Further Directions

Capillary electrophoresis has proven potential for carbohydrate analysis. High efficiencies and short analysis times are its advantages compared with other analytical separation methods. A variety of different separation modes can be applied to separate complex carbohydrate mixtures. It has been shown that, depending on the properties of the sample, different separation methodologies can be applied and optimized.

The electrophoretic analysis of carbohydrates is still under development. In particular the analysis of biochemical compounds, such as glycoconjugates, is still a challenge and can give new information about cell mechanisms, structure of antibodies, etc. This article has focused on the basic aspects of capillary electrophoresis of carbohydrates. The carbohydrates described were confined to simple model compounds. For more specific information, the Further Reading list should be consulted.

See also: **III/Ion Analyses: Capillary Electrophoresis.**

Further Reading

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Gas Chromatography and Gas Chromatography–Mass Spectrometry

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Overview of Derivatization of Sugars for GC, GC-MS or GC-MS-MS Analysis

Table 1 overviews many of the important events in chromatographic and mass spectrometric analysis of sugar monomers. These will be discussed at appropriate points in this review. It was not until the years 1961–1963 that gas chromatography (GC) was applied to the quantitative analysis of mixtures of neutral and amino sugar monomers, even though individual sugars had been derivatized earlier. Carbohydrate analysis by GC lagged behind the analysis of many other compounds because of the difficulty in producing volatile derivatives. Two schools of thought developed, relating to whether the anomeric centre should be retained or destroyed in derivatization. Both persist to this day. Sugars exist in equilibrium betweeen ring and straight chain forms. If the anomeric centre is not eliminated, derivatization fixes the sugars in the anomeric ring forms. Thus, two to four peaks will be produced from each L or D sugar (two from furanose and two from pyranose anomers). Interpretation of chromatograms becomes complicated and quantitation difficult. Acidic sugars (i.e. generally with carboxyl groups) require additional derivatization steps. The carboxyl moiety may be converted to a lactone, ester or reduced to an alditol.

The Alditol Acetate Procedure

The alditol acetate procedure was the first developed in which the anomeric centre is eliminated. The anomeric centre is converted by borohydride reduction (although later borodeuteride was introduced)

Table 1 Important advances in analysis of carbohydrates

1960–1965	Fundamental articles on preparation of sugar derivatives for gas chromatography (GC)
1970s	Introduction of gas chromatography–mass spectrometry (GC-MS) for structure analysis of carbohydrates
Late 1970s–early 1980s	Introduction of selected ion monitoring (SIM) GC-MS for trace analysis of derivatized carbohydrates
1980s	Development of anion exchange liquid chromatography/pulsed amperometric detection of native sugars
1990s	Introduction of liquid chromatography–electrospray mass spectrometry for analysis of underivatized sugars
1995–1996	Introduction of gas chromatography–tandem mass spectrometry for trace analysis of derivatized sugars
1995–1996	Introduction of liquid chromatography–tandem mass spectrometry for identification of native sugars

to an alcohol prior to acetylation (to produce a volatile derivative). Unfortunately, borate derived from the added borohydride, or borodeuteride, inhibits the subsequent acylation step. The classical procedure employs a multi-step evaporation with methanol–acetic acid to remove borate as tetramethyl borate gas in between the reduction and acetylation steps (**Figure 1**).

In the 1960s, sodium acetate and pyridine were introduced as catalysts for acetylation, pyridine being more efficient than sodium acetate. However, in both cases borate must be removed prior to acetylation. Subsequently, several catalysts (including methylimidazole) were described that allowed acetylation without removal or borate or moisture. Unfortunately, many catalysts (including both pyridine and methylimidizole) often generate side-reaction products (from reaction with acetic anhydride) that produce chromatograms contaminated with extraneous peaks. Furthermore, in the presence of moisture and/or borate, acetylation can be somewhat unpredictable. When using sodium acetate as a catalyst following removal of borate and moisture, these problems do no occur but unfortunately, the process to remove borate and moisture is tedious to perform. An automated evaporator has been developed to perform the multiple cycles of methanol–acetic acid addition/evaporation for borate removal in the alditol acetate procedure. More recently, an automated derivatization instrument has been developed to automate the entire alditol acetate procedure.

The Trimethylsilyl Procedure

Also in the early 1960s, other workers described a simple one-step trimethylsilyl derivatization of hydroxyl groups. This remains one of the most common procedures used for GC analysis. Derivatization is readily achieved. However, complex chromatograms are produced due to anomer formation noted above. Also, analysis must be prompt since the derivatives

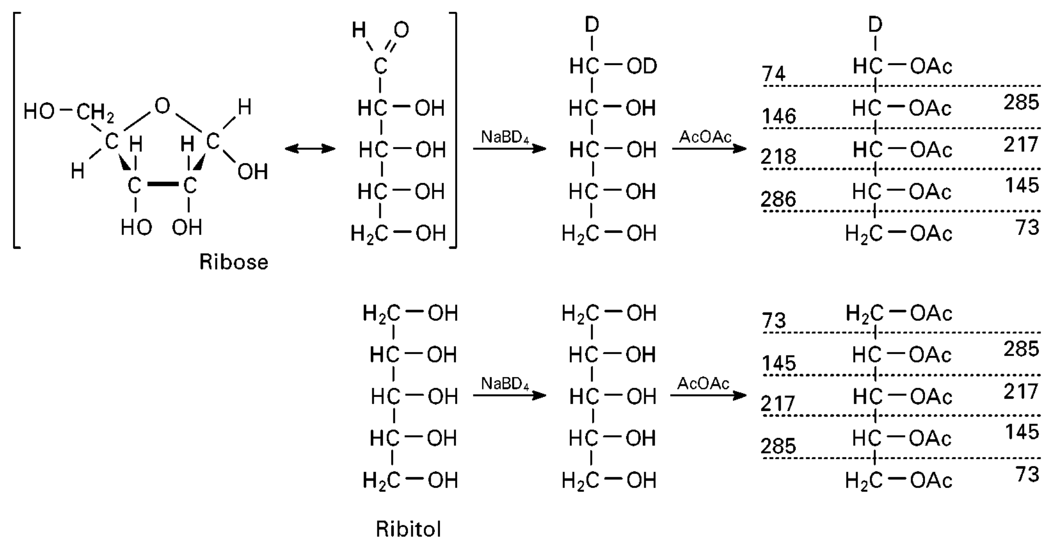

Figure 1 An example of an alditol acetate derivatization reaction employing sodium borodeuteride in the reduction step. Differences in the mass spectra of ribose and ribitol illustrate the distinctness of mass spectra of alditols and aldoses.

decompose in the presence of moisture, complicating clean-up of complex samples.

The Aldononitrile Acetate and *O*-Methyloxime Acetate Procedures

Procedures developed in the 1970s involving destruction of the anomeric centre were particularly concerned with proceeding directly to the final acylation step. Such procedures generally employed acetylation as the final acylation step due to the stability of acetylated sugar derivatives. Such methods included the aldononitrile acetate and the *O*-methyloxime acetate procedures. To generate the former, the aldehyde is reacted with hydroxylamine to produce an oxime which is converted to a nitrile on acetylation. *O*-methyloxime acetates, on the other hand, are prepared by destruction of the anomeric centre by reaction with *O*-methyl-hydroxylamine, also followed by acetylation. Unfortunately, *O*-methyloxime acetates display a new isomeric centre and two products, syn- and anti, are generated for each sugar.

An advantage of both aldononitrile and *O*-methyloxime acetate methods is that aldoses produce distinct peaks from alditols. However, substitution of borodeuteride for borohydride reduction (in alditol acetate formation) labels the aldose group, whilst alditols remain unlabelled. Thus, aldoses and alditols can be distinguished by gas chromatography–mass spectrometry (GC-MS) analysis. Alternatively, alditols do not contain an anomeric centre and can be acetylated without prior derivatization steps (e.g. reduction) and are simply analysed. This also allows discrimination of alditols from aldoses. For further details see the section on GC-MS analysis of alditol acetates, below.

The Trifluoroacetyl Procedure

Trifluoroacetyls, like trimethylsilyl derivatives, are also formed by a simple one-step derivatization procedure. However, as with trimethylsilyl derivatives, multiple peaks are generated for each sugar and are unstable on exposure to moisture. Therefore, samples must be analysed soon after derivatization. As noted above, acetate derivatives are generally stable indefinitely.

Chiral Derivatives

Methods developed have followed two approaches: after conventional derivatization (e.g. permethylation) enantiomers (L and D isomers) can be separated on chiral (e.g. cyclodextrin) GC columns; alternatively, glycosidation with chiral reagents (e.g. an optically active alcohol such as butanol) produces diastereoisomers that can be resolved on conventional nonchiral capillary columns.

Choice of Derivatives for Instrumental Detection

Most reports in the 1960s and 1970s employed the flame ionization detector (FID). While the FID is useful as a GC detector, it lacks specificity. The use of the mass spectrometer (MS) or tandem mass spectrometer (MS-MS) as a GC detector dramatically expands the capability of the analysis. GC-MS, using electron impact ionization (EI) or chemical ionization (CI) followed by positive ion detection, employs the same derivatives as in FID but provides specificity that the FID lacks.

There have been a few reports using the electron-capture detector (ECD) offering the possibility of increased sensitivity if a halogenated (electron-capturing) derivative such as trifluoroacetyl, pentafluoropropyl or heptafluorobutyl is employed. Unfortunately, such derivatives are unstable in the presence of moisture. Furthermore, compounds other than sugars may be converted to halogenated derivatives, resulting in increased background. Thus, it is unclear whether the increased sensitivity can be utilized. An unusual derivative not widely used is the *O*-pentafluorobenzyl oxime acetate derivative. For this derivative, pentafluorobenzyl oxime replaces the aldehyde as the electron-capturing group prior to acetylation. Unlike other halogenated derivatives, the *O*-pentafluorobenzyl oxime acetate is stable to moisture. Alternatively, such derivatives might be appropriate for use with MS in the negative ion mode. Like ECD, negative ion chemical ionization (NI-CI) GC-MS also necessitates an electron-capturing derivative. Use of GC-MS-MS might take further advantage of the increased sensitivity of NI-CI since background is essentially eliminated.

The first benchtop GC-MS instruments were introduced in the late 1970s and had only EI capability. Later instruments could perform both EI and CI with both positive and negative ion detection capabilities. In the past 3–4 years, comparably priced benchtop GC-MS-MS instruments, also with EI-CI and positive/negative ionization capability, have been introduced. Most GC-MS and GC-MS-MS analyses of sugars are still performed with EI in positive ion detection mode. These instruments are simple to operate and maintain and are run by Windows-based PCs.

Preparation of Alditol Acetate Derivatives of Sugars Present in Complex Matrices

Steps in the analysis of complex sugar mixtures include release of the sugar by hydrolysis, addition of internal standard, derivatization and instrumental

analysis. Examples of analyses performed with the alditol acetate procedure will be used here to illustrate these steps.

Hydrolysis

There have been numerous articles on the selection of hydrolysis conditions. Parameters requiring consideration include temperature, duration and the type and strength of acid (primarily hydrochloric, sulfuric and trifluoroacetic acids). Optimization is highly dependent on the sugar(s) of interest to be released from a particular polymeric matrix. If the hydrolysis conditions are too gentle, there is an inadequate release of the sugar monomers. Conversely, if the conditions are too harsh, then destruction of certain sugars occurs. Neutral sugars are often easier to release and destroy than aminosugars. Invariably, a compromise must be made in the analysis of a mixture of neutral and aminosugars in a complex matrix. It must also be recognized that it is not the absolute amount of polymeric sugar present in the matrix that is being determined, but the amount released as monomers under the selected hydrolysis conditions.

Following hydrolysis and prior to derivatization, the acid must be removed. Trifluoroacetic and hydrochloric acids are generally removed by evaporation. Under these conditions further destruction of sugars is possible. Sulfuric acid can be removed by neutralization with a solution of barium hydroxide or a suspension of barium carbonate. Unfortunately, on neutralization with a barium hydroxide solution, it is difficult to remove the sugar from the precipitate of barium sulfate. The use of barium carbonate is not practical because a coat of barium sulfate forms, protecting a large portion of the particulate barium carbonate from reacting. Thus, large quantities of barium carbonate are needed for neutralization. A simple alternative involves neutralization with a solution of an organic base (*N*,*N*-dioctylmethylamine) in chloroform. Sugars remain in the aqueous phase and sulfate is removed in the organic phase.

Choice of Internal Standard

There is a great deal of variability of sugar monomers that can be present in complex biological matrices. Quantitation of each sugar present in a profile is desirable, but it is not practical to select one internal standard for each sugar. Generally, internal standards are selected for groups of sugars based on structural similarities. On SP-2330 columns, neutral sugars elute much earlier than aminosugars. Relative standard deviations (RSD) for multiple samples on GC analysis for late-eluting aminosugars are high if an early eluting neutral sugar (e.g. arabinose) is used as the internal standard. Selection of a late-eluting aminosugar (e.g. methylglucamine) as an internal standard for amoinosugars dramatically increases precision. However, on less polar columns, such as the DB-5ms, certain high molecular weight neutral sugars (e.g. heptoses) tend to elute in the same region as aminosugars (e.g. aminohexoses). In the analysis of heptoses, when using arabinose as the internal standard (by comparing relative peak areas), the RSD for D-glycero-D-mannoheptose and L-glycero-D-mannoheptose was found to be 25.3% and 30.7%, respectively. Using methylglucamine, RSD was lowered to 11.0 and 7.2%. This suggests that, when selecting an internal standard, the relative retention time of the eluting sugars, rather than similarity in structure, may be more critical. Heptoses are highly characteristic of Gram-negative bacteria.

The aldoses L-glycero-D-mannoheptose and D-glyceroheptose are not available commercially. On reduction they are converted into their respective heptitols. However, the ketose heptulose, which is available commercially, on reduction generates both L-glycero-D-mannoheptitol and D-glycero-D-mannoheptitol. Hydrolysates of *Escherichia coli* contain L-glycero-D-mannoheptose but not D-glycero-D-mannoheptose. Thus, by comparing chromatograms of standards containing heptulose and hydrolysates of *E. coli* (as alditol acetates) it is possible to identify the two heptose peaks. **Figure 2** shows a typical separation of a mixture of neutral and amino sugar standards (including both heptose peaks) on a DB-5ms fused silica capillary column and a hydrolysate of *E. coli* (containing L-glycero-D-mannoheptose but not D-glycero-D-mannoheptose).

The Automated Derivatization Instrument for Preparation of Alditol Acetates

The manually performed alditol acetate derivatization procedure described elsewhere has been used for many years. This multi-step procedure can now be performed sequentially by a computer-controlled instrument. The procedure, which previously took $2\frac{1}{2}$ working days, is performed in an automated fashion, requiring a total of 90 min manual work. The samples are processed in four stages:

1. evacuation/hydrolysis (3 h 15 min, automated);
2. prederivatization clean-up (1 h, manual);
3. alditol acetate derivatization (23 h, automated);
4. post-derivatization clean-up (30 min, manual).

The core of the machine, where chemical manipulations and reactions are performed, consists of a custom-built manifold with 21 glass chambers, to each of which a test tube is attached. The manifold is seated in a movable heating block. A series of electrically driven solenoid valves are attached in-line with

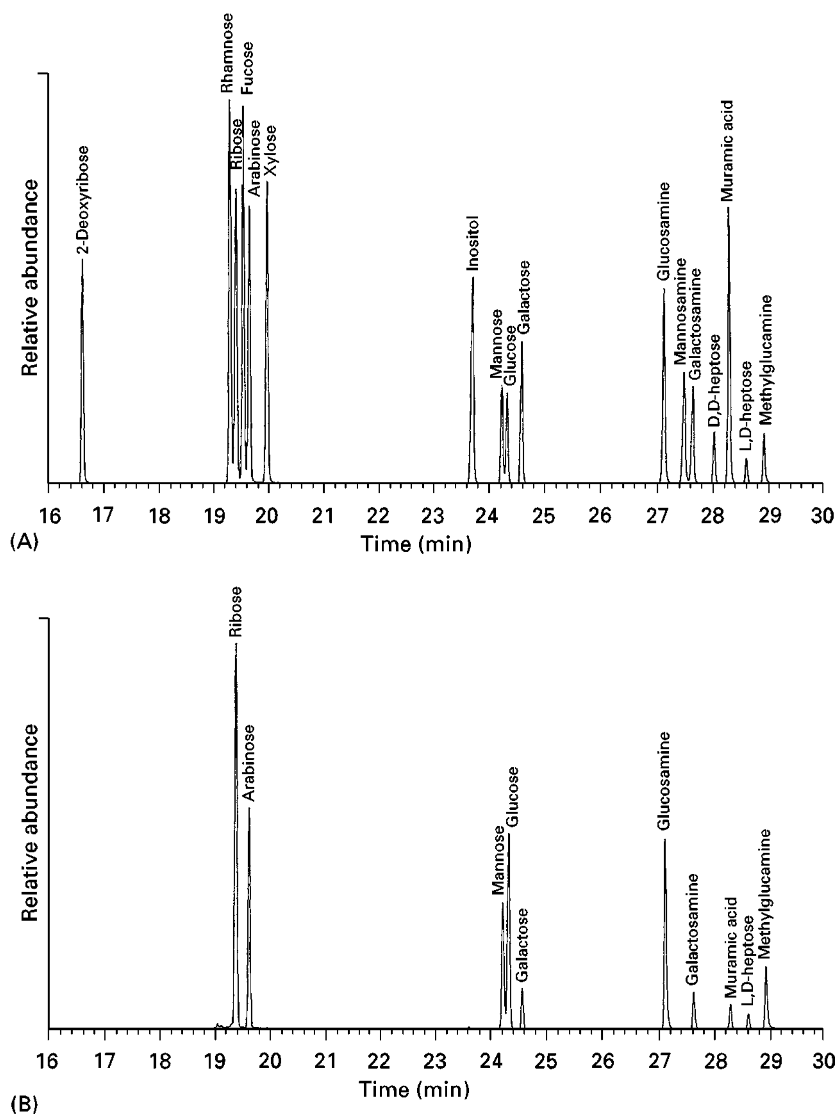

Figure 2 Selected ion monitoring (SIM) GC-MS chromatograms of alditol acetates (A) sugar standards and (B) hydrolysate of *Escherichia coli*. The following m/z were monitored: 160 (deoxyribose); 171 (rhamnose and fucose); 145 (ribose, arabinose and xylose); 199 (inositol, 199); 290 (mannose, glucose and galactose); 318 (glucosamine, galactosamine, mannosamine); 362 (D,D-heptose and L,D-heptose); 168 (muramic acid); 327 (methylglucamine).

the manifold. A set of solvent valves control the input of solvent and/or nitrogen gas to each sample chamber. A set of gas valves controls output to atmosphere or vacuum. Additionally, closure of all valves allows the samples to be sealed in a closed chamber.

Computer control of the individual stages of the derivatization process is a major feature of the system. Ten mg of each sample, in 1 mL of 2 mol L^{-1} sulfuric acid, is placed in each of the custom test tubes. The samples are attached to the manifold and the program started. Oxygen is evacuated by repeated alternate exposure of nitrogen and vacuum. After evacuation, the program sets the heating block to 100°C for hydrolysis. Heating continues for 3 h under nitrogen.

Following hydrolysis, internal standards are added, the samples are removed from the instrument, neutralized with 2 mL 50% *N*,*N*-dioctylmethylamine (Fluka, Buchs, Switzerland) in chloroform, and then centrifuged. The aqueous phase containing the sugar monomers is removed and passed through C_{18} columns (J&W, Folsom, CA) into 21 new sample tubes via the evacuated 21-chamber manifold described above. Aqueous sodium borodeuteride 200 μL (25 mg mL^{-1}) is then added to each sample.

The derivatization procedure is entirely under computer control. After a 2 h delay, in which sample reduction occurs at room temperature, methanol–acetic acid (200 : 1 v/v) is added by activation of a solenoid valve connected to a reagent reservoir. The

program then sets the heating block to 60°C, and evaporation under N_2 occurs for 30 min. This step (solvent addition and evaporation) is automatically repeated servaral times to remove borodeuteride as tetramethyl borate gas. After the last addition, the system is evacuated by activation of the attached vacuum pump, and the samples are dried for 4 h at room temperature. Acetic anhydride is then added to the samples from another reservoir, and the samples acetylated for 13 h at 100°C. Finally, the samples are evaporated to dryness under N_2, and chloroform is added from a third reservoir.

The final post-derivatization clean-up (taking 30 min) is performed manually but also uses the 21-sample manifold, alleviating the necessity for additional equipment. Samples are passed through a pair of connected Chem-Elut columns (Varian, Walnut Creek, CA), the first pre-treated with 2 mol L^{-1} acetic acid and the second with 14.8 mol L^{-1} ammonium hydroxide. The chloroform eluent is evaporated under N_2, and samples reconstituted for analysis.

