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Development of a LC/MS/MS method to analyze butyrylcholinesterase inhibition resulting from multiple pesticide exposure☆

Jinchun Sun[a,b], Bert C. Lynn[b,*]

[a] Division of Systems Toxicology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, United States
[b] Department of Chemistry, University of Kentucky, Lexington, KY 40506-0055, United States

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ABSTRACT

A hybrid LC/MS/MS and proteomics method was developed for the assessment of multiple pesticide exposure. The methodology was based on the analysis of tryptic peptides resulting from inhibited butyrylcholinesterase (BChE) after exposure to pesticides including organophosphates (OPs) and carbamates (CBs). The primary advantage of the assay was its ability to simultaneously examine multiple pesticide exposures in a single analytical experiment. Application of tandem and MS^3 techniques provided identities of the inhibiting pesticide, confirmation and localization of the site of inhibition and relative quantification of phosphorylated peptides present in tryptic digests of equine BChE (eBChE).

1. Introduction

Frequent and worldwide use of pesticides increases the potential for multiple chemical exposures [1,2]. With the identification of Gulf War Syndrome [3], the issue of multiple chemical exposures poses an ever increasing military and civilian threat. Therefore, detection and quantification of pesticide exposure in clinical and forensic samples demands sensitive and effective analytical techniques. At the very least, analytical methods must determine compelling evidence of chemical intoxication.

Analysis of intact pesticides including organophosphates (OPs) and carbamates (CBs) has involved many analytical techniques such as capillary electrophoresis [4], biosensors [5], matrix-assisted laser desorption/ionization (MALDI) MS [6], ion mobility time of flight (IM-TOF-MS) [7], gas chromatography coupled with diverse detection systems [8,9] and many more. The major common drawback of these approaches is time-consuming and laborious sample preparation. Most importantly, the detection of intact pesticides is strictly restricted to the time frame of a few hours to days due to the hydrolysis and elimination processes occurred in the biosystems. In contrast, OP- or CB-enzyme adducts can be more stable with a half-life of 10–16 days [10] in the organism. Thus, a method was developed to investigate multiple chemical exposures based on the analysis of inhibited butyrylcholinesterase (BChE) using LC/MS/MS techniques. An *in vitro* study was designed to examine qualification and the relative quantification of BChE inhibitors using equine BChE (eBChE) as a model system.

Organophosphates and carbamates pesticides inhibit BChE through covalent modification of the active site serine (Ser198) resulting in the formation of an inhibited enzyme (Fig. 1A). Therefore, a detailed analysis of inhibited BChE can be employed to detect exposure to pesticides and possibly other nerve agents. In recent years, a number of studies [11–15] have been specifically developed to analyze alkyl-phosphorylated peptides caused from OPs or nerve agent exposure. These methods involve isolation of inhibited BChE from plasma followed by pepsin, trypsin or chymotrypsin digestion [11–15]. Among these, a method based on the reactivation of non-aged phosphorylated enzyme and quantitative conversion of the phosphoryl moiety to a GC/MS detectable phosphorofluoridate was developed by Polhuijs et al. [15]. Another method based on LC/MS examination of specific nonapeptide adducts after pepsin digestion of inhibited human BChE (hBChE) developed by Fidder et al. [11] surpasses the limitation of the Polhuijs method. Noort et al. [14] further developed a generic mass spectrometry-based method for detection of phosphorylated hBChE through β-elimination in the presence of mild base followed by Michael-addition with a functionalized thiol or amine group. Tsuge and Seto [13] detected a hBChE-nerve gas adduct—an ethylphosphonylated serine contain-

Abbreviations: LC/MS, liquid chromatography/mass spectrometry; BChE, butyrylcholinesterase; eBChE, equine butyrylcholinesterase; OP, organophosphates; CB, carbamates; MALDI, matrix-assisted laser desorption/ionization.

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* Corresponding author at: UK Mass Spectrometry Facility, University of Kentucky, A052 ASTeCC Bldg., Lexington, KY 40506-0286, United States.
Tel.: +1 859 218 6529; fax: +1 859 257 2489.
E-mail address: bclynn2@uky.edu (B.C. Lynn).

