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Optimization of SPE method for the extraction of 12 neurotransmitters from sheep brain

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Abstract. The present paper presents our attempts concerning the development of an extraction method for catecholamines. In order to achieve the extraction of all the selected solutes using a single SPE cartridge, several types of support were tested, among them: cation exchange supports, hydrophilic-lipophilic supports, C18 supports and PGC supports. As unfortunately none of the supports tested offered us the possibility of carrying out the extraction of 12 catecholamines from our standard mixture, we chose to use a coupling of two different cartridges: Oasis HLB and PGC which together ensure the extraction of all the compounds of the mixture with good extraction yields and with simple protocols. The selected cartridges were successfully tested for the extraction of a sample spiked from sheep brain with the 12 catecholamines in our mixture. The SPE method that we have developed allows the purification of the samples (a significant part of the components of the matrix is eliminated during this step) and also a preconcentration of the samples.

Keywords: Catecholamine; Oasis HLB; PGC; SPE; Sheep brain extract.

1. Introduction

Catecholamines and indolamines are biological compounds accomplishing many different vital functions in both humans and animals, they can play the role of neurotransmitters and hormones, and are involved in numerous neurophysiological processes like stress, anxiety, or depression [1]. The quantification of these compounds in different biological samples can facilitate a better understanding of the mechanism of many diseases (e.g.: pheochromocytoma, paraganglioma, schizophrenia, Parkinson's, and Alzheimer's disease) [2-5]. In this context, numerous high performance liquid chromatography (HPLC) systems have been optimized for catecholamines separation: reversed phase liquid chromatography with [6 – 9] and without ion pairing agents addition [9–11] and hydrophilic interaction liquid chromatography (HILIC) [12–15]. Concerning the detection modes employed, electrochemical detection is one of the most popular [16–20], but UV [10, 21] and fluorescence [22, 23] detection were also mentioned in the literature. Lately, mass spectrometry has gained lots of interest due to the fact that it offers supplementary structural information as well as high sensitivity [5, 14, 24–27].

The greatest challenge when analyzing these compounds comes from the fact that their concentrations in the different biological samples are very low, for example, there are 0.5 µg/g of dopamine and 0.1 µg/g of noradrenaline in the rat brain [28]. Also when analyzing biological samples, different matrix components can interfere masking the analytes signal [8, 29, 30]. Thus effective sample preparation methods are required in order to solve these problems. Literature presents methods that have been developed for the extraction of catecholamine from various complex biological matrices [9, 28, 31, 32], among them: liquid–liquid extraction (LLE) [33] or solid phase microextraction (SPME) [34]. The most popular pretreatment and/or extraction technique proved to be solid phase extraction (SPE) owing to the fact that it is more sensitive, environmentally friendly and faster [35]. Thus different SPE sorbents have been used for catecholamine extraction from biological samples, like: C18 [9, 36], alumina [37, 38], ion exchanging supports [7, 24, 25, 39], boronate compounds [40, 41], crown ether-modified polymers [34], hydrophil-lipophil supports [42, 43] and even specially created molecular imprinted polymers [32]. However, most of the proposed methods were developed for a small number of compounds that had similar properties [9, 25, 36, 39].

This paper presents the development of a simple and rapid SPE method for the extraction of the twelve compounds (catecholamines, indolamines, their precursors and metabolites, and an internal standard) from sheep brain extract. To this end, we have tested several commercially available SPE cartridges: ion-exchange supports, hydrophilic-lipophilic supports, C18 grafted supports, and PGC support. The optimized method necessities the use of two SPE cartages: Oasis HLB and PGC in order to retain all the selected compounds.

2. Experimental

2.1. Chemicals and reagents

Adrenaline (A), dopamine (DA), 3,4-dihydroxy-phenylacetic acid (DOPAC), 3,4-

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dihydroxyphenylalanine (DOPA), 5-hydroxyindole-3-acetic acid (5HIAA), homovanillic acid (HVA), 3-methoxytyramine (3-MT), noradrenalin (NA), serotonin (S), tryptophan (Trp) and tyrosine (Tyr), nonafluoropentanoic acid (NFPA), tridecafluoroheptanoic acid (TDFHA) and pentadecafluorooctanoic acid (PDFOA) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). The 3,4-dihydroxybenzylalanine (DHBA), ammonium formate ($HCOONH_4$), 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 methanol (MeOH) and acetonitrile (MeCN) were purchased from J.T. Baker (Noisy le Sec, France). All the analytes solutions and mobile phases were prepared using deionized water, purified using an Elgastat UHQ II system (Elga, Antony, France).

2.2. Standards and sample preparation

1000 µg/mL stock standard solutions were prepared by dissolving the needed amount of compound in 0.2 mol/L perchloric acid ($HClO_4$). All stock solutions were stored at -80 °C.

The sheep brain extract was prepared as previously described [11]. Just before analysis, the brain extract was filtered through a 0.45 µm syringe filter (Millipore) and used as so or an aliquot (500 µL) of the filtrate was mixed in 500 µL of an aqueous solution of NFPA 1.25 mM.

