Microchemical Journal 208 (2025) 112438

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Ultrasound assisted membrane-assisted solvent extraction for the determination of antidepressants in pericardial fluid by gas chromatography-mass spectrometry

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ARTICLE INFO

Keywords:
Membrane-assisted solvent extraction
Pericardial fluid
Gas chromatography-mass spectrometry
Antidepressants
Membrane

ABSTRACT

The current trend in the development of analytical methods focuses on the miniaturization of sample preparation techniques, avoiding the consumption of large volumes of organic solvents and replacing the more harmful ones with less toxic solvents. In order to bring Forensic Toxicology closer to this trend, a simple method based on membrane-assisted solvent extraction (MASE) has been developed for the first time for the determination of three antidepressants (ATDs) using pericardial fluid as an alternative biological sample to blood. The analytes under study were later determined by gas chromatography-mass spectrometry (GC–MS). The optimized extraction procedure consisted of using 400 μL of n-hexane as an extractant solvent, placed inside a polypropylene (PP) membrane, and 4 mL of pericardial fluid (pH adjusted at 11). Ultrasound assistance (50–60 Hz, room temperature, 30 min) was used to favor analytes mass transfer from the aqueous phase to the organic phase. The n-hexane extract was further evaporated to dryness and reconstituted with 40 μL of methanol, achieving a preconcentration factor of 100. Validation was performed in accordance with FDA guidance as a reference. Lower limit of quantification (LOQ) values of 5 ng/mg were achieved for each ATD under study. The recoveries were higher than 90 % and the values of accuracy and precision did not exceed the maximum allowed error of 20 % for the LOQ or 15 % for the remaining concentration levels. Finally, the method was successfully applied to 14 real cases.

1. Introduction

The identification and quantification of drugs and drugs of abuse in biological samples is one of the most important objectives to clarify the cause of death in Forensic Toxicology. The most used postmortem samples are blood and urine, especially peripheral blood, since it is less affected by different factors, such as cadaveric phenomena and postmortem redistribution. Therefore, it is of great importance to bear in mind that blood analysis is fundamental in this field since it allows to know the toxicological status of the deceased at the time of death. However, the collection of blood samples is sometimes impossible due to several factors, such as exsanguination or advanced putrefaction. In these cases, other biological samples, such as pericardial fluid (PF), can offer complementary information. It is a pale yellow, serous fluid present in the pericardial cavity, which confers greater preservation of the sample without significant contamination and less influence of PMR (postmortem redistribution) compared to other biological samples. In addition, PF is free of haemolysis and has a lower protein concentration, unlike blood, making it a sample with less matrix effect [1]. Usually, a volume of 15–60 ml of sample is available, so it does not have the limitations shown by other alternative biological samples. The use of PF is still very limited in the forensic field, and few studies have been published. Some publications focused on the development of a method for the determination of drugs and drugs of abuse in this unconventional matrix [2–7], while others have proposed studies on postmortem redistribution (PMR) [1,8–11] showing a good correlation between the

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https://doi.org/10.1016/j.microc.2024.112438
Received 2 October 2024; Received in revised form 26 November 2024; Accepted 10 December 2024
Available online 13 December 2024

target levels found in blood and those found in PF, suggesting that these concentrations are useful to estimate the degree of intoxication. Table S1 showed more detailed information from the studies mentioned above.

Mental illnesses are very common in today's society, and the use of ATDs combined with psychological treatment is a current practice to mitigate and treat the symptoms associated to depression. These drugs are also frequently detected in postmortem samples, and their determination is relevant to understand the cause of death [12,13]. There is a wide variety of ATDs available on the market, with notable differences between them (side effects, cost, drug-drug interaction…). Tricyclic antidepressants were the first drugs used to treat this type of mental disorders. They have a high efficacy, but they are associated with strong side effects. For this reason, they are often replaced by ATDs that cause fewer side effects (new generation of ATDs), such as selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SSNRIs), and noradrenergic and specific serotoninergic antidepressants (NaSSAs) [14].

One of the most critical stages when assessing drugs in biological samples is the sample pre-treatment procedure. Several extraction methods have been proposed in the scientific literature for ATDs isolation, such as conventional liquid–liquid extraction (LLE) and solid-phase extraction (SPE) [13,15–20] processes, as well as more novel methods, referred as microextraction techniques, such as dispersive liquid–liquid microextraction [5,21,22], air-disperser liquid–liquid microextraction [23], bar adsorptive microextraction [24], stir bar filled magnetic ionic liquids [25], and hollow-fiber drop-to-drop solvent [26]. Among microextraction techniques, membrane-assisted solvent extraction (MASE) is a miniaturized version of LLE that uses a hydrophobic polymeric membrane to separate the aqueous phase (donor phase) from the organic phase (acceptor phase), so that the two phases are not in direct contact. The organic analytes present in the aqueous phase are first dissolved in the polymer, thus passing to the organic phase. Typically, the membranes are made of a hydrophobic polymeric material such as polypropylene (PP) and low-density polyethylene (LDPE) [27]. This technique has been used for the treatment of environmental [28–48] and food samples [49–51]. However, its application in the field of forensic toxicology is still very limited, focusing only on the analysis of urine samples [27,52]. To our knowledge, there are no publications in the scientific literature describing the application of MASE for the determination of ATDs in PF. Table S2 provided a review of MASE applications.

