Optimization of the SPME Parameters and Its Online Coupling with HPLC for the Analysis of Tricyclic Antidepressants in Plasma Samples

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Abstract

Solid-phase microextraction (SPME)–liquid chromatography (LC) is used to analyze tricyclic antidepressant drugs desipramine, imipramine, nortriptyline, amitriptyline, and clomipramine (internal standard) in plasma samples. Extraction conditions are optimized using a 2^3 factorial design plus a central point to evaluate the influence of the time, temperature, and matrix pH. A Polydimethylsiloxane–divinylbenzene (60-µm film thickness) fiber is selected after the assessment of different types of coating. The chromatographic separation is realized using a C_{18} column (150 × 4.6 mm, 5-µm particles), ammonium acetate buffer (0.05 mol/L, pH 5.50)–acetonitrile (55:45 v/v) with 0.1% of triethylamine as mobile phase and UV–vis detection at 214 nm. Among the factorial design conditions evaluated, the best results are obtained at a pH 11.0, temperature of 30°C, and extraction time of 45 min. The proposed method, using a lab-made SPME–LC interface, allowed the determination of tricyclic antidepressants in in plasma at therapeutic concentration levels.

Introduction

The effective treatment of depression was introduced in the 1950s with the development of antidepressants, which have been traditionally classified in two groups: tricyclic antidepressants (TCAs) and the monoamine oxidase inhibitors.

TCAs are one of the groups of drugs used as reference for the treatment of psychiatric disorders, mainly major depression. These drugs act by inhibiting the reuptake of the neurotransmitters norepinephrine (as in the case of desipramine, nortriptyline, and protriptyline secondary amines) and serotonin (as in the case of amitriptyline, imipramine, clomipramine, and doxepine tertiary amines) in the central nervous system (1–10).

These drugs are highly liposoluble, being metabolized in the liver, mainly in hydrosoluble compounds that are eliminated by the kidneys. Their biotransformation involves one or more metabolic steps, and metabolites can have a higher or lower pharmacological activity than the original compound or can be inactive. Only a low drug percentage is eliminated in the original form (11). The chemical structures and other characteristics of TCAs are shown in Table I.

Several analytical techniques have been employed for the determination of TCAs in plasma. The conventional sample pretreatment technique, liquid–liquid extraction, is laborious, time-consuming, difficult to automate, and requires relatively large amounts of organic solvents, which are often expensive, toxic, carcinogenic, and hazardous to the environment (10,12,13). Several drawbacks of the classical techniques have been reduced using solid-phase extraction (SPE) (14–17) in cartridges or disks. SPE consumes less solvent, but it is still laborious and requires sample concentration, possibly resulting in the loss of volatile compounds of the sample.

Solid-phase microextraction (SPME), developed by Pawliszyn et al., is a sample preparation technique that integrates sampling, extraction, concentration, and sample injection consuming less or no solvent (18–20).

Most recently, SPME has been successfully applied to the analysis of drugs in biological samples, mainly by coupling SPME to gas chromatography (GC). When SPME is coupled to GC, the desorption process of the analytes occurs directly in the injector. The SPME needle is introduced into the GC injector, the fiber is exposed to the heated chamber, and the analytes are thermally desorbed (25).

On the other hand, the desorption process, by online coupling of SPME to high-performance liquid chromatography (HPLC) (SPME–LC), requires an appropriate interface. A commercial interface has already been developed and is composed of a six-port valve and chamber where the desorption takes place (25–29). However, this interface presents some problems, such as low reproducibility, band broadening, leaking, and high cost. To overcome these problems, the Laboratory of Chromatography at the Institute of Chemistry of São Carlos,

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(University of São Paulo) has developed a novel system. This labmade interface has a heating system that has not yet been described in the literature. It increases the analytes desorption and method sensitivity, and it decreases the carryover effect, a common concern in SPME.

The extraction process involves analyte partitioning among the matrix sample, headspace, and extraction phase. At equilibrium, the extracted amount is proportional to the partition coefficient and the analyte concentration in the sample. The partition coefficient is determined by the analyte–matrix and analyte–extraction phase interactions. Extraction is considered complete when the analyte concentration reaches the distribution equilibrium between the extraction phase and the matrix (18,19).

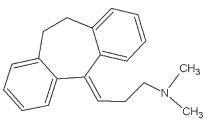
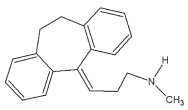
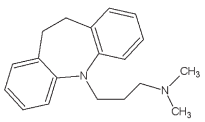
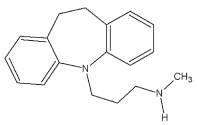
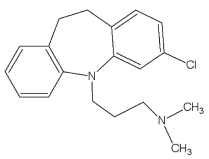
The amount of analyte extracted in SPME can be influenced by many parameters, including extraction-phase characteristics, temperature, extraction time, medium ionic strength, pH, and stirring speed. The optimization of these parameters is essential to achieve the best performance of the technique (30).

Currently, the most employed strategy to develop an experimental optimization is to evaluate each parameter independently, varying only one at a time. In this case, all parameters are fixed, except the parameter that will be varied until finding the best result. After this procedure, the optimized parameter is maintained and all others are varied, one at a time, until finding the best value for each parameter. In this type of procedure, incomplete interpretations can occur because the interaction effects between the variables are not explored.

Therefore, the best way to perform such an optimization is to vary all the parameters at the same time systematically, promoting a multivariate optimization. This optimization can be performed employing a factorial design, in which the number of experiments is generally smaller, with the advantage of evaluating the main effect and all interactions (31–33).

In this paper, factorial design optimization of the SPME procedure is described for analyses of tricyclic antidepressant drugs in human plasma, using online SPME–LC.

Table I. Characteristics of TCAs Drugs

Analytes	Molar mass	Molecular structure	pK_a	Therapeutic concentration range (ng/mL)
Amitryptyline	277.41		9.40	80–200
Nortryptyline	263.38		10.08	50–150
Imipramine	280.41		9.50	125–250
Desipramine	266.39		10.20	115–300
Clomipramine	314.30		9.30	100–400

Experimental

Reagents and analytical standards

Analytical standards were obtained as hydrochloride salts from different suppliers as follow: imipramine (IMI) and desipramine (DESI) was obtained from Ciba Geigy (São Paulo, Brazil); amitriptyline (AMI) and clomipramine (CLOMI) (used as internal standard) were from Sigma Aldrich Chemical Co. (Steinhein, Germany); and nortriptyline (NOR) was from Sandoz (São Paulo, Brazil).

Stock and working standard solutions were prepared in methanol. TCA stock solutions (2 mg/mL) were prepared by weighing 10 mg of pure drug and dissolving them in a volumetric flask with 5 mL of methanol. Working solutions were prepared by diluting an adequate aliquot of stock solution with an appropriate volume of methanol. Methanol, acetonitrile (HPLC grade), ammonium acetate, and triethylamine were purchased from Mallinckrodt (Paris, Kentucky). Sodium carbonate was obtained from Synth (Diadema, São Paulo, Brazil). Purified water was obtained from a Milli-Q system (Millipore, Billerica, MA).

SPME device

A manual fiber holder for SPME was obtained from Supelco (Bellefonte, PA), as well as the following SPME coated fibers: carboxen (CAR)–polydimethylsiloxane (PDMS) (75-µm film thickness), PDMS (100-µm film thickness), polyacrylate (PA) (85-µm film thickness), and PDMS–divinylbenzene (DVB) (60-µm film thickness). All fibers were conditioned by soaking in the mobile phase for 30 min.

Description of the SPME–LC lab-made interface

A lab-made interface was designed and built in our laboratory to couple SPME to HPLC. The interface consisted of a six-port and two-position valve (C6UW), obtained from Valco (Houston, TX), and a 60-µL desorption chamber. In this interface, the desorption process can be performed either in dynamic mode (with the mobile phase eluting through the fiber) or in static mode (in which the fiber is

maintained in the interface in contact with some volume of mobile phase, or another organic solvent, in order to desorb the analytes). When the desorbtion time elapses, the fiber is removed from the chamber and the valve is switched from load to injection position, sending the analytes to be separated by the chromatographic column. Figure 1 illustrates the lab-made interface with the valve and the desorption chamber. Figure 2 shows a schematic diagram in which the two valve positions (load and injection) are detailed.