Instrumental Analysis of Alditol Acetate Derivatives

Columns of GC Analysis

There was a great deal of work in the early days in the selection of packed columns for separation of a complex mixture of neutral and amino sugars by GC. This work was readily extrapolated to the vastly improved fused silica columns introduced later. As an example, excellent capillary GC separation of neutral and aminosugar mixtures is obtained on relatively polar SP-2330 columns. However, aminosugars require high final temperatures and/or extended run times for elution and this column tends to display poor temperature stability under such conditions. Furthermore, irreversible adsorption of aminosugars is a significant problem, causing poor sensitivity of the aminosugars relative to neutral sugars. More recently, nonpolar DB-5ms columns have been used which have not exhibited these problems. In complex mixtures, sugars are observed as sharp peaks with almost baseline resolution. It would be highly desirable if a commercial column were developed that displayed the stability of the DB-5ms column but had the resolving capacity of the SP-2330 column.

GC-MS Analysis of Alditol Acetates (Total Ion Spectra and SIM)

Using total ion GC-MS, carbohydrate monomers can readily be identified by their characteristic ion spectra. Once the sugar has been identified, a particular ion or set of ions characteristic of that monomer can be chosen for selected ion monitoring (SIM). Once retention time has been established, SIM chromatograms allow for clean, easily quantifiable peaks for each monomer. **Figures 3** and **4** compare total ion and SIM GC-MS analysis of two bacterial hydrolysates (*Bacillus subtilis* strains 168 and W23). In the total ion chromatograms there are a number of tiny background peaks (e.g. the glucose and methylglucamine peaks both have shoulders). The extraneous peaks are eliminated in the SIM chromatograms. Furthermore, sensitivity is dramatically increased in SIM analysis. This is illustrated in that galactosamine is readily detected in the SIM – but not the total ion chromatogram – for strain 168. In such analyses, generally μg amounts of sugars are present but, in our experience, SIM GC-MS can be used to detect as little as 100–250 ng in complex samples (10–20 mg of starting sample). However, background peaks become increasingly more of a problem as the concentration of sugar is decreased.

As noted above, a native sugar forms 2–4 anomers upon acylation, thus creating multiple peaks from a single sugar and complicating chromatograms. The anomeric centre is usually first destroyed. If hydroxylamine and *O*-methylhydroxylamine are used in the acylation of carbohydrate monomers, aldononitrile acetates and *O*-methyloxime acetates, respectively, generate distinct chromatographic peaks for aldoses and alditols. This is not the case for alditol acetates.

Sugars are generally reduced using sodium borohydride or borodeuteride prior to acylation to eliminate anomer formation. For example, during reduction of aldoses the C_1 aldehyde is converted to an alcohol (i.e. aldose to an alditol), whereas alditols remain chemically unchanged. Using sodium borohydride, in the formation of alditol acetates, aldoses and alditols cannot be differentiated. However, when using sodium borodeuteride, two deuteriums are added to the aldehyde moiety, one of which remains after acylation. Thus, there is a one mass unit shift in ions containing C_1. Fragments lacking C_1 generate ions of the same m/z for deuterated and nondeuterated samples.

As an example, *B. subtilis* W23 is readily discriminated from *B. subtilis* 168 by the presence of a ribitol containing polysaccharide (teichoic acid). Total ion and SIM chromatograms of carbohydrates derived from these two bacterial strains are shown in Figures 3 and 4 respectively. During hydrolysis, ribitol is released from the teichoic acid. Unfortunately, RNA releases ribose upon hydrolysis which is converted to ribitol upon reduction, thus hindering the detection of ribitol originating from teichoic acid. However, ribitol from teichoic acid is also partially

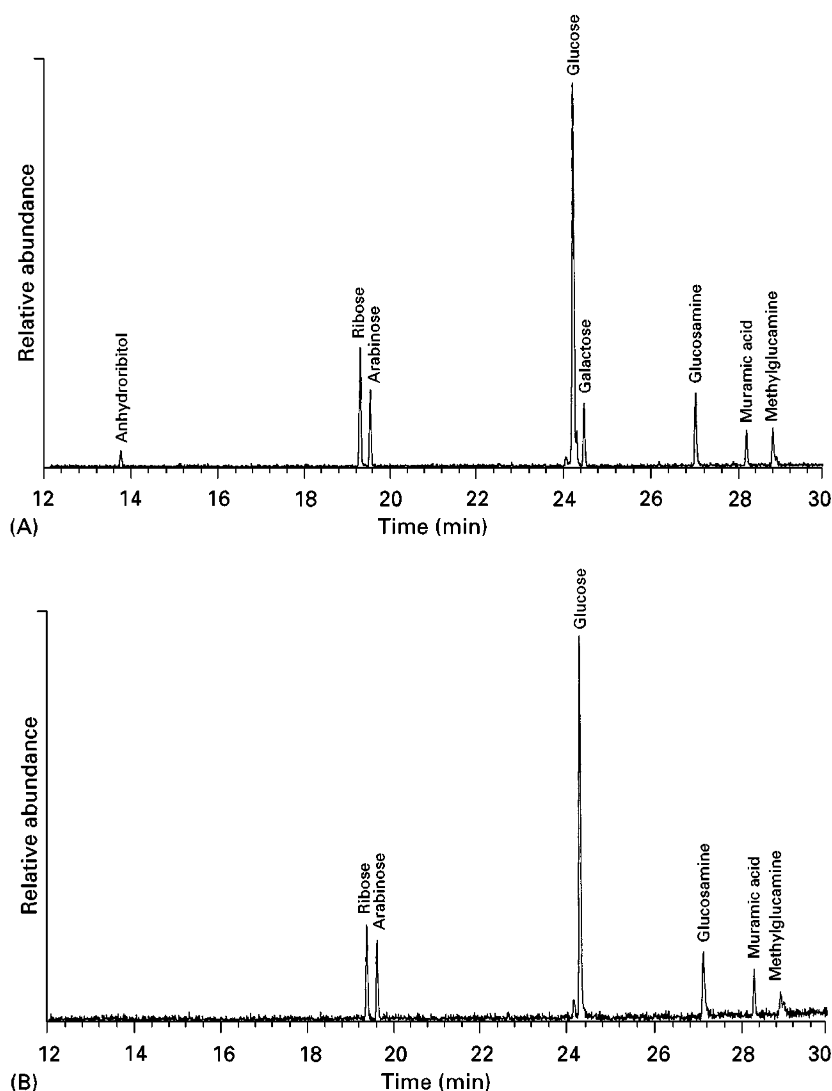

Figure 3 Total ion GC-MS chromatograms of alditol acetates of hydrolysates of *Bacillus subtilis* (A) strain W23 and (B) strain 168. Ribose is present at 1.13% of the dry weight of the sample of W23 analysed (total 10 mg), i.e. 113 μg.

converted to anhydroribitol during hydrolysis. Strain W23 can therefore be readily distinguished from strain 168 by the presence of anhydroribitol. The mass spectrum of anhydroribitol and its structure are shown in **Figure 5**. The molecular weight of anhydroribitol is 260; *m/z* 187 (M-73, breakage between C_4 and C_5, which leaves the ring intact), *m/z* 127 (loss of acetic acid, 60) and *m/z* 85 (loss of ketene, 42).