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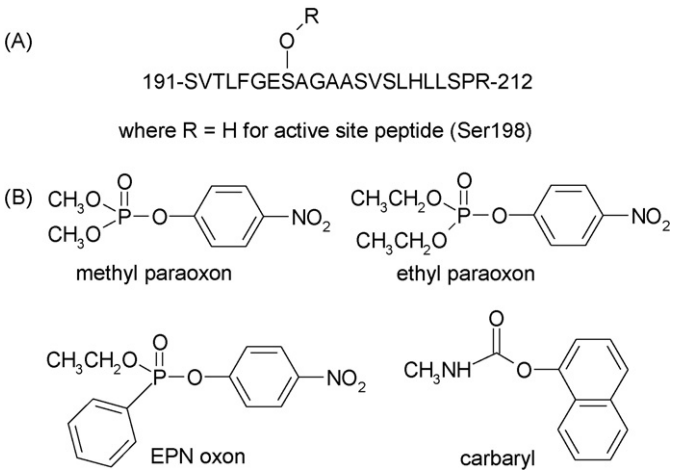

Fig. 1. (A) The active site peptide obtained from the trypsin digestion eBChE (Ser198 peptide) is shown. The doubly charged ion from this peptide would be observed at m/z 1100^{2+}. (B) Structures of compounds used to inhibit eBChE. Methyl paraoxon, ethyl paraoxon and EPN oxon are oxons derived from organophosphate pesticides, carbaryl was used as a model for all *N*-methyl carbamate pesticides.

ing peptide fragment, after electrophoresis, chymotrypsin in-gel digestion and LC/MS. Analytical methods have also been developed to investigate carbamylated peptides resulting from CBs exposure. The fast reactivation (half-life ~2 h) of the CB-inhibited BChE makes the investigation more challenging. Li et al. [16] reported a LC/MS/MS method to positively and rapidly identify carbofuran exposure through monitoring the carbamylated peptide from trypsin digestion of hBChE, which was partially purified from 1 mL human serum. In our previous studies [17], we used a MALDI-TOF-MS method to detect and quantify both phosphorylated or carbamylated active site peptide of inhibited eBChE.

All of the above mentioned methods are designed for the detection of individual pesticide or nerve agent exposures. Therefore, our goal was to develop an improved LC/MS/MS method to assess multiple pesticide exposures. To our knowledge, this is the first report to investigate multiple pesticide exposures based on LC/MS and a proteomics approach. Three OP compounds (including methyl paraoxon, ethyl paraoxon, and EPN oxon) and one CB (carbaryl), were used in this study (Fig. 1B). The resulting alkyl-phosphorylated peptide mixture or *N*-methyl carbamylated peptides from the inhibited BChE were analyzed using LC/MS/MS methodologies. Using these techniques, improved target-ion detection sensitivity and isolation were obtained. Our results demonstrated that multiple pesticide exposures could be determined and relatively quantified by analyzing phosphorylated peptide mixtures.

2. Experimental

All chemicals (OPs, EPN and CB), eBChE and sequencing grade modified porcine trypsin were the same as described in our previous work [17]. Methods for producing EPN oxon, eBChE inhibition and eBChE trypsin digestion were also described previously [17]. The 574 amino acid sequence of the eBChE protein plus 28 amino acid signal peptide sequence for a total of 602 amino acids are in the primary Swiss Prot accession No. Q9N1N9-1. The active serine position is numbered as 198 without including the first 28 signal amino acids.

2.1. LC/MS analysis

All mass spectrometric experiments were conducted on a Finnigan LCQ quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a standard electrospray interface. Separations were performed with an Agilent Hewlett Packard 1100 HPLC (Santa Clara, CA) modified with a custom splitter to deliver mobile phase at 4 μL/min to a custom C18 capillary column (300 μm id × 15 cm, packed in-house with Macrophere 300 Å 5 μm C18 from Alltech Associates, Deerfield, IL). A linear gradient was employed to elute peptides from the capillary column. Starting gradient conditions were 95% (v/v) solvent A [water + 0.1% formic acid] and 5% solvent B [acetonitrile + 0.1% formic acid] at a flow rate of 0.2 mL/min. The concentration of solvent B was increased to 20% over 8 min and then to 90% over 25 min, maintaining at this concentration for an additional 8 min. Approximately 9 μL of digested sample (~19 pmol) was injected onto the C18 capillary column. The heated inlet capillary temperature of the mass spectrometer inlet was 175 °C. The scan range was from m/z 150 to 2000 for the tandem experiments. The MS/MS parameters included an ion isolation width 5 (m/z), a normalized collision energy of 35%, an activation Q of 0.25 and an activation time of 30 msec.

