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Review

Recent progress in the analysis of sugar monomers from complex matrices using chromatography in conjunction with mass spectrometry or stand-alone tandem mass spectrometry

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Abstract

Analysis of trace levels of carbohydrate monomers in complex matrices requires excellent discrimination of the peaks of interest from background noise. Minimizing contaminating peaks introduced during sample preparation and chromatography is extremely important. However, the exquisite selectivity of the mass spectrometer is essential as a chromatographic detector in this regard. Traditionally gas chromatography–mass spectrometry (GC–MS) has been the method of choice for trace analysis of derivatized carbohydrates. Recent improvements in commercial tandem mass spectrometers (MS–MS) are encouraging the use of GC–MS–MS for improved specificity in trace analysis. There has also been an explosion in applications of electrospray ionization (ESI) for sensitive introduction of polar molecules (including sugars) into the mass spectrometer. This has encouraged ongoing developments in high-performance liquid chromatography–mass spectrometry (LC–MS) and MS–MS of underivatized carbohydrates. This has the potential to dramatically simplify sample preparation. However, as yet LC–MS and MS–MS do not match the sensitivity of GC–MS or GC–MS–MS. Developments in analysis of sugar monomers from complex matrices using chromatography (GC/LC) in conjunction with mass spectrometry (MS, MS–MS) or stand-alone MS–MS are discussed.

Contents

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1. Introduction

Much of our work has been concerned with developments in the alditol acetate procedure for analysis of carbohydrates involving gas chromatography (GC) and mass spectrometry (MS). Of particular concern has been the differentiation of bacterial species based on neutral and amino-sugar profiles of whole cell hydrolysates using GC [1] and GC–MS [2–4]. More recently, we have begun to address whether electrospray ionization (ESI) MS–MS and LC–MS analysis of underivatized sugars can provide complementary information to GC–MS [5]. These analyses would considerably reduce sample preparation and instrumental analysis time. Other work has involved trace detection of bacterial carbohydrates in more complex matrices including mammalian tissues and body fluids [6,7], and environmental samples (e.g. organic dust) [8,9]. This work has primarily involved GC–MS [6–8] and more recently GC–MS–MS [9]. The primary focus in these trace studies has been to decrease the limits of detection. Muramic acid (MA) is a sugar of particular interest since it is one of the few substances common to almost all eubacteria (as a component of peptidoglycan, PG) while not present in non-bacterial matter [9,10]. The current review summarizes earlier analytical developments, involving established GC and GC–MS in relationship to current research, employing rapidly advancing instrumentation, including MS–MS, LC–MS, and GC–MS–MS.

2. Selection of a chromatographic and/or mass spectrometric system

Considerations in choosing a system for carbohydrate analysis include the following. First, ease of sample preparation. Second, the difficulty in detecting the sugar of interest amongst other components of the mixture; this is particularly critical when the sugar is at a much lower concentration than other components present in complex mixtures.

The detection limit is affected by both sensitivity and selectivity. GC analysis of sugars is commonly performed with a universal detection (flame ionization detection, FID) [11–16]. Similarly LC analysis of carbohydrates often employs pulsed amperometric detection (PAD) [17–19]. Both FID and PAD are extremely sensitive. However, they lack the exquisite specificity of MS [2–4,6–8] or preferably tandem mass spectrometry (MS–MS) [9].

Carbohydrates require derivatization prior to GC analysis. GC and GC–MS [11–16] are well established as routine tools for carbohydrate analysis. Indeed, bench-top GC–MS instruments have been available since the early 1980s. In the 1990s user-friendly GC–MS–MS instruments have become available allowing detection limits to be lowered for trace analysis [9]. Derivatization (for GC, GC–MS and GC–MS–MS) is still time-consuming. Simplification and automation will be important in the more wide-spread use of these approaches.

Alternatively, LC with PAD requires no derivatization [17–19]. LC–MS and MS–MS when formed in conjunction with ESI also do not require derivatization. In both cases, direct spectral identification from aqueous solution is possible [5,20–22]. Technical advances allowing LC–MS and MS–MS analysis of native carbohydrates are showing considerable promise. However, it should be recognized that LC–MS and MS–MS profiling of carbohydrate monomers in complex matrices are still in their developmental stages. LC–MS and MS–MS analyses still exhibit poor sensitivity compared to GC–MS or GC–MS–MS, especially in complex samples [3–5,7–9].

3. Derivatization for GC, GC–MS and GC–MS–MS

Monosaccharide analysis by GC (with FID, MS, or MS–MS detection) requires derivatization to increase their volatility and decrease interaction with the analytical system. There are several well established derivatization methods. The most popular methods are the alditol acetate and trimethylsilyl procedures. The aldononitrile acetate and trifluoroacetate procedures have also

been commonly utilized. Methyloxime acetates are also occasionally employed. These methods are primarily concerned with neutral and amino-sugars. Acidic sugars require additional manipulations since carboxyl groups remain underivatized by standard methods. Excellent reviews have been published on trimethylsilyl, trifluoroacetyl, aldononitrile acetate and O-methyloxime acetate methods and these will only briefly be discussed here [11–16]. The alditol acetate procedure will be discussed in greater detail.

Aldoses and ketoses exist in aqueous solution in dynamic equilibrium between ring and straight chain forms. In the straight chain form, an aldehyde (or ketone) group is accessible; in the ring forms, this moiety is part of a hemiacetal ring (pyranose and/or furanose forms). Two anomers (α and β) can be formed on cyclization (for both pyranose and furanose forms) in which the newly formed hydroxyl group is above or below the plane of the ring. If the anomeric center is not destroyed, acylation of the aldose ring freezes the structure in the α or β anomeric form producing multiple peaks on GC analysis. Certain sugars exist only in pyranose or furanose forms and for other sugars both forms can occur. Thus either 2 or 4 multiple chromatographic peaks are produced (2 from pyranose and 2 from furanose anomers) on acylation. Since these anomers are usually resolved by capillary GC, complicated chromatograms often result. Multiple peaks may confound both qualitative identification and quantitative measurement [11–16].

For the aldononitrile acetate procedure, the aldose is reacted with hydroxylamine generating an oxime. During the subsequent acetylation, the oxime is converted by dehydration to a nitrile. Although the anomeric center is eliminated, an additional functional group generated during the derivatization (the nitrile group) can cause adsorption to GC columns. Contaminating peaks are also produced by reaction of hydroxylamine with the acylating agent (acetic anhydride) [12,23,24].

For the O-methyloxime acetate procedure, the anomeric center is destroyed by reaction with O-methyl hydroxylamine to generate O-methyloximes followed by acetylation. Unfortunately (on reaction of the aldehyde with O-methyloxime) a new isomeric center is generated and 2 products are produced per sugar (*syn*- and *anti*-isomers). Thus complicated chromatograms can still result [14].

Trifluoroacetyl and trimethylsilyl derivatives are usually prepared without destruction of the anomeric center. This allows simplified sample work-up. However, as noted above, multiple peaks in chromatograms are observed. Both derivatives are unstable in the presence of moisture; thus they decompose on storage [11,13]. Post-derivatization clean-up is also difficult since sugars decompose on exposure to water. Thus it is difficult to analyze sugars present in complex matrices because of excessive contaminating background peaks.

Alditol acetate derivatives are extremely stable and simple chromatograms are observed. Reduction (with sodium borohydride or borodeuteride) eliminates the anomeric center of aldoses or ketoses converting them to alditols. Elimination of the anomeric center greatly clarifies chromatograms as each alditol produces only one chromatographic peak. Reduction with borodeuteride is often preferred since the asymmetry of the molecule is retained by deuterium labeling. Following reduction and removal of the borate, the hydroxyl and amino moieties are heated in acetic anhydride. Carboxyl groups found in acidic sugars are not derivatized and thus these compounds do not produce chromatographic peaks. MA (unique to bacterial peptidoglycan, PG), however, forms a lactam (a cyclic amide) by internal dehydration between its carboxyl and amino groups and thus can be analyzed by the alditol acetate procedure [15,16].

