Studii şi Cercetări Ştiinţifice 2020, **21** (1), pp. 059 – 069
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

DETERMINATION OF CATECHOLAMINES AND RELATED MOLECULES IN BRAIN EXTRACT USING A HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY MASS SPECTROMETRY METHOD

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Received: September, 12, 2019
Accepted: February, 19, 2020

Abstract: The present paper presents a hydrophilic interaction chromatography (HILIC) coupled to tandem mass spectrometry method for the analysis of 11 catecholamines, precursors and metabolites in a sheep brain extract. The LODs were between 0.5 ng·mL^{-1} (3-MT) and 250 ng·mL^{-1} (DOPAC). The method accuracy has been evaluated in brain extract by recovery studies with samples spiked at a concentration of 100 ng·mL^{-1}. For the spiked brain extract, the mean recovery values ranged from 81 % to 101 %, with precision < 8 %.

Keywords: *HILIC*, *neurotransmitters, sheep brain extract*, *tandem mass spectrometry*, *TSK gel Amide 80*

INTRODUCTION

Catecholamines and indolamines represent an important category of biogenic amines that are involved in the neurotransmission mechanism. Adrenaline (A) (epinephrine), dopamine (DA) and noradrenaline (NA) (norepinephrine) are the 3 *in vivo* catecholamines, that are produced from tyrosine (Tyr) and 3,4-dihydroxy-phenylalanine (DOPA) [1] and metabolized principally in homovanillic acid (HVA), 3-methoxytyramine (3-MT) and 3,4-dihydroxy-phenylacetic acid (DOPAC). Serotonin (S) is one of the most important indolamine, tryptophan (Trp) and 5-hydroxyindole-3-acetic acid (5HIAA) are its precursor and metabolite, respectively.

The concentration levels of catechoamines and indolamines in different biological fluids or tissues can provide important information about the state of health of an individue [2 – 5]. Thus, various high performance liquid chromatography (HPLC) systems have been used for catecholamines separation. Reversed phase liquid chromatography (RPLC) [6 – 8], ion pairing chromatography (IPLC) [9 – 11] and hydrophilic interaction liquid chromatography (HILIC) [12 – 15] have been applied for the catecholamine analysis.

Due to its rather high sensitivity and selectivity, electrochemical detection is one of the most popular detection modes used for catecholamines [9, 16 – 20]. However, UV [7, 21] and fluorescence [22, 23] detection were also mentioned in the literature. On the other hand, mass spectrometric detection is becoming extensively used [6, 11, 24 – 29] as it can offer supplementary structural information as well as high sensitivity.

In this view, for the analysis of neurotransmitters, coupling HILIC separation and MS detection is beginning to seem attractive for the analysis of neurotransmitters [29, 30]. Thus, a HILIC-APPI-MS/MS assay has been developed on a diol column to separate both acetylcholine, dopamine, DHEA (dehydroepiandrosterone) and GABA (γ-aminobutyric acid) [8]. A capillary HILIC-ESI-MS/MS system using a polyhydroxyethyl-aspartamide column has been proved capable of detecting and quantifying *in vivo* acetylcholine, serotonin, dopamine, GABA, glutamate and aspartate [14]. In 2008, Gu Q. *et al.* [12] assessed a HILIC-MS/MS method using cyano silica column for the analysis of eight catecholamines and metabolites (Tyr, DA, DOPA, A, NA, 3-MT, normetanephrine (NMN) and metanephrine (MN)) in adrenal gland, with limits of detection (LOD) between 0.5 and 20 $ng \cdot mL^{-1}$. However, under these chromatographic conditions, even though MS quantification of these eight compounds was demonstrated to be feasible thanks to the high specificity of the MS detection mode, the selectivity towards polar catecholamines remains poor with coelution between A, DA, MN and NMN resulting from weak retention.

The present study aims to determine the concentration of the selected catecholamines in the sheep brain extract using an HILIC-MS method. For that, an already optimized HILIC method [15] was coupled to MS detection (the optimization of the MS parameters is also presented).