3. Results and discussion

Our previously reported MALDI-MS method [17] was quite successful when used for single pesticide exposure analysis. Unfortunately, multiple pesticide exposures were problematic using the MALDI-MS technique due to the lack of online-separation as well as ion suppression in the peptide mixture. The goal of this study was to develop an enhanced method for detection and quantification of eBChE inhibition following exposure to multiple pesticides using LC/MS/MS techniques. The primary advantage of this LC/MS/MS approach lies in its ability to simultaneously investigate exposure to several pesticides in a single analytical assay.

Tryptic peptide containing Ser198 (the active site of BChE) was of special interest since OPs and CBs reacted with the hydroxyl group of the serine residue. This tryptic peptide containing the active site serine will be referred to as the Ser198 peptide for the remainder of this discussion. Fig. 1A shows the amino acid sequence of the Ser198 peptide which is typically observed at m/z 1100^{2+}. Fig. 1B displays the structures of methyl paraoxon, ethyl paraoxon and EPN oxon (oxons derived from organophosphate pesticides), and the structure of carbaryl as a model for all *N*-methyl carbamate pesticides.

3.1. Identification and sequencing of OP-modified Ser198 peptides using LC/MS/MS

Three OPs were reacted with eBChE individually and digested with trypsin. The resulting tryptic peptides containing Ser198 (Fig. 2A) were identified by LC/MS and LC/MS/MS using optimized instrumental conditions. Two modified Ser198 peptides resulting from methyl paraoxon inhibition were observed at m/z 1147^{2+} (aged peptide, further loss of methyl group from methyl paraoxon-modified Ser198 peptide) and m/z 1155^{2+}. Similarly, ethyl paraoxon and EPN oxon modified Ser198 peptides were detected at m/z 1168^{2+} and 1184^{2+}, respectively. These modified Ser198 peptides were identified based on the mass difference from the intact Ser198 peptide. For instance, a mass shift of 47 was observed between the aged Ser198 peptide at m/z 1147^{2+} (resulting from methyl paraoxon) and the intact Ser198 at m/z 1100^{2+}. This mass shift corresponded to the addition of *O*-methyl phosphoric acid $(HO)_2P(O)(OCH_3)$ (112 Da) with the concomitant loss of water during the phosphorylation of Ser198 (thus addition of 94 Da which converts to 47 on a double charged ion).

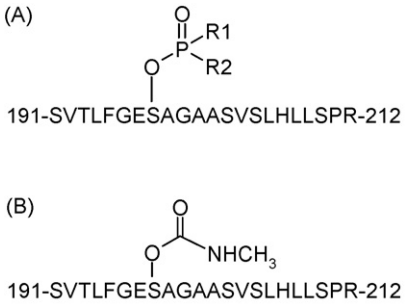

Fig. 2. (A) Structure of the oxon inhibited Ser198 peptide where R1 = MeO, R2 = OH, for the aged methyl paraoxon inhibited Ser198 peptide, m/z 1147^{2+}; R1 = R2 = MeO, for the methyl paraoxon-modified Ser198 peptide, m/z 1155^{2+}; R1 = R2 = EtO, for the ethyl paraoxon-modified Ser198 peptide, m/z 1168^{2+}; and R1 = EtO, R2 = phenyl, for the EPN oxon modified Ser198 peptide, m/z 1184^{2+}. (B) Structure of the carbaryl-modified Ser198 peptide.

Tandem mass spectrometry was conducted on the four ions mentioned above to further confirm the identities of OP-modified peptides. Tandem mass spectra for these four precursor ions showed similar fragmentation pattern which all MS^2 spectra contained a dominant fragment ion at m/z 1091^{2+}. Fig. 3A shows a representative MS/MS spectrum of the organophosphorylated peptide (calculated MH^{2+} at m/z 1168^{2+}) generated from the tryptic digest of the ethyl paraoxon inhibited eBChE. A dominant fragment ion at m/z 1091^{2+} was observed, corresponding to the loss of *O*,*O*-diethyl phosphoric acid $HOP(O)(OCH_2CH_3)_2$ (154 Da). As shown in Fig. 3E, the neutral loss produced a dehydroalanine residue at the active site Ser198. This ion corresponded to the neutral fragment loss of alkyl phosphoric acid from the phosphorylated precursor ion. As such, different alkyl-phosphorylated peptides can be identified by their neutral losses.

Unfortunately, the low abundance of other fragment ions in the tandem spectrum made elucidation the amino acid sequence difficult. To obtain sequence information for the phosphorylated peptide ions, MS^3 spectra (precursor $\rightarrow$ m/z 1091^{2+} $\rightarrow$ products) were obtained. Fig. 3B shows the MS^3 spectrum from m/z 1168^{2+} that clearly contained sequence informative fragmentation. In comparison, the tandem spectrum of the unmodified Ser198 peptide is shown in Fig. 3C. The *y*-ions labeled in the fragment mass spectra are numbered from the C-terminal side of the Ser198 peptide, e.g. *y*15 is SAGAASVSLHLLSPR (where the underlined serine is the active site serine 198 in the total eBChE protein sequence). While the intensities vary, a series of *y*-ions common to both spectra (*y*3–*y*14) were observed. Mass shifts of 18 Da were noted for *y*16–*y*19 ions (Fig. 3B), compared with the *y*-ions from the unmodified Ser198 peptide. The mass shift was due to the elimination of alkyl phosphoric acid on the catalytic serine resulting in a dehydroalanine residue (Fig. 3E).

Based on the above mentioned discussion, the precursor ion at m/z 1168^{2+} was indeed an OP-modified Ser198 peptide. Furthermore, the OP modification can be determined by the neutral loss of (77 Da) from the doubly charged precursor and producing the dominant fragment ions at m/z 1091^{2+}. In this case, the neutral loss identified *O*,*O*-diethyl phosphate (154 Da) as the modification. Additionally, the phosphorylation site on Ser198 was confirmed by localization of the dehydroalanine residue.

3.2. Identification and sequencing of the CB-modified Ser198 peptide using LC/MS/MS

Carbaryl was reacted with eBChE followed by trypsin digestion, and analyzed by LC/MS and LC/MS/MS using optimized instrumental conditions. Fig. 2B shows the structure of carbamylated Ser198 peptide. Fig. 3D displays the MS/MS spectrum of the doubly charged CB-modified Ser198 peptide at m/z 1129^{2+}. The pattern of the tandem mass spectrum was different from that of the phosphorylated peptides but surprisingly similar to the unmodified Ser198 peptide. Instead of eliminating *N*-methylcarbamic acid, the tandem spectrum contained sequence specific fragmentation information nearly identical to the unmodified Ser198 peptide. An identical set of unmodified fragment ions corresponding to *y*14–*y*3 was observed. Mass shifts of 57 Da, due to the attached carbamylate group ($O{=}C{-}NHCH_3$, 57 Da) at the active site serine residue, were observed for *y*15Δ–*y*19Δ ions (Fig. 3D). The result also confirmed that the modification occurred at Ser198.

