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Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector

--Manuscript Draft--

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Abstract:	A new analyte separation and preconcentration method for the trace determination of antidepressant drugs, Fluoxetine (FLU) and Citalopram (CIT) in urine and wastewaters, was developed based on HPLC-DAD analysis after magnetic solid phase extraction (MSPE). In the proposed method, FLU and CIT were retained on the newly synthetized magnetic sorbent (Fe 3 O 4 @PPy-GO) in the presence of buffer (pH 10.0) and then were desorbed into a lower volume of acetonitrile prior to the chromatographic determinations. Before HPLC analysis, all samples were filtered through a 0.45 µm PTFE filter. Experimental parameters such as interaction time, