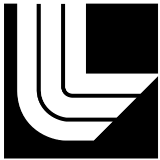

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# Forensic attribution profiling of Russian VX in food using Liquid Chromatography-Mass Spectrometry

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**Part 2: Forensic attribution profiling of Russian VX in food using Liquid Chromatography-Mass Spectrometry**

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**Abstract**

This work is part two of a three-part series in this issue of a Sweden-United States collaborative effort towards the understanding of the chemical attribution signatures of Russian VX (VR) in synthesized samples and complex food matrices. In this study, we describe the sourcing of VR present in food based on chemical analysis of attribution signatures by liquid chromatography-tandem mass spectrometry (LC-MS/MS) combined with multivariate data analysis. Analytical data was acquired from seven different foods spiked with VR batches that were synthesized *via* six different routes in two separate laboratories. The synthesis products were spiked at a lethal dose into seven food matrices: water, orange juice, apple purée, baby food, pea purée, liquid eggs and hot dog. After acetonitrile sample extraction, the samples were analyzed by LC-MS/MS operated in MRM mode. A multivariate statistical calibration model was built on the chemical attribution profiles from 118 VR spiked food samples. Using the model, an external test-set of the six synthesis routes employed for VR production was correctly identified with no observable major impact of the food matrices to the classification. The overall performance of the statistical models was found to be exceptional (94%) for the test set samples retrospectively classified to their synthesis routes.

*Keywords:* Attribution profile, CWA, Russian-VX, VR, LC/MS.

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## Introduction

Nerve agents are highly toxic organophosphorus compounds developed for use as chemical warfare agents (CWAs). These agents target the peripheral and central nervous system by inhibiting the acetylcholinesterase enzyme at the junctions between nerve cells. The inhibition causes the accumulation of acetylcholine in the nerve synapses that leads to an overstimulation of the nervous system, muscle cramps and eventually respiratory failure [1]. Clinical symptoms of nerve agent intoxication are; decrease in respiratory rate and depth, miosis, salivation and muscle cramps [2]. The G-agents; Tabun, Sarin and Soman, were the first nerve agents developed in Germany during the 1930s and 1940s [3, 4]. During the Cold war, the development of a new class of nerve agents took place in the UK and the former Soviet Union. The V-agents; VX, O-Ethyl S-2-diisopropylaminoethyl methylphosphonothiolate and Russian VX (VR), O-Isobutyl S-2-diethylaminoethyl methylphosphonothiolate (Figure 1), are more toxic and resistant towards hydrolysis than the G-agents [4]. The V-agents are colorless liquids with low vapor pressure which allow them to persist for several weeks under most environmental conditions. VR is reported to be more resistant towards hydrolysis than VX and it has a reported half-life in water of 12.4 days compared to 4.8 days for VX [3]. VX has an estimated dermal $LD_{50}$ = 40 µg/kg [5], supported by recent data from intra-muscular exposure of pig; $LD_{50}$ IM=17 µg/kg [6]. The clinical effect of VX is slower than for the G-agents and it may take even hours before any visible symptoms [2]. To our knowledge there is no human toxicity data published on VR, but it can be assumed to be comparable to that of VX.

A terrorist attack involving nerve agents is a cause of major concern to society with its consequences aptly illustrated by the sarin gas attack in the Tokyo subway system 20$^{th}$ of March 1995 [7]. The attack was perpetrated by the religious cult *Aum Shinrikyo* and despite its hastily and ill prepared nature, the attack killed 13 people and injured over 6,000 others (the number of severely injured was likely in the hundreds) [7]. The sarin used in the Tokyo subway attack was made from an improvised synthesis method on the 18$^{th}$ of March 1995, just two days before the attack [7-10]. This method gave rise to crude sarin, later described as “brown sarin” [7]. When the Japanese police raided the *Aum Shinrikyo* production site in Kamikuishiki on March the 22$^{nd}$, no stockpile of sarin was found [8]. However, traces

of sarin and *N,N*-diethylaniline (DEA) together with other degradation products were detected in samples taken from the confiscated synthesis equipment and from this attribution profile the sarin synthesis route was determined [9]. The chemical attribution profile matched the samples from the Tokyo subway and DEA was believed to be the cause of the brown color of the end product.

The *Aum Shinrikyo* also produced small quantities of VX (about 200 - 400 grams). The cult used VX in six attacks towards opponents between September 1994 and January 1995, causing one death [7, 8]. The victim was sprinkled with VX on his neck, leaving him in a deep coma and he died 10 days after the attack. The cause of death was not determined until cult members confessed to the killing after being arrested for the Tokyo subway attack several months later. Post mortem analysis revealed VX metabolites in the victim's serum [11]. This was the only documented lethal attack using VX, until the assassination of Kim Jong Nam, the half-brother of the North Korean leader Kim Jong Un that took place at the Kuala Lumpur Airport on the 13$^{th}$ of February 2017 [12, 13].

