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Development of an orbital shaker-assisted fatty acid-based switchable solvent microextraction procedure for rapid and green extraction of amoxicillin from complex matrices: Central composite design

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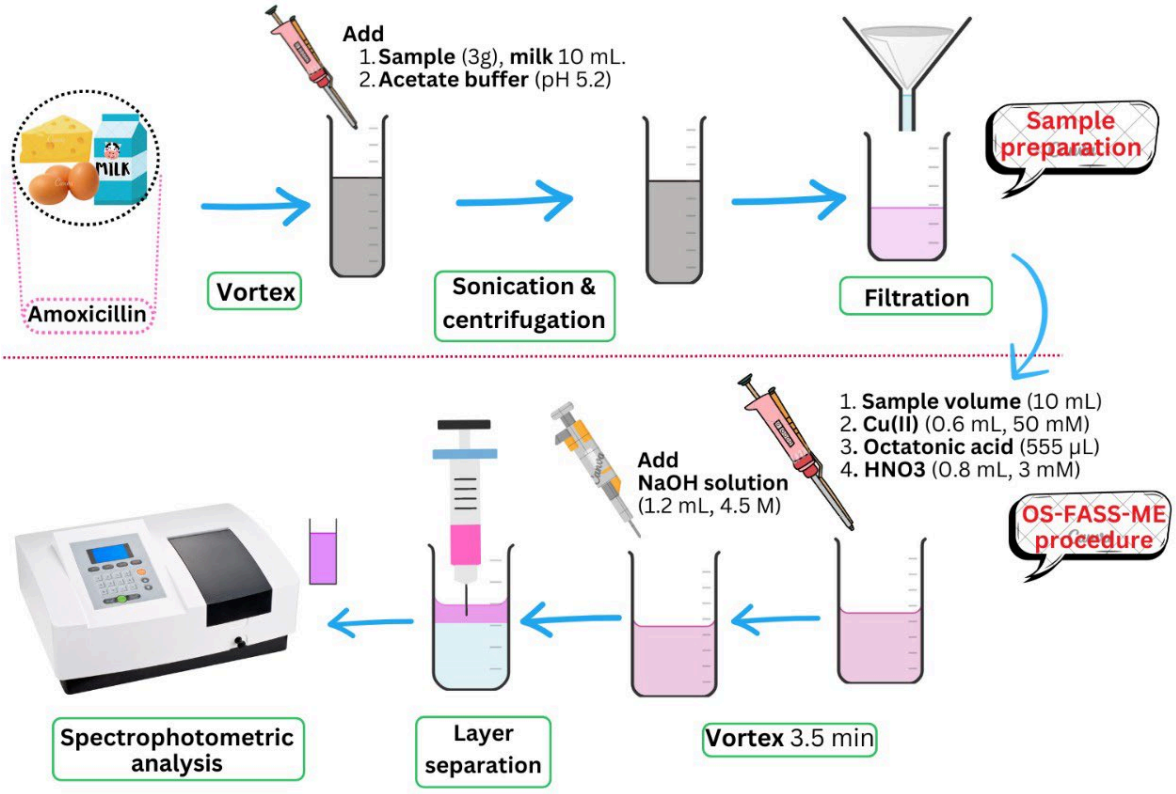
Abstract

In this study, a cheap, fast and simple orbital shaker-assisted fatty acid-based switchable solvent microextraction (OS-FASS-ME) procedure was developed for the extraction of amoxicillin (AMOX) in dairy products, pharmaceutical samples and wastewater prior to its spectrophotometric analysis. Fatty acid-based switchable solvents were investigated for extracting AMOX. The key factors of the OS-FASS-ME procedure were optimized using a central composite design. The linearity of OS-FASS-ME procedure was in the range 5-600 ng mL^{-1} with a correlation coefficient of 0.991. In five replicate experiments for 20 ng mL^{-1} of AMOX solution, the recovery and relative standard deviation were 95.8% and 2.2%, respectively. Limits of detection and quantification were found 1.5 ng mL^{-1} and 5 ng mL^{-1}, respectively. The accuracy, precision, robustness and selectivity of the OS-FASS-ME procedure were investigated in detail under optimum conditions. The OS-FASS-ME procedure was applied to milk, cheese, wastewater, syrups and tablets. A comparison of the results obtained from the reference method and the OS-FASS-ME method showed that the OS-FASS-ME procedure can be successfully applied to complex matrices.



Keywords: Switchable solvent, Amoxicillin, Central composite design, Antibiotic analysis, Microextraction

Highlights

- Fatty acid-based switchable solvents were investigated for extraction of amoxicillin.
- The optimised procedure does not include heating and centrifugation steps.
- The linearity was in the range of 5-600 ng mL^{-1} (R^2:0.991).
- The LOD of the method was 1.5 ng mL^{-1} with an extraction time of 3.5 min.

1. Introduction

Amoxicillin (AMOX) is a commonly used antibiotic antimicrobial agent belonging to the penicillin class and has bactericidal activity (Sodhi et al., 2021). AMOX is used for the treatment of bacterial diseases in living organism. However, amoxicillin can be transferred to the environment and biological specied through various sources and causes environmental

implications (Grenni et al., 2018). Additionally, the AMOX is used in animal food production which may cause some undesirable residues, especially in dairy products (Bacanlı & Başaran, 2019). Additionally, high levels of AMOX in biological fluids cause a variety of adverse effects, such as nausea, vomiting, rashes, and antibiotic-associated colitis (Cunha, 2001). For these reasons, it is important to develop fast and inexpensive methods to monitor trace levels of the AMOX in different food, wastewater and pharmaceutical samples.

In trace analysis of analytes, matrix effect is an important factor, even if sensitive techniques such as high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry are used in the measurement step (Cortese et al., 2020; Van Eeckhaut et al., 2009). To for better selectivity and high sensitivity extraction/preconcentration are performed (López-Lorente et al., 2022). Therefore, the development of environmentally friendly sample preparation methods with high selectivity for selected analyte is important inanalytical chemistry. In microextraction small volume chemicals are used to separate and pre-concentrate the target analyte in leading to low chemical consumption. Liquid-phase microextraction (LPME) (Elik et al., 2021) and solid-phase microextraction (SPE) (Tüzen et al., 2022; Chen et al., 2022) are most commonly used methods for sample preparation methods. The efficiency of the LPME method depends on the selection of suitable extraction solvent. In LPME studies, ionic liquids (Altunay et al., 2020), surfactants (Gürkan & Altunay, 2015), deep eutectic solvents (Haq, Bibi et al. 2022;Altunay et al., 2023), SUPRAS (Altunay & Elik, 2021) and switchable solvent (Altunay, 2022) are the most frequently used solvents for extraction. Among these, switchable solvents are frequently used in the microextraction of organic and inorganic analytes since they can exist in both hydrophobic and hydrophilic forms in the sample solution (Bazel et al., 2020). Switchable solvents are defined as solvents that suddenly

and reversibly change their physical properties. This property occurs as a result of a reversible reaction in response to external stimuli such as temperature change, addition of acid-base, or addition-removal of a gas, and thus the changed solvent can return to its original state (Darabi et al., 2016; Lasarte-Aragonés et al., 2015). Switchable solvents are considered environmentally friendly and have significant separation properties which make them preferable ovet the traditional organic solvents (Musarurwa & Tavengwa, 2021).

