

QuEChERS-Based Analytical Method Developed for the Simultaneous Detection of Antidepressant Drugs from a Biological Matrix Using LC-MS/MS

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Abstract: Antidepressant drugs are a diverse group of compounds used to treat various mental health disorders, including depression, anxiety, and obsessive-compulsive disorder. They act by altering the levels of neurotransmitters in the brain, such as serotonin, norepinephrine, and dopamine. It is, therefore, necessary to develop an analytical strategy for adoption in forensic science laboratories. A simple LC-MS/MS was developed for the simultaneous determination of Sertraline (SHC), Trazodone (TDH), and Fluoxetine Hydrochloride (FHC) from saliva samples. The drugs were extracted using an optimized QuEChERS method using dispersive solid phase extraction (DSPE) at six different concentrations (5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, and 200 ng/ml) followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis for identification and quantification. The detection of drugs was performed on a triple quadrupole tandem mass spectrometer. The injection volume of the sample and the temperature of the column were maintained at 10 µl and 40°C, respectively. The method was linear, with a range of 5-200 ng/ml. The method was validated according to SWGTOX (Scientific Working Group of Toxicology) guidelines and showed good linearity, sensitivity, precision, and accuracy. The method is advantageous due to its quick and easy sample preparation and rapid total run time of 8 minutes per analysis, making it a practical and efficient method for the analysis of antidepressants in forensic samples.

Keywords: antidepressant drugs; QuEChERS; simulated matrices; LC-MS/MS; LOD; LOQ; SWGTOX.

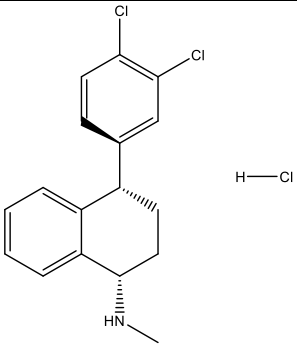
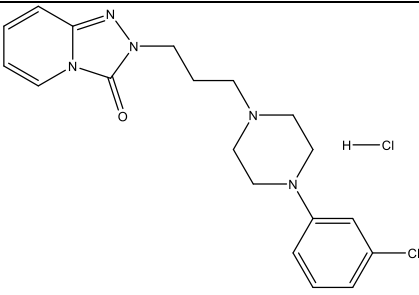
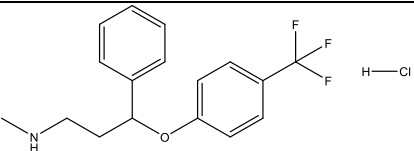
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1. Introduction

Depression is indeed a very common mental disorder that affects millions of people worldwide [1]. It is a complex condition with a wide range of symptoms, including persistent feelings of sadness, hopelessness, helplessness, loss of interest in activities, and changes in appetite or sleep patterns [2]. Depression can significantly impact a person's ability to function, including their work performance, social relationships, and overall quality of life [3]. Moreover, depression can lead to suicide, which is a tragic outcome of this illness [4]. Antidepressant drugs are the first treatment choice for moderate to severe illnesses like stress and anxiety disorders and obsessive-compulsive disorder [5]. Antidepressants cover various

drugs with different modes of action towards neurotransmitter receptors and transporters [6]. They are often characterized as 1st, 2nd, or 3rd new generation [7], depending on when they have developed. The highly prescribed classes of these drugs are selective serotonin reuptake inhibitors (SSRIs) and serotonin receptor antagonist reuptake inhibitors (SARIs) [8]. The commonly administered drugs of these classes are (SHC), (FHC), and (TDH) as shown in Table 1. The chemical structure of SHC is a complex organic molecule that belongs to the class of drugs known as selective serotonin reuptake inhibitors (SSRIs) [9]. It consists of a central ring structure called a naphthalene, which is attached to a side chain containing several other functional groups, including an amine, an ether, and a substituted benzyl group [10]. SHC inhibits CNS neuronal reuptake of serotonin with a steady-state plasma concentration for approximately 1 week [11]. It is widely distributed throughout body tissue and is highly protein-bound (less than 98%). Peak plasma concentration (Cmax) occurs between 4.5 and 8.5 hours after ingestion of a single 100 mg dose [12]. The average elimination half-life is approximately 26 hours and is excreted as ketone and alcohol conjugates undergo metabolism by the action of cytochrome P450 enzymes [13]. It has a longer half-life and higher plasma concentration than the parent drug. Additionally, FHC, widely recognized by its chemical name (3RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy]propane-1-amin hydrochloride, serves as a noteworthy compound in various applications [14]. It has been shown that it inhibits the uptake of serotonin in presynaptic neurons, is administered as a racemic mixture, and is extensively metabolized in the liver to its metabolite norfluoxetine, which has a chiral center and exhibits similar potency [15].