2.3. HPLC analysis

For the HPLC analysis we have chosen a previously optimized method that allows the separation of the 12 selected compounds [8]. Thus, separations were carried out on the octadecyl-bonded monolithic silica column Onyx (Phenomenex) (LxØ = 100 × 4 mm). The mobile phase was composed of MeOH and NFPA 1.2 mM aqueous solution in gradient elution mode: 0 to 5 min 5 % of MeOH, from 5 to 8 min from 5 to 20 % of MeOH, from 8 to 12 min from 20 to 40 % of MeOH, in 0.1 min back to the initial conditions for column reequilibration. The chromatographic system was composed of a Merck-Hitachi quaternary pump model Lachrom L-7100 (Darmstadt, Germany), a Rheodyne (Cotati, CA, USA) model 7725 injection valve fitted with a 20 L loop, column oven Jet Stream 2 Plus and a 785A UV-visible HPLC Detector (Applied Biosystems, Courtaboeuf, France), set at 280 nm in order to obtain maximal absorbance for all the compounds. The chromatographic data handling was accomplished using EZChrom Server software (Merck, Darmstadt, Germany)

2.4. SPE

The extraction procedures were carried out on a 12-Port Visiprep vacuum manifold (Supelco, Sigma–Aldrich, St. Quentin-Fallavier, France). The main characteristics of the tested SPE cartridges are presented in the table below.

Table 1. Characteristics of the tested SPE supports

Cartridge/ Producer name	Support type	Specific surface [m^2/g]	Particles diameter [µm]	Pores diameter [Å]	Sorbent weight [mg]
Oasis HLB/ Waters	Polymer	810	-	80	30
Oasis MCX/ Waters	Polymer	-	60	80	200
OASIS WCX/ Waters	Polymer	-	30	80	30
Bond Elut Plexa PCX/ Varian	Polymer	450	45	120	60
Bond Elut Plexa AccuCAT/ Varian	Polymer	450	45	120	200
C18/ Sigma Aldrich	Polymer	475	45	60	100
PGC/ Thermo Fisher	Porous graphitic carbon	-	30	250	25 and 50

Different extraction protocols were tested in order to obtain the extraction of the selected compounds from test solutions. They are listed in the table below.

Table 2. Extraction protocols on the different tested SPE supports

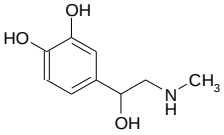
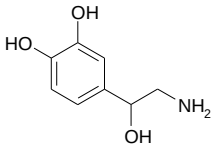
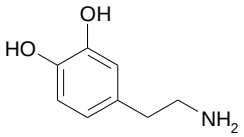
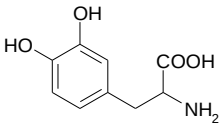
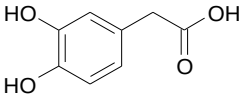
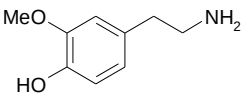
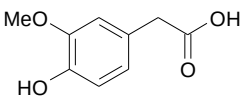
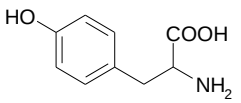
Protocol number	Conditioning	Load	Wash	Elution
I	6 mL MeOH 6 mL 5% $HCOONH_4$ in MeOH 6 mL H_2O	3 mL standard solution of DA, DOPA and DOPAC 10 µg/mL in $HClO_4$ 0.2 M	3 mL $HClO_4$ 0.1 M (aqueous solution) 3 mL MeOH	3 mL 5% $HCOONH_4$ in MeOH
II	6 mL MeOH 6 mL 5% $HCOONH_4$ in MeOH 6 mL H_2O	3 mL standard solution of NA, A, DA, DOPA, DHBA, 3-MT, S Tyr, Trp 10 µg/mL in $HClO_4$ 0.2 M	3 mL $HClO_4$ 0.1 M (aqueous solution) 3 mL MeOH	3 mL 1% $HCOONH_4$ in MeOH
III	2 mL MeOH 2 mL H_2O	1 mL standard solution of 12 catecholamine 10 µg/mL in $HClO_4$ 0.2 M	3 mL H_2O	0.5 mL MeOH
IV	2 mL MeOH 5 mL prefluorinated acid 1% in H_2O	1 mL standard solution of 12 catecholamine 10 µg/mL in $HClO_4$ 0.2 M	3 mL H_2O	1 mL MeOH

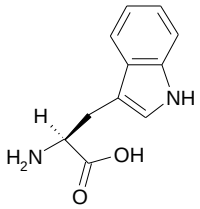
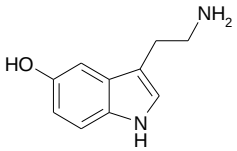
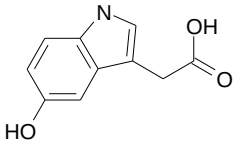
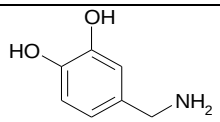
Protocol number	Conditioning	Load	Wash	Elution
V	6 mL MeOH 8 mL NFPA 10 mM	1 mL standard solution of catecholamine 10 µg/mL in $HClO_4$ 0.2 M and NFPA 5 mM	4 mL NFPA 1.25 mM	0.5 mL TFA 0.1% in MeOH
VI	3 mL MeOH 4 mL NFPA 20 mM	1 mL standard solution of 3 catecholamine 10 µg/mL in $HClO_4$ 0.2 M	4 mL H_2O	0.5 mL TFA 5% in MeOH 0.5 mL TFA 5% in MeCN
VII	3 mL MeOH 4 mL NFPA 1.25 mM (aqueous solution)	1 mL standard solution of 6 catecholamine 10 µg/mL in $HClO_4$ 0.2 M / NFPA 1.25 mM (1:1 v/v)	2 mL NFPA 1.25 mM (aqueous solution)	2 x 0.5 mL TFA 0.1% in MeOH
VIII	3 mL MeOH 5 mL NFPA 10 mM (aqueous solution)	1 mL standard solution of 6 catecholamine 10 µg/mL in $HClO_4$ 0.2 M / NFPA 5 mM (1:1 v/v)	2 mL NFPA 1.25 mM (aqueous solution)	0.5 mL TFA 0.1% in MeOH