Optimization of analytical parameters is need to get high selectivity and sensitivity for targeted analyte. In general, univariate and experimental design (DoE) are used for optimization approaches (Benedetti et al., 2022; Jiang, 2021). The DoE strategy is based on an experimental model in which all variables vary statistically differently. DoE correlation determine the correlation between different variables (Altunay et al., 2021). While the univariate strategy evaluates only linear interactions, the DoE approach introduces a quadratic interaction between parameters (Mousavi et al., 2018). The DoE strategy can determine the optimum values of variables with a small number of experimental runs. Therefore, the use of DoE strategy in the optimization is time saving and cost effective.

In this research, an easy, fast and simple orbital shaker-assisted switchable solvent based microextraction (OS-FASS-ME) procedure was developed for the extraction of AMOX from dairy products, drugs and wastewater. Fatty acid-based switchable solvents were tested for extracting AMOX. Significant parameters of the OS-FASS-ME method were investigated and optimized by experimental design. Absorbance measurments were performed using a double-beam UV-Vis spectrophotometer. The OS-FASS-ME is a simple procedure and does not include any heating or centrifugation steps. In this way, the extraction time is quite short (3.5 min). According to our research, this research is the first report using fatty acid-based switchable

solvents for the extraction of AMOX. The method was found effective in different matrices with high selectivity for AMOX. The OS-FASS-ME method contains significant innovations compared to similar UV-Vis spectrophotometric methods as it does not include additional experimental steps that cause energy consumption, such as heating and centrifugation.

2. Materials and Methods

2.1. Reagents

All reagents used in experimental studies were of analytical grade and no further purification procedures were applied. Ultra-pure water was obtained from a Milli-Q purification system (Millipore, USA). Fatty acids such as decanoic acid (≥99.5%), hexanoic acid (≥99%), octanoic acid (≥99%) and dodecanoic acid (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Base solutions include sodium hydroxide (NaOH), ammonia (NH_3), magnesium hydroxide ($Mg(OH)_2$), and potassium hydroxide (KOH) were obtained from Merk (Darmstadt, Germany). Sulfuric acid (H_2SO_4, ≥98%) and nitric acid (HNO_3, 70%) were purchased from Sigma, while formic acid (HCOOH, ≥96%) and hydrochloric acid (HCl, 37%) were purchased from Merck. Appropriate concentrations of base and acid solutions were prepared in the water. A reagent of amoxicillin (AMOX) was purchased from Merck. A stock solution of AMOX (100 mg L^{-1}) was obtained by dissolving the appropriate amount of its reagent in water at room temperature (~25 ^oC). Working and extraction solutions of AMOX were prepared by successive daily dilutions of the stock solution.

2.2. Equipment

Preparation of the real samples was achieved using an SK5210LHC model ultrasonic bath (Shanghai Kedao Ultrasonic Instrument Co., Ltd., Shanghai, China). The mixing step was provided by an Multi Bio RS-24 model orbital shaker (BioSan, ProfiLab24 GmbH, Landsberger

Berlin, Germany). A double-beam UV–visible spectrophotometer (UV-1800, Shimadzu, Tokyo, Japan) was used for measuring of analytical signal of AMOX in the final solution. Design-Expert software version 12.0.1 (Stat-Ease, Minneapolis) was used for chemometric analyses and ANOVA.

2.3. Samples and sample preparation

In this study, real sample were prepared and used for extraction of AMOX using OS-FASS-ME. Dairy products including milk, egg and cheese were collected from dairies in Sivas/Türkiye. Waste water was collected from the industrial area in Sivas. Tablet and syrup samples containing the AMOX were purchased from the pharmacy in Sivas. The waste-water samples were filtered using a membrane filter before applying the extraction procedure. The extraction procedure was applied directly to the syrup samples without applying any sample preparation steps. 1 g of the tablet samples was dissolved in 100 mL of water, and then the extraction procedure was applied to this solution. Sample preparation steps applied to dairy products are as follows (Igualada et al., 2022). First, cheese and egg samples were homogenized using a vortex. Then, the milk (10 mL), cheese (3 g), and egg (3 g) samples were added to centrifuge tubes containing 10 mL of acetonitrile. Then, the pH of the mixture was adjusted at pH=5.2 with a 0.2 M acetate buffer solution. The samples were placed in the ultrasonic bath and sonicated for 10 min. Following the centrifugation step at 4000 rpm for 5 min, the resulting solution was filtered using a membrane filter of 0.45 µm (Gelman Sciences, Ann Arbor, MI, USA). In the final stage, the OS-FASS-ME procedure was applied to the final solution for selective extraction of AMOX

2.4. Optimization strategy

In microextraction studies, experimental design is the most frequently used approach for the effective and rapid optimization of important factors. In this study, a four-variable five-level central composite design (CCD) was applied for the optimization of the OS-FASS-ME procedure. The optimized parameters were orbital shaking time (X_1), octanoic acid volume (X_2), HNO_3 amount (X_3) and NaOH amount (X_4). The ranges tested for parameters X_1, X_2, X_3 and X_4 were 3-18 min, 200-800 µL, 2-12 mol L^{-1} and 3-15 mol L^{-1}, respectively. In addition to these ranges, experiments were carried out at starting points (±1.2 α) selected above and below the lowest and upper limits of each parameter. A total of 30 extraction studies were performed and 6 of them were centre studies. The CCD details for the parameters were presented in the electronic file Table S1.

2.5. OS-FASS-ME procedure

The OS-FASS-ME procedure for the extraction of AMOX from the collected samples includes the following experimental steps. First, 10 mL of sample solution was added to the centrifuge tubes. 0.6 mL of 50 mmol L^{-1} Cu(II) solution was added to form the complex of AMOX in the sample solution. Following this, 555 µL of octatonic acid (as fatty acid-based switchable solvent) was added to extract the AMOX-Cu complex from the sample solution. For the octatonic acid in the sample solution to break down the fatty acids and provide hydrophilicity, 3 mmol L^{-1} of HNO_3 solution (0.8 mL) was added to the resulting mixture and vortex was applied for 3.5 min. At this stage, a cloudy solution was obtained. In the last step, 1.2 mL of NaOH solution (4.5 mol L^{-1}) was added to the sample solution to restore the hydrophobicity of the octatonic acid in the solution. In this step, the AMOX-Cu complex along with the octatonic acid was collected on top of the sample solution. The octatonic acid phase

containing the AMOX was separated from the sample solution using a syringe and transferred to the micro cuvettes. In the measurement step, the micro cuvettes were placed in a dual-beam UV-Vis spectrophotometer. Absorbance measurements were performed at 305 nm.

3. Results and Discussion

3.1. Preliminary experiments

To ensure selective and rapid extraction of the AMOX, the extraction efficiency of the method should be maximum. To achieve this, preliminary experiments were carried out to select important parameters for the optimization step by CCD. Recovery % was taken as a reference for the selection of parameters. Recovery % was calculated according to the formula below.

Recovery % = 100 x [Amount of AMOX in solution after extraction]/[Amount of AMOX added to solution]

3.1.1. Selection of type of extraction solvent

The main purpose of microextraction studies is to extract the target analyte from the sample solution quickly, selectively and safely. The key factor to achieve this goal is the selection of suitableextraction solvent. In general, the interaction between the extraction solvent and the analyte must be high for the analyte to be easily separated from the sample solution. In recent years, the use of fatty acids as green switchable solvent are getting great attention due to its ecofriendly nature, high selevtivity and easy preparation and applications,. For the OS-FASS-ME procedure, fatty acid-based switchable solvents including decanoic acid, hexanoic acid, octanoic acid and dodecanoic acid were tested as extraction solvents. According to the results in Figure 1a, the best recovery of AMOX was obtained using octanoic acid. Based on these results, octanoic acid was chosen as the extraction solvent for the CCD step.

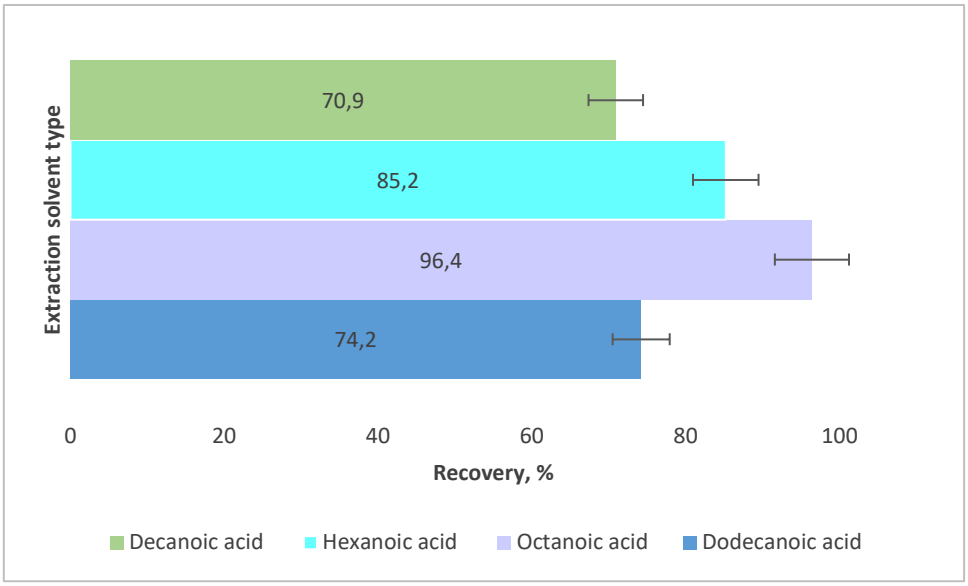


Figure 1a. Effect of type of extraction solvent on the recovery of AMOX

3.1.2. Selection of type of base solution

Chain fatty acids switch between hydrophobic and hydrophilic forms in the sample solution depending on pH. In microextraction studies using fatty acid-based switchable solvents, base solutions are used to dissolve the extraction solvent in the sample solution. When a base solution is added to fatty acids, they can be converted into their hydrophilic forms. As a result, fatty acids decompose and a homogeneous solution is formed in the sample solution.. Therefore, the effect of base solutions such as NaOH, NH_3, $Mg(OH)_2$, and KOH on the recovery of AMOX was investigated. The results in Figure 1b show that the most suitable base solution for the quantitative extraction of AMOX is NaOH. The recovery of AMOX was not quantitative when the weak base NH_3 was used. The probable reason for this is that the NH_3 solution cannot provide the degradation necessary for the fatty acid to gain hydrophilicity. Based on these results, NaOH was chosen as the base solution solvent for the CCD step

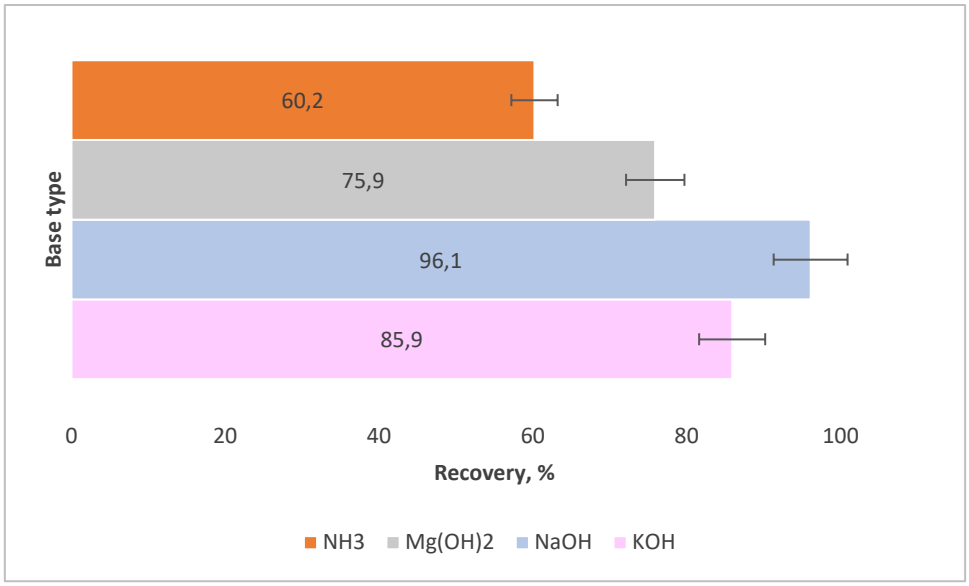


Figure 1b. Effect of type of base solution on the recovery of AMOX

3.1.3. Selection of type of back-extraction acid

In microextraction studies using fatty acids, adding acid solution to the homogeneous solution obtained by adding base solution transforms the fatty acid in the sample solution into a hydrophobic form. In this way, the hydrophobic fatty acid containing the analyte is separated from the aqueous solution. To achieve this, the acid solutions used are called back-extraction acid solutions. The effect of HCOOH, H_2SO_4, HNO_3 and HCl on the recovery of AMOX was investigated as a back-extraction acid solvent. According to Figure 1c, the best recovery of AMOX was obtained using HNO_3 solution. Additionally, it was observed that quantitative phase separation could not be achieved when weak acid (HCOOH) was used. Based on these results, HNO_3 was chosen as the back-extraction acid solvent for the CCD step.

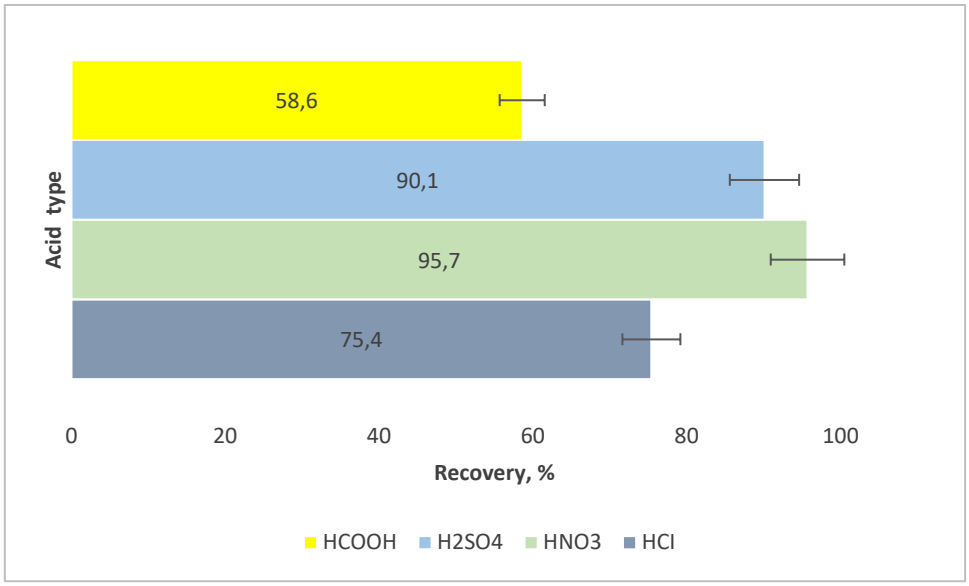


Figure 1c. Effect of type of acid solution on the recovery of AMOX

3.1.4. Effect of amount and type of metal ions

Forming a hydrophobic complex of AMOX accelerates its quantitative and selective extraction in the sample solution. To achieve this, the complex formation of AMOX with some metal ions (Fe(III), Zn(II) and Cu(II)) was investigated. The aim of using these metal ions is to form complexes of amoxicillin in the sample solution and then extract the formed complex into the extraction phase. Secondly, Fe(III), Zn(II) and Cu(II) are non-toxic and frequently available in the form of different salts. As a result of the studies carried out in the literature, metal ions can form complexes with amoxicillin with low toxicity. In addition, these metal ions form colored complexes and could be easily analyzed using a spectrophotometer. Based on the results in Figure 1d, Cu(II) ion was chosen to complex AMOX. For the complexation of AMOX with the Cu(II) ion to be completed, the amount of Cu(II) ions added to the sample solution must be sufficient. Therefore, the effect of the volume of Cu(II) solution (50 mmol L^{-1}) on the recovery of AMOX was investigated in the volume range of 0-2 mL. Experimental studies showed that the highest recovery was achieved at 0.6 mL Cu(II) solution, and recovery was

stable at volumes above this volume. Therefore, 0.6 mL of 50 mmol L^{-1} Cu(II) solution was used in subsequent studies.

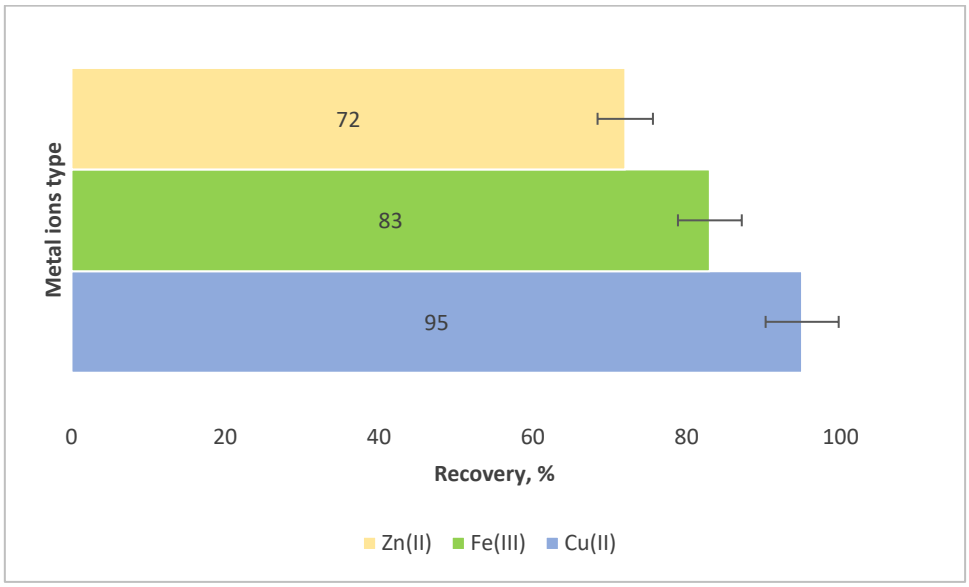


Figure 1d. Effect of type of metal ions on the recovery of AMOX

3.2. Central composite design

The experimental steps designed by CCD for the extraction of AMOX were carried out in three replicates. The average recovery obtained for each run was given in the electronic file Table S2. These obtained data were processed by CCD and ANOVA analysis (see electronic file Table S3) was performed. According to ANOVA, for a variable to be significant for the established model, its p-value must be less than 0.05 at the 95% confidence level. Analyzing electronic file Table S3 based on this explanation, all parameters including linear, quadratic and binary (p-value < 0.05) are significant for the recovery of AMOX. Another parameter evaluated in ANOVA analysis is the F-value. F-value explains the degree of influence of factors on the extraction step. As the F-value increases numerically, its effect on the extraction step also increases. Based on these explanations, the linear, binary and quadratic factors that had the highest impact on the recovery of AMOX were X_1 (F: 168.01), X_1X_4 (F: 316.84) and X_3 (F: 479.28), respectively.

