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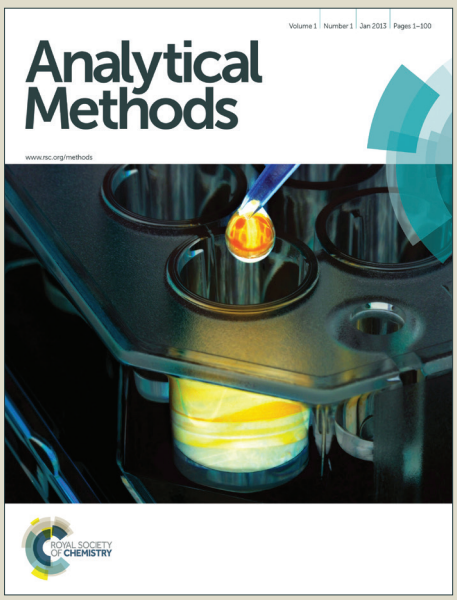

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Determination of seven anticoagulants rodenticides in human serum by ultra-performance liquid chromatography–mass spectrometry

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A rapid, sensitive and selective method for simultaneous determination of seven anticoagulants rodenticide residues (warfarin, coumatetralyl, diphacinone, chlorophacinone, brodifacoum, bromadiolone, and flocoumafen) in human serum by ultra performance liquid chromatography–mass spectrometry (UPLC-MS) has been developed and validated. Serum sample preparation was carried out rapidly and effectively by one-step protein precipitation and analytes extraction using methanol containing 10% acetone. Chromatographic separation was achieved within 10 min using a BEH C18 column (1.7μm, 2.1 mm ×100 mm) and gradient elution with the mobile phase of 5 mM ammonium acetate aqueous solution–methanol. Good linearity was achieved over three orders of magnitude with a correlation coefficient (r^2) of 0.9924–0.9994. The limits of detection for the seven rodenticides ranged from 0.06 μg L^{-1} (flocoumafen) to 1.5 μg L^{-1}

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(diphacinone). The intra- and inter-day relative standard deviations (RSDs) of the seven analytes at three spiked levels (2×LOQ, 5×LOQ and 10×LOQ) were in the range of 0.6–10.3% and 5.1–15.1%, respectively. The mean recoveries for the seven analytes at the three spiked levels (1×LOQ, 5×LOQ and 10×LOQ) were in the range of 77.3–98.2% with RSDs of 0.58–11.1 %. The proposed method was successfully applied for the analysis of the seven anticoagulant rodenticides in human serum.

Keywords: Ultra-performance liquid chromatography; Mass spectrometry; Anticoagulants rodenticide; Human serum

Introduction

Anticoagulants are biocides widely used as pest control agents in agriculture, urban infrastructures, and domestic applications for the control of rodents [1]. Mice and rat populations are commonly controlled by anticoagulant rodenticides(ARs). 4-hydroxycoumarin rodenticides and indandione rodenticide are commonly used to control field mice and house mice. Humans can be poisoned by rodenticides, which were among the most frequently used chemicals in deliberate selfpoisonings [2], and in childhood poisonings [3]. In recent years, anticoagulant rodenticides poisoning cases occur repeatedly, threatening human health. Sensitive and selective residue analysis of ARs in human body fluids is necessary for successful diagnosis of poisoning.

Most of the analytical methods for the determination of ARs in biologic matrices are based on liquid chromatography (LC). Liquid chromatography–UV detection has been used for analysis of 1-4 diphacinone sodium in food poisoning samples [4], rodenticides in biological fluids, such as warfarin in human plasma [5,6], bromadiolone, warfarin, and coumatetralyl in whole blood [7], and four rodenticides in urine[8,9]. However, these LC methods have lower sensitivity. Liquid chromatography–mass spectrometry (LC-MS) has high sensitivity and was used for analysis of bromadiolone in fox faeces [10], chlorophacinone and diphacinone [11], warfarin enantiomers in human plasma [12,13], as well as bromadiolone, flocoumafen and brodifacoum in whole blood [14]. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) were applied for the determination of 13 rodenticides in wastewater[15], bromadiolone in whole blood [16], bromadiolone and brodifacoum in human hair [17], warfarin in plasma [18], and coumatetralyl in human serum [19], valone in serum [20,21], bromadiolone and brodifacoum in human blood [22], as well as 4-hydroxycoumarin rodenticides and one indandione rodenticide in animal plasma [23]. The detection limit of the targets for these methods was in the range of 0.07–3.21μg L^{-1}. Recently, a LC-MS/MS method presented allows for simultaneous qualitative identification of brodifacoum, bromadiolone, chlorphacinone, dicumarol, difenacoum, diphacinone, and warfarin in blood, serum, and plasma using ESI in the negative mode [24].

The six rodenticides in whole blood specimens were analyzed by HPLC high resolution MS-MS, and their limits of detection were 10 μg L^{-1} or better, but with extremely low recovery for warfarin [25]. None of these methods seem to be completely suitable for the highly sensitive multiresidue analysis of 4-hydroxycoumarin and indandione rodenticide in biological fluids.

The aim of this work was to develop a simple, rapid, and sensitive method for multiresidue determination of the most commonly used ARs. The proposed ultra performance liquid chromatography–mass spectrometry (UPLC-MS) method was validated applied for the simultaneous determination of the seven ARs including two indandiones (coumatetralyl and warfarin) and five 4-hydroxycoumarins (diphacinone, chlorophacinone, bromadiolone, flocoumafen and brodifacoum) in human serum.

Experimental

Chemicals and reagents

Anticoagulant rodenticides warfarin, coumatetralyl, diphacinone, chlorophacinone, bromadiolone, flocoumafen and brodifacoum (purity: >99% for each) were purchased from Dr. Ehrenstorfer GmbHX (Augsburg, Germany).

All chemicals and reagents were of analytical grade except specific statements. Methanol, acetone, acetonitrile, formic acid, and ammonium acetate were HPLC grade, and obtained from Beijing Chemical Factory

(Beijing, China). Doubly deionized water was used throughout. The methanol was filtered through a 0.22-μm microporous membrane of polyvinylidene fluoride before use.

Single stock standards of 400 mg L^{-1} were prepared in methanol. The stock solutions stored at –20 °C were stable for at least six months. Mixed standard working solutions were prepared by diluting the standard stock solution with methanol just before use.

Instrumentation

UPLC-ESI-MS analyses of serum samples were performed on a Xevo Triple Quadrupole (TQ) system (Waters, USA). This system consisted of an autosampler, a binary pump, a solvent degasser, a BEH C18 stainless steel cartridge column (100 mm × 2.1 mm i.d., 1.7μm; Waters) equipped with a guard column at 40°C. The mass spectrometer used was a triple quadruple equipped with an ESI interface operating in the positive or negative mode. A TGL-16M centrifuge (Xiangyi Centrifuge Co. Hunan, China) was used in sample treatment.

Sample preparation

Serum samples were collected from a patient in the First Central Hospital of Baoding, who consented to provide samples for this study. All experiments were performed in compliance with the relevant laws and institutional guidelines.

These experiments have been approved by The Ethic Committee of the

First Central Hospital of Baoding. Serum (200 μL) was added into a polypropylene centrifuge tube, and extracted using 500μL methanol solution containing 10% acetone under shaking for 1 min. After centrifugation at 4 ºC and 10,733 ×g for 3 min, the upper organic layer was transferred to a disposable glass tube for UPLC-MS analysis.

Conditions of UPLC-MS

Seperation of rodenticides was carried out with a BEH C18 column at 40°C. Flow rate was 0.2 mL min^{-1} and sample injection volume was 2 μL. The mobile phase consisted of 5 mM ammonium acetate aqueous solution (A) and methanol (B). The gradient elution was as follows: 40–55% B at 0–4 min; 55–90% B at 4–10 min; 90–40% B at 10–12 min. The TQ parameters were as follows: source temperature, 150°C; capillary voltage, 3.0 kV; desolvation temperature, 500°C; desolvation flow (N_2), 900 L h^{-1}; collision gas flow (Ar), 0.19 mL min^{-1}. Table 2 shows the ion and collision energy of all analytes.