A number of methodological changes which simplify the classical alditol acetate derivatization method [25,26] have been reviewed in depth elsewhere [15,16] and therefore only a brief description of the significant changes is presented here. The aim of each change has been simplification of manual sample handling, while at the same time allowing processing of large batches of samples. Descriptions and photographs of several custom-built devices which have significantly

simplified sample handling, including a vacuum manifold (for simultaneous evacuation of multiple samples) and an automated evaporator (for borate removal) are given elsewhere [15,16].

Other modifications include substantially improved chemical clean-up steps, both before and after derivatization. Each stage of the procedure [hydrolysis, pre-derivatization (hydrophobic) clean-up, reduction, acetylation, and post-derivatization (hydrophobic) clean-up] has been optimized to decrease background noise and allow analysis of large batches of samples. However, the current procedure still requires two and one-half days of sample preparation prior to GC analysis [15,16].

After reduction, it is generally necessary to remove the reducing agent, as remaining borate would inhibit the subsequent acylation reaction [25,26]. The use of certain acylation catalysts which are not inhibited by borate, such as 1-methylimidazole, eliminates the need to remove borate [23]. Such catalysts can greatly reduce the amount of time necessary for acylation (from hours to minutes). However, generation of extraneous peaks from side-reactions between both pyridine and 1-methylimidazole with acetic anhydride discourages their use in trace analysis [27,28]. More recently, it has been suggested that a mixture of N,N-dimethylaminopyridine and pyridine (used as a catalyst for acylation) when used at ambient temperature minimizes production of extraneous peaks [29]. However, others have found it necessary to use this reagent under more stringent conditions (80°C) for trace analysis of sugars in complex matrices [30]. The generation of side reaction products between pyridine and its analogs with acetic anhydride increases with extended temperature and heating time [27,28].

The use of sodium acetate as a catalyst essentially eliminates side reactions [27,28]. In this instance, borate is subsequently removed as tetramethylborate gas by multiple evaporations with methanol–acetic acid. Classically methanol–acetic acid is added and the sample evaporated to dryness; this is repeated 4 additional times. This is extremely time-consuming. A recently developed automated evaporator, mentioned above, replaces these tedious steps. Sets of 21 samples are held in a heated carousel with a constant flow of nitrogen delivered to each sample, simultaneously. The carousel rotates the samples past a stationary solvent reservoir containing methanol–acetic acid (200:1, v/v), which is dropwise added to each sample at a rate of ca. 5 ml/h. The time for a given sample to rotate all the way around the carousel is adequate for evaporation of one solvent drop before addition of the next [16].

The procedure also incorporates a number of clean-up steps. Hydrolysis in sulfuric acid is first performed to release sugars monomers. The acid is neutralized with an organic base (N,N-dioctylmethylamine in chloroform). Pre-derivatization clean-up is accomplished by passing the aqueous phase through a C_{18} column (Alltech, Deerfield, IL, USA), which removes hydrophobic substances (e.g. fatty acids). The C_{18} columns also serve to filter any remaining particulate matter. After reduction and acetylation, post-derivatization clean-up involves both acid and base extractions. Water is added to decompose acetic anhydride generating acetic acid. Chloroform is added and after shaking the aqueous acidic phase is discarded. Ammonium hydroxide is then added followed by passage through disposable magnesium sulfate columns (Chem Elut, Varian, Harbor City, CA, USA). The aqueous, alkaline phase is adsorbed by the column along with remaining hydrophilic compounds and the chloroform phase is collected. The combination of pre-derivatization (hydrophobic) and post-derivatization (hydrophilic) clean-up steps are highly selective. Neutral and aminosugars remain in the hydrophilic phase prior to derivatization, but in the hydrophobic phase after derivatization [15,16].

