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pH-Dependent Piecewise Linear Correlation of ^{1}H,^{31}P Chemical Shifts: Application in NMR Identification of Nerve Agent Metabolites in Urine Samples

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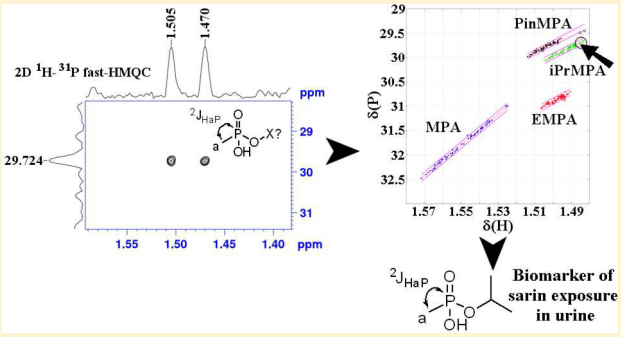
Supporting Information

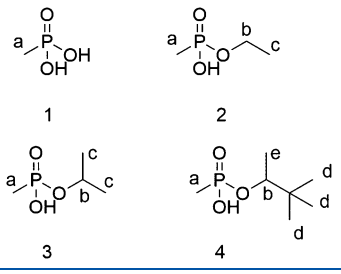
ABSTRACT: The NMR-observable nuclei of the acidic and basic compounds experience pH dependence in chemical shift. This phenomenon can be exploited in NMR titrations to determine pK_a values of compounds, or in pH measurement of solutions using dedicated pH reference compounds. On the other hand, this sensitivity can also cause problems in, for example, metabolomics, where slight changes in pH result in significant difficulties for peak alignment between spectra of set of samples for comparative analysis. In worst case, the pH sensitivity of chemical shifts can prevent unambiguous identification of compounds. Here, we propose an alternative approach for NMR identification of pH-sensitive analytes. The ^{1}H and X (^{13}C, ^{15}N, ^{31}P, ...) chemical shifts in close proximity to the acidic or basic functional group should, when presented as ordered pairs, express piecewise linear correlation with distinct slope, intercept, and range. We have studied the pH dependence of ^{1}H and ^{31}P chemical shifts of the CH_3−P moiety in urinary metabolites of nerve agents sarin, soman and VX using 2D ^{1}H−^{31}P fast-HMQC spectroscopy. The ^{1}H and ^{31}P chemical shifts of these chemicals appear in very narrow range, and due to subtle changes in sample pH the identification on either ^{1}H or ^{31}P chemical shift alone is uncertain. However, if the observed ^{1}H and ^{31}P chemical shifts of the CH_3−P moiety of individual compounds are presented as ordered pairs, they fall into distinct linear spaces, thus, facilitating identification with high confidence.



Due to recent escalation of the civil war in Syria, there has been several incidents where organophosphorus nerve agent sarin has been used as chemical weapon. The UN-OPCW joint mission has needed to conduct inspections in the Middle East, and take samples that can be transported to the designated laboratories of the OPCW for verification of the alleged use of chemical weapons.[1] Due to access restrictions and the safety concerns the inspectors have had limited changes to collect environmental samples on-site, therefore they have also taken biomedical samples like urine and blood samples from the identified victims and refugees. The OPCW reporting criteria for biomedical samples indicate the identification must be reported with two analytical, preferably spectrometric methods,[2] but currently there are only criteria available for mass spectrometry (MS) based techniques. Therefore, it is important to develop alternative methods for the analysis of biomedical samples to complement the established MS techniques.

We have studied the possibility to identify the metabolites of nerve agents VX, sarin, and soman, namely, ethyl methylphosphonate (EMPA), isopropyl methylphosphonate (iPrMPA), pinacolyl methylphosphonate (PinMPA), and methylphosphonic acid (MPA; Chart 1) in urine samples using nuclear magnetic resonance (NMR) spectroscopy. The challenge with the urine samples is the extremely low concentration of the analytes. As an example, the level of urinary metabolite of sarin, iPrMPA,

Chart 1. Structures and Group Numbering of the Nerve Agent Metabolites Methylphosphonic Acid (MPA, 1), Ethyl Methylphosphonate (EMPA, 2), Isopropyl Methylphosphonate (iPrMPA, 3), and Pinacolyl Methylphosphonate (PinMPA, 4)