3. Results and discussion

The chemical structures of the selected compounds are presented in Table 3. The reason for these compounds selection is that among the biogenic amines that are involved in the neurotransmission mechanism there are: adrenaline (epinephrine), dopamine and noradrenaline (norepinephrine) – they are produced from tyrosine and 3,4-dihydroxy-phenylalanine and metabolized mainly, in homovanillic acid, 3-methoxytyramine and 3,4-dihydroxy-phenylacetic acid. The most popular compound selected as internal standard for the catecholamine determination is 3,4 dihydroxybenzylalanine (DHBA) [7, 20, 32, 43], and thus we also used it. On the other hand, one of the most important indolamine, serotonin, is obtained from tryptophan and has 5-hydroxyindole-3-acetic acid as main metabolite. Subsequently, for simplicity reasons, all the selected compounds will be addressed as catecholamines.

Table 3. Selected compounds structure and characteristics

Compound	Chemical structure	Molar weight [g/mol]	pK_a*	log P**
A		183	pKa_1= 8.66 pKa_2= 9.95	0.33
NA		169	pKa_1= 8.64 pKa_2= 9.7	-0.08
DA		153	pKa_1= 8.9 pKa_2= 10.6	0.85
DOPA		197	pKa_1= 2.32 pKa_2= 8.72 pKa_3= 9.96 pKa_4= 11.79	0.58
DOPAC		167	pKa= 4.4	1.11
3-MT		167	pKa= 9.6	0.88
HVA		181	pKa_1= 4.43 pKa_2=7.85	1.54
Tyr		181	pKa_1= 2.20 pKa_2= 9.11 pKa_3= 10.13	0.87

Compound	Chemical structure	Molar weight [g/mol]	pK_a*	log P**
Trp		204	pKa_1= 2.43 pKa_2= 9.44	1.25
S		176	pKa_1= 9.80 pKa_2= 11.1	1.23
5HIAA		191	pKa_1= 4.51 pKa_2= 15.59 pKa_3= 9.92	1.49
DHBA		139	pKa= 8.4	0.59

* pKa values from [42, 44] or calculated with Marvin 4.1.11 software
** log P values were calculated with Marvin 4.1.11 software

As it can be seen in Table 3, the selected compounds can be divided in three groups: amines (DA, NA, A, 3-MT, S and DHBA), amino acids (Tyr, DOPA and Trp) and carboxylic acids (HVA, DOPAC and 5HIAA). To prevent degradation of analytes, their handling was mostly realized under acidic conditions. In aqueous solutions at pH 3, the six amines are positively charged (protonated), HVA, DOPAC and 5HIAA, containing carboxylic functions, with pKa values around 4.5, are only partially dissociated, thus bearing a partial negative charge. For the three amino acids, the amine functions are protonated whereas the carboxylic functions are principally deprotonated resulting in the presence of zwitterionic compounds (global nominal net charge equal to zero) [13].

Our previous research on the catecholamines, indolamines and their metabolites and precursors quantification in the brain extract has revealed the need for an SPE method that would allow the sample clean up in order to removing matrix components that interfere with the analytes detection [8, 14].

3.1. Ion exchange cartridges

As we have mentioned earlier, an important number of publications describe for catecholamines extraction ion exchange SPE methods [7, 24, 25, 39], however in those cases more heterogeneous groups of compounds were analyzed. Thus, considering that nine of the selected compounds bear a positive charge (in acidic pH conditions) we have decided to test first a cation exchange support. For this purpose, we have used the Oasis MXC, which is a mixed mode support type, offering retention possibilities both through cation exchange and reversed phase mechanisms (Figure 1).

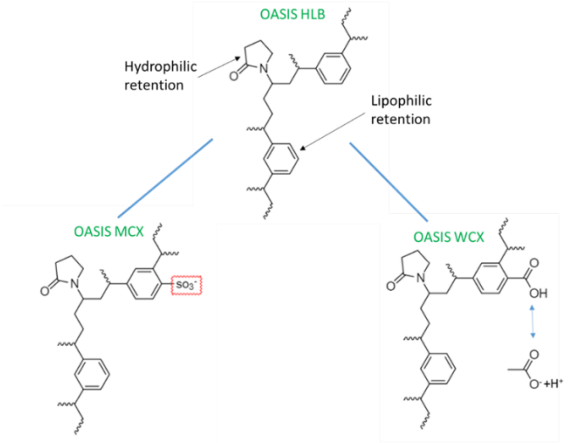


Figure 1. Oasis sorbents structure [45].

For Protocol I we have selected DA, DOPA and DOPAC as test compounds representative for each of the three groups. The fraction recovered after elution step is evaporated to dryness under a light nitrogen flow. After evaporation a significant amount of salt crystallized on the flasks walls. The residue recovered after evaporation is solubilized in 0.5 mL of 1.25 mM aqueous NFPA solution, and then analyzed in the chromatographic system. The presence of a large amount of salt in the reconstituted solution after evaporation has the effect of modifying the retention times of the solutes. For example, for DA we recorded a difference of 1.5 min between its retention time as standard prepared directly in an aqueous solution of 1.25 mM NFPA (7.72 min) and that of the reconstituted solution in an aqueous solution of 1.25 mM NFPA after elution on the cartridge and evaporation to dryness (6.17 min). Moreover DOPAC was not recovered in the elution fraction but in the wash one, proving that this negatively charged compound was only retained

through reversed phase mechanism and was dislocated from the support by the MeOH (wash step).