The results in Table 1 showed that the quadratic model was suitable for the recovery of AMOX. According to the quadratic model, the p-value of CCD was < 0.0001. Moreover, the impact of uncertain errors on the recovery of AMOX is evaluated based on the Lack of Fit p-value. The Lack of Fit p-value was 0.6887. This result indicates that uncertain errors are insignificant for the recovery of AMOX. The reliability of the results obtained depends on adjusted-R^2 and predicted-R^2. As these values approach 1.0, the reliability of the results increases. Additionally, the difference between the two values should not be more than 0.2. The adjusted-R^2 (0.9903) and predicted-R^2 (0.9799) results supported these explanations. As a result of all these explanations, the quadratic model explained the effect of factors on the recovery of AMOX according to the equation below.

$$\text{Recovery, \%} = 77.76 - 2.98X_1 + 1.36X_2 + 1.48X_3 - 1.81\ X_4 + 2.18\ X_1X_2 + 3.03X_1X_3 + 4.45X_1X_4 - 1.04\ X_2X_3 - 2.02X_2X_4 + 3.78X_3X_4 + 0.9365X_1^2 - 4.69X_2^2 + 9.62X_3^2 - 4.51X_4^2$$

Table 1. Fit parameters of the CCD for the OS-FASS-ME procedure

Source	Sequential p-value	Lack of Fit P-value	Adjusted R^2	Predicted R^2	
Linear	0.2832	< 0.0001	0.0447	-0.2825	
2FI	0.0045	0.0001	0.4910	0.5542	
Quadratic	**< 0.0001**	**0.6887**	**0.9903**	**0.9799**	**Suggested**
Cubic	0.8713	0.2871	0.9860	0.8022	

Surface response graphs were drawn to evaluate the effect of the binary interactions of the parameters on the recovery of AMOX. The effect of NaOH concentration and octanoic acid volume on the recovery of AMOX was determined (see electronic file Figure S1a), the recovery was found maximum in almost all octanoic acid volumes when the NaOH concentration was lower than 9 mol L^{-1}. NaOH was used to break down the fatty acids bound to octanoic acid and transition it to the hydrophilic form. These results showed that a NaOH concentration of less than 9 mol L^{-1} was sufficient for effective extraction. The effect of HNO_3 concentration and octanoic acid volume on the recovery of AMOX is presented in the electronic file Figure S1b.

Nitric acid was used to restore the hydrophobicity of octanoic acid, which converted to hydrophilic form, and to separate it from the aqueous solution. When the HNO_3 concentration was greater than 4 mol L^{-1}, the recovery of AMOX was low at almost all octanoic acid volumes. Hydrophobicity could not be achieved because high HNO_3 concentrations disrupted the structure of fatty acids. The effect of orbital shaking time and NaOH concentration on the recovery of AMOX was presented in the electronic file Figure S1c. After adding NaOH to the sample solution, orbital shaking was applied to increase its interaction with the extraction solvent. According to the results, good recoveries were obtained at 4.5-9.0 mol L^{-1} NaOH concentrations using orbital shaking over 3 min.

According to CCD, the theoretical recovery of AMOX was 92.9% using orbital shaking time (3.5 min), octatonic acid volume (555 µL), HNO_3 concentration (3 mol L^{-1}), NaOH concentration (4.5 mol L^{-1}). As a result of five repeated measurements under these conditions, the average recovery was calculated as 91.6%. Since no significant difference was observed between theoretical recovery and experimental recovery, these values were selected as optimum for the parameters in the validation and application steps.

3.3. Validation and application of the OS-FASS-ME procedure

Various analytical figures of merit of the OS-FASS-ME procedure, including linear range, correlation coefficient (R^2), limit of detection (LOD) and quantification (LOQ), recovery, and relative standard deviation (RSD), enrichment factor (EF), selectivity, robustness, precision and accuracy were determined using optimized conditions (3.5 min of orbital time, 555 µL of octatonic acid volume, 3 mol L^{-1} of HNO_3 concentration, 4.5 mol L^{-1} of NaOH concentration).

3.3.1. Linearity, LOD, LOQ and EF

Analytical and validation parameters including linearity range, LOD, LOQ, RSD, recovery, and EF were estimated for model samples with and without applying the OS-FASS-ME procedure. Different concentrations of standard AMOX solution were added to model samples, the linearity range for OS-FASS-ME procedure was in the range of 5-600 ng mL^{-1} (R^2:0.991). Without the OS-FASS-ME the linearity range was 1000-15000 ng mL^{-1} (R^2:0997), respectively. LOD (1.5 ng mL^{-1}) and LOQ (5.0 ng mL^{-1}) were calculated from the ratio of 3 and 10 times the standard deviation obtained from ten replicate analyzed of blank solutions to the slope of the calibration graphs, respectively. From 5 replicate studies for 20 ng mL^{-1} concentration of AMOX solution, RSD and recovery were calculated as 2.2% and 95.8%, respectively. The EF was calculated as 146 from the ratio of the slopes of the calibration curves with and without the OS-FASS-ME procedure. Analytical data are presented in Table 2.

Table 2. Analytical figures of merit of the OS-FASS-ME procedure.

Figures	With the OS-FASS-ME procedure	Without the OS-FASS-ME procedure
LR, ng mL^{-1}	5–600	1000-15000
R^2	0.991	0.997
LOD, ng mL^{-1}	1.5	303
LOQ, ng mL^{-1}	5.0	1000
RSD,% (for 20 ng mL^{-1}, N=5)	2.2	-
Recovery, % (for 20 ng mL^{-1})	95.8	-
EF	146	--

LR: Linear range; EF: Enrichment factor; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation; R^2: Correlation coefficient

3.3.2. Selectivity

With optimized conditions for analytical method there are still chances of possible interferences of chemical species present in foods, water drug samples that could affect the extraction of AMOX. In light of these facts, the tolerance limits of some species that may affect the extraction recovery of AMOX in the presence of these species were investigated. The

investigated species and the obtained data are presented in Table 3. In this study, the investigated species were added to model samples with different concentrations and the OS-FASS-ME procedure was applied for the extraction of AMOX (100 ng mL^{-1}). The tolerance limit is the concentration corresponding to a ±5% change in the analytical signal of AMOX of the investigated species. The data in Table 3 show that the method exhibits high tolerance limits even in the presence of species with similar chemical structures. In addition, the recovery values were at acceptable levels (99.4-91.2%). These results show that the optimized method was selective for AMOX.

