

## A New Molecularly Imprinted Polymer for In-Tube SPME/UHPLC-MS/MS of Anandamide in Plasma Samples

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Parkinson's disease is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons. There is substantial evidence that the endocannabinoid system modulates the dopaminergic activity in the basal ganglia, a forebrain system that integrates cortical information to coordinate motor activity regulating signals. In this article, a fused-silica capillary with a molecularly imprinted polymer was developed for in-tube solid-phase microextraction of the endocannabinoid anandamide in plasma samples from Parkinson's disease patients and further analysis by ultra-high-performance liquid chromatography with tandem mass spectrometry. The molecularly imprinted polymer capillary presented recognition sites with complementary shape, size, and functionality to anandamide. Scanning electron micrographs and Fourier transform infrared spectra illustrated the physical and chemical modification of the printed and non-printed capillary surface. The in-tube solid-phase microextraction ultra-high-performance liquid chromatography with tandem mass spectrometry method presented a linear range from 0.1 to 20 ng mL<sup>-1</sup>, precision with coefficient of variation values ranging from 1.2 to 13%, and relative standard deviation accuracy ranging from -3.6 to 7.5%. The method developed herein can adequately determine anandamide in plasma samples from Parkinson's disease patients. By applying the standard addition approach, the anandamide plasmatic concentration in these samples was found to range from 0.2 to 0.4 ng mL<sup>-1</sup>.

**Keywords:** anandamide, molecularly imprinted polymer, in-tube SPME-UHPLC-MS/MS

### Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by motor symptoms that include bradykinesia, rest tremor, and rigidity.<sup>1</sup> The PD neuropathological hallmark is dopaminergic neuronal loss in the substantia nigra pars compacta (SNpc) and dopamine (DA) depletion in the striatum.<sup>2</sup>

The endocannabinoid system, which comprises a set of endogenous molecules known as endocannabinoids (eCBs), cannabinoid receptors (CBs), and metabolic enzymes, underlies numerous cellular signaling mechanisms involved in various neurodegenerative diseases. This system regulates many physiological neurotransmission pathways, including the dopaminergic system.<sup>3,4</sup> *N*-Arachidonylethanolamine,

or anandamide (AEA), is one of the main eCBs and was the first eCB molecule to be characterized.<sup>5</sup> AEA is synthesized on demand by receptor-stimulated cleavage of lipid precursors in neural membranes and is extracellularly released, acting as a partial agonist of CB1 and CB2.<sup>6</sup> Therefore, the levels of eCBs, especially AEA, in biological samples have been investigated to elucidate how the eCB and dopaminergic systems are related.

Conventional sample preparation techniques, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE), followed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) have been applied to quantify AEA levels in plasma, serum, brain, stem cells, cerebrospinal fluid, and urine samples.<sup>7-12</sup> Despite the great merits and developments reported for these analytical methods, developing highly sensitive chromatographic methods that can quantify AEA in peripheral fluids (e.g., plasma) is challenging because

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AEA is present at trace levels in these biological fluids.<sup>13</sup> Limitations of conventional sample preparation techniques include the fact that SPE sample extracts are insufficiently clean, and that LLE is poorly selective, which prevents adequate lower limit of quantifications (LLOQ) from being achieved.

Currently, microextraction techniques have been used to reduce the volume of organic solvent and biological sample without extraction efficacy being lost. Moreover, developing selective sorbents for these techniques may increase the sensitivity of the HPLC-MS/MS methods and minimize matrix effects. In this context, in-tube solid-phase microextraction (in-tube SPME) is noteworthy.

In-tube SPME is a miniaturized sample preparation technique in which a fused-silica capillary column, packed or coated with a stationary phase, is used as extraction device. In this technique, the sample solution flows through the extraction capillary, and the selective sorbent promotes effective enrichment of the sample with the analytes and sample cleanup. The extraction capillary can be used in the offline mode, or it can easily be coupled to a chromatographic system (online mode).<sup>14</sup> The advantage of the offline mode is that the system is simple: the in-tube SPME procedure can be manual. On the other hand, the online mode fully automates the preconcentration step, increasing the analytical reproducibility. However, this mode requires additional chromatographic pumps and valves. The sorbent chemical properties determine the extraction efficiency during in-tube SPME enrichment. We have reported new in-tube SPME stationary phases including crosslinked polymeric ionic liquid (PIL) and restricted access material (RAM) containing octyl inner surface.<sup>15,16</sup> These sorbents exploit different extraction mechanisms to preconcentrate eCBs from rat brain samples and cerebrospinal fluid.<sup>15,16</sup> Compared to cerebrospinal fluid, plasma samples contain a higher number and concentration of endogenous molecules, so plasma is an extremely complex matrix. For this reason, the successful enrichment of plasma samples with AEA requires a biocompatible sorbent and a selective extraction mechanism.