The analysis results show that a rather low extraction yield (only 60%) was obtained for the extraction of a standard solution of DA prepared at a concentration of 10 µg/mL and for which the yield is calculated by relating the area of the chromatographic peak obtained for the reconstituted solution to the area of the peak obtained for the standard solution before loading. On the other hand, if the yield calculated relative to a solution of DA prepared in a 5% methanolic solution of $HCOONH_4$ that was submitted to an evaporation step then and then to the reconstitution in 0.5 mL of NFPA, better extraction yields were obtained (greater than 95%). Taking these results into account we concluded that a significant loss of solutes took place during the evaporation step. Therefore, for the remainder of our ion exchange study, all extraction yields were calculated relative to standard evaporated and reconstituted solutions.

Considering the problems caused by the salt presence in the samples after elution we have tested its influence on the extraction yields. To this end 3 salt concentrations (1, 2.5 and 5%) in the eluting solvent were tested. The results obtained showed only a small decrease of the extraction yield from 102.5 % to 99 % when the salt concentration decreases from 5 % to 1%, thus the salt concentration in the elution solvent can be reduced to a minimum value of 1%. Protocol II that we used subsequently keeps the first three steps (conditioning, loading and washing) identical to the previous one, only one change occurs at the level of the last step, the elution is carried out with 3 mL of a methanolic solution of 1% $HCOONH_4$.

Taking these results into account, we removed the three acidic compounds from our mixture, and extracted a standard mixture of 9 compounds (NA, A, DOPA, DHBA, Tyr, DA, Trp, 3-MT and S) potentially retained on the Oasis MCX media. Figure 2 shows the chromatogram obtained for the solution recovered after elution in SPE (green curve) and for comparison the chromatograms of two standard mixtures of the 9 solutes prepared either directly in an aqueous solution of NFPA (black curve), or in a 1% $HCOONH_4$ methanolic solution evaporated then solubilized in an NFPA solution (pink curve).

As can be seen in Figure 2 the presence of salt in the injected sample has the effect of reducing the retention times for the 5 compounds eluted first, and a loss of symmetry for these chromatographic peaks, which makes it difficult to calculate the extraction yield (problems of peaks integration) for these compounds. On the other hand, for the 4 compounds eluted last (DA, S, 3-MT and Trp), a perfect superposition of the chromatographic peaks is observed regardless of the method of preparation. Peak integrations can be performed under good conditions.

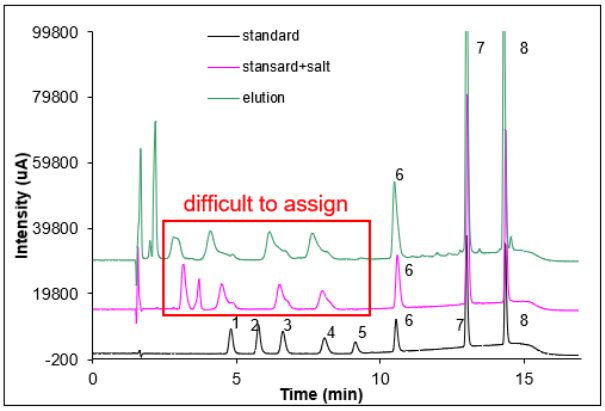


Figure 2. Analysis of a mixture of 9 catecholamines prepared in three different ways: standard mixture prepared in NFPA, standard mixture prepared with added salt and solution recovered after SPE elution: 1. NA, 2. DOPA, 3. A, 4. DHBA, 5. Tyr, 6. DA, 7. S, 8. 3-MT, 9. Trp

The chromatographic system used, being the one with which we obtained the best results in HPLC-MS coupling, the optimized extraction method must therefore be compatible with these chromatographic conditions. Consequently, in order to adapt our extraction conditions, we tested the influence on the retention and peak symmetry of other volatile salts (ammonium acetate, formate and carbamate) and soluble in MeOH. However, no significant amelioration was observed.

Similar results were obtained with another type of strong cation exchange support (Bond Elut Plexa PCX) and no amelioration were obtained by the use of a weak cation exchange support (Oasis WCX). We also tested a mixt mode anion and cation exchange cartridge (Bond Elut AccuCAT) that is presented by its producer as suitable for the extraction of acidic, neutral and basic compounds. However, in the case of our compounds simultaneous retention of differently charged compounds couldn't be achieved. So we have concluded that ion exchange approach is not appropriate for our selected compounds.

3.2. Oasis HLB Cartridge

Previously we have seen that the Oasis MCX cartridge retained not only the cationic compounds of our mixture but also the negatively charged ones through revers phase mechanism. As the Oasis HLB cartridge has the same back bone as Oasis MCX (Figure 1) we decided to test this support hoping it could retain all our analytes.

Protocol III was applied to this cartridge, the elution fraction was evaporated and resolubilized in 0.5 mL of the mobile phase. Under these conditions, out of the 12 selected compounds, only 5 are found in the elution fraction: DOPAC, Trp, HVA, 5HIAA and S. The other compounds of our mixture are not retained on the support and they are found either in the fractions recovered after cartridge loading and / or washing steps. Note that the 5 solutes retained on the HLB cartridge are the most hydrophobic (log P > 1) (Table 3) and they have different charges, which implies that a reverse phase type retention mechanism is mainly responsible for the retention of these solutes under these conditions.