On examination of SIM chromatograms, the ribitol/ribose peak cannot be used to distinguish the two organisms. However, this can be accomplished by full scan (GC-MS) analysis. As noted above, on borodeuteride reduction, aldoses (e.g. ribose) gain two deuteriums, one of which remains after acylation, while alditols (e.g. ribitol) remain unchanged. For aldoses, pairs of peaks are generated, from the C_1 end labelled with deuterium and the other, unlabelled end. For alditols, C_1 is not labelled, resulting in single peaks. Thus, the mass spectrum of ribose contains pairs of ions of nearly equal abundance (i.e *m/z* 115/116, 145/146, 187/188 and 217/218) generated from the two ends of the molecule, while ribitol would be dominated by single ions (i.e. *m/z* 115, 145, 187 and 217). Therefore, for strain W23 where ribitol, in addition to ribose, is present, the mass spectrum contains a greater abundance of the lower mass ion of each pair than for strain 168 (where ribose alone is present). **Figure 6** clearly demonstrates that standard ribose has a mass spectrum indistinguishable from the ribose peak derived from the hydrolysate of strain 168. However, the peak for strain W23, containing a mixture of ribose and ribitol, is readily distinguished by the dominance of

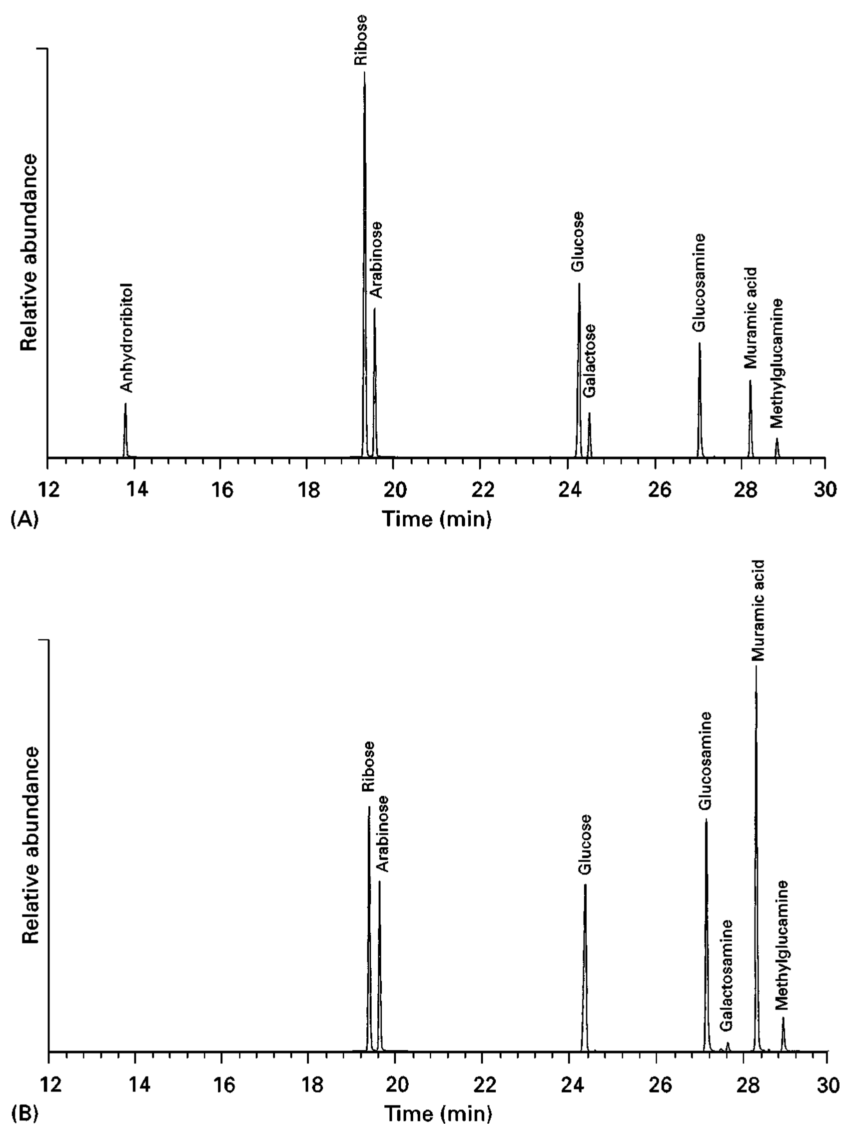

Figure 4 Selected ion monitoring GC-MS chromatograms of alditol acetates of hydrolysates of *Bacillus subtilis* (A) strain W23 and (B) strain 168. Same ions as Figure 2 plus 187 (anhydroribitol).

115 over 116, 145 over 146, 187 over 188 and 217 over 218.

The base peak in EI mass spectra of alditol acetates is generally dominated by the acetylinium ion (*m/z* 43). Many primary fragments are produced by cleavage between sequential carbon atoms. Secondary fragmentation results from losses of acetic acid (*m/z* 60), acetoxyl groups (*m/z* 59) and ketene (*m/z* 42). Generally, mass spectra of aminosugars are relatively simple since cleavage preferentially occurs between the carbon with attached acetamido group and adjacent acetylated carbons.

Mass spectra of stereoisomers of alditol acetates contain ions of the same *m/z*. On casual observation the mass spectra appear similar. However, certain isomers display differences in relative ion abundances which can be quite striking. Aminodideoxyhexoses, quinovosamine and fucosamine (found in legionellae), have been noted to display distinct mass spectra. Differences in mass spectra among isomers are accentuated by the use of borodeuteride. Aldoses are asymmetric since there is an aldehyde on C_1. Asymmetry is retained after borodeuteride but not borohydride reduction since C_1 is labelled. It has been proven that all eight hexoses can be differentiated by a combination of distinct mass spectra and/or retention times.

Muramic acid, 3-O-lactyl glucosamine, is an unusual sugar which additionally contains a carboxyl group in ether linkage. A lactam (a cyclic amide) is formed by internal dehydration between its carboxyl and amino groups on derivatization. In contrast to

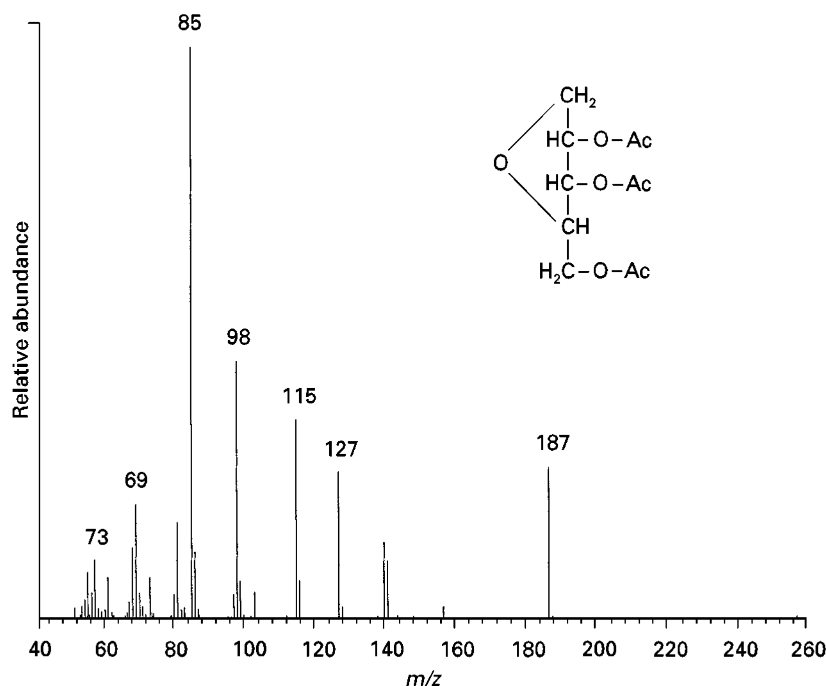

Figure 5 Mass spectrum of alditol acetate of anhydroribitol derived from hydrolysate of *Bacillus subtilis* W23.