The food production sector is a potential target for a chemical or biological terrorist attack [14] and contaminated food could rapidly reach over 100,000 consumers as illustrated by the simulated attack with botulinum toxin on the Californian milk supply chain [15, 16]. In December 2013, more than 1,000 people were poisoned in Japan after consumption of food products tainted with high levels of Malathion [17, 18]. The pesticide was added in the food production process by an employee at the company Aqli Foods and the outcome of this incident could have been much more severe if a more potent chemical threat agent (CTA) had been used. Most historical cases of deliberate poisoning of food with CTA´s have been targeting smaller groups of people [19], as in the Wakayama curry arsenide poisoning [20] and cases of intentional addition of Tetramethylenedisulfotetramine (TETS) rodenticide to food in China [21-23].

Protecting the public from such attacks is difficult, not only because of the complexity of the food production process, but also due to the challenge to rapidly detect threat agents present as unexpected contaminants at low levels in food matrices [14]. In the event of a chemical attack, the main focus is to detect the chemical agent while minimizing or fully preventing the exposure of the consumers to it.

Once the CTA has been identified, a more detailed analysis of the sample can be performed at the forensic laboratory in order to gather additional information that can be used to link the contaminated food sample to other confiscated material source and/or directly to the perpetrator. The specific chemical attribution profile of a CTA, which includes byproducts and other inherent contaminants from the starting materials employed in its production/synthesis, can be used in the arduous task of sample matching and to identify the method of CTA production (i.e. sourcing).

This work is part two of a three-part series in this issue of a Sweden-United States collaborative effort towards the understanding of the chemical attribution signatures of VR in synthesized samples and complex food matrices. In this study, we describe the sourcing of VR present in food based on chemical analysis of attribution signatures by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The aim of this work was to identify the production method of VR by comparing attribution profiles from six different synthesis methods that were chosen to closely mimic those that would likely to be employed in a terrorist production scenario (i.e. sub-weapons grade).

## 2. **Material and methods**

### 2.1. Chemicals

Ammonium acetate (98%) was purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile (HPLC grade) was from Fisher Scientific (Loughborough, UK). Acetonitrile (hypergrade) and methanol (hypergrade) for LC/MS/MS analysis were bought from Merck (Darmstadt, Germany). Water was purified with a Milli-Q Plus ultra-pure water purification system (Millipore, Bedford, MA, USA). VR was produced in parallel by the Lawrence Livermore National Laboratory (LLNL) and the Swedish Defence Research Agency (FOI) via six different synthesis routes [24] (this issue).

### 2.2. Food matrices

The following food matrices were used in this study and used as received: purified bottled drinking water, apple purée, a mixed potato, tomato and beef purée (baby food), and orange juice (no pulp) were

purchased locally and supplied by FOI. Purified bottled drinking water, pea purée, liquid eggs, and hot dogs were purchased commercially and supplied by LLNL.

## 2.3. Sample preparation

All liquid samples (Milli-Q water, drinking water, apple purée, pea purée, egg and orange juice) were spiked to an estimated concentration of 50 ppm from each of the six synthesis solutions (from both SWE and US synthesis). Semisolid food (baby food and hot dog) were prepared in the range of 32 - 48 ppm. All food matrices were used unprocessed, except for the hot dog which was prepared as follows; one hot dog link was weighted and placed into a blender and half of the weight of the hot dog in water was added and the sample was processed for two minutes.

The samples spiked at LLNL were frozen at - 20°C and shipped to FOI for analysis along with blank matrices where they were stored at - 20°C for approximately one year before analysis. The blank US matrices were spiked with Swedish and US synthesis solutions at FOI in order to produce more replicates from the US foods. All samples prepared at FOI were analyzed immediately after thorough mixing. All samples were process as follows; 100 µL of the food sample was diluted with 300 µL acetonitrile, then mixed, extracted and subjected to centrifugation at 10000 rpm for 10 min. The supernatant was diluted 1:2 in Milli-Q water prior to LC/MS/MS analysis.

## 2.4. Stability study

The stability of the markers was evaluated by incubating all six synthesis routes (FOI synthesis) in water, orange juice and baby food in the dark at room temperature (20°C) for 21 days. The samples were prepared as described in section 2.3. Aliquots were analyzed with LC/MS/MS on day 1, 7 and 21.

