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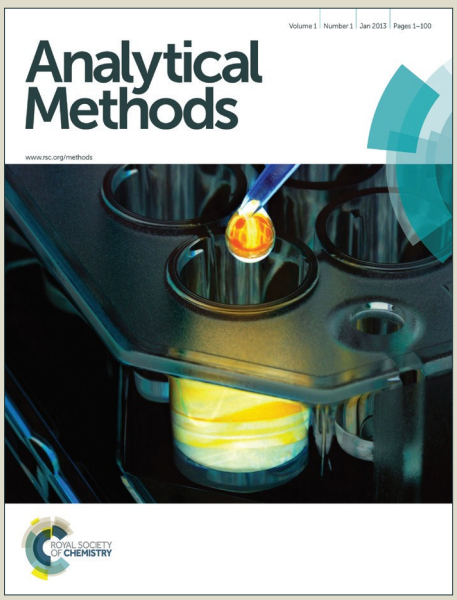

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Simultaneous determination of 14 biogenic amines and metabolites in bullfrog blood using UFLC-MS/MS with SPE

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Abstract: In this work, a reliable, accurate, and sensitive method based on a simple extraction with trichloroacetic acid at 5% and a clean-up step by means of Strata-X cartridge SPE prior to analysis of 14 biogenic amines (BAs) and metabolites by ultra-fast liquid chromatography-tandem quadrupole mass spectrometry (UFLC-MS/MS) was developed. The 14 BAs and metabolites could be separated by LC column under conditions of gradient elution within 6.0 min and simultaneously determined without interference from contaminants in biological samples. The results showed that the Strata-X cartridge SPE used in this method is more effective than C18 SPE and HLB SPE cartridges for cleanup of BAs and metabolites in bullfrog blood. The obtained results demonstrated the higher extraction capacity of Strata-X cartridge SPE with recoveries between 73.2-102%. The limits of quantification for

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the 14 BAs and metabolites ranged from 0.20-34.3 μg·L^{-1}. The developed Strata-X cartridge SPE UFLC-MS/MS method had been successfully applied to 15 real samples, and it was confirmed that the Strata-X cartridge SPE was a kind of highly effective clean-up method for BAs and metabolites.

Keywords: Ultra-fast liquid chromatography-tandem quadrupole mass spectrometry (UFLC-MS/MS); Biogenic amines (BAs); Solid-phase extraction; Bullfrog blood

1. Introduction

Biogenic amines (BAs) have important metabolic and physiological roles, such as the regulation of growth, control of blood pressure, and neural transmission[1]. The catecholamine, dopamine (DA), nore-pinephrine (NE) and epinephrine (E), together with their precursors, *i.e.*, putrescine (PUT), cadaverine (CAD), spermidine (SPD), spermine (SP), tyramine (TYR), phenylethylamine (PHE), histamine (HIS), and tryptamine (TR) and metabolites, *i.e.*, 5-hydroxy-3-indoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) have been implicated in the pathophysiology of various neurological and psychiatric conditions [2,3]. Given their role in the control and regulation of principal functions and behaviours, these biogenic amines (BAs) and metabolites can serve useful biomarkers for disease development and targets for the development of new therapeutic leads [4-7]. Thus, it is of major interest to a broad neuroscience field for developing a rapid and accurate method to measure BAs and metabolites in a wide variety of biological and environmental matrices.

Recently, several high performance liquid chromatography (HPLC) methods with online

sensors, such as fluorescence detectors (FD), electrochemical detectors (ECD) and mass spectrometry (MS), have been proposed for the determination of BAs [8-20]. In the HPLC-FD and HPLC-ECD methods, a derivatization step was usually performed with derivatizing agents such as dansyl chloride, O-phthalaldehydes and fluorenyl-methyl chloroformate, etc. Because direct application of a single chromatographic approach is not feasible for the simultaneous determination of BAs, which are lack of common chromophore or fluorophore in their structure. However, derivation reactions are time consuming and increase the risk of low recovery, analyte loss and contamination. Liquid chromatography (LC) coupled to MS is becoming the most commonly used methods for detecting BAs. Because it has the advantage that no derivatisation process is required and a relatively easy sample preparation is enough. Furthermore, MS detectors provide more structural information and can conform to the specifications for confirmatory methods included in the European Commission Decision 2002/657/EC. Therefore, LC-MS/MS is an attractive alternative due to its simplicity, separation efficiency and excellent sensitivity and selectivity for simultaneous determination of BAs and metabolites in complex matrix. Only a few published LC-MS/MS methods propose a direct analysis of BAs. F. Gosetti et al. proposed a LC-MS/MS method to determine eight BAs in cheese [21]. In another study, a hydrophilic interaction MS/MS method using a hybrid triple quadrupole/linear ion trap to determine seven BAs in cheese [22]. Recently, Magnes proposed a rapid and robust online SPE-coupled to LC-MS/MS method to determine eight polyamines in various biological samples [23]. According to our knowledge, no published articles are reported in the literature about the direct analysis of 14 underivatised BAs and metabolites in bullfrog blood.