Table 1. Chemical structure of SHC, FHC, and TDH.

Name	Chemical name	Structure
Sertraline hydrochloride	1S,4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine hydrochloride	
Trazodone hydrochloride	2-[3-[4-(3-Chlorophenyl)piperazin-1-yl]propyl][1,2,4]triazolo[4,3-a]pyridin-3(2H)-one	
Fluoxetine hydrochloride	(3RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy]propane-1-amin hydrochloride	

And lastly, TDH, which is known as 2-[3-[4-(m-chlorophenyl)-1-piperazinyl]-propyl]-1, 2, 4-triazolo-[4,3, a] pyridine-3(2H)-one hydrochloride, is a psychoactive compound which belongs to the group of second-generation non-tricyclic antidepressants [16]. It selectively

inhibits serotonin uptake by brain synaptosomes and enhances the behavioral changes induced by the serotonin precursor, 5-hydroxytryptophan [17]. Oral administration with mean peak plasma levels was obtained within 0.5 to 2 hours after ingestion. The mean plasma elimination half-life ranges between 4.4 and 7 hours for the first 3 to 10 hours after a dose and between 10 and 34 hours [18].

The development of analytical techniques for the analysis of drugs from biological matrices is an ongoing process, and researchers are continually striving to improve these techniques to overcome the challenges posed by complex biological matrices [19,20]. Saliva collection is a non-invasive and easy technique that can be performed in a variety of settings, including clinical, research, and forensic contexts. Saliva samples can be collected through various methods, including passive drool, simulated saliva, or swabbing of the oral cavity. Compared to blood and urine samples, saliva collection is less invasive and does not require specialized training or equipment. In addition, saliva collection eliminates the possibility of sample exchange or adulteration, which can occur with urine samples. Because saliva samples can be observed directly during collection, it is less likely that the sample will be tampered with or substituted. Overall, saliva collection is a convenient, reliable, and non-invasive method for obtaining biological samples for various applications [21–23].

Additionally, LLE may not be selective enough to extract only the target analytes and may also extract unwanted compounds. Solid phase extraction (SPE) [19,24] can also be time-consuming and labor-intensive and requires careful attention to the selection of appropriate sorbents and elution conditions for the target analytes [25,26]. It can also be prone to matrix effects, which can affect the accuracy and precision of the results. Solid-liquid extraction (SLE) can suffer from similar issues as LLE, such as the need for large volumes of solvents and a lack of selectivity for the target analytes. Additionally, SLE can be challenging to automate and requires careful optimization of extraction conditions to achieve good recovery and selectivity [27,28].

New sample preparation techniques have been developed in recent years, such as dispersive solid phase microextraction techniques (dSPE), to overcome these limitations. One of the important developments in sample preparation is the QuEChERS method, which has been a significant development in sample preparation for various analytical applications, particularly in food safety and environmental analysis [29,30]. The QuEChERS method involves a simple two-step extraction process that combines salting out and dispersive solid-phase extraction to efficiently extract and clean up a wide range of analytes from complex matrices [28]. The QuEChERS method's first step involves adding a mixture of salts to the sample matrix, which induces the partitioning of the analytes into an organic solvent [31]. This step is known as the salting-out effect and helps to increase the extraction efficiency of the method. The second step involves dispersive solid-phase extraction (d-SPE) to remove any interfering substances from the organic extract. This step typically involves adding a mixture of sorbents to the extract, which selectively adsorbs interfering compounds while leaving the analytes of interest in the solvent [32]. The QuEChERS method has revolutionized sample preparation in many analytical fields by providing a simple, efficient, and cost-effective alternative to traditional sample preparation techniques. Its versatility and adaptability make it attractive. In previous literature, many hyphenated techniques, including Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography coupled with tandem mass spectrometry (LC-MS/MS) [33,34], have been employed. LC-MS/MS is considered a hyphenated technique and represents some advantages such as sensitivity, selectivity, and less

