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Analysis of a stable halogenated derivative of muramic acid by gas chromatography–negative ion chemical ionization tandem mass spectrometry

Michael P. Kozar, Alvin Fox*

Department of Microbiology and Immunology, University of South Carolina, School of Medicine, Columbia, SC 29208, USA

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Abstract

Muramic acid (Mur) is present in the cell wall of *Eubacteria* and serves as a chemical marker for the trace detection of bacteria and bacterial cell wall debris in complex matrices. There have been numerous studies using a variety of derivatives of Mur, particularly in combination with gas chromatography–tandem mass spectrometry (GC–MS–MS) where the detection limit has been steadily lowered. A stable, halogenated derivative, the pentafluorobenzyl oxime (PFBO) acetate of Mur, has been developed by others and successfully used for GC with electron-capture detection. The current report is the first use of this derivative for GC–MS–MS analysis of Mur, or indeed any other carbohydrate, using negative ion chemical ionization (NICI) with GC–MS–MS. Mur was readily detected in settled surface dust (166 ng/mg), as well as dust collected from indoor air (1.4–5.9 ng/mg). Analyses of Mur as a PFBO acetate by GC–NICI-MS–MS or as alditol acetates by electron impact GC–electron impact ionization MS–MS serve as complementary approaches for trace detection in complex matrices.

Keywords: Dust; Muramic acid; Carbohydrates; Lactylglucosamines; Amino sugars

1. Introduction

Muramic acid (Mur), 3-*O*-lactylglucosamine, is an amino sugar present in the cell wall peptidoglycan (PG) of bacteria. It is found in all *Eubacteria*, except *Mycoplasma* and *Chlamydia* [1] and not elsewhere in nature. Thus, Mur has been widely used as a chemical marker for trace detection of bacteria and bacterial cell wall remnants in complex biological matrices. Mur has been assayed at minute concentrations in both environmental [2,3] and clinical samples [4,5].

There have been numerous methodologies employed in the analysis of Mur including spectrophotometry [6,7], high-performance liquid chromatography (LC) where Mur is detected by fluorescence as a dansyl derivative [8], or without derivatization using pulsed amperometric detection (PAD) [9]. LC–mass spectrometry techniques have also been employed [10]. However, none have been able to match the combined sensitivity and specificity of capillary gas chromatography–tandem mass spectrometry (GC–MS–MS) of derivatized Mur [3,5,11].

*Corresponding author. Tel.: +1-803-733-3288; fax: +1-803-733-3192.

E-mail address: afox@med.sc.edu (A. Fox).

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GC, GC–MS and GC–MS–MS analysis have included the use of non-halogenated derivatives (including alditol acetate [12], aldononitrile acetate [13], *O*-methyloxime acetate [14], methyl ester *O*-methyl acetate [11] and trimethylsilyl [15]) and halogenated derivatives (including trifluoroacetyl [16], pentafluoropropyl [17], and isobutyl heptafluorobutyl [18]). Unlike acetates, all other derivatives, whether halogenated or not, have been found to be unstable in the presence of moisture. Analyses of many environmental and clinical samples require post-derivatization clean-up (through extractions with an aqueous phase) to minimize interfering background, which adversely affects the detection limit.

In most instances, GC–MS or GC–MS–MS analysis of Mur has employed electron impact (EI) ionization. Potentially increased sensitivity, after GC separation, can be achieved by the use of electron-capture detection (ECD) or negative ion chemical ionization (NICI) MS or MS–MS analysis. As noted above, classical halogenated derivatives are unstable in the presence of moisture. It is only within the last few years that the first water-stable, halogenated derivative of Mur and other sugars was developed [19–22]. This derivative is unusual in that the halogenated group is attached to the C_1 aldehyde by reaction with *O*-pentafluorobenzyl hydroxylamine to form the pentafluorobenzyl oxime (PFBO) of Mur, which is then acetylated.

Indeed, in Chiesa et al.'s novel work, increased sensitivity in GC analysis with an ECD was demonstrated using the PFBO acetate derivative [21]. These workers speculated that GC–NICI-MS might allow even lower detection limits because of greatly increased specificity. In the current work, the utility of the PFBO acetate derivative of Mur for GC–MS–MS analysis was explored.