Molecularly imprinted polymers (MIPs) are synthetic sorbents with highly specific receptor sites for the target molecules.<sup>17</sup> To synthesize a MIP, a template molecule (analyte or compound with a similar structure), a functional monomer, and a crosslinking agent are needed. In general, MIPs are synthesized from functionalized monomers via radical polymerization, to give materials that are cheaper, physically stronger, more robust, and more chemically stable than immunosorbents.<sup>18,19</sup>

In this study, a biocompatible MIP sorbent chemically bound to the internal surface of a fused-silica capillary

was synthesized by *in situ* polymerization, aiming at its use in in-tube SPME. The in-tube SPME procedure was evaluated in the offline mode, and analyses were performed on an ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system. The enrichment step based on this open tubular capillary exhibited lower backpressure and did not require frits, avoiding the adsorption of macromolecules and interferents from the matrix. The in-tube SPME and UHPLC-MS/MS method was fully validated and successfully applied to determine AEA in plasma samples from PD patients selectively. To the best of our knowledge, this is the first time that a selective MIP for AEA has been reported.

## Experimental

### Reagents and standards

All the reagents were analytical grade or HPLC-grade. AEA and AEA-*d*<sub>4</sub> (internal standards, IS) and arachidoyl ethanolamide (template) were purchased from Cayman Chemical (Michigan, USA). Vinyltrimethoxysilane (VTMS), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), NaCl, HCl, NaOH, toluene, and formic acid were acquired from Sigma-Aldrich (São Paulo, SP, Brazil). The initiator, 2,2-azobisisobutyronitrile (AIBN), was supplied by Merck (São Paulo, SP, Brazil). Acetonitrile and methanol (HPLC grade) were acquired by JT Baker (Phillipsburg, USA). The fused-silica capillary was purchased from NanoSeparation Technologies (São Carlos, Brazil). Aqueous solutions were prepared with ultrapure water obtained from a Milli-Q (18 MΩ cm) system (Millipore, São Paulo, Brazil). The AEA and AEA-*d*<sub>4</sub> working standard solutions were prepared by diluting stock solutions in acetonitrile. All the AEA solutions were stored at -80 °C, which ensured their stability for at least six months.

### Plasma samples

Plasma samples spiked with AEA and AEA-*d*<sub>4</sub> were used to optimize the in-tube SPME variables and to evaluate the analytical validation parameters of the in-tube SPME/UHPLC-MS/MS method. The plasma samples were collected from PD patients in agreement with the criteria established by the Ethics Committee of the Faculdade de Medicina de Ribeirão Preto and Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil (Process No. of ethics committee 3.036.243). All the participants provided a written informed consent to participate in this study.

## MIP synthesis

A fused-silica capillary (0.53-mm internal diameter, 5.0-cm length) was activated with NaOH solution (1.0 mol L<sup>-1</sup>), followed by HCl solution (0.1 mol L<sup>-1</sup>). Then, the capillary was washed with ultrapure water and dried at 60 °C for 12 h. Next, the capillary inner surface was silanized with VTMS at 85 °C for 2 h.<sup>20</sup> After that, the capillary was washed with methanol.

The MIP was synthesized via the precipitation polymerization method.<sup>21</sup> To prepare the MIP, 5 mg of arachidoyl ethanolamide (template) were mixed with 5 mL of toluene (porogen solvent), followed by the addition of 50 µL of MAA (functional monomer) in a screw-capped glass vial. Next, 100 µL of crosslinker (EGDMA) and 100 µL of initiator (AIBN) were added to the polymerization solution. After sonication, the solution was degassed with a nitrogen stream for 10 min. Then, the pretreated capillary was filled with the polymerization solution with the aid of a syringe. The capillaries were sealed with silicone rubber at both ends and were allowed to polymerize at 60 °C for 4 h. The coated capillary was washed with ethanol to remove the synthesis residues.

The non-imprinted polymer (NIP) was also synthesized by using the method described above, without adding the template.

## MIP and NIP physical and chemical characterization

The MIP and NIP were characterized by scanning electron microscopy (SEM). A Zeiss EVO 50 scanning electron microscope (Cambridge, UK) operating at an acceleration voltage of 20 kV and nominal resolution of 30 nm generated the SEM micrographs.

The chemical groups in these polymers were characterized by Fourier-transform infrared (FTIR) spectroscopy on an ABB Bomem series MB100 Spectrometer (Quebec, Canada). The spectra (in KBr pellets) were recorded from 400 to 4000 cm<sup>-1</sup>. One data point *per* 2 cm<sup>-1</sup> and 32 scans were collected.

## Plasma sample pretreatment

The proteins of the plasma samples (200 µL) were

precipitated with 400 µL of acetonitrile. After vortexing (1 min), the mixture was centrifuged (15 °C and 15 min) at 9000 rpm. The supernatant (350 µL) was dried in a vacuum concentrator (Eppendorf®, Hamburg, Germany), and the dry residue was resuspended in 200 µL of ammonium acetate buffer (pH = 9.0)/acetonitrile 90:10 (v/v) for the in-tube SPME procedure.

## UHPLC-MS/MS conditions

As described in a previous paper by our group,<sup>22</sup> the UHPLC-MS/MS analysis was carried out in a Waters ACQUITY UPLC H-Class system coupled to the Xevo® TQD tandem quadrupole (Waters Corporation, Milford, USA) mass spectrometer equipped with a Z-spray source operating in the positive electrospray ionization (ESI+) and selected reaction monitoring (SRM) mode. The samples were kept in the autosampler at 10 °C, and 10 µL of sample was injected into a Kinetex C18 column (Phenomenex®, USA; 100 mm × 2.1 mm × 1.7 µm) at 40 °C, under isocratic conditions. The mobile phase consisted of an aqueous solution containing 0.5% formic acid (eluent A)/acetonitrile (eluent B) 30:70 (v/v) at 0.4 mL min<sup>-1</sup>.

The MS/MS parameters were capillary voltage of 3.20 kV, source temperature of 150 °C, desolvation temperature of 350 °C, and desolvation gas flow of 700 L h<sup>-1</sup> (N<sub>2</sub>, 99.9% purity). Argon (99.9999% purity) was used as the collision gas. The dwell time was established for each transition separately, and the interscan delay was set to the automatic mode. Two specific transitions were optimized for the analyte and internal standard (IS). The first transition helped to quantify the analyte, whilst the second SRM transition aided qualitative identification. The fragments, energy of the cone, and collision energy (Table 1) were determined by the Intellistart Program, which automatically optimizes parameters. The data were acquired by using the MassLynxV4.1 software.

## In-tube SPME/UHPLC-MS/MS procedure

For the in-tube SPME procedure, the MIP capillaries were coupled in a conventional HPLC microsyringe (250 µL), as described by Souza *et al.*<sup>23</sup>

**Table 1.** MS/MS transition (SRM), optimal declustering potential (DP), optimal collision energy (CE), and retention time for AEA and AEA-*d*<sub>4</sub> (internal standard)

Analyte	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Qualifier ion ( <i>m/z</i> )	Declustering potential / V	Collision energy / eV	Retention time / min
AEA	348.3	61.8	90.8	14	18/50	1.86
AEA- <i>d</i> <sub>4</sub>	352.4	65.9	47.8	26	18/34	1.84

AEA: anandamide.

Initially, the MIP capillary was conditioned twice with 150  $\mu\text{L}$  of water followed by 150  $\mu\text{L}$  of ammonium acetate buffer solution (50  $\text{mmol L}^{-1}$ ). The influence of the sample pH on AEA extraction was the first variable to be evaluated. The dry extract ("Plasma sample pretreatment" sub-section) was reconstituted with 200  $\mu\text{L}$  of ammonium acetate buffer solution (50  $\text{mmol L}^{-1}$ ) at three different pH values (4.0, 7.0, and 9.0). Ammonium acetate buffer solution with 10% acetonitrile was also evaluated to verify whether it increased the AEA solubility in the sample and consequently improved its sorption on the MIP sorbent. Next, the resuspended plasma sample was manually drawn through the MIP sorbent and ejected one or more times to preconcentrate AEA. Before AEA was eluted, different solutions (water/methanol 95:5 (v/v), 1% acetic acid solution, 1% ammonium hydroxide solution, and pure water) and volumes (50, 100, and 150  $\mu\text{L}$ ) were evaluated for removing the endogenous compounds adsorbed on the stationary phase.

The elution process was assessed by means of different acetonitrile draw/eject cycles (1  $\times$  100  $\mu\text{L}$ , 2  $\times$  100  $\mu\text{L}$ , 3  $\times$  100  $\mu\text{L}$ , and 4  $\times$  100  $\mu\text{L}$ ). Acetonitrile was dried under vacuum, and the dry extract was resuspended in 50  $\mu\text{L}$  of acetonitrile/0.5% formic acid aqueous solution 70:30 (v/v).

#### Analytical validation

The in-tube SPME/UHPLC-MS/MS method was validated on the basis of current guidelines issued by Agência Nacional de Vigilância Sanitária (ANVISA) RDC No. 27.<sup>24</sup> Analytical validation was carried out with blank plasma samples spiked with the IS (10  $\text{ng mL}^{-1}$ ) and standard AEA solutions at concentrations ranging from 5 to 20  $\text{ng mL}^{-1}$ . A calibration curve was generated by plotting the relative peak areas (AEA/IS) vs. the AEA concentration in the plasma samples. The analytical signals (peak area of endogenous AEA) of the blank plasma sample were subtracted from the AEA area obtained with the spiked plasma samples. The standard addition approach was applied to determine the AEA concentration in the plasma samples.

Using the standard addition curves, the lower limit of quantification (LLOQ) was calculated by applying the equation  $\text{LLOQ} = 10 \times (\text{SD}/b)$ , where SD is the standard deviation of the intercepts of the calibration curve and b is the mean of the slope of linearity plot.<sup>25</sup>

Matrix effects were evaluated by using eight lots of blank plasma obtained from individual volunteers. The matrix factor (MF) was determined for each matrix lot, by calculating the ratio between the AEA peak area in the presence of the matrix (measured by analyzing the spiked

blank matrix after AEA extraction) and the AEA peak area in the absence of the matrix (pure AEA solution). The IS-normalized MF was also calculated by dividing the AEA MF by the IS MF. The coefficient of variation (CV) of the IS-normalized MF calculated from the eight matrix lots should not be greater than 15%. Carryover was assessed by injecting three aliquots of the same blank plasma sample, one before and two after the plasma sample spiked with AEA at the concentration corresponding to the upper limit of quantification (ULOQ: 20  $\text{ng mL}^{-1}$ ) was analyzed. Carryover in the blank plasma sample evaluated immediately after the ULOQ sample should not be greater than 20% of the analyte signals in the LLOQ chromatogram, and not greater than 5% of the IS signals.

Three new MIP capillaries were synthesized from different batches. Then, the reproducibility of the *in situ* polymerization procedure was evaluated by comparing the extraction efficiency of these capillaries.

## Results and Discussion

### MIP sorbent synthesis

To synthesize the polymers, the internal surface of the fused-silica capillary was initially functionalized with VTMS. This step generated vinyl groups, which allowed radical polymerization of the MIP chemically bound on the capillary internal surface.<sup>20</sup>

The MIP was synthesized by using an analogue molecule (arachidoyl ethanolamide) as template-AEA bears polymerizable groups that can copolymerize with the functional monomer and crosslinker (Figure S1, Supplementary Information (SI) section).

MAA was chosen as a functional monomer because it has chemical groups that interact with the template (arachidoyl ethanolamide) through hydrogen bonding, a strong intermolecular interaction. The interactions between the template and the functional monomer are essential for the pre-polymeric complex to form and for the molecular imprinting process.<sup>26</sup> As shown in Figure S2 (SI section), the capillary, synthesized by using a template/functional monomer molar ratio of 1:5, presented higher sorption capacity. However, excess functional monomer generated non-specific sites in the polymer and consequent loss of selectivity, which increased the matrix effect. Thus, the polymer synthesized by using a template/functional monomer molar ratio of 1:3 was the polymer of choice for the in-tube SPME method because it presented good selectivity and lower standard deviation.

Using EGDMA as crosslinker stabilized the imprinted binding sites and improved the mechanical stability of the

polymer matrix. The template (arachidoyl ethanolamide) is soluble in toluene, the solvent employed in the MIP synthesis. Toluene favored polar non-covalent interactions (hydrogen bonding) during the pre-polymeric complex formation.<sup>27</sup> AIBN is a cheap radical initiator that proved suitable for polymerization at 60 °C.

#### MIP and NIP chemical and morphological characterization

Figure 1 shows the SEM micrographs of the synthesized polymers. The images show that the MIP (Figure 1a) and NIP (Figure 1b) were formed and attached on the capillary internal surface compared to the empty capillary (Figure 1c), demonstrating that the synthesis process was efficient. The MIP presented larger and apparently more porous particles than the NIP. The higher MIP porosity can be attributed to the presence of selective cavities, which are absent in the NIP. The porous structure facilitates the mass transfer and benefits analyte adsorption.

