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Abstract

A derivatization protocol based on the acylation of pinacolyl alcohol (PA), an important marker for the nerve agent Soman, is presented. The procedure provides a convenient means of detecting, by Gas Chromatography-Mass Spectrometry (GC-MS), PA when present at a low concentration in a complex glycerol/alcohol-rich matrix. While there are only two reports describing the specific analysis of PA in matrices at low concentrations, the protocol described herein represents the first of its kind in the analysis of PA in a highly-reactive matrix. Two alternative paths for the protocol's execution are presented. The first involves the direct derivatization of the PA with either acetyl or benzoyl chloride; both reactions yield ester products with significantly different retention times than those of the interferences of the reactive glycerol-rich matrix and in areas of the GC-chromatogram featuring lower levels of matrix interferences. A second procedure involved an initial diethyl ether/aqueous extraction of the matrix; while the extraction was found to substantially remove many of the hydrophilic matrix components and improve the overall derivatization, it also led to some loss of PA available for the derivatization. Both protocols were applied to the successful derivatization and analysis of PA by GC-MS when present at a 5 $\mu g \cdot mL^{-1}$ concentration in a glycerol-rich matrix sample administered during the 48th Proficiency Test administered by the Organisation for the Prohibition of Chemical Weapons (OPCW).

Keywords: Pinacolyl alcohol, Soman, acetylation, benzoylation, GC-MS.

Introduction

Analysis and detection of chemical warfare agent (CWA) degradation products using Gas Chromatography-Mass Spectrometry (GC-MS) continues to play a central role in the field of analytical chemistry dealing with these notoriously toxic substances [1]. Relative to Liquid Chromatography-Mass Spectrometry (LC-MS) methods, GC-MS analyses are often preferred, because of their ease of implementation and the existence of extensive mass spectral libraries. Thus, GC-MS is an important, first-line analytical technique to examine a sample for the presence of CWAs or their degradation products that, particularly when detected together, can be used as markers for their retrospective identification. As most CWAs, specifically the organophosphorus-based nerve agents (OPNAs), are hydrophobic in nature, their direct analysis and detection by GC-MS often does not represent a problem. Nonetheless, the chances of encountering a nerve agent in its intact state in an environmental or biological sample are poor and it is in these instances that the analyst must focus on finding its degradation products. Yet in carrying out these analyses, one must consider the highly polar nature of these products that in most cases makes them extremely difficult to detect directly, at low concentrations, by GC-MS techniques. In these instances, derivatization reactions in the form of silylations [2-4], methylations [5-9] and acylations [10] have become invaluable tools in the analysis of these compounds. Furthermore, the fact that different derivatives can be detected from fractions originating from the same sample extract enhances the confidence in the correct identification of the CWA degradation product.

Pinacolyl alcohol (PA, 3,3-dimethyl-2-butanol, **1** in Figure 1a) is a key component in the synthesis, and correspondingly the degradation, of the nerve agent Soman (GD, 1-methyl-2,2-dimethyl-propylmethylphosphonofluoridate). Therefore, the presence of PA in a sample can be indicative of the past presence of this nerve agent. Even though PA represents an important indicator of chemical weapons use, itself marked as a Schedule II chemical in the Chemical Weapons Convention (CWC), few derivatization protocols exist for its analysis by GC-MS. This is partly because PA can be detected in its native form by GC-MS, however, this direct detection can be insurmountably difficult to accomplish when PA is present in a sample not only at low levels but in the presence of much more abundant matrix interferences. One notable PA derivatization technique that appeared in 2010 involved the use of *p*-tolyl isocyanate to form a carbamate with a unique retention time and mass spectral profile [11]. However, this report dealt with the derivatization of an PA in isolated form and not when present in any particular matrix. Another method, published by our laboratory in 2014, involved the silylation of PA using chlorodimethylphenylsilane (CDPMS) using N-methylimidazole (NMI) as a promoting base to yield a unique derivative [12]. In that work, the silylation proved to be extremely useful in providing a PA derivative that was qualitatively detected when present at an approximately 10 $\mu g{\cdot}mL^{-1}$ concentration in a hydrocarbon-rich matrix.