4. GC and GC–MS: established techniques for carbohydrate profiling

Conventionally, microbes are identified by morphological and metabolic characteristics. For example, the Gram stain and other microscopic tests can readily differentiate bacteria and fungi.

However, in recent years there has been considerable interest in applying chromatography and mass spectrometry for bacterial characterization based on chemical composition. Carbohydrate profiling of bacterial cells serves as a practical illustration of the power of GC and GC–MS.

Legionella species are environmental opportunists which cause legionellosis, a form of pneumonia (Legionnaires' disease), which can be life-threatening, and a "flu-like" illness (Pontiac fever) which is self-limiting after exposure to contaminated aerosols. Members of this family are ubiquitous inhabitants of freshwater and reside in natural as well as man-made sources. Disease results from the inhalation of contaminated water vapor (from sources as diverse as showers to fog over ponds). Classical isolation and identification of this family of organisms is problematic due to stringent nutritional requirements and slow growth rates. This group of organisms represents a prime example for the development and utilization of nonculture-based detection methods.

We first differentiated *Legionellae* by analyzing their carbohydrate content, using gas chromatography with FID detection [1]. Increased sensitivity and selectivity for carbohydrate detection was achieved using selected reaction monitoring (SRM) GC–MS. Analysis of whole bacterial cells by GC–MS noted the almost universal presence of unusual sugars (aminodideoxyhexoses). Two of the uncommon sugars we previously discovered in the *Legionellae* (X1 and X2) [1,2] have subsequently been identified as quinovosamine and fucosamine, respectively [3]. Total cellular carbohydrates can distinguish strains of *L. pneumophila* which always contain the aminodideoxyhexose quinovosamine, from *T. micdadei* which contains the isomer fucosamine [3].

Bacillus anthracis is a respiratory pathogen associated with animal products (especially hides), *B. cereus* causes food poisoning, and *B. thuringiensis* is a "natural" pesticide, which can be released into the environment. Like other bacilli, these are soil organisms and can be transmitted by the airborne route. These 3 species are phenotypically and genotypically extremely closely related. *B. subtilis* (a nonpathogenic soil contaminant) is more distantly related. The carbohydrate profiles of each of the four species are quite distinct between vegetative and spore form. However, *B. anthracis* was readily distinguished from *B. cereus* and *B. thuringiensis* by carbohydrate profiles [4,31]. Spores of many strains of all 4 species contained characteristic unusual carbohydrates including O-methylated sugars uncommon in nature (2- and 3-O-methyl rhamnose). The rare sugar quinovose (2-deoxyglucose) is characteristic of *B. subtilis*.

5. Electrospray LC–MS and MS–MS: developing technologies for carbohydrate profiling

When the carbohydrate is present in high concentration, LC (with PAD) and GC (with FID) both allow ready detection of the sugar of interest. LC, unlike GC, does not require derivatization which offers considerable advantage by reducing sample handling and chemical manipulation. Analysis of carbohydrates by LC has made great progress in the past five years. For example, a dedicated LC carbohydrate system is marketed by Dionex (Sunnyvale, CA, USA) and employs anion-exchange chromatography with PAD. This LC system has been successfully used for the separation and identification of amino, neutral, and acidic sugars present in hydrolysates of purified bacterial macromolecules [19]. Chromatography is performed in concentrated NaOH. At this high pH, interaction of partially ionized hydroxyl groups with the anion-exchange resin produces excellent sugar separations. The electrochemical detector also detects carbohydrates with excellent sensitivity at alkaline pH. Accordingly, all LC parts that come in contact with solvent must be resistant to alkali. Development of an LC–MS procedure (for the more exact task of analysis of profiling of sugars in bacterial whole cell hydrolysates) remains to be performed.

Attempts to combine on-line anion-exchange liquid chromatography–mass spectrometry (LC–

MS) employing thermospray ionization [20] or ESI [21] require an ion suppressor to be interfaced between the LC and MS. Sodium ions interfere with ionization efficiency and strong alkali is incompatible with current MS ion sources. The ion suppressor exchanges hydrogen for sodium ions, replacing sodium hydroxide with water. Unfortunately aminosugars do not pass through the suppressor [21]. Neutral sugars are generally detected on MS analysis as adducts; this is achieved by post-column addition of an appropriate reagent. After complexing with NH_4^+ or Li^+ (derived from ammonium or lithium acetate), sugars can be detected in the positive ion mode [21] or after complexing with chlorine (derived from chloroform) in the negative ion mode [32]. Certain alternative ion exchange columns can be used at neutral pH (eliminating the need for an ion suppressor) making them more compatible with MS detection, but chromatographic resolution can be compromised [32,33].

In those instances where the separation of sugar isomers is not vital, chromatography may be replaced by MS–MS. Selective identification is accomplished by using the first MS as an ion filter to selectively pass compounds to the second MS for mass spectral identification. This creates a greatly simplified analytical system and allows essentially instantaneous instrumental analyses. Additionally, suppression of ionization by substances generated from the LC separation system is avoided. However, suppression by components of the sample matrix can still adversely affect sensitivity [5]. Furthermore, carbohydrates exist in multiple isomeric forms, which can not be readily differentiated by MS–MS, and require chromatographic resolution.

Certain carbohydrate markers are present at high concentration in bacterial cells. Thus, ESI-MS–MS can be used for the analysis of underivatized carbohydrates in whole cell hydrolysates [5]. Adapted from the alditol acetate procedure, MA was released from whole bacterial cells by hydrolysis in 1 *M* sulfuric acid followed by neutralization with N,N-dioctylmethylamine in chloroform followed by hydrophobic clean-up (by passage through a C_{18} column). The subsequent derivatization steps of the alditol acetate method were eliminated. Sample processing time was reduced to ca. 4 h (for MS–MS) compared to two and one-half days (for GC–MS). Additionally, the elimination of chromatography reduces instrumental analysis from 30–60 min to 1–2 min per sample. Samples were introduced into the MS–MS using a Harvard syringe pump with a 50% acetonitrile–water mobile phase. Therefore, batches of multiple samples can be accomplished in a few hours rather than days. MA contains an amino and carboxyl group and is readily detected by ESI-MS without the need for adduct formation. In the positive ion mode the M + 1 ion (NH_3^+ form) predominates [5], whilst in the negative ion mode the M − 1 (CO_2^- form) is observed (unpublished).

Analysis of MA in whole cell hydrolysates by ESI-MS–MS with NI detection is given here as an illustration of the potential for acidic sugar analysis (Fig. 1). The samples were prepared in a similar fashion as described earlier for positive ion analysis [5]. The molecular ion for MA (*m/z* 250 [$(M - H)^-$]) generates a characteristic lactic acid fragment (*m/z* 90). This is consistent with earlier work demonstrating the ease of loss of lactic acid from MA [34,35]. The ESI-MS spectrum of the bacterial hydrolysate is considerably more complicated than the standard (Fig. 1A and C, respectively), whereas product spectra of the bacterial hydrolysate and standard are very similar (Fig. 1B and D) allowing rapid visual identification of MA. ESI-MS–MS has considerable potential for sugar profiling of whole cell hydrolysates.