For the 5 retained compounds, we carried out a repeatability study of the extraction yields (Table 4). For DOPAC, HVA, 5HIAA and Trp it can be seen that very good extraction yields (greater than 90%) and acceptable coefficients of variation (CV) (most of them less than 5%) were obtained. On the other hand, for S, the extraction yield is less than 75% with a greater variability (CV greater than 8%). These results are in agreement with those presented in the literature for similar compounds and protocols [3, 46].

Table 4. Repeatability of the extraction yields on the Oasis HLB cartridge

Compound	Extraction yield (%)						Average (%)	Standard deviation	RSD (%)
	1	2	3	4	5	6			
DOPAC	97.0	102.4	100.9	93.9	95.0	91.8	96.8	3.7	3.9
HVA	96.7	103.6	100.9	95.0	95.4	90.7	97.0	4.2	4.3
5HIAA	91.3	95.2	83.9	89.0	94.7	87.2	90.2	4.0	4.4
TRP	90.0	100.2	97.9	88.0	91.8	88.0	92.6	4.7	5.1
S	68.1	83.6	71.9	81.9	68.1	73.7	74.5	6.2	8.3

In order to retain a larger number of the selected compounds, we tried three other reverse phase type protocols, based on conditioning and loading no longer in acidic medium but at neutral pH. To test these protocols, we chose NA as the model solute of the compounds not retained on the HLB cartridge with the previous protocol. Under these new conditions the results remain unsatisfactory, as the NA could not be retained. Therefore, a reverse phase protocol does not allow the retention of catecholamines.

Previously we showed that the retention of amines was increased, on the two types of media tested (C18 grafted silica and PGC), when ion pairing agents are added (perfluorinated carboxylic acid type) in the mobile phase [8]. Thus, we attempted to increase catecholamine retention on the HLB cartridge by adding an ion-pairing agent to the cartridge conditioning solvent. The hydrocarbon chains present on the support (Figure 1) could be able to ensure the fixation of the fluoro-carbon chains of the perfluorinated ion pairing agents (Protocol IV). The tested perfluorinated acids we used were NFPA, TDFHA and PDFOA. Unfortunately, the presence of any of these ion-pairing agents did not significantly increase the interactions with the support of the seven solutes not retained under the previous conditions.

As a result, the Oasis HLB cartridge cannot be used for the simultaneous extraction of all the catecholamines from our mixture. It may, however, be used if the solutes of interest are the 5 compounds retained or in combination with another cartridge which will be able to ensure the retention of the solutes not retained on Oasis HLB support.

3.3. C18 and PGC cartridges

The retention mechanisms in SPE are very similar to those in HPLC, thus as we previously showed that catecholamines were retained and separated on C18 support only in the presence of ion paring agents [8], we tested the extraction capabilities of C18 cartridges by adding NFPA as paring agent in the first 3 steps of the SPE protocol (Protocol V) to endorse the retention of our solutes on the C18 support.

The compounds selected to test the efficiency of Protocol V were: A, NA, DHBA, DOPA, DA, Tyr, Trp and S, compounds having an amino functional group capable of forming the ion pair with the perfluorinated acid. This protocol did not allow us to retain on the cartridge 4 solutes (NA, A, DHBA and DOPA) recovered in load and washing fractions. Significant amounts of DA and Tyr were also lost during the washing step. In contrast, S and Trp were extracted with satisfactory yields (greater than 85%).

This approach of retention on C18 support in ion pairing mode was not satisfactory since only two catecholamines can be retained, so we turned to PGC support which proved more retentive than C18 support in HPLC [8, 47]. Rinne *et al.* [10] have tested a PGC capillary precolumn for NA, DA, A, and S extraction on an in-line SPE-HPLC system. For precolumn loading, they used a mobile aqueous phase containing 0.1% pentafluoropropionic acid (PFPA) as an ion pairing agent. Based on these results and those we obtained in ion pairing chromatography on PGC support [8], we tested the protocol VI for a standard mixture of Trp, DHBA, and 5HIAA. These solutes are selected as representative of the three classes of compounds in our mixture: amino acids, amines, and acids, respectively.

If the retention and elution of Trp, under these conditions (protocol VI), does not pose a problem, in the case of DHBA a significant portion was lost upon the loading step and only a small portion is recovered at elution. Moreover, as we had previously observed in HPLC [8], the elution of 5HIAA from the PGC support is not possible due to its planar structure associated with its anionic character. Thus for further research, 5HIAA will no longer be introduced into the loaded mixture, due to its excessive retention, as well as the other two acidic compounds (HVA and DOPAC).

We then modified the cartridge conditioning step by increasing the concentration of the NFPA solution from 20 mM to 100 mM. With this new protocol we tried to extract a mixture of 6 solutes: DHBA, Tyr, DA, 3-MT, NA and A. Again some of the compounds are not well retained on the cartridge: DHBA, NA, DA and a significant amount of A is recovered in the washing fraction. As the increase in the concentration of the ion paring agent, only during cartridge conditioning, didn't improve the retention of the test solutes, we evaluated the effect of the pairing agents with a longer carbon chain (TDFHA and PDFOA), but without success. We therefore retained the NFPA for our subsequent trials and in order to facilitate the formation of the ion pair between our solutes and the perfluoric acid we added the ion paring agent directly in the solution loaded on the cartridge (loading solution) and replaced the water washing step from Protocol VI with a NFPA aqueous solution washing step, in order to maintain an acidic pH

during the entire extraction. The new protocol (Protocol VII) was applied to the mixture of the 6 selected compounds.