Quantification was carried out by using matrix-matched standards calibration curves based on peak area toward concentration in 7 concentration points.

Results and discussion

Optimization of LC–MS conditions

Because of the wide variety of molecular structures of rodenticides, the

development of a considerable number of chromatographic separation methods was needed for their successful analysis. The composition and mobile phase additives not only affect the retention time and peak shape of the target compound, but also affect their ionization efficiency, thus affecting the sensitivity. Our initial test showed that use of isocratic elution could not separate the seven analytes. The gradient elution with the mobile phase of 5 mM ammonium acetate aqueous solution (A) and methanol or acetonitrile (B) was investigated and compared, as shown in Fig. 1.

Figure 1

It can be seen that higher chromatographic response for diphacinone, chlorophacinone, bromadiolone, and brodifacoum was achieved using methanol than acetonitrile. Thereby gradient elution with 5 mM ammonium acetate aqueous solution (A)–methanol (B) was used.

Using flow injection pump for continuous sampling, mass spectrometric conditions of each herbicide were optimized. Obvious protonated molecules $[M+H]^+$ at *m/z* 309.2 for warfarin and *m/z* 292.9 for coumatetralyl were observed using positive ion mode, and obvious deprotonated molecules $[M–H]^-$ at *m/z* 338.9, *m/z* 372.9, *m/z* 525.1, *m/z* 541.0, and *m/z* 520.9 for diphacinone, chlorophacinone, bromadiolone, flocoumafen, and brodifacoum, respectively, were observed using negative ion mode. So that both positive ion and negative ion modes were used, and these $[M+H]^+$ and $[M–H]^-$ ions were selected for the quantification.

According to the different retention times and response of compound, two channels were set up. The collision energy of different target compounds was optimized. The experiment selected higher abundance of parent/daughter ion for monitoring to ensure that each peak has at least 15 collection points. The observed retention time, parent ions, and daughter ions as well as used cone voltage and collision energy (CE) are listed in Table 1. Total ion chromatogram and extracted ion chromatogram are shown in Fig. 2. MS spectra with the fragmentation mechanism for six rodenticides are shown in Fig.3, whereas the fragmentation mechanism of brodifacoum needs to be studied further.

Table 1 Figure 2 Figure 3

Optimization of extraction conditions

The effect of different conditions on extraction efficiency was investigated via recovery test. Three replicates of each extraction experiment were carried out, and the average values of recoveries were calculated as a ratio of the peak areas of the analyte to the standard sample of corresponding concentration.

The selection of a suitable extraction solvent is the first challenge. The polarity of the extraction solvent should closely match that of the target compounds. In this work, the recoveries for simultaneous extraction of the seven rodenticides from a spiked serum sample were investigated using methanol or acetonitrile as extraction solvent. The data in Table 2 show that

use of methanol or acetonitrile as extraction solvent obtained low extraction efficiency (49.6–77.6% or 55.1–70.1%) for the seven compounds. The presence of acetone in methanol or acetonitrile can increase extraction efficiency. When used methanol containing 10% acetone as extraction solvent, good recovery of 81.7–97.3% was achieved, achieving one-step protein precipitation and analytes extraction.

Table 2

Matrix effect

Matrix effects are a major concern in biological analysis. They can be a serious problem as they can severely compromise qualitative and quantitative analysis of the target compounds at trace levels as well as method reproducibility, especially when electrospray ionization is used. The standard solutions were prepared in the aqueous solvent and in blank serum extract at 1 mg L^{-1}. They are compared to determine if the ionization of analytes at the MS source was enhanced or suppressed by the matrix. The results showed that the signal intensity of the analytes obtained for matrix-matched standard was lower that in the aqueous solvent standard. This phenomenon indicated that there is the signal suppression of the analytes. A highest value of the suppression was 27% for chlorophacinone, and the suppression value was in the range of 10–20% for other 6 analytes. In order to obtain more reliable results, matrix-matched standard calibration curve was used for quantification in this work.