When required to demonstrate the presence or absence of PA in complex matrices (*e.g.* soils, decontamination solutions, reaction mixtures, etc…) that contain interferences capable of reacting with the derivatizing agent to yield products that will obscure the derivatized PA, one must have several ways of cleverly modifying the PA in such mixture for its detection. An alternate approach that we recently came across and found highly useful in the derivatization of this unique alcohol was acylation. Acylation itself as a means for derivatizing alcohols, including PA, specifically for GC-MS analyses has been used in the past but only when these analytes are in an isolated form or spiked in a non-reactive matrix (*e.g.* hydrocarbons) [12]. Conversely, there are no reports describing the use of this type of derivatization for PA or other alcohols in matrices composed of highly reactive interferences. During the 48th Proficiency Test of the Organisation for Prohibition of Chemical Weapons (OPCW), our group at the Forensic Science Center (FSC) dealt with such a scenario, where several analytes, one of which was PA, were spiked at low levels in a liquid, glycerol/ethanol rich matrix. In this report we describe our work to establish acylation as a solid alternative method for the derivatization of PA along with its successful application in the unambiguous detection and identification in this highly reactive, complex liquid matrix.

Materials and methods

General method description for direct derivatization OPCW PT sample 481/17 (50 μL) containing pinacolyl alcohol at 5 $\mu g{\cdot}mL^{-1}$ concentration (in a glycerol/ethanol matrix) was placed in an autosampler vial equipped with a micro stir bar. To the colorless mixture, triethylamine (378 μL) and acetyl chloride (192 μL) were added via pipette and stirring of the solution was done at ambient temperature for 2 hours. Addition of the acetyl chloride results is an exothermic reaction (even upon cooling with an ice bath) as evidenced by the generation of bubbles presumably from the boiling of ethanol and other low volatiles present in the glycerol matrix. After 2 hours, the mixture was partitioned with diethyl ether (100 μL) and deionized water (100 μL) and transferred to another autosampler vial with an insert, capped tightly and vortexed for 30 seconds. The top ethereal layer was collected and analyzed by GC-MS (and other techniques). The same protocol was followed for the benzoylation reaction of PA, using benzoyl chloride (314 μL) instead of acetyl chloride.

General method description for the extraction of PA from glycerol-rich matrix OPCW PT sample 481/17 (400 μL) was transferred to an autosampler vial. To the colorless mixture, deionized water (800 μL) and diethyl ether (400 μL) were added sequentially via pipette. The vial was tightly capped and vortexed for 15 seconds. The top ethereal layer (300 μL) was transferred to another using a pipette to an autosampler vial equipped with a micro stir bar and treated with triethylamine (304 μL, 2.17 mmol) and the acylating agent (2.17 mmol; 155 μL for acetyl chloride and 252 μL for benzoyl chloride) via pipette and stirred at ambient temperature for 2 hours. The reaction was then quenched with the addition of deionized water (300 μL) and stirring was done for 1 min. The top ethereal layer (100 μL) was dried over anhydrous sodium sulfate (1 mg), transferred to an autosampler vial equipped with an insert and analyzed by GC-MS.

GC-MS Analysis Method A 6890 Agilent GC with 5975 MS detector equipped with a split/splitless injector was used for the analysis. The GC column used for the analysis was an Agilent HP- 5ms UI capillary column (30 m × 0.25 mm id × 0.25 μm film thickness). Ultra-high purity helium, at 0.8 mL/min, served as the carrier gas. The inlet was operated in pulsed splitless mode (25 psi for 1 minute, followed by a 50 mL/min purge flow), with the injector temperature set at 250 °C and the injection volume was 1 μL. The oven temperature program was as follows: 40 °C, held for 3 min, increased at 8 °C/min to 300 °C, held for 3 min. The MS ion source and quadrupole temperatures were 230 °C and 150 °C, respectively. Electron ionization (EI) was used with an ionization energy of 70 eV. The MS was operated to scan from *m/z* 29 to 600 in 0.4 s. with a solvent delay of 3.5 min.