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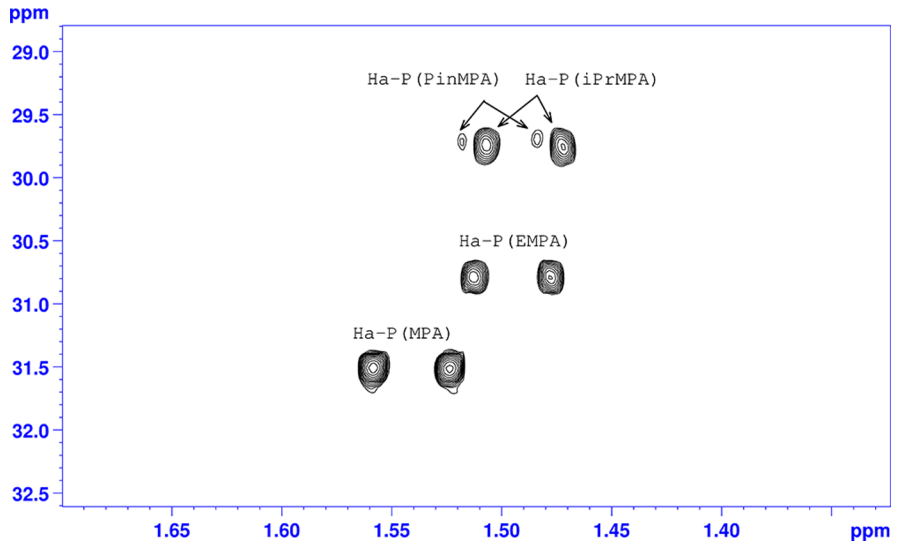

Figure 1. Expansion of the 2D $^{1}H-^{31}P$ fast-HMQC spectrum from a urine sample spiked with 700 ppb of nerve agent metabolites and reconstituted in MeOH-d_4.

was found to decrease from 760 ppb (5.5 μM) to 10 ppb (72 nM) within 7 days from the sarin poisoning.[3] The low concentration and the complex sample matrix must be addressed with sample enrichment and purification methods prior to the analysis to improve sensibility. The observation of the proton resonances of the analytes with ^{1}H NMR may still be difficult due to the residual urine sample matrix overlap, but the organophosphorus nerve agent metabolites can be selectively observed using 1D/2D $^{1}H-^{31}P$ correlation techniques.[4−6] Unfortunately, while the CH_3-P moiety should be able to be observed with good sensitivity even in the low concentration range, the R−O−P moiety, which would be important to distinguish the nerve agent metabolites from each other, is very often not observed due to poor sensitivity caused by small/nonexistent J_{HP} coupling and the J_{HH} coupling evolution within the spin system during the pulse sequence.[7] Furthermore, if the pH of the sample is close to the pK_a values of the analytes, minute changes in the pH can change the proton chemical shift significantly, making the identification on the basis of proton chemical shift uncertain against reference sample spectra or library spectra, which are the established identification options with environmental samples.[8] However, it is known that the pH dependence of chemical shifts follow very accurately Henderson−Hasselbalch based functions, and hence this principle is routinely utilized in the precise pH titration.[9] Because of this discrete behavior, we have considered the possibility to exploit the pH dependence of chemical shifts for the identification purposes.

The pH dependence of a monoprotic acid[10] can be expressed with an equation:

$$\delta_i = \frac{\delta_{1i} + (\delta_{2i} \cdot 10^{(\mathrm{pH}-\mathrm{p}K_a)})}{10^{(\mathrm{pH}-\mathrm{p}K_a)} + 1}$$

where δ_{1i} and δ_{2i} values are the chemical shifts of nucleus i in the acid form and the conjugate base of the monoprotic acid, respectively. Interestingly, if we consider the relation of nucleus j to a nonequivalent nucleus k of the same compound, they follow a linear equation as follows:

$$\delta_k = a \cdot \delta_j + b$$

where

$$a = \frac{(\delta_{1k} - \delta_{2k})}{\delta_{1j} - \delta_{2j}}$$

$$b = \frac{\delta_{1j} \cdot \delta_{2k} - \delta_{2j} \cdot \delta_{1k}}{\delta_{1j} - \delta_{2j}}$$