2. Experimental

2.1. Samples

Settled dust was collected from surfaces in an open, unheated, turn-of-the-century wooden barn located on a 70-acre farm 5 miles from Barnwell, SC, USA. The dust was freeze dried and homogenized before storage and kept frozen at −20 °C prior to analysis. Airborne dust was collected in a university laboratory for 115 h using 37-mm preweighed PTFE filters, 1–2 μm in pore size (SKC, Eight Four, PA, USA) at an airflow rate of 51 l/min. Commercially available polystyrene filter holders and electrically driven pumps were used.

2.2. Sample preparation

Aqueous standards of Mur (Sigma–Aldrich, St Louis, MO, USA) were prepared in advance. [^{13}C]Mur was prepared by hydrolyzing 40 mg of ^{13}C-labeled cyanobacteria (Isotec, Miamisburg, OH, USA). The bacteria were 0.4% Mur on a dry mass basis. A 285-ng amount of [^{13}C]Mur was added to each sample as an internal standard. Additionally, 50 μg of glucose was added as a carrier. External standards consisted of a known amount of Mur and constant amounts (285 ng) of ^{13}C-labeled Mur. Blanks consisted of water spiked with ^{13}C-labeled Mur.

Samples were first hydrolyzed to release Mur from PG by the addition of 1 ml of 1 *M* sulfuric acid (Fisher Scientific, Atlanta, GA, USA) to 20 mg of dust or half of a PTFE filter for 3 h at 100 °C and internal standard added. Samples were then neutralized by mixing with 2 ml of *N*,*N*-dioctylmethylamine (Fluka, Buchs, Switzerland) in chloroform (Fisher Scientific) (50:50, v/v), and centrifuged. The aqueous phase was removed and passed through C_{18} columns (J&W Scientific, Folsom, CA, USA) to remove hydrophobic contaminants. The aqueous samples were then dried in a water bath at 60 °C, under a stream of N_2, with the constant addition of methanol to enhance evaporation. Once dry, an oxime was obtained by reaction with 15 mg of *O*-pentafluorobenzyl hydroxylamine (Fluka) in 0.2 ml of pyridine (Alltech, Deerfield, IL, USA) at 80 °C for 20 min. Samples were cooled to room temperature, and then 1.0 ml of acetone was added to each. Acetone reacts with excess derivatizing agent to generate a volatile oxime [21]. After 15 min at room temperature, samples were dried a second time under a stream of N_2 at 60 °C with the constant addition of methanol. Following this step, samples were dried under vacuum for 3 h. The oximes were acetylated with the addition of 0.3 ml of acetic anhydride

(Alltech) or [^{2}H]acetic anhydride (Fisher Scientific) and 5 mg of sodium acetate (J.T. Baker, Phillipsburg, NJ, USA) added as a catalyst at 100 °C overnight (16 h). The stability of the PFBO acetate derivative allowed the use of both acidic and alkaline extractions to remove polar contaminants. Acetic anhydride was decomposed with 0.75 ml of water and shaken for 1 h at room temperature. A 1-ml volume of chloroform was added and after mixing the aqueous phase discarded. A 0.8-ml volume of ammonium hydroxide (Fisher Scientific) in water (80:20, v/v) was added, and the organic phase was removed, evaporated to dryness under a stream of N_2, and reconstituted in 25–30 μl of chloroform prior to analysis.

2.3. Instrumentation

Samples were analyzed on a VG Quattro 1 triple quadrupole tandem mass spectrometer (Micromass, Boston, MA, USA) coupled to a Fisons 8000 GC system equipped with an automated sample injector (A200s) and a non-polar, DB-5ms fused-silica capillary column 30 m×0.25 mm I.D.×0.25 μm film thickness (J&W Scientific).

2.4. Electron impact ionization conditions

The injection port of the gas chromatograph was maintained at 250 °C while the source was maintained at 225 °C. Initial oven temperature was 160 °C for 1 min with the split valve closed, then a ramp of 20 °C/min to 270 °C and hold for 1 min with split open. Helium was used as a carrier gas with a constant gas velocity of 40 cm/s. EI ionization was performed with a standard five-coil filament with electron energy of 70 eV and emission current at 200 μA.

2.5. Positive ion chemical ionization (PICI) conditions

GC conditions remained the same. The standard five-coil filament was replaced with a ribbon filament (Scientific Instrument Services, Ringoes, NJ, USA) at 70 eV and emission current at 200 μA with a source temperature of 130 °C. Anhydrous ammonia of 99.999% purity was used as the reagent gas with a source pressure of 2.3×10^{-4} mbar.