Since the matrix of whole blood sample is extremely complex, the pretreatment of the sample is also important. Up to now, a number of pretreatment methods such as the phenylboronic acid (PBA), alumina and cation-exchange SPE have been reported to extract and enrich E and NE from biological samples [24-26]. Strata-X cartridge SPE has been used for extraction eight polyamines in various biological samples [23]. In fact, the stationary phase of Strata-X contains free nitrogen that forms hydrogen bond with unprotonated BAs, and aromatic structure that forms π–π interaction with metabolites. Therefore, Strata-X has been used for extraction of 14 BAs and metabolites in this work.

The aim of this work was to set up a new analytical method for analysing 14 underivatised BAs, *i.e.*, DA, NE, E, PUT, CAD, SPD, SP, TYR, PHE, HIS, TR and metabolites, *i.e.*, 5-HIAA, DOPAC and HVA in bullfrog blood using a solid phase extraction (SPE) followed by LC–MS/MS analysis. The proposed bioanalytical method was validated and its application to the analysis of BAs and metabolites in bullfrog blood samples was demonstrated.

2. Experimental

2.1 Reagents and materials

BAs, *i.e.*, spermidine trihydrochloride ($C_7H_{17}N_3 \cdot 3HCl$, >98%), 2-phenyl-ethylamine hydrochloride ($C_8H_{11}N \cdot HCl$, >98%), cadaverine dihydrochloride ($C_5H_{14}N_2 \cdot 2HCl$, >98%), spermine tetrahydrochloride ($C_{10}H_{26}N_4 \cdot 4HCl$, >98%), putrescine dihydrochloride ($C_4H_{12}N_2 \cdot 2HCl$, >98%), histamine dihydrochloride ($C_5H_9N_3 \cdot 2HCl$, >99%), tyramine hydrochloride ($C_8H_{11}NO \cdot HCl$, >98%), tryptamine hydrochloride ($C_{10}H_{12}N_2 \cdot HCl$, >99%), dopamine hydrochloride ($C_8H_{11}NO_2 \cdot HCl$, >98%), epinephrine hydrochloride

($C_9H_{13}NO_3$·HCl, >98%), norepinephrine bitartrate ($C_8H_{11}NO_3$·C_4H_6O·H_2O, >97%), homovanillic acid ($C_9H_{10}O_4$, >98%), 3,4-dihydroxyphenylacetic acid ($C_8H_8O_4$, >98%), and 5-hydroxyindoleacetic acid ($C_{10}H_9NO_3$, >98%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile, trichloroacetic acid (TCA) and formic acid of HPLC grade were purchased from Merck Company (Darmstadt, Germany). Deionized water was purified using a Millipore water purification system (Millipore, Billerica, MA). Strata™ X Cartridge SPE was acquired from Phenomenex (Madrid, Spain).

The whole blood was collected from the bullfrog. Three batches of bullfrogs (five samples for each batch) were acquired from local markets, *i.e.*, Gaotang. Cuibai and Shuangdongfang markets (Ningbo, China). And the weight of bullfrogs is in the range of 220~250g.

2.2 Equipment

Ultra-fast liquid chromatography-tandem quadrupole mass spectrometry (UFLC-MS/MS) analyses were performed using a Prominence UFLC XR system equipped with a DGU-20A3 degasser, a LC-20AD pump, a CTO-20AC column oven, a SIL-20AC autosampler (Shimadzu Corp., Tokyo, Japan) and an AB SCIEX TRIPLE QUADTM 5500 mass spectrometer (Applied Biosystems, Foster City, CA). The UFLC-MS/MS system was controlled and data were analyzed on a computer equipped with Applied Biosystems/MDS Sciex Analyst 1.5.1 (Applied Biosystems, Foster City, CA).

2.3 UFLC-QqQ-MS/MS analysis

UFLC analysis was performed on a Shim-pack XR-ODS□(100 mm×2.0 mm i.d., 2.2 μm). Analytes were separated by UFLC using 0.1% formic acid (v/v) in acetonitrile as eluent (A) ,

and 0.01% formic acid (v/v) water as eluent (B). The linear gradient was: 0→2.00 min, 5.00→40.0% A (95.0→60.0% B); 2.00→3.00 min, 40.0→90.0% A (60.0→10.0% B); 3.00→4 min, 90.0% A (10.0% B) and 4.01→6.00 min, 5.00% A (95.0% B). Chromatographic separation of the analytes was accomplished at a constant flow of 0.40 mL/min and the injection volume was 10.0 μL. The column was thermostated at 40°C to increase the retention time reproducibility. Mass spectrometry analysis was performed using an electrospray ionization source in positive mode. The operation conditions were as follows: ion spray voltage, 5500 V; curtain gas (CUR), 40 psi and interface heater was on; collision gas, medium; nebuliser gas (gas 1) and heater gas (gas 2), 50 and 50 psi; the turbo spray temperature, 500 °C; entrance potential (EP), 10 V; collision cell exit potential (CXP), 10 V. Nitrogen was used in all cases. Multiple-reaction monitoring (MRM) mode was used for quantification. The results of the precursor ion, product ion are shown in Table 1. Applied Biosystems/MDS Sciex Analyst software (versions 1.5.1) was used for data acquisition and processing.