Performance of the method

Specificity

The effective baseline separation of the analytes in serum samples was observed, and no interfering peaks were found at the retention times of the seven analytes (Fig. 4). Chromatographic separation of seven analytes was achieved within 10 min. There was no interference from impurity peaks. Based on the above results, better specificity and selectivity were achieved

Figure 4

Linearity and detection limit

The matrix-matched standard calibration was performed using linear regression based on the peak-area of the target compounds and their at least seven concentrations in the range of 0.06–1500 μg L^{-1}. The correlation coefficient (r^2) of linear calibration curves was given in Table 3. A linear range for all rodenticides were in three orders of magnitude with r^2 of 0.9924–0.9994. It can be seen that the linearity is very satisfactory.

Table 3

The limit of detection (LOD) was determined as the sample concentration that produced a peak with a height three times the level of the baseline noise, and the limit of quantification (LOQ) was calculated as the sample concentration that produced a peak with a height 10 times the ratio of signal to noise. The data in Table 4 shows that the LOD ranged from 0.06 μg L^{-1} (flocoumafen) to 1.50 μg L^{-1} (diphacinone), and their LOQs ranged from

0.20 to 4.9 μg L^{-1}. The proposed method has higher sensitivity than HPLC–UV methods [4-9] and some HPLC–MS methods [10,12, 13, 18,19, 23, 25].

Repeatability

The precisions of the method were investigated by analyzing the four analytes in spiked blank serum sample. Under the optimized conditions the intra-day precisions (in terms of the relative standard deviation, RSD) of peak area for 7 determinations and inter-day RSD for one determination for each day within 7 days were investigated. The intra- and inter-day RSDs of the seven analytes at three spiked levels (2×LOQ, 5×LOQ and 10×LOQ) were in the range of 0.6–10.3% and 5.1–15.1%, respectively. It was shown that the repeatability of the method is satisfactory for the residue determination of the studied analytes in real sample. .

Analysis of spiked samples

Using matrix-matched standards calibration carried out quantification for warfarin, coumatetralyl, diphacinone, chlorophacinone, bromadiolone, flocoumafen, and brodifacoum in three serum samples. No studied analytes were detected in serum samples. To examine accuracy, the recoveries were investigated based on three parallel measurements. The results are listed in Table 4.

Table 4

The mean recoveries for the seven ARs at the three spiked levels, 1×LOQ, 5×LOQ and 10×LOQ, were in the range of 77.3–92.9 % with RSDs of 3.45–10.2 %, 81.7–94.8 % with RSDs of 1.06–11.1 %, and 78.4–98.2% with RSDs of 0.58–6.93 %, respectively. These results show the accuracy of the method is satisfactory.

Conclusion

A new UPLC-MS method for the multiresidue analysis of anticoagulant rodenticides in serum was developed. Using one-step protein precipitation and analytes extraction with methanol containing 10% acetone can achieve rapidly and effectively serum sample preparation, without further cleanup. Chromatographic separation of seven analytes can be achieved within 10 min using a BEH C18 column (1.7μm, 2.1 mm ×100 mm) and gradient elution with the mobile phase of 5 mM ammonium acetate aqueous solution–methanol. The method has the characteristic of being speediness, high sensitivity and accuracy as well as low consumption of reagents. A linear range for all rodenticides were in three orders of magnitude. The LODs and RSDs make the method possible to detect a low concentration of rodenticides. The developed and validated method can be applied for multiresidue detection of anticoagulant rodenticides in biological fluid in suspected poisoning cases.