2.6. Negative ion chemical ionization (NICI) conditions

GC conditions remained the same. The standard five-coil filament was used in the source with 35 eV electron energy and 275 μA electron current. The source was maintained at 100 °C. The amount of reagent gas introduced into the source was determined experimentally for optimum ionization and the source pressure was maintained at 2.3×10^{-4} mbar. Collision-induced dissociation (CID) of precursor ion m/z 415 for Mur and m/z 424 for ^{13}C-labeled Mur was performed at a collision energy of 11.0 V using argon as the collision gas with a gas cell pressure of 3.8×10^{-4} mbar. Product ion masses monitored were m/z 124 for Mur and m/z 127 for ^{13}C-labeled Mur. Quantitation was based upon the peak area ratio of Mur to the internal standard (^{13}C-labeled Mur) in the sample compared with the peak area ratio in the external standard mixture (containing a known amount of Mur and ^{13}C-labeled Mur).

3. Results

Mur was converted to the PFBO acetate derivative as described by Biondi and coworkers [20,21]. In initial experiments 4-*N*,*N*-dimethylaminopyridine (DMAP) was used as the catalyst for acylation (80 °C, 15 min). As previously noted for pyridine [23] and methylimidazole [24], DMAP formed an oily brown side-reaction product with acetic anhydride which was not entirely removed by post-derivatization clean-up. When using sodium acetate, it has been previously noted [12,25] that an increased acetylation time is needed (16 h and temperature 100 °C), but following post-derivatization clean-up, samples are essentially colorless. Thus, sodium acetate was selected for use as a catalyst in the current study. Using DMAP or sodium acetate as catalyst under rigorous acetylation conditions, one major and one minor chromatographic peak were observed for

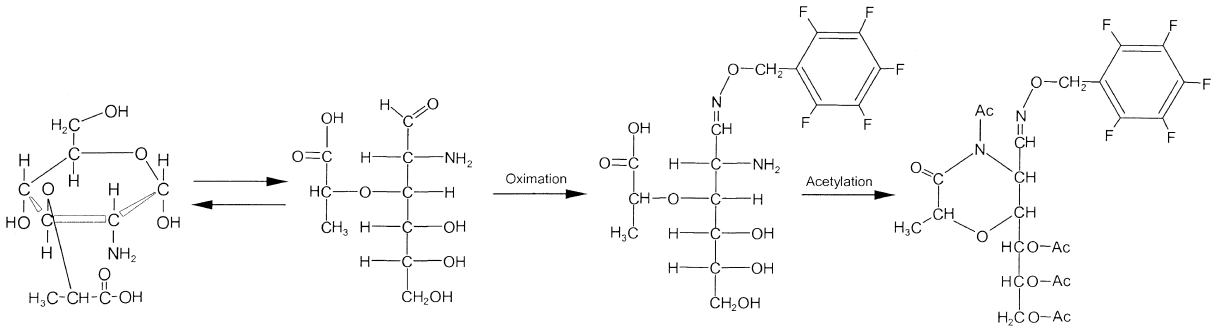

Fig. 1. Scheme for formation of the PFBO acetate derivative of Mur.

the PFBO acetate of Mur (syn and anti isomers). Conversion of Mur to the PFBO acetate derivative is shown in Fig. 1.

The PFBO acetate derivative of Mur was analyzed by GC–EI-MS, GC–PICI-MS and GC–NICI-MS. Total ion chromatograms with the corresponding spectra are shown in Fig. 2. Both peaks gave the same fragment masses in similar relative abundances. The spectra show the same characteristic fragments as previously observed [20]. The base peak at m/z 181 is attributed to the loss of the $C_7H_2F_5$ head group [22]. The molecular ion is observed at m/z 596 while the m/z 554 fragment results from the loss of a ketene (M-42) (Fig. 2A). Using ammonia as the reagent gas in PICI, the PFBO acetate derivative of Mur displayed a large molecular ion at m/z 614 (M+18) (Fig. 2B).

In NICI with ammonia as the reagent gas, the mass spectrum of the PFBO acetate derivative of Mur exhibited excessive fragmentation [26]. A relatively small abundance of the molecular ion, m/z 596, was observed. Base peaks were m/z 178 $[C_7F_4H_2O]^-$ and m/z 196 $[C_7F_5HO]^-$; these are also the two most abundant fragments in methane NICI of PFBO derivatives of prostaglandins [26]. The two major high mass ions were m/z 521 and m/z 415. No dominant fragments were observed in the product ion spectra of m/z 521. Thus, m/z 415 (M-181 $[C_7F_5H_2]^-$) was selected for MRM (multiple reaction monitoring) experiments. Pentafluorobenzyl derivatives of prostaglandins and steroids have been observed previously to undergo dissociative electron-capture in the gas phase to generate negative ions through the loss of a pentafluorobenzyl radical [27].

The product ion spectrum of m/z 415 is shown in Fig. 3. The base peak is observed at m/z 124. Thus the transition 415→124 was chosen for MRM experiments. Parent ion scans revealed m/z 415, 355, and 295 as sources for m/z 124; m/z 355 and 295 result from successive losses of 60 (acetic acid) from m/z 415.

The PFBO acetate derivatives of unlabeled Mur and ^{13}C-labeled Mur, differ by nine mass units (M_r 596 and 605, respectively). In the mass spectrum of unlabeled Mur, the precursor ion m/z 415 corresponds to m/z 424 for the ^{13}C-labeled analog (Table 1) indicating that the nine Cs from the sugar backbone and lactyl side-chain remain. The corresponding product ions are m/z 124 and 128, respectively, indicating four of these nine Cs are present (Table 1). The molecular ion of the PFBO acetate of Mur (m/z 596), when prepared with [^{2}H]acetic anhydride, results in a 12 mass unit shift (m/z 608, the addition of four acetate groups). Thus the precursor ion (m/z 415, 596-181) must also contain four acetate groups. m/z 415 corresponds to m/z 427 in ^{2}H-acetylated Mur confirming the presence of four acetate groups (Table 1). The product ion, m/z 124 corresponds to m/z 127 indicating that only one of the acetate groups remain. This product ion, $C_6H_6NO_2$ or $C_6H_8N_2O$, has at least two possible structures since either a N or C remains acetylated (Fig. 4).

Using the transitions 415→124 for Mur and 424→128 for ^{13}C-labeled Mur (internal standard) in MRM experiments, a linearity study was performed. Levels were analyzed in duplicate at 0, 2.5, 5, 10, 50, 100, 500, 1000, 2500, and 5000 ng. The entire curve is shown in Fig. 5. Even at the low end of the curve

