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A COMPARATIVE ASSESSMENT OF DIFFERENT NERVE AGENT VX STOCKS USING CHOLINESTERASE INHIBITION ASSAYS

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Abstract: *Biochemical methods for the detection of nerve agents are highly sensitive, rapid, and based on the ability of organophosphorus compounds to irreversibly inhibit enzymes from the cholinesterase family. The decrease in enzyme activity after exposure to a sample is directly proportional to the concentration of the nerve agent, making these methods powerful analytical tools, both for field detection and quantitative analysis. Nerve agents undergo various degradation pathways during long-term storage, and the complex mixture of potentially interfering compounds that accumulate over the years can affect the accuracy of enzymatic assays. In this work, a comparative analysis of the two VX samples with significantly different degradation profiles was performed using inhibition assays with electric eel acetylcholinesterase (AChE) and equine serum butyrylcholinesterase (BChE). The resulting linear regression analysis, alongside the independent GC-MS benchmark, were used to evaluate the overall inhibitory potency of the samples, screen for matrix interferences and assess the applicability of this biochemical method for quantifying residual VX in long-term stored stockpiles.*

Keywords: *cholinesterases, nerve agent VX, enzymatic method, quantitative analysis.*

1. INTRODUCTION

The interactions of highly toxic organophosphates (OPs) with enzymes from the cholinesterase family (ChEs) have been the subject of scientific research for almost a century. In this area, numerous scientific studies are focused on the development of methods for the detection and quantitative analysis of these toxic compounds. Biochemical quantification of OPs relies on the irreversible nature of ChE inhibition by OPs and is based on measuring the residual enzyme activity after a certain time of reaction with the inhibitor. Under defined experimental conditions, the reduction in enzyme activity directly correlates with both the concentration of the inhibitor and the duration of its interaction with enzyme. In certain regimes, when the inhibitor is in large excess, the inhibition reaction follows pseudo-first-order kinetics and this correlation corresponds to equation:

$$\ln (v_t/v_0) = - k_i \cdot [I] \cdot t \qquad (1)$$

where k_i is the bimolecular inhibition constant, $[I]$ is the inhibitor concentration, v_0 is the initial velocity of the enzymatic reaction and v_t is the velocity after the inhibition period t. The bimolecular inhibition constant k_i is a characteristic property of a specific irreversible inhibitor and its efficacy against a target enzyme, which serves as a key metric for comparing the inhibitory potency of different compounds [1].

Different OPs show significant differences in inhibitory potency towards ChEs from different sources. The most potent OPs known are the lethal nerve agents, which are considered to be among the deadliest chemical warfare agents to date [2]. Although their development and stockpiling as weapons are strictly prohibited by the Chemical Weapons Convention (CWC), the use of nerve agents in research, testing and development within the field of chemical defense is permitted under strict compliance with the CWC's obligations, as it remains vital for advancing protective and medical countermeasures. Consequently, possessing reliable methods for their quantitative determination is essential to maintain safety and monitor these processes effectively. While various methods for the quantification of nerve agents have been described, they often require laborious sample preparation and expensive, high-resolution analytical devices. Compared to these sophisticated techniques, biochemical methods based on ChE inhibition are rapid, simple and cost-effective [3], offering a convenient and advantageous supplementary analytical tool for many laboratory applications.

Although there are few compounds with an inhibitory potency comparable to that of nerve agents, the results of enzymatic analysis can be affected by various chemical and environmental factors. Sampling and analysis of nerve agents from different storage containers led to the identification of a large number of impurities, the type

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and quantity of which depended on various factors, such as the quality and type of precursors used in production, the stabilizers applied, the manufacturing process and the numerous reaction pathways driving various degradation processes during storage [4][5]. Any potential biological activity that these compounds might exhibit towards the enzyme could affect the accuracy of enzyme inhibition-based methods. This is particularly important when the analysis is performed under conditions where the concentration of potentially interfering compounds is many times higher than the concentration of the target analyte, which is the case with highly degraded inhibitors.

Nerve agent VX (O-ethyl S-(2-diisopropylamino)ethyl methylphosphonothiolate) is one of the deadliest chemicals known. For chemical forensics, the researchers analyzed three unstabilized VX samples using NMR, LC-HRMS, and GC-MS (CI and EI) and detected over 40 compounds, including synthesis by-products, degradation products, unreacted precursors, and contaminants such as phosphonic acids, phosphonates, phosphonothioates, alkyl sulfides, aminothioalkanes, and unidentified species [4]. Consequently, enzymatic tests must take these impurities into account, especially the possibility that the samples contain a compound known as EA 2192 (diisopropylaminoethyl methylthiophosphonic acid), which is potent ChE inhibitor and almost as toxic as VX itself.

In this work, a comparative analysis of the two VX samples with significantly different purity was performed using inhibition assays with acetylcholinesterase (AChE) from electric eel and butyrylcholinesterase (BChE) from equine serum. Additionally, GC-MS analysis was performed to obtain approximate data on the quantitative ratio of the remaining VX content in the tested samples. The aim was to investigate the impact of the impurities present on the quantitative comparison of VX stocks with different degradation profile by ChE inhibition assays and to consider the application of this biochemical method for the assessment of residual VX content in long-term stored samples.

2. MATERIALS AND METHODS

Lyophilized equine serum BChE and electric eel AChE (type V-S), as well as butyrylthiocholine iodide (BTCh), acetylthiocholine iodide (ATCh) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. All other reagents were of analytical grade, purchased from Sigma, Fluka or Riedel de Haën. VX samples were made available by the Military Technical Institute (Belgrade, Republic of Serbia).

Stock solutions of AChE and BChE were prepared by dissolving lyophilized enzymes in 50 mM phosphate buffer pH 7.2 (PB) and stored at 4 °C. Dilutions were made daily to obtain enzyme solutions with an approximate activity of 1 U/mL. A solution of DTNB (4.8 mM) was prepared in PB. Substrate solutions, ATCh (30 mM) and BTCh (60 mM), were prepared in distilled water. Stock solutions of VX samples (10 % v/v) were prepared in 2-propanol weekly and stored at 4 °C. On the day of the experiment, stock solutions were kept at room temperature for 1 h and than appropriately diluted in 2-propanol to obtain VX sample solutions for use in the inhibition assays.

2.1. Inhibition assays

The AChE and BChE activity measurements were performed essentially according to the method of Ellman [6], using ATCh or BTCh as the substrate, respectively, and DTNB (Ellman's reagent) as the indicator. In brief, the enzyme solution (75 µL) was added to a cuvette containing PB (2.6 mL) pre-incubated at 25 °C, followed by addition of the tested VX sample solution (25 µL). After mixing, the content was incubated for 20 min at 25 °C. The enzymatic reaction was then started by the addition of DTNB (200 µL) and corresponding substrate (100 µL). The formation of the yellow anion obtained from the reaction between Ellman's reagent and the thiocholine generated by enzymatic hydrolysis of the substrate was monitored on a Cary 60 UV/VIS spectrophotometer (Agilent Technologies) by measuring the increase in absorbance at 412 nm for 2 min at 12 s intervals. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate per minute under the described conditions.

For each enzyme, a range of dilutions of both VX samples was used to cover a broad spectrum of different inhibition levels, and results that included residual enzyme activity between 10 % and 80 % were considered for data processing. To determine the initial enzyme activity (100 %), measurements were carried out as described above, except that 2-propanol was used as the sample instead of VX solution.

Considering that the VX concentrations in the tested samples were unknown, a more convenient way to process the experimental data was to present the dependence described by equation (1) as a function of the mass concentration of the VX sample instead of the concentration of VX in the incubation mixture, which after corresponding transformation and rearrangements gives the following relationship:

$$\log(100 \cdot \frac{A_r}{A_0}) = -\frac{k_i \cdot Q \cdot t \cdot 10^{-6}}{2{,}303 \cdot R \cdot M_{VX}} \cdot C_m + 2 \qquad (2)$$

where A_r and A_0 are the residual and initial enzyme activities, k_i is the bimolecular inhibition constant ($M^{-1}min^{-1}$), Q is the purity coefficient of VX in the tested samples, t is the inhibition time (20 min), R is the dilution factor of the VX sample solutions in the incubation mixture (108), M_{VX} is the molar mass of VX (267.38 $gmol^{-1}$), and C_m is the mass concentration of the VX sample solutions (ng/mL).

Because the VX samples were a complex mixture rather than a pure compound, the obtained data points were fitted via linear regression in MS Excel using the empirical equation:

$$\log(A_r\%) = \boldsymbol{B} \cdot C_m + \boldsymbol{A} \qquad (3)$$

where A_r% is the percentage of residual enzyme activity.

The resulting regression outputs were used to evaluate and compare the overall inhibitory potency among the tested samples and to screen for potential matrix interferences caused by impurities.

2.2. GC-MS analysis

Each of the two VX samples was prepared at two nominal concentration levels, approximately 4.7 and 9.0 μg/mL, using acetonitrile as the solvent. The lower- and higher-concentration preparations were designated **S1** and **S1h** for **VX1** sample, and **S2** and **S2h** for **VX2** sample, respectively. Semiquantitative estimation of the VX content was performed using naphthalene-d8 as an internal standard at a final concentration of 2 μg/mL. The samples were analyzed using a Shimadzu GCMS-QP2020 instrument, and the chromatograms and peak heights were evaluated using LabSolutions software. To screen the chromatograms for VX and VX-related degradation products, the extracted-ion chromatogram (EIC) at m/z 114 was examined. This ion is a characteristic, high-abundance fragment of VX and related compounds containing the 2-(diisopropylamino)ethyl moiety. The instrumental conditions are summarized in **Table 1**. Splitless injection was used to improve the detection of minor sample components and to facilitate comparison of their chromatographic responses.

Table 1. GC-MS operating conditions

GC conditions	
Parameter	**Value**
Initial oven temperature	40 °C (3 min)
Oven temperature program	40-150 °C at 10 °C/min; 150-280 °C at 25 °C/min; hold at 280 °C for 10.8 min
Injector temperature	250 °C
Injection mode	Grob-type splitless injection; splitless time 0.75 min
Column	DB-5
Column dimensions	30 m × 0.25 mm i.d. × 0.25 µm
Carrier gas	Helium; linear velocity 32 cm/s
MS conditions	
Parameter	**Value**
Transfer-line temperature	280 °C
Ion-source temperature	230 °C
Detector temperature	150 °C
Electron energy	70 eV
Scan range	m/z 35-500
Scan rate	3.15 scans/s
Library	NIST 05 Mass Spectral Database

3. RESULTS AND DISCUSSION

3.1. Inhibition assays

The enzyme inhibition assays were used for comparative analysis of two VX samples from different long-term stored stocks with distinct storage-induced degradation profiles. Sample **VX1** was taken from a frequently pipetted stock stored in a glass bottle with screw cap that was opened and closed periodically, while the sample **VX2** was from the flame-sealed glass ampoule. The residual AChE and BChE activities within the range of 20–80 % after 20 min inhibition were determined using 8 different mass concentrations of each VX sample for each enzyme. The obtained data points and their linear fits according to equation (3) are shown in **Figure 1** and **Figure 2**. The resulting semi-logarithmic plots confirmed a monotonically decreasing trend in concentration-dependent residual activity for both enzymes and both tested samples. Visually, a superior inhibitory potency was observed for the **VX2** sample, while more pronounced data scattering was evident for the **VX1** sample in both enzymatic assays. This pronounced scattering is likely attributed to the presence of interfering impurities, which may dynamically interact with the enzyme or the inhibitor, destabilizing the inhibition kinetics.

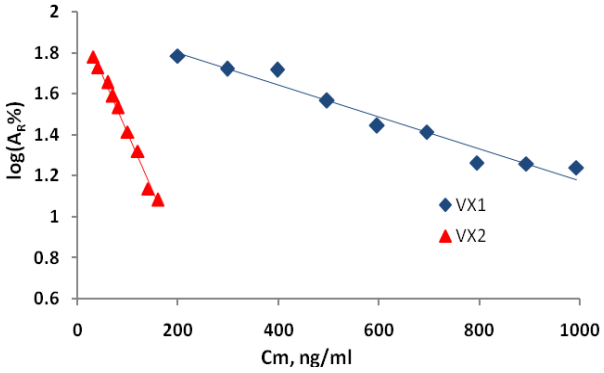


Figure 1. Semi-logarithmic plot of AChE inibition by **VX1** and **VX2** samples

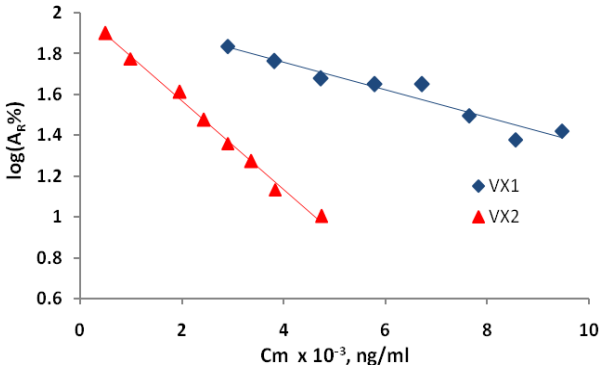


Figure 2. Semi-logarithmic plot of BChE inibition by **VX1** and **VX2** samples

The visual observations are in agreement with the expected quality of the tested samples, since the **VX1** sample originated from a source whose contents were more susceptible to handling-induced degradation and chemical alteration. In addition, AChE was more susceptible to inhibition by both tested samples, requiring much lower inhibitor concentrations to achieve the same reduction in activity as in BChE. This reveals that the AChE-based system provides a significantly higher sensitivity for the analysis of different VX samples compared to the BChE-based inhibition assay.

To further evaluate these effects quantitatively, we focused on the linear regression outputs for model presented by equation (3), which are shown in **Table 2**. The parameter ***B*** corresponds to the slope of the regression line and accounts for both the inhibitory potency and the purity factor of the tested samples, while the parameter ***A*** denotes the intercept on the ordinate and corresponds to the initial enzyme activity in the absence of the inhibitor, with theoretical value of 2. The calculated values for both parameters highly supported the visual findings and reflected the distinct inhibition profiles influenced by the purity-induced factors of the each sample. The obtained intercepts (***A***) for both enzymatic systems showed minor deviations from the theoretical value, confirming that the model closely reflects the initial enzyme activity before the inhibition. The slope values (***B***) corresponded to a higher sensitivity of AChE compared to BChE and a superior potency of **VX2** sample over **VX1**, the latter implying a higher purity of the **VX2** sample. The lower coefficients of determination (R^2) and higher standard errors (***SE***) associated with **VX1** sample successfully quantified the higher degree of experimental noise and data scattering observed visually, likely driven by the presence of interfering impurities. Conversely, the high R^2 values and lower ***SE*** values observed for **VX2** across both enzymes pointed towards less interference caused by impurities compared to **VX1**.

Table 2. The linear regression outputs: the slope (***B***), the intercept (***A***), standard errors of ***B*** and ***A*** (SE_B, SE_A) and the coefficient of determination (R^2)

ChE/sample	$B \pm SE_B$	$A \pm SE_A$	R^2
BChE/VX1	$(-6.77\pm0.77)\times10^{-5}$	2.029±0.050	0.93
BChE/VX2	$(-21.68\pm0.67)\times10^{-5}$	2.003±0.020	0.99
AChE/VX1	$(-7.76\pm0.62)\times10^{-4}$	1.953±0.040	0.96
AChE/VX2	$(-56.26\pm2.03)\times10^{-4}$	1.969±0.019	0.99

Linearization of the data for concentration-dependent inhibition was based on the model represented by equation (2), which was derived assuming pseudo-first order conditions, the presence of only one active inhibitory species (VX), and the absence of any interference caused by impurities in VX samples. If all of the above assumptions were satisfied, the slope ratio of samples **VX2** and **VX1** (B_{VX2}/B_{VX1}) would be the same for both ChE assays, as would the slope ratio determined by AChE and BChE assays (B_{AChE}/B_{BChE}) for each tested sample. This follows from the variables that define the slope in ideal conditions according to equations (2) and (3), since the expression for the slope ratio would reduce to the purity ratio (Q_{VX2}/Q_{VX1}) in the first case and to the bimolecular inhibition constant ratio ($k_{i(AChE)}/k_{i(BChE)}$) in the second.

To evaluate whether these enzyme inhibition assays can be used to quantitatively compare the VX content in tested samples, the ratio of the regression slopes B_{VX2}/B_{VX1} was calculated for each enzyme. Under ideal pseudo-first-order kinetics with a single active inhibitory component and no matrix interference, this ratio should directly reflect the relative purity of the two samples and remain constant regardless of the enzyme used. However, the experimental data (**Table 3**) contradict this ideal model, as they revealed a significant discrepancy between the values obtained by AChE and BChE assays. The facts that AChE-based test yielded a slope ratio that was more than twice the value obtained by BChE (7.25 vs. 3.20) and that the slope ratios were enzyme-dependent, strongly indicate that the ideal kinetic assumptions are not met. This discrepancy provides definitive evidence that the VX sample impurities do not behave as inert diluents, but actively engage in enzyme-specific interactions or interfere with the primary inhibitors bioavailability. These findings demonstrate that ChE inhibition assays cannot be universally and reliably applied for the direct quantitative cross-comparison of VX content across the samples of different and unknown purity, as the unpurified samples matrices actively interferes with the inhibition kinetics in an enzyme-specific manner.

Table 3. Comparative presentation of regression-derived slope ratios and the resulting calculated values: relative purity of **VX1** sample versus **VX2** sample (Q_{VX1}), and the relative k_i of BChE versus AChE ($k_{i(BChE)}$).

Enzyme	B_{VX2}/B_{VX1}	Q_{VX1} (%)
AChE	7.25	13.79
BChE	3.20	31.21
Sample	B_{AChE}/B_{BChE}	$k_{i(BChE)}$ (%)
VX1	11.46	8.72
VX2	25.95	3.85

Since the bimolecular inhibition constant is a characteristic property of each individual enzyme-inhibitor system, a biochemical fingerprint for the identification of an unknown irreversible inhibitor can be obtained by assessing its inhibitory potency against a group of structurally different or related enzymes. The inhibition spectrum (or relative inhibitory response profile), obtained by normalizing all k_i values for the enzymes in the group as a percentage of the highest value within that set, serves as a unique multi-enzyme barcode for identifying inhibitors.

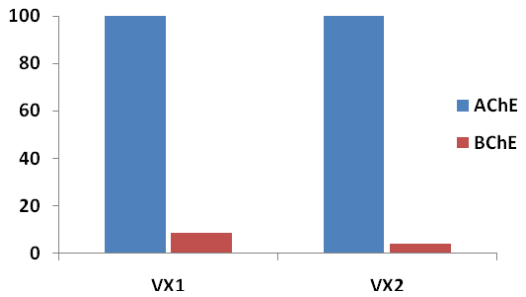


Figure 3. Inhibition spectrum of **VX1** and **VX2** samples

The two-enzyme inhibitory spectrum was established for each VX sample using relative k_i values derived from the ratio of the regression slopes between AChE and BChE. Although this approach mathematically does not provide new independent information, the graphical comparison

of these spectra **(Figure 3)** allows for visual observation of asymmetry between the two tested samples.

3.2. GC-MS analysis

The chromatogram of the **S1h** sample is shown in **Figure 4**. The total ion chromatogram (TIC) is shown in black, whereas the extracted-ion chromatogram at m/z 114 is shown in pink. Only a very small VX peak was observed at a retention time of approximately 17.350 min. Two additional relevant compounds were tentatively identified by comparison with the NIST library: *bis*[2-(diisopropylamino)ethyl] sulfide at 18.832 min, with an 81% library match, and *bis*[2-(diisopropylamino)ethyl] disulfide (DES) at 21.175 min, with an 88 % library match. The markedly lower VX response relative to the DES response qualitatively indicates extensive degradation of the **S1h** sample, only a small residual amount of the parent compound remaining.