Nuclear Magnetic Resonance Spectra were obtained using a Bruker Avance III 600 MHz instrument equipped with a Bruker QNP 5 mm cryoprobe (Bruker Biospin, Billerica, MA) at 30.0 ± 0.1°C. NMR data is reported as follows: chemical shift (δ) (parts per million, ppm); multiplicity: s (singlet), d (doublet), t (triplet), q (quartet) and tt (triplet of triplets); coupling constants (J) are given in Hertz (Hz). ^{1}H NMR (600 MHz) chemical shifts are calibrated with respect to residual chloroform in $CDCl_3$ centered at 7.26 ppm, whereas for ^{13}C NMR (151 MHz), the center peak for $CDCl_3$, centered at 77.0 ppm, was used for the calibration. ^{13}C NMR DEPT-135 was used to identify the nature (*i.e*. 1°, 2°, 3° or quaternary) of the carbon atoms in the standards.

Chemical synthesis of acylated standards Pinacolyl alcohol (PA, **1**, 400 μL, 3.18 mmol) was taken up in dichloromethane (DCM, 3 mL) in a 20-mL scintillation vial equipped with a stir bar. To this solution, triethylamine (535 μL, 3.8 mmol, 1.2 equiv.) followed by the acyl chloride (3.8 mmol, 1.2 equiv. to PA) was added dropwise and the resulting solution was stirred overnight at ambient temperature. The following day, the solution was diluted with diethyl ether (10 mL) and partitioned with deionized water (5 mL). The organic phase was extracted with water (2 x 5 mL), dried over Na_2SO_4 and evaporated *in vacuo* to give a solid residue that was purified by flash column chromatography (hexanes → 1:1 EtOAc:hexanes) to give the acylated products as colorless oils (Figure 1a), acetyl-PA (**2**, 398 mg, 87%), benzoyl-PA (**3**, 432 mg, 66%). Acetyl-PA (**2**): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 4.73 (q, J = 6.0 Hz, 1H), 2.17 (s, 3H, OAc), 1.15 (d, J = 6.0 Hz, 3H), 0.90 (s, 9H); ^{13}C NMR (151 MHz) δ 168.3, 77.8 ($\mathit{C}(CH_3)_3$), 34.3, 25.8 ($C(\mathit{CH_3})_3$), 21.3, 15.2 (CH_3); R_f = 0.60 (1:9 EtOAc:hexanes); Benzoyl-PA (**3**): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.04 (d, J = 1.3 Hz, 2H), 7.55 (tt, J = 8.7, 1.3 Hz, 1H), 7.44 (t, J = 7.4 Hz, 2H), 4.92 (q, J = 6.5 Hz, 1H), 1.27 (d, J = 6.5 Hz, 3H), 1.00 (s, 9H); ^{13}C NMR (151 MHz) δ 166.3 (C=O), 132.8, 129.7, 128.5, 78.5 ($\mathit{C}(CH_3)_3$), 34.6, 25.9 ($C(\mathit{CH_3})_3$), 15.1 (CH_3); R_f = 0.85 (1:9 EtOAc:hexanes).

Results and Discussion

Even though pinacolyl alcohol (PA) can be detected in its native form by GC-MS, its early elution near (or under) the solvent front (attributed to its low molecular weight of 102 amu), the fact that its mass spectrum features ions that are shared by other hydrocarbons, and its potential presence at low levels (< 10 ppm) make its direct detection and clear identification in complex matrices a significantly challenging task. For these reasons, the ability to form unique derivatives of PA provides additional means of identification. From an OPCW Proficiency Test (PT) standpoint, a participating laboratory needs to identify a reportable analyte by at least two analytical techniques; with underivatized and derivatized versions of a compound completely acceptable [13]. Interestingly, reports describing derivatization approaches for its subsequent GC-MS analysis do not abound with only two known reports directly linked to its derivatization for GC-MS purposes [11, 12]. Participation in annual proficiency tests administered by OPCW, continuously exposes our laboratory to encounter a range of matrices where specific analytes related to CWAs, such as PA, have been spiked at low levels (~1-10 ppm). During these PTs, it is important to both detect and unambiguously identify an analyte by comparing its chromatographic retention time and its mass spectrum to those of a known reference chemical [13]. During the 48th OPCW PT, we encountered a liquid sample rich in glycerol and ethanol, among other alcohol components, where the PA was initially detected by GC-MS in its native form. Initially, silylation using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added to the list of used methods for its derivatization, however, this approach was abandoned due to the fact that the silylated PA derivative signal was effectively obscured by the reagent's signal which is commonly used in large excess.

To this end, we explored acylation as a viable derivatization strategy for PA in this complex matrix. Despite the anticipated high reactivity of the matrix towards acylating agents, we decided that this approach might be successful if the resulting PA derivative featured a retention time that was shifted away from derivatized matrix components and other interferences. Thus, we looked at the reaction of PA with two commonly employed acylating agents: acetyl chloride and benzoyl chloride. In principle, these two reactions would produce derivatives with two distinct retention times and thus two additional means of reporting the analyte during the OPCW PT. The reactions between PA and these acylating agents were found to occur smoothly at ambient temperature and both products arising from the reactions, acetyl-PA (**2**) and benzoyl-PA (**3**) (Figure 1b), could be observed within 2 hours. Interestingly, analysis of the reaction mixtures after overnight stirring, revealed that significantly more of the benzoyl-PA had accumulated relative to the first 2 hours of the reaction. This was presumably due to the slower reaction of PA with benzoyl chloride, which is a reagent sensitive to steric effects due to its large size relative to that of acetyl chloride which is a more non-discriminating acylating agent (Figure 1b) [14]. This observation is in agreement with the isolated yields resulting from the syntheses of the standards, where the synthesis of the benzoyl-PA standard produced a 66% yield compared to the 87% yield obtained for its acetyl counterpart. No heating was attempted during the standard synthesis or the sample treatment preparations as we wanted to assess the ability of the protocol to accomplish the derivatization under the mildest conditions possible in order to avoid any loss of temperature-sensitive analytes in the mixture. Armed with this information, we proceeded to seek the use of both acylating agents in the OPCW PT sample labeled 481/17. Sample 481/17 is a liquid matrix that was composed mainly of glycerol and ethanol, a composition prepared to mimic that of commonly-used hand sanitizers. Therefore, reactions aimed at the specific derivatization of PA in this matrix would prove particularly challenging due to the fact that the matrix itself will be highly reactive towards our derivatizing agents. Again, we conjectured that if we were able to modify the PA to produce a derivative featuring a retention time away from the glycerol and other alcohols in the matrix, then this would represent a significant aid in the analysis and detection of this analyte.