In addition to the sample pre-treatment method, the instrumental

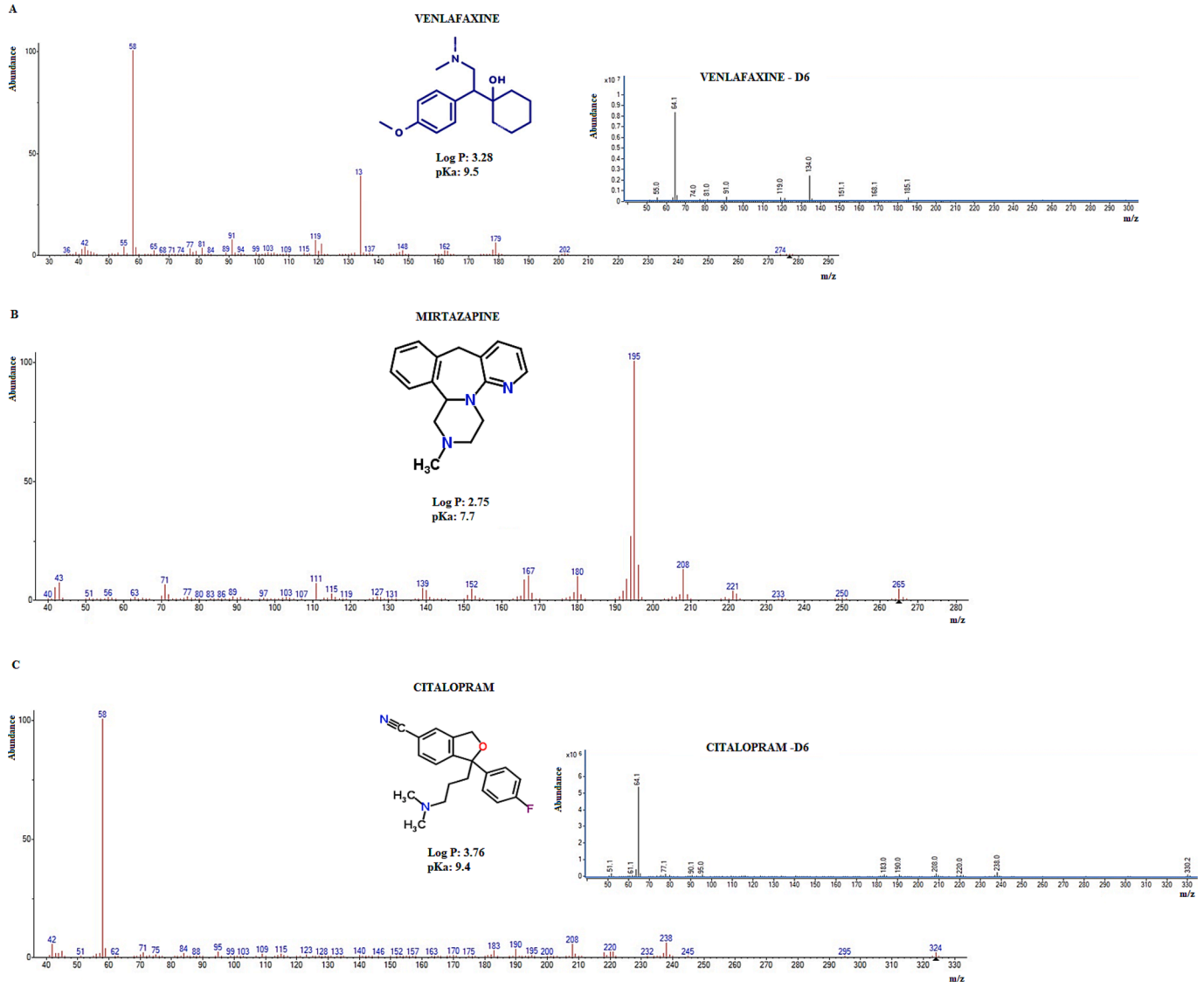

Fig. 1. Venlafaxine (chemical structure and mass spectra) (A), mirtazapine (chemical structure and mass spectra) (B) and citalopram (chemical structure and mass spectra) (C). Source: PubChem.

technique is also essential since the use of biological matrices is a challenge for analytical laboratories due to their complexity and incompatibility with many of the instrumental techniques [53]. Several chromatographic methods have been used for ATDs assessment in biological samples, such as gas chromatography-tandem mass spectrometry (GC–MS/MS) [13], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [52,54], high-performance liquid chromatography-UV–Vis detector [25], liquid chromatography with diode array detector (LC-DAD) [55], gas chromatography-flame ionization detector (GC-FID) [56,57], liquid chromatography-mass spectrometry (LC-MS) [58], and gas chromatography-mass spectrometry (GC–MS) [5,14,19,20,22,24,26,53,59,60]. However, there are scarce literature regarding ATDs determinations in PF.

Therefore, the main objective of this study was to explore the possibilities of ultrasonication-accelerated MASE (US-MASE) in the forensic laboratory using PF as an alternative biological sample for those cases in which blood was not available. The ATDs under study have been venlafaxine, mirtazapine and citalopram, whose chemical structures are shown in Fig. 1. The identification and quantification of the ATDs was performed by GC–MS. The optimized method was applied to real forensic cases after method validation in accordance with international guidelines, and results have demonstrated the high potential of the proposed procedure.

2. Material and methods

2.1. Chemicals

Ultrapure water was processed through a Milli-Q water system (Millipore, Bedford, MA, USA). Methanolic solutions of venlafaxine hydrochloride (≥98 %), mirtazapine, and citalopram at 1.0 mg mL^{-1} were from Cerilliant Corporation (Round Rock, TX, USA). Methanolic solutions of deuterated analogues (internal standards) of 0.1 mg mL^{-1} of venlafaxine D3 and citalopram D3 were also from Cerilliant. The stock standards and their diluted methanolic solutions were stored at −20 °C. Methanol, sodium hydroxide, n-hexane, chloroform, cyclohexane, dichloromethane, diethyl ether, and sodium chloride were purchased from Merck® (Darmstadt, Germany). Other solvents were 1-butanol, and 1-octanol (PanReac, Barcelona, Spain).

Drugs isolation was performed with a MASE system consisting of 10 mL glass vials equipped with polypropylene (PP) membrane (4 cm × 6 mm i.d., wall thickness of 0.03 mm), stainless-steel funneld, PTFE rings, Viton rings, and metallic ring caps (Gerstel, Mülheim, Germany).