acetylation of other aminosugars, which produce amides, muramicitol pentaacetate (acetylated muramic acid) has an imido group in which two acyl groups, lactyl and acetyl respectively, are linked to the nitrogen atom. Formation of the imido moiety requires harsh conditions (higher temperatures and longer heating times).

Naturally occurring *O*-methylated sugars exist in bacteria and in eukaryotes. The fragmentation pattern of methylated sugars is distinctive. Fragmentation between the *O*-methylated carbon and the adjacent acetylated carbon atoms dominates the spectra. Additional secondary ions can be produced by loss of methanol (*m*/*z* 32) and formaldehyde (*m*/*z* 30).

GC-MS-MS Analysis of Alditol Acetates (Multiple Reaction Monitoring and Product Ion Spectra)

High resolution chromatographic separations coupled with selective clean-up steps are important in improving the specificity of the detection of chemical markers (e.g. muramic acid as a marker for bacterial infection) in complex matrices. However, chromatographic separation is not sufficient to eliminate extraneous peaks when nonselective detectors are employed. The use of the mass spectrometer as a selective GC detector (i.e. GC-MS analysis in SIM), helps greatly in diminishing background noise by focusing only on ions that are present in the compound of interest. However, even when using SIM, it is not uncommon to find extraneous background peaks. The tandem mass spectrometer, as a GC detector, provides even greater specificity in detecting trace amounts of chemical markers in complex matrices. Tandem mass spectrometry has the added advantage of generating a total ion spectrum from a selected precursor ion (product ion spectrum). The resulting product ion spectrum can be used for a definitive identification of the compound of interest at trace levels.

Multiple reaction monitoring (MRM) and generation of product ion spectra both involve three discrete mass analysis steps. The first stage involves selection of a precursor ion. This instrumental clean-up removes other ions. The precursor ion is then fragmented by collision-induced dissociation (CID) using an inert gas. In the third stage, all precursor ions can be collected (product ion spectrum) or a single product ion is selected for monitoring (MRM). Both SIM GC-MS and MRM GC-MS-MS analysis allow excellent quantitation of such chemical markers, but the latter provides much greater confidence in trace analysis.

Two types of GC-MS-MS instruments are primarily used in such analysis: ion traps and triple quadrupoles. In triple quadrupole instruments, the three stages of analysis are performed using three distinct quadrupole mass analysers. There is some decrease in sensitivity due to loss of ions in transmission through the three quadrupoles. In ion trap tandem mass spectrometers, the three stages occur in the same mass analyser. This dramatically simplifies the instrument and its cost. Furthermore, sensitivity of MS-MS

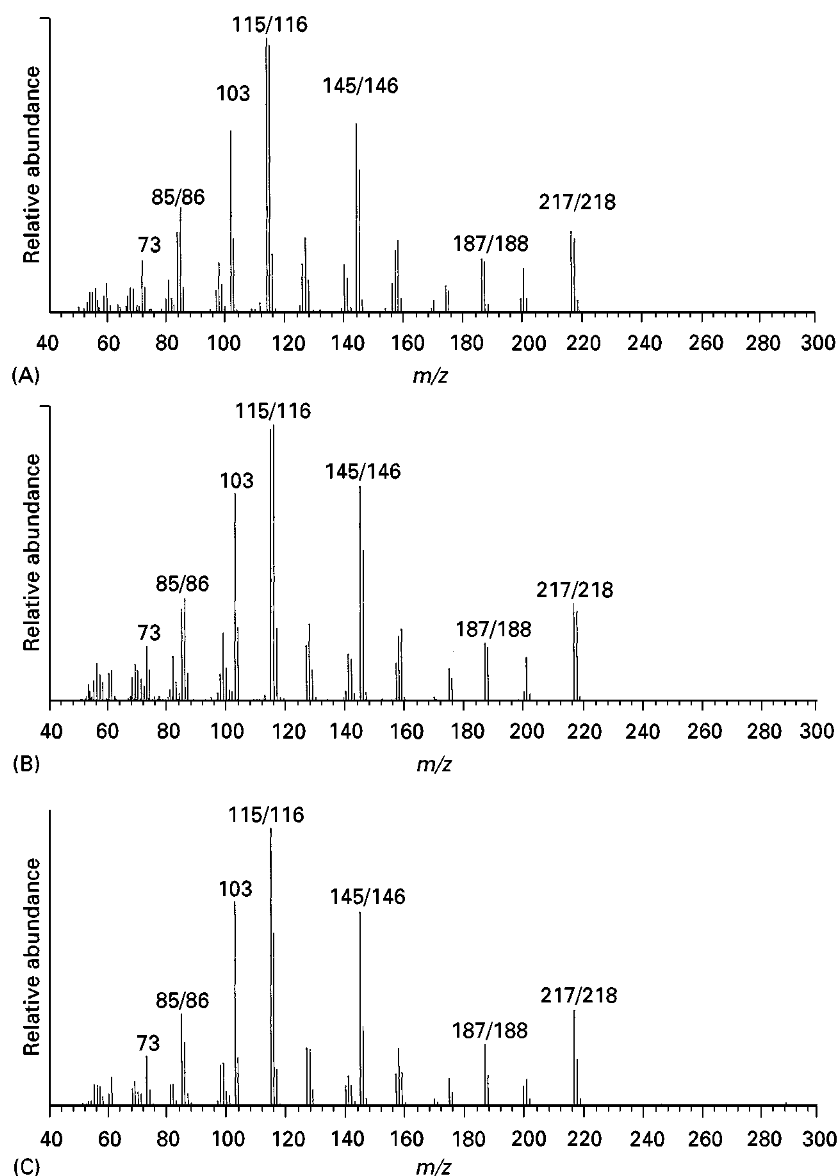

Figure 6 Mass spectra of alditol acetates (A) ribose standard (B) ribose present in hydrolysate of strain 168 (C) a mixture of ribitol and ribose present in hydrolysates of *Bacillus subtilis* W23. Ions derived from the C_1 end are labelled with deuterium (e.g. m/z 146). Ions generated from the C_6 end are not labelled and thus m/z is one less (e.g. m/z 145).

analysis is improved, particularly in product ion spectrum mode. However, in trace quantitative analysis of carbohydrates, in MRM mode, it has been observed that the ion trap is less precise than the triple quadrupole. However, the low cost, ease of use of the ion trap and its power for absolute identification (product ion spectrum) make its use extremely attractive for diagnostic applications.

It is important to note that heavy isotope-labelled internal standards for many sugars are unavailable as pure compounds. Whole ^{13}C-labelled bacterial cell hydrolysates may be used as a cheap alternative source. As an example, in quantitative studies, both methylglucamine and ^{13}C-labelled muramic acid have been used as internal standards in the analysis of muramic acid (a unique chemical marker for bacteria not found elsewhere in nature). While quantitation is better with the latter, there is a possibility of contamination of the ^{13}C-labelled muramic acid with non-labelled muramic acid. Thus, at the detection limit of the procedure, where the major issue is to prove the presence or absence of muramic acid, methylglucamine is preferred. At higher levels, where quantitation is the major issue, ^{13}C-labelled muramic acid is preferred. Obviously an internal standard with both characteristics would be optimal and this is currently under investigation. Isomuramic acid has been described as a natural component of the

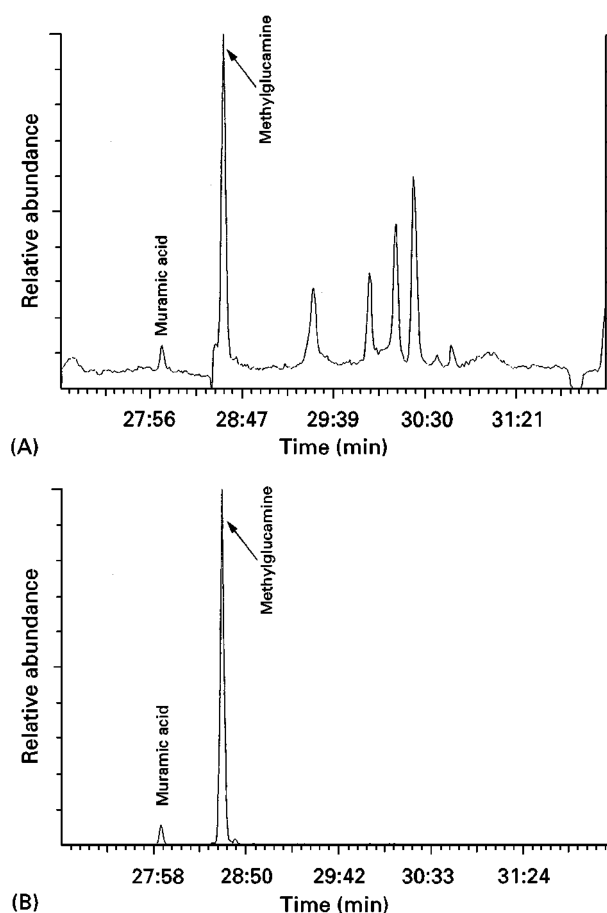

Figure 7 GC-MS-MS shows chromatograms of cerebrospinal fluid from a patient with bacterial meningitis. (A) Full scans (precursor ion *m/z* 403 for muramic acid and *m/z* 327 for methylglucamine) would have a similar appearance to a SIM GC-MS chromatogram (*m/z* 403 and *m/z* 327 respectively); (B) multiple reaction monitoring (*m/z* 403 to *m/z* 198 transition). 250 μL of cerebrospinal fluid was analysed and found to contain a total of 13.6 ng muramic acid. Methylglucamine 500 ng (internal standard) was present in the sample.

lipopolysaccharide of certain bacteria and also has been chemically synthesized. Whether muramic acid and isomuramic acid can be adequately chromatographically resolved has not yet been addressed.

Application of GC-MS-MS in Trace Analysis

Ion trap GC-MS-MS has been used for absolute identification of trace levels of muramic acid in human body fluids. This is the only report to date using GC-MS-MS to detect muramic acid or any other marker for bacteria in a human/animal body fluid or tissue. Product ion mass spectra (upon MS-MS analysis) of muramic acid peaks ($\geq 30\ \text{ng mL}^{-1}$) found in infected human body fluids were identical to those of pure muramic acid. An illustration of the detection of muramic acid in human cerebrospinal fluid is shown (approximately $5\ \text{ng mL}^{-1}$). A powerful feature of the ion trap is the utility of the product ion spectrum for absolute identification of trace levels in complex matrices. This is likely to have particular utility for studies of clinical specimens to determine the presence of bacteria (that are difficult to culture) or their nonviable cell wall components (not detectable by culture). Sterile body fluids and tissues from healthy humans or animals do not generally contain muramic acid.

Figure 7 shows GC-MS-MS chromatograms of cerebrospinal fluid from a patient with bacterial meningitis. The full scan (precursor ion *m/z* 403 from muramic acid and *m/z* 327 for, the internal standard, methylglucamine) would be similar in appearance to a SIM GC-MS chromatogram (*m/z* 403 and *m/z* 327 respectively). Muramic acid is readily detected, but there are clearly numerous background peaks, particularly in the *m/z* 327 window. For comparison, using multiple reaction monitoring (*m/z* 403 to *m/z* 198 transition) background peaks are eliminated. The sample contained a total of 13.6 ng of muramic acid. **Figure 8**, for comparison, shows full scan and MRM

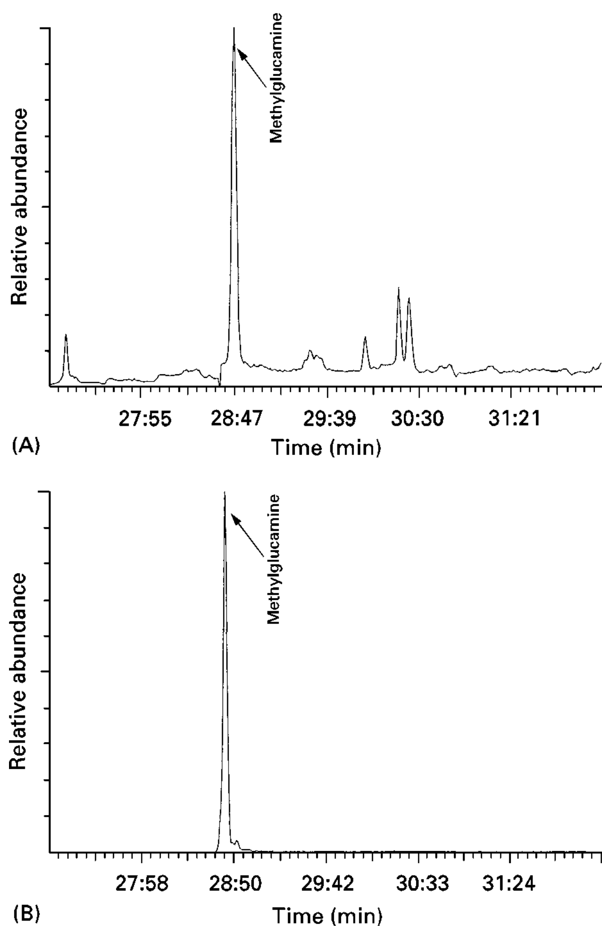

Figure 8 GC-MS-MS chromatograms of uninfected cerebrospinal fluid from a patient with otitis media. (A) Full scan of precursor *m/z* 403 (equivalent in appearance to SIM GC-MS); (B) multiple reaction monitoring (*m/z* 403 to *m/z* 198 transition).

