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Review

Carbohydrate profiling of bacteria by gas chromatography–mass spectrometry and their trace detection in complex matrices by gas chromatography–tandem mass spectrometry

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Abstract

Bacterial cellular polysaccharides are composed of a variety of sugar monomers. These sugars serve as chemical markers to identify specific species or genera or to determine their physiological status. Some of these markers can also be used for trace detection of bacteria or their constituents in complex clinical or environmental matrices. Analyses are performed, in our hands, employing hydrolysis followed by the alditol acetate derivatization procedure. Substantial improvements have been made to sample preparation including simplification and computer-controlled automation. For characterization of whole cell bacterial hydrolysates, sugars are analyzed by gas chromatography–mass spectrometry (GC–MS). Simple chromatograms are generated using selected ion monitoring (SIM). Using total ion GC–MS, sugars can be readily identified. In more complex clinical and environmental samples, markers for bacteria are present at sufficiently low concentrations that more advanced instrumentation, gas chromatography–tandem mass spectrometry (GC–MS–MS), is preferred for optimal analysis. Using multiple reaction monitoring, MS–MS is used (replacing more conventional SIM) to ignore extraneous chromatographic peaks. Triple quadrupole and ion trap GC–MS–MS instruments have both been used successfully. Absolute chemical identification of sugar markers at trace levels is achieved, using MS–MS, by the product spectrum.

Keywords: Reviews; Bacteria; Carbohydrates

Contents

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1. Introduction

Analysis of carbohydrates present at high concentrations in isolated macromolecules (e.g., polysaccharides and glycoproteins) using liquid chromatography (LC) in conjunction with conventional detectors are now well established. The most widely used approach involves separation of underivatized sugars using high-performance anion-exchange chromatography and detection using the pulsed amperometric detector [1–3]. When GC is performed in conjunction with mass spectrometry (i.e., GC–MS), the increased selectivity of detection allows ready analysis for less-purified samples (e.g., whole bacterial cell hydrolysates) [4–6]. LC–MS and LC–MS–MS for analysis of bacterial sugars in complex matrices show great promise but are still in the developmental stage [7–10].

GC–MS methods are well established and have been used routinely since the early 1980s [5,6]. GC–MS–MS methods, although only recently introduced for trace detection of bacterial chemicals in complex matrices [11–14] have been readily adapted from existing GC–MS technology. Both triple quadrupole and ion trap GC–MS–MS instruments have been successfully employed. A major argument against the use of GC-based methods is the laborious nature of derivatization methods which up to this time have been performed manually. Recently, most of the manual steps of the alditol acetate procedure have been eliminated employing a computer-controlled automated derivatization instrument [15]. The alditol acetate procedure is the only procedure used extensively in the analysis of bacterial carbohydrates in complex matrices using both GC–MS and GC–MS–MS and is the first method to be automated.

Some chemical markers (such as aminodideoxyhexoses common among the family, *Legionellaceae*) [16–18] and quinovose present in spores of bacilli [19–21]) are not widely distributed among different bacterial species or genera. GC–MS can be used to differentiate bacterial species by profiling the carbohydrate composition of whole cell hydrolysates. Other sugar markers (notably muramic acid) are present in high concentrations in most bacteria, but are uncommon in higher life forms such as animals or fungi. Table 1 summarizes some of the carbohydrate markers identified in bacteria as alditol acetates on GC–MS analysis. For detection of these microbial constituents, as trace contaminants, in complex samples (e.g., animal body fluids/tissues or airborne dust) GC–MS can be used but GC–MS–MS is preferred [11,12].

2. Manual/automated preparation of alditol acetates and their analysis

In the alditol acetate procedure, neutral and amino sugars are released by hydrolysis and then the

Table 1
Carbohydrate markers, identified in bacteria as alditol acetates, on GC–MS analysis

Compound	Source	Organism
Muramic acid (3-*O*-lactylglucosamine)	Peptidoglycan	Bacteria but not elsewhere in nature
Heptoses (L-glycero-D-mannoheptose [L,D-heptose] D-glycero-D-mannoheptose [D,D-heptose])	Lipopolysaccharide	Gram-negative but not Gram-positive bacteria
L,D-Heptose but not D,D-heptose	Lipopolysaccharide	*Salmonella typhimurium* other Enterobacteriaceae
D,D-Heptose and L,D-heptose	Lipopolysaccharide	*Pasteurella multocida*
Quinovose (6-deoxyglucose)	Spore polysaccharide	*Bacillus subtilis* but not *Bacillus cereus*
Galactose	Vegetative cell wall polysaccahride	*Bacillus anthracis* but not *B. cereus*
Fusosamine (2-amino-2,6-dideoxygalactose)	Lipopolysaccharide	*Tatlockia* But not *Legionella*
Quinovosamine (2-amino-2,6-dideoxyglucose)	Lipopolysaccharide	*Legionella* but not *Tatlockia*
Quinovosamine	Lipopolysaccharide	*Brucella abortis*, *B. suis* and *B. melitiensis* but not *B. canis*
Yersiniose A (3,6-dideoxy-4-hydroxyethyl-D-xylohexose)	Lipopolysaccharide	*Tatlockia*

aldehyde group is reduced (to eliminate the anomeric center) and hydroxyl and amino moieties subsequently acetylated [22,23]. Elimination of the anomeric center simplifies chromatograms dramatically as most sugars produce one chromatographic peak. Muramic acid, 3-*O*-lactylglucosamine is an unusual sugar which additionally contains a carboxyl group. A lactam (a cyclic amide) is formed by internal dehydration between its carboxyl and amino groups. In contrast to acetylation of other amino sugars, which produces amides, muramicitol pentaacetate has an imido group (where two acyl groups, lactyl and acetyl, respectively, are linked to the nitrogen atom). Formation of the lactam ring and the imido moiety require harsh conditions (higher temperatures and longer heating times) [24]. Formation of a new, stable, halogenated derivative of muramic acid (*O*-pentafluorobenzyloxime acetate) also requires extreme acylation conditions [25]. Carboxyl groups, such as found in acidic sugars or amino acids, appear not to be derivatized under any of these reaction conditions and thus these compounds do not produce chromatographic peaks.