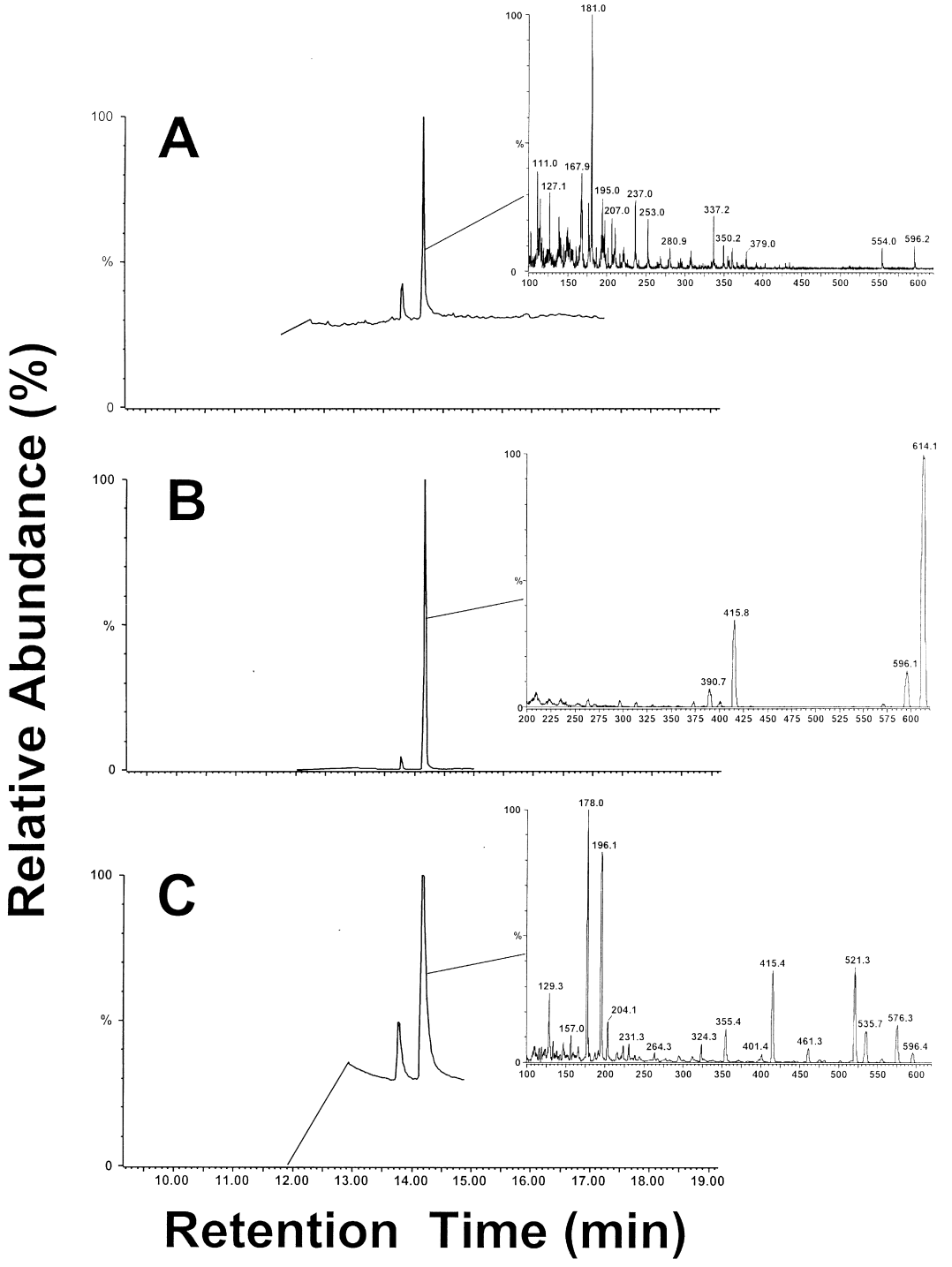

Fig. 2. Total ion MS chromatograms of the PFBO acetate derivative of Mur: (A) EI, (B) PICI, and (C) NICI. The mass spectra of both peaks were nearly identical and represent the syn and anti isomers of the oxime.

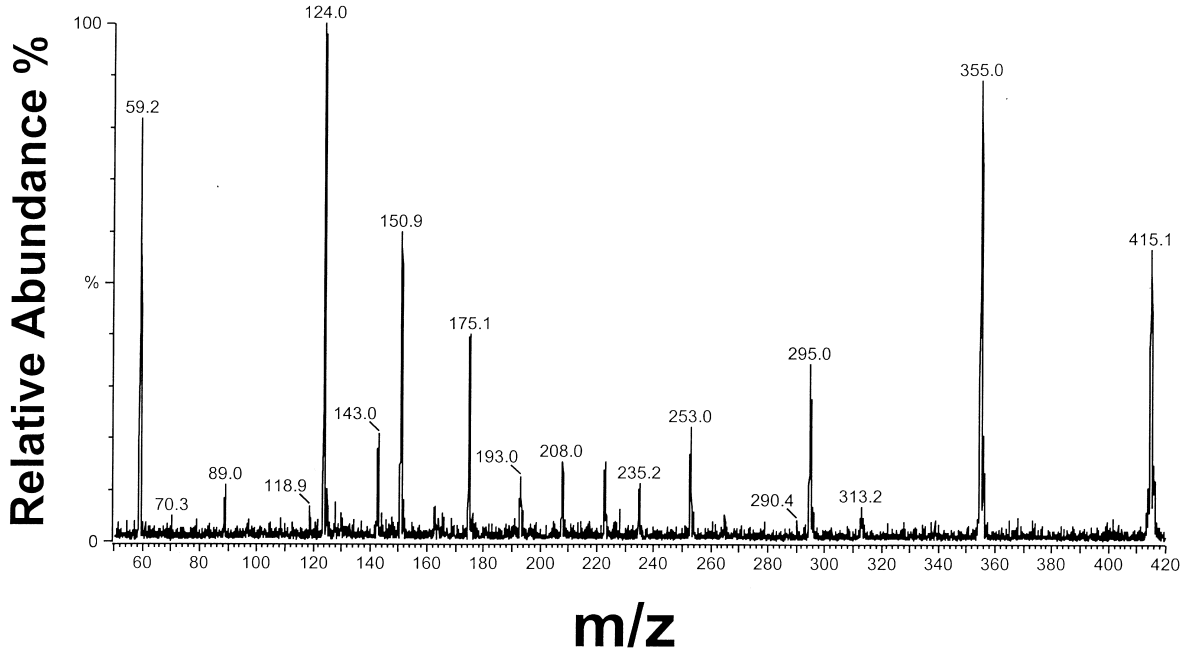

Fig. 3. NICI product ion spectra of m/z 415.