## 2.5. LC/MS/MS method

A Waters Xevo TQ triple quadrupole instrument was used together with an Acquity UPLC system from Waters (Milford, MA, USA). LC/MS/MS data were acquired and processed with the MassLynx 4.1 software. The mass spectrometer was operated in positive electrospray mode and the capillary voltage

was set to 3.5 kV. Cone voltage and collision energy were optimized for each compound (Table 1). Ten µL samples were injected on an Acquity UPLC BEH C18, 1.7 µm, 2.1 x 50 mm column (Waters, Milford, MA, USA) at a flow rate of 300 µL/min. The following eluents were used: (A) 10 mM ammonium acetate and (B) 10 mM ammonium acetate in 98 % methanol, 2% water. A linear gradient was used from 5% B to 100% B in 4 min where it was held for 1.0 min before returning to 5% B.

Table 1. ES+ MS/MS parameters of the final LC/MS/MS method on the Waters Xevo TQ triple quadrupole instrument.

| Marker compound | Parent Ion | Daughter Ion | Cone voltage | Collision Energy |
|---|---|---|---|---|
| **A** | 101.9 | 57.9 | 20 V | 20 eV |
| **B** | 125.0 | 97.0 | 20 V | 11 eV |
| **C** | 152.0 | 96.2 | 20 V | 20 eV |
| **D** | 181.0 | 96.9 | 20 V | 20 eV |
| **E** | 189.0 | 100.0 | 20 V | 20 eV |
| **F** | 196.0 | 100.0 | 26 V | 20 eV |
| **G** | 225.9 | 100.0 | 26 V | 20 eV |
| **H** | 236.0 | 86.1 | 25 V | 20 eV |
| **I** | 240.1 | 72.0 | 20 V | 32 eV |
| **J** | 247.0 | 102.0 | 24 V | 40 eV |
| **K** | 252.1 | 100.0 | 26 V | 20 eV |
| **L** | 268.2 | 72.0 | 20 V | 32 eV |
| **M*** | 286.0 | 230.1 | 25 V | 20 eV |
| **N** | 289.8 | 100.0 | 26 V | 20 eV |
| **O** | 300.1 | 122.9 | 20 V | 20 eV |
| **P** | 316.1 | 139.1 | 20 V | 20 eV |

* Two peaks in chromatogram

## 2.6. Statistical methodology

SIMCA 14 from Umetrics (Umeå, Sweden) was used for the Partial Least Squares Discriminant Analysis (PLS-DA). PLS-DA is a supervised statistical method that uses information about the data in order to extract maximum amount of variation. The combined data of 118 samples, including synthesis replicates from two separate laboratories of all six synthesis routes spiked to seven foods (water and baby food in replicate) was used as a calibration set (Table 2) to build the PLS-DA models. Thirty five samples constituted an external test set (Table 3), used to evaluate the predictive power of the model.

Both the calibration and the test set contained spiked food samples from LLNL and FOI. The significance of the models was evaluated by cross validation statistics, where $Q^2Y$ describes the predictive ability of the model (goodness of prediction) [25]. $R^2X$ and $R^2Y$ describes the fraction of the variation of the variables (X and Y), explained by the model (goodness of fit) [25]. Values close to 1 indicate both good prediction ability and fit. The external test set was treated as "unknown" samples and were prepared in the same way as the samples in the model. Prior to PLS-DA modeling, the data (chromatographic peak area) was log transformed to improve the normal distribution and then scaled to unit variance. In order to get the most accurate prediction for a synthesis method, the predictions were performed in a hierarchic order combining multiple PLS-DA models. All statistical results are presented as the number of correct predictions from the external test set of unknown samples.

## 3. Results and Discussion

The aim of this study was to enable the determination of the synthetic route employed for the production of the organophosphorus nerve agent VR found in various spiked food matrices. The VR used in this study was produced by chemical syntheses *via* six different synthesis routes performed in parallel, by two laboratories (LLNL and FOI). Each synthetic route produced its unique chemical attribution profile, as demonstrated by GC-MS analysis of the synthetic solutions followed by multivariate statistical data analysis [24] (this issue).