The alditol acetate procedure is still generally performed manually. Due to the time-consuming and complicated nature of derivatization, carbohydrate profiling of bacteria have not routinely been performed outside a few specialist analytical microbiology laboratories. The alditol acetate procedure currently takes 2.5 working days, when performed manually, to prepare a batch of samples. Earlier developments in the chemistry of the alditol acetate procedure have been previously reviewed [5,6]. In brief, the major steps in the procedure include acid hydrolysis (under vacuum or nitrogen) to release sugars. Internal standards are added. The aqueous hydrolysates are then converted to neutrality; H^+ ions, presumably followed by SO_4^-, are extracted with a chloroform solution of an organic base (*N*,*N*-dioctylmethylamine). Hydrophobic materials are removed pre-derivatization (when sugars are hydrophilic) by passage through a second disposable column packed with C_{18} phase. The sugars (including aldoses) are then converted to sugar alcohols (alditols) by reduction (using sodium borohydride or borodeuteride to label C1). Between the reduction and acylation steps the reducing agent (borohydride/borodeuteride) is removed (as tetramethylborate gas) by multiple evaporations with methanol/acetic acid which is quite time consuming. This is necessary since borate would inhibit the subsequent acylation reaction. After drying, the alditols are then acetylated (with acetic anhydride) and converted to alditol acetates. The alditol acetates are then passed through a second set of two disposable columns containing $MgSO_4^-$ (pre-treated with solutions of acetic acid and ammonium hydroxide, respectively) to remove hydrophilic contaminants. The latter columns also dry the samples before GC analysis.

The two sets of clean-up steps to remove hydrophobic materials pre-derivatization (when sugars are hydrophilic) and hydrophilic compounds post-derivatization (after sugars have been converted to alditol acetates) are highly selective. The pre-derivatization clean-up steps remove hydrophobic substances (e.g., fatty acids) before acylation of hydroxyl/amino groups. Post-derivatization clean-up steps remove hydrophilic compounds (e.g., acetylated amino acids that have free carboxyl groups). Thus, in the analysis of complex biological samples the procedure is highly selective for neutral and amino sugars that remain in the hydrophilic phase prior to derivatization, but in the hydrophobic phase post-derivatization.

Automation could help enormously in popularizing the alditol acetate, and indeed other derivatization, techniques. As noted above, during reduction with sodium borohydride, borate is generated which if not removed inhibits the subsequent acylation reaction. Classically, borate removal involves five cycles of manual addition of methanol–acetic acid followed by evaporation. This is extremely tedious and time consuming. Alternatively, an automated evaporator can be used [26] that simplifies the removal of borate. A photograph of the evaporator can be seen elsewhere [6]. Batches of samples, in reaction vials, are seated in a heating bath and are continuously rotated past a syringe pump containing a reservoir of methanol–acetic acid. Methanol–acetic is pumped into the samples (over a period of 2–3 h) as they rotate past the syringe pump outlet. Nitrogen is also passed into each sample through a manifold connected to a nitrogen tank.

More recently, an automated derivatization instrument (15) has been developed which automates the alditol acetate derivatization reaction. Hydrolysis under nitrogen (3 h) followed by pre-derivatization clean-up (30 min) is first performed. The 24–26-h

procedure is then performed unattended in the automated derivatization instrument. The various stages of the derivatization process are performed sequentially by computer-controlled opening of the appropriate electrically driven solenoid valves. The instrument has a central unit consisting of 21 individual glass/PTFE/reaction chambers. The reaction chambers are seated in a heating block. The chambers can be evacuated or placed under a nitrogen atmosphere. If the chambers are open to atmosphere nitrogen or vacuum-assisted evaporation occurs. If the chambers are sealed, then derivatizaton reactions (e.g., reduction, removal of borate or acetylation) can be performed Solvent exchange is achieved by automated evaporation of the first solvent followed by adding solvent into the samples under nitrogen pressure. As noted above, after hydrolysis and pre-derivatization clean-up, under computer control, methanol–acetic acid is pumped into the samples. At this stage the samples are heated. Liquid flow stops and nitrogen flow begins with the chambers open to atmosphere causing evaporation. This is repeated multiple times. The chambers are evacuated and dried further. Acetic anhydride is pumped into the samples from another reservoir. The samples are evaporated to dryness under nitrogen. Chloroform is finally pumped, from a third reservoir, into each dried sample. The final post-derivatization clean-up (taking 30 min) is performed manually.

Excellent capillary GC separation of mixtures of neutral and amino sugars are obtained on relatively polar SP-2330 columns. However, amino sugars require high final temperatures and/or extended run times for elution Unfortunately, these columns tend to display poor temperature stability under such conditions. Furthermore, irreversible adsorption of amino sugars is a significant problem; poor sensitivity of amino sugars relative to neutral sugars is often observed [27]. More recently, non-polar DB5-MS columns have been used which have not exhibited these problems. In complex mixtures, sugars are observed as sharp peaks with almost base-line resolution [12,28].

3. Bacterial differentiation by whole cell profiling

Classical methods to identify and classify microorganisms are based on their morphology and physiological characteristics. These methods, although useful, are tedious, time consuming and sometimes provide ambiguous results. Molecular biology methods offer an exciting alternative where genes are characterized; this includes the use of DNA/RNA probes or gene amplification by the polymerase chain reaction (PCR). These methods are generally based on gel electrophoresis. Molecular methods are rapidly being introduced into the routine diagnostic laboratory but simple cheap alternatives to electrophoresis are needed. Analytical chemical approaches particularly using mass spectrometry offer great opportunities in this area but are currently in their infancy [29,30].