Because the linear equation is valid in the range of $\delta_{1j} \geq \delta_j \geq \delta_{2j}$, it will form a piecewise linear function. Hence, for the CH_3-P moiety of the monoprotic acids EMPA, iPrMPA, and PinMPA, the δ_H versus δ_P piecewise correlation can be expressed as follows:

$$\begin{cases} \delta_P = \dfrac{(\delta_{1P} - \delta_{2P})}{\delta_{1H} - \delta_{2H}} \cdot \delta_H + \dfrac{\delta_{1H} \cdot \delta_{2P} - \delta_{2H} \cdot \delta_{1P}}{\delta_{1H} - \delta_{2H}}, \\ \text{if} \quad \delta_{1H} \geq \delta_H \geq \delta_{2H} \end{cases}$$

The slope, intercept, and range of this linear correlation should be unique for each compound. For MPA, which is a diprotic acid, the piecewise linear correlation is more complex, and can be expressed as follows:

$$\begin{cases} \delta_P = \dfrac{(\delta_{1P} - \delta_{2P})}{\delta_{1H} - \delta_{2H}} \cdot \delta_H + \dfrac{\delta_{1H} \cdot \delta_{2P} - \delta_{2H} \cdot \delta_{1P}}{\delta_{1H} - \delta_{2H}}, \\ \text{if} \quad \delta_{1H} \geq \delta_H > \delta_{2H} \\ \delta_P = \delta_{2P}, \quad \text{if} \quad \delta_H = \delta_{2H} \\ \delta_P = \dfrac{(\delta_{2P} - \delta_{3P})}{\delta_{2H} - \delta_{3H}} \cdot \delta_H + \dfrac{\delta_{2H} \cdot \delta_{3P} - \delta_{3H} \cdot \delta_{2P}}{\delta_{2H} - \delta_{3H}}, \\ \text{if} \quad \delta_{2H} > \delta_H \geq \delta_{3H} \end{cases}$$

Table 1. pK_a Values of the Analytes Cited in Literature

comp	ref 18	ref 19	ref 20
MPA			pK_{a1} 2.3 pK_{a2} 7.9
EMPA	2.75	2.19	
iPrMPA	2.38	2.22	
PinMPA	2.50	2.34	

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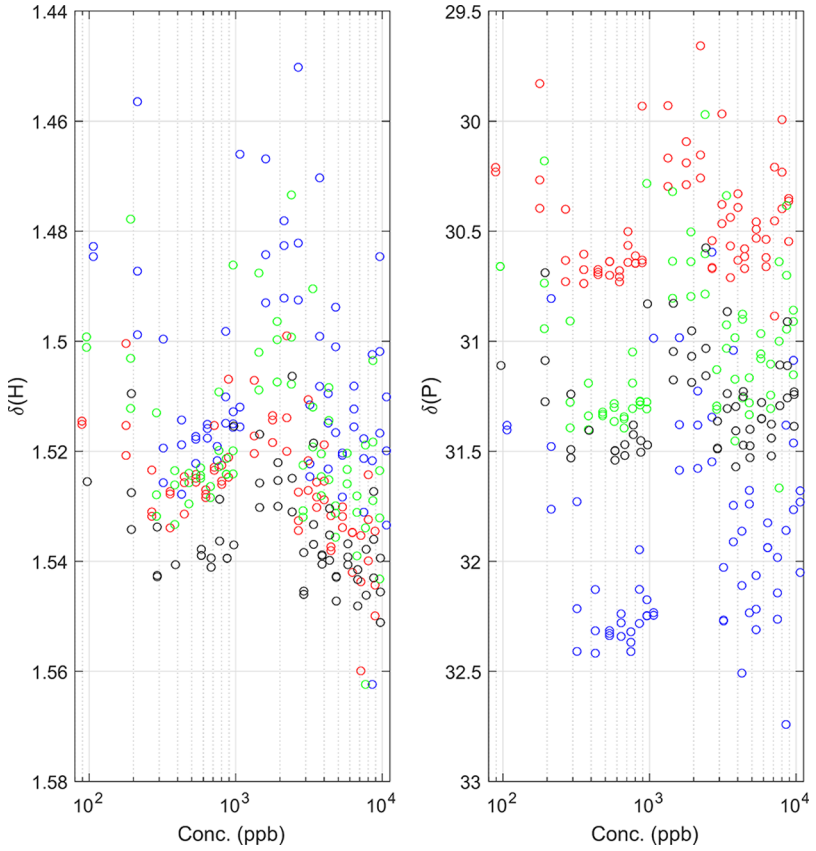

Figure 2. Spiking concentration vs Ha and P shifts of MPA (blue), EMPA (red), iPrMPA (green), and PinMPA (black) in urine samples spiked with mixture of nerve agent metabolites and reconstituted in D_2O.