The six different VR synthesis solutions were spiked to seven different foods at an estimated lethal level (32 - 50 ppm). Chemical analysis of VR attribution markers was performed after acetonitrile extraction of the spiked food samples. In aqueous sample matrices, as in most foods, many of the chemicals present in the VR synthesis cocktail hydrolyzed to produce a modified set of markers [4, 26]. Although VR and most of the synthesis by-products can be readily analyzed using gas chromatography-mass spectrometry (GC-MS) [24, 26] (this issue), the more polar hydrolysis products require an alternative analysis method such as electrospray LC-MS/MS. In addition, GC-MS analysis of food samples requires extensive sample preparation such as solid phase extraction (SPE) whereas LC-MS/MS only require a protein precipitation protocol [27]. In this study, we describe an acetonitrile-based sample preparation method

combined with a LC-MS/MS-based analytical approach for detecting chemical attribution markers related to the VR synthesis routes. In a separate publication, an alternative approach to detect chemical attribution signatures in food is presented [28] (this issue).

3.1. LC/MS/MS analysis

For initial method development, the VR synthesis mixtures were spiked into water and 56 potential markers were selected based on data from a full scan LC/MS analysis. For each of the candidates, two LC-MS/MS multiple reaction monitoring (MRM) transitions were optimized (data not shown). Based on chromatographic behavior, MRM response (peak area < 250) and/or reproducibility, a number of marker candidates were excluded, reducing the total number to 40. When more complex food matrices were introduced into the study, sample preparation was required prior to LC-MS/MS analysis. Given that a sample extraction and protein precipitation using acetonitrile is a generic sample preparation method yielding high recoveries of chemical threat agents in many different foods, this protocol was applied in our overall process. The method has previously been evaluated with 108 compounds (covering biological toxins, pesticides, toxic industrial chemicals, rodenticides, narcotics and pharmaceuticals) representing a wide range of physicochemical properties within 19 different food matrices [29].

The method's performance of the forty chemical attribution markers was evaluated by LC-MS/MS analysis of water, baby food and apple purée, spiked with VR. The MRM data from the acetonitrile extracts of VR-spiked food samples showed that not all of the markers were detectable in all matrices and based on this result, the number of markers was further reduced to seventeen. The disappearance of some of these marker candidates was possibly due to ion-suppression caused by matrix-related chemicals. The ESI+ MS/MS parameters of the final method are presented in Table 1 and the chromatograms of water samples spiked with VR from the six synthetic routes, are shown in Figure 3.

Table 2. Samples used in the construction of the model (n = 118). Synthesis solution produced at the Lawrence Livermore National Laboratory (LLNL) and the Swedish Defence Research Agency (FOI) were spiked into seven different foods.

Number of samples in food included in the model

| Synthesis route # | Water | | Orange juice | | Apple purée | | Baby food | | Pea purée | | Liquid egg | | Hot dog | | Σ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LLNL | FOI | LLNL | FOI | LLNL | FOI | LLNL | FOI | LLNL | FOI | LLNL | FOI | LLNL | FOI | |
| R1 | 2* | 5* | | 1 | | 1 | | 3* | 1 | 1 | 1 | 1 | 1 | 1 | 18 |
| R2 | 2* | 5* | 1 | 1 | 1 | 1 | 1 | 3* | | 1 | 1 | 1 | 1 | 1 | 20 |
| R3 | 1 | 5* | | 1 | | 1 | | 3* | 1 | 1 | 1 | 1 | 1 | 1 | 17 |
| R4 | 3* | 5* | | 1 | | 1 | | 3* | 1 | 1 | 1 | 1 | 1 | 1 | 19 |
| R5 | 4* | 5* | 1 | 1 | 1 | 1 | 1 | 3* | | 1 | 1 | 1 | 1 | 1 | 22 |
| R6 | 4* | 5* | 1 | 1 | 1 | 1 | 1 | 3* | | 1 | 1 | 1 | 1 | 1 | 22 |

* Independently spiked and extracted samples

Total number of samples in the model = 118

Table 3. Samples included in an external test set. US (LLNL) and Swedish (FOI) synthesis solution samples were spiked in each respective laboratory.

Number of samples in food included in the test set

| Synthesis route # | Water | | Orange juice | | Baby food | | Pea purée | | Liquid egg | | Hot dog | | Σ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LLNL | FOI | LLNL | FOI | LLNL | FOI | LLNL | FOI | LLNL | FOI | LLNL | FOI | |
| R1 | 1 | 1 | | 1 | | 1 | 1 | | 1 | | 1 | | 7 |
| R2 | | 1 | | 1 | | 1 | | | 1 | | 1 | | 5 |
| R3 | 1 | 1 | | 1 | | 1 | | | 1 | | 1 | | 6 |
| R4 | 1 | 1 | | 1 | | 1 | 1 | | 1 | | 1 | | 7 |
| R5 | | 1 | | 1 | | 1 | | | 1 | | 1 | | 5 |
| R6 | | 1 | | 1 | | 1 | | | 1 | | 1 | | 5 |