Acknowledgements

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List of figures

Table 1 Mass spectral data parameters

Compound	Retention time (min)	Cone voltage (V)	Parent ions (m/z)	Daughter ions (m/z)	CE (V)
Warfarin	3.38	30	309.2	163	15
				251	15
Coumatetralyl	3.61	30	292.9	91	25
				175	20
Diphacinone	5.77	-25	338.9	167	-25
				172	-20
Chlorophacinone	7.38	-35	372.9	144.9	-20
				200.9	-20
Bromadiolone	8.58	-54	525.1	92. 9	-48
				250.1	-40
Flocoumafen	9.53	-35	541.0	161.3	-35
				381.9	-25
Brodifacoum	9.97	-35	520.9	134.9	-35
				186.9	-35

Table 2 Recoveries of the rodenticides from serum spiked at 5×LOQ level using different solvents (n=3, %)

Compound	MeOH	MeOH–5% ACTN	MeOH–10% ACTN	MeOH–15% ACTN	ACN	ACN–5% ACTN	ACN–10% ACTN	ACN–15% ACTN
Warfarin	77.6	81.3	91.2	89.6	69.9	73.6	89.9	91.2
Coumatetralyl	68.1	78.3	91.3	88.4	70.1	83.9	93.4	90.3
Diphacinone	49.6	77.1	97.3	92.2	55.1	77.1	91.9	92
Chlorophacinone	55.3	80.1	94.2	93.5	61.2	70.1	92.4	89.9
Bromadiolone	59.8	77.9	94.8	89.7	55.4	80	90.6	89.2
Flocoumafen	66.2	79.9	94.8	91	75.3	80.2	88.7	92.3
Brodifacoum	70.1	86.2	81.7	77.2	56.9	69.3	78.5	78.1

Note: MeOH—methanol, ACN—acetonitrile, ACTN—acetone

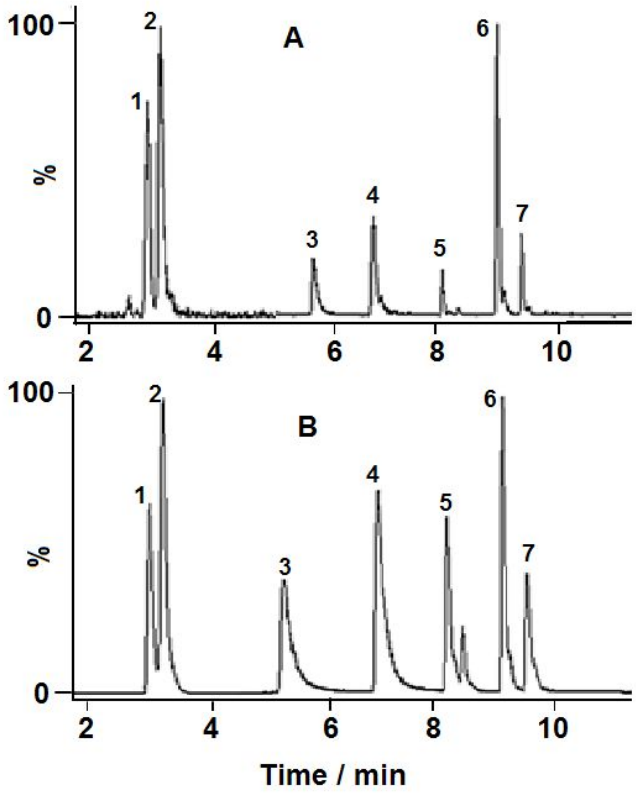
–1

Table 3 Analytical performance of the method

Compound	Linear range ($\mu g\ L^{-1}$)	Correlation coefficient (r^2)	LOD ($\mu g\ L^{-1}$)	LOQ $(\mu g\ L)^{-1}$
Warfarin	0.25–250	0.9992	0.25	0.83
Coumatetralyl	0.25–250	0.9994	0.25	0.83
Diphacinone	1.5–1500	0.9950	1.50	4.9
Chlorophacinone	0.25–250	0.9924	0.25	0.83
Bromadiolone	0.5–500	0.9949	0.50	1.7
Flocoumafen	0.06–60	0.9990	0.06	0.20
Brodifacoum	0.25–250	0.9927	0.25	0.83

