Forensic Chemistry 35 (2023) 100526

Contents lists available at ScienceDirect

Forensic Chemistry

journal homepage: www.sciencedirect.com/journal/forensic-chemistry

On-site detection and laboratory verification of the presence of nerve agent biomarkers using dried blood spots

Mirjam de Bruin-Hoegée [a,b,*], Alex Fidder [b], Tomas van Groningen [b], Marcel J. van der Schans [b], Daan Noort [b,1], Arian C. van Asten [a,c]

[a] van 't Hoff Institute for Molecular Sciences, Faculty of Science, University of Amsterdam, P.O. Box 94157, 1090GD Amsterdam, The Netherlands
[b] TNO Defence, Safety and Security, Dep. CBRN Protection, Lange Kleiweg 137, 2288GJ Rijswijk, The Netherlands
[c] CLHC, Amsterdam Center for Forensic Science and Medicine, University of Amsterdam, P.O. Box 94157, 1090GD Amsterdam, The Netherlands

ARTICLE INFO

Keywords:
Chemical warfare agents
Novichok nerve agents
Biomarkers
Mass spectrometry
Protein adducts
Forensic chemistry

ABSTRACT

The analysis of biomedical samples is important for the investigation of the alleged exposure to chemical warfare agents. The present study aims to use dried blood spots for portable detection and laboratory verification of organophosphate poisoning. After in-vitro incubation of blood with Novichok A-230, A-232 and A-234 and sarin, a volume of 25 and 50 μL was spotted on a protein saver card. Subsequently, the dried spots were extracted and analyzed by a mobile cholinesterase test kit. In addition, butyrylcholinesterase (BChE) was isolated and digested by pepsin followed by analysis with liquid chromatography tandem mass spectrometry (LC-MS/MS). The fluoride-activated samples were analyzed by gas chromatography tandem mass spectrometry (GC–MS/MS) and LC-MS/MS. It was found that the CWA induced cholinesterase inhibition was remarkably stable in dried blood spots. Even after at least one month storage under ambient conditions, the same linear reduction was visible as function of nerve agent exposure. Additionally, nonapeptide adducts were identified by LC-MS/MS one month after exposure. Also, intact Novichok nerve agents and regenerated sarin were observed by GC–MS/MS. In addition to the stability of the sample, important benefits of the proposed method include the less invasive sample collection and safer and easier shipping and storage conditions. In conclusion, this study shows the feasibility of using on-site detection and state-of-the-art laboratory analysis of dried blood spots for unambiguous verification of nerve agent exposure.

Introduction

Despite the efforts of the Organisation for the Prohibition of Chemical Weapons (OPCW) to achieve a world free of chemical weapons, there is still an ongoing threat of chemical warfare agent (CWA) use. For example, nerve agents have recently been used in large-scale attacks during the conflict in the Syrian Arab Republic [1–3], and to assassinate individuals in Malaysia [4], and the United Kingdom [5,6]. In addition to evidence such as witness statements, video footage, and satellite imagery, chemical analysis is important for the investigation of the alleged use of toxic chemicals.

Nerve agents are mostly organophosphorus compounds and are prohibited under the Chemical Weapons Convention. Their toxicological mechanism of action is acetylcholinesterase inhibition, resulting in overstimulation of the cholinergic pathway [7]. The nerve agent sarin (GB) is one of the most potent chemical weapons, affecting the nervous system [8]. A 30-minutes exposure to 4 $\mu g/m^3$ already gives mild effects, such as pinpoint pupils, blurred vision, runny nose, and shortness of breath [9]. An increased concentration of 0.19 mg/m^3 is lethal or gives severe health effects, which can include muscular twitching, fluid accumulation within the airways, and cessation of breathing. Besides, Novichoks, considered the fourth generation of CWAs, exert the same mechanism of action as other organophosphate nerve agents, although they are expected to be even more potent [10,11]. Accordingly, the median lethal concentration (LCt_{50}) is estimated to be eight times lower than it is for sarin [12].

Since CWAs are highly reactive chemicals, the analysis is mainly focused on degradation products in environmental samples or

* Corresponding author at: van 't Hoff Institute for Molecular Sciences, Faculty of Science, University of Amsterdam, P.O. Box 94157, 1090GD Amsterdam, The Netherlands.
E-mail address: mirjam.debruin@tno.nl (M. de Bruin-Hoegée).
[1] Current address: Organisation for the Prohibition of Chemical Weapons, Johan de Wittlaan 32, 2517 JR The Hague, The Netherlands.

https://doi.org/10.1016/j.forc.2023.100526
Received 27 June 2023; Received in revised form 8 August 2023; Accepted 22 August 2023
Available online 23 August 2023

metabolites and protein adducts in biomedical matrices. An overview of major pathways is given in Figs. S1 and S2 in the Supplemental Information. Typical on-site detection techniques are based on ion mobility spectrometry, flame photometry, Raman and IR spectroscopy, and mass spectrometry [13]. Most commonly applied laboratory techniques are gas and liquid chromatography combined with mass spectrometry [14].

One of the remaining challenges for forensic examinations concerns the stability of the actual sample during collection, transportation, storage, and analysis [15]. A possible way of preserving the CWA signature might be through drying of blood plasma or applying whole blood on filter paper. This fixation might maintain the reactive components into a more stable matrix and could potentially also slow down degradation of other relevant components. Advantages of using dried blood spots (DBS) include reduced sample volume, safer handling, and less stringent storage conditions [16]. Also, less invasive sample collection is required since only a small amount of blood is obtained by a finger puncture instead of venous blood sampling which usually needs to be performed by a medical practitioner. For instance, sulfur mustard adducts of human serum albumin can be detected in dried plasma samples weeks after drying [17]. Also, nerve agents and simulants thereof have been detected in dried sample spots as free agent [18,19], hydrolysis product [20–22], tyrosine adduct [23], and butyrylcholinesterase (BChE) adducts [15,24]. Another category of chemical threat agents that have been studied through detection in dried blood spots are very powerful synthetic opioids like fentanyl [25–35]. For all compounds, the most commonly applied analytical technique was liquid chromatography-tandem mass spectrometry (LC-MS/MS), but also paper spray mass spectrometry (PS-MS) [22,26,29], various thermal desorption methods [19,35], online solid phase extraction (SPE) tandem mass spectrometry [30,32], and the Ellman assay [24] have been proven to be valuable methods.

Currently, sophisticated analytical methods have to be used to evaluate the DBS for organophosphate poisoning. Because of the life-threatening effects of a nerve agent intoxication, first responders or military personnel may need fast results which cannot easily be obtained by laboratory analysis. To overcome this limitation, on-site tests may be used, such as the user-friendly cholinesterase (ChE) mobile test kit, which was designed for whole blood [36]. The toxicological mechanism of organophosphate poisoning is acetylcholinesterase (AChE) and BChE inhibition [7], which is the basis of the detection principle used by the field kit. The application of the Ellman photometric assay produces a yellow color after reaction with AChE or BChE [37,38]. It is expected that this portable technique is not only suitable for whole blood, but also for DBS analysis.

Chemical threat agents that have been scarcely studied are the Novichok nerve agents, which have only recently been added to Schedule 1 of the Chemical Weapons Convention (CWC). In 2021 two studies identified biomarkers for Novichok compounds in blood

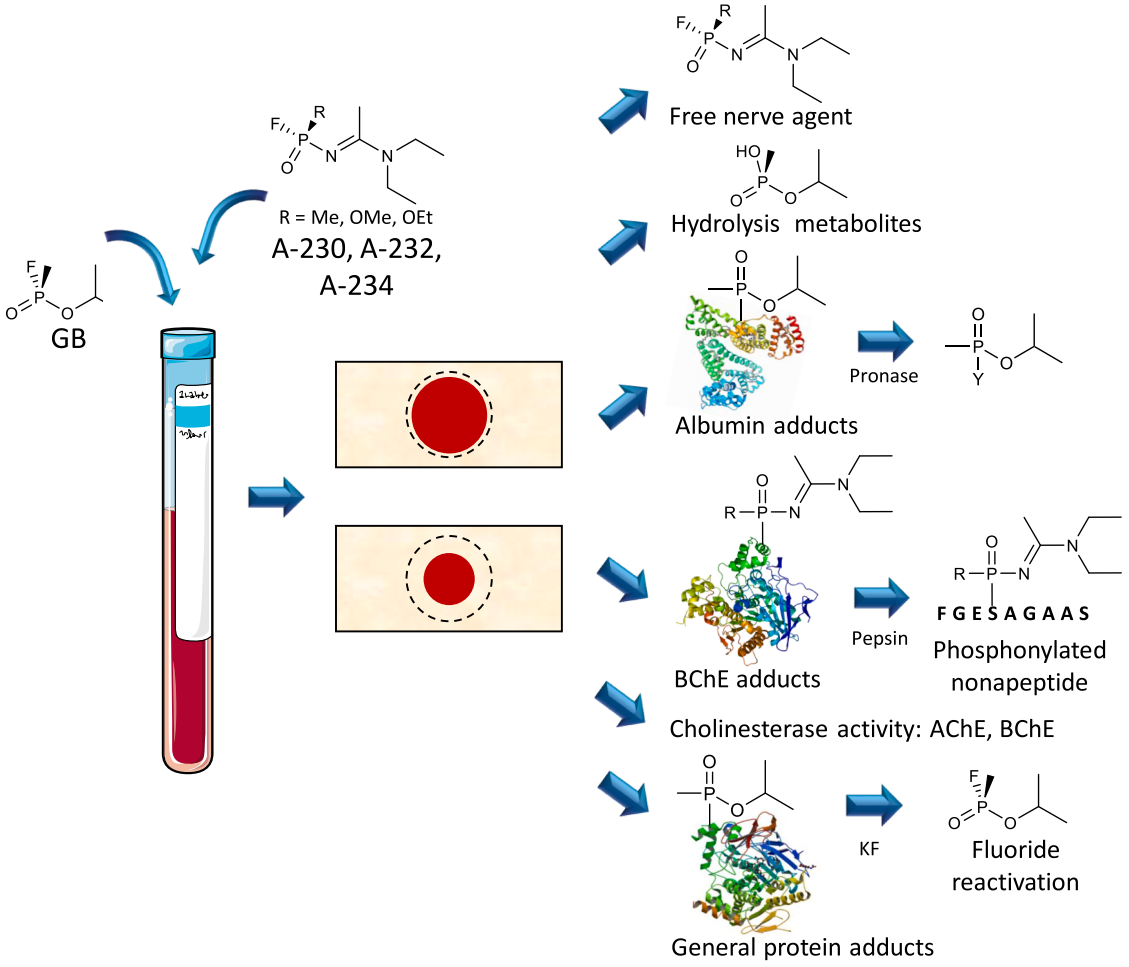

Fig. 1. Method for biomarkers analysis in dried blood spots after nerve agent incubation of human whole blood. Blood was incubated with sarin (GB), Novichok A-230, A-232, or A-234. Subsequently, 25 and 50 μL blood was spotted on a protein saver card and dried. Various biomarkers could potentially be detected. 1) The nerve agents itself and its 2) hydrolysis metabolites; 3) tyrosine adducts in albumin; 4) nonapeptide with serine adduct in butyrylcholinesterase (BChE); 5) the acetylcholinesterase (AChE) or BChE activity; and finally, 6) the protein adducts can be reactivated by fluoride resulting in the release of the original nerve agent.

samples, based on mass spectrometric analysis of a nonapeptide adduct [39,40]. In addition, human serum albumin protein modifications of Novichok A-234 in blood can be detected by LC-MS/MS [41,42] while the intact or regenerated nerve agent can be identified by both GC–MS/MS and LC-MS/MS [42]. Lastly, degradation products in urine could be found by LC-MS/MS after derivatization [43] or by hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) [44]. Up to now, to our knowledge, no studies have been published concerning the detection of Novichok agents in dried blood spots or by using these samples for on-site point-of-care diagnosis by measuring the cholinesterase activity.

Therefore, the aim of this research is to explore whether dried blood spots can be used for both on-site and laboratory analysis of nerve agent biomarkers. A variety of analytical techniques were used for the identification of the specific agent (Fig. 1). After incubation of blood with sarin (GB) and three types of Novichok nerve agents at various concentrations, dried blood spots were prepared and processed according to the procedure for the particular biomarkers. LC-MS/MS was used to analyze phosphonylated tyrosine adducts, hydrolysis metabolites, and BChE adducts by applying the nonapeptide assay. Both LC-MS/MS and gas chromatography-tandem mass spectrometry (GC–MS/MS) were utilized for analyzing the intact nerve agent either present as free agent in the sample or as reactivated organophosphate after fluoride reactivation. In this article a toolbox is provided for the verification of exposure to nerve agents by combining a portable technique and state-of-the-art laboratory analysis to enable fast and reliable diagnosis after potential organophosphate intoxications.

Experimental

Safety

Due to the potent nature of nerve agents, all experiments were performed by trained personnel in the dedicated High-Tox facility at TNO (Rijswijk, The Netherlands), which is allowed under the Chemical Weapons Convention to synthesize and handle chemical warfare agents for research purposes. The chemicals were handled in solution and the concentrations were kept as low as possible to reduce the hazard. Precautions were taken to prevent accidental exposure, including the use of gloves and eye protection.

Chemicals

The nerve agents GB (o-Isopropyl methylphosphonofluoridate, CAS: 107–44-8), Novichok A-230 (methyl-(1-(diethylamino)ethylidene) phosphonamidofluoridate, CAS: 2387496–12-8), A-232 (Methyl (1-(diethylamino)ethylidene)phosphoramidofluoridate, CAS: 2387496–04-8), and A-234 (Ethyl (1-(diethylamino)ethylidene)phosphoramidofluoridate, CAS: 2387496–06-0) were synthesized in the high-tox facility of The Netherlands Organization for Applied Scientific Research (TNO, Rijs-wijk, The Netherlands). The purities of the synthesized compounds exceeded 95% as demonstrated by nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC–MS), and liquid chroma tography-mass spectrometry (LC-MS). Butyrylcholinesterase monoclonal antibodies 3E8, diethyl ether, dynabeads protein G, and hydrochloric acid were obtained from Thermo Scientific (Waltham, MA, USA). Acetic acid, ammonium bicarbonate (ABC), dimethyl pimelidate (DMP), ethyl acetate, formic acid, pepsin from porcine gastric mucosa (≥2,500 units/mg protein), phosphate buffered saline with 0.05% Tween-20 (PT buffer), phosphate buffered saline (PBS), potassium fluoride, protease from Streptomyces griseus (pronase, ≥3.5 units/mg solid), sodium bicarbonate, triethanolamine buffer (0.2 M), and tris buffered saline, were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Additionally, dichloromethane (DCM, Biosolve), isopropanol (IPA, Acros Organics), and water (MilliQ, SimPak® 1) were employed. Acetone, acetonitrile, chloroform, and hexane were purchased from Biosolve (Valkenswaard, The Netherlands). The purities of the chemicals exceeded 97%. Human blood containing EDTA anticoagulant was obtained from Sanquin (Amsterdam, The Netherlands).

Preparation of dried blood spots

For each nerve agent two incubated samples were prepared with different concentrations. A solution of GB, A-230, A-232, or A-234 (100 μL, 1 μM) in IPA was added to 3 mL of human blood (final concentration 33 nM) to achieve an inhibition of 100% (assuming 3 nM AChE and 30–45 nM BChE in human whole blood). It should be noted that a 100% inhibition is based on the average level of ChE in human blood. Because there is some variation between people, the inhibition can be lower than 100% or there can be a small excess of nerve agent that does not react. To make it more likely that all ChE has reacted, another sample was prepared with a large excess of nerve agent. In this case, either 100 μL of 13 μM or 40 μM nerve agent in IPA was added to 3 mL of blood (final concentration 0.4 μM or 1.3 μM). The samples were incubated overnight with gentle shaking at 50 rpm. Afterwards solutions of 25, 50, and 75% nerve agent inhibited samples were made, by mixing the blank and the 100% inhibited sample in various ratios. Five blood spots with a volume of 25 and 50 μL of each sample were spotted on Whatman 903™ protein saver cards and dried overnight under ambient conditions. Next the cards were stored for 3 days up to 3 months at 20–25 °C and 50–70% relative humidity. The size of the DBS that was further processed was dependent on the analysis method. Either a 25 μL spot was cut in half (12.5 μL), or a 25 or 50 μL spot was collected by using a small pair of nail scissors.

On-site analysis of dried blood spots

For the portable assessment of cholinesterase inhibition, the ChE check mobile test kit was used, based on a reference method developed by Worek et al. [38]. The suggested protocol included the use of 10 μL whole blood, which could be obtained by using a finger-prick device. In this research initial experiments were performed with 10 μL liquid blood as well. This was later extended to the application of half a spot (12.5 μL) created from a volume of 25 μL whole blood, which was cut and brought into a vial with 2 mL buffer, that was provided with the ChE check mobile test kit. The DBS was extracted for one hour with gentle shaking. Afterwards, the cap was replaced for a reagent cap and either the AChE or BChE activity was measured by the ChE check mobile test kit (Securetec, Neubiberg, Germany).

Protein isolation and digestion from dried blood spots

Human butyrylcholinesterase adducts

The 25 μL spots, inhibited with an excess of 0.4 μM, were extracted in 0.5 mL PBS for 30 min with gentle shaking. A volume of 200 μL was added to the KingFisher magnetic particle processor (mL system, Thermo Fisher, Waltham, MA, USA) for isolation of human BChE using anti-HuBChE antibodies conjugated to magnetic beads according to the method as described by D. Noort et al. [39]. After digestion with pepsin the nonapeptide adduct could be analyzed with LC-MS/MS as described in section 2.6.

Tyrosine adducts

In addition, samples were digested by pronase using a method designed for the analysis of tyrosine adducts after sarin exposure [3]. A volume of 100 μL whole blood or a 50 μL-based dried blood spot extracted in 0.5 mL PBS was added to a 2 mL Eppendorf vial with 0.6 mL of acetone for protein precipitation. After centrifugation at 14000 rpm for 5 min (Eppendorf 5417R), the acetone layer was discarded, and the protein precipitate was allowed to dry in the air at ambient temperature for 30 min. Then, the isolated protein was dissolved in 400 μL of aqueous ABC buffer (50 mM) and an aqueous solution of 100 μL of pronase (10

mg/mL in 50 mM ABC). The samples were incubated for 90 min in a Thermoshaker (Grant-bio PHMT) at 50 °C and 500 rpm. Subsequently, the digest was purified using a reversed phase solid phase extraction (SPE) C18 column (Bakerbond SPE™) [45]. After drying under nitrogen, the sample was dissolved in 50 µL water with 0.2% v/v formic acid and analyzed with LC-MS/MS as described in Section 2.6.

Phosphonic acid metabolites

Additionally, the samples were prepared for phosphonic acid analysis, based on sample preparation procedures for blood plasma. First 400 µL of the PBS extract of a 50 µL-based DBS and 100 µL 10 M HCl to protonate the phosphonic acids, were mixed in a 4 mL vial. Then, the sample was extracted four times with 2 mL diethyl ether/acetonitrile (85/15 %v/v). Finally, all extracts were combined and dried under nitrogen. Subsequently, the residue was dissolved in 50 µL water with 0.2% v/v formic acid and analyzed with LC-MS/MS as described in Section 2.6.

Intact agent analysis and fluoride-induced reactivation of protein adducts

For intact agent analysis, liquid extraction was performed with 300 µL DCM for 200 µL whole blood or a 50 µL DBS. The extract was subsequently analyzed by GC–MS as described in Section 2.6.2. For the fluoride reactivation [3,42] a 4 mL vial was filled with 400 µL liquid blood or a 400 µL PBS extract of a 50 µL dried blood spot and 1.2 mL acetate buffer (0.2 M, pH = 3.5), and placed in a water bath at 25 °C. After 10 min, 75 µL potassium fluoride solution (5.25 M) was added resulting in a final fluoride concentration of 250 mM. The vial was kept in the water bath for 15 additional minutes at 25 °C. Afterwards, the sample was loaded on the conditioned cartridge and washed with 0.5 mL, 0.8 M sodium bicarbonate solution. Then the sample was eluted with 2 mL chloroform and collected in a centrifugal tube. The water was frozen by holding the tube in dry ice mixed with acetone. The organic layer was collected and added to a 4 mL vial together with 150 µL ethyl acetate. The sample was concentrated under a gentle stream of nitrogen until a final volume of 100–150 µL was reached and subsequently analyzed by GC–MS/MS, as described in Section 2.6.3. Conditioning of the cartridge was performed as follows: a NEXUS LRC cartridge (Agilent Technologies) with a bed mass of 60 mg and a volume of 10 mL was conditioned with subsequently 2 mL hexane, two times 4 mL ethyl acetate, two times 4 mL chloroform, and two times 5 mL water.

Instrumental analysis

LC-MS/MS

The nonapeptide and tyrosine adducts and the hydrolysis products of GB, Novichok A-230, A-232, and A-234 were analyzed on a Waters Acquity ultra-high pressure liquid chromatographic (UPLC) system equipped with a Waters Acquity HSS T3 C18 column (1.8 µm, 2.1 × 100 mm). Samples were kept at 8 °C in the autosampler and the injection volume was 5 µL. The analysis was performed at room temperature with a gradient flow of 100 µL/min. Eluent A was 0.2 v% formic acid in MilliQ water and eluent B was 0.2 v% formic acid in acetonitrile. The optimized gradient elution settings are specified in Section 2 of the Supplemental Information. The UPLC system was coupled to a Waters (Milford, MA, USA) Xevo TQ-S triple-quadrupole mass spectrometer, equipped with electrospray Ionization for detection of the analytes in positive ionization mode. A capillary voltage of 3.5 kV was used with a nitrogen cone gas flow of 150 L/h and an argon collision gas flow of 0.19 mL/min. For each category of biomarkers, all nerve agents could be analyzed with a single chromatographic method [39]. Data was first acquired in full scan mode and afterwards in multiple reaction monitoring (MRM) mode. The monitored mass transitions can be found in Section 2 of the Supplemental Information. Compounds were identified by comparison with reference standards, in accordance with OPCW guidelines for biomedical analysis [39].

GC–MS

The intact agent was analyzed on an Agilent 7890B GC equipped with an Agilent VF-5 ms column (5% phenylmethyl polysiloxane, 30 m × 0.25 mm × 0.25 µm). One microliter sample was injected using an autosampler (Combi Pal, Ctc analytics). Helium was used as the carrier gas at a constant flow of 1 mL/min. The GC injector was operated in splitless mode at 275 °C. The split vent was opened at 0.5 min at 50 mL/min. The oven temperature was maintained at 90 °C for 1 min., then ramped at 10 °C/min. to 325 °C, and held for 5 min. Detection was performed with an Agilent 5977A MS, which operated in electron ionization (EI) mode with an ionization potential of 70 eV and a scan range of 25–550 mass units. A solvent delay time of 2.6 min was applied. Compounds were identified by comparison with reference standards.