Analytical microbiology (chemotaxonomy-based) methods, using chromatography and/or mass spectrometry, are primarily used in research or reference laboratories. Microorganisms are classified based on their chemical characteristics, i.e., the presence of endogenous chemical constituents. Most commonly low molecular mass fatty acid monomers are released by methanolysis and converted to fatty acid methyl esters (FAMEs) for analysis by GC using flame ionization detection (FID) [31]. This allows universal bacterial speciation. With this chromatographic method the basis of identification is the retention time.

Profiling of whole cell hydrolysates for their carbohydrate composition also allows bacterial speciation. This is less commonly performed than FAMEs because derivatization of sugars has been more time consuming. Sugars are released by hydrolysis and converted to their alditol acetates. Analysis can be performed by GC–FID but GC–MS is preferred. Ionization is generally achieved by electron impact (EI). In the selected ion monitoring (SIM) MS mode, simple chromatograms free of background interferences from other components of the bacterial cell (e.g., amino acids, fatty acids and nucleotides) are generated. Total ion spectra can also be used to identify each sugar component. The use of SIM in bacterial analysis is illustrated in Fig. 1, which shows carbohydrate profiles of several species of brucellae. *Brucella canis* is readily discriminated from other brucellae by the absence of quinovosamine. Fig. 2 illustrates the identification of quinovosamine by comparison of the mass spectra of the chromatographic peak isolated from brucellae

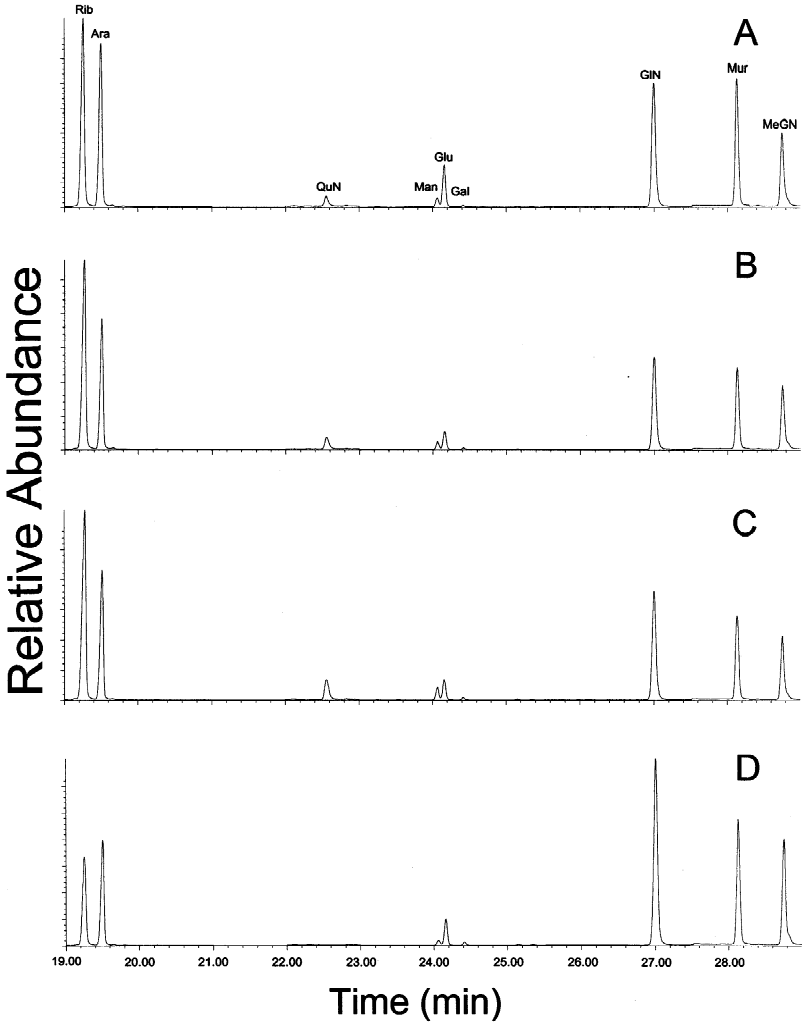

Fig. 1. Carbohydrate profiles of alditol acetates analyzed by SIM GC–MS (A) *B. abortus* (30 101) (B) *B. melitensis* (31 242) (C) *B. suis* (23 444) and (D) *B. canis* (30 201). Rib, ribose; Ara, arabinose (internal standard); QuN, quinovosamine; Man, mannose; Glu, glucose; Gal, galactose; GlN, glucosamine; Mur, muramic acid; MeGN, methylglucamine (internal standard). Reproduced with permission from Ref. [28].

with quinovosamine previously identified in *Legionella pneumophila* [28]. The masses (actually mass/charge where the charge is invariably 1) represent fragments of the alditol acetate of quinovosamine (^{2}H labeled on C1). The molecular mass of derivatized quinovosamine is 376. Loss of carbon (C) one (74 mass units) generates the molecular ion 302. Breakage of the bond between C2 and C3 generate the other primary fragment of mass 145. The dominant mass 85 is generated from mass 145 by loss of acetic acid (60 mass units). Secondary fragments from 302 include prominent peaks of mass 260 (loss of 42, ketene) and 201 (loss of 42 and 59, the acetoxyl group) [28]. For general information on interpretation of mass spectra of alditol acetates see Section 3.3 below.