Table 4 Recoveries and RSDs of the rodenticides (n=3)

Analyte	Added 1×LOQ (μg L^{-1})		Added 5×LOQ (μg L^{-1})		Added 10×LOQ (μg L^{-1})	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Warfarin	89.1	9.6	91.2	4.34	92.7	4.15
Coumatetralyl	84.0	7.78	91.3	4.86	96.2	5.26
Diphacinone	92.9	10.2	97.3	6.10	98.2	4.53
Chlorophacinone	90.5	9.93	94.2	11.1	94.5	6.93
Bromadiolone	89.6	4.90	94.8	6.03	95.2	0.58
Flocoumafen	85.5	5.52	94.8	2.98	98.1	1.92
Brodifacoum	77.3	3.45	81.7	1.06	78.4	4.84



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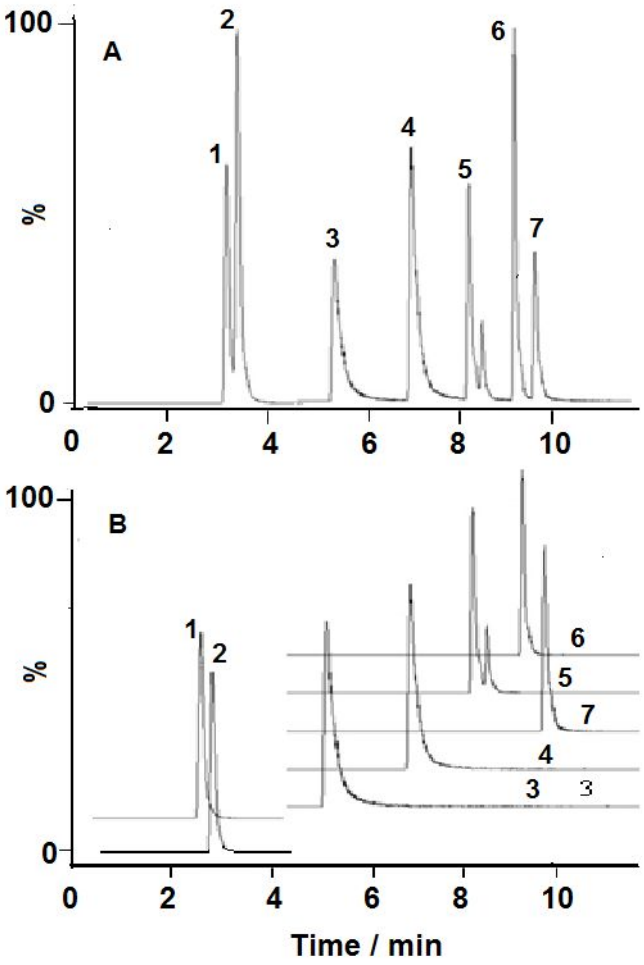

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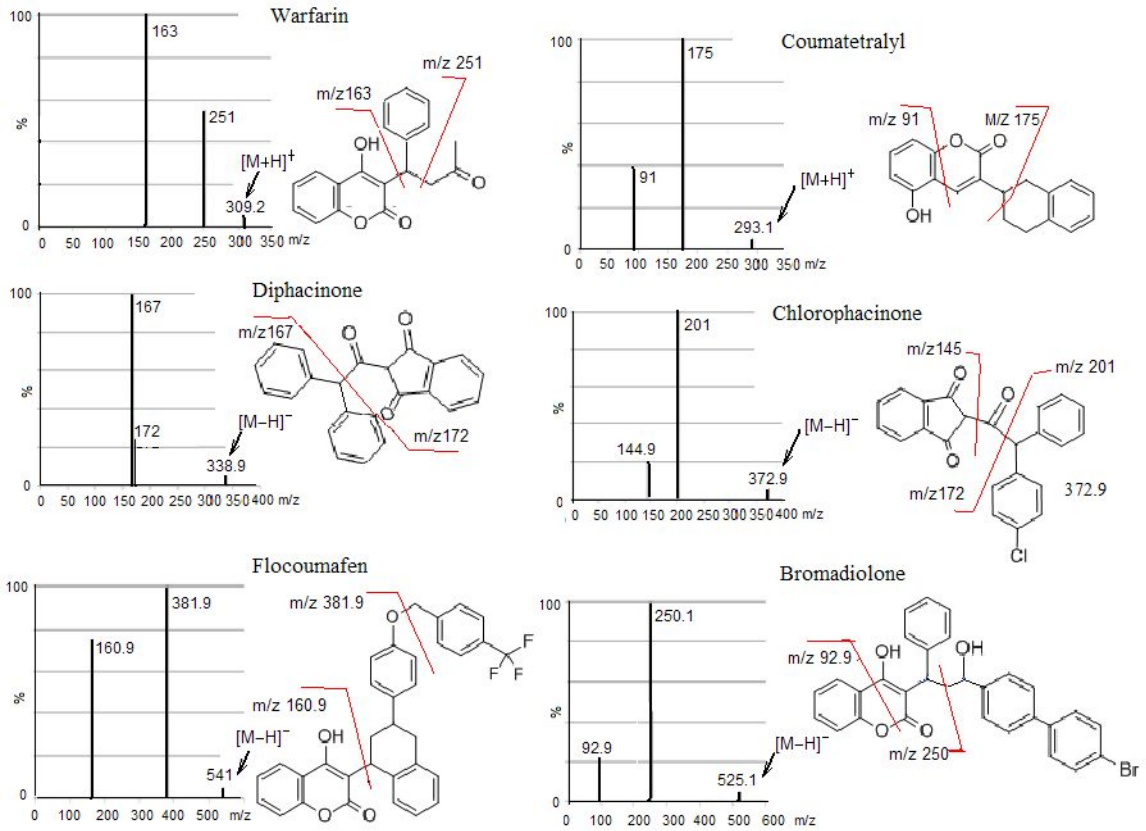

Figure 3

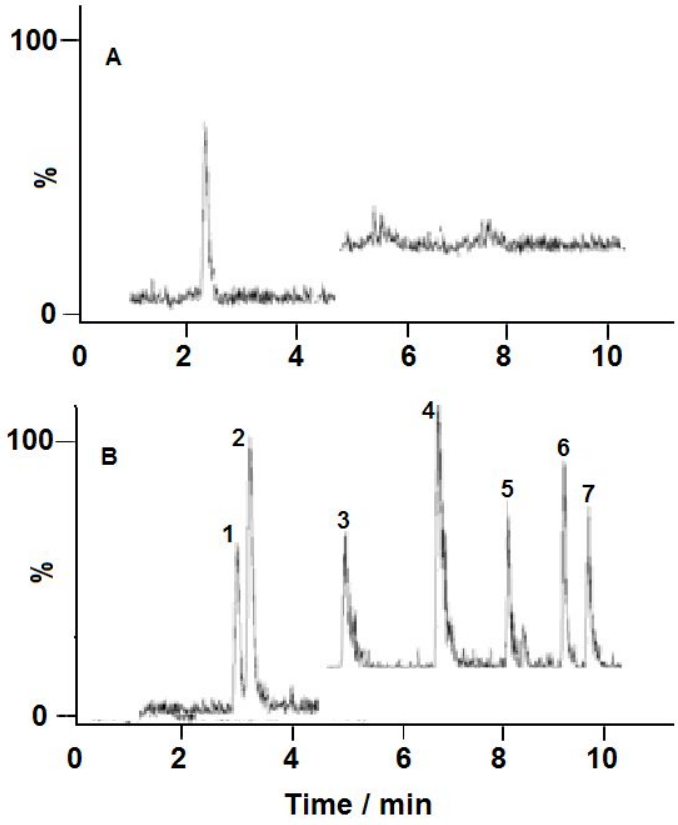

Figure 4