GC–MS/MS

The free nerve agent and the reactivated organophosphates after fluoride addition were also measured on an Agilent 8890 GC with an Agilent VF-5 ms column (5% phenylmethyl polysiloxane, 30 m × 0.25 mm × 1 µm). A volume of 1 µL was injected with a PAL RTC autosampler. A helium column flow of 1 mL/min was used. The GC injector was operated in splitless mode at 280 °C. The split vent was opened at 0.5 min with a flow of 50 mL/min. The oven temperature was maintained at 40 °C for 1 min, then ramped at 10 °C/min to 240 °C, and then increased at 100 °C/min to 280 °C, which was maintained for 3 min. The GC system was coupled to an Agilent 7000D triple-quadrupole mass spectrometer, which was operated in electron ionization (EI) mode. Nitrogen was used as a collision gas at 1.5 mL/min and a quench flow of 2.25 mL/min helium was applied. The solvent delay time was 3 min. Data were acquired in MRM mode. The monitored mass transitions and quantification method are described in Section 2 of the Supplemental Information. The method was in agreement with the OPCW guidelines for biomedical analysis [46].

Results & discussion

On-site analysis of cholinesterase inhibition

Fig. 2 shows the reduced cholinesterase activity after nerve agent inhibition of blood. As expected, a negative linear correlation was observed after exposure to GB or Novichok nerve agents. For GB, blood was exposed to an excess of nerve agent (1.3 µM), to enable occupation of all accessible protein sites. It was expected that the remaining non-reacted GB would be hydrolyzed after one day and would be incapable of reacting with the non-exposed blood when mixing exposed and non-exposed blood in various ratios. For the Novichoks a 40 times lower concentration was applied (33 nM), because intact nerve agent remained in the sample, as described in section 3.3. This would otherwise react with the non-exposed blood when mixing in various ratios resulting in a lower ChE activity (Fig. S4 in the Supplemental Information). For all Novichok variants tested the same degree of inhibition was observed, hence it was decided to present the average value for all variants in Fig. 2. Because of the limited number of supplies for the ChE check mobile test kit, only three concentrations were evaluated for AChE inhibition of Novichok nerve agents. Fig. 2 illustrates activities measured in dried blood spots one month after storage of the dried blood spots at ambient conditions. The activities shown for non-exposed blood correspond well with reference values for AChE and BChE activity of respectively 33 – 49 U/gHb and 1623 – 3861 U/L, reported by Worek et al. based on blood analysis of 242 volunteers [36]. For BChE similar behavior for liquid blood and dried blood spots is observed, although the dried blood spot consistently yields slightly lower activity. However, the AChE activity in dried blood spots was considerably lower than the activity in whole blood. This might be due to loss of AChE in vesicles that are generated during the concentration of red blood cells, which results in a reduced enzymatic activity [47]. Therefore, the AChE activity measurement of DBS does not provide a reliable indicator of CWA

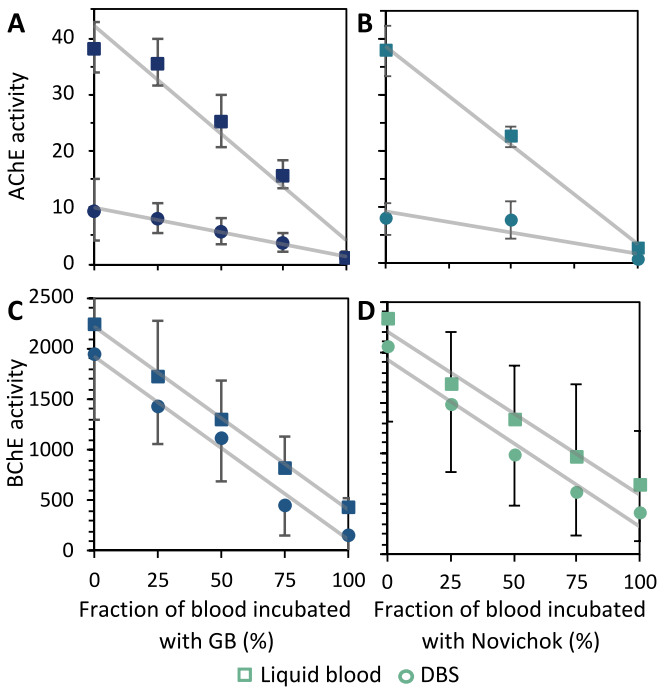

Fig. 2. Cholinesterase activity of 10 μL liquid blood (□) and half a 25 μL-based DBS (12.5 μL) (○), one month after storage of the dried spots at ambient conditions. For GB an excess of 1.3 μM incubation was assumed to give 100% inhibition. For Novichok the fraction of blood incubated with 33 nM Novichock nerve agent is shown. A) AChE activity after GB inhibition (n = 7), B) Average AChE activity after Novichok inhibition A-230, A-232, and A-234 (n = 3), C) BChE activity after GB inhibition (n = 8), D) Average BChE activity after Novichok A-230, A-232, and A-234 inhibition (n = 9). Error bars represent ± 1 standard deviation (for clarity only positive error bars are shown for BChE activity of liquid blood and negative error bars for DBS).

exposure. Interestingly, the activities measured in dried blood spots did not significantly change over a period of three months (Fig. S5, Supplemental Information), which makes it a promising tool for retrospective nerve agent diagnosis.

Detection of BChE nonapeptide adducts by LC-MS/MS

A more specific method to verify nerve agent exposure and identify the agent is by utilizing a semi-targeted human butyrylcholinesterase nonapeptide assay. The nonapeptide adducts of sarin and Novichok nerve agents (incubation level 0.4 μM) were identified in dried blood spots, one month after storage of the dried spots at ambient conditions, as illustrated in Fig. 3. The biomarkers were verified with the use of references of Novichok spiked in plasma. Although the assessment of Novichok exposure in human plasma has been investigated before [39], the current research demonstrated for the first time that these BChE adducts can also detected in dried blood spots.

In addition, the sensitivity of the method was assessed. Fig. 4 shows the extracted ion chromatograms of the nonapeptide adduct of A-234 for various percentages of inhibited blood. In a 25 μL dried blood spot with 50% inhibition, the biomarker could clearly be identified, while at 25% inhibition its presence is near the detection limit. The presence of the biomarker did not show a linear trend, which is likely due to losses which occurred during the extensive sample preparation procedure. In future research, this might be resolved by using an internal standard, that could be added prior to the sample preparation. To give an indication how the DBS method relates to the direct detection of the nonapeptides in blood, a reference of Novichok A-234 was spiked in 12.5 μL plasma. The BChE was isolated with the same batch of magnetic beads and subsequently digested with the same pepsin solution. This should