labor that is required for less sample compared to other analytical techniques. The present study was developed to combine the performance of LC-MS/MS detection strategies and the utility of QuEChERS techniques to enable the rapid and simultaneous quantitative detection of SHC, FHC, and TDH. The resultant protocol only requires tiny samples and easy-to-implement pretreatment procedures, offering a reliable approach to therapeutic drug monitoring. Direct detection of these samples is usually challenging due to the complexity of the matrices. For instance, salts, urea, proteins, and other foreign substances inhibit the analysis and increase the matrix effect when present in gastric fluid, saliva, and urine [30]. In addition to *in vitro* analysis, simulated matrices are employed to overcome these obstacles since they produce results that are comparable to those of biological matrices while also being more accurate.

In this work, antidepressants were concurrently extracted and quantified from simulated biological samples. The QuEChERS extraction technique was used to separate SHC, FHC, and TDH, and the quantification step was completed by LC-MS/MS. SWGTOX (Scientific Working Group of Forensic Toxicology) ensures that toxicology testing is conducted scientifically and reliably and that the results are reported accurately and appropriately [35].

2. Materials and Methods

2.1. Chemicals and reagents.

In this research work, only analytical-grade chemicals and reagents were used. The NIAM lab provided the SHC, TDH, and FHC standards. Methanol, water, methanoic acid (formic acid), and ammonium formate solution were purchased from Sigma Aldrich, St. Louis, MO, USA, for HPLC (High-Performance Liquid Chromatography). When it comes to dispersive solid phase extraction (d-SPE), an EN QuEChERS salt pouch (Agilent: 5982-0650 Agilent Technologies, Inc., Santa Clara, CA, USA), sodium citrate tribasic-dihydrate (1 gram), and sodium citrate dibasic-sesquihydrate (0.5 g). In addition, undamped magnesium sulfate, sodium chloride, sodium acetate, and primary secondary amine (PSA) were also obtained from Sigma Aldrich lab, St. Louis, MO, USA. The standard solution of drugs 0.1 gram was prepared and serially diluted in methanol at six different concentrations, i.e., (5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, and 200 ng/ml) respectively, and stored at -20°C.

2.2. Sample preparation and extraction procedure.

The standard solutions (stock, work, and calibration) were prepared in methanol at 0.1 mg/L and stored at -20°C. Drugs were serially diluted from the stock solution at six distinct concentration levels to create working saturation solutions, which were then diluted in methanol according to their concentrations in simulated saliva at therapeutic levels for all medicines, 5, 10, 20, 50, 100, and 200 ng mL⁻¹. Simulated saliva was prepared according to Pietrzynska et al. (2017) by dissolving the entire content in distilled water to make a volume of 1 L [22]. QuEChERS Extraction protocol consists of two steps: Step 1, a partitioning step was used where a mixture of Milli Q water, methanol, and the sample spiked with SHC, TDH, and FHC was homogenized using a wrist action shaker. The QuEChERS salt pouch was added to the mixture, followed by centrifugation to obtain a supernatant layer. In Step 2, the obtained mixture was cleaned up using magnesium sulfate and primary and secondary amine (PSA). After vortexing and centrifugation, the supernatant was further cleaned using MgSO₄ before being analyzed by LC-MS/MS [36,37].

2.3. Analytical condition.

Analysis was performed using LC-MS/MS (Agilent 6470B, Agilent Technologies, Inc., Santa Clara, CA, USA) in positive-ion mode with the following settings for precursor ion scans: 100–500 m/z, 0.3–2.0 V ramping collision energy (Smart Frag), 4 amu precursor ion isolation width, and analysis of one MS and one MS/MS spectra. The analytical column Poroshell 120 with bonded phase EC-C18 (2.7 m, 3 mm x 150 mm) with the flow rate set at 0.4 mL/min, 10 µL of the sample solution was injected, and the temperature was kept at 40°C. To evaluate and quantify SHC, FHC, and TDH, the MS/MS data were calculated using the MRM (multiple reaction monitoring) modes, whose transitions and collision energies had been adjusted.