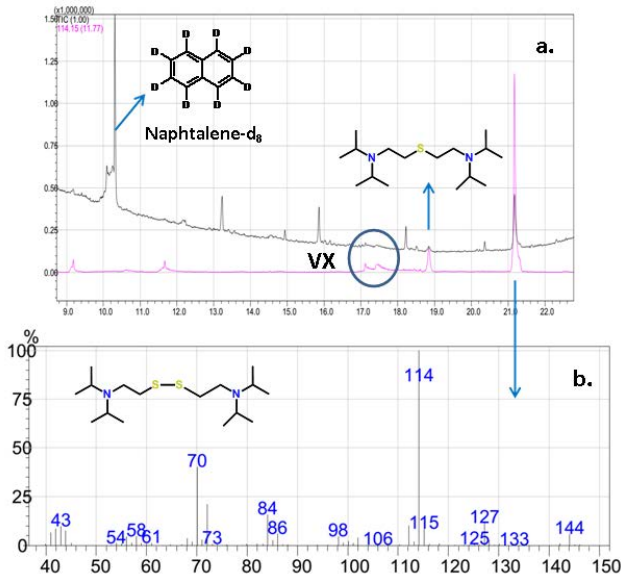


Figure 4. (a) Chromatogram of the **S1h** sample: TIC (black) and EIC at m/z 114 (pink); **(b)** mass spectrum of *bis*[2-(diisopropylamino)ethyl] disulfide (DES).

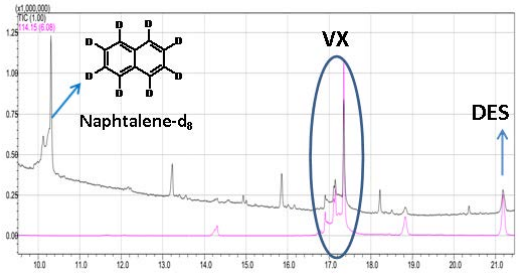


Figure 5. Chromatogram of the **S2h** sample: TIC (black) and EIC at m/z 114 (pink).

In the **S2h** chromatogram (**Figure 5**), VX was the dominant component, accompanied by smaller peaks tentatively assigned to DES and the corresponding sulfide. Pronounced tailing of the VX peak was observed, likely reflecting non-optimized chromatographic conditions. To assess whether the relative VX contents could be compared semi-quantitatively, the VX-to-internal-standard peak-height ratios were calculated for the **S2** and **S2h** samples. The ratio between these responses was consistent with the ratio of the nominal sample concentrations, supporting the use of this approach for a rough relative comparison under the applied conditions. On this basis, the residual VX contents in **S1** and **S1h** samples were estimated to be approximately one-fifth of those in the corresponding **S2** and **S2h** samples, indicating a relative purity of 20 % for the **VX1** sample compared to the **VX2** sample (Q_{VX1}). Several additional peaks observed in both chromatograms at retention times of approximately 13.2, 15.9, and 18.2 min did not contain the m/z 114 ion and were tentatively attributed to long-chain hydrocarbon contaminants originating from the GC column or inlet septum.

3.3. The practical relevance of ChE inhibition assays and future perspectives

The quantitative ratio of the remaining VX content in the tested samples determined by the slope-ratio method using AChE, BChE and by GC-MS analysis was found to be 7.25, 3.20 and 5, respectively, corresponding to a relative purity of the **VX1** sample of 14 %, 31 % and 20 %.

Despite the observed discrepancies between enzymatic assays and reference GC-MS analysis, the obtained results have significant practical and predictive value for analytical applications. This significance is manifested trough two key aspects, the first of which relates to the validity of the method as a screening tool, and the second to the dependence of the assay accuracy on the VX content ratio in the samples and their absolute purity.

Regarding the former, both applied enzymatic assays consistently identified the **VX1** sample as unusable for R&D applications. The results show that VX content in the **VX1** sample is below 31 % relative to the sample of **VX2**, so its absolute purity cannot exceed 31 %, the maximum value that can be reached if the **VX2** sample were 100 % pure. From an analytical perspective, whenever the determined quantitative ratio between samples exceeds 3, unambiguous information regarding the unsuitability of at least one sample is generated. In this context, the numerical discrepancies between the methods lose relevance, as the primary objective of rapid screening of inappropriate samples is successfully achieved. Therefore, these enzymatic assays provide a clear, rapid diagnostic signal to disqualify low-purity samples without immediately relying on cost-intensive chromatographic techniques.