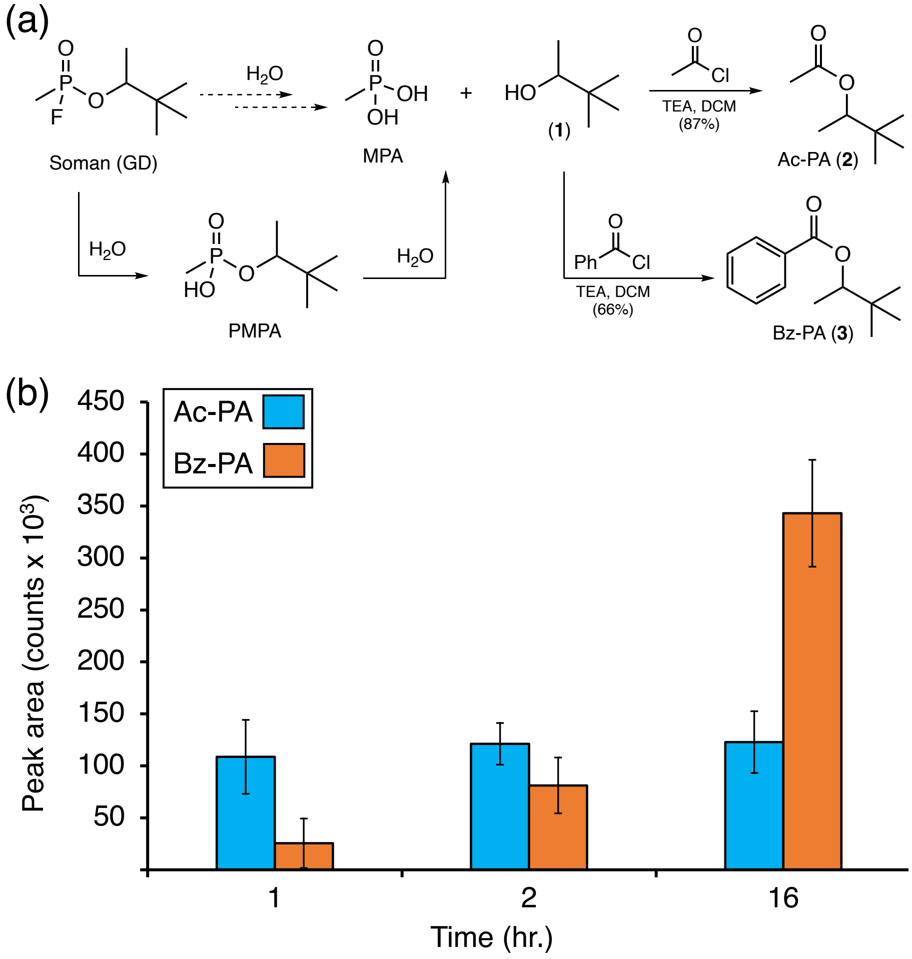

Fig. 1 (a) Degradation of Soman to yield methylphosphonic acid (MPA) and pinacolyl alcohol (PA, **1**) through the intermediacy of pinacolyl methylphosphonic acid (PMPA). These unique phosphonic acids when detected along with PA in a matrix, can serve as indicators for the past presence of Soman; (b) average (n=6) peak areas (± the standard deviation) for the acylation of pinacolyl alcohol with acetyl (blue columns) and benzoyl chloride (orange columns) at ambient temperature using triethylamine

An initial approach we sought to test was to directly treat the 481/17 sample with the acyl chloride using triethylamine as the scavenging base. To this end, we were gratified to find that this direct approach resulted in the derivatization of PA in this complex matrix and more importantly, that the resulting derivatives featured retention times different from those of the matrix interferences. Therefore, treatment of the matrix with excess acetyl chloride resulted in the generation of acetyl-PA (**2**) whose retention time of t = 6.56 minutes lies in an area of the chromatogram that is clear of any other interferences and also well separated from the solvent front (between 3-4 min) (Figure 2a). By comparison, underivatized PA features a retention time of t = 3.30 minutes. As a side note, derivatization using trifluoroacetic anhydride was also investigated, however the retention time exhibited by the trifluoroacetyl-PA was found to be very close to that of the solvent front (t = 3.24 min.), and for this reason, this alternate acylation was abandoned (data not shown). Another protocol investigated involved the use of benzoyl chloride since it would provide another acyl derivative of PA with a significantly different retention time than the acetyl-PA analog. Again we were gratified to find that this benzoylated analog (**3**) exhibited a retention time of t = 17.7 minutes, which fortuitously lies in a fairly-cleared area of the glycerol-rich matrix studied (Figure 2c). An added benefit of both acylated PA derivatives is the fact that their mass spectral data are present in the commercially-available NIST spectral library, making it convenient to qualitatively screen for their presence when using other GC columns and analysis conditions (Figures 2b and 2d) [15].