Table 3. Effect of some chemical species on the recoveries of AMOX

Species	Concentration, mg L^{-1}	Recovery ± SD (%)
NO_3^-	15000	99.4±3.1
F^-	15000	99.2±3.2
PO_4^{3-}	15000	98.6±3.7
Mg^{2+}	10000	98.5±2.5
SO_4^{2-}	10000	98.8±2.9
Ca^{2+}	5000	98.1±3.1
Fe^{2+}	2500	97.9±2.7
Pb^{2+}	1500	97.6±3.8
Sn^{2+}	1500	96.2±3.3
Ni^{2+}	750	96.7±2.7
Co^{2+}	750	95.9±2.4
Cephalexin	100	95.8±3.3
Levofloxacin	100	95.2±2.9
Lansoprazole	50	94.7±2.4
Clavulanic acid	30	91.2±3.8

3.3.3. Robustness

The robustness of an analytical method refers to its ability to remain unaffected by small variations in experimental parameters. Robustness of the method was tested by comparing the results obtained for the target analyte using optimized conditions with minor modification. In this study, the effect of the variables on the extraction of AMOX was investigated with a ±10% change in the optimized conditions. The minor changes for the parameters and the results obtained by the OS-FASS-ME procedure applied in these changes

are given in the electronic file Table S4. The data in the electronic file Table S4 show that the RSD and recovery values obtained as a result of the changes made to the optimized conditions were acceptable. Additionally, the largest difference in RSD and recovery was obtained with the change in orbital duration. Already, ANOVA results have shown us that the parameter that has the most impact on the extraction of AMOX is the orbital shaking time.

3.3.4. Precision

The precision of the OS-FASS-ME procedure was investigated with intraday and inter-day studies. In these studies, three levels of standard solutions of AMOX were spiked into the model solutions. In the intraday study, the OS-FASS-ME procedure was applied to five replicates samples per day, while in the interday study, the method was applied in five replicates in three consecutive days. The interday and intraday precision experiments were performed for 25, 200 and 500 ng mL^{-1} concentrations of AMOX solution. The RSDs of AMOX solutions for intraday and interday studies were in the range of 2.1-2.9% and 2.4-3.3%, respectively. These calculated results showed that the OS-FASS-ME procedure exhibited good precision. Analytical data are given in the electronic file Table S5.

3.3.5. Accuracy

The accuracy of the OS-FASS-ME procedure was tested by standard addition method. In this context, 25, 200 and 500 ng mL^{-1} concentrations of AMOX were added to milk, egg, cheese, wastewater, syrup and tablets. Then, the OS-FASS-ME procedure was applied separately to the samples added for each concentration. The recoveries are given in Table 5. Quantitative recoveries obtained as a result of the application of three concentration levels of AMOX solution supported the accuracy of the OS-FASS-ME procedure. Moreover, these results mean that the OS-FASS-ME procedure exhibits a low matrix effect in the analysis of real samples.

Table 4. Accuracy of the OS-FASS-ME method in real samples

Samples	Recovery values (%) of added AMOX amount		
	Recovery for 25 ng mL^{-1}	Recovery for 200 ng mL^{-1}	Recovery for 500 ng mL^{-1}
Milk-1	94±3	96±3	97±2
Milk-2	95±4	97±3	98±3
Egg	91±3	94±4	94±4
Cheese	96±5	97±5	96±3
Wastewater	92±4	93±3	95±3
Syrup-1	97±2	95±4	97±4
Syrup-2	96±4	98±3	98±2
Tablet-1	95±6	95±4	97±4
Tablet-2	92±6	95±5	96±3
Tablet-3	94±4	96±3	98±3

3.3.6. Analysis of real samples

Following the necessary validation studies, the applicability of the OS-FASS-ME procedure was investigated for the extraction of AMOX from milk, egg, cheese, wastewater, syrup and tablets samples. After the microextraction step, the amount of AMOX in the final solution was determined using a UV-Vis spectrophotometer. To test the reliability of the results calculated with the OS-FASS-ME procedure, a refrence method (Hamran & Khudhair, 2020) was used to evaluate the validity of the new method. The reference method is based on the formation of a dense yellowish brown complex by the interaction between the analytical reagent and AMX in ethanol at pH 4.06 and subsequent extraction of this complex to the Triton x-114 phase at 40 °C. Finally, spectrophotometric analysis was performed at 415 nm. The results obtained from the two methods are presented in Table 5. The reliability of the results was investigated by applying a student t-test at a 95% confidence level. Here, the t-critical for the four degrees of freedom at the 95% confidence level was 2.78. All t-exp values obtained from the analysis of samples were smaller than the t-critical. These results show us that there is no significant difference between the results obtained with both methods and that the OS-FASS-ME procedure we have developed can be safely applied to real samples.

Table 5. Analysis results obtained from real samples using OS-FASS-ME method (t-tab=2.78, P = 0.05; SD=4)

Samples	OA-FASS-ME method	Reference method	*t-exp
Milk-1	n.d**	n.d	-
Milk-2	n.d	n.d	-
Egg	n.d	n.d	-
Cheese	n.d	n.d	-
Wastewater	n.d	n.d	-
Syrup-1	187±7***	193	1.07
Syrup-2	242±8	247	1.45
Tablet-1	381±14	396	1.61
Tablet-2	488±22	493	0.94
Tablet-3	861±37	884	1.35

*$t_{exp} = (x_o - x_r)/ S_{pooled} \times [(n_1 + n_2)/n_1 \times n_2]^{1/2}$ and $S_{pooled} = [(n_1-1)\, S_o^2 + (n_2-1)\, S_r^2 /(n_1 + n_2-2)]^{1/2}$

**could not be determined

***Mean ± SD

Syrup-1 (200 mg AMOX), Syrup-2 (250 mg AMOX), Tablet-1 (400 mg AMOX), Table-2 (500 mg AMOX), Table-3 (875 mg AMOX)

3.4. Comparison with literature

Results and analytical parameters of the OS-FASS-ME procedure were compared to different extraction and analytical methods reported in the literature (Gebretsadik et al., 2023;Soleimanirad et al., 2022; Sürücü et al., 2022; Shirani et al., 2021; Shirani et al., 2020; Hamran & Khudhair, 2020; Zare Khafri et al., 2019; Pirsaheb et al., 2019; Buszewski et al., 2011). The comparison parameters included linearity range (LR), LOD, RSD%, extraction time and EF. Comparison data are presented in the electronic file Table S6. The OS-FASS-ME procedure has a wide linearity range compared to other extraction procedures. Also, the RSD (2.9%) is the lowest. Since the OS-FASS-ME procedure does not include heating and centrifugation steps, the extraction time (3.5 min) is shorter than other procedures. Compared to HPLC techniques, which are more sensitive than spectrophotometry, the LOD (1.5 ng mL^{-1}) and EF (146) values obtained from the method were comparable. Furthermore, the method does not required any complicated instrumentation or analytical tools.