Bacteria are divided into Gram-positive and -negative categories (based on Gram-staining characteristics). Gram-positive bacteria have polysaccharides covalently bound to the cell wall skeleton (peptidoglycan). These covalently bound polysaccharides are absent in Gram-negative bacteria. However, Gram-negative bacteria have an additional outer membrane which contains a lipopolysaccharide anchored by its

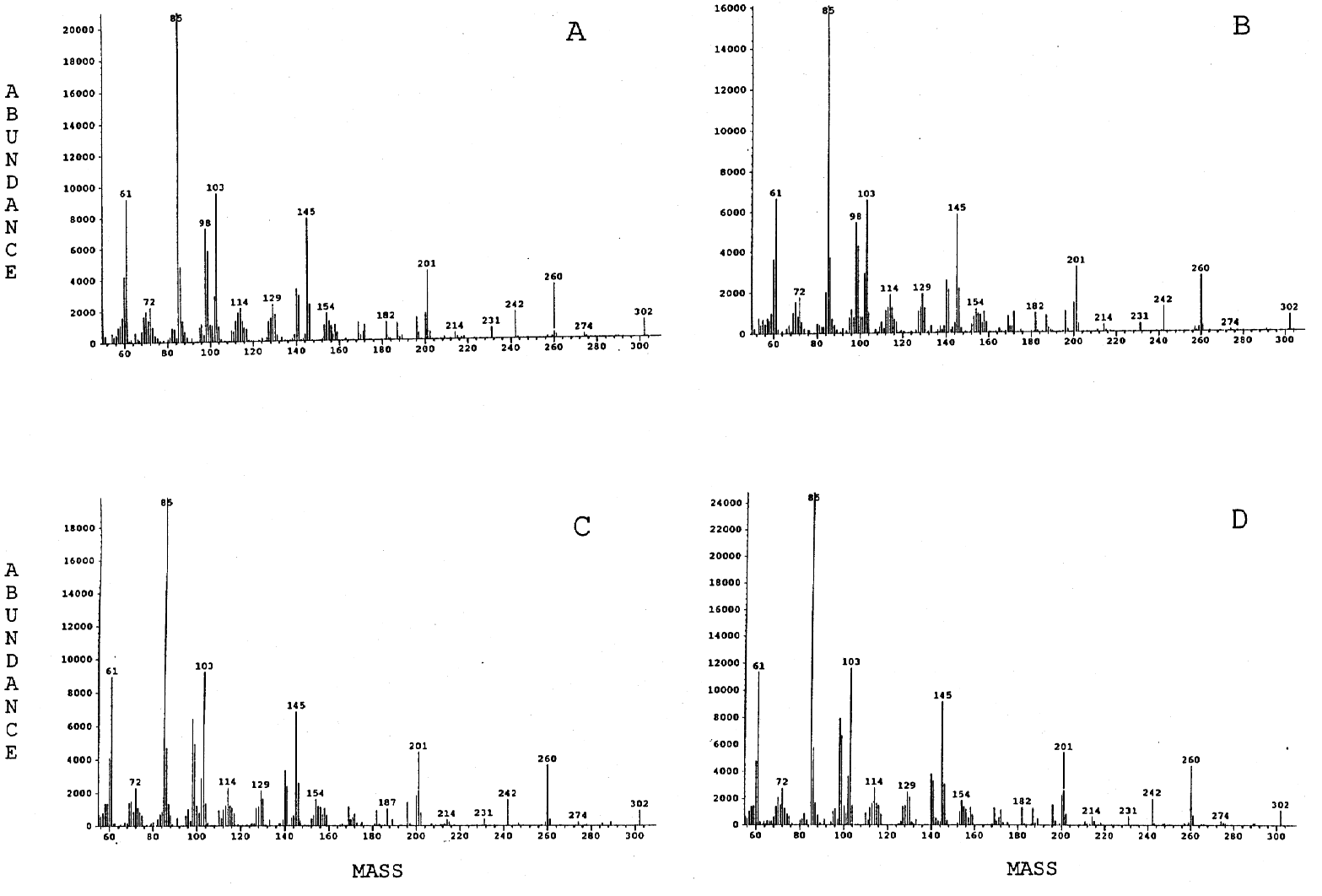

Fig. 2. Mass spectra of alditol acetates of quinovosamine from (A) *B. melitensis*, (B) *B. abortus*, (C) *B. suis* and (D) *L. pneumophila*. Reproduced with permission from Ref. [27].

lipid end in the membrane. Lipopolysaccharides are characterized by the presence of L,D-heptose and D,D-heptose which are generally absent in Gram-positive bacteria [16]. Both Gram-positive and -negative bacteria can have a loose polysaccharide layer outside the cell wall or outer membrane, respectively, referred to as a capsule. There is a great diversity of sugar monomers present in these bacterial carbohydrates.

Our work has successfully demonstrated that bacterial sugars can be readily identified in whole bacterial hydrolysates using total ion monitoring GC–MS. The abundances of these sugars are quite high, around 0.1–2.0%, or higher, of the dry mass. In addition, sample size is not limited, allowing analysis of 5–10 mg of sample. The purpose of using SIM is to improve visual discrimination of chromatograms by eliminating background. However, it is possible to obtain mass spectra of sugars that are present at quite low concentrations (<0.001%).

3.1. Taxonomic characterization of bacteria

A primary focus of research at USC is to develop profiling strategies that have widespread applicability among diverse taxonomic groups. Examples from our work follow with two bacterial groups; the genus *Bacillus*, a Gram-positive organism [19–21], and the *Legionellaceae* [16–18], a Gram-negative family of organisms. In both instances there are numerous unresolved questions regarding the inter-relationships within and among the constituent species/genera.