Table 1
Observed m/z values for precursor and product ions of PFBO acetate derivative of Mur

Reagents	Molecular ion	Precursor ion	Product ion
Acetic anhydride/muramic acid	596	415	124
Acetic anhydride/[^{13}C]muramic acid	605	424	128
[^{2}H]Acetic anhydride/muramic acid	608	427	127

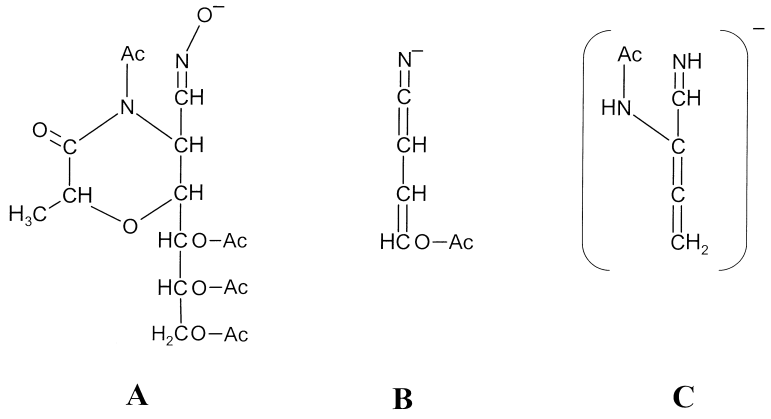

Fig. 4. Proposed structures for the parent ion m/z 415 (A) and two possible structures for the product ion, m/z 124 (B) $C_6H_6NO_2$ or (C) $C_6H_8N_2O$. Ac, acetate.

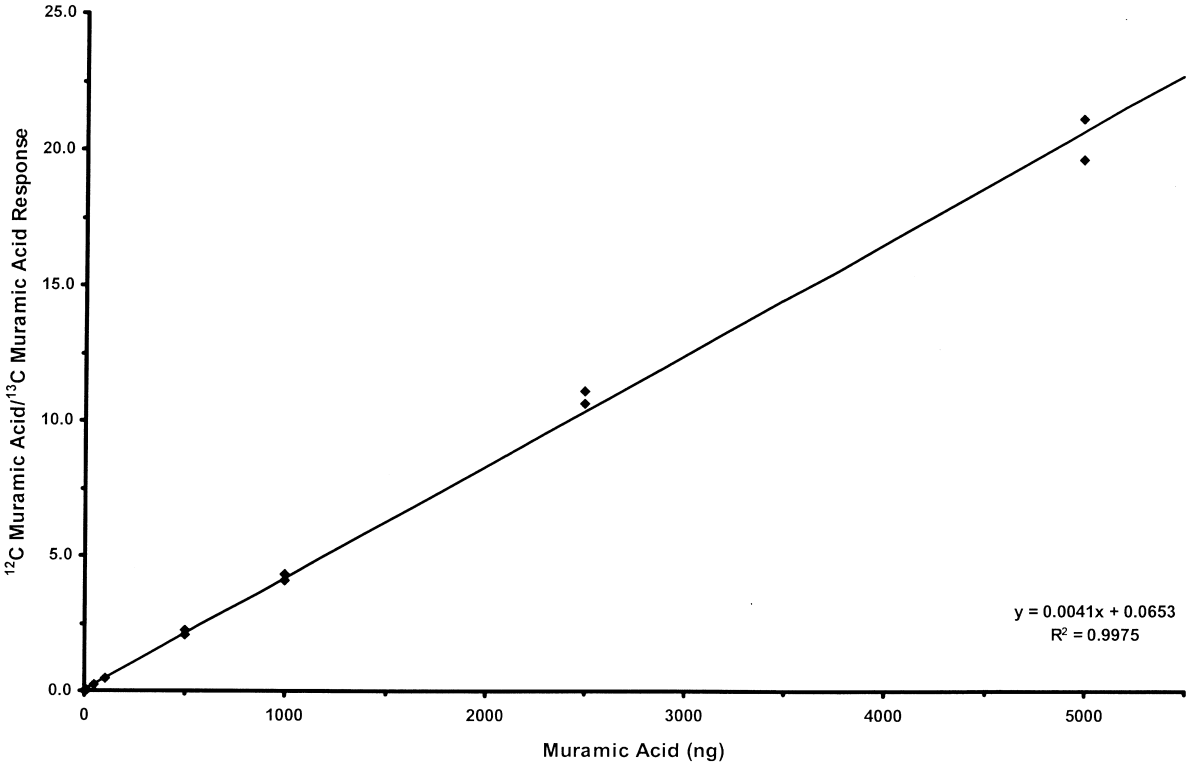

Fig. 5. Standard curve, GC–NICI-MS–MS for Mur as its PFBO acetate derivative demonstrating linearity. Levels were performed in duplicate and included 0, 2.5, 5, 10, 50, 100, 500, 1000, 2500 and 5000 ng.