MATERIALS AND METHODS

Chemicals and Reagents

Adrenaline (A), 3,4-dihydroxy-phenylacetic acid (DOPAC), 3,4-dihydroxy-phenylalanine (DOPA), dopamine (DA), 5-hydroxyindole-3-acetic acid (5HIAA), homovanillic acid (HVA), 3-methoxytyramine (3-MT), noradrenalin (NA), serotonin (S), tryptophan (Trp) and tyrosine (Tyr) were purchased from Sigma-Aldrich (St.-Quentin-Fallavier, France). The internal standard (3,4 dihydroxybenzylalanine - DHBA), ammonium acetate and acetic acid were obtained from Fluka (St.-Quentin-Fallavier, France). The perchloric acid ($HClO_4$) was from VWR Prolabo (Darmstadt, Germany). HPLC-grade acetonitrile (MeCN) was purchased from J.T. Baker (Noisy le Sec, France). All the solutions analytes and mobile phases were prepared using deionised water, purified using an Elgastat UHQ II system (Elga, Antony, France).

Chromatographic and spectrometric conditions

The chromatographic system used to optimize the chromatographic separation consisted of an Agilent 1100 series (Waldbronn, Germany) system with: pump, auto sampler with 5 µL loop, column oven (temperature set at 20 °C) and DAD detector (set at 280 nm). The chromatographic software was Chemstation version A.08.03 (Waters). The selected stationary phase was an amide bounded silica column, TSK gel Amide 80 (250 x 2 mm i.d.) (Tosoh Bioscience, USA). The mobile phase was previously optimized [13] and consisted of a 80:20 v/v mixture of MeCN and 20 mM CH_3COONH_4 buffered at *p*H 3.
The mass spectrometric analyses were carried out on a Quatro Ultima (Micromass Ltd, Manchester, UK) triple quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionization source. The analysis was conducted in the multiple reaction monitoring (MRM) mode. With the exception of DOPAC and HVA, which were detected in negative ionization mode, all the other compounds were analyzed in the positive mode. The mass spectrometer settings were optimized in order to obtain the maximal value for the signal to noise ratio, as follows: cone voltage - 35 V, capillary voltage - 3500 V, source temperature - 100 °C, desolvation temperature - 400 °C, cone gas flow - 70 $L \cdot h^{-1}$, desolvation gas flow - 650 $L \cdot h^{-1}$, collision gas - Ar. The collision energy was optimized for each compound and the values are registered in Table 1. The dwell time was set at 200 ms and both the first (Q1) and the third (Q3) quadrupoles operated in open resolution mode. The data were processed with MassLynx 4.0 software. Significant fragmentation of the analytes occurred already in the ionization source, with the result that the molecular ion $[M+H]^+$ is not the base peak for the A, NA, DA, DOPAC, S, DHBA and 3-MT spectra. For these compounds, the transitions from the base peak to the main fragment obtained were selected. Table 1 report all the MRM transitions followed for the identification and quantification of the targeted compounds.

Table 1. *Mass spectrometric transitions and LODs (in mobile phase or in brain extract) for catecholamines, indolamines, metabolites and precursors*

Analyte	Molecular ion [m/z]	Selected transition (base ion > main fragment) [m/z]	Collision Energy [eV]	LOD in the mobile phase [µg·L^{-1}]	LOD in the matrix [µg·L^{-1}]
	[M+H]$^+$				
DHBA	140	123 > 95	20	50	200
DA	154	136 > 91	20	50	200
3-MT	168	151 > 91	20	1	0.5
NA	170	152 > 107	15	10	1
S	177	160 > 115	25	2.5	1
TYR	182	182 > 136	15	10	Already present
A	184	166 > 123	25	10	5
5HIAA	192	192 > 146	15	5	0.5
DOPA	198	198 > 152	15	100	35
TRP	205	205 > 188	10	2.5	Already present
	[M-H]$^-$				
DOPAC	167	123 > 94	20	500	250
HVA	181	181> 123	15	1000	200

Standards and sample preparation

1000 µg·mL^{-1} stock standard solutions were prepared by dissolving the needed amount of compound in 0.2 mol·L^{-1} perchloric acid ($HClO_4$). All stock solutions were stored at -80 °C. The injected standards were diluted in a mixture of acetonitrile/buffer in order to have a composition as close as possible to that of the mobile phase.