Figure S3 (SI section) illustrates the MIP and NIP FTIR spectra. The specific cavities formed in the MIP did not cause the FTIR spectral profiles of the MIP and NIP to be significantly different because both polymers have the same functional groups incorporated into the polymeric structures. The bands at 1728 and 3507  $\text{cm}^{-1}$  respectively referred to the carbonyl (C=O) and hydroxyl (OH) groups of EGDMA and MAA incorporated into the polymer structure.<sup>19</sup> The characteristic band of the C–H bond present in polymers appeared at 2988  $\text{cm}^{-1}$ .<sup>19</sup> The spectra also exhibited bands ascribed to bending vibrations (1456  $\text{cm}^{-1}$ ), symmetric (1259  $\text{cm}^{-1}$ ) and asymmetric (1162  $\text{cm}^{-1}$ ) ester C–O stretching bands of EGDMA incorporated into the polymer structure, OH harmonics (1637  $\text{cm}^{-1}$ ), and vinylic C–H out-of-plane bending vibration (960  $\text{cm}^{-1}$ ).<sup>19</sup>

#### Determination of in-tube SPME variables

The in-tube SPME variables (offline method) such as sample pH, washing step, and desorption conditions were

optimized so that the highest extraction efficiency and sample cleanup would be achieved.

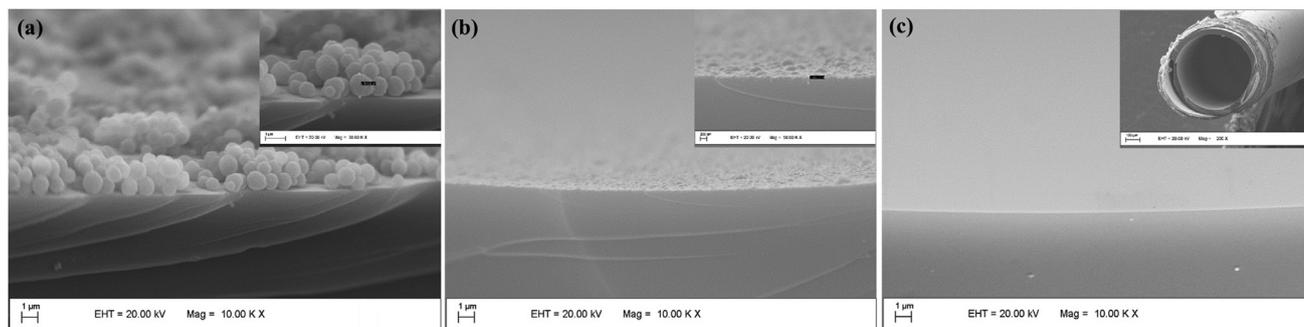
Sample pH is important for analytes that bear a pH-dependent dissociable group. As shown in Figure 2a, plasma samples resuspended with ammonium acetate at pH 9 afforded the highest extraction efficiency. In the alkaline medium, site selectivity (*in situ* polymerization) was favored, and intermolecular interactions between the MIP and AEA were maximized. However, the MIP selectivity decreased under acid conditions because AEA can establish effective hydrogen bonding with non-selective sites.<sup>23</sup> As a fatty acid derivative, AEA is lipophilic. However, due to the presence of the amide and glycerol groups, AEA is sparingly soluble in water. Thus, AEA is soluble in both protic and aprotic organic solvents (e.g., acetonitrile). Ten percent of acetonitrile was added to the ammonium acetate buffer (pH 9) to improve AEA solubility in the aqueous medium.

After AEA was adsorbed on the MIP phase, a washing step was evaluated to exclude the residual endogenous compounds. This procedure decreased the plasma matrix effect during LC-MS/MS analysis. The washing process consisting of a single draw/eject cycle of 100  $\mu\text{L}$  of methanol/water 5:95 (v/v) efficiently removed the endogenous compounds without the analyte being lost (Figure 2b).

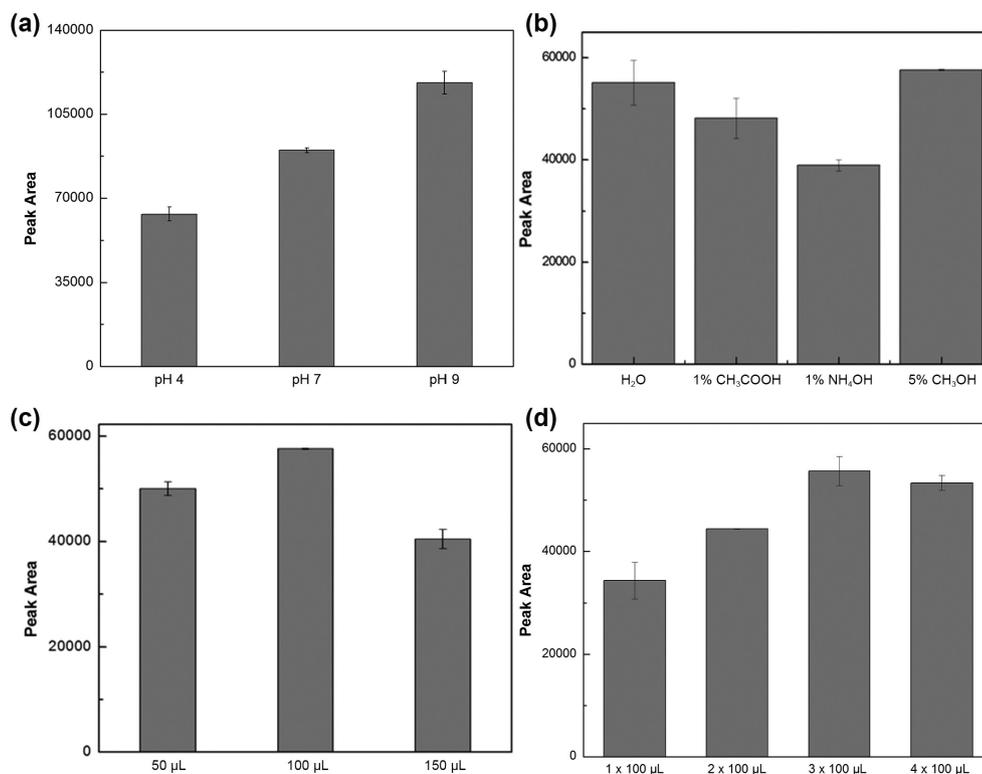
Acetonitrile was employed as a desorption solvent because AEA is highly soluble in it. The highest extraction efficiency was obtained with three draw/eject cycles of 100  $\mu\text{L}$  of acetonitrile (Figures 2c and 2d).