2.2. Pericardial fluid samples

The optimization and validation of the method was performed using drug-free PF samples, which were verified by analysis of the corresponding urine and peripheral blood. The samples were taken during autopsies performed by forensic physicians, and they were stored at −20 °C until analysis. Those samples positive for ATDs were used for the application of the method, resulting in fourteen real cases.

2.3. Sample preparation

Cleaning of the MASE pieces (PP membranes, Teflon rings, and conic metallic components) was necessary to avoid possible cross-contamination. For this, they were washed and sonicated with n-hexane for 10 min. The cleaning process was then repeated using methanol as a solvent. The pieces were left to dry at room temperature.

The MASE procedure was performed with 4 mL of drug-free pericardial fluid (pretreated by ultracentrifugation at 14000 rpm for 5 min), spiked with 40 μL of a 10 μg mL^{-1} internal standard solution (venlafaxine D6 and citalopram D6) in 10 mL vials. The samplés pH was adjusted to 11 with sodium hydroxide solution to promote the analytes to the neutral state. Analyte transfer into the organic phase (400 μL of n-hexane inside the PP membrane) was favored by the addition of 20 mg of sodium chloride (salting out effect). Ultrasonication (50–60 Hz) was then applied for 30 min at room temperature. After extraction, the acceptor phase (n-hexane) was collected and transferred to a conical glass tube. The organic solvent was evaporated under a stream of nitrogen in a heated aluminum block at 40 °C (VLM GmbH, HP series). The dried residue was redissolved with 40 μL of methanol prior to injection of a 1 μL aliquot into the GC–MS system.

2.4. Instrumentation

Chromatographic analyses were carried out using gas chromatography (Gas Chromatograph model 7890B) coupled to mass spectrometry (Mass selective detector, MSD, model 5977B), both from Agilent Technologies® (Las Rozas, Spain). Electronic impact was used as the ionization method. The column used was an HP5-MS capillary column (30 m × 250 mm i.d., 5 μm film thickness; also, from Agilent Technologies®) with helium as carrier gas (1 mL min^{-1}) to achieve the chromatographic separation. The injector temperature was set at 250 °C and a purge time of 2 min was used. Samples were injected in the spitless mode. The temperature gradient program used for the chromatographic separation of the compounds has been slightly modified with respect to a previous work [5]. It was finally as follows: the initial column temperature was set at 100 °C for 1 min; then the ramp was set at 30 °C min^{-1} until reaching 200 °C for 2 min, followed by a ramp at 7 °C min^{-1} up to 280 °C and held for 1 min (a total chromatographic separation time of 18.7 min). Finally, the temperature was increased to 290 °C for 5 min to clean the column. The MSD was kept at 300 °C, the ion source at 250 °C, and the quadrupole at 150 °C.

An ultrasound cleaner bath (50–60 Hz, 360 W) from Selecta (Cham, Switzerland) was used for speeding up the MASE process.

2.5. Identification of compounds

Compound identification was performed by injecting the standards (neat standards of ATDs and internal standards, IS) into the GC/MS working in SCAN mode, ranging from 50 to 550 amu. The mass spectra obtained for each analyte allowed the selection of quantifier and qualifier ions, based on their abundances and mass-to-charge ratios (m/z) (Fig. 1). The quantifier and qualifier ions and retention times for each analyte were listed in Table 1. Upon ions selection, the mass spectrometry was run in selected ion monitoring mode (Fig. 2).

2.6. Method validation

The validation study was performed according to Food and Drug Administration (FDA) guidelines [61] in terms of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), intra-day and inter-day precision and accuracy tests, and recovery.

The **selectivity** study was performed by analyzing six drug-free PF samples from different sources to confirm that the assay is free of potential interfering substances, including endogenous matrix components, metabolites, etc. **Linearity** was assessed by constructing six calibration curves at the 9 concentration levels between LOQ to ULOQ analyzed from different days. A linear regression model was applied based on the analyte versus IS peak area ratio. The **LOD** and **LOQ** were

Table 1
Retention time and ions selected for monitoring.

Compounds	Quantifier ions	Qualifier ions	Retention time, min
Venlafaxine	58	179, 162	13.34
Venlafaxine D6	64	185,168	13.29
Mirtazapine	195	167,180	15.65
Citalopram	58	71, 324	17.32
Citalopram D6	64	77, 330	17.28

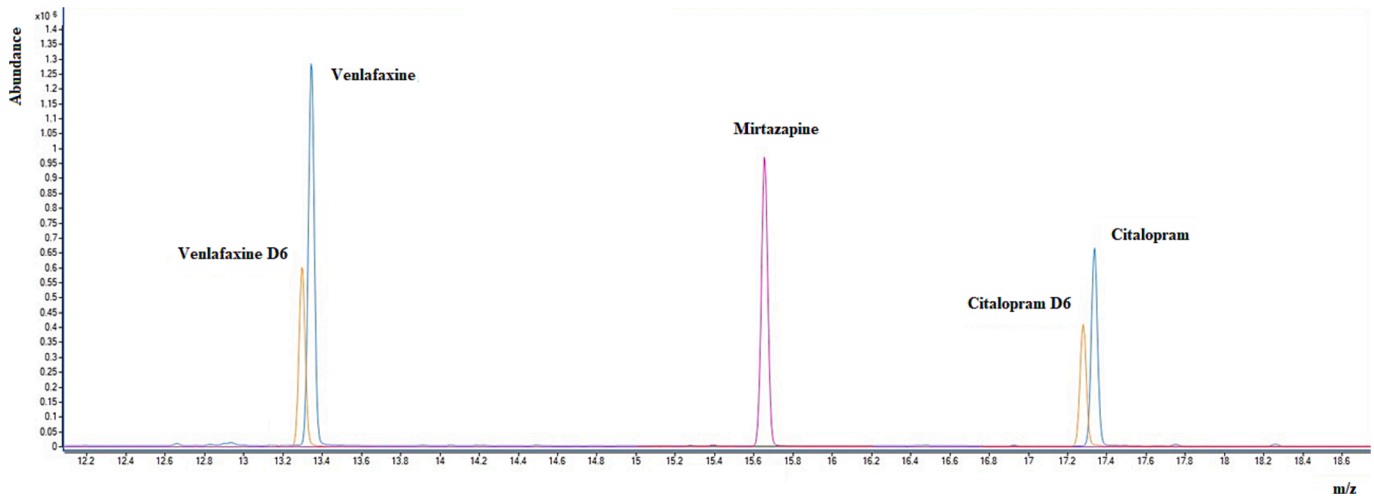

Fig. 2. Ion extracted chromatograms of all analytes.