The δ_i and pK_a values will change between different solvent systems like from aqueous matrix to a protic organic solvent like MeOH.[11] In addition, these values are also dependent on the ionic strength.[12] The contribution of the ionic strength to the observed chemical shifts is a critical part in the approach, but if the solvent and the ionic strength are regulated with appropriate sample preparation, these dependencies can be controlled. Consequently, it should be possible to determine and validate the linear correlation equations and the prediction bounds for the identification of the nerve agent metabolites in samples prepared with a regularized sample preparation method.

MATERIALS AND METHODS

Isopropyl methylphosphonate (iPrMPA) was purchased from Cerilliant, U.S.A. Ethyl methylphosphonate (EMPA) was provided by Spiez laboratory, Switzerland. Methylphosphonic acid (MPA) was purchased from Fluka, Switzerland. Pinacolyl methylphosphonate (PinMPA), D_2O (99.9 d%), NH_4OH, and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4, 98 d%) were purchased from Aldrich, U.S.A. HPLC grade acetonitrile and methanol were purchased from Merck KGaA, Darmstadt, Germany. MeOH-d_4 (99.9 d%) was purchased from Euriso-Top, France. Hydrogen chloride (HCl, 25%) was purchased from Oy FF-Chemicals Ab, Finland. Hydrogen chloride gas (99.5%), which was used in acidification of MeOH-d_4, was purchased from Praxair (Oevel, Belgium). Strata SI-1 (500 mg/6 mL) SPE cartridges were purchased from Phenomenex, Torrance, CA. BondElut SCX (500 mg) SPE cartridges were purchased from Agilent Technologies, Lake Forest, CA. Water was purified with a Millipore Direct-Q (Merck KGaA, Darmstadt, Germany) purification system.

Pooled urine was collected from four healthy volunteers, fractionated to 1.5 mL aliquots and kept in freezer at −21 °C. Urine samples were slowly thawed in refrigerator before use. A total of 23 triplicate 1 mL fractions of urine were spiked with mixture of nerve agent metabolites before sample preparation with average concentrations ranging from 10000 to 100 ppb. Additional 19 of 1 mL fractions of urine were spiked with individual nerve agent metabolites with concentrations ranging from 1000 to 400 ppb. Each urine fraction was diluted with 5 mL of acetonitrile and loaded to a SI cartridge conditioned with 4 mL of 25% water in acetonitrile and 3 mL of acetonitrile. Cartridges were washed with acetonitrile (2 × 1 mL) and 10% water in acetonitrile (2 × 2 mL). Analytes were eluted with 2.5 mL of 30% water in acetonitrile. Eluate was evaporated to ca. 0.3 mL in a Genevac EZ-2 Plus centrifuge evaporator (Gardiner, NY) using factory preloaded method for HPLC samples (method 09). The concentrates were diluted with 3 mL of 0.14% (v/v) NH_4OH solution (pH 8) and loaded to a SCX cartridge conditioned with 1 mL of methanol and 2 mL of water. In order to improve the yield of the analytes the cartridge was further flushed with 3 mL of 0.14% (v/v) NH_4OH solution. The eluate was evaporated to dryness in the centrifuge evaporator using factory preloaded method for aqueous samples (method 03). For the reconstitution of the residue acidic MeOH-d_4 was prepared as follows: a vessel (Erlenmeyer flask) containing clean MeOH-d_4 was placed in an ice bath. Gaseous HCl was leaded to the solvent in a moderate rate (ca. 5 bubbles/sec) until the solvent was saturated

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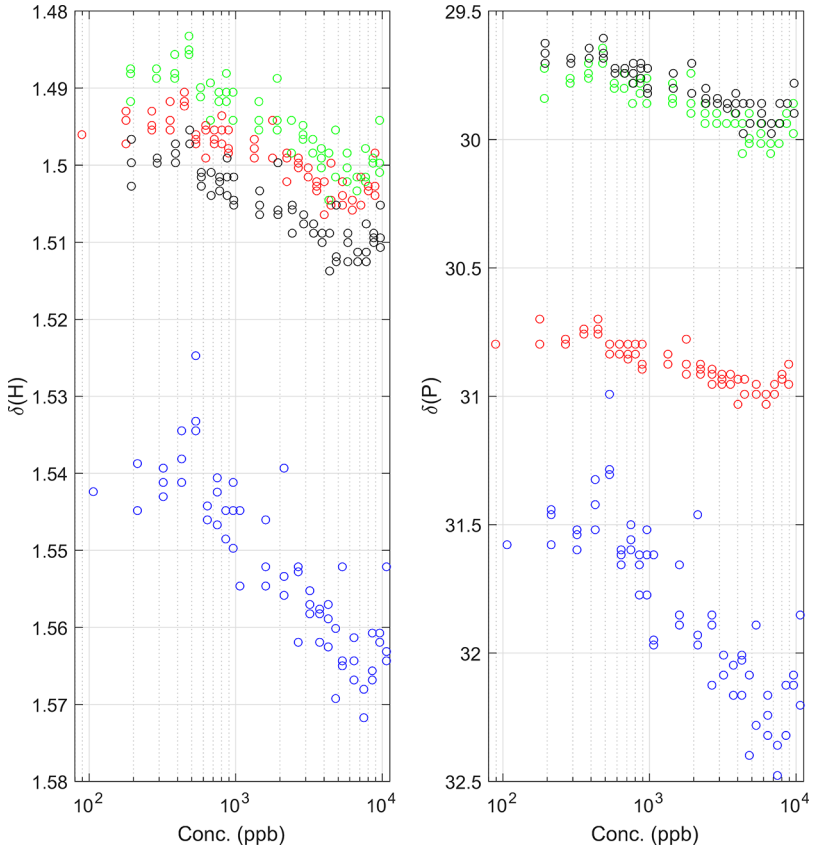

Figure 3. Spiking concentration vs Ha and P shifts of MPA (blue), EMPA (red), iPrMPA (green), and PinMPA (black) in urine samples spiked with mixture of nerve agent metabolites and reconstituted in MeOH-d_4.

(MeOH-d_4 became turbid). The molarity of acidic MeOH-d_4 solution was measured by traditional acid/base titration using 1 M sodium hydroxide solution, and diluted for target molarity by addition of pure MeOH-d_4. The residue was dissolved with 50 μL of 1 N acidic MeOH-d_4 and transferred to a 1.7 mm NMR tube (Bruker BioSpin, Rheinstetten, Germany). The yield of the sample preparation was determined with selected samples against external quantification reference sample using PULCON method,[13] and was determined to be in average 61 ± 12%. The second sample set was prepared using the same sample preparation with exception that the final evaporation residue was dissolved in 40 μL D_2O with 0.025% HCl and 2.5 mg/mL TSP-d_4.

NMR measurements were performed using Bruker Avance III 500 MHz spectrometer equipped with a 1.7 mm TXI (^{1}H, ^{13}C, ^{31}P) probe head. Measurement temperature was 17 °C. The ^{1}H NMR spectrum was measured with presaturation on residual water peak. The samples reconstituted in MeOH-d_4 were referenced with respect to the lock signal, resulting in that partially protonated MeOH-d_4 signal was at δ_H = 3.31 ppm. The samples reconstituted in D_2O were referenced with respect to TSP-d_4. The 2D ^{1}H−^{31}P fast-HMQC spectrum was recorded using an Ernst pulse angle optimization as described elsewhere.[6] Phosphorus dimension was recorded using spectral width of 40 ppm and 200 increments, and referenced using unified scale method (Ξ = 40.480 742% for ^{31}P).[14] The data analysis was performed with MATLAB R2016a (MathWorks, Inc., Natick, MA, U.S.A.).