Analytical instrument

A Shimadzu HPLC system LC-10AVP (Kyoto, Japan), consisting of two pumps (LC-10Ai), an oven (CTO-10ASVP), a fixed wavelength UV–vis detector (SPD-10AVP/10AVVP), an autosampler (Sil-10Ai), a system controller (SCL-10AVP), a degasser (DGU-14A), and acquisition data software (Class-VP), was employed in this study.

Chromatographic conditions

The chromatographic separation was achieved using an RP-18 column obtained from Shimadzu (150 × 4.6 mm, 5-µm particles), preceded by a guard column packed with RP-8 particles obtained from Agilent Technologies (Palo Alto, CA). The mobile phase was an isocratic mixture of ammonium acetate buffer (0.05 mol/L) plus 0.1% of triethylamine (pH 5.50)–acetonitrile (55:45 v/v) at a flow rate of 0.4 mL/min. The UV–vis detector was set at 214 nm and the oven temperature at 35°C.

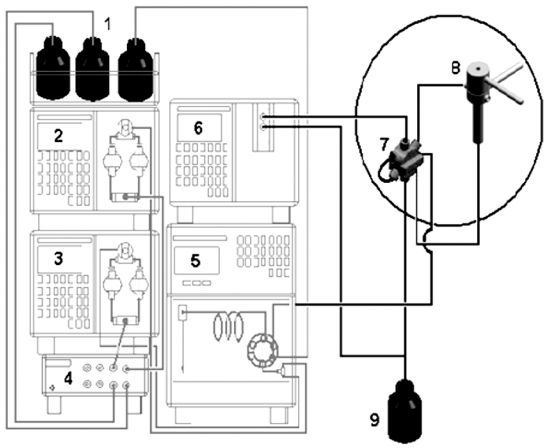


Figure 1. Diagram illustrating the HPLC system and the SPME lab-made interface: solvents, 1; pumps, 2,3; degasser, 4; autoinjector, 5; UV detector, 6; six-port valve, 7; lab-made interface, 8; and waste, 9.

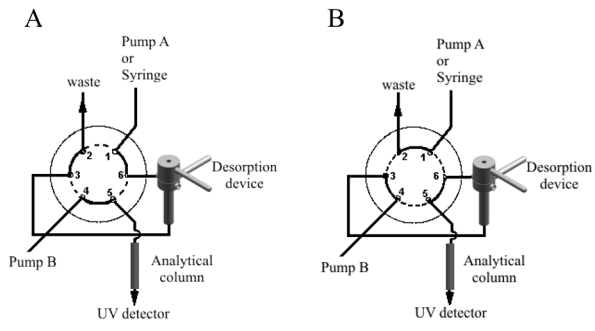


Figure 2. Schematic diagram of the six-port valve showing: static desorption mode (A) and injection mode (B).

SPME analytical procedure

Initially, different SPME fibers were compared in order to choose the appropriate coating for this application. The selected coating was employed in the optimization of other extraction parameters.

Extraction was performed in a 5-mL conical glass vial sealed with a silicone septum using a small, triangular-shaped stir bar. Into the vial was added 1 mL of drug-free plasma sample spiked with all TCAs at 1.0 µg/mL and 4.0 mL of sodium carbonate buffer (0,06 mol/L) with different pH values (pH 9.0, 10.0, and 11.0). This mixture was vortexed for 20 s before extraction. The fiber was then immersed in the mixture and heated up to 30°C under a magnetic stirring rate of 1200 rpm. After extraction, the analytes were desorbed (for 20 min) in either off-line mode in 200 µL of mobile phase (during the choice of the best fiber coating) or online mode in the 60-µL chamber of the developed interface (during the optimization of the extraction). After the desorption, the fiber was cleaned by immersion in 5 mL of a water–methanol mixture (50:50 v/v) for 5 min.