Legionellae are important environmental agents that often colonize hot water towers. After airborne transmission, these microbes can initiate disease in susceptible individuals. *Legionella pneumophila* is the major pathogen in the family *Legionellaceae* and the causative agent of Legionnaire's disease. Because of their poor growth characteristics it has been difficult to use conventional biochemical tests in the differentiation of members of the *Legionellaceae*. We first differentiated legionellae by analysis of their carbohydrate content using GC–FID [16]. Total ion mode GC–MS was used to detect a number of unusual sugars, including a branched octose [17,18] which was subsequently identified by others as yersiniose A [32]. Increased sensitivity and selectivity for carbohydrate detection was subsequently achieved using SIM. Two of the uncommon amino-dideoxyhexoses we discovered in the legionellae were later identified as quinovosamine and fucosamine, respectively [33].

Recently, we have focussed on another group of environmental pathogens, the Gram-positive bacilli. These organisms unlike many other bacteria when grown in high nutrient content display typical cellular morphology (referred to as vegetative). Under nutrient deprivation there is a change in morphological form to a spore. The spore-form is a dormant cell capable of resisting harsh environmental conditions. Many aspects of the taxonomic characterization and clinical identification of bacilli remain unresolved. As an example, differentiation of the environmental organisms, *Bacillus thuringiensis*, *B. anthracis* and *B. cereus*, presents a taxonomic challenge for they display few distinguishing physiological characteristics and share a significant degree of genetic similarity (including high degree of 16S ribosomal RNA and 16S–23S interspace rRNA sequence relatedness) [20,34]. As a result of the extreme similarity within this group these organisms have been referred to as the *B. cereus* group. Two of these species are human pathogens (*B. anthracis*, the causative agent of anthrax, and *B. cereus*, a food-poisoning organism). A distinguishing characteristic of *B. thuringiensis* is its ability to produce a class of insecticidal proteins, known as crystallins or δ-toxins. *B. subtilis*, and related organisms including *B. atrophaeus*, in contrast, are not human or insect pathogens and are readily differentiated from the *B. cereus* group providing a closely related but distinct organism for study [20,35].

Using GC–MS, sugar profiles of vegetative cells are similar for *B. cereus* and *B. thuringiensis*. *B. anthracis* contains high levels of galactose which generally distinguish it from *B. cereus*/*B. thuringiensis*, while *B. subtilis* is distinguished from the *B. cereus* group by low mannosamine levels. Spore profiles differ from vegetative profiles in all four species. Like vegetative profiles, spore profiles are distinctive for *B. cereus*/*B. thuringiensis*, *B. anthracis*, and *B. subtilis*. *B. cereus* and *B. thuringiensis* spores both contain rhamnose, fucose, 2-*O*-methylrhamnose and 3-*O*-methylrhamnose, unlike *B. anthracis* spores that contain only rhamnose and 3-*O*-methylrhamnose. *B. subtilis* strains are

heterogeneous with some resembling *B. anthracis* and others *B. cereus*/*B. thuringiensis*, although *B. subtilis* strains typically contain the rare sugar quinovose [19,20].

3.2. Characterization of bacterial cells in different physiological states

When bacilli are grown under phosphate-rich conditions they synthesize phosphate-rich polysaccharides. These polymers of ribitol or glycerol phosphate are referred to as teichoic acids. Under conditions of phosphate deprivation bacilli switch to synthesis of an alternative polysaccharide—teichuronic acid. The teichuronic acids of certain strains of bacilli lack ribitol but are rich in glucuronic acid. In both cases morphologically the cells are present in their vegetative form. GC–MS has been used to confirm the classical observation of switching from teichoic acid (phosphate-rich conditions) to teichuronic acid (phosphate-deprived conditions). It is unnecessary to purify polysaccharides prior to GC–MS analysis. The presence of anhydroribitol (a dehydration product of ribitol), in whole cell hydrolysates, indicates teichoic acid production. Most interestingly it was demonstrated that certain spore sugars (presumably part of a spore polysaccharide) are not produced in vegetative cells grown under either phosphate-rich or phosphate deprivation conditions. Spore markers (methylpentoses, *O*-methylpentoses and quinovose) are not seen in vegetative cells and conversely vegetative markers (e.g., ribitol) are not seen in spores [21].

3.3. Identification of bacterial sugars by total ion GC–MS

GC, in conjunction with MS in the total ion mode, is a powerful tool for identification of sugars in bacterial cells. Bacterial species/genera often contain unusual sugars that are rarely found in nature. Commercial standards are, commonly, unavailable for these compounds. In these instances, interpretation of EI mass spectra from first principles is essential.

A native sugar forms two to four anomers upon acylation, thus creating multiple peaks from a single sugar and complicating chromatograms. Thus, as noted above, sugars are generally reduced using sodium borohydride or borodeuteride prior to acylation to eliminate anomer formation. For example, during reduction of aldoses the C1 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 C1. Fragments lacking C1 are not different between deuterated and non-deuterated samples.

The base peak in EI mass spectra of alditol acetates is 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, amino sugar mass spectra are relatively simple since cleavage preferentially occurs between the carbon with attached acetamido group and adjacent acetylated carbons [36].

Mass spectra of isomers of alditol acetates contain ions of the same m/z. On casual observation the mass spectra thus 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 [17]. Differences in mass spectra among isomers are accentuated by the use of borodeuteride. Aldoses are asymmetric since there is an aldehyde on C1. Asymmetry is retained after borodeuteride but not borohydride reduction since C1 is labeled. All eight hexoses can be differentiated by a combination of distinct mass spectrum and/or retention time [37].

Naturally occurring *O*-methylated sugars are not common in bacterial cell wall polysaccharides. 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). As noted above, 3-*O*-methyl- and 2-*O*-methylpentoses have been identified in members

of the *B cereus* group (*B. anthracis*, *B. cereus*. and *B. thuringiensis*) as well as *B. subtilis* [19,20].