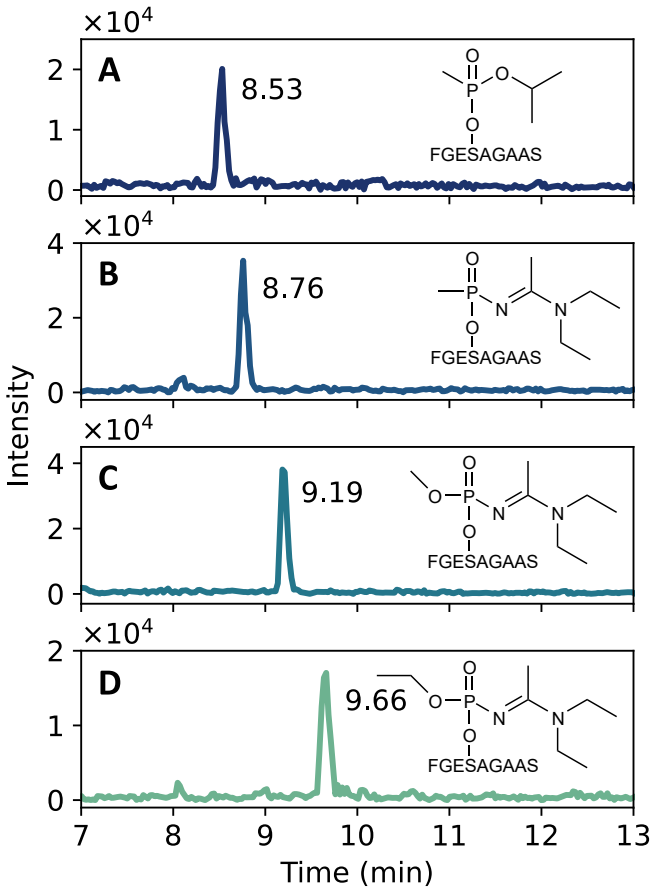

Fig. 3. Extracted ion chromatograms of human BChE adducts measured in 100% inhibited dried blood spots (25 μL, 0.4 μM), one month after storage of the dried spots at ambient conditions. A) Sarin (m/z 916.3 → 778.3), B) Novichok A-230 (m/z 970.4 → 778.3), C) Novichok A-232 (m/z 986.4 → 778.3), D) Novichok A-234 (m/z 1000.4 → 778.3).

then in principle give a similar biomarker concentration as for the 25 μL DBS (assuming 55% of plasma in blood). As demonstrated in Fig. 4E-F, a 1.5-fold reduction in sensitivity is visible for DBS compared to plasma.

Analysis of free and regenerated nerve agent by GC–MS/MS and LC-MS/MS

The unambiguous verification of nerve agent exposure requires the identification of multiple biomarkers or the application of at least two analytical techniques [46]. A suitable supplementary method is the analysis of nerve agents after fluoride-induced reactivation by GC–MS/MS and LC-MS/MS. Earlier research demonstrates that very low concentrations, even below toxicologically relevant exposure levels, could be monitored in plasma and serum using this reactivation procedure [48,49]. Therefore, the current study evaluates whether this method could be extended to dried blood spots as well. Fig. 5 demonstrates that regenerated sarin was detected by GC–MS/MS using dried blood spots. The same result was found for liquid whole blood (Fig. S6 in the Supplemental Information). No intact sarin was detected in dried spots of human whole blood, exposed to this nerve agent, without KF addition. This confirms that the analyzed sarin is the result of regeneration after KF addition and hence was bound to the biomatrix. A concentration of 5 ng/mL was established (n = 3) after processing of a 50 μL dried blood spot. This is a higher level than expected based on the available ChE sites. A partial explanation would be the formation of other protein adducts besides ChE-adducts. In addition, it is likely that some solvent was evaporated during the sample preparation, resulting in a more

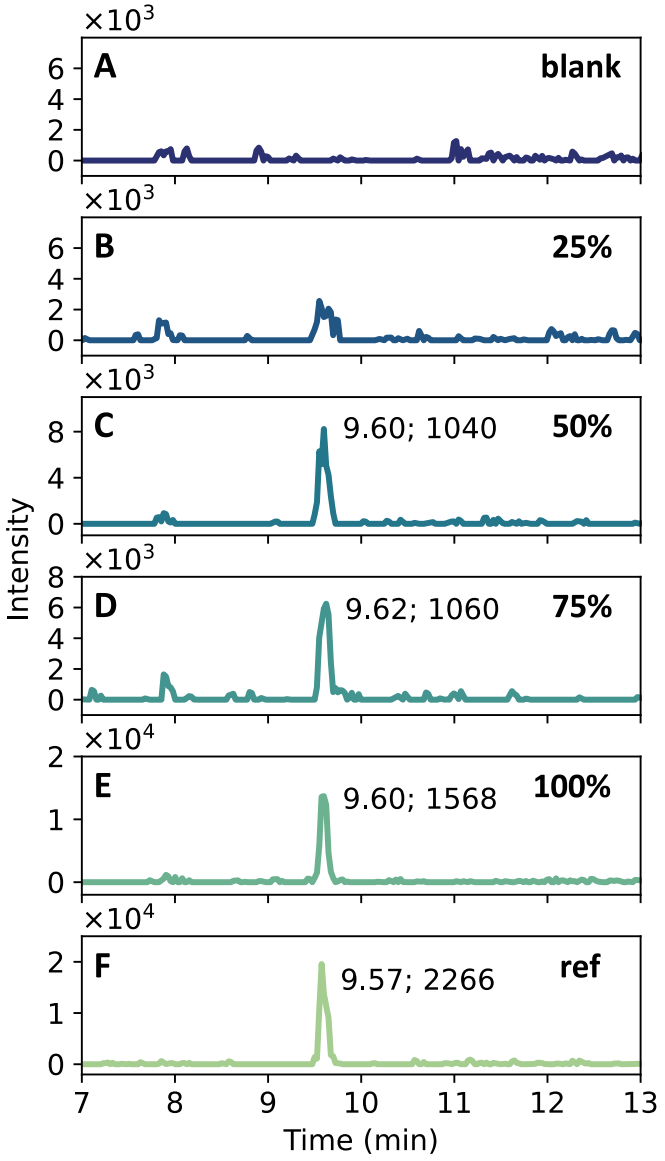

Fig. 4. Extracted ion chromatograms of human BChE adducts of Novichok A-234 measured in 25 μL dried blood spots (excess exposure of 0.4 μM), one month after storage of the dried spots at ambient conditions (m/z 1000.4 → 778.3). Corresponding retention times and peak areas are shown. A) sample preparation blank, B) 25%, C) 50%, D) 75%, E) 100% inhibited blood and F) Reference of Novichok A-234 spiked in 12.5 μL plasma (100% inhibition).

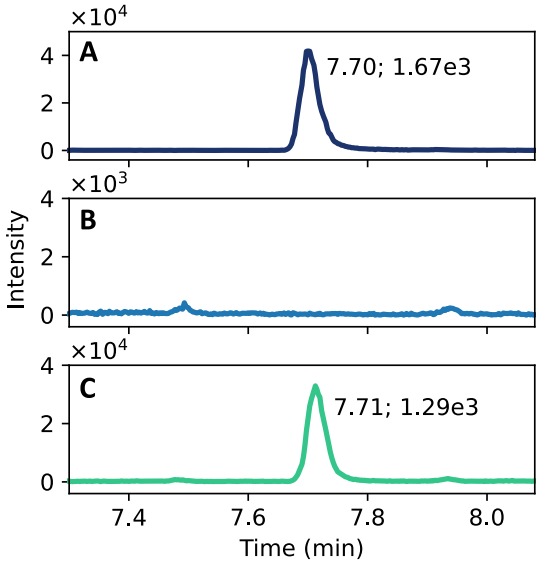

Fig. 5. Extracted ion chromatograms of regenerated sarin in dried blood spots (50 μL, 33 nM) after fluoride reactivation analyzed by GC–MS/MS, three days after storage of the dried spots at ambient conditions (m/z 99 → 81). Corresponding retention times and peak areas are shown. A) Reference standard of sarin, B) Dried blood spots exposed to sarin without KF addition (control), C) DBS exposed to sarin with KF addition.

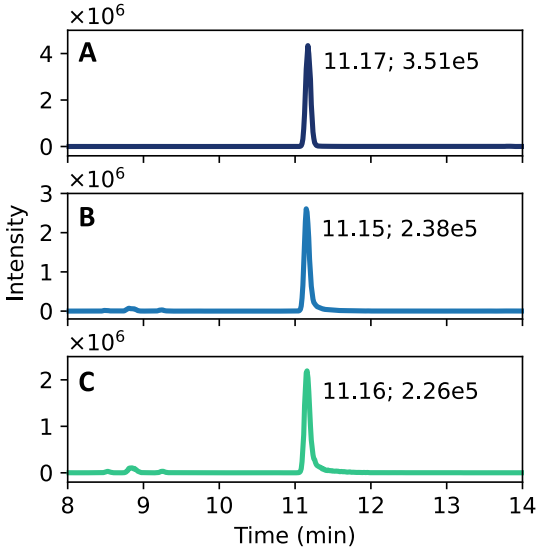

Fig. 6. Extracted ion chromatograms of intact Novichok A-232 (211 → 73.8) in dried blood spots (50 μL, 33 nM) analyzed by LC-MS/MS, three days after storage of the dried spots at ambient conditions. Corresponding retention times and peak areas are shown. A) Reference standard, B) Dried blood spots exposed to nerve agent without KF addition (control), C) DBS exposed to nerve agent with KF addition.

concentrated sample.

The same procedure was applied to the Novichok nerve agents. Both GC–MS/MS and LC-MS/MS were employed, but the LC-MS/MS method appeared to be more sensitive since a 1 ng/mL sample was not detected by GC–MS/MS, while LC-MS/MS still showed a clearly visible peak. It should be noted that the current study mainly focuses on qualitative research, therefore future validation should verify this finding. Fig. 6 shows the LC-MS/MS chromatograms of A-232 analyzed in dried blood spots without KF addition (control), and with KF addition (KF), compared to a reference standard. The peak areas of the nerve agents are similar or higher in the control samples than in the KF-regenerated samples. The same result was found for A-230 and A-234 in dried blood spots and for the Novichok nerve agents in liquid whole blood (Section S5 and S6 in the Supplemental Information). No excess of Novichok agents (33 nM) was added during the in-vitro exposure, which suggests that not all available cholinesterase sites have been occupied and that the remaining chemical is not hydrolyzed. The latter is in accordance with previous research stating that the hydrolysis rate of Novichoks is considerably slower than for sarin [50]. Since intact nerve agent was detected at the same or higher level in both liquid whole blood and dried blood spots without KF addition compared to the samples with KF addition, it appears that no fluoride regeneration has occurred. This is a somewhat surprising observation, since the current study does not support the conclusions of Mirbabaei et al. [42]. In this previous study no control samples without KF addition were shown. Although two methods were used for the removal of free A-234, this might have been ineffective. The observed A-234 in the KF samples

could then originate from free agent instead of the regenerated agent, similar to the findings in the current study for both liquid and DBS samples of A-230, A-232, and A-234. Therefore, it is more relevant to use the intact Novichok nerve agent for retrospective analysis of Novichok nerve agent exposure in comparison with the fluoride reactivation method.

Limitations of dried blood spot analysis

In addition to phosphonylated serine adducts, tyrosine adducts in blood have also been used as marker to verify sarin poisoning, although lower concentrations were reported for this biomarker [3]. Other studies confirm the presence of such adducts after the processing of a 400 μL dried blood spot [23]. However, in the current study no tyrosine adducts have been detected after the sample preparation of a 50 μL dried blood spot. Since the primary ChE targets will interact first and then the secondary albumin sites, experiments were also performed with blood incubated to an excess (1.3 μM) of chemical agent to make it more likely that all available protein adduct sites were occupied. However, after excess addition no albumin adducts were detected. This is most likely due to the limited sample volume handled with DBS, which results in an adduct concentration below the detection limit in the final extract.

The dried blood spots exposed to excess sarin (1.3 μM) were also screened for the hydrolysis products IMPA and MPA using LC-MS/MS. The latter was not detected and IMPA could not unambiguously be identified because only one mass transition was found at a low level (Fig. S10 in the Supplemental Information). Phosphonic or phosphoric acids of Novichok nerve agents were also not detected in the extracted ion chromatograms at m/z 193.1, 209.1, and 223.1. The subsequent hydrolysis products methyl phosphonic acid, monomethyl, and monoethyl phosphate could also not be detected at m/z 97.0, 113.0, and 126.0, respectively. Shaner et al. demonstrated the possibility to analyze organophosphate hydrolysis products in dried blood spots by hydrophilic interaction liquid chromatography (HILIC) [20]. Future research could focus on applying this method to dried blood spots exposed to Novichok nerve agents as well.

Conclusions

A toolbox of analytical techniques was developed for the retrospective analysis of nerve agent exposure using dried blood spots. We have demonstrated the possibility of on-site point-of-care diagnosis after sarin and Novichok nerve agent exposure using the ChE mobile test kit for BChE activity measurements. An advantage of using DBS compared to liquid whole blood is the less invasive sample collection and the possibility to store the sample under ambient conditions and conduct the analysis at a later stage. In addition, laboratory analysis of nonapeptide adducts with LC-MS/MS enabled a sensitive and selective manner to verify exposure and identify the agent involved. Additionally, the analysis of fluoride-induced regenerated sarin by GC–MS/MS provided a suitable supplementary method for verification purposes. For the first time the feasibility of detecting regenerated sarin in dried blood spots was demonstrated. For the Novichok nerve agents, no regenerated Novichok was detected. However, the intact chemical would be a valuable biomarker for unambiguous verification. This work shows that the corresponding nerve agent biomarkers in dried blood spots are very stable and can be detected at least one month after application on a protein saver card and storage under ambient conditions. Furthermore, the implementation in forensic practice is relatively straightforward due to the less invasive sample collection and safer and easier shipping and storage conditions. Future research should focus on the improvement of the sample preparation methods to further lower the detection limit and fully validate the quantitative method. In conclusion, the on-scene analysis of biomarkers in dried blood spots followed by laboratory verification is a promising analysis scheme to verify nerve agent exposure. This should however be confirmed in practice with regular and dried blood spot samples from an alleged exposure victim or animal model exposure studies. Fortunately, CWA exposure incidents are relatively rare which can hamper field validation.

CRediT authorship contribution statement

Mirjam de Bruin-Hoegée: Methodology, Validation, Formal analysis, Writing – original draft, Visualization. **Alex Fidder:** Methodology, Validation, Formal analysis. **Tomas van Groningen:** Methodology, Validation, Formal analysis. **Marcel J. van der Schans:** Writing – review & editing, Supervision. **Daan Noort:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Arian C. van Asten:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work is part of the Forensic Attribution for CWA INtelliGence (FACING) project, a collaboration between the Van 't Hoff Institute for Molecular Sciences (HIMS) of the University of Amsterdam and TNO Defence, Safety & Security. The FACING project is financed by the DO-AIO fund of the Dutch Ministry of Defence; part of the research was performed under grant V1802.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2023.100526.