RESULTS AND DISCUSSION

We adopted an SPE based sample preparation method[15] to enrich the urine sample to a 50 μL volume, which was then analyzed with a microcoil NMR probe head with high mass sensitivity.[16] The NMR sample solvent was selected to be acidic, as based on our previous studies[17] pH conditions between 1 and 3 provided good solvation in the sample reconstitution and sufficient dispersion of the chemical shifts of the analytes. As shown in Figure 1, the CH_3−P moieties of the nerve agent metabolites give distinctive and well separated doublet correlation peaks in the 2D ^{1}H−^{31}P fast-HMQC spectrum. The challenge with the acidic conditions is that the pH is very close to the pK_a values of the studied compounds (Table 1). The small volume of the sample means that the pH and the ionic strength cannot be kept equal between the samples, thus there was certain variation in the proton and phosphorus chemical shifts of the analytes (Figures 2 and 3; see also Supporting Information). The dispersion of the chemical shifts was clear in the samples reconstituted in D_2O, and the range of the variation clearly exceeded the acceptable tolerances between sample and reference data (δ_H = ±0.05 and δ_P = ±0.2) given in the OPCW reporting criteria for the environmental samples.[8] In the samples reconstituted in MeOH-d_4 the variation was less severe, and MPA was able to be distinguished from the other alkyl methylphosphonates. The most likely explanation for this observation is that while acidified methanol has been used efficiently for elution of methylphosphonic acid and methylphosphonates,[21] methanol has lower solvation efficiency for inorganic salts in comparison with water.[22] Consequently, the ionic strength is lower in samples reconstituted in MeOH-d_4, and corresponding affects to the observed chemical shifts are less pronounced, leaving the pH dependency to be the dominant factor in chemical shift variation.

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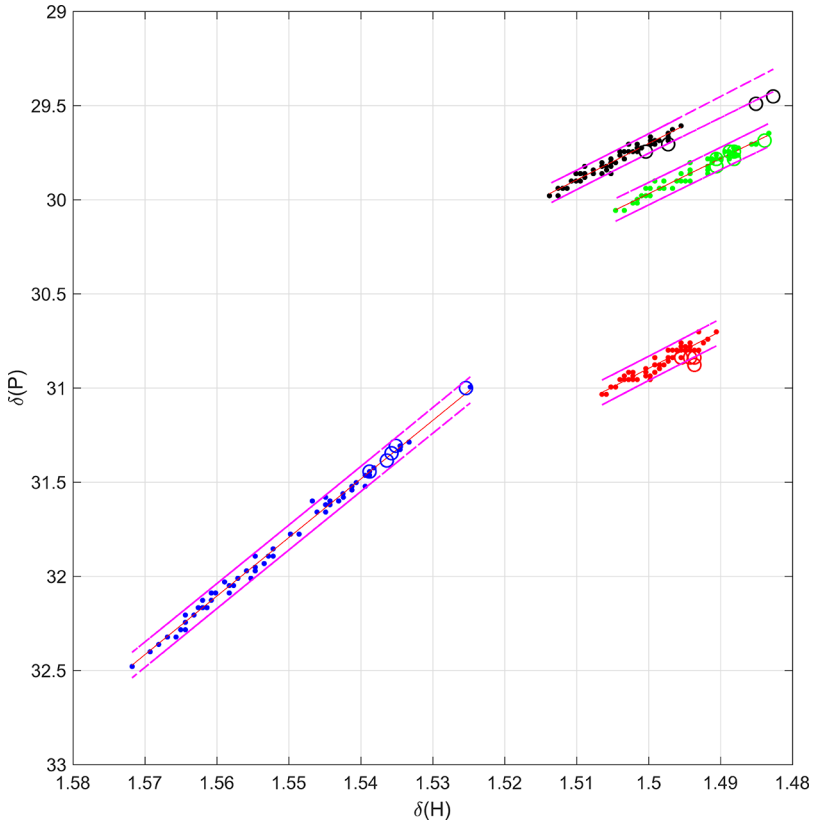

Figure 4. Ha vs P correlation of MPA (blue), EMPA (red), iPrMPA (green), and PinMPA (black) in prepared urine samples reconstituted in MeOH-d_4. The filled and open circles are the mixture samples and single compound samples, respectively. The 95% simultaneous prediction bounds are calculated using the data from the mixture samples.