Under these conditions the retention of the 6 compounds was obtained, without loss at the level of the loading and washing steps. We were also able to observe that the second elution (0.5 mL 0.1% TFA in MeOH) was not necessary, since no peak was detected in this second elution volume. With the exception of NA, for all other compounds the recovery yields exceed 60%. Ion pair formation between the solute and the ion paring agent prior to loading on the support is therefore essential for retention.

In order to improve the extraction yields, we studied the influence of the NFPA concentration in both the solution used for cartridge conditioning and the loading solution. Figure 3 summarizes the results obtained.

For the influence of NFPA concentration in the load solution, we kept constant the NFPA concentration in the cartridge conditioning solution (1.25 mM). Extraction yields appear to be very little affected by the increase in NFPA concentration (between 1.25 and 50 mM) in the load solution (Figure 3.a). Thus we can state that the presence of the ion-paring agent in the load solvent is essential for the ion pair with the catecholamines to be formed. However, we can see that the maximum yield is obtained with 5 or 10 mM NFPA (depending on the compound), beyond that concentration a loss of yield is again observed for all solutes. We therefore retained a 5 mM NFPA concentration in the load solution for the rest of our work.

Figure 3.b shows the influence of NFPA concentration in the conditioning solution. It reveals that even in the absence of NFPA, catecholamine retention is sufficient, proving once more that the ion pair is predominantly formed in the mobile phase at the time of the loading step, before being adsorbed on the stationary phase. With the exception of NA, for all other compounds the best extraction yields are obtained with a concentration of 10 mM NFPA in the conditioning solution.

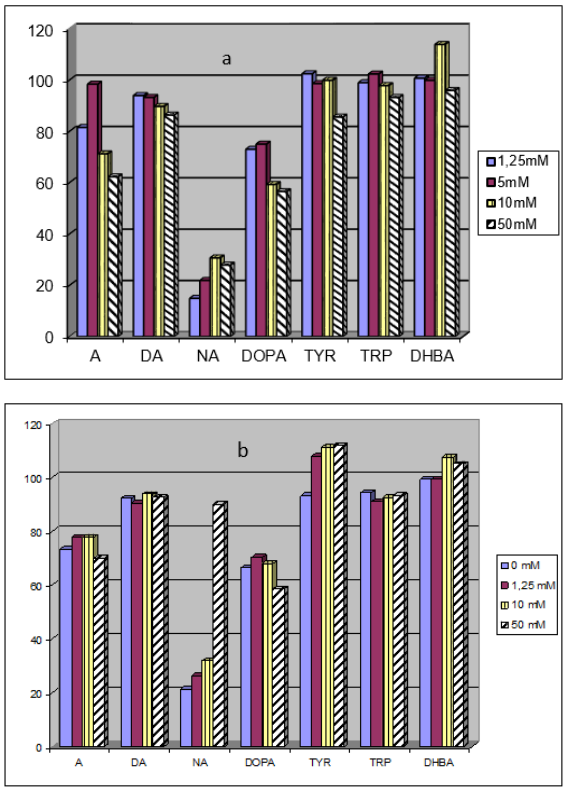


Figure 3. Influence of the concentration of NFPA in the load solution (a) and the conditioning solution (b) on the extraction yield

Considering these findings, the new protocol (Protocol VIII) lead to good extraction yields (exceeding 60%) for the majority of solutes. For NA the yield remains below 50%, but the use of a PGC cartridge containing 50 mg of phase instead of 25 mg lead to an improvement in its the extraction yield for NA (> 80%).

We conducted a repeatability study of the extraction yields for the following 7 solutes: A, NA, DA, DHBA, Trp, Tyr, and S. The results presented in Table 5, show that very good extraction yields (above 90%) and acceptable coefficients of variation (CV) (most below 5%) were obtained for NA, DA, DHBA, Tyr and Trp. On the contrary, for S and A, extraction yields inferior to 80% are obtained and also with greater variability (CV > 7%).

Table 5. Repeatability of the extraction yields on the PGC cartridge

Compound	Extraction yield (%)				Average	Standard deviation	RSD (%)
	1	2	3	4			
A	64.0	60.2	77.6	86.1	72.0	10.4	14.5
DA	96.5	88.5	97.5	99.8	95.6	4.3	4.5
NA	97.0	91.9	97.3	96.4	95.7	2.2	2.3
TYR	102.4	96.4	106.0	92.9	99.4	5.1	5.1
TRP	100.5	93.1	100.7	98.8	98.3	3.1	3.1
S	76.6	87.6	75.6	70.7	77.6	6.2	7.9
DHBA	102.3	98.3	103.8	102.6	101.7	2.1	2.1

In conclusion, the PGC cartridge used under ion paring conditions provides retention for compounds with an amine group in their structure. Also, this system is not suitable for the extraction of acidic compounds, as they cannot be eluted from the cartridge under these conditions. However, this sample preparation system, although not suitable for all of our 12 catecholamines, is complementary to the system developed on the Oasis HLB hydrophilic-lipophilic polymer cartridge (Protocol III). A combination of these two systems can ensure the extraction of all selected compounds.