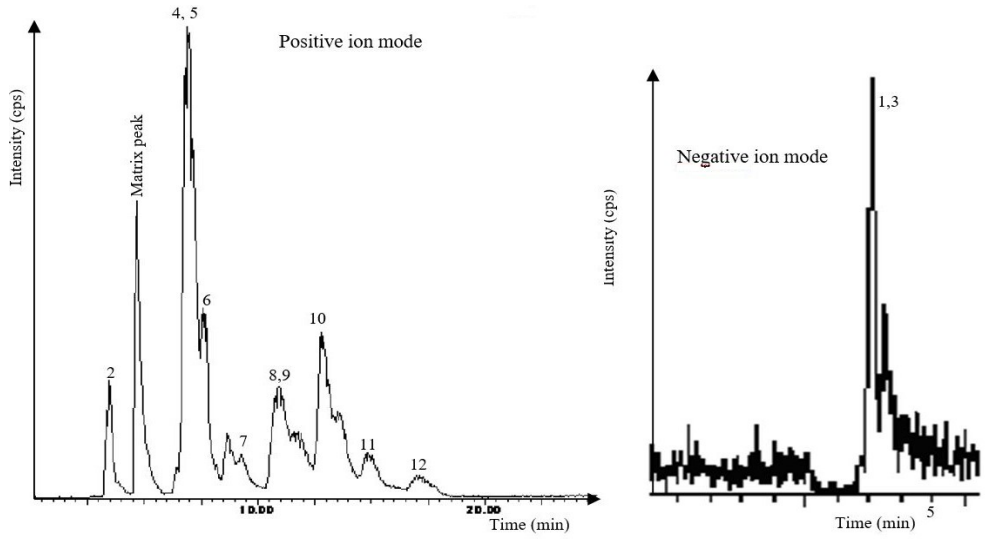
For the calibration curves, intermediate stock standard solutions were prepared by diluting the corresponding stock standard solutions in 0.2 mol·L^{-1} $HClO_4$. The concentration of each intermediate solution was as follows: 5 µg·mL^{-1} for DOPAC and HVA; 10 µg·mL^{-1} for NA, A, Tyr, 5HIAA, Trp, DHBA and 3-MT; 20 µg·mL^{-1} for DA; 10 µg·mL^{-1} and 500 µg·mL^{-1} for DOPA. Five calibration solutions (from 25 - 200 ng·mL^{-1} to 300 - 800 ng·mL^{-1} depending on the LOD of the solute analyzed as reported in Table 1) were obtained by diluting these intermediate stock standard solutions with the optimized mobile phase. To establish the calibration curves, DHBA was used as internal standard and added to the different calibration solutions at a constant concentration of 200 µg·mL^{-1}.

The sheep brain extract was prepared as previously described [11]. Just before analysis, the brain extract was filtered through a 0.45µm syringe filter (Millipore) and an aliquot (100 µL) of the filtrate was diluted in 900 µL of a mixture composed of 100 µL ammonium acetate buffer and 800 µL of acetonitrile in order to obtain a final solvent composition as close as possible to that of mobile phase. Thus, the final analyzed sample corresponds to 20 mg of tissue by mL of solution.

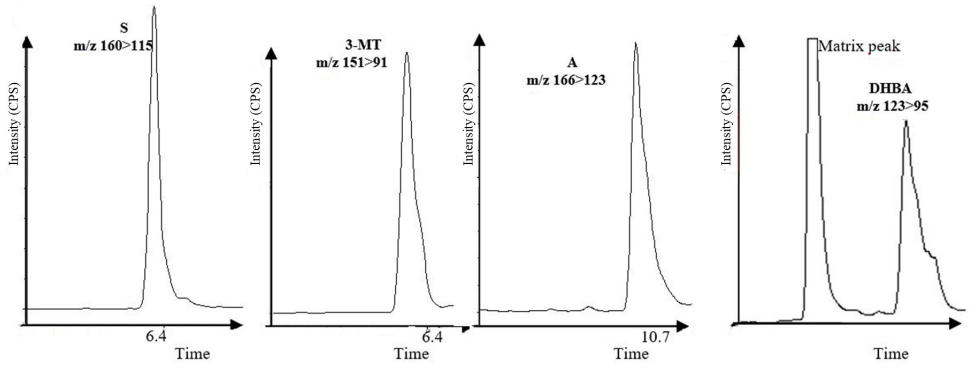
RESULTS AND DISCUSSION

HPLC-MS tandem

The mass spectrometric detection was carried out in both negative and positive ionization modes. DOPAC and HVA responded better in the negative ionization mode, while all the other compounds gave better responses in the positive ionization mode, as already observed by Tornkvist *et al.* [31]. Figure 1 shows the MRM of 12 catecholamines obtained by coupling the optimized HILIC system to tandem MS and shows the extracted ion currents (XIC) of several ion transitions monitoring. The sample analyzed corresponds to a brain extract spiked with the same concentration (1 $\mu g \cdot mL^{-1}$) of each catecholamine. At a given concentration, not all the solutes have the same MS response. A significantly lower response in electrospray negative ion mode than in positive ion mode can be observed on Figure 1.