<Insert Table 1>

2.4 Sample preparation

For the analysis, a total of 0.5 mL of bullfrog blood samples was homogenised for one minute with 3 mL of TCA 5% using an Ultra Turraxmixer. The obtained homogenate was decanted into centrifuge tubes and centrifuged (20000×g, 5 min, 4 ◦C). After removing the extracts, 3 mL of TCA 5% was added to the remaining solid and the process was repeated. Then, both extracts were combined and collected in a plastic vial.

For Strata X cartridge-SPE purification, the column was conditioned sequentially with 5.0 mL methanol followed by 4 mL of Milli-Q water using a vacuum system. Then, the sample extracts with a pH adjusted to 8.5 with NH_4OH 28% were passed through the cartridges at a flow rate of 1.0 mL/min. After sample loading was complete, the sample tube was washed by 5.0 mL methanol/H_2O (5:95, v/v) and dried under vacuum for 5 min. Then the Strata X cartridge was eluted by 5.0% formic acid/methanol (3×2 mL, v/v). And the eluting solutions concentrated to dryness with a nitrogen stream and was redissolved with 100.0 μL of initial mobile phase and filtered using a 0.22 μm polytetrafluoroethylene (PTFE) membrane prior to its injection into the UFLC-MS/MS system.

2.5 Method validation

Individual stock standard solutions were prepared at 1000 $mg \cdot L^{-1}$ level by exact weighing and dissolution in 0.1% formic acid/water (v/v). The stock mixture standard solution (10.0 mg/L) was prepared by appropriate dilution of the stock solutions with water-methanol (1:1, v/v). Calibration standards in initial mobile phase with concentration in the range of 0.4-40 $\mu g \cdot L^{-1}$ for HIS, SPD, E and TR, and 1.0-100 $\mu g \cdot L^{-1}$ for SP, NE, DA, TYR and PHE, and 2.0-200 $\mu g \cdot L^{-1}$ for CAD, PUT and 5-HIVV, and 4.0-400 $\mu g \cdot L^{-1}$ for DOPAC, and 40.0-4000 $\mu g \cdot L^{-1}$ for HVA. The matrix-matched calibration curves made by peak area vs concentration ($\mu g \cdot L^{-1}$) were used to calibrate spike samples in the recovery experiments.

Spike recoveries were performed at concentrations of 0.16, 0.8, and 4.0 $\mu g \cdot L^{-1}$ for HIS, SPD, E and TR, and 0.4, 2.0 and 10 $\mu g \cdot L^{-1}$ for SP, NE, DA, TYR and PHE, and 0.8, 4.0 and 20 $\mu g \cdot L^{-1}$ for CAD, PUT and 5-HIVV, and 1.6, 8.0 and 40 $\mu g \cdot L^{-1}$ for DOPAC, and 16, 80 and 400 $\mu g \cdot L^{-1}$ for HVA, respectively. Due to the difficulty of obtaining blank bullfrog blood

samples, each sample was previously analysed for UFLC-MS/MS optimization studies. The amount of BAs and metabolites found was subtracted from spiked samples. For each spiked sample, a stock mixture solution of the standards was added to 0.5 mL of sample, which was free from the target compounds. The spiked samples prepared were stored at 4°C for about 12 h to let the analytes permeate uniformly into the samples.

The method was evaluated by linearity, LOD, LOQ, precision and accuracy. Calibration standards with concentrations ranging from 0.4 to 4000 $\mu g \cdot L^{-1}$ were prepared for the calibration curves. Calibration curves of peak area of quantitative ion pairs (as shown in Table 1) against the analyte concentration were used to calibrate spike samples in recovery experiments. LOD and LOQ were determined based on a signal-to-noise ratio of 3 (S/N=3) and 10 (S/N=10), respectively. Both the method accuracy and precision were estimated by HIS, SPD, E and TR spiked at concentrations of 0.16, 0.8, and 4.0 $\mu g \cdot L^{-1}$, and SP, NE, DA, TYR and PHE spiked at 0.4, 2.0 and 10 $\mu g \cdot L^{-1}$, and CAD, PUT and 5-HIVV spiked at 0.8, 4.0 and 20 $\mu g \cdot L^{-1}$, and DOPAC spiked at 1.6, 8.0 and 40 $\mu g \cdot L^{-1}$, and HVA spiked at 16, 80 and 400 $\mu g \cdot L^{-1}$ in samples, respectively. The method accuracies were expressed as the recoveries, and the method precisions were expressed as the intra-day and inter-day relative standard deviations (RSDs). The intra-day RSDs were obtained by repeating the three levels of spiked samples six times within a day, and the inter-day RSDs were obtained by repeating the three levels of spiked samples in triplicate on six separate days within a 2-week period.

3. Results and Discussion

3.1 Optimization of UFLC-MS/MS conditions

For optimization of the detection of 14 BAs and metabolites by MS, a standard solution of

analytes (100 μg·L^{-1}) in methanol was infused directly into the MS. The objective of this set was to select representative ions (precursor and product ions) and to obtain values of DP, EP, CEP, CE and CXP for their detection. In the work, UFLC-ESI-MS/MS methods of 14 BAs and metabolites have investigated three ways to chose precursor ions including $[M+Na]^+$, $[M+H]^+$ and $[M-H]^-$. When using $[M+H]^+$ as the precursor ion of 11 BAs, the ion was much more abundant peak in the mass spectra than others. In case of 3 metabolites, the ion abundance of $[M-H]^-$ was much better. Finally, We chose $[M+H]^+$ as the precursor ion because of the low sensitivity of positive and negative switching mode. The final MS/MS conditions are detailed in Table 1.