Factorial design

A 2^3 complete factorial design plus central point (three replicates) was performed in order to evaluate the influence of time, temperature of extraction, and pH. Table II presents the set values of the evaluated parameters at high, medium, and low levels. These levels are presented in Table III as +1, 0, and –1, respectively. A total of 11 experiments were performed during the optimization.

An empirical mathematical model was built using Statistica 6.0 software (Tulsa, OK). The data were modeled in a quadratic equation, and the results were evaluated by analysis of variance. Response surfaces were built in the optimized condition.

Results and Discussion

Currently, there are different types of SPME fiber coatings available. Because the equilibrium time of the analytes between the matrix sample and fiber coating depends on the diffusion

Table II. Levels of the Parameters Evaluated During Optimization

	Levels		
Parameters	Low	Medium	High
Time (min)	10	30	45
Temperature (°C)	30	45	60
pH	9	10	11

into the coating, as well as on the coating thickness, it is essential to select an appropriate coating in order to maximize the SPME efficiency (18,19).

Table III. Factorial Planning Matrix (23) Plus a Central Point and the Area Values with PDMS–DVB (60 µm) for the Analyses of TCAs.

Experiment	Temperature (°C)	Time (min)	pH	DESI	NOR	IMI	AMI	CLOMI
				Area values				
1	–1	–1	–1	46237	71563	24111	39595	50844
2	+1	–1	–1	242460	365159	119031	187882	166874
3	–1	+1	–1	197558	288772	97752	160302	171676
4	+1	+1	–1	322078	482704	165327	274188	321736
5	–1	–1	+1	62092	95368	35396	64509	74287
6	+1	–1	+1	163102	226179	71005	114844	115229
7	–1	+1	+1	471487	767543	234555	477159	571149
8	+1	+1	+1	344308	475805	164617	252861	277351
9	0	0	0	158184	227487	66868	116304	126480
10	0	0	0	152675	212019	69286	112175	121437
11	0	0	0	167107	229945	73394	121277	134770

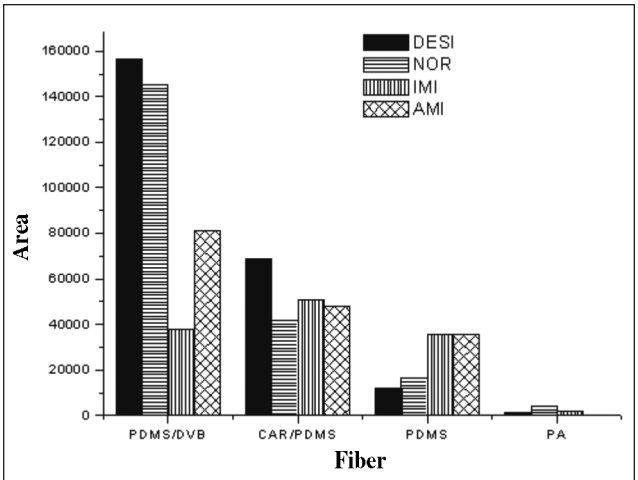


Figure 3. Peak-area values obtained for the different fiber coatings.

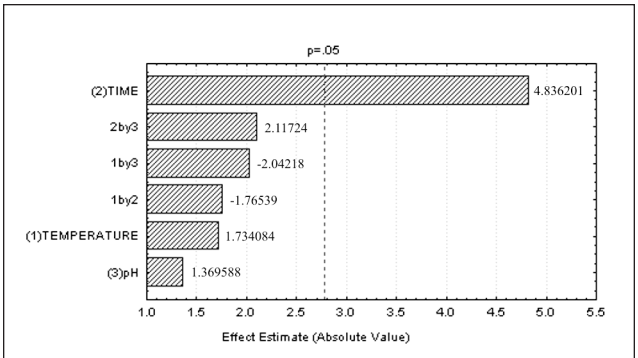


Figure 4. A pareto diagram illustrating the influence of the parameters temperature, time, pH, and their interactions over DESI extraction. The results were calculated by Statistica 6.0 based on the 2^3 factorial planning plus central point.

Evaluation of the SPME coating

Selection of a fiber coating was the first step in the development of an SPME methodology. The polarity of analytes to be determined and the physicochemical properties of the coating should both be carefully considered during the optimization. As concluded in earlier studies, the selectivity of the coating for the analyte were improved by a modification in the chemical structure of the polymer, as is commonly done in the stationary phase for GC. Thus, if polar sorbents were coated onto the fiber, more polar compounds could be better extracted (18). Currently, there are two types of commercially available coatings: pure polymeric layer coatings (liquid coating) (such as PDMS and PA) that extract analytes via absorption and heterogenic polymeric layer coatings (such as PDMS–DVB, CW–DVB, CAR–PDMS, DVB–CAR–PDMS, and CW–TPR) that extract via a mix of absorption and adsorption mechanism. Figure 3 presents the peak-area values obtained for the four different fiber coatings to SPME.

The selection of the coating was mainly based on the principle that "like dissolves like". Considering that the tricyclic antidepressants were semipolar compounds, the PA (the most polar phase) and PDMS coatings (the most nonpolar phase) did not demonstrated a good performance for the extraction of TCAs. On the other hand, the mixed-phase fiber coatings, such as PDMS–DVB and CAR–PDMS, were the coatings that presented the best results in the extraction of the TCAs. However, when both coatings were compared, the results were in favor of PDMS–DVB. The polarity of PDMS was modified by the insertion of divinylbenzene groups, and, based upon the experimental results, the PDMS–DVB fiber (60-µm film thickness) was selected to analyze the TCAs.

SPME optimization

Upon selecting the best fiber coating, an optimization was performed in order to obtain the best conditions to analyze TCAs in plasma. Table III shows the matrix combination of the evaluated parameter for the 11 performed experiments, as well as the peak areas of the TCAs obtained in each experiment. After obtaining the area values for each analyte, the results were evaluated using Statistica 6.0 software.

Figure 4 shows a Pareto diagram that presents the influence of the evaluated parameters, as well as their interactions, on the extracted amount. The dashed line indicates the region above which the effect was significant (with a confidence limit of 95%). In this kind of graphic, the longer the bar is, the higher the influence (positive or negative) on the response of interest. As the obtained results for the other analytes presented the same profile as for DESI, only the diagram for this analyte is presented. The percentage of the explained variation ranged from 84% to 91%, allowing an adequate evaluation of the parameters of influence.

The extraction time was the parameter, according to the model, that most influenced the extraction. It acted positively,