calculated at a signal-to-noise ratio of 3 (S/N = 3) and 5 (S/N = 5), respectively. **Precision** was established through the evaluation of relative standard deviation (%RSD). Blank samples were fortified with three quality control (QC) samples at low, medium and high level, within the linear range of the calibration curve of the analytes. Analysis was carried out using 5 replicates of each QC sample on the same day (intraday precision) and 5 replicates on 5 different days (interday precision). **Accuracy** was calculated through relative error (RE, %), following the same schedule than precision. In order to meet the validation criteria, the error of accuracy and precision should not exceed 15 % for each calibration standards, except for LOQ, where 20 % of error is accepted. **Recovery** (R, %) of the method was examined by comparing the analytical results for extracted samples at three concentrations levels (low, medium and high) five times on the same day (intra-day study) and on five different days (inter-day study) with theoretical concentration that represent 100 % recovery.

3. Results and discussion

The proposed method presents two important novelties such as the use of PF as an alternative forensic sample and the application of MASE as an extractive technique for isolating ATDs.

MASE has several advantages over other extraction techniques, such as simplicity of operation, high degree of selectivity, reduced consumption of organic solvents and sample, and ability to use complex samples because the membrane prevents large compounds and particles

Table 2
Comparison of MASE with other extraction techniques.

	Proposed article	[5]	[12]	[1]
Compounds	Citalopram, venlafaxine and mirtazapine	ATDs (including citalopram, venlafaxine and mirtazapine)	ATDs (including citalopram, venlafaxine and mirtazapine)	Several drugs of abuse and drugs (including mirtazapine)
Biological samples	PF	PF	PF, Bl, muscle, urine and HV	PF, Bl and BMA
Extraction technique	MASE	DLLME	LLE	LLE
Detection technique	GC–MS	GC–MS	UHPLC-MS/MS	GC–MS
LOQ, ng/mL	5	Venlafaxine and mirtazapine: 20 Citalopram: 40	Citalopram: 3 Mirtazapine: 1 Venlafaxine: 5	–
Recovery, %	Citalopram: 97.3–117 Mirtazapine: 90.2–109.2 Venlafaxine: 97.4–119.3	Citalopram: 93–105 Mirtazapine: 85–97 Venlafaxine: 92–99	Citalopram: 64–72 Mirtazapine: 65–73 Venlafaxine: 68	–
Application to real cases	Mirtazapine was the most detected ATD. Some venlafaxine concentrations were high	Venlafaxine was one of the most frequently detected ATD. Some concentrations were high.	ATDs most detected: citalopram and mirtazapine Concentration of venlafaxine above the therapeutic range and one of them in extremely high concentration	–
Correlation with blood samples	Not studied	Not studied. The authors suggest the use of PF as an alternative sample to blood, based on the findings of other studies.	Good correlation	Significant correlation of drug concentration in right heart blood, PF and BMA.

ATD: antidepressant.
Bl: blood.
BMA: bone marrow aspirate.
DLLME: dispersive liquid–liquid microextraction
GC–MS: gas chromatography-mass spectrometry.
HV: vitreous humour.
LLE: liquid–liquid extraction.
PF: pericardial fluid.
MASE: membrane-assisted solvent extraction.
UHPL-MS/MS: ultra-high-performance liquid chromatography – triple-quadrupole mass spectrometry.

in the sample (aqueous phase) can reach the organic solvent inside the membrane [52,53]. Table 2 showed a comparison of this technique with other extraction methods used in the scientific literature for the determination of at least one of the ATDs under study using the PF [1,5,12]. In addition to the use of PF, Tominaga et al [1] used blood, muscle, urine and HV while Leere et al [12] employed blood and bone marrow aspirate. In terms of extraction techniques, the authors mentioned above used liquid–liquid extraction (LLE) [1,12], compared to the DLLME used by our research group in a previous work [5]. Tominaga et al. [1] and Cabarcos-Fernández et al. [5] used GC–MS for analyte identification, separation and quantification. However, Leere et al. [12] used UHPLC-MS/MS, a more sensitive technique that allowed them to slightly improve the sensitivity of the method.

To achieve a good use of the technique, several parameters needed to be optimized for each analyte in the selected matrix. The main parameters to be studied are the properties of the analytes with respect to the extraction solvent, and parameters such as sonication (extraction) time, ionic strength, sample and organic solvent volume and pH.

3.1. Optimization of the ultrasound assisted MASE procedure

3.1.1. Properties of the analytes under study

Analytes move from the aqueous phase to the organic phase by passive diffusion and depending on their distribution coefficients. Hydrophobic (apolar organic compounds) are required to be retained in solvents of apolar nature (low solubilities in water) in order to avoid losses through the membrane. The suitability of the analytes has been investigated as a function of log P values, being more favorable the extraction of targets with log P values higher than 3 (Fig. 1).