1a.



1b.

Figure 1. *LC-ESI-MS/MS analysis of a brain extract doped with the 12 neurotransmitters and related compounds under isocratic conditions*

Peaks: (1) HVA; (2) 5HIAA; (3) DOPAC; (4) 3-MT; (5) S; (6) Trp; (7) DA; (8) A; (9) DHBA; (10) Tyr; (11) NA; (12) DOPA.

a. Total ion current (TIC) obtained with ion source operated in a positive or negative ionization mode
b. Extracted ion currents (XICs) of the co-eluted analytes (S, 3-MT) and (A, DHBA)

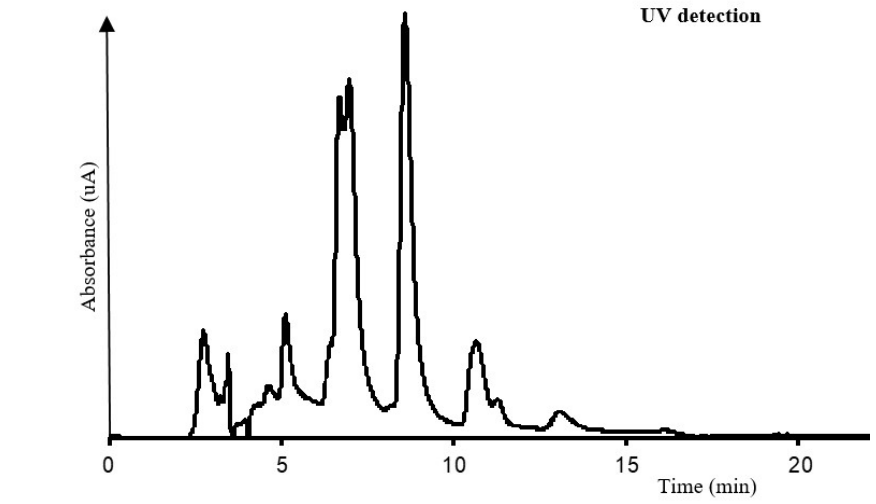
From the XIC, we can observe that 3-MT and S on the one hand and, A and DHBA on the other hand, are co-eluted but the specificity of mass spectrometry enables them to be detected due to the difference of their base and product ions. From these observations, it has been concluded that it is possible to detect the presence in brain extract of all the target compounds under HILIC-ESI-MS/MS conditions.

Brain extract analysis

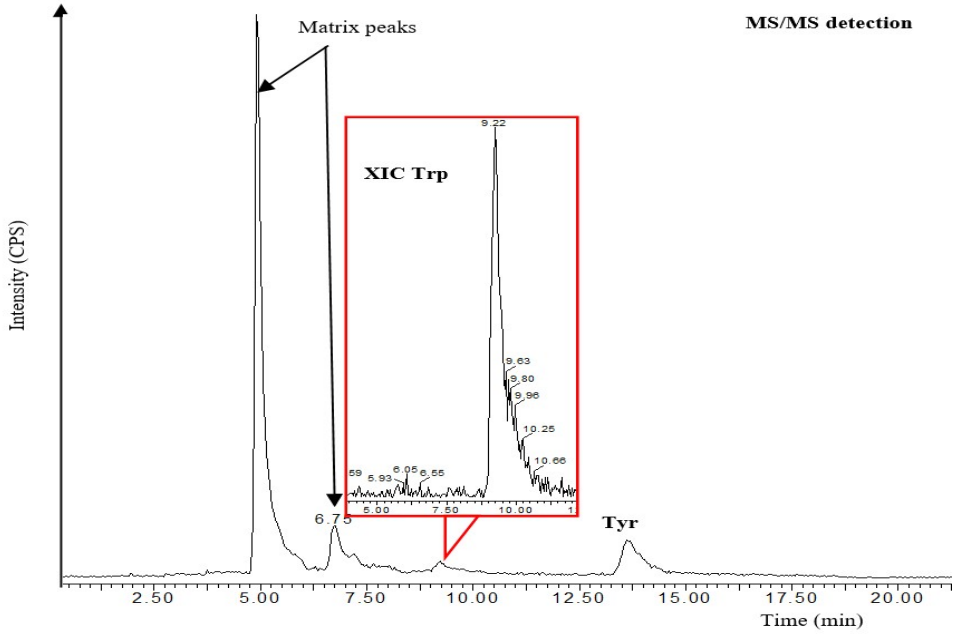
The complexity of the matrix and its low content in neurotransmitters make impossible to obtain any information about the catecholamines present in the extract by simple UV analysis. Some matrix components also absorb at 280 nm, with the result that their signals are added to those of the solutes (Figure 2a). This is not the case with mass spectrometric detection, thanks to its high specificity, especially in the MRM mode. As can be seen from the MRM analysis (Figure 2b), Trp and Tyr can be identified in the brain extract sample due to their retention time and ESI-MS spectra which are identical to those of the reference standards. Nonetheless, even when using a selective detector such as mass spectrometry, matrix interference can still be observed. Two very intense peaks at approximately 5 min and 6.7 min are detected on the transitions of DHBA and DA respectively, showing yet again the importance of chromatographic separation in the analysis of complex matrices, as the retention time correlated to the selected ion transitions for a solute helps to identify it unambiguously.

Detection limits and linearity

The detection limits (LODs) were determined in mobile phase and in brain extract for quantitative purposes and in order to assess the matrix effect. The corresponding values (signal to noise ratio of 3) are reported in Table 1. Most of the LOD values evaluated in mobile phase are between 1 and 50 $ng{\cdot}mL^{-1}$ for the analytes ionized in a positive ion mode (except DOPA) and above 500 $ng{\cdot}mL^{-1}$ for those ionized in a negative ion mode. These LODs are in good agreement with the values obtained previously by Gu Q. *et al.* [12] on a cyano column with a mobile phase composed of MeCN and HCOOH solution (*p*H 3) (60:40, v/v) and a linear trap spectrometry detection. As can be seen in table 1, LODs determined in brain extract were different from those in mobile phase. The matrix effect is not the same for all the compounds, resulting either in an increase in ion signal (for A, DOPA, 3-MT, NA, S, HVA, DOPAC and 5HIAA) or in an ion suppression (for DA and DHBA).
The calibration curves were first established with standard solutions prepared in mobile phase and performed for nine compounds detected in a positive ion mode over a range of concentrations from 25 - 200 $ng{\cdot}mL^{-1}$ to 300 - 800 $ng{\cdot}L^{-1}$ (depending on the limit of quantification of each solute, Table 1). The curves were determined at five evenly spaced concentrations using DHBA as internal standard. The internal ratios (solute peak area versus internal standard peak area) were calculated for each point and standard curves were constructed using least square linear regression analysis of internal ratios over solute concentration. Results are displayed in Table 2.



a.



b.

Figure 2. *Analysis of a brain extract (20 mg·mL^{-1} of tissue)*
a. UV detection at 280 nm
b. TIC of the MS/MS detection

The calibration curves were found to be linear with an average determination coefficient (r^2) higher than 0.994 except for Trp and DOPA (0.976 and 0.983 respectively). The accuracy of the assay was determined by comparing the nominal concentrations of quality control samples prepared at median concentration level in mobile phase, with the corresponding calculated ones. Acceptable accuracies were obtained for most of the catecholamines within 96 and 105 % of theoretical values except for 5-HIAA (62 %), with a precision below or close to 5 % except for Tyr (14.7 %). This weak precision value for Tyr could be explained by a possible poor correction of its MS signal variations by the internal standard.