4. Conclusions

An orbital shaker-assisted fatty acid-based switchable solvent microextraction (OS-FASS-ME) followed by spectrophotometric detection was developed and successfully used for the analysis of AMOX in milk, egg, cheese, wastewater, syrup and tablets. Key parameters that could affect the OS-FASS-ME procedure were investigated and optimized using a central composite design (CCD). The method was found highly sensitive with LOD=1.5 ng mL^{-1} and LOQ=5.0 ng mL^{-1}. Spike experiments and interday/intraday experiments show that OS-FASS-ME procedure is accurate and precise. Acceptable recovery results (94-98%) and low RSD values (≤3.3%) were obtained. The OS-FASS-ME procedure was successfully used for the fater, reliable and accurate determination of AMOX in the real samples. The analysis results of these samples were validated by comparing the results with the reference method. The results obtained showed that the OS-FASS-ME procedure can be safely applied for the extraction of AMOX in a wide range of products, such as pharmaceuticals, waters, and foods.

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Development of an orbital shaker-assisted fatty acid-based switchable solvent microextraction procedure for rapid and green extraction of amoxicillin from complex matrices: Central composite design

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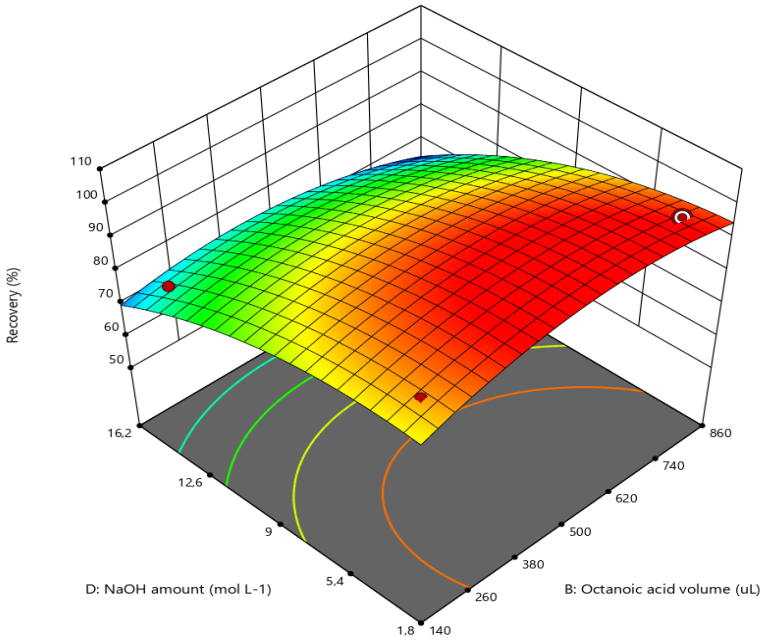

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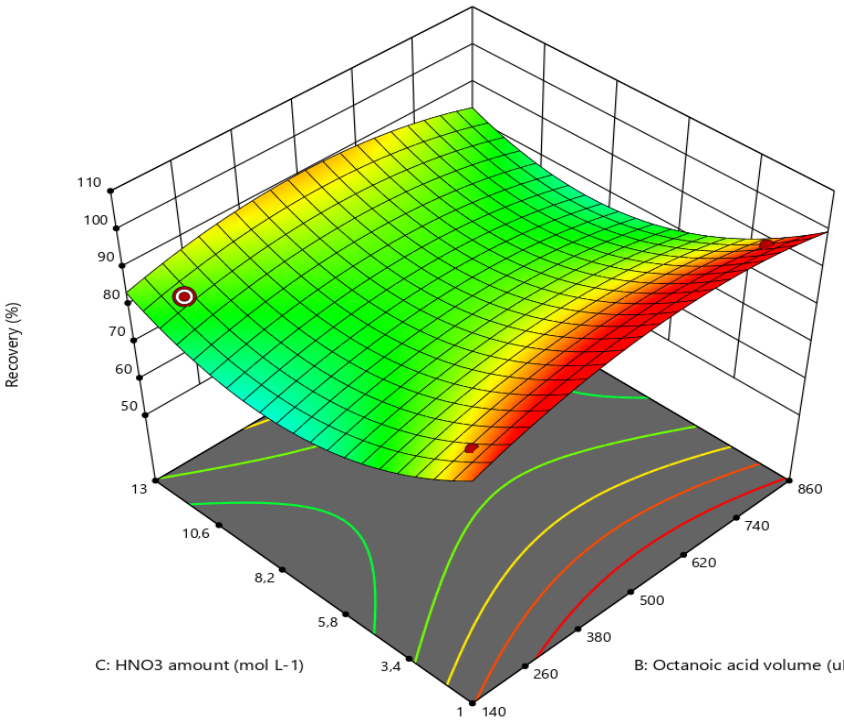

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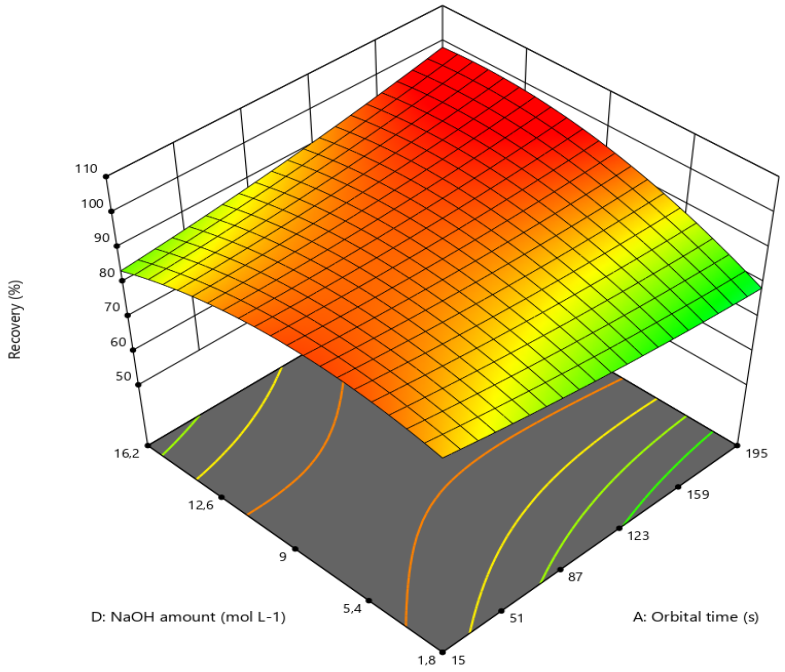