3.1.2. Selection of solvent

The selection of the extractant solvent is one of the most important points for obtaining satisfactory results. The solvents must meet a series of requirements: good selectivity for the analytes under study, polarity like analyte's polarity, immiscible with the sample, compatible with the instrumental technique, good chromatographic behavior, and high purity, among others. The organic solvents studied in this work were n-hexane, cyclohexane, chloroform, 1-butanol, 1-octanol, dichloromethane, and diethyl ether. Dichloromethane and diethyl ether were not taken into account, as they diffuse through the membrane. Experimental tests with the other solvents during the development of the method were carried out in duplicate, with n-hexane and cyclohexane being the solvents with the best results. The final choice of the solvent used in the procedure was made on the basis of the calculation of the ratio n-hexane/cyclohexane. The optimal solvent for venlafaxine and mirtazapine was n-hexane, with 2.5 and 1.8-time better recoveries, respectively. For citalopram, the n-hexane/cyclohexane ratio was slightly lower using n-hexane. However, the LOD and LOQ achieved in the method validation justified the correct use of this solvent even if it did not provide the best recoveries, as it was able to quantify sub-therapeutic levels, according to the tables consulted [62,63]. Therefore, n-hexane was finally selected as acceptor phase because of its good extraction capabilities, PP membrane compatibility, stability during the extraction process and fast evaporation during the further drying process.

3.1.3. Multivariate optimization of variables affecting the ultrasound assisted MASE procedure

The use of experimental designs helps the researcher to select the optimal values for the variables affecting a process/procedure. These techniques are appealing alternative tools to classical (single factor) optimization methods since they reduce the time and cost of the optimization procedure, as well as they offer more information by considering the potential interactions between factors (variables).

The main difficulty of this methodology was to design a reduced experimentation that allowed obtaining all information with a reduced number of experiments. The statistical program StatGraphics Centurion 19 has been used throughout this work.

Initially, a fractional factorial design (two-level, five components factorial design, 2^5-1, randomly conducted with two central points, aiming to minimize the impact of noise factors and systematic errors; P value of 0.05; 18 experiments) was carried out with a 95 % confidence level, since a full factorial design required many experiments, exceeding the available resources. The experimental factors (independent variables) and the dependent variables defined in this design were listed in Table 3 (independent variables were fixed at high and low levels). In general, microextraction techniques requires small sample volumes, thus requiring shorter times to reach equilibrium. Therefore, the variable sample volume was fixed at 2.5 mL (low level) and 5.0 mL (high level) whereas, n-hexane volume was fixed at 0.4 and 0.8 mL for the low and high levels, respectively (the volume of the extractant solvent will depend on the amount of analyte to be extracted and the capacity of the membrane). The addition of salts to the aqueous phase causes an increase in the ionic strength of the medium, aiding analytes transfer into the organic phase. In this work, amounts of NaCl ranging from 8 mg (low level) to 30 mg (high level) were tested. In addition, the pH also affects the dissociation equilibrium of some analytes in aqueous media and sample must be buffered to keep the analytes at the neutral state for better extraction. Due to the basic character of the compounds under study, the pH range was established between 11 (low level) and 13 (high level). Being a non-exhaustive technique, MASE requires longer extraction times to achieve diffusion of analytes across the membrane by a concentration gradient (higher concentration in the donor phase, usually the aqueous phase, and low concentration in the acceptor phase, organic solvent) [52,53]. Sonication reduces the time needed to reach equilibrium, since it favors the transfer of analytes to the organic phase, and sonication (extraction) times were fixed at 5.0 min (low level) and 30 min (high level).

After analysis and statistical evaluation, results were plotted in Figs. 3 and 4, which showed the Pareto charts of the standardized effects and the main effects plot, respectively. As shown in Fig. 3, there were no variables affecting statistically the extraction of mirtazapine and citalopram. However, variables such as sample volume and pH, as well as various variables interactions involving the salt amount have been found to influence significantly the venlafaxine recovery.

Fig. 4 showed the plots of the main effects, which will indicate how each factor will influence the recovery of the analyte. Based on these results, the extraction of the three ATDs seemed to be improved by using low pH values and small amounts of salt, while the use of large sample volumes was found to be more suitable. Regarding the volume of extractant solvent and sonication time, the results showed opposite values for venlafaxine and citalopram. In the case of mirtazapine, none of these factors, apart from pH, have been found to influence the response obtained. In view of the results obtained, an adequate conclusion was not possible to draw, and a Response Surface Design (RSM) with the same experimental factors and low and high values (Table 3) was required. The response to the main effects (Fig. 5) showed that the analytes had a better response when high sample volumes and low pH values were tested. Regarding the amount of NaCl, targets recoveries were more favorable when intermediate values were used, the most frequent value being 20 mg of NaCl. With respect to the sonication (extraction) time and the volume of extractant solvent, the values

Table 3
Experimental factors and dependent variables defined in the StatGraphics Centurion 19 statistical program.

Dependent variables	Experimental factors
Venlafaxine Mirtazapine Citalopram	Sample volume (2.5–5 mL) Extractant solvent volume: 0.4–0.8 mL NaCl amount (8–30 mg) Sonication time (5–30 min) pH (11–13)