Table 2. Shift Parameters and 95% Simultaneous Prediction Bounds for the Ha versus P Correlation

comp	δ_H range	δ_P	δ_P 95% prediction bounds	R^2
MPA	1.525−1.572	$31.09 \times \delta_H - 16.4$	$31.1119 \times \delta_H - 16.4969$:$31.0719 \times \delta_H - 16.3017$	0.9938
EMPA	1.491−1.507	$19.72 \times \delta_H + 1.315$	$19.7219 \times \delta_H + 1.2478$:$19.7190 \times \delta_H + 1.3816$	0.911
iPrMPA	1.483−1.505	$18.67 \times \delta_H + 1.961$	$18.6890 \times \delta_H + 1.8748$:$18.6525 \times \delta_H + 2.0482$	0.9474
PinMPA	1.483−1.514	$19.33 \times \delta_H + 0.7103$	$19.4691 \times \delta_H + 0.4450$:$19.1852 \times \delta_H + 0.9757$	0.955

The phosphorus chemical shift was sufficiently distinctive to separate the analytes further from each other in samples reconstituted in MeOH-d_4, but still phosphorus chemical shifts of iPrMPA and PinMPA were too close to be distinguishable using the OPCW criteria given in for the environmental samples. Interestingly, a logarithmic correlation between the concentration and the chemical shifts was observed with the samples reconstituted in MeOH-d_4, which is most likely due to the affect of the analytes to the sample pH. When the concentration of the analytes is higher in the sample, the sample itself is more acidic, causing the change in the proton and phosphorus shifts of the phosphonates.[10,17]

If the proton and phosphorus chemical shifts of the CH_3−P moieties of the analytes are presented as ordered pairs, they show a linear trend as the theory indicates. With the samples reconstituted in D_2O (cf. Supporting Information) the correlation is not very clear, and R^2 values are from 0.8556 to as low as 0.4437. However, in the samples reconstituted in MeOH-d_4 the shift pairs fall into distinct linear spaces (Figure 4). Based on the statistical analyses the linear regression model explained well the total variation in the data (Table 2, Supporting Information).

In order to assess the usability of the obtained data from the mixture samples to determine linear correlation equations and the prediction bounds for the identification of the nerve agent metabolites in samples, the data was subjected to further statistical analysis. The proton-phosphorus correlation data was processed in MATLAB to calculate simultaneous observation bounds for the prediction of the new observation. The 95% simultaneous observation bounds calculated from the mixture sample results gave good separation boundaries even between iPrMPA and PinMPA correlation peaks. These prediction bounds were used to assess the confidence to identify the individually spiked urine samples. In a few cases, particularly with PinMPA, the observed proton-phosphorus correlation peak chemical shifts did differ quite much from the mixture sample observations, which can be again explained by the affect of the analyte to the sample pH. Despite this, the correlation peaks did follow well the 95% simultaneous prediction bounds (Figure 4), demonstrating the potential of the approach for reliable identification.

The limit of detection, that is, the lowest spiking concentration where the analyte cross peak was still observed with S/N > 3:1 on the F2 trace, was 108, 90, 195, and 193 ppb, for MPA, EMPA, iPrMPA and PinMPA, respectively. As such, the method has potential for a complementary identification technique in cases of severe nerve agent poisoning when the

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urine sample from a victim is analyzed soon after the exposure. The optimization of the sample preparation with conjunction of the use of technologies that provide higher sensitivity, such as ultra-high-field NMR spectrometers equipped with a microcryoprobe,[23] would offer means to further lower the limit of detection to facilitate the analysis of urine samples after several days from the exposure.

CONCLUSIONS

As the sensitivity of the NMR has significantly improved in recent years, it has started to become a noteworthy complementary technique usable in trace level analyses, which have so far been the realm of mass spectrometry.[24,25] Therefore, it is, important to establish international collaboration to proceed with the development of generally accepted NMR identification criteria for trace level analyses.

The presented approach should be also usable as a supporting identification criterion of naturally occurring plant or animal metabolites. Some of the metabolites possess the same spin systems and very similar proton and carbon chemical shifts, but still distinctly different chemical shift pH dependencies (e.g., glutamine vs glutamate). The pH-dependent linear correlation of $^{1}H,^{13}C$ chemical shifts could potentially assist to unequivocally identify observed metabolites, giving additional paradigm to measure the confidentiality of metabolite identification.[26]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b01308.