(c)

Figure S1. The effect of binary interactions of variables on the recovery of AMOX (a) NaOH volume and octanoic acid volume, (b) HNO_3 amount and octanoic acid volume, (c) NaOH amount and orbital time

Table S1. Optimization design of key parameters of the OS-FASS-ME procedure with CCD

Variables	Units	Abbreviation	Levels of variables				
			-α	-1	0	+1	+α
Orbital time	min	X_1	1.5	3	10.5	18	19.5
Octanoic acid volume	µL	X_2	140	200	500	800	860
HNO_3 amount	mol L^{-1}	X_3	1	2	7	12	13
NaOH amount	mol L^{-1}	X_4	1.8	3	9	15	16.2

Table S2. Experimental plan for optimization of the OS-FASS-ME procedure

Experimental	X_1	X_2	X_3	X_4	Recovery (%)
1	19.5	500	7	9	75.2
2	18	800	12	3	78.6
3	10.5	500	1	9	89.2
4	10.5	500	13	9	93.3
5	18	200	12	15	86.7
6	18	200	2	3	65.9
7	10.5	860	7	9	72.8
8	18	200	2	15	68.1
9	10.5	500	7	9	77.2
10	3	800	12	3	82.7
11	18	800	2	3	79.6
12	10.5	500	7	16,2	68.9
13	10.5	500	7	9	79.2
14	10.5	500	7	9	78.1
15	10.5	140	7	9	68.5
16	3	200	2	3	91.5
17	3	200	2	15	76.0
18	18	200	12	3	70.2
19	1.5	500	7	9	82.3
20	3	800	2	3	96.4
21	10.5	500	7	9	77.8
22	10.5	500	7	9	78.9
23	18	800	2	15	73.3
24	3	800	2	15	71.1
25	3	200	12	3	83.2
26	3	200	12	15	81.5
27	10.5	500	7	9	77.2
28	3	800	12	15	74.9
29	18	800	12	15	87.1
30	10.5	500	7	1,8	72.9

Table S3. ANOVA data of the OS-FASS-ME procedure

Source	Sum of Squares	F-value	p-value	
Model	1727.85	212.40	< 0.0001	significant
X_1-Orbital time	168.01	289.13	< 0.0001	
X_2-Octanoic acid volume	35.15	60.49	< 0.0001	
X_3-HNO_3 amount	41.29	71.06	< 0.0001	
X_4-NaOH amount	61.95	106.62	< 0.0001	
X_1X_2	75.69	130.26	< 0.0001	
X_1X_3	146.41	251.97	< 0.0001	
X_1X_4	316.84	545.27	< 0.0001	
X_2X_3	18.49	31.82	< 0.0001	
X_2X_4	65.61	112.91	< 0.0001	
X_3X_4	228.01	392.40	< 0.0001	
X_1^2	4.54	7.82	0.0136	
X_2^2	113.92	196.05	< 0.0001	
X_3^2	479.28	824.83	< 0.0001	
X_4^2	105.64	181.80	< 0.0001	

Table S4. Robustness of the OS-FASS-ME procedure

Variables	Optimum value	Minor changes, ±10%	RSD, %	Recovery, %
Orbital time	3.5 min	3.9 min	2.9	91±3
		3.2 min	2.7	92±5
Octanoic acid volume	555 µL	610 µL	2.1	94±6
		500 µL	2.2	93±7
HNO_3 amount	3.0 mol L^{-1}	3.3 mol L^{-1}	2.0	94±3
		2.7 mol L^{-1}	2.2	96±6
NaOH amount	4.5 mol L^{-1}	5 mol L^{-1}	1.9	95±7
		4 mol L^{-1}	1.7	93±6

Table S5. Precision of the OS-FASS-ME procedure for intra/inter day studies

Studies	Spiked, the AMOX	RSD, %	Recovery, %
Intraday (N=5)	25 ng mL^{-1} (Low)	2.1	96±2
	200 ng mL^{-1} (Medium)	2.6	97±5
	500 ng mL^{-1} (High)	2.9	97±4
Interday (N=5x3)	25 ng mL^{-1} (Low)	2.4	94±5
	200 ng mL^{-1} (Medium)	3.0	95±5
	500 ng mL^{-1} (High)	3.3	91±6

Table S6. Comparison of the OS-FASS-ME procedure with other reported methods

Samples	Extraction procedure	LR, ng mL^{-1}	LOD, ng mL^{-1}	RSD,%	EF	Time, min	References
Drug, Milk, Cheese, waters	OS-FASS-ME	5-600	1.5	2.9	146	3.5	This study
Drug	-	50-300	17	1.2	-	15	(Gebretsadik et al., 2023)
Urine and hair	DMSPE	3.5-1000	0.6	4.9	250	32	(Soleimanirad et al., 2022)
Milk	CPE	10-900	2.98	4.2	42.1	55	(Sürücü et al., 2022)
Chicken, egg, honey	USA-DLLME-SFO	12-750	2.8	4.0	31.3	7	(Shirani et al., 2021)
Milk	UA-MDSPE	2.5-750	0.5	3.1	44	9	(Shirani et al., 2020)
Drug	CPE	25-6500	45	-	-	20	(Hamran & Khudhair, 2020)
Pharmaceuticals and water samples	HF-LPME	0.5–10	0.2	7.3	240	30	(Zare Khafri et al., 2019)
Hospital sewage	VALPME–SDES	3-600	1	3.8	164	15	(Pirsaheb et al., 2019)
Plasma	SPME	1-20	1.21	5.9	-	15	(Buszewski et al., 2011)

CPE: Cloud point extraction; DMSPE: Dispersive micro solid-phase extraction; SPME: Solid phase microextraction; VALPME–SDES: Vortex-assisted liquid-phase microextraction based on the solidification of the deep eutectic solvent; HF-LPME: Hollow fiber liquid-phase microextraction; USA-DLLME-SFO: Ultrasound-assisted dispersive liquid–liquid microextraction based on solidification of floating organic drop; UA-MDSPE: ultrasound-assisted magnetic dispersive solid-phase extraction;

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