Thermospray l.c.-m.s. also has the capability of generating mass spectra without any external source of electrons, *e.g.*, in the filament-off mode. This mode, although much less sensitive than the filament-on mode of operation for analysis of carbohydrates, is sometimes used because it causes less fragmentation. In a separate experiment, ammonium acetate-enhanced d.l.i.-m.s. analysis was performed without a filament emission current in the hope of duplicating thermospray in the filament-off mode, but this technique failed to yield spectra suitable for structural analysis.

GENERAL DISCUSSION

D.l.i.-m.s. has proved to be a valuable technique for the structural analysis of some per-*O*-alkylated carbohydrates and peptides[1,3]. The addition of ammonium acetate to the solvent stream during d.l.i.-m.s. has allowed recording of excellent chemical-ionization mass spectra of such compounds that had previously not been amenable to d.l.i.-m.s.

Ammonium acetate is easily added to the mobile phase entering the mass spectrometer. Even if l.c. conditions preclude the use of ammonium acetate on the column, a solution containing ammonium acetate may be added post-column, *via* a low dead-volume mixing-tee[12]. The presence of ammonium acetate helped considerably in the interpretation of the mass spectrum, by increasing the abundance of p.m.i. or a.m.i.c., thereby increasing the structural information available in the mass spectra. Furthermore, ammonium acetate in d.l.i.-m.s. simplifies the task of interpreting the mass spectra by depressing the formation of rearrangement ions that result from the elimination of internal glycosyl residues during c.i.-m.s. of per-*O*-alkylated oligosaccharides[11].

The purification and analysis of complex carbohydrates by l.c. is becoming increasingly important as carbohydrates having biological regulatory properties are being discovered[13,14]. The biologically active carbohydrates found to date have been obtained in complex mixtures, and are often available in only small quantities. Thus, it is imperative that one be able to analyze l.c. effluents for the biological activity of complex carbohydrates, as well as to analyze them by mass spectrometry. D.l.i.-m.s. is an excellent procedure for routine analysis of such biologically active carbohydrate molecules; it provides structural information and a measure of the purity, but, in contrast to thermospray l.c.-m.s., requires only a small portion of l.c. effluents of standard l.c. columns, leaving the remainder for the analysis of biological activity and for other analytical needs.

Biologically active carbohydrates might best be fractionated and analyzed as their per-*O*-acetyl derivatives, because these derivatives provide increased sensitivity, compared to underivatized carbohydrates, in ammonium acetate-enhanced d.l.i.-m.s. Per-*O*-acetylation of carbohydrates is easily accomplished[15], and after the molecules have been separated and analyzed by d.l.i.-m.s., the acetyl groups may be easily removed for assay of biological activity[16].

Another advantage of ammonium acetate d.l.i.-m.s. is that it allows p.m.i. or a.m.i.c. information to be collected even when the mass spectrometer is operated at relatively higher source temperatures (200–250°, compared to 150–175° in the absence of ammonium acetate), which allows carbohydrates of higher molecular weight to be volatilized and analyzed. Furthermore, high source temperatures lessen contamination of the source, thereby increasing the instrument operating-time available between the time-consuming source cleanings. Because ammonium acetate also increases the abundance of p.m.i. or a.m.i.c., deterioration of the source response due to contamination is not so serious as in d.l.i.-m.s. conducted without ammonium acetate-enhancement. Therefore, the ammonium acetate-increased abundance of a.m.i.c. or p.m.i. also increases the operating time between necessary source-cleanings, that is, before the a.m.i.c. or p.m.i. can no longer be identified.

These benefits (increased sensitivity for p.m.i. and a.m.i.c., ability to analyze larger carbohydrates, ability to analyze a smaller portion of an l.c. effluent, and increased operating time between source cleanings), coupled with the simplicity of using ammonium acetate for the enhancement of mass spectra of carbohydrates and peptides during d.l.i.-m.s. analysis, make this method comparable to, and as useful as, thermospray m.s.

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