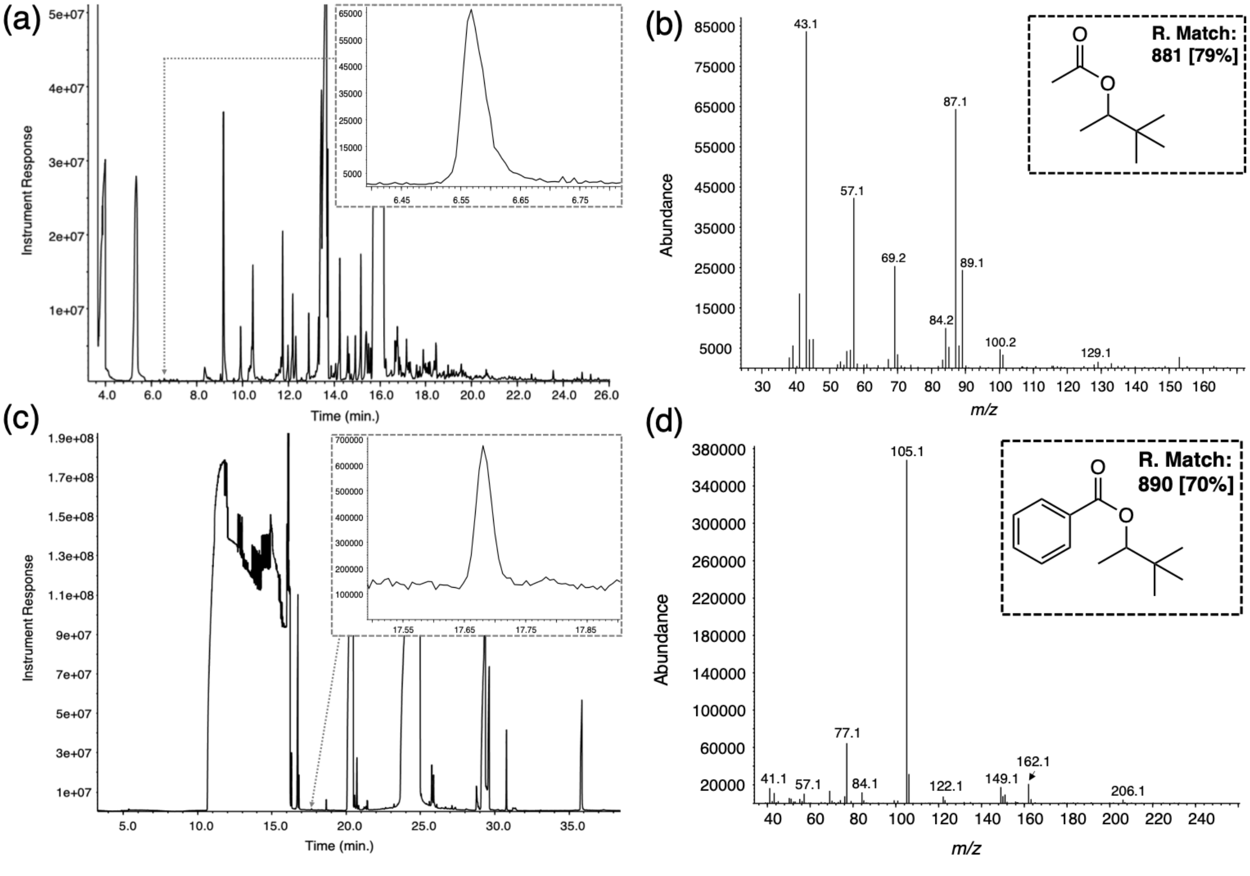

Fig. 2 Identification of pinacolyl alcohol ($5\ \mu g{\cdot}mL^{-1}$) as its acetyl derivative in the OPCW PT sample 481/17. (a) GC total ion chromatogram of mixture showing the acetyl-PA product (inset); (b) mass spectrum of acetylated sample in matrix. The retention time for the acetyl-PA standard is t = 6.40 min., while in the matrix under these conditions, it shifts to t = 6.56 min. (c) GC chromatogram of mixture showing the benzoyl-PA product (inset); (d) mass spectrum of benzoylated sample in matrix. The retention time for the benzoyl-PA standard is t = 17.1 min., while in the matrix under these conditions, it shifts to t = 17.7 min