Total number of samples in the test set = 35

## 3.2. Multivariate analysis

The final set of 17 markers in the optimized LC/MS/MS method was used to analyze the six different synthesis routes spiked into seven different food matrices. The number of markers present at significant levels in the different routes varied from 7 to 14. The combined data of 153 samples was evaluated with Partial Least Squares-Discriminant Analysis (PLS-DA), which is a multivariate statistical classification method that can be used when the data is not of full rank [25]. A total of 118 samples were used as a calibration set to build the PLS-DA model (Table 2) and 35 samples constituted an external test set (Table 3), used to evaluate the predictive power of the model. The initially method development was performed on water and baby food samples, and as more foods were added these matrixes were repeated

thus generating more replicates for water (n = 5) and baby food (n = 3). Consequently, the calibration set is 57 % water and baby food samples. To avoid any bias towards these matrices, 57 % of other foods were included in the external test set. For the same reason, foods with few samples in the calibration set (i.e. pea purée and orange juice) were included in the external test set. Both the calibration and the test set had spiked food samples from LLNL and FOI. The calibration set have a larger number of Swedish synthesis solutions (66 %). To detect any bias in the models towards Swedish synthesis solutions, the external test set had an even distribution of Swedish (51 %) and US synthesis solutions.

The initial PLS-DA model M0, was built with 17 dependent Y variables (attribution markers) and six explanatory variables (classes = synthetic routes). However, this model (Figure 4) was not able to fully attribute the test set samples to their correct synthesis route (data not shown). Nevertheless, it was concluded that, independent of the food matrices, the calibration samples in the model clustered into two groups containing routes 2 and 5 (A1) and routes 1, 3, 4 and 6 (A2), respectively. Therefore, a dedicated PLS-DA model (M1) was used to separate the two classes A1 and A2, described above, leading to the score plot shown in Figure 5.

In order to further resolve the clusters, a hierarchic decision tree was constructed including in total five PLS-DA models (Figure 6), thereby reducing the complexity of the dataset. The first PLS-DA model M1 of the tree will classify samples in the full test set A0 as members of the classes A1 or A2, as described above. Both A1 and A2 represent a reduced variation compared to A0 and this is expected to improve the subsequent route classifications. The second model, M2, built on R2 and R5 calibration samples, will predict if the test set samples in A1 belongs to synthesis route 2 or 5 (Figure 7). The high complexity in the A2 class (R1, R3, R4 and R6) could not be resolved in a single model. Therefore, a hierarchic design with three additional models; M3, M4 and M5, was required to achieve full separation (Figure 6). Thus, the third model, M3 was built to separate route 1 from samples of synthesis routes 3, 4 or 6 (B2) and correspondingly the fourth model, M4 was built to separate route 3 from samples of routes 4 and 6 (C2) and finally the fifth model, M5 was constructed based on R4 and R6 calibration samples to separate the remaining test set samples into individual routes. The score plots of the three models M3, M4 and M5 are shown in Figure 8.

The strategy of using a top down hierarchic clustering approach in combination with PLS-DA modeling was successfully used to perform classification of the synthesis routes of VR spiked to food. When evaluated by cross validation statistics, all of the PLS-DA models had high prediction ability for the model classes (goodness of prediction) with a $Q^2Y > 0.87$ for M1 - 4 (Table 4) [25]. Model M5 had a lower $Q^2Y$-value of 0.75 and this indicated the challenge to resolve the closely related synthetic routes R4 and R6. Small differences (< 0.2) between the $Q^2Y$ and the fraction of the explained Y-variation ($R^2Y$) indicate valid models and no outliers in the training data [25]. The $R^2X$-values of 0.48 - 0.86 (Table 4) represent the fraction of the variation of the X variables explained by the models.

Table 4. Model statistics of the five PLS-DA models included in the decision tree. The cross validation parameter $Q^2Y$ describes the predictive ability of the model (goodness of prediction). $R^2Y$ and $R^2X$ describes the variation in Y and X, explained by the model (goodness of fit). Values close to 1 indicate good prediction ability and model fit.

| Model name | Number of principal components | $Q^2Y$ | $R^2Y$ | $R^2X$ |
|---|---|---|---|---|
| M1 | 4 | 0.96 | 0.97 | 0.67 |
| M2 | 4 | 0.97 | 0.99 | 0.86 |
| M3 | 2 | 0.87 | 0.90 | 0.49 |
| M4 | 2 | 0.88 | 0.91 | 0.49 |
| M5 | 2 | 0.75 | 0.87 | 0.48 |

Table 5. Summary of the predictions of the external test set in the five PLS-DA models included in the decision tree. An YPred value above 0.65 indicate that a test set sample belong to the class. Full data in Table S-2.