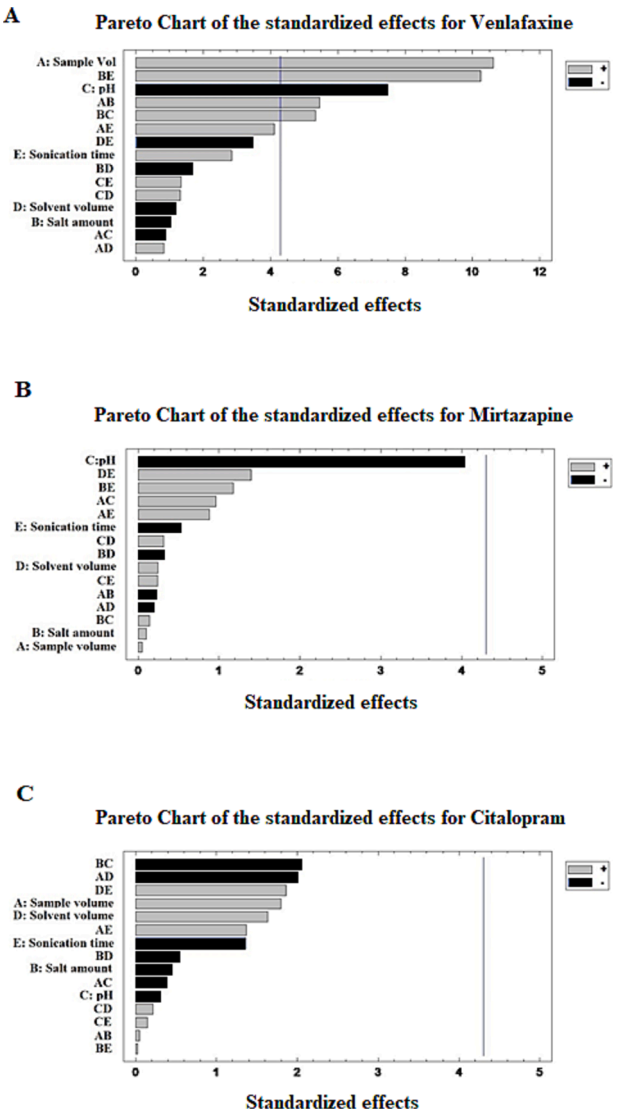

Fig. 3. Pareto chart of the standardized effects for venlafaxine (A), mirtazapine (B) and citalopram (C). Fractional factorial design.

obtained differed among the compounds, being necessary to reach a compromise condition.

Therefore, the following conditions were established for the ultrasound assisted MASE procedure: 4 mL of PF, 20 mg of NaCl and pH adjusted at 11, 400 μL of organic phase (n-hexane), and sonication (extraction) for 30 min.

3.2. Performance parameters and validation

In order to evaluate the proposed method, US-MASE-GC/MS, the validation parameters described in a previous section have been determined.

3.2.1. Selectivity

Drug-free PF samples from six different sources were analyzed to ensure that there is no matrix effect throughout the application of the method. The extracted ion chromatogram of blank PF spiked with internal standards was shown in Fig. S1. The results showed no interfering peaks from endogenous substances at the retention time of the analytes of interest and IS.

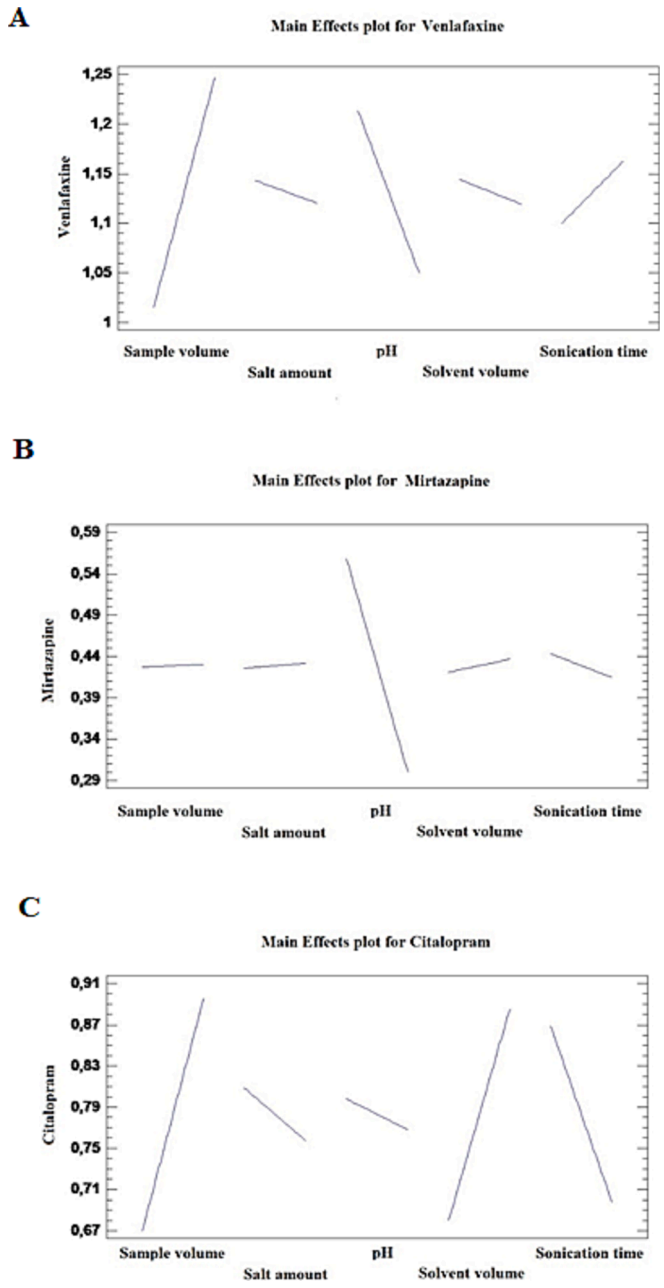

Fig. 4. Main effects plot for venlafaxine (A), mirtazapine (B) and citalopram (C). Fractional factorial design.

3.2.2. Linearity

The calibration curves were created using blank PF samples spiked with venlafaxine and mirtazapine at concentrations of 5–1000 ng mL^{-1} and citalopram at concentrations of 5–2000 ng mL^{-1}. All standard samples were spiked with 40 μL of a 10 μg mL^{-1} solution of internal standards (venlafaxine D6 and citalopram D6). The curves were obtained by fitting the ratio of the peak areas of ATDs to that of IS versus concentrations. The correlation coefficients (r^2) were higher than 0.99 in all cases, demonstrating good linearity. Calibration curves for each of the analytes under study were shown in Fig. S2.

3.2.3. Sensitivity

The sensitivity of the method was determined by calculation of the LOD and the LOQ. The LOD and LOQ were calculated at a signal-to-noise ratio of 3 (s/n = 3) and 5 (s/n = 5), respectively [61], and they were listed in Table 4. The sensitivity achieved in this study significantly

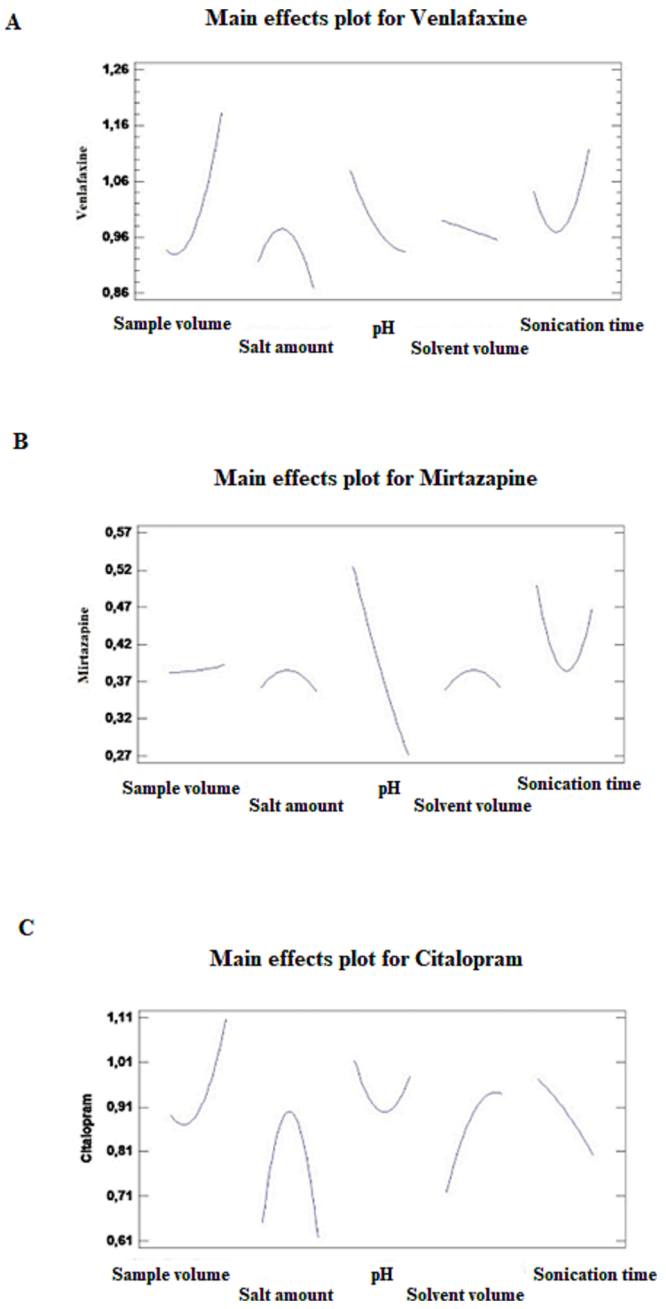

Fig. 5. Main effects plot for venlafaxine (A), mirtazapine (B) and citalopram (C). Response Surface Design.

Table 4
Limit of detection, limit of quantification and calibration results for the antidepressants studied. *LOD: limit of detection; LOQ: limit of quantification; C. coef.: correlation coefficient.*

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	C. coef.	Range of calibration (ng mL^{-1})
Venlafaxine	1.8	5	0.99	5–1000
Mirtazapine	1.3	5	0.99	5–1000
Citalopram	4	5	0.99	5–2000

exceeded that achieved in a previous study in which the same analytes were analyzed using dispersive liquid–liquid microextracion (DLLME) as the extraction technique [5]. The sensitivity achieved in the method proposed by Leere et al. [12] slightly improved these values, using a more sensitive technique (UHPLC-MS/MS) than the one proposed here (Table 2).

3.2.4. Precision and accuracy

The results of intraday and interday precision (expressed in relative standard desviation, RSD) and accuracy (expressed in Relative Error, RE) were shown in Table 5. The intraday and interday precission were all less than 20 %, and the accuracy of intra and interday were in the range of 0.69–19.3 %. Data presented in Table 5 satisfied the international validation rules.

3.2.5. Recovery

The recoveries of the ATDs at three levels of concentrations were presented in Table 5. The data obtained showed that the proposed extraction procedure was satisfactory, providing recovery values ranging between 90 and 119 % for all compounds, as those obtained in other studies [5,52], and even improving those achieved by other authors such as Leere et al [12].

3.2.6. Method applicability

The method developed was used to analyze fourteen PF samples received at the Forensic Toxicology Service of the Institute of Forensic Sciences of Santiago de Compostela. The PFs analyzed belonged to deceased people whose clinical history included a prescription for the drugs under study. They were cases of suicide, accidental and natural deaths. Table 6 showed some of the data from the cases analyzed, together with the concentrations of ATDs determined using the proposed method. Blood ATDs concentrations were also added when the sample amount was sufficient for determination.

Mirtazapine was the most prescribed ATD in the selected cases, and therefore the most detected in the PF analyses, which is consistent with the study by Leere et al [12]. Considering the therapeutic, toxic and lethal ranges for blood samples published in the scientific literature for the three ATDs [63,62], we found in the PF cases analyzed two samples with high therapeutic levels of venlafaxine and one case with toxic levels (cases number 4, 6 and 14 in Table 6), which is in agreement with previous determinations made by our research group [5] and those made by Leere et al [12]. Three cases were detected with negative results for the three ATDs studied, despite having some of them prescribed (the authors assumed that these results were due to a lack of continuity of treatment). One case of suicide (number 5) was also detected in which a non-prescribed ATD was identified, presumably due to a possible prior prescription. The results obtained in PF could only be compared with those determined in blood in 4 cases, due to the absence of the sample, with very similar results. Due to the scarce research related to the use of

Table 5
Intra-day, inter-day precision (RSD; relative standard deviation), accuracy (RE; relative error) and recovery (R) of the method.