2.4. Method optimization and validation

The efficacy of the QuEChERS method depends on several factors, such as the choice of extraction solvent, the sample-to-solvent ratio, the pH of the extraction solution, the type of agitation used, the quantitation of partitioning salts, and the cleaning of sorbents [38]. For instance, the selection of the extraction solvent is crucial because it determines the selectivity and efficiency of the extraction process. Commonly used solvents include acetonitrile, methanol, and ethyl acetate, which have different polarities and properties. The sample-to-solvent ratio also affects extraction efficiency, as using too little or too much solvent can lead to incomplete or excessive extraction. The pH of the extraction solution is another critical factor because it can affect the stability and solubility of the target analytes. Depending on the nature of the analytes, acidic or basic conditions may be necessary to optimize extraction efficiency [39]. The type of agitation used during the extraction process can also influence the extraction efficiency, as it affects the mass transfer of analytes from the sample matrix to the solvent [40]. Common agitation methods include shaking, vortexing, and sonication. Based on the information provided, the QuEChERS extraction technique was applied in two steps, and a design experiment was conducted to optimize the extraction conditions. Methanol was chosen as the solvent for the extraction step due to its ability to mildly precipitate proteins and its miscibility with water. The second step involved using a d-SPE (dispersive solid-phase extraction) process to remove co-extracted components, using sorbents such as PSA and a commercial substance called EMR-lipid, along with MgSO₄ to remove any residual water or salt in the sample [41,42]. To evaluate the effectiveness of the extraction technique, a preliminary assessment was conducted by comparing the amplitude and statistical significance of parameters related to the response of the chromatographic peak regions.

3. Results and Discussion

3.1. Implementation of LC-MS/MS system.

By diffusing clean standard solutions directly into the LC-MS/MS system, the ideal chromatographic and spectrometric conditions for SHC, FHC, and TDH were established. After observing each precursor ion, each sample's two distinct product ions were identified using a range of collision energy voltages, with one of the transitions acting as the qualifier ion and the peak with the second-highest intensity acting as the quantifier ion. The settings were then automatically changed by employing multiple reaction monitoring (MRM) transitions and dwell times. The concentration and pH of the buffer solution, which impact the mobile phase's

composition and the peak shapes of the analytes in chromatography and the analytes' ionization efficiency in MS, are critical factors in enhancing quantitation specificity in addition to the MS settings [43]. When compared to water, the peak's breadth was dramatically reduced by 5 mM ammonium acetate and became more symmetrical. Formic acid (0.1%) significantly improved the signal responses of SHC, FHC, and TDH, respectively. After a series of tests, the mobile phase was selected from methanol, 5 mM ammonium acetate, and formic acid with a ratio of 35:65:0.1 (v/v/v) to ensure consistent sensitivity and high retention of SHC, FHC, and TDH, respectively. Under ideal LC conditions, the retention durations of SHC, FHC, and TDH for saliva were discovered to be 4.349, 4.733, and 4.973, respectively. Using 100 ng/mL of optimized solution in both positive and negative ionization modes, the MS parameters for SHC, FHC, and TDH were tuned. The precursor and product mass ions were measured for SHC, FHC, and TDH. The result of the LC-MS/MS data displayed in Figure 1 illustrates the simultaneous response and retention time of the antidepressant medications in simulated saliva samples at concentrations ranging from 5 to 200 ng/mL. By generating calibration curves from peak area to standard internal ratios of analytes and six concentration levels using the statistical program GraphPad Prism V9.0, it was demonstrated that the proposed approach is linear. The concentration range for the study encompassed the therapeutic concentration for all medications, and the working range was 10 ng/mL. A signal-to-noise ratio of 3:1 and 10:1 was used to evaluate the LOD and LOQ of the LC-MS/MS system. While the R^2 value for linear regression is more than 0.9999, the model adjustment fit is considered to be acceptable.

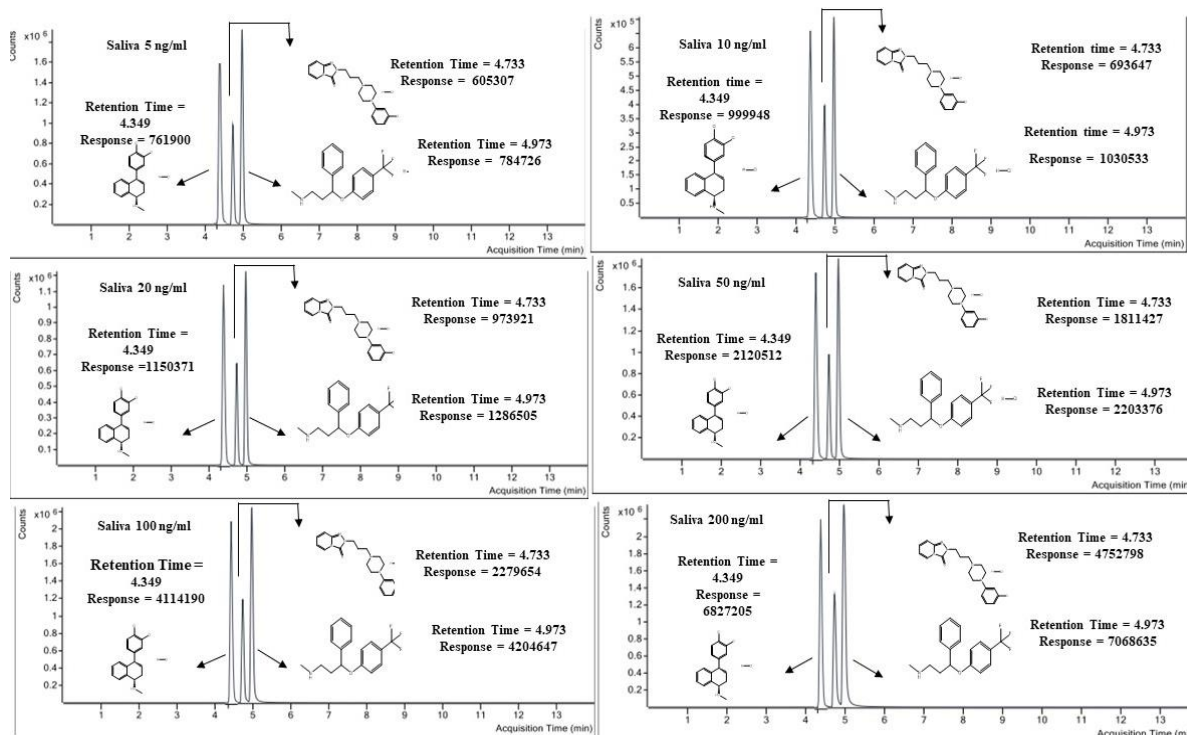


Figure 1. LC-MS/MS chromatogram of simultaneous detection of saliva samples of SHC, TDH, and FHC at six different concentrations (5,10,20,50,100, and 200 ng/ml).

3.2. Method validation.

According to SWGTOX (Scientific Working Group for the Analysis of Seized Drugs), several validation parameters were evaluated, including linearity, limit of detection (LOD), and limit of quantification (LOQ). To assess the linearity, calibration curves were constructed at

six different concentrations to determine the relationship between response and concentration. This information can be used to confirm that the method produces a linear response across a range of concentrations. The LOD and LOQ were also determined as part of the validation process. The LOD is the lowest concentration at which the method can reliably detect a compound, while the LOQ is the lowest concentration at which the method can accurately quantify the compound. These values are important indicators of the sensitivity of the method. Overall, validating a method according to SWGTOX guidelines involves a rigorous process of evaluating multiple parameters to ensure that the method is accurate, precise, and sensitive enough to be used for forensic analysis. The LOD and LOQ values are given in Table 2 and Figure 2, respectively.