3.3. Quantification by LC/MS/MS

In three parallel experiments, eBChE was inhibited with 1.2 μM methyl paraoxon, 0.5 μM ethyl paraoxon, and 0.2 μM EPN oxon and subsequently digested with trypsin. Equivalent amount of tryptic digests (0.9 μg/μL) of each inhibited eBChE was combined and the mixture was further analyzed by LC/MS/MS. Initial experiments utilized data dependent protocols. Unfortunately, when a data dependent acquisition was used, the peptide phosphorylated by EPN oxon was consistently omitted (data not shown) due to its low concentration in the mixture. Data dependent acquisition is typically set up to analyze the most abundant ions in each duty cycle. Because the four phosphorylated peptides eluted from the capillary column during a short time interval (about 2.23 min for elution all four selected ions, see Fig. 4) and suppression from other abundant ions, not all of the ions of interest were selected for MS/MS. To address this problem, a time-segmented LC/MS/MS protocol was evaluated. In time-segmented analyses, the MS method was set up to specifically analyze one species of precursor ions during a certain time window while ignoring all other ions in the matrix. For example, only precursor ions at m/z 1147^{2+} were monitored during 23.5–24.90 min, while ions at m/z 1155^{2+} were monitored at 24.90–25.90 min. Using the time-segmented LC/MS/MS protocol, all OP-modified peptides from the mixture of the tryptic digests of inhibited eBChE were baseline separated and observed (Fig. 4).

As described above, all OP-modified Ser198 peptides had a similar product ion mass spectrum, which contained one dominant fragment ion at m/z 1091^{2+}. As such, the phosphorylated peptides could be identified by neutral loss from the precursor ions. For instance, the neutral loss in panel A (Fig. 4) was 56, which was the mass difference between the doubly charged precursor ion and the doubly charged fragment ion at m/z 1091^{2+}. The precursor ion was determined to be m/z 1147^{2+} corresponding to the modified peptide with an *O*-methyl phosphate group (112 Da). Similarly, all the precursor ions in panels B–D (Fig. 4) were deduced based on the neutral loss of *O*,*O*-dimethyl phosphoric acid (126 Da), *O*,*O*-diethyl phosphoric acid (154 Da), and *O*-ethyl phenyl phosphoric acid (186 Da), respectively. Precursor ions at m/z $1147^{2+}$and 1155^{2+} were determined to correspond to aged Ser198 peptide and methyl paraoxon-modified Ser198 peptide, respectively. Similarly, precursor ions at m/z 1168^{2+} and 1185^{2+} corresponded to OP-modified Ser198 peptides by ethyl paraoxon and EPN oxon, respectively.

Since peaks of various OP-modified peptides were baseline separated, it was possible to quantify multiple pesticide exposures in a single analytical assay. The ratios of the phosphorylated peptides were obtained by integrating peak intensities. It was assumed that all the alkyl-phosphorylated peptides had similar electrospray efficiencies. Triplicate analyses of the synthetic mixture produced normalized peak areas of AA1147:AA1155:AA1168:AA1185 = 2.5:4.3:2.7:1 (AA is the average peak area). Normalized areas from AA1147 were added to AA1155 to obtain a total value, which demonstrated the total extent of exposure to methyl paraoxon. As such, the relative ratio of the extent exposure to each OP was methyl paraoxon:ethyl