Concentrations (ng mL^{-1})	Intra-day study ($n = 5$)			Inter-day study ($n = 5$)		
	RE, %	RSD, %	R, %	RE, %	RSD, %	R, %
Venlafaxine						
5	12.6	16.6	103.7	19.3	9.84	119.3
200	14.0	12.6	114.0	10.8	10.9	110.8
1000	2.56	11.1	97.4	1.12	1.89	101.1
Mirtazapine						
5	12.5	7.9	90.2	9.24	11.9	109.2
200	6.17	1.60	106.2	5.40	11.1	105.4
1000	6,70	10.5	93.3	0.69	5.09	100.7
Citalopram						
5	7.03	10.5	117.0	19.3	20.0	111.3
200	2.71	3.10	97.3	2.53	4.25	102.5
2000	1.71	2.57	101.7	3.92	2.56	103.9

Table 6
Real cases. *F: female; M: male.*

Case n°	Age, years	Sex	Cause of death	General information and treatment	Concentration in PF, [ng mL^{-1}]	Concentration in blood, [ng mL^{-1}]
1	35	F	Choking	Erythromycin, ciclopirox, clonacepam, tripiramate, omeprazole, fluoxetine, clotiapine, paliperidone, quetiapine, aridiprazole, furosemide, malbutamol, terbutaline, **mirtazapine, citalopram**	Mirtazapine [138] Citalopram [35]	Mirtazapine [110] Citalopram: not determined
2	21	M	Natural	Bisoprolol, atorvastatin, pantoprazole, **mirtazapine**, allopurinol, telmisartan, hydrochlorothiazide, amlodipine, duloxetine	Mirtazapine [112]	Mirtazapine [100]
3	23	M	Accidental	Bromazepam, **mirtazapine**, **citalopram**	Mirtazapine [< LOQ] Citalopram [146]	
4	21	M	Accidental	**Venlafaxine**, oxcarbazepine, olanzapine, ziprasidone, amisulpride, amikacin, atorvastatin.	Venlafaxine [564]	
5	22	M	Suicide (Hanging)	Lorazepam, simvastatin, **venlafaxine**, quetiapine, **mirtazapine**, bilastine, dutasteride	Mirtazapine [70.4] Venlafaxine [62.5] Citalopram [165.7]	
6	21	F	Suicide (Submersion)	Simvastatin, enalapril maleate, propanolol, primidone, valproic acid, quetiapine, **venlafaxine**	Venlafaxine [251.7]	
7	23	M	Suicide (intoxication)	Pregabalina, sertralina, bromazepam, zolpidem, **mirtazapine**, quetiapina	Mirtazapine [73.2]	
8	24	F	Accidental	**Citalopram**	Negative	
9	68	F	Natural	Pregabalin, sertraline, bromazepam, zolpidem, **mirtazapine**, quetiapine	Mirtazapine [58]	
10	23	M	Natural	Bisoprolol fumarate, levetiracetam, **mirtazapine**, paroxetine, clonazepam, lorazepam	Negative	
11	22	M	Suicide (hanging)	Betamethasone, atorvastatin, lorazepam, acenocoumarol, paracetamol, silodosin, lansoprazole, brinzolamide, furosemide, sertraline, simethicone, lactulose, folic acid, umeclidinium bromide, vilanterol, ethinylestradiol, drospirenone, levosulpiride, clobetasol propionate, alprazolam, **mirtazapine**	Negative	
12	23	M	Accidental	**Mirtazapine**, fluoxetine, omeprazole, bromazepam, clobazam, dexketoprofen	Mirtazapine [84.8]	
13	22	M	Accidental	Allopurinol, lormetazepam, paroxetine, oxacarbazepine, quetiapine, **mirtazapine**, dipotassium clorazepate, oxacepam	Mirtazapine [103.4]	Mirtazapine [130]
14	75	F	Suicide	Acetylsalicylic acid, trazodone, levothyrosine, omeprazole, **venlafaxine**, quetiapine, metamizole, ferrous sulfate	Venlafaxine [> ULOQ]	Venlafaxine [>ULOQ]

PF, further studies will be necessary to establish a possible correlation between plasma concentrations and those found in PF, which can support the interpretation of the results obtained. Studies such as those developed by Tominaga et al [1] and Leere et al [12] suggested a significant correlation of the concentrations determined in PF with those present in blood, being necessary to consider the chemical and pharmacokinetic properties, especially in drugs such as antidepressants, with high values of the volume of distribution (Vd). The use of PF as a biological sample in the field of forensic toxicology opens the way for research on the influence of postmortem redistribution mechanisms on the death process, which could lead to a better interpretation of the results [64].

4. Conclusions

Ultrasound assisted-MASE proved to be a valid technique for application in forensic toxicology, achieving simplicity, selectivity, good recoveries and providing adequate cleanup, especially useful in complex matrices. The determination of drug concentrations in blood is essential for toxicological analysis. However, the interpretation can be difficult due to the cadaveric processes that this biological sample undergoes, or even the impossibility of collection due to other reasons [6]. Therefore, it is of special interest to look for possible alternative samples such as PF, which has been little used to date in forensic toxicology. It is worth mentioning the novelty of this work, since there are no publications in scientific literature on the determination of ATDs in pericardial fluid using MASE. Despite the scarcity of studies on the use of MASE in the forensic field, the results obtained in the present proposal were promising. The proposed method was subjected to the validation parameters study in accordance with FDA guidelines, achieving limits of quantification of 5 ng mL^{-1} for the analytes under study, precision and accuracy values with errors below 20 % and recoveries between 90 and 119 %. Therefore, the proposed method has proven to be suitable for application in forensic practice with a high degree of selectivity, sensitivity and precision. Finally, the method was applied to fourteen real cases, with mirtazapine being the most frequently detected ATD. However, venlafaxine was the ATD with the highest concentrations.

CRediT authorship contribution statement

P. Cabarcos-Fernández: Writing – original draft, Validation, Methodology, Investigation. **L. Gesia:** Investigation. **A. Moreda-Piñeiro:** Writing – review & editing, Methodology. **A. Fernández-Liste:** Resources. **I. Álvarez-Freire:** Writing – review & editing, Methodology. **M.J. Tabernero-Duque:** Writing – review & editing, Conceptualization. **A.M. Bermejo-Barrera:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.112438.

Data availability

The data that has been used is confidential.

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