(from 0 to 50) the r^2-value was 0.9972 indicating excellent linearity. At levels less than 2.5 ng it became increasingly difficult to identify the signal from background indicating that the limit of detection for the PFBO acetate derivative of Mur is in the same range as the alditol acetate derivative analyzed by GC–MS–MS [3,5].

The levels of Mur in settled dust and dust collected from indoor air were analyzed as the PFBO acetate derivative by GC–NICI-MS–MS. Mur was present at levels of 166 ng/mg settled dust. The levels of Mur in airborne dust ranged from 1.4 to 5.9 ng/mg. This is illustrated in Fig. 6. In each case, the top panel shows the MRM chromatogram for the internal standard, ^{13}C-labeled Mur (295 ng). The bottom panel shows the chromatogram for Mur naturally present in dust. For comparison, a 50-ng standard of Mur is also shown.

4. Discussion

Biondi et al. developed the first stable halogenated derivative of carbohydrates (PBFO acetates) suitable for GC–MS or GC–MS–MS analysis. Initially, derivatization conditions for neutral sugars were described [22]. Subsequently, the more difficult derivatization of amino sugars was accomplished [19] and finally Mur was successfully derivatized [20]. Mur (which has both amino and carboxyl moieties) requires harsh conditions for successful acetylation due to the necessity for formation of a lactam ring [24]. The latest study using PFBO acetate derivatives described the use of acetone to eliminate excess halogenated reagent as a volatile oxime allowing the use of GC with ECD [21]. In this latter report, the authors speculated that improved sensitivity/specificity might be achieved by GC–