References

[1] OPCW, First Report by the OPCW Investigation and Identification Team (IIT) Pursuant to Paragraph 10 of Decision C-SS-4/Dec.3 "Addressing the Threat From Chemical Weapons Use" Ltamenah (Syrian Arab Republic) 24, 25, and 30 March 2017, (2020). https://www.opcw.org/sites/default/files/documents/2020/04/s-1867-2020%28e%29.pdf (accessed February 5, 2023).
[2] OPCW, Report of the OPCW Fact-Finding Mission in Syria Regarding an Alleged Incident in Khan Shaykhun, Syrian Arab Republic April 2017, (2017). https://www.opcw.org/sites/default/files/documents/Fact_Finding_Mission/s-1510-2017_e_.pdf (accessed February 6, 2023).
[3] H. John, M.J. van der Schans, M. Koller, H.E.T. Spruit, F. Worek, H. Thiermann, D. Noort, H. John, Fatal sarin poisoning in Syria,, forensic verification within an international laboratory network, Forensic Toxicol. 36 (2018) (2013) 61–71, https://doi.org/10.1007/s11419-017-0376-7.
[4] T. Nakagawa, A.T. Tu, Murders with VX: Aum Shinrikyo in Japan and the assassination of Kim Jong-Nam in Malaysia, Forensic Toxicol. 36 (2018) 542–544, https://doi.org/10.1007/s11419-018-0426-9.
[5] OPCW, Summary of the report on activities carried out in support of a request for technical assistance by the United Kingdom of Great Britain and Northern Ireland, (2018). https://www.opcw.org/sites/default/files/documents/S_series/2018/en/s-1612-2018_e__1_.pdf (accessed February 6, 2023).
[6] OPCW, Summary of the report on activities carried out in support of a request for technical assistance by Germany, (2020). https://www.opcw.org/sites/default/files/documents/2020/10/s-1906-2020%28e%29.pdf (accessed February 6, 2023).
[7] V. Aroniadou-Anderjaska, J.P. Apland, T.H. Figueiredo, M. De Araujo Furtado, M. F. Braga, Acetylcholinesterase inhibitors (nerve agents) as weapons of mass destruction: History, mechanisms of action, and medical countermeasures, Neuropharmacology 181 (2020), 108298, https://doi.org/10.1016/j.neuropharm.2020.108298.
[8] OPCW, What is a Chemical Weapon, (n.d.). https://www.opcw.org/our-work/what-chemical-weapon (accessed June 12, 2022).
[9] The National Institute for Occupational Safety and Health (NIOSH), Sarin (GB): Nerve Agent, (2011). https://www.cdc.gov/niosh/ershdb/emergencyresponsecard_29750001.html (accessed June 12, 2022).