| Class | YPred | Model |
|---|---|---|
| A1 | 0.84 - 1.14 | M1 |
| A2 | 0.76 - 1.14 | |
| R2 | 0.90 - 1.04 | M2 |
| R5 | 0.94 - 1.02 | |
| B1 | 0.80 - 0.97 | M3 |
| B2 | 0.65 -1.25 | |
| C1 | 0.71 - 1.05 | M4 |
| C2 | 0.78 - 1.21 | |

| R4 | 0.58 - 1.11 | M5 |
|---|---|---|
| R6 | 0.74 - 1.16 | |

Table 6. Summary of predictions from the test set using the hierarchic model. N represents the number of samples in each synthesis class. Deviations from the diagonal indicate incorrect predictions. Green cells correspond to correct predictions and the yellow cell corresponds to insignificant classification of samples with YPred values below 0.65.

| True class belonging | | | Predicted class belonging | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | S1 | S2 | S3 | S4 | S5 | S6 | % Correct |
| | R1 | 7 | 7 | | | | | | 100 |
| | R2 | 5 | | 5 | | | | | 100 |
| | R3 | 6 | | | 6 | | | | 100 |
| | R4 | 7 | | | | 5 2 | | | 71 |
| | R5 | 5 | | | | | 5 | | 100 |
| | R6 | 5 | | | | | | 5 | 100 |

The prediction power of the statistical models was evaluated by an external test set of 35 food samples spiked with VR produced at FOI or LLNL in six different food matrices (Table 3). The use of an external test set for the assessment of the models' predictive abilities is more reliable than cross validation within the calibration set [30]. The correct synthesis route class membership was determined based on the predicted values of the Y variable (YPred) which describes the class membership (synthesis route) for each observation in the external test set. An Ypred value of 1 define a complete match to the model and values above 0.65 were interpreted as positive classifications while values below 0.65 were considered as a negative classification result [31]. The test set predictions are summarized in Table 5 and 6. The models were able to correctly predict samples from VR synthesis routes 1, 2, 3, 5 and 6 to 100% in all tested food matrices. Five out of the seven route 4 samples were correctly assigned in model M5, while two samples had weaker R4 class membership prediction (YPred 0.64 and 0.59). The samples were US R4 synthesis spiked into hot dog and bottled water at LLNL and they also had YPred values for R6 class membership at 0.36 and 0.41 respectively, making the classification of those samples ambiguous (Table

6 and S-2). The VR synthetic routes 4 and 6 utilize the same reagents but the reaction conditions are different. The small differences between the chemical attribution profiles of these routes combined with the variation in synthesis between laboratories [24] (this issue), makes the separation of the R4 and R6 routes difficult in food samples. In order to resolve the R4 and R6 classes, a larger sample set is probably required. Even though the classification of synthesis route 4 test set samples was not perfect, the models in this study have shown strong predictive power for the classification of synthesis routes 1, 2, 3, 5 and 6 and most of the route 4 samples. The distribution of YPred values of the model samples from cross-validation (Table S-1) and test set samples (Table S-2) is shown in Figure 9. The high prediction power of M1-M3 is illustrated by the tight YPred distribution of model and test set samples. Model M4 and M5 have wider Ypred distributions and the lower prediction power of M5 is indicated by the presence of test set samples with YPred < 0.65. Data of the reverse predictions by the models are shown in Table S-2.

The stability of the selected markers in food was a major concern when developing the LC/MS/MS method. V-type nerve agents are known to hydrolyze in water [4, 26] and the high water content in some of the tested foods could potentially cause degradation of the selected markers over time. The stability data test-set (n = 54) was predicted for synthesis route membership in the calibration models (M1 - M5) as described above. The models were able to correctly predict all the stability samples based on the YPred values from the six VR synthesis routes in all three foods tested, except for a synthesis route R3 sample. This sample was an orange juice incubated for 21 days and it had an YPred value of 0.62 in model M4. The tested stability samples were correctly classified after 21 days incubation which indicates that the selected markers are stable in food over the tested timespan (Table S-3). No general drift due to instability that affect the classification of samples could be detected over the tested time.

## 4. Conclusions

In this proof of concept study, the potential of using attribution markers related to VR synthesis to allow for chemical attribution profiling was evaluated. The attribution profiling data was utilized to determine the synthesis method of VR in spiked food samples. A generic sample preparation method followed by LC-MS/MS analysis and a comprehensive PLS-DA analysis allowed the classification of VR to 94%

correct synthesis route. The chemical stability of the markers in food has been a selection criterion and consequently the markers are stable in food for at least 21 days in room temperature.