3.4. SPE of brain extract on the combined Oasis HLB and PGC cartridges protocol

For the analysis of biological samples, we selected the two supports (Oasis HLB and PGC) which together should ensure the extraction of the 12 selected compounds. Considering the different extraction protocols optimized on the two cartridges, for their coupling, it is obvious to place the HLB cartridge first, because this cartridge ensures the retention of acidic compounds that cannot be eluted from the PGC cartridge. First we performed separate extractions on each of the two cartridges of sheep brain doped with catecholamines in order to verify firstly the influence of the presence of the matrix on the extraction yields and secondly, the efficiency of the SPE methods in eliminating the "undesirable" components of the matrix.

First, we performed the SPE of the brain extract to check whether matrix constituents other than catecholamines are removed prior to the elution step. Extraction was performed according to Protocol III. The fractions recovered after each SPE step were analyzed by HPLC-UV at 2 different wavelengths (λ): 280 nm ($\lambda_{max\ of\ catecholamines}$) (Figure 4) and 254 nm (less specific λ).

On the chromatogram of the non-extracted matrix (blue trace) it can be seen that a significant part of the constituents of the matrix is eluted between 1 and 4 min. Almost all of these compounds are removed during the load and washing steps thus demonstrating the effectiveness of our SPE system in terms of sample purification. In the elution fraction mainly a peak at 10.6 min appeared, its retention time corresponds to the retention time of 5HIAA, but given that the fact that its intensity at 254 nm (data not shown) is twice as large, it is not a catecholamine, but another component of the matrix retained by the cartridge under the conditions of extraction of our solutes.

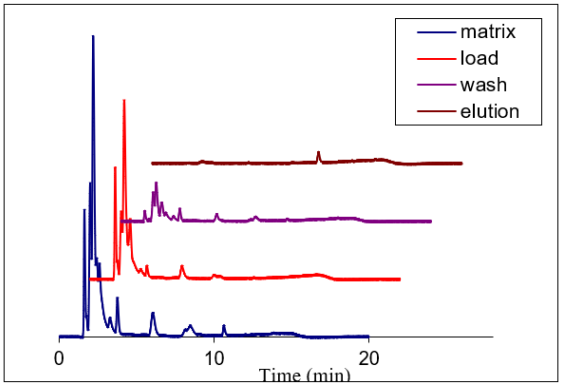


Figure 4. HPLC analysis of each Oasis HLB SPE fraction for sheep brain extract

We then proceeded to extract a sample of brain extract doped at 10 µg/mL with DOPAC, 5HIAA, HVA, TRP and S. Table 6 shows the results we obtained in terms of extraction yields for the same solution deposited on two cartridges in parallel.

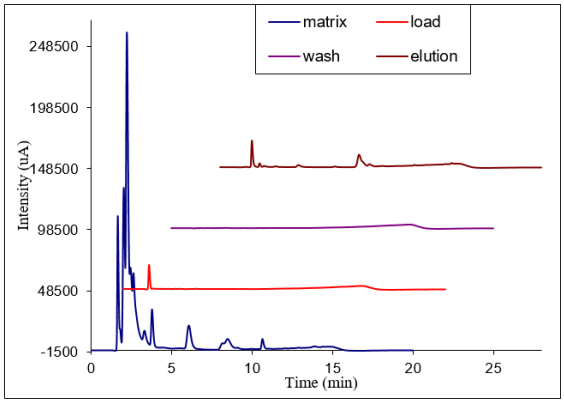
Table 6. Extraction yields of the compounds from the doped sheep brain extract on Oasis HLB

Compound	Extraction yield 1st series (%)	Extraction yield 2nd series (%)	Average	Standard deviation	RSD
DOPAC	94.8	94.1	94.4	0.4	0.4
HVA	94.1	91.8	92.9	1.1	1.2
5HIAA	105.9	104.7	105.3	0.6	0.5
TRP	84.6	81.1	82.9	1.8	2.2
S	63.7	59.5	61.6	2.1	3.4

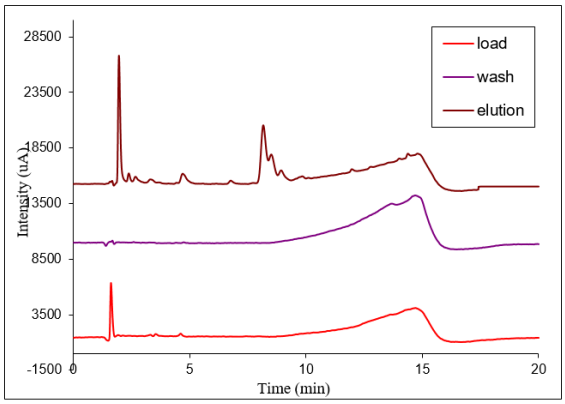
It can be seen that the extraction yields of DOPAC and HVA are very close to those obtained for the extraction of a standard mixture (97%) (Table 4). For 5HIAA the higher yield than that obtained for the standard mixture was most likely caused by the matrix peak which was eluted at the same retention time. The presence of the matrix has no negative effect on the expected retention of the acidic compounds in the mixture. For S and Trp lower yields compared to those observed in standard mixture are observed, which reflects a loss of these molecules during SPE, probably caused by matrix constituents removed from the cartridge at the time of loading and / or washing. This phenomenon might not be harmful as these two compounds are also retained on the PGC cartridge.