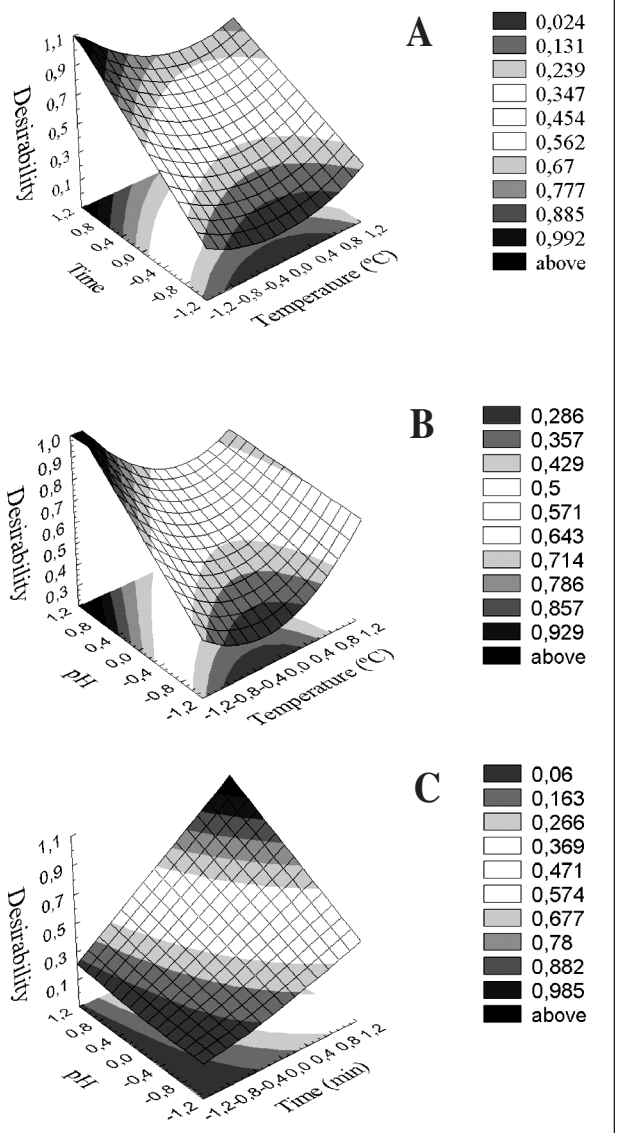


Figure 5. Response surfaces for all TCAs at 1.0 µg/mL obtained with the 2^3 factorial planning plus central point: time and temperature effects for pH 11.0 (A), pH and temperature effects extraction time of 45 min (B), and pH and time effects for temperature of 30°C (C).

increasing the extracted amount. The area value obtained after 45 min of extraction was higher than the one obtained after 10 min. The optimum extraction time varied for each analyte, depending on its solubility in water and its molar mass. However, 45 min proved to be enough for all.

Temperature presented a positive effect upon the extraction. However, according to the models, this effect was not significant for all compounds. Temperature acts on the extraction by increasing the diffusion of the analytes and, as a consequence, increasing the extraction. On the other hand, it decreases the partition coefficient between the analyte and the fiber coating, decreasing the extraction efficiency. For TCAs, the temperature influence on the diffusion process seemed to stand out because the temperature presented a positive effect, as demonstrated by the Pareto diagram. Although temperature was not significant, it collaborated with the extraction of the analytes.

Depending on the type of SPME coating, predominantly neutral forms of analytes show efficient extractions. Thus, ionizable compounds, such as TCAs, require a matrix pH control (19,24,25). Thus, according to the literature, the matrix pH should show a positive effect on the extraction of these kinds of analytes when they are weak bases. The observed pH effect (Figure 4) corroborated the literature. However, according to the evaluated model, this effect was also not significant.

During the performed factorial planning, the PDMS–DVB (60 µm) fiber was employed. This fiber has a pH range from 2 to 11, and higher pH values are not allowed. At pH 11.0, antidepressants IMI and AMI (pK_a 9.5 and 9.4) and DESI and NOR (pK_a 10.2 and 10.08) were predominantly in the neutral form, yielding a better extraction.

The interaction between time and pH was positive, meaning that extraction increased when time and pH increased together. Interactions between temperature and pH and between temperature and time were negative. When those parameters were increased or decreased together, the extraction efficiency decreased.

Figure 5 shows the response surfaces built with the obtained data. As shown in Figures 5A and 6, temperature should be set at a low point. Even with a positive effect (Figure 4), the best choice is a low temperature value when temperature interacts with time. This fact occurs because, when time is set at a