## 5. Acknowledgements

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## 6. References

[1] S. Reutter, Hazards of chemical weapons release during war: New perspectives, Environmental Health Perspectives 107(12) (1999) 985-990.
[2] H. Thiermann, F. Worek, K. Kehe, Limitations and challenges in treatment of acute chemical warfare agent poisoning, Chemico-Biological Interactions 206(3) (2013) 435-443.
[3] D.H. Ellison, Handbook of Chemical and Biological Warfare Agents, Second Edition ed., CRC Press, New York, 2008.
[4] K. Kim, O.G. Tsay, D.A. Atwood, D.G. Churchill, Destruction and Detection of Chemical Warfare Agents, Chemical Reviews 111(9) (2011) 5345-5403.
[5] N.B. Munro, K.R. Ambrose, A.P. Watson, TOXICITY OF THE ORGANOPHOSPHATE CHEMICAL WARFARE AGENTS GA, GB, AND VX - IMPLICATIONS FOR PUBLIC PROTECTION, Environmental Health Perspectives 102(1) (1994) 18-38.
[6] J.L. Langston, T.M. Myers, VX toxicity in the Gottingen minipig, Toxicology Letters 264 (2016) 12-19.
[7] R.D. Danzig, M. Sageman, T. Leighton, L. Hough, R. Yuki, R. Kotani, Z.M. Hosford, Aum Hinrikyo Insights Into How Terrorists Develop Biological and Chemical Weapons, 2012. www.cnas.org/publications/reports/aum-shinrikyo-second-edition-english.
[8] A.T. Tu, Overview of sarin terrorist attacks in Japan, in: A.T. Tu, W. Gaffield (Eds.), Natural and Selected Synthetic Toxins: Biological Implications2000, pp. 304-317.
[9] Y. Seto, N. Tsunoda, M. Kataoka, K. Tsuge, T. Nagano, Toxicological analysis of victims' blood and crime scene evidence samples in the sarin gas attack caused by the Aum Shinrikyo Cult, in: A.T. Tu, W. Gaffield (Eds.), Natural and Selected Synthetic Toxins: Biological Implications2000, pp. 318-332.
[10] Y. Ogawa, Y. Yamamura, A. Ando, M. Kadokura, T. Agata, M. Fukumoto, T. Satake, K. Machida, O. Sakai, Y. Miyata, H. Nonaka, K. Nakajima, S. Hamaya, S. Miyazaki, M. Ohida, T. Yoshioka, S. Takagi, H. Shimizu, An attack with sarin nerve gas on the Tokyo subway system and its effects on victims, in: A.T. Tu, W. Gaffield (Eds.), Natural and Selected Synthetic Toxins: Biological Implications2000, pp. 333-355.