As discussed in detail below muramic acid (3-*O*-lactylglucosamine) is an important sugar marker present in bacteria. This sugar is present is one of the two sugars making up the backbone polysaccharide of peptidoglycan. It is not generally present elsewhere in nature. Thus muramic acid serves as a marker for the presence of bacteria. The mass spectrum of the alditol acetate of muramic acid (3-*O*-lactylglucosamine) was more difficult to interpret, since it contains an internal, cyclic amide. The molecular mass of muramicitol pentaacetate is 463. Loss of water allows the formation of an amide bond between the C2 nitrogen and the carboxyl group of the lactyl moiety producing muramicitol pentaacetate lactam (molecular mass 445). The four hydroxyl moieties and the amino group are acetylated, the latter existing as an imide, thus forming the pentaacetate [12,24].

4. Trace analysis in complex matrices using GC–MS–MS

During the past few years we introduced the use of GC–MS–MS for trace detection of chemical markers for bacteria and their constituents in complex clinical and environmental matrices [11,12]. Absolute identification is achieved using the product spectrum and levels of chemical markers for bacteria determined by multiple reaction monitoring (MRM) [13].

Certain compounds serve as chemical markers for the trace detection of bacteria in complex matrices. Culture is by far the most widely used procedure of assessing both environmental biopollution or clinical infection. However, an important portion of the bacterial population may remain undetected using conventional microbiological culture. A non-traditional approach for non-culture-based determination of biocontamination involves chemical monitoring of components of bacteria. With such assays, there is no discrimination as to whether the components are derived from viable or non-viable organisms. This is achieved currently with optimal sensitivity and specificity using GC–MS–MS. LC–MS–MS (with electrospray ionization) has considerable potential in simplifying analysis but currently does not match the sensitivity of GC–MS–MS for this purpose [7].

Muramic acid is one of the few chemical markers that is unique to bacteria. Muramic acid is found in most bacterial pathogens [in the cell wall peptidoglycan (PG)], but not elsewhere in nature including animal or plant cells or fungi. Thus muramic acid levels serve as a qualitative marker for the presence of PG and for quantitation.

High-resolution chromatographic separations coupled with selective clean-up steps are important in improving the specificity of detection of chemical markers (e.g., muramic acid) in complex matrices. However, chromatographic separation is not sufficient to eliminate extraneous peaks when non-selective detectors are employed. The use of the mass spectrometer, as a selective GC detector (e.g., GC–MS analysis in the SIM mode), helps greatly in diminishing background noise. However, even using SIM it is not uncommon to find extraneous background peaks [38]. The specificity of the tandem mass spectrometer in MRM mode as a GC detector provides even further specificity in detection at trace levels in complex matrices. Both SIM GC–MS [38,39] and MRM GC–MS–MS [11–14] analysis allow excellent quantitation of chemical markers for bacteria but the latter allows much greater confidence in assignment of peaks as the compound of interest. The exquisite specificity of MRM GC–MS–MS analysis is illustrated in Fig. 3. In analysis of muramic acid in organic dust, extraneous peaks are essentially eliminated.

The first use of GC–MS–MS for detection of a chemical marker for bacteria, published in 1995, employed a triple quadrupole instrument and was used for trace analysis of the alditol acetate of muramic acid in organic dust [11]. Subsequently, successful use of more modestly priced and user friendly ion trap MS–MS instruments in this laboratory [12] and one other [14] have demonstrated the potential for expanding the use of GC–MS–MS outside of a few specialist analytical microbiology laboratories

When present at relatively high levels it is possible to categorically identify muramic acid in a chromatographic peak by the ‘total ion mass spectrum’ (GC–MS analysis). For example, in a 1980 report using GC–MS (after systemic administration of streptococ-

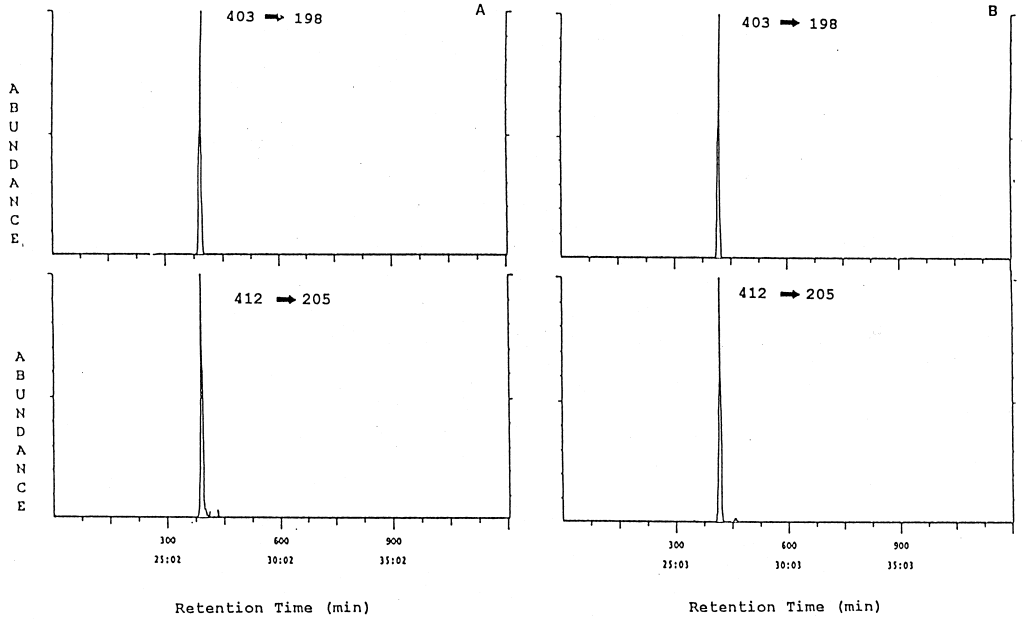

Fig. 3. MRM chromatogram after GC–MS–MS analysis of alditol acetates of (A) standard muramic acid (B) muramic acid present in airborne dust. One fragmentation transition was monitored for muramic acid (*m*/*z* 403–198) and one for [^{13}C]muramic acid (*m*/*z* 412–205). Reproduced with permission from Ref. [12].

cal cell wall components) a peak at the retention time for muramic acid found in rat spleen (70 μg/g wet mass of tissue) had an identical 'mass spectrum' to that of standard muramic acid. In joints of cell wall injected rats, a peak was observed, at 1 μg/g levels using SIM), at the retention time for muramic acid but a peak was not present in normal joints used as negative controls. However, at the low levels present in these biological it proved impossible to obtain a full mass spectrum for 'absolute' identification [39].