Detailed linear regression analyses (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) OPCW–UN Joint Mission. https://opcw.unmissions.org/ (20 November, 2017).

(2) Work Instruction for the Reporting of the Results of the OPCW Biomedical Proficiency Tests. *QDOC/LAB/WI/BioPT04*; 2016; Issue No. 1, Revision No. 1, 28 December 2016.

(3) Nakajima, T.; Sasaki, K.; Ozawa, H.; Sekijima, Y.; Morita, H.; Fukushima, Y.; Yanagisawa, N. *Arch. Toxicol.* **1998**, *72*, 601–603.

(4) Meier, U. C. *Anal. Chem.* **2004**, *76*, 392–398.

(5) Albaret, C.; Lœillet, D.; Auge, P.; Fortier, P. *Anal. Chem.* **1997**, *69*, 2694–2700.

(6) Koskela, H.; Grigoriu, N.; Vanninen, P. *Anal. Chem.* **2006**, *78*, 3715–3722.

(7) Koskela, H.; Hakala, U.; Vanninen, P. *Anal. Chem.* **2010**, *82*, 5331–5340.

(8) Work Instruction for the Reporting of the Results of OPCW Proficiency Tests. *QDOC/LAB/WI/PT04*; 2017; Issue No. 2, Revision No. 3, 7 April 2017.

(9) Gift, A. D.; Stewart, S. M.; Kwete Bokashanga, P. *J. Chem. Educ.* **2012**, *89*, 1458–1460.

(10) Robitaille, P. L.; Robitaille, P. A.; Gordon Brown, G.; Brown, G. G. *J. Magn. Reson.* **1991**, *92*, 73–84.

(11) Rived, F.; Rosés, M.; Bosch, E. *Anal. Chim. Acta* **1998**, *374*, 309–324.

(12) Tynkkynen, T.; Tiainen, M.; Soininen, P.; Laatikainen, R. *Anal. Chim. Acta* **2009**, *648*, 105–112.

(13) Wider, G.; Dreier, L. *J. Am. Chem. Soc.* **2006**, *128*, 2571–2576.

(14) Harris, R. K.; Becker, E. D.; Cabral de Menezes, S. M.; Pierre, G.; Hoffman, R. E.; Zilm, K. W. *Pure Appl. Chem.* **2008**, *80*, 59–84.

(15) Mawhinney, D. B.; Hamelin, E. I.; Fraser, R.; Silva, S. S.; Pavlopoulos, A. J.; Kobelski, R. J. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2007**, *852*, 235–243.

(16) Koskela, H.; Vanninen, P. *Anal. Chem.* **2008**, *80*, 5556–5564.

(17) Koskela, H.; Anđelković, B. *Magn. Reson. Chem.* **2017**, *55*, 917–927.

(18) Bossle, P. C.; Martin, J. J.; Sarver, E. W.; Sommer, H. Z. *J. Chromatogr.* **1983**, *267*, 209–212.

(19) Mercier, J.-P.; Morin, Ph.; Dreux, M.; Tambute, A. *Chromatographia* **1998**, *48*, 529–534.

(20) Corbridge, D. E. C., Ed. *Studies in Inorganic Chemistry. Phosphorus. An Outline of its Chemistry, Biochemistry and Technology*, 4th ed. Elsevier: Amsterdam, 1990; p 250.

(21) Verweij, A.; Degenhardt, C. E. A. M.; Boter, H. L. *Chemosphere* **1979**, *8*, 115–124.

(22) Pinho, S. P.; Macedo, E. A. *J. Chem. Eng. Data* **2005**, *50*, 29–32.

(23) https://www.rd100conference.com/awards/winners-finalists/699/increasing-nmr-sensitivity/.

(24) 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance; notified under document number C(2002) 3044).

(25) WADA Technical Document – TD2015IDCR: Minimum criteria for chromatographic-mass spectrometric confirmation of the identity of analytes for doping control purposes.

(26) Everett, J. E. *Comput. Struct. Biotechnol. J.* **2015**, *13*, 131–144.

DOI: 10.1021/acs.analchem.8b01308
Anal. Chem. 2018, 90, 8495–8500