#### MIP and NIP extraction efficiencies and MIP selectivity

Figure 3a shows the MIP and NIP extraction efficiencies evaluated by using blank plasma samples spiked with AEA at 100  $\text{ng mL}^{-1}$ . The MIP presented higher extraction efficiencies than the NIP (which extracted AEA by non-specific interactions). This evidenced that the MIP was more selective and demonstrated that the molecular imprinting process was successful.



**Figure 1.** SEM images of (a) fused-silica capillary coated with the MIP phase, (b) fused-silica capillary coated with the NIP phase, and (c) uncoated fused-silica capillary.

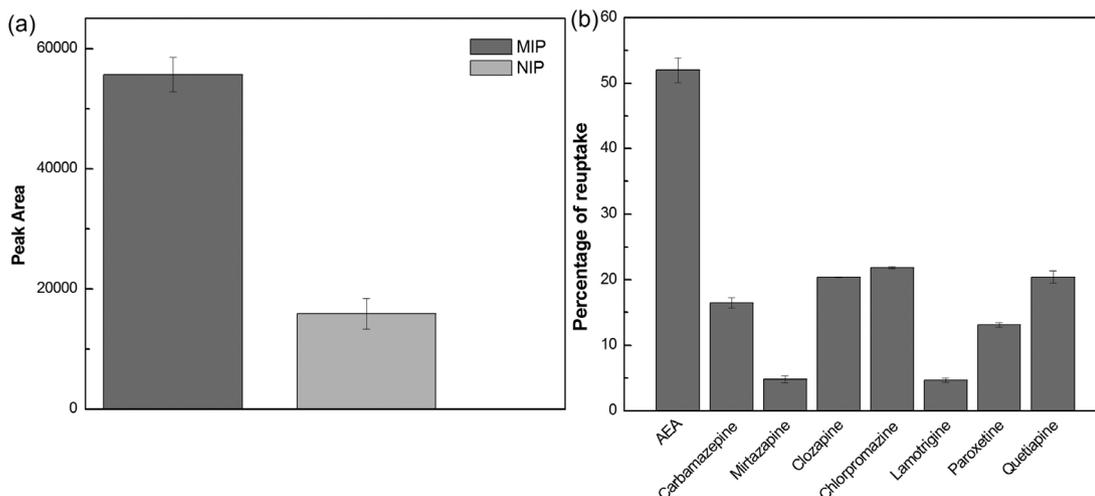


**Figure 2.** Optimization of the in-tube SPME variables (a) sample pH, (b) washing step solution, and (c) desorption conditions; (d) draw/eject cycles.

The selectivity of the MIP and NIP sorbents was evaluated by in-tube SPME/UHPLC-MS/MS analysis using blank plasma samples spiked with AEA and different drugs at 100 ng mL<sup>-1</sup>. As shown in Figure 3b, the highest AEA recovery (51%) obtained by using the MIP confirmed that this sorbent was selective. The other compounds adsorbed on the MIP through non-selective binding sites. The imprinting factor is defined as the ratio between the AEA area in the MIP and NIP. In this study, the AEA imprinting factor was 4 (Figure S4, SI section).

#### Analytical validation of the SPME/UHPLC-MS/MS method

The selectivity of the SPME/UHPLC-MS/MS method was demonstrated by representative chromatograms of a blank plasma sample and a human plasma sample spiked with AEA at a concentration corresponding to the LLOQ. The chromatogram of the blank plasma sample attested that endogenous compounds in the plasma sample were effectively removed during the sample preparation step: interfering peaks were absent at the AEA retention time.



**Figure 3.** (a) MIP and NIP extraction efficiency. Blank plasma spiked with AEA at 100 ng mL<sup>-1</sup> was used; (b) investigation of the selectivity of the MIP phase by using blank plasma samples spiked with AEA and different drugs (100 ng mL<sup>-1</sup>).

The presence of a small peak at the AEA retention time is referred to endogenous AEA. Plasma samples obtained from six different sources were also evaluated (Figure S5, SI section).

The SPME/UHPLC-MS/MS method was linear from 0.1 ng mL<sup>-1</sup> to the upper limit of 20 ng mL<sup>-1</sup>. The coefficient of determination was higher than 0.994. The points of the calibration curves were performed in replicate (n = 5) and presented CV values lower than 15% (Table 2).

The accuracy of the method ranged from -3.6 to 7.5% (intra-assay accuracy) and from -4.4 to 5.4% (inter-assay accuracy). The precision of the method presented CV values ranging from 1.2 to 13% (intra-assay precision) and from 3.1 to 11.7% (inter-assay precision). Carryover in the blank sample, evaluated after the ULOQ sample was analyzed, was not greater than 20% of the AEA signal in the case of the chromatogram of the LLOQ sample and not greater than 5% of the signal in the case of the IS. The CV of the IS normalized matrix effect (MF) calculated from the five matrix lots was not greater than 15%. This assay (n = 3) was carried out at the LLOQ and ULOQ concentrations (Table 2).

To ensure that the *in situ* synthesis procedure (MIP capillary) was reproducible, three new MIP capillaries were synthesized and evaluated by using plasma samples spiked with AEA at 100 ng mL<sup>-1</sup> (n = 3). The CV values ranged from 7.0 to 10% (intra-synthesis) and from 5.0 to 14% (inter-synthesis). Each MIP capillary was reused in multiple extractions (over 100 times) without the extraction

efficiency changing significantly. These results attested to the MIP capillary robustness.

Sample preparation techniques based on different sorbent phases have been reported (Table 3). Among all the sorbents, MIP provides the best selectivity in terms of extraction mechanism. Compared to PILs, MIPs are easier to synthesize because a purification step is not required. Additional advantages of in-tube SPME using the MIP as sorbent include smaller sample volume and shorter analysis time.

Analyses of plasma samples obtained from Parkinson's disease patients

AEA is an endogenous molecule and, because obtaining a blank or surrogate plasma matrix is impossible, the standard addition method was used to quantify this analyte in plasma samples from PD patients. The plasma samples were enriched with the analyte at 5, 10, 12, and 15 ng mL<sup>-1</sup>. The absolute value on the x-axis, obtained from the calibration curve when the value on the y-axis was equal to zero, refers to the amount of AEA found in the analyzed plasma sample. Table S1 (SI section) presents the calibration curves (standard addition method) obtained for each sample. All the calibration curves exhibited a coefficient of determination higher than 0.99 and LLOQ values of 0.1 ng mL<sup>-1</sup>. Figure 4 presents the chromatograms obtained for each analyzed sample. The endogenous AEA

**Table 2.** Analytical validation of the SPME/UHPLC-MS/MS method

Analyte	Amount spiked / (ng mL <sup>-1</sup> )	Precision (CV) / %		Accuracy (RSD) / %		Matrix effects
		Inter-assay	Intra-assay	Inter-assay	Intra-assay	
AEA	5.0	8.3	6.4	7.5	5.4	
	12.0	1.2	3.7	-1.0	0.7	13.9
	10.0	13.2	11.7	2.5	-1.5	
	15.0	4.2	3.1	-3.6	-4.4	5.8
	20.0	5.5	4.6	3.0	2.5	

AEA: anadamide; CV: coefficient of variation; RSD: relative standard deviation.