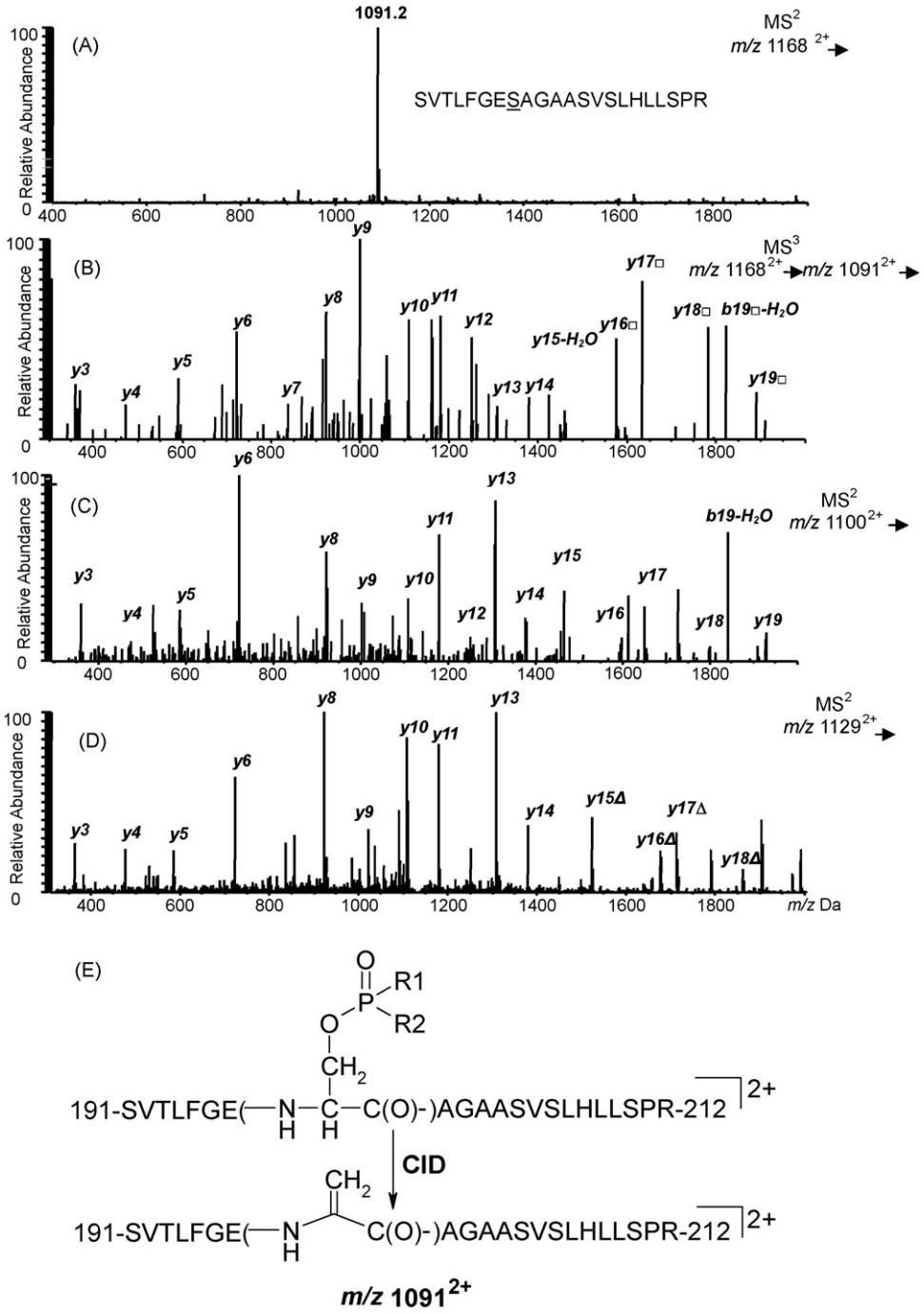

Fig. 3. (A) Tandem mass spectrum of the ethyl paraoxon-modified Ser198 peptide at m/z 1168^{2+}; (B) MS^3 spectrum of the ethyl paraoxon-modified Ser198 peptide (m/z 1168^{2+}→ m/z 1091^{2+}→ products); (C) tandem mass spectrum of the unmodified Ser198 peptide at m/z 1100^{2+}; (D) tandem mass spectrum of the carbaryl-modified Ser198 peptide at m/z 1129^{2+}; (E) shows the fragmentation pathway for the neutral loss of alkyl phosphoric acid resulting in a dehydroalanine residue at the active Ser198 (m/z 1091^{2+}). Note: fragment ions with neutral loss of alkyl phosphoric acid are indicted by □, while fragment ions with the attached carbamylate group are labeled with Δ. The *y*-ions labeled in the fragment mass spectra are numbered from the C-terminal side of the Ser198 peptide, e.g. *y*15 is SAGAASVSLHLLSPR, where the active site serine is underlined.

paraoxon:EPN oxon = 6.8:2.7:1. Results from this method were consistent with the actual incubation concentration ratios, which were methyl paraoxon:ethyl paraoxon:EPN oxon = 6.0:2.5:1. The relative standard error was 8% between the detected and the actual values. The difference was likely caused by the different inhibition efficiencies of OPs. Based on the results from our previous work [17], the inhibition ability of methyl paraoxon was lower than that of ethyl paraoxon and EPN oxon. The lower inhibition efficiency caused reduced inhibition of eBChE by methyl paraoxon, compared to ethyl paraoxon and EPN oxon. It has to be emphasized that the quantification here was not absolute, because no internal standard was used. However, a relative quantification was obtained, and was consistent with the actual pesticide concentrations.