We also performed the extraction of the undoped matrix on PGC support. We performed two SPEs according to the PGC-optimized protocol (Protocol VIII). For the first SPE we directly loaded 1 mL of brain extract prepared in 0.2 M $HClO_4$ and for the second SPE the loading was performed after mixing 500 µL of brain extract prepared in the $HClO_4$ and 500 µL of a 10 mM aqueous solution of NFPA. Figure 5 a and b clearly show that the addition of NFPA to the sample prior to loading does not change the overall composition of the various solutions analyzed (no additional peaks in Figure 5 b were observed), only the dilution effect is noticed. Compared to the HLB support where most of the components of the matrix are removed during loading and washing steps, on the PGC these components are completely retained on the support, since they are found only in a very small proportion in the elution fraction and that they are not present in the loading nor in the washing fractions. Nevertheless, in the elution fraction, three groups of peaks of non-negligible intensity are identified: the first group eluted in the void volume does not cause difficulties during the analysis of the doped extract since under the chromatographic analysis conditions catecholamines have higher retention times. The other two groups correspond to solutes that have retentions comparable to those of NA, Tyr, and DHBA. The presence of these constituents in the matrix may lead to difficulties in the quantification of these three catecholamines by HPLC-UV analysis of the doped matrix due to probable peak deformations for those 3 compounds. Extraction yields

of NA, DHBA, and Tyr can't be accurately calculated (difficult integration of chromatographic peaks for these compounds). Table 7 shows the results we obtained in terms of extraction yield of catecholamines extracted from the matrix doped with 10 µg/mL NA, A, DOPA, DA, DHBA, S, Tyr and Trp.



a.



b.

Figure 5. HPLC analysis of each Oasis HLB SPE fraction for sheep brain extract: a. load 1 mL sheep brain extract; load 1 mL sheep brain extract diluted with NFPA 1/1 v/v.

Table 7. Extraction yields of the compounds from the doped sheep brain extract on PGC

Compound	Extraction yield (%)
NA	120.5
DOPA	61.2
A	78.2
DHBA	128.9
TYR	123.4
DA	90
S	86.6
Trp	93

For 5 compounds (A, DA, Trp, DOPA, S) the extraction yields obtained are similar to those of the standard mixture, indicating a good recovery of these compounds and the absence of matrix effects. As expected, the extraction yields of the doped matrix for NA, Tyr and DHBA are higher than those obtained for the standard mixture (Table 5), the interference with the matrix peaks was well confirmed. However, the mass spectrometric analysis should allow us to overcome the influence of these matrix constituents and realize a more accurate quantification.

Finally, we proceeded to the extraction of a sheep brain extract doped at 10 ppm with the 12 catecholamines from our mixture using the two selected cartridges. 1 mL of doped brain extract was loaded on the Oasis HLB cartridge, the fractions recovered after the loading (~ 1 mL) and the washing (~ 3 mL) were pooled and mixed with 4 mL aqueous solution NFPA 10 mM before being loaded on the PGC cartridge. Extractions were further performed on each cartridge according to the optimized protocols, with the elution fractions being recovered in a single flask and then evaporated. Table 8 shows the average extraction yields obtained for extraction on both supports. Since the coelution of 3-MT and S makes it impossible to calculate the extraction yield of each of these solutes, we present an overall yield for these two compounds.

Table 8. Extraction yields of the compounds from the doped sheep brain extract on Oasis HLB and PGC

Compound	Extraction yield (%)
NA	34.0
DOPA	66.3
A	69.6
DOPAC	94.7
DHBA	**149.5**
TYR	**207.9**
DA	86.0
5HIAA	99.1
HVA	106.7
S	76.4
3-MT	
Trp	92.3

With the exception of NA for which a low extraction yield is obtained (loss of NA during washing on PGC), for the rest of the compounds the extraction yields similar to those obtained for SPEs carried out separately on each of the cartridges were obtained. As noted earlier, the coelution of DHBA and Tyr with other components of the matrix makes their LC-UV assay impossible. The specificity of mass spectrometry may solve this problem as well as that of the correlated compounds of S and 3-MT.

4. Conclusions

The present paper presents the development of a method for the catecholamines extraction from a brain sample. Several types of support were tested in order to achieve the extraction of all the selected solutes using a single SPE cartridge, among them: cation exchange supports, hydrophilic-lipophilic supports, C18 supports and PGC supports.

We have seen that the weak cation exchange cartridges do not offer sufficient retention for catecholamines, but that on the other hand, the excessive retention of strong cation exchange supports requires us to use high salt concentrations in the elution solvent, with negative effects on the appearance of the chromatogram of the elution fraction. Hydrophilic-

lipophilic supports provide sufficient retention only for compounds with log P > 1 (DOPAC, HVA, 5HIAA, S and Trp). C18 and PGC type media only provide retention in ion pairing mode. Under these conditions, the best results are obtained on the PGC cartridge which ensures the retention of a greater number of compounds than the C18.

As unfortunately none of the supports tested offered us the possibility of carrying out the extraction of 12 catecholamines from our standard mixture, we chose to use a coupling of two different cartridges: Oasis HLB and PGC which together ensure the extraction of all the compounds of the mixture with good extraction yields and with simple protocols.

The selected cartridges were successfully tested for the extraction of a sample spiked from sheep brain with the 12 catecholamines in our mixture. The SPE method that we have developed allows the purification of the samples (a significant part of the components of the matrix is eliminated during this step) and also a preconcentration of the samples.

Conflict of interest

The authors have no conflicts of interest to declare regarding this research article.

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