Another alternative route that was investigated at the sample preparation stage during the PT was to incorporate an extraction step to potentially obtain a sample with less matrix interferences. Glycerol present in the sample matrix was particularly challenging due to its quantity and reactivity during various derivatizing reactions. If glycerol could be selectively removed from the matrix, direct analysis of PA would be possible and thus increase the overall efficiency of any subsequent derivatizations. To this end, we partitioned the PT sample between diethyl ether and deionized water followed by vortexing to yield a biphasic mixture. This procedure was efficient in removing most of the glycerol and low molecular weight alcohols out of the matrix. Derivatization of the resulting residue after careful removal of the diethyl ether with a slow nitrogen stream provided very clean chromatograms where the detection of the acetyl and benzoyl derivatives of PA were accomplished after derivatization of these were performed (Figures 3a and 3b). An interesting point of comparison between the two protocols (direct acylation vs. extraction followed by acylation) is that examination of the extracted ion chromatogram was required for detection, rather than the total ion chromatogram. This is perhaps due to a loss of PA through the additional extraction step. In contrast, the signals arising from both acylated products could be clearly discerned, albeit very low in concentration relative to the interfering signals, in the direct acylation protocol (Figures 2a and 2c). Thus, it is believed that the extraction step, although performed to remove most of the matrix interferences, it also acted deleteriously to decrease the initial PA concentration available for derivatization.

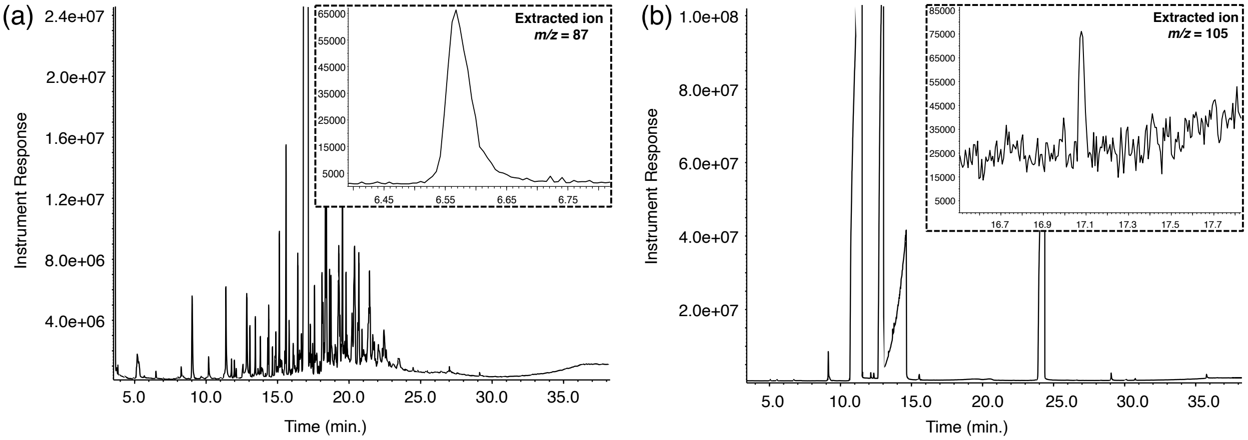

Fig. 3 Identification of PA as its ester forms in the 481/17 matrix after an initial extraction of the matrix with diethyl ether. Acylation was carried out on the concentrated organic phase with (a) acetyl chloride

and (b) benzoyl chloride. Extracted ion chromatograms (insets) were used to obtain clearer peaks of the products

Conclusion

The derivatization of pinacolyl alcohol (PA) in a highly complex glycerol matrix to produce acylated derivatives that can be detected by GC-MS is reported. The acetylation and benzoylation of PA occur successfully at ambient temperature and are completed within two hours yielding products that can be unambiguously identified by their unique mass spectra and subsequent corroboration with the commercially available NIST library. Both modes of derivatizations are complementary to each other and provide derivatives of PA with significantly different retention times that conveniently flank the more abundant interferences arising from the glycerol-rich matrix. The method was used to reproducibly identify PA in the 48th OPCW PT sample 481/17, composed of glycerol, ethanol, and other low-molecular alcohols of equal or superior reactivity than PA, which was present at a 5 $\mu g \cdot mL^{-1}$ concentration. As there are only two reports describing the specific analysis of PA in matrices at low concentrations, the protocol described herein represents the first of its kind in the analysis of PA in a highly-reactive matrix. Therefore, we anticipate that this derivatization protocol will become a useful alternative to analytical chemists involved in the analysis of PA present at low levels in highly-reactive matrices such as glycerol and other alcohol-based media, whether it is during routine or real world sample analysis.

Acknowledgements

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