Categorical identification at trace levels has awaited the development of more advanced GC–MS–MS instrumentation [13]. Ion trap GC–MS–MS has been used for 'absolute' identification at trace levels of muramic acid in human body fluids (see Fig. 4). This is the only report to date using GC–MS–MS to detect muramic acid or indeed any other marker for bacteria in a human/animal body fluid or tissue. Product spectra of muramic acid peaks (≥30 ng/ml) in infected human body fluids were identical to those of pure muramic acid. The origin of the precursor ion (403) and its fragmentation into the product ion 198 is interpreted in Fig. 5. The generation of two other precursor ions (228 and 168) and their fragmentation into product ion transitions (114 and 96, respectively) are also interpreted. For further information on the basis of these interpretations see Ref. [12].

Both triple quadrupole and ion-trap GC–MS–MS instruments have been used successfully for trace analysis of muramic acid in environmental and clinical samples. In routine use, sensitivity of the ion trap for absolute identification of muramic acid (product spectra mode) substantially exceeds that of the triple quadrupole. This might be anticipated based on theoretical considerations of instrument design ('tandem in time' versus 'tandem in space') [40]. However, it is important that this has been experimentally demonstrated in trace detection in complex matrices. The utility of the product spectrum, for absolute identification at trace levels in complex matrices, readily obtained with the ion trap, is a powerful feature. However, quantitation, is substantially better using the triple quadrupole mass spectrometer. It is worthy of note that in most clinical or environmental applications of the type described there the reproducibility obtained with the ion trap would more than suffice. The ease of use,

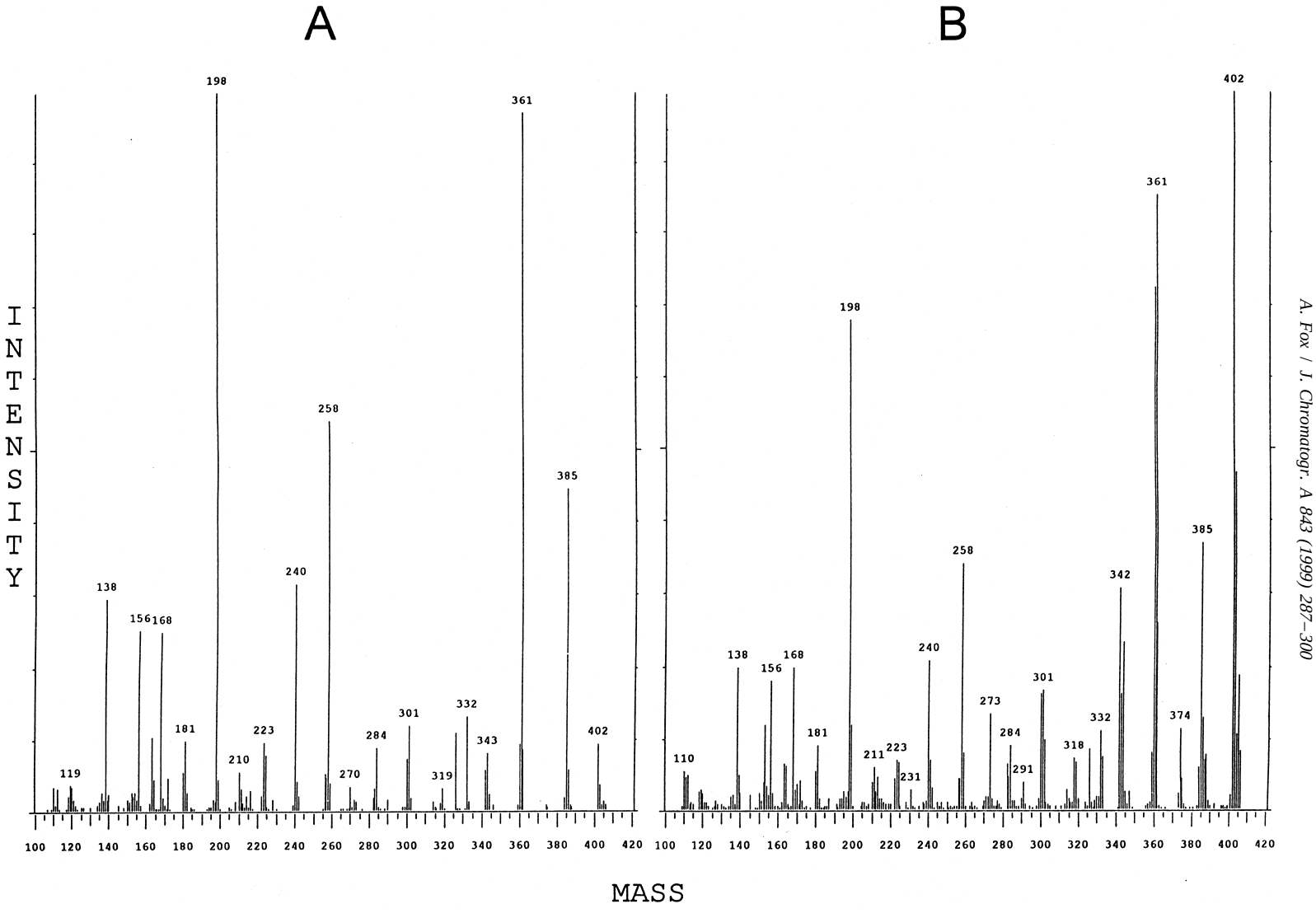

Fig. 4. Product spectra of chromatographic peaks of alditol acetates of (A) pure muramic acid and (B) muramic acid isolated from a synovial fluid of a patient with septic arthritis. The molecular mass of the alditol acetate of muramic acid is 445 and loss of ketene (mass 42) generates 403. Reproduced with permission from Ref. [13].