In order to achieve an optimal chromatographic separation, the gradient elution and the effects of AmAc and formic acid concentration on the chromatographic separation were studied. Firstly, a series of aqueous mobile phase were composed of different concentration of AmAc prepared at 1.0, 2.5, 5.0, 10.0 and 20.0 mmol·L^{-1} levels by appropriate dilution of the stock AmAc solution with water, respectively. And the results showed that the peak width at half-height of the analytes ranged of 0.2 min to 0.4 min was not improved with the increasing of the concentration of AmAc. Therefore, a series of aqueous mobile phase, which were composed of different concentration of formic acid prepared at 0.01%, 0.02%, 0.05%, 0.1% and 0.2% (v/v) levels, respectively, were used for further improvement the chromatographic retention and peak shape of the 14 BAs and metabolites. The results showed that the peak shape was prominently improved with the increasing of the concentration of formic acid from 0.01% to 0.1% (v/v), without tailed peak and time-lag in the chromatograph peak by using 0.1% formic acid (v/v) aqueous solution as the mobile phase.

Finally, an UFLC-MS/MS method was established to determine the presence of 14 BAs and metabolites by MS, and the Shim-pack XR-ODS□(100 mm×2.0 mm i.d., 2.2 μm) was employed to achieve the maximum sensitivity and the satisfactory peaks by using acetonitrile-0.1% formic acid (v/v) aqueous solution as the mobile phase system in gradient elution. And the extract ion chromatograms (XIC) of 14 BAs and metabolites were shown in Fig.1.

<Insert Fig. 1>

3.2 Optimization of sample extraction procedure

Owing to the different chemical structures (aromatic, hetero-cyclic and aliphatic structures) of BAs and metabolites, simultaneous determination of these analytes in bullfrog blood can be challenging. In addition, the complexity of bullfrog blood matrix can lead to some problems during the extraction. In order to obtain a more rapid and sensitive method for analysing BAs and metabolites in bullfrog blood, a SPE–UFLC–MS/MS method without derivatisation step was developed. For injecting into UFLC–MS/MS system a final clean eluate containing BAs and metabolites, various SPE cartridges were investigated, *i.e.*, C18-SPE, HLB-SPE, and Strata™ X-SPE). And the effectiveness of the three different SPE cartridges on analyte recovery were studied with samples spiked at a concentration of 0.8 $\mu g \cdot L^{-1}$ for HIS, SPD, E and TR, and 2.0 $\mu g \cdot L^{-1}$ for SP, NE, DA, TYR and PHE, and 4.0 μg/L for CAD, PUT and 5-HIVV, and 8.0 $\mu g \cdot L^{-1}$ for DOPAC, and 80 $\mu g \cdot L^{-1}$ for HVA, respectively. The average recoveries and relative standard deviations (RSDs) of the studied analytes are shown in Fig. 2.

As can be seen in Fig. 2, polar analytes, *i.e.*, HIS, CAD, PUT, SPD and SP could not be retained well on C18-SPE resulted in low recoveries. This phenomenon can be explained that C18 sorbent (silica based) is more hydrophobic than other 2 sorbents resulted in low recoveries for polar analytes. Both HLB and Strata™ X sorbents contain highly electronegative nitrogen and oxygen atoms which form hydrogen bonding with the unprotonated BAs, while the phenyl functional group forms π–π interaction with the analytes containing aromatic rings. The HLB cartridges yielded acceptable results for NE, E, DA, DOPAC, PHE, TYR, TR, 5-HIAA and HVA, but low recoveries for HIS, CAD, PUT, SPD and SP. However the Strata™ X SPE cartridges provided better overall recoveries ranged from 78.2% to 99.5% with RSDs less than 10%, thus was selected for the purification of 14 BAs and metabolites in bullfrog blood.

<Insert Fig. 2>

3.3 Method linearity, accuracy, LOD and LOQ

The linearity of the calibration curves made by peak area vs concentration ($\mu g \cdot L^{-1}$) was studied using calibration standards in initial mobile phase. The response function was found to be linear with a determination coefficient (r^2) higher than 0.9980 in the tested range listed in Table 2 for the BAs and metabolites.

The method accuracies were expressed as the recoveries, and the method precisions were expressed as the intra- and inter-day relative standard deviations (RSDs). The results are summarized in Table 2. It shows that the majority of mean recoveries were in the range of

73.2-102% with the intra-day RSDs ranging from 2.2 to 7.6% and inter-day RSDs ranging from 2.6 to 7.6%.

<Insert Table 2>

The limits of detection (LODs) and limits of quantification (LOQs) for the analyzed BAs and metabolites are shown in Table 2. The LODs and LOQs, which were calculated on the analysis of 14 BAs and metabolites spiked at a concentration of 0.16 μg/L for HIS, SPD, E and TR, and 0.4 μg/L for SP, NE, DA, TYR and PHE, and 0.8 μg/L for CAD, PUT and 5-HIVV, and 1.6 μg/L for DOPAC, and 16 μgL for HVA, respectively, in blank samples that yielded a signal-to-noise (S/N) ratio of 3 and 10, were in the range of 0.066-10.4 μg/L and 0.20-34.3 μg/L, respectively. LOQs are significantly lower than other reported LC-MS/MS -based methodologies [21,22].