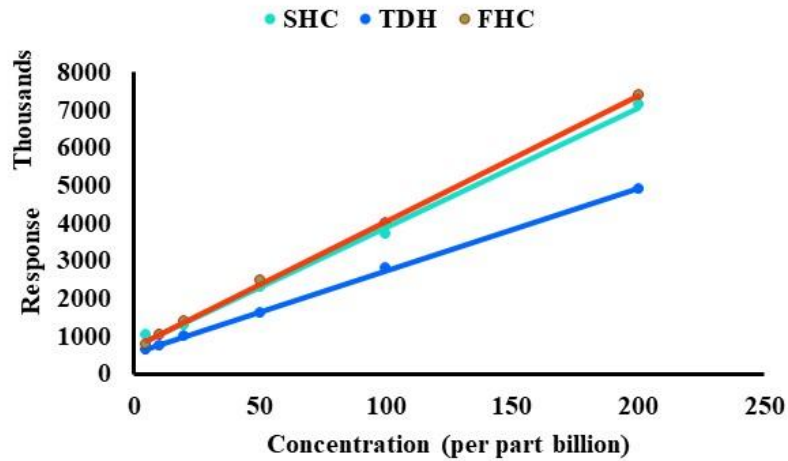


Figure 2. Calibration curve of SHC, TDH, and FHC in a saliva sample.

Table 2. Data of R², Slope, Intercept, LOD, LOQ, Precursor, Production, and RT of SHC, TDH, and FHC.

Drug	Biological matrices	R ²	Slope	Intercept	LOD	LOQ	Precursor	Product ion	RT
SHC	Saliva	0.9949	31591	635238	10.07	29.23	341.053	346.05	4.349
TDH		0.9965	22604	477400	7.06	22.13	407.13	412.13	4.733
FHC		0.9859	22364	483872	6.32	19.08	345.11	348.11	4.973

3.3. Recovery percentage.

To calculate the recovery percentage. Two sets of samples were prepared, one in a blank matrix spiked after extraction (AE) and the other spiked in the blank matrix before extraction [44]. The recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{BE}{AE} * 100 \quad (1)$$

Table 3. The recovery percentage of SHC, TDH, and FHC in the saliva sample.

Concentration (ng/ml)	SHC	TDH	FHC
5	90	73	76
10	83	76	110
20	96	101	93
50	108	110	91
100	94	89	103
200	98	96	94

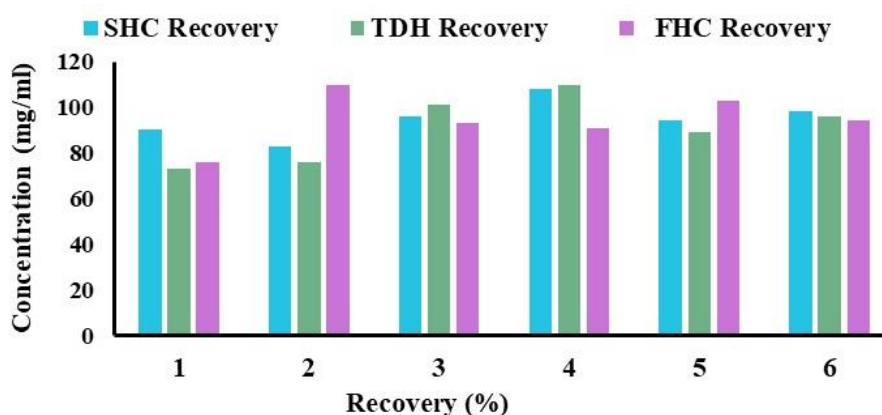


Figure 3. Recovery (%) of saliva at six different concentrations.

It was analyzed that the recovery percentage lies between 73% and 110% at six different concentrations. It was also analyzed that even at low concentration, i.e., 5 ng/ml, it gave good recovery as QuEChERS proved to be effective in providing significant recovery as shown in Table 3 and Figure 3, respectively.

3.4. Interference, carryover, and matrix effect.

The analytes had matrix effects at two different concentration levels: lower quality control (LQC) at 5 ng/L and higher quality control (HQC) at 200 ng/L. It was observed that matrix effects for all analytes for both quality control levels were less than 20%, which is acceptable data and complies with validation criteria.

4. Conclusions

A modified QuEChERS and LC-MS/MS analysis method that can quickly and easily quantify 3 different antidepressants in saliva samples. The method was optimized using a DSPE approach with low sample and solvent consumption. The best conditions were determined to be the use of acetonitrile as a solvent for extraction, a combination of sodium acetate and magnesium sulfate salts for the salting-out effect, vortex or homogenizer agitation, and PSA with magnesium sulfate for sorbent clean-up. The entire method was validated to ensure its specificity and selectivity for these antidepressants. The method was successfully applied to saliva samples and demonstrated its potential as an alternative routine use in forensic laboratories.

Author Contributions

All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

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Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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