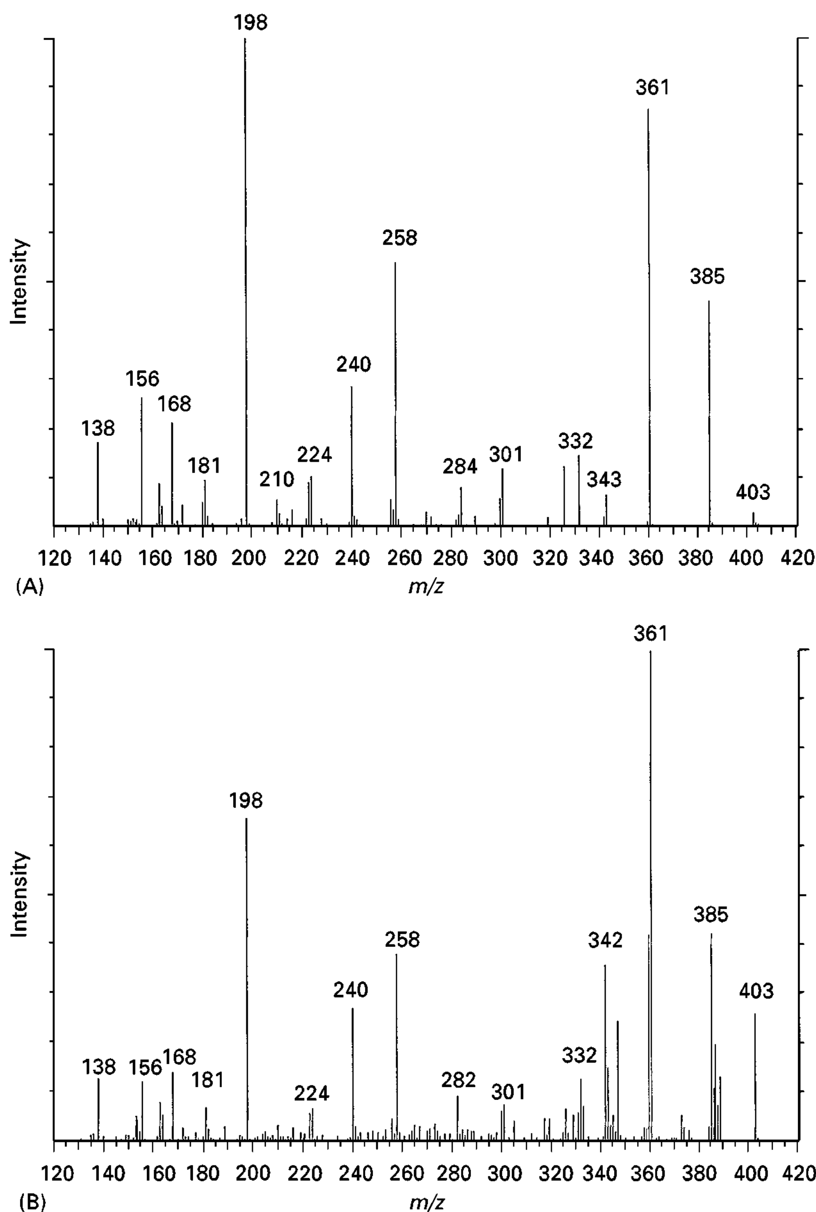

Figure 9 Product ion spectrum (GC-MS-MS) of *m/z* 403 (A) muramic acid standard (250 ng total) and (B) cerebrospinal fluid from patient with bacterial meningitis (2.7 ng in 400 μL of cerebrospinal fluid analysed).

of cerebrospinal fluid from a patient with otitis media (inner-ear infection). In this instance, culture indicated the absence of bacteria in the sample. As expected, there is no peak at the retention time of muramic acid; this serves as a negative control.

A product ion spectrum of muramic acid present in a cerebrospinal fluid (2.7 ng total) and a muramic acid standard (250 ng) are compared in **Figure 9**. Muramic acid is clearly categorically identified from the spectrum of the patient's sample, although background ions are obvious. Clearly, this represents an analysis close to the detection limit, for GC-MS-MS of this type of complex biological sample.

MRM analysis has great utility for determining the levels of bacterial contamination for clinical and environmental analyses. For example, muramic acid levels have been demonstrated to serve as a useful measure of biocontamination of air. This has relevance to our understanding of the 'sick building phenomenon' and assessing the quality of indoor air. The product spectrum is of limited use in the analysis of environmental samples, since bacteria are invariably present. However, as noted above, it is a powerful tool for absolute detection of bacteria in human body fluids and tissues which are sterile in the absence of infection.

Gas Chromatography and Liquid Chromatography

In the 1980s successful analysis of underivatized sugars using high performance liquid chromatography was introduced. Eliminating derivatization dramatically reduces sample handling and chemical manipulation. After separation on anion exchange columns (particularly the PA1 column) sugars are detected with a pulsed amperometric detector (PAD). This LC system has been successfully used for the separation and identification of amino, neutral and acidic sugars. Chromatography is performed in concentrated NaOH. At high pH, interaction of ionized hydroxyl groups with the anion exchange resin produces excellent sugar separations. The electrochemical (PAD) detector also detects carbohydrates with excellent sensitivity at alkaline pH. Unfortunately, in the analysis of sugars in complex matrices, the nonselective PAD detector often has inadequate specificity to discriminate sugars of interest from background noise.

When chromatography is performed in conjunction with the mass spectrometer (e.g. GC-MS or LC-MS), the increased selectivity of detection allows analysis of less purified samples. For example, whole cell hydrolysates can be analysed. With LC-PAD analysis, structural components such as glycoproteins or polysaccharides must be purified prior to analysis. LC-MS-MS has been used for quantitation of sugars in complex environmental matrices. LC-MS and LC-MS-MS for the analysis of carbohydrates show great promise but are still in the developmental stage. Currently, GC-MS and GC-MS-MS have considerably lower detection limits than LC-MS and LC-MS-MS. Automation of sugar derivatization for GC eliminates many of the advantages of LC-based techniques over the GC-based ones but the only derivatization procedure for carbohydrate analysis by GC that has been automated, at this time, is the alditol acetate procedure.

Conclusions

GC-MS and GM-MS-MS are mature techniques which may be used in the analysis of carbohydrate monomers in complex samples. In SIM GC-MS and MRM GC-MS-MS, sugars are readily detected and quantitated in complex samples, although use of MRM dramatically lowers the detection limit. Using total ion (MS) and product ion spectra (MS-MS), identification at trace levels is readily performed. The detection limit is much lower for the latter when the ion trap GC-MS-MS is used. Substantial improvements have been made to sample preparation, including simplification and computer-controlled automation of derivatization reactions for GC analysis. This is likely to help GC-based techniques to remain competitive with their LC competitors where derivatization is usually eliminated. The limit of detection is still currently much lower with GC-based procedures.

See also: **II/Chromatography: Gas:** Detectors: Mass Spectrometry. **III/Carbohydrates:** Electrophoresis; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Polysaccharides:** Liquid Chromatography.

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