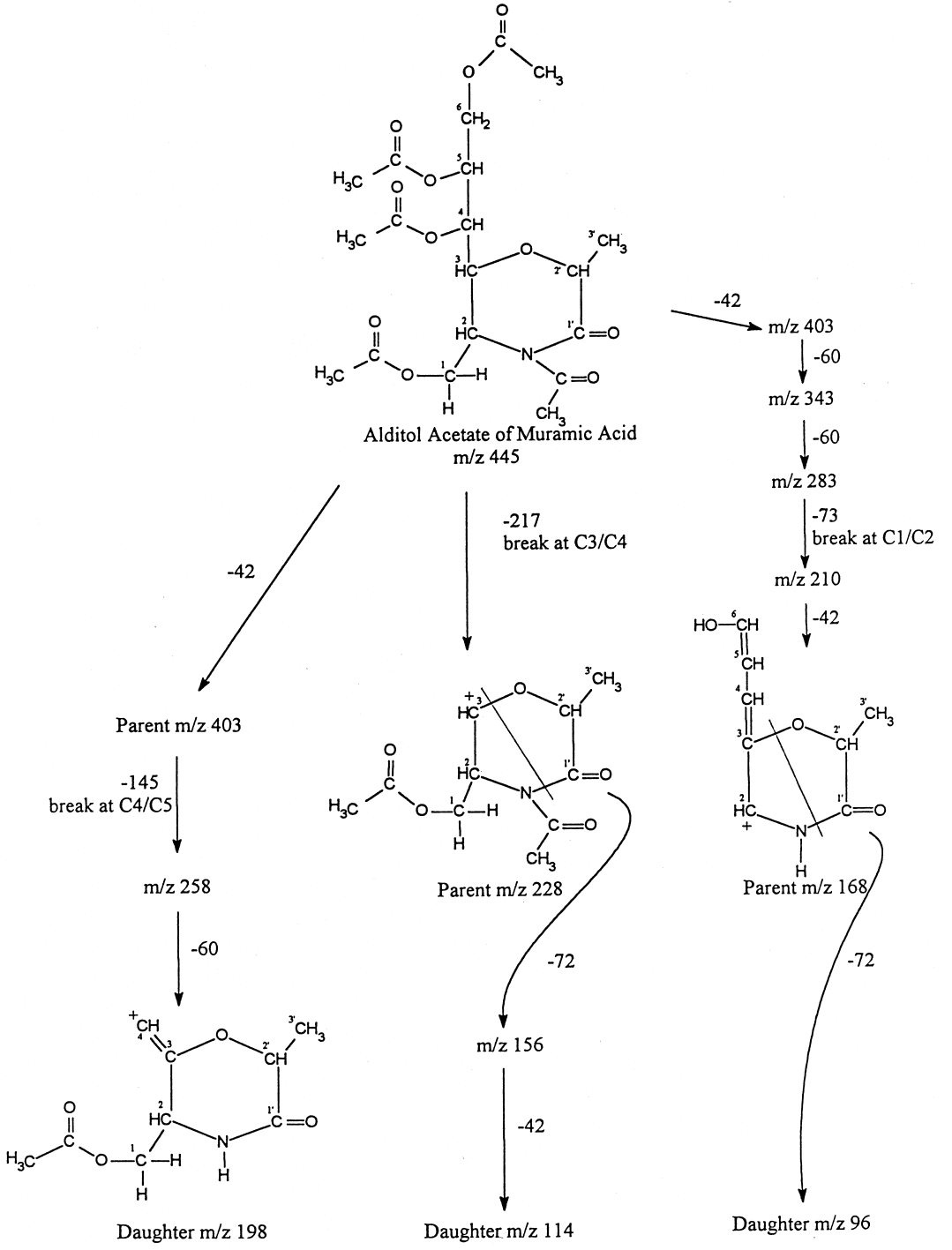

Fig. 5. Interpretation of structures of precursor and product ions generated from muramicitol pentaacetate on GC–MS–MS analysis. Reproduced with permission from Ref. [12].

low cost and ready maintenance of the ion trap makes it extremely attractive despite this limitation.

5. Concluding statement

Over the past 20 years we have continued to optimize the alditol acetate procedure for GC–MS and more recently introduced GC–MS–MS analysis. Both triple quadrupole and ion GC–MS–MS instruments have been used successfully. Application of the newly developed automated derivatization instrument, developed at USC, has greatly simplified the existing method. A fully automated procedure is a realistic possibility. The procedure as currently performed allows both identification of bacteria (GC–MS) and their trace detection in complex matrices (GC–MS–MS). The alditol acetate procedure is a reliable method for identification of novel sugars in whole bacterial cells allowing chemotaxonomic differentiation or determination of physiological status. Trace detection in complex matrices of bacteria or their products is also readily achievable. There is great potential for the use of GC–MS–MS to assess the levels of bacterial contamination in clinical and environmental matrices. The automation and simplification of the alditol acetate procedure recently achieved could encourage widespread application of the method.

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