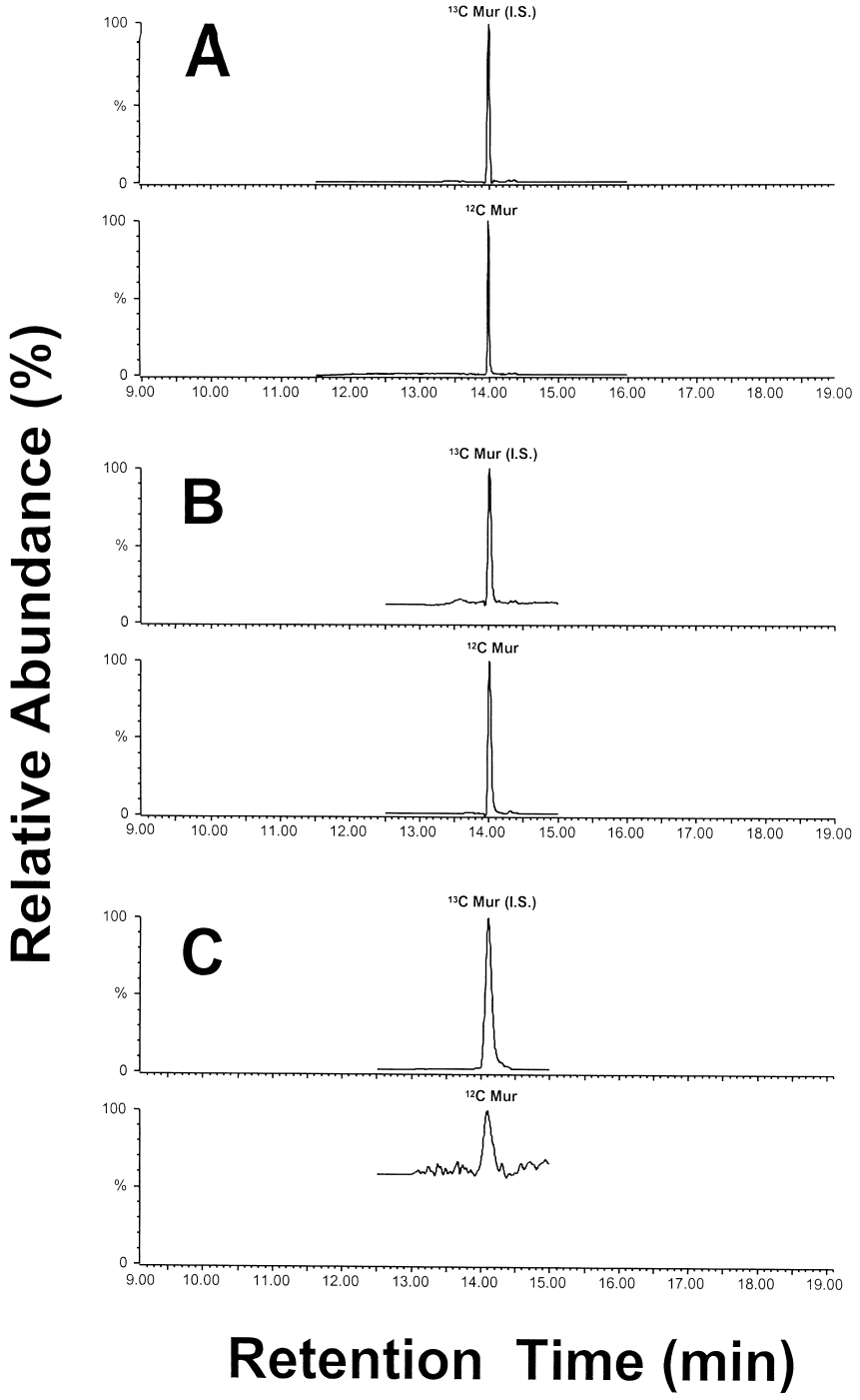

Fig. 6. GC–NICI-MS–MS of Mur. (A) 50 ng standard, (B) hydrolysate of settled dust, and (C) hydrolysate of airborne dust collected on a PTFE filter. In each chromatogram, the upper window depicts monitoring of ^{13}C-labeled Mur, the internal standard. The lower windows depict monitoring for natural [^{12}C]Mur.

NICI-MS detection. The current report is the first use of the stable, PFBO acetate derivative of Mur, or indeed any other carbohydrate, for trace analysis using GC–NICI-MS or GC–MS–MS.

Using GC–NICI-MS–MS, Mur was readily detected in settled house dust and airborne dust. Sensitivity was approximately 2.5 ng total in mg quantities of dust (i.e. 150 pg on-column). This level of sensitivity was achieved previously, in the EI mode, when analyzing Mur as an alditol acetate [3,5] or methyl ester *O*-methyl acetate [11] also using GC–MS–MS.

As noted above, GC–NICI-MS–MS might be anticipated to provide considerably higher sensitivity than GC–EI-MS–MS. However, this was not found to be the case. The low sensitivity is unlikely to be because of low yield of the pentafluorobenzyl oxime acetate derivative. This derivatization consists of two steps. The conditions for oximation were as described by Biondi et al., who extensively optimized this reaction [20]. Acetylation employed the exhaustive conditions, as used for alditol acetates [12]. The source temperature can also dramatically affect sensitivity in GC–NICI-MS–MS analysis. A variety of source conditions were used. However, there was no substantial improvement in sensitivity. Thus non-optimal source temperature is also unlikely to provide an explanation.

In the NICI mode, the mass spectrum of the PFBO acetate derivative of Mur exhibited excessive fragmentation. A relatively small abundance of the molecular ion m/z 596 was observed. Base peaks were m/z 178 $[C_7F_4H_2O]^-$ and m/z 196 $[C_7F_5HO]^-$; these are also the two most abundant ions in GC–NICI-MS of various PFB derivatives of prostaglandins [26]. In this study, a PFB-containing moiety was placed on carbonyl and aldehyde groups resulting in formation of an ester or oxime, respectively. Mass spectra of the PFB oxime exhibited excessive fragmentation. However, mass spectra of the PFB ester exhibited minimal fragmentation and the molecular ion was M-PFB$^-$. The difference in sensitivity for the PFB ester was two orders of magnitude greater than PFB oxime (800 vs. 2 pg on column). The sensitivity observed in NICI mode for the PFB oxime was comparable in the EI and NICI modes. These results are entirely consistent with our observations.

In conclusion, GC–NICI-MS–MS analysis employing the PFBO acetate derivative is comparable in sensitivity to GC–MS–MS in the EI mode. Thus, the method provides a useful means of confirming the presence of Mur in a complex sample, especially at the detection limit. Two different derivatives (e.g. alditol acetate and PFBO acetate) can be analyzed in two different ionization modes (EI and NICI, respectively) providing confirmatory information. However, due to excessive fragmentation, the potential sensitivity in NICI mode for the PFBO acetate derivative could not be exploited. Future experiments might involve formation of a PFB ester of Mur.

Acknowledgements

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