[10] S. Costanzi, G.D. Koblentz, Controlling Novichoks after Salisbury: revising the Chemical Weapons Convention schedules, Nonproliferation Rev. 26 (2019) 599–612, https://doi.org/10.1080/10736700.2019.1662618.
[11] D. Steindl, W. Boehmerle, R. Körner, D. Praeger, M. Haug, J. Nee, A. Schreiber, F. Scheibe, K. Demin, P. Jacoby, R. Tauber, S. Hartwig, M. Endres, K.U. Eckardt, Novichok nerve agent poisoning, Lancet 397 (2021) 249–252, https://doi.org/10.1016/S0140-6736(20)32644-1.
[12] T.C.C. Franca, D.A.S. Kitagawa, S.F. de A. Cavalcante, J.A.V. da Silva, E. Nepovimova, K. Kuca, Novichoks: The dangerous fourth generation of chemical weapons, Int. J. Mol. Sci. 20 (2019) 1222. 10.3390/ijms20051222.
[13] A.H. Plamboeck, S. Stöven, R. Duarte, E. Fykse, M. Griffiths, M. Nieuwenhuizen, C. Rivier, M. Van Der Schans, Trends in Analytical Chemistry Laboratory analysis of CBRN-substances : Stakeholder networks as clue to higher CBRN resilience in Europe, Trends Anal. Chem. 85 (2016) 2–9, https://doi.org/10.1016/j.trac.2016.03.006.
[14] Z. Witkiewicz, S. Neffe, Chromatographic analysis of chemical warfare agents and their metabolites in biological samples, TrAC Trends Anal. Chem. 130 (2020), 115960, https://doi.org/10.1016/j.trac.2020.115960.
[15] E.I. Hamelin, T.A. Blake, J.W. Perez, B.S. Crow, R.L. Shaner, R.M. Coleman, R.C. Johnson, Bridging the gap between sample collection and laboratory analysis: using dried blood spots to identify human exposure to chemical agents, Proc. SPIE 9863, Smart Biomedical and Physiological Sensor Technology XIII. 9863P (2016). 10.1117/12.2223796.
[16] M.T. Ackermans, V. de Kleijne, F. Martens, A.C. Heijboer, Hematocrit and standardization in DBS analysis: A practical approach for hormones mainly present in the plasma fraction, Clin. Chim. Acta 520 (2021) 179–185, https://doi.org/10.1016/j.cca.2021.06.014.
[17] H. John, S. Willoh, P. Hörmann, M. Siegert, A. Vondran, H. Thiermann, Procedures for Analysis of Dried Plasma Using Microsampling Devices to Detect Sulfur Mustard-Albumin Adducts for Verification of Poisoning, Anal. Chem. 88 (2016) 8787–8794, https://doi.org/10.1021/acs.analchem.6b02199.
[18] L. Yishai Aviram, D. Loewenthal, A. Weissberg, D. Marder, S. Gura, S. Chapman, R. Gez, S. Lazar, S. Dagan, Determination of free G-type nerve agents in blood: in situ derivatization on a dried blood spot (DBS) paper followed by LC–MS/MS analysis, Forensic Toxicol. 38 (2020) 327–339, https://doi.org/10.1007/s11419-019-00516-8.
[19] D. Marder, S. Dagan, L. Yishai-Aviram, D. Loewenthal, S. Chapman, R. Adani, S. Lazar, A. Weissberg, S. Gura, Instantaneous monitoring of free sarin in whole blood by dry blood spot–thermal desorption–GC–FPD/MS analysis, J. Chromatogr. B 1136 (2020), 121911, https://doi.org/10.1016/j.jchromb.2019.121911.
[20] R.L. Shaner, R.M. Coleman, N. Schulze, K. Platanitis, A.A. Brown, C. Seymour, P. Kaplan, J. Perez, E.I. Hamelin, R.C. Johnson, Investigation of dried blood sampling with liquid chromatography tandem mass spectrometry to confirm human exposure to nerve agents, Anal. Chim. Acta 1033 (2018) 100–107, https://doi.org/10.1016/j.aca.2018.06.049.
[21] L. Yishai Aviram, M. Magen, S. Chapman, A. Neufeld Cohen, S. Lazar, S. Dagan, Dry Blood Spot sample collection for post-exposure monitoring of chemical warfare agents – In vivo determination of phosphonic acids using LC-MS/MS, J. Chromatogr. B. 1093–1094 (2018) 60–65. 10.1016/j.jchromb.2018.06.035.
[22] J. McKenna, E.S. Dhummakupt, T. Connell, P.S. Demond, D.B. Miller, J. Michael Nilles, N.E. Manicke, T. Glaros, Detection of chemical warfare agent simulants and hydrolysis products in biological samples by paper spray mass spectrometry, Analyst 142 (2017) 1442–1451, https://doi.org/10.1039/c7an00144d.
[23] N.L. Koryagina, T.I. Aliushina, G.V. Karakashev, E.I. Savel'eva, N.S. Khlebnikova, A.S. Radilov, Determination of Phosphonylated Tyrosine as a Marker of Exposure to Nerve Agents in Dried Blood Plasma Spots by HPLC–HRMS/MS, J. Anal. Chem. 74 (13) (2019) 1341–1348.
[24] J.W. Perez, B.G. Pantazides, C.M. Watson, J.D. Thomas, T.A. Blake, R.C. Johnson, Enhanced stability of blood matrices using a dried sample spot assay to measure human butyrylcholinesterase activity and nerve agent adducts, Anal. Chem. 87 (2015) 5723–5729, https://doi.org/10.1021/acs.analchem.5b00893.
[25] M. Massano, C. Incardona, E. Gerace, P. Negri, E. Alladio, A. Salomone, M. Vincenti, Development and validation of a UHPLC-HRMS-QTOF method for the detection of 132 New Psychoactive Substances and synthetic opioids, including fentanyl, in Dried Blood Spots, Talanta 241 (2022), 123265, https://doi.org/10.1016/j.talanta.2022.123265.
[26] C. Skaggs, L. Kirkpatrick, C. Nguyen, S. Dowling, H. Zimmerman, G. Ren, N. Manicke, Simultaneous optimization of paper spray substrates and solvents for hydrophilic and hydrophobic molecules, Int. J. Mass Spectrom. 470 (2021), 116705, https://doi.org/10.1016/j.ijms.2021.116705.
[27] L.M. Rocca, N. L'episcopo, A. Gordiani, M. Vitali, A. Staderini, A 'dilute and shoot' liquid chromatography-mass spectrometry method for multiclass drug analysis in pre-cut dried blood spots, Int. J. Environ. Res. Public Health 18 (2021) 1–20, https://doi.org/10.3390/ijerph18063068.
[28] C. Seymour, R.L. Shaner, M.C. Feyereisen, R.E. Wharton, P. Kaplan, E.I. Hamelin, R.C. Johnson, Determination of fentanyl analog exposure using dried blood spots with LC-MS-MS, J. Anal. Toxicol. 43 (2019) 266–276, https://doi.org/10.1093/jat/bky096.
[29] J. McKenna, R. Jett, K. Shanks, N.E. Manicke, Toxicological drug screening using paper spray high-resolution tandem mass spectrometry (HR-MS/MS), J. Anal. Toxicol. 42 (2018) 300–310, https://doi.org/10.1093/jat/bky001.
[30] R.L. Shaner, N.D. Schulze, C. Seymour, E.I. Hamelin, J.D. Thomas, R.C. Johnson, Quantitation of fentanyl analogs in dried blood spots by flow-through desorption coupled to online solid phase extraction tandem mass spectrometry, Anal. Methods 9 (2017) 3876–3883, https://doi.org/10.1039/c7ay00532f.
[31] D. Chepyala, I.L. Tsai, H.W. Liao, G.Y. Chen, H.C. Chao, C.H. Kuo, Sensitive screening of abused drugs in dried blood samples using ultra-high-performance liquid chromatography-ion booster-quadrupole time-of-flight mass spectrometry, J. Chromatogr. A 1491 (2017) 57–66, https://doi.org/10.1016/j.chroma.2017.02.037.
[32] R. Verplaetse, J. Henion, Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS, Drug Test. Anal. 8 (2016) 30–38, https://doi.org/10.1002/dta.1927.
[33] S. Odoardi, L. Anzillotti, S. Strano-Rossi, Simplifying sample pretreatment: Application of dried blood spot (DBS) method to blood samples, including postmortem, for UHPLC-MS/MS analysis of drugs of abuse, Forensic Sci. Int. 243 (2014) 61–67, https://doi.org/10.1016/j.forsciint.2014.04.015.
[34] C.F. Clavijo, J.J. Thomas, M. Cromie, B. Schniedewind, K.L. Hoffman, U. Christians, J.L. Galinkin, A low blood volume LC-MS/MS assay for the quantification of fentanyl and its major metabolites norfentanyl and despropionyl fentanyl in children, J. Sep. Sci. 34 (2011) 3568–3577, https://doi.org/10.1002/jssc.201100422.
[35] D. Li, Z. Li, B. Xu, J. Chen, J. Xue, S. Hu, L. Wen, L. Guo, J. Xie, G. Jiang, Thermal desorption bridged the gap between dielectric barrier discharge ionization and dried plasma spot samples for sensitive and rapid detection of fentanyl analogs in mass spectrometry, Analyst 147 (2022) 4187–4196, https://doi.org/10.1039/d2an00946c.
[36] F. Worek, M. Schilha, K. Neumaier, N. Aurbek, T. Wille, H. Thiermann, K. Kehe, On-site analysis of acetylcholinesterase and butyrylcholinesterase activity with the ChE check mobile test kit-Determination of reference values and their relevance for diagnosis of exposure to organophosphorus compounds, Toxicol. Lett. 249 (2016) 22–28, https://doi.org/10.1016/j.toxlet.2016.03.007.
[37] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961), https://doi.org/10.1016/0006-2952(61)90145-9.
[38] F. Worek, U. Mast, D. Kiderlen, C. Diepold, P. Eyer, Improved determination of acetylcholinesterase activity in human whole blood, Clin. Chim. Acta 288 (1-2) (1999) 73–90.
[39] D. Noort, A. Fidder, D. van der Riet-van Oeveren, R. Busker, M.J. van der Schans, M.J. Van Der Schans, Verification of Exposure to Novichok Nerve Agents Utilizing a Semitargeted Human Butyrylcholinesterase Nonapeptide Assay, Chem. Res. Toxicol. 34 (8) (2021) 1926–1932.
[40] W.H. Jeong, J.Y. Lee, K.C. Lim, H.S. Kim, Identification and study of biomarkers from novichok-inhibited butyrylcholinesterase in human plasma, Molecules 26 (2021), https://doi.org/10.3390/molecules26133810.
[41] J.H. Lee, W.E. Jang, J.H. Park, H.B. Mohammad, J.-Y. Lee, W.-H. Jeong, M.-S. Kim, Identification of organophosphate modifications by high-resolution mass spectrometry, Bull. Kor. Chem. Soc. 43 (2022) 444–449, https://doi.org/10.1002/bkcs.12478.
[42] F. Mirbabaei, A. Mohammad-Khah, M.T. Naseri, M. Babri, S.M. Faraz, S. E. Hosseini, D. Ashrafi, Unambiguous identification and determination of A234-Novichok nerve agent biomarkers in biological fluids using GC–MS/MS and LC–MS/MS, Anal. Bioanal. Chem. 414 (2022) 3429–3442, https://doi.org/10.1007/s00216-022-03964-1.
[43] A. Yamaguchi, H. Miyaguchi, M. Tokeshi, Dimethoxytriadinylation LC-MS/MS of Novichok A-Series Degradation Products in Human Urine, Anal. Chem. 94 (2022) 4658–4665, https://doi.org/10.1021/acs.analchem.1c04634.
[44] M. Otsuka, A. Yamaguchi, H. Miyaguchi, Analysis of degradation products of Novichok agents in human urine by hydrophilic interaction liquid chromatography–tandem mass spectrometry, Forensic Toxicol. (2022), https://doi.org/10.1007/s11419-022-00656-4.
[45] M. de Bruin-Hoegée, L. Lamriti, J.P. Langenberg, R.C.M. Olivier, L.F. Chau, M. J. van der Schans, D. Noort, A.C. van Asten, Verification of exposure to chemical warfare agents through analysis of persistent biomarkers in plants, Anal. Methods 15 (2023) 142–153, https://doi.org/10.1039/d2ay01650h.
[46] OPCW Technical Secretariat, Work Instruction for the Reporting of the results of the OPCW Biomedical Proficiency Tests, 2021.
[47] J.K.F. Leal, M.J.W. Adjobo-Hermans, R. Brock, G.J.C.G.M. Bosman, Acetylcholinesterase provides new insights into red blood cell ageing in vivo and in vitro, Blood Transfus. 15 (2017) 232–238, https://doi.org/10.2450/2017.0370-16.
[48] C.E.A.M. Degenhardt, K. Pleijsier, M.J. Van Der Schans, J.P. Langenberg, K. E. Preston, M.I. Solano, V.L. Maggio, J.R. Barr, Improvements of the Fluoride Reactivation Method for the Verification of Nerve Agent Exposure, J. Anal. Toxicol. 28 (2004), https://doi.org/10.1093/jat/28.5.364.
[49] K.E. Holland, M.I. Solano, R.C. Johnson, V.L. Maggio, J.R. Barr, Modifications to the Organophosphorus Nerve Agent-Protein Adduct Refluoridation Method for Retrospective Analysis of Nerve Agent Exposures, J. Anal. Toxicol. 32 (2008), https://doi.org/10.1093/jat/32.1.116.
[50] M. Noga, K. Jurowski, What do we currently know about Novichoks? The state of the art, Arch. Toxicol. 97 (2023) 651–661, https://doi.org/10.1007/s00204-022-03437-5.