6. Trace analysis of microbial products by GC–MS and GC–MS–MS

Sugar markers for microbes have been detected at trace levels in diverse samples (from animal and human body fluids and tissues to airborne dust). Currently, GC–MS sensitivity for trace detection is superior by several orders of magnitude over ESI-MS–MS. For example, in GC–MS studies MA has been detected in serum

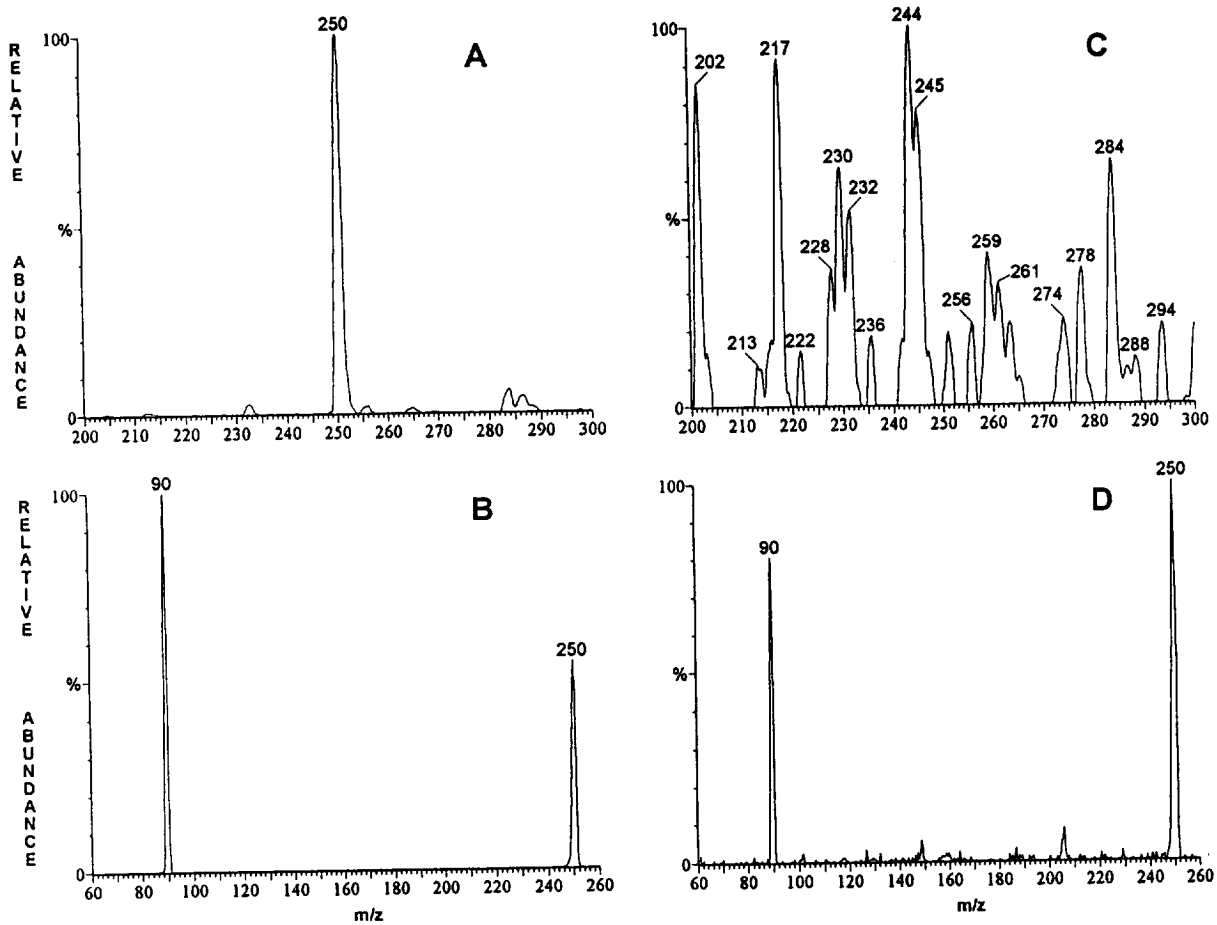

Fig. 1. Analysis of muramic acid after ionization by electrospray followed by separation by MS–MS and negative ion detection: (A) an MS spectrum of a standard; (B) a product spectrum (MS–MS) of a standard; (C) an MS spectrum of a hydrolysate of *Staphylococcus aureus*; (D) an MS–MS spectrum of a hydrolysate of *S. aureus*. Note the similarity between the product ion spectra of the standard and the bacterial hydrolysate.

from animals injected with the cell wall building block, muramyl dipeptide, at concentrations of 100 ng/ml (1 part in 10 million) [7]. MA (as a component of naturally present bacteria) was detected at levels of 4 ng/mg (1 part in 250 000) in house dust using GC–MS [8] and GC–MS–MS [9].