Results indicated that the LC/MS/MS technique was a powerful tool for the isolation of the organophosphorylated peptide ions. In addition, these results show that this method could be used for the detection of CB exposure, although carbamylated-eBChE spontaneously reactivates very quickly [18].

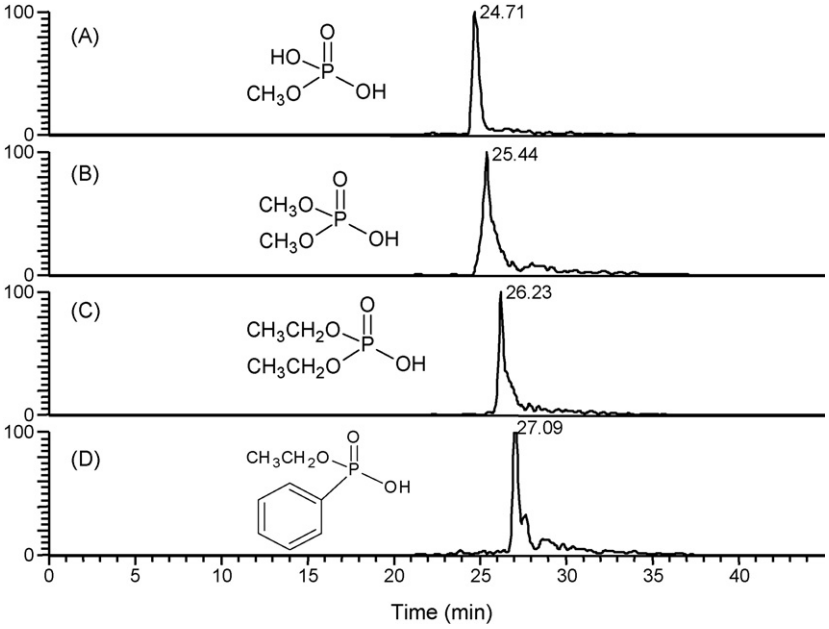

Fig. 4. Extracted ion chromatograms of the common fragment ion (m/z 1091^{2+}) resulting from inhibition of eBChE by the three oxons. The structure of the modifying group was determined by neutral losses from precursors to the common ion at m/z 1091^{2+}. (A) Chromatogram of m/z 1091^{2+} resulting from the neutral loss of 56 Da, corresponding to $CH_3OP(O)(OH)_2$ (112 Da), from the aged methyl paraoxon inhibited eBChE (precursor ion m/z 1147^{2+}); (B) chromatogram of m/z 1091^{2+} resulting from the neutral loss of 63 Da, corresponding to $(CH_3O)_2P(O)OH$ (126 Da), from methyl paraoxon inhibited eBChE (precursor ion m/z 1155^{2+}); (C) chromatogram of m/z 1091^{2+} resulting from the neutral loss of 77 Da, corresponding to $(CH_3CH_2O)_2P(O)(OH)$ (154 Da), from ethyl paraoxon inhibited eBChE (precursor ion m/z 1168^{2+}); and (D) chromatogram of m/z 1091^{2+} resulting from the neutral loss of 93 Da, corresponding to $(CH_3CH_2O)(C_6H_5)P(O)(OH)$ (186 Da), from EPN oxon inhibited eBChE (precursor ion m/z 1184^{2+}).

4. Conclusion

A method was developed for the identification and baseline separation of a mixture of OP-modified Ser198 peptides using LC/MS/MS. Sequence information and the location of the modification site of phosphorylated peptides were obtained by MS^3 experiments through the analysis the common fragment ion m/z 1091^{2+}. Finally, a mixture of the phosphorylated peptides was baseline separated, detected, identified, and quantified in a single analytical measurement through time-segmented LC/MS/MS. Thus inhibition of eBChE resulting from multiple pesticide exposures (including OPs and CB) can now be examined by mass spectrometry. In future experiments, the newly developed LC/MS/MS approach will be extended towards the analysis of multiple pesticide exposure in human serum samples.

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