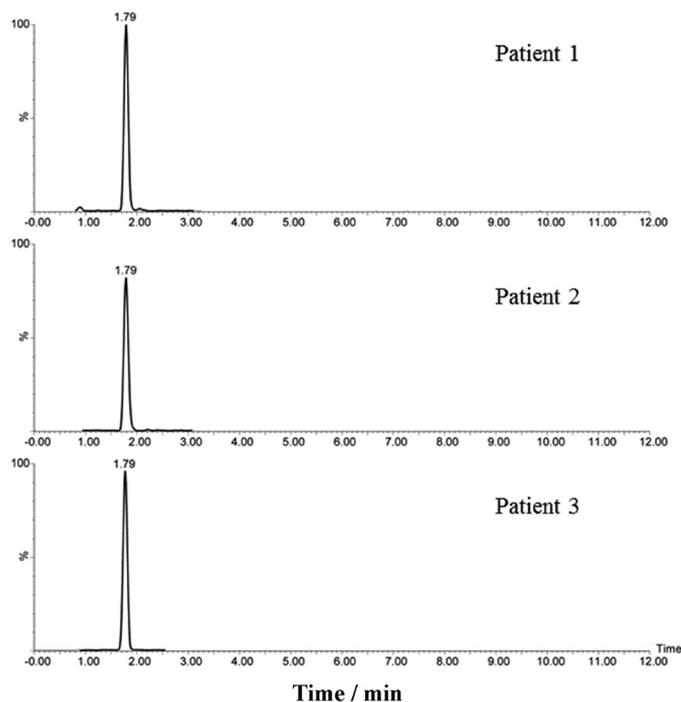
**Table 3.** Comparison of HPLC-MS/MS methods to determine AEA in plasma samples

Sample volume / $\mu$ L	Sample preparation technique (sorbent)	Chromatographic system	LLOQ <sup>a</sup> / (ng mL <sup>-1</sup> )	Total analysis time <sup>b</sup> / min	Real samples analyzed	Reference
300	SPME (HLB) <sup>c</sup>	nanoESI-MS/MS <sup>d</sup>	1	130	-	28
400	in-tube SPME <sup>e</sup> (PIL) <sup>f</sup>	UHPLC-MS/MS	0.1	25	6	15
1000	SPE <sup>g</sup> (C8)	HPLC-MS/MS	20	26	23	29
250	LLE <sup>h</sup> + column switching (C8-RAM) <sup>j</sup>	UHPLC-MS/MS	0.1	30	10	22
200	in-tube SPME (MIP) <sup>i</sup>	UHPLC-MS/MS	0.1	20	3	this study

<sup>a</sup>LLOQ: lower limit of quantification; <sup>b</sup>time includes the sample preparation procedure and chromatographic run; <sup>c</sup>HLB: hydrophilic-lipophilic-balanced;

<sup>d</sup>ESI: electrospray ionization; <sup>e</sup>SPME: solid-phase microextraction; <sup>f</sup>PIL: polymeric ionic liquid; <sup>g</sup>SPE: solid-phase extraction; <sup>h</sup>LLE: liquid-liquid extraction;

<sup>i</sup>MIP: molecularly imprinted polymer; <sup>j</sup>RAM: restricted access material.



**Figure 4.** In-tube SPME/UHPLC-MS/MS chromatograms of AEA in plasma samples from PD patients.

concentration ranged from 0.2 to 0.4 ng mL<sup>-1</sup> (Table S1). These values agreed with the values reported in the literature,<sup>11</sup> demonstrating that the method is reliable.

## Conclusions

The characterization of the MIP and NIP phases by the SEM and FTIR techniques showed important aspects of the morphological and chemical characteristics of the structure of the synthesized sorbents. *In situ* polymerization was successfully applied to obtain the MIP sorbent chemically bound to the internal surface of the fused-silica capillary. During the in-tube SPME extractions, the MIP sorbent preconcentrated AEA with higher specificity than the NIP sorbent. Arachidoyl ethanolamide acted as an adequate template, creating selective molecular imprinted sites with a high capacity for recognizing AEA. Optimization of the in-tube SPME parameters ensured that extraction was efficient in a simple and fast way; small volumes of plasma sample (200 µL) and organic solvent were used. The MIP sorbent presented excellent mechanical strength, which allowed the capillaries to be reused over 100 times without extraction efficiency is lost. The in-tube SPME/UHPLC-MS/MS method achieved outstanding performance in all the parameters required by international guidelines for analytical validation.

The applicability of the in-tube SPME/UHPLC-MS/MS was proven by successful AEA quantification in plasma samples from PD patients.

## Supplementary Information

Supplementary information (chemical structures of analytes, bar graphs of optimization of template/functional monomer molar ratios, FTIR spectra of the MIP and NIP, chromatograms of the in-tube SPME/UHPLC-MS/MS analysis of blank plasma samples, and plasma sample spiked with AEA) is available free of charge at <http://jbcbs.sbq.org.br> as PDF file.

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## Author Contributions

Mônia A. Lemos Pinto was responsible for conceptualization, investigation, formal analysis, data curation, laboratory analysis, and writing of original draft manuscript; Israel D. de Souza for investigation, formal analysis, and writing of original draft manuscript; Luis Felipe C. Miranda for conceptualization, investigation, formal analysis, data curation, and laboratory analysis; Maria Eugênia C. Queiroz for conceptualization, investigation, laboratory supervision, and revision of original draft manuscript.

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