Considering the second aspect, it is reasonable to expect that the convergence of results among different methods will increase as the difference in the quantitative inhibitor ratio decreases. However, this trend is a direct function of the absolute purity of the tested samples, which dictates the profile and variance of the interfering matrix impurities. Assuming that the ratio of residual VX content in the tested samples was exactly 5, the feasibility of using these enzyme assays to obtain analytically acceptable results can be evaluated through two extreme hypothetical scenarios, which are presented below.

In the high-purity scenario (e.g., samples of **VX2** and **VX1** had purity of 95 % and 19 %), the difference in the impurity matrix was extreme (5 % vs. 81 %), explaining the pronounced deviations in the enzymatic assays. In this case, samples with a lower quantitative ratio (< 5), regardless of their absolute purity, may only have a reduced difference in impurity levels. Therefore, it can be predicted that their testing would inherently lead to analytically acceptable agreement between the two enzyme assays, at the point when their quantitative ratio falls below a certain limit.

In the low-purity scenario (e.g., samples of **VX2** and **VX1** had purity of 5 % and 1 %), the impurity fractions were inherently similar (95 % vs. 99 %). Reducing the quantitative ratio would reduce the difference in impurity levels, but only if the absolute purity of the tested samples remain at the level of the low purity scenario. However, samples with higher absolute purity and the same quantitative ratio of 5 may only have an increased difference in impurity levels and consequently a greater difference in enzyme assay results. In this case, the performance of assays at ratios below 5 for a highly purified sample remains an open question.

Given that GC-MS analysis confirmed a significantly higher abundance of degradation products in **VX1** sample, the low-purity scenario can be rejected. This finding, supported by the results of ChE inhibition tests which pointed at high impurity loads in **VX1** sample, implies that the test conditions were closer to the high-purity scenario. Accordingly, the assumption that lower quantitative ratios of the tested VX samples will yield improved correlation in the enzymatic assays is well-founded. To fully define the accuracy windows and practical boundaries of enzymatic inhibition assays in VX sample analysis, further systematic investigations and development of matrix-matching calibration strategy are required. The focus of future work should be on establishing a robust calibration framework and selecting the most resilient and suitable single enzyme for high-throughput, rough quantitative profiling of partly-degraded VX samples.

5. CONCLUSION

The aim of this work was to examine the influence of impurities, present in two VX samples with different degradation profiles, on the results of ChE inhibition assays and their application in the quantitative comparison of residual VX content in stocks that have undergone distinct storage-induced decomposition.

The experimental results strongly supported the initial assumption regarding the varying degrees of purity between the two tested samples. The linear regression analysis for the inhibition of AChE and BChE yielded distinct profiles regarding both inhibitory potency and data consistency, driven by purity-related matrix interferences in each sample. The calculated relative purity values for **VX1** sample determined by AChE and BChE inhibition assays correctly classified the sample as heavily contaminated and unsuitable for application, a finding fully supported by preliminary GC-MS data. The dual-enzyme screening approach demonstrated excellent feasibility for ensuring the accuracy and reliability of enzyme-based assays during the quantitative determination of target inhibitors in non-homogeneous samples. The combination of regression metrics and slope ratio divergence across two distinct enzymes served as an effective tool to detect the presence of interfering impurities, assess relative sample heterogeneity and evaluate the susceptibility of different target enzymes to matrix effects.

The proposed methodology provides a robust and effective analytical framework for sample triage, preliminary quality assessment and semi-quantitative estimation of relative inhibitor content prior to definitive chromatographic analysis. While GC-MS remains indispensable for the final, precise quantification of highly heterogeneous matrices, the enzymatic approach serves as an excellent first-line screening method. Definitive mapping of the applicability threshold, which includes working with a broader sample library with varying purity gradients, a wider panel of alternative enzymes, and systematic cross-referencing with GC-MS data, will optimize this inhibition assay into a standardized screening protocol, fully capable of achieving a high degree of accuracy within analytically acceptable results.

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