[11] H. Tsuchihashi, M. Katagi, M. Nishikawa, M. Tatsuno, Identification of metabolites of nerve agent VX in serum collected from a victim, Journal of Analytical Toxicology 22(5) (1998) 383-388.
[12] R. Wingfield-Hayes, Kim Jong-nam killing: VX nerve agent found on his face, BBC News, 2017.
[13] J. Diamond, North Korea's Chemical Weapon: Inside Deadly VX Nerve Agent Attack, Rolling Stone Magazine, 2017.
[14] M.J. Alvarez, A. Alvarez, M. De Giacomo, M.C. De Maggio, R. Onori, A. Oses, J.M. Sarriegi, R. Setola, M. Trombetta, A risk assessment of the food supply chain: vulnerability against terrorist or criminal, International Journal of Food Safety, Nutrition and Public Health 4(1) (2011).
[15] L.M. Wein, Y.F. Liu, Analyzing a bioterror attack on the food supply: The case of botulinum toxin in milk, Proceedings of the National Academy of Sciences of the United States of America 102(28) (2005) 9984-9989.
[16] B. Alberts, Modeling attacks on the food supply, Proceedings of the National Academy of Sciences of the United States of America 102(28) (2005) 9737-9738.
[17] D. Exil, Tainted Frozen Food in Japan Leaves 1,000 People Sick, FOOD WORLD NEWS, 2014.
[18] Kyodo, Massive amount of pesticide found in frozen croquette, The Japan Times News, 2014.
[19] G.R. Dalziel, Food Defence Incidents 1950-2008, A cronology and analysis of incidents involving the malicious contamination of the food supply chain, S. Rajaratnam School of Int Studies, Nanyang Tech Univ, Singapore, 2009.
[20] O. Suzuki, H. Seno, K. Watanabe-Suzuki, A. Ishii, Situations of poisoning and analytical toxicology in Japan, Forensic Science International 113(1-3) (2000) 331-338.
[21] K.S. Whitlow, M. Belson, F. Barrueto, L. Nelson, A.K. Henderson, Tetramethylenedisulfotetramine: Old agent and new terror, Annals of Emergency Medicine 45(6) (2005) 609-613.
[22] E. Eckholm, Man Admits Poisoning Food in Rival's Shop, Killing 38 in China, New York Times, 2002.
[23] T. Thornhill, Boy, six, dies after eating lollipop covered in illegal rat poison - 100 times more toxic than cyanide, Daily Mail, 2015.
[24] K. Höjer Holmgren, C.A. Valdez, R. Magnusson, A.K. Vu, S. Lindberg, A.M. Williams, A. Alcaraz, C. Åstot, H. S., N. R., Sourcing Russian VX by multivariate statistics of chemical attribution signatures, Talanta This issue (2017).
[25] L. Eriksson, T. Byrne, E. Johansson, J. Trygg, C. Vikström, PLS, Multi- and Megavariate Data Analysis: Basic Principles and Applications MKS Umetrics AB, Malmö, Sweden, 2013, pp. 55-88.
[26] D.K. Rohrbaugh, Characterization of equimolar VX-water reaction product by chromatography mass spectrometry, Journal of Chromatography A 809(1-2) (1998) 131-139.
[27] H.G.J. Mol, P. Plaza-Bolanos, P. Zomer, T.C. de Rijk, A.A.M. Stolker, P.P.J. Mulder, Toward a Generic Extraction Method for Simultaneous Determination of Pesticides, Mycotoxins, Plant Toxins, and Veterinary Drugs in Feed and Food Matrixes, Analytical Chemistry 80(24) (2008) 9450-9459.
[28] A.M. Williams, A.K. Vu, B.P. Mayer, S. Hok, C.A. Valdez, A. Alcaraz, Extraction and Detection of Russian VX and its Chemical Attribution Signatures in Food using Solid Phase Extraction, Gas Chromatography-Mass Spectrometry and Liquid Chromatography- Mass Spectrometry, Talanta ((this issue)) (2017).
[29] A. Herrmann, J. Rosen, D. Jansson, K.-E. Hellenas, Evaluation of a generic multi-analyte method for detection of > 100 representative compounds correlated to emergency events in 19

food types by ultrahigh-pressure liquid chromatography-tandem mass spectrometry, Journal of Chromatography A 1235 (2012) 115-124.
[30] B. Worley, R. Powers, Multivariate Analysis in Metabolomics, Current Metabolomics 1(1) (2013) 92-107.
[31] User Guide to SIMCA, Version 13, MKS Umetrics, 2012, p. p. 352.

## Figure Captions

Figure 1. Structure of VR and VX.

Figure 2. Schematic description of data reduction process.

Figure 3. The total ion chromatograms of the selected seventeen markers for the six VR synthesis routes in water. For security reasons, no peak identities are given so as to not to disclose any critical information regarding the synthetic performance of the six different synthesis routes.

Figure 4. Score plot for the PLS-DA model, M0, for prediction of synthetic routes (R1 - R6) based on 17 variables (attribution markers).

Figure 5. Score plot for the PLS-DA model M1 predicting the class membership of the two groups A1 and A2, consisting of R2 + R5 and R1 + R3 + R4 + R6, respectively.

Figure 6. The hierarchic decision tree for prediction of synthesis method of VR by the use of five PLS-DA models; M1, M2, M3, M4 and M5. A0 represent the full test set, A1 and A2, reduced test sets including samples of synthesis route R2 + R5 and R1 + R3 + R4 + R6, respectively. The B2 and C2 test sets contain the remaining routes after the divergence of R1 and R3, respectively.

Figure 7. Score plot of PLS-DA model M2. The results show the separation between samples of the synthesis routes R2 and R5, constituting the reduced test set A2.

Figure 8. Score plot of PLS-DA model M3 (top), M4 (middle) and M5 (bottom), used for the classification of the reduced test set A2 samples into the individual synthesis routes R1, R3, R4 and R6. The reduced test sets; B2 and C2 corresponds to samples of R3 + R4 + R6 and R4 + R6, respectively.

Figure 9. Prediction of synthesis route-class A1, R2, R3 and R4 in models M1 - M5, respectively. YPred distribution of the model samples (circles, grey columns in Table S-1) and external test set samples (crosses, grey columns in Table S-2). Models M1 - M5 and sample classes as described by the decision tree in Figure 6. X-axis represents the predicted values and Y-axis the true class.