Sensitive and selective detection of trace bacterial carbohydrates (or other compounds) in complex matrices has almost exclusively employed GC–MS until recently. Bacterial markers (such as MA) are present at very low abundances. Ions contributed by the marker sugar must be distinguished from considerable background noise. Replacing the mass spectrometer with a tandem spectrometer allows dramatic improvements in specificity with improved detection in complex matrices (GC–MS–MS) [9]. As far as we are aware, there have been no previous published reports on the use of GC–MS–MS for trace detection of bacterial sugar markers in complex matrices.

If either sensitivity or specificity is adversely affected, then the limit of detection is affected. Whether sensitivity or specificity are more important depends on the particular analytical problem. In this study, GC–MS was clearly much more sensitive than GC–MS–MS (approxi-

mately two orders of magnitude greater signal). In multiple reaction monitoring (MRM) 1 parent ion is selected (from ions generated in the first MS) for production of a secondary ion to be analyzed in MS2. There are further losses in transmission of ions from MS1 to MS2. However, GC–MS–MS provides much greater specificity. Dust samples, so far analyzed, contain at the low end of the range around 5 ng of MA/mg of dust to 30–50 ng/mg at the high end. Using GC–MS it was impossible to reliably differentiate dust chromatograms that contained low concentrations of MA from negative controls (for plants and fungi). GC–MS–MS chromatograms of dust were always readily differentiated from controls. Thus for this type of analysis MRM is preferable to SRM. This suggests, in this instance, that specificity and not sensitivity is the limiting factor in determining the detection limit. However, these analyses were clearly performed at the current limits of sensitivity for MRM.

As an illustration of the power of GC–MS–MS, Fig. 2 compares SRM and MRM for trace detection of derivatized bacterial MA in organic dust. A fungal sample, *Aspergillus fumigatus*, known as not to contain MA [9], was used as a negative control. Although in SRM there are

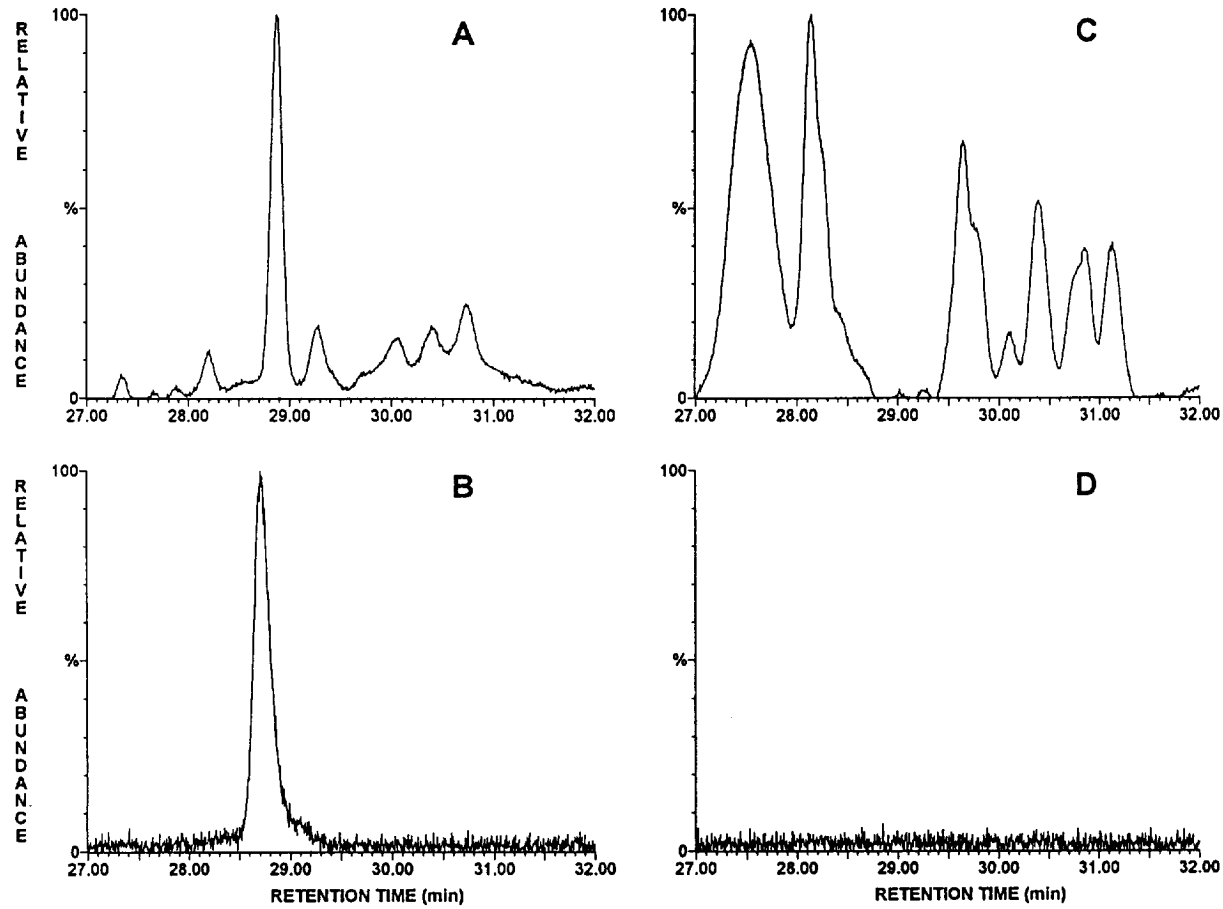

Fig. 2. Analysis of muramic acid by GC–MS using selected reaction monitoring (SRM) and GC–MS–MS using multiple reaction monitoring (MRM): (A) SRM of kitchen dust; (B) MRM of kitchen dust; (C) SRM of a fungus, *Aspergillus fumigatus*; (D) MRM of *A. fumigatus*. Note the clear visual identification of muramic acid based on MRM from the house dust and absence from the fungal sample, compared to more complicated SRM tracings.

multiple peaks which make detection of MA difficult, MRM clearly identifies MA from organic dust with no signal in the fungal sample.

In order to further decrease the detection limit (i.e. to decrease the amount of sample [below 10 mg currently analyzed] or to analyze concentrations at the pg/mg level) it will be necessary to maintain the current specificity whilst further increasing sensitivity. This might involve the use of detection using negative ion chemical ionization (NICI) [36–38] or ion trap MS–MS instruments [30]. However, MA was readily detected in dust at trace levels by GC–MS–MS (with electron impact ionization) using a triple quadrupole instrument. There is great potential for GC–MS–MS in detection of other chemical markers with environmental, medical and biotechnology applications.

7. Conclusions

In terms of simplicity, LC with PAD offers advantages over GC with FID detection for analysis of purified polymers (such as glycoproteins and polysaccharides). However, for analyses of carbohydrates present at low levels in complex matrices, GC–MS offers vastly superior selectivity and much lower detection limits. Preparation of alditol acetates for GC–MS analysis currently involves a lengthy derivatization procedure, although full automation is feasible. LC–MS and MS–MS for analysis of native sugars in complex matrices, although in the developmental stage, show promise for greatly simplified sample preparation. There is considerable potential in terms of specificity (e.g. GC–MS–MS) and sensitivity (e.g. ion trap instrumentation) for improving the limits of detection as applied to trace analysis. Thus, it appears that there are still numerous paths to be explored in the development and application of analytical methods for identification and trace detection of carbohydrates.

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