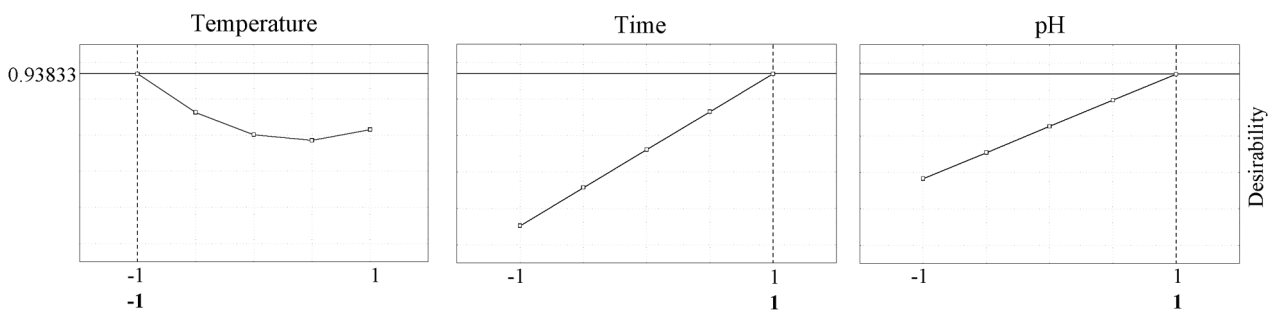


Figure 6. Response profile built by Statistica 6.0 software showing the influence of each parameter on the extracted amount, as well as the best extraction conditions.

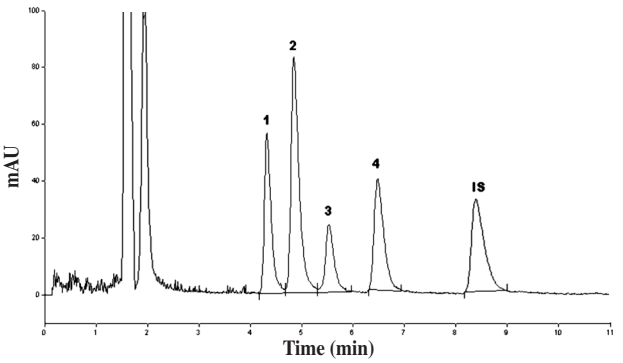


Figure 7. Typical chromatogram of TCAs using online SPME–LC. Peak numbers are: DESI, 1; NOR, 2; IMI, 3; AMI, 4; CLOMI, 5 (internal standard) (analytical standards at 1 µg/mL and internal standard at 3 µg/mL).

high point, there is enough time for diffusion of the analytes into the extraction phase. Figures 5B and 6 show that pH should be set at a high point. This fact was expected because of the basic characteristic of the TCAs. The interaction between pH and temperature was negative (Figure 4) when the influence of pH in the extraction amount was predominant at a low temperature (see Figure 5B). This interaction could be caused by the fact that, at a low temperature, the partition coefficient is higher. Therefore, when the pH is elevated and the analytes are neutralized, the extraction amount is predominantly increased when compared with the same situation at a high temperature. In the same way, Figure 5C shows that, when the time is sufficiently high, the analytes neutralized by the elevated pH are better extracted. In summary, these diagrams show that, in order to increase the extraction yield, it is necessary to increase time and pH and decrease temperature.

Moreover, Figure 6 presents a response profile diagram at the optimized conditions, grouping all the analytes together. The vertical dashed line indicates the best temperature, time, and pH. The calculation, evolved by the Statistica 6.0 software, indicated that more than 90% of desirability was obtained with the set parameters when all of the analytes were computed at the same time.

Among the evaluated conditions, the best results were obtained at pH 11.0, temperature of 30°C, and time of 45 min. A typical chromatogram, obtained the optimized SPME optimization, is shown in Figure 7.

Conclusion

The SPME–LC method optimized in this work has proved to be suitable to analyze TCAs in plasma samples, with the advantage of using a small amount of solvent. The employed factorial design allowed for simple and organized optimization of the evaluated parameters, which more information with a smaller number of experiments yielded a reduction of the time for optimization of the experiment, and a decrease in the consumption of materials and samples. The PDMS–DVB (60-µm film thickness) fiber was selected after comparison with other types of coating. Among the investigated SPME conditions, the best, obtained with the factorial design, were a pH of 11.0, temperature of 30°C, and time of 45 min.

The SPME–LC interface, designed and built in our laboratory, showed good performance when analyzing TCAs in plasma samples. Preliminary results demonstrate a linear range between 50 and 500 ng/mL, with a relative standard deviation in accordance with the values approved by the U.S. Food and Drug Administration (34). Thus, according to these results, this method could be employed for therapeutic drug monitoring.

Acknowledgments

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