3.4 Application to real samples

Three batches of bullfrog (five samples for each batch) were analyzed by the developed method. The results showed HIS and SP was detected in 5 of the 15 collected samples in a concentration range of 15.29-38.2 $\mu g \cdot L^{-1}$ and 11.16-26.33 $\mu g \cdot L^{-1}$, respectively, SPD was found in all 15 samples with an average concentration of 58.22 $\mu g \cdot L^{-1}$ in a range of 36.16-96.33 $\mu g \cdot L^{-1}$, TR was found in 3 of the 15 collected samples in a concentration range of 160.2-200.5 $\mu g \cdot L^{-1}$, TYR was found in 5 of the 15 collected samples in a concentration range of 62.26 -120.5 $\mu g \cdot L^{-1}$, and other BAs and metabolites were not detected because of lower

than the LOQs in the analyzed samples. The MRM chromatogram for one examined samples was shown in Fig. 3.

<Insert Fig. 3>

4. Conclusions

In this work, an efficient Strata-X Cartridge SPE coupled with UFLC-MS/MS method was optimized for the 14 BAs and metabolites. The easiness-to-handle of the extraction method was definitely in favour of the UFLC–MS/MS procedure, since the extraction was faster and involves less intermediate steps. Acceptable recoveries for the studied BAs and metabolites were obtained in the range of 73.2-102%. The results demonstrate that the accuracy and precision of the proposed method are satisfactory for analysis of the BAs and metabolites in bullfrog blood samples.

References

[1] S. Lee, M. Yoo, D. Shin, LWT-Food Sci. Technol. 62 (2015) 350.

[2] F.I. Tarazi, M.J. Kaufman, Neural principles of neurological and psychiatric disorders, in: F.I. Tarazi, J.A. Schetz (Eds.), Current Clinical Neurology: Neurological and Psychiatric Disorders: From Bench to Bedside, Humana Press Inc, Totowa, NJ, 2005, p. 3.

[3] J.A. Schetz, Pharmacotherapeutic principles of neurological and psychiatric disorders, in: F.I. Tarazi, J.A. Schetz (Eds.), Current Clinical Neurology: Neurological and Psychiatric Disorders: From Bench to Bedside, Humana Press Inc, Totowa, NJ, 2005, p. 29.

[4] D. V. Dam, Y. Vermeiren, T. Aerts, P. P. De Deyn, J. Chromatogr. A 1353 (2014) 28.

[5] B. Mravec, A. Tillinger, I. Bondar, G. M. Nagy, M. Palkovits, R. Kvetnansky, N. Y. Ann, Acad. Sci. 1148 (2008) 196.

[6] E. M. Cotella, I. M. Lascano, G. M. Levin, M. M. Suarez, Int. J. Neurosci. 119 (2009) 664.

[7] G. Zhang, Y. Zhang, C. Ji, T. McDonald, J. Walton, E. A. Groeber, R. C. Steenwyk, Z. Lin, J. Chromatogr. B 895/896 (2012) 186.

[8] J. Bicker, A. Fortuna, G. Alves, A. Falcão, Anal. Chim. Acta. 768 (2013) 12.

[9] L. Mercolini, G. Gerra, M. Consorti, L. Somaini, M.A. Raggi, Talanta 78 (2009) 150.

[10] L. Yang, M. F. Beal, Methods Mol. Biol. 793 (2011) 401.

[11] A.T. Nguyen, T. Aerts, D. Van Dam, P. P. De Deyn, J. Chromatogr. B 878 (2010) 3003.

[12] L. I. B. Silva, F. D. P. Ferreira, A. C. Freitas, T. A. P. Rocha-Santos, A. C. Duarte, Talanta 80 (2009) 853.

[13] Y. Sakaguchi, H. Yoshida, T. Hayama, M. Itoyama, K. Todoroki, M. Yamaguchi, H. Nohta, J. Chromatogr. A 1218 (2011) 5581.

[14] H.-M. Tseng, D. A. Barrett, J. Chromatogr. A 1216 (2009) 3387.

[15] H. Mao, B. Chen, X. Qian, Z. Liu, Microchem. J. 91(2009) 176.

[16] R. N. Goyal, S. Bishnoi, Talanta 84 (2011) 78.

[17] A. M. Piasta, A. J. bska, M. P. Krzemiński, T. M. Muzioł, and E. Szł yk, Anal. Chim. Acta 834 (2014) 58.

[18] M. Notou, A. Zotou, P. D. Tzanavaras, D. G. Themelis, J. Chromatogr. A 1356 (2014) 272.

[19] R. M. Ramos, I. M. Valente, J. A. Rodrigues, Talanta 124 (2014) 146.

[20] Y.-Q. Wang, D.-Q. Ye, B.-Q. Zhu, G.-F. Wu, C.-Q.Duan, Food Chem. 163 (2014) 6.

[21] F. Gosetti, E. Mazzucco, V. Gianotti, S. Polati, M. C.Gennaro, J. Chromatogr. A 1149 (2007) 151.

[22] V. Gianotti, U. Chiuminatto, E. Mazzucco, F. Gosetti, M. Bottaro, P. Frascarolo, M. C. Gennaro, J. Chromatogr. A 1185 (2008) 296.

[23] C. Magnes, A. Fauland, E. Gander, S. Narath, M. Ratzer, T. Eisenberg, F. Madeo, T. Pieber, F. Sinner, J. Chromatogr. A 1331 (2014) 44.

[24] A.H. Wu, T.G. Gornet, Clin. Chem. 31 (1985) 298.

[25] E. Hollenbach, C. Schulz, H. Lehnert, Life Sci. 63 (1998) 737.

[26] K. Nyyssönen, M.T. Parviainen, Clin. Chem. 33 (1987) 1938.

Figures and Tables Captions

Fig. 1 MRM chromatograms for blood sample spiked with 14 BAs and metabolites at 0.8 μg/L for HIS, SPD, E and TR, and 2.0 μg/L for SP, NE, DA, TYR and PHE, and 4.0 μg/L for CAD, PUT and 5-HIVV, and 8.0 μg/L for DOPAC, and 80 μg/L for HVA, respectively, under Strata-X Cartridge SPE extraction procedure

Fig. 2 The effect of three different SPE cartridges on analyte recovery

Fig. 3 The MRM chromatogram for one examined samples

Table 1 Q1/Q3 ion pairs, declustering potential (DP), collision energy (CE) of MRM and retention time for the optimized UFLC-MS/MS method

Table 2 Validation parameters obtained for the analytes at three concentration levels in bullfrog blood

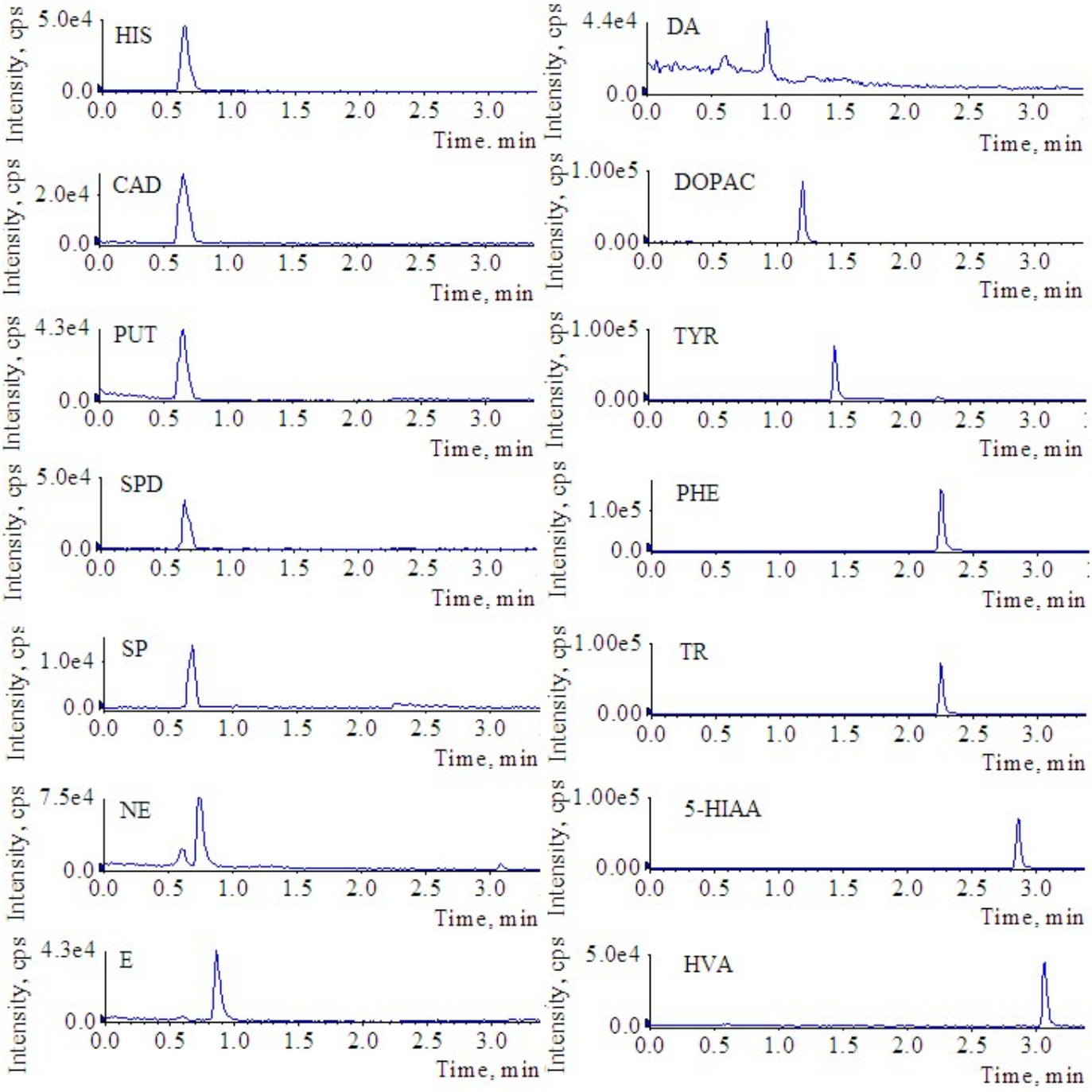
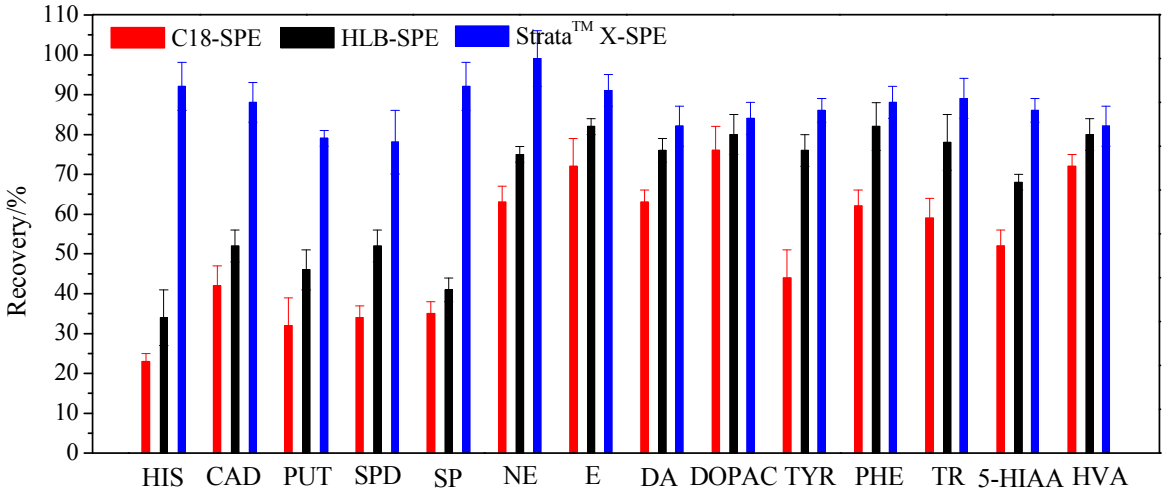


Fig. 1 MRM chromatograms for blood sample spiked with 14 BAs and metabolites at 0.8 μg/L for HIS, SPD, E and TR, and 2.0 μg/L for SP, NE, DA, TYR and PHE, and 4.0 μg/L for CAD, PUT and 5-HIVV, and 8.0 μg/L for DOPAC, and 80 μg/L for HVA, respectively, under Strata-X Cartridge SPE extraction procedure.



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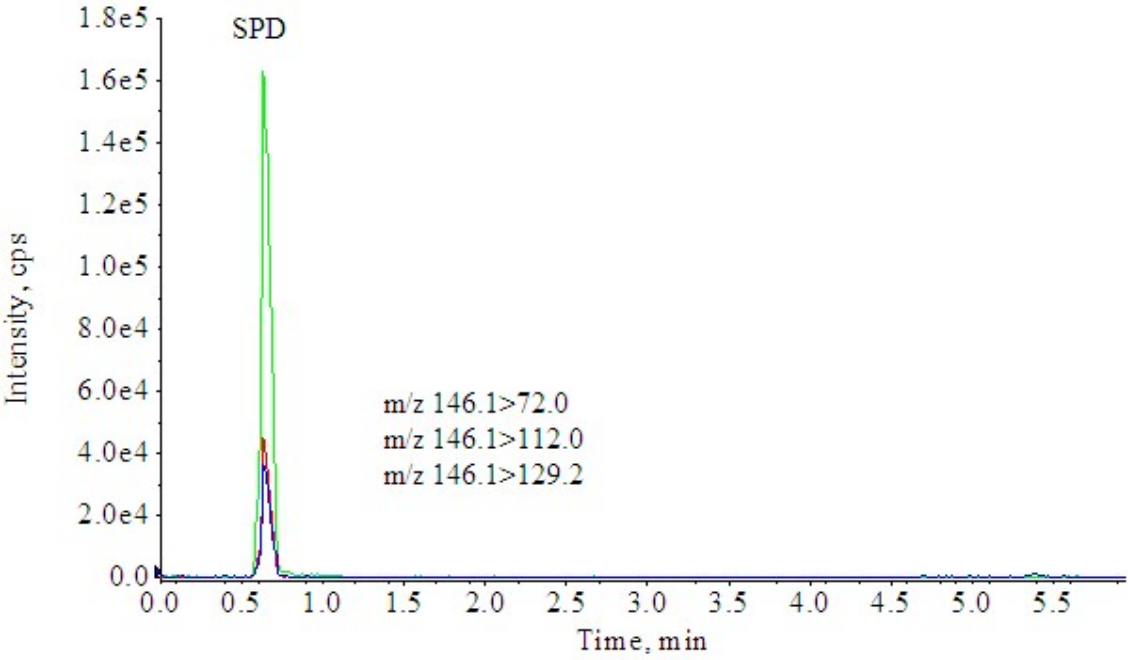