Table 2. *Linearity parameters in the mobile phase*

Analyte	Range [µg·L^{-1}]	Linear regression	r²	Accuracy	RSD [%]
DA	100 – 300	y = 0.0002+0.01	0.9953	105.4±2.5	1.2
3-MT	10 – 100	y = 0.0254x+0.0942	0.9956	112.5±6.1	2.8
NA	50 – 200	y = 0.0114x+0.0818	0.9974	97.0±4.4	4.3
S	25 – 200	y = 0.0051x+0.1559	0.9979	103.04±5.4	5.3
TYR	50 – 250	y = 0.0067x+0.1528	0.9972	105.2±15.5	14.7
A	50 – 200	y = 0.0066x+0.1528	0.9969	99.5±4.5	4.5
5HIAA	50 – 200	y = 0.0067x+0.1528	0.9941	61.7±3.2	1.2
DOPA	300 – 800	y = 0.002x+0.0979	**0.9758**	102.8±5.8	1.1
TRP	25 – 200	y = 0.007x–0.1528	**0.9830**	96.5±6.1	5.5

The potential matrix effect on the catecholamine signal was then tested by analyzing a brain extract sample spiked with 6 catecholamines (DA, 3-MT, NA, S, A and DOPA) at median concentration levels. Back concentrations calculated from equations of linearity established with standards prepared in mobile phase, exhibit a low accuracy (below 70 % for most of the solutes). This suggests that a significant bias was introduced in the quantification. For A, co-eluted with DHBA, the internal standard, the accuracy exceeds 90 % of the theoretical value. These results confirm that interferences with other matrix components occurred during the detection step. Furthermore, in the absence of stable isotopically labelled analogues of the compounds, the choice of an internal standard with similar properties in terms of chromatographic behavior and ionization to those of the corresponding analytes enables the accuracy of the quantitative method to be increased.

A new set of calibration standards was then prepared in brain extract spiked with catecholamines (3-MT, NA, S, A, 5HIAA, DOPA, Trp) in the same concentration ranges as the calibration samples in mobile phase. Results are shown in Table 3.

Table 3. *Linearity parameters in the matrix*

Analyte	Range [µg·L^{-1}]	Linear regression	r²	Accuracy	RSD [%]
3-MT	10 – 100	y = 0.0184x–0.2364	0.9961	95.4±0.8	3.9
NA	50 – 200	y = 0.0083x–0.1362	0.9955	**82.4**±4.5	**5.1**
S	25 – 200	y = 0.0043x+0.1559	0.9927	100.9±7	7
A	50 – 200	y = 0.008x+0.0906	0.9975	96.6±2.3	2.2
5HIAA	50 – 200	y = 0.0067x+0.1528	**0.9588**	93.1±4.5	3.6
DOPA	300 – 800	y = 0.0005x+0.0637	**0.954**	86.5±2.7	4.9
TRP	25 – 200	y = 0.0021x+0.2922	0.9943	95.04±8.3	7.7

The calibration curves were found to be linear with an average determination coefficient r^2 of 0.995 for (3-MT, NA, S, A, Trp) and higher than 0.95 for 5HIAA and DOPA. The accuracy determined by comparing the nominal concentrations of quality control samples prepared at median concentration level in matrix, with the corresponding calculated ones was satisfactory (within 81 % and 101 % with a precision below 5 % for most of solutes). Better results in recovery terms were obtained for the calibration curves prepared in matrix (Table 3). These results mean that calibration must be

preferentially carried out directly in a matrix blank spiked with a known concentration of analytes to improve the accuracy of the quantitative method.
As can be seen from Figure 5b, two analytes, Trp and Tyr, have been identified in the brain extract due to their retention time and ESI-MS spectra which are identical to those of the reference standards. Their concentrations evaluated from the external calibration method and expressed by g of brain were respectively 0.6 $\mu g \cdot g^{-1}$ for Trp and 13 $\mu g \cdot g^{-1}$ for Tyr.

CONCLUSION

The method provides good linearity for the calibration curves prepared both in the mobile phase and in the matrix. The poor accuracies obtained for the spiked brain when using the calibration curves prepared in the mobile phase were improved with the calibration curves prepared in the matrix.
The detection limits under the developed method were about 0.5 and 200 $ng \cdot mL^{-1}$. Considering the extremely low levels of catecholamines in some areas of the brain, higher sensitivity would be required to extend this application to neurochemical studies. Future developments will be: (i) to lower the detection limits using Narrow-bore HILIC column coupling a special trap column which can enrich the solutes and also remove potential matrix interferences as much as possible; (ii) to increase the accuracy of the quantitative method by using some stable isotopically labeled analogues of the compounds.

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