Fig. 3 The MRM chromatogram for one examined samples

Table 1 Q1/Q3 ion pairs, declustering potential (DP), collision energy (CE) of MRM and retention time for the optimized UFLC-MS/MS method

Analytes	Retention time/min	Precursor ion (Q1, *m/z*)	Product ion (Q3, *m/z*)	DP/V	CE/eV
HIS	0.63	112.0	95.0*,68.0	100,100	22,28
CAD	0.64	103.0	86.0*	60	14
PUT	0.64	89.2	72.0*	60	14
SPD	0.65	146.1	72.0*,112.0,129.2	90,90,90	20,18,14
SP	0.68	203.1	112.0*,129.0	140,140	26,17
NE	0.73	170.0	152.0*	60	12
E	0.86	184.1	166.0*	70	15
DOPAC	0.93	169.0	151.0*	70	15
DA	1.19	154.0	137.0*	70	15
TYR	1.44	138.0	121.0*	70	15
PHE	2.25	122.1	105.0*	110	15
TR	2.32	160.9	144.1*	60	15
5-HIAA	2.90	192.0	146.1*	110	22
HVA	3.10	183.0	137.0*	80	15

* quantitative ion

Table 2 Validation parameters obtained for the analytes at three concentration levels in bullfrog blood

Analytes	Linear equation[a]	Added/ μg L^{-1}	Recovery, (RSD, %) Intra-day[b]	Inter-day[c]	LOD/ μg L^{-1}	LOQ/ μg L^{-1}
HIS	$Y=4.28\times10^4X+2.58\times10^3$	0.16	87.0(3.8)	90.3(5.6)	0.072	0.24
		0.8	90.6(2.3)	82.2(4.9)		
		4.0	80.2(2.3)	83.6(4.6)		
CAD	$Y=3.98\times10^3X+3.78\times10^2$	0.8	90.2(5.3)	102(5.9)	0.60	1.98
		4.0	86.8(3.8)	93.2(3.6)		
		20	86.0(4.8)	88.3(6.8)		
PUT	$Y=4.19\times10^3X+3.76\times10^2$	0.8	82.2(2.9)	82.4(3.8)	0.58	1.91
		4.0	79.4(3.7)	86.6(6.5)		
		20	80.2(4.7)	76.2(6.2)		
SPD	$Y=4.60\times10^4X-6.10\times10^3$	0.16	80.0(3.2)	73.2(5.2)	0.066	0.20
		0.8	82.5(2.6)	80.7(3.6)		
		4.0	81(4.8)	81.8(5.6)		
SP	$Y=1.72\times10^4X+3.47\times10^3$	0.4	78.2(7.6)	86.2(6.9)	0.12	0.38
		2.0	86.3(6.0)	79.2(6.7)		
		10	82.2(3.9)	86.2(7.3)		
NE	$Y=1.12\times10^4X+3.47\times10^3$	0.4	100(2.2)	96.2(2.6)	0.20	0.66
		2.0	94.5(3.9)	93.0(5.0)		
		10	90.2(2.3)	96.7(4.2)		
E	$Y=3.69\times10^4X+2.54\times10^3$	0.16	92.6(4.2)	92.1(6.2)	0.078	0.26
		0.8	90.6(4.4)	89.6(5.7)		
		4.0	92.9(3.2)	82.8(6.1)		
DA	$Y=2.89\times10^4X+3.60\times10^3$	0.4	97.0(2.8)	92.3(3.6)	0.28	0.92
		2.0	91.6(3.3)	88.0(5.9)		
		10	86.2(4.3)	88.2(4.2)		
DOPAC	$Y=1.01\times10^3X-6.19\times10^2$	1.6	82.0(3.3)	90.2(6.9)	1.2	3.96
		8.0	76.9(4.8)	83.2(7.6)		
		40	82.0(5.8)	80.3(6.0)		
TYR	$Y=1.98\times10^4X-3.81\times10^3$	0.4	83.0(3.9)	86.4(3.8)	0.26	0.86
		2.0	79.6(2.7)	89.2(4.5)		
		10.0	90.5(5.7)	86.1(6.6)		
PHE	$Y=2.63\times10^4X+2.15\times10^3$	0.4	80.2(2.9)	87.2(5.3)	0.082	0.27
		2.0	90.1(3.2)	92.2(7.6)		
		10	84.5(2.9)	83.0(3.0)		
TR	$Y=3.40\times10^4X+5.83\times10^3$	0.16	90.6(3.3)	96.1(5.2)	0.10	0.33
		0.8	82.6(3.2)	82.1(5.2)		
		4.0	86.1(4.4)	81.3(6.7)		
5-HIAA	$Y=7.84\times10^3X+3.53\times10^2$	0.8	90.9(4.2)	92.8(5.1)	0.54	1.78
		4.0	96.5(2.9)	90.0(4.0)		
		20	91.3(2.6)	96.5(3.2)		
HVA	$Y=2.05\times10^2X+22$	16	82.6(5.2)	90.1(6.2)	10.4	34.3
		80	91.6(4.1)	86.3(5.3)		
		400	87.3(2.2)	81.8(3.1)		

[a]Y: peak area, X: mass concentration;[b] Intra-day, (n = 6); [c]Inter-day, n = 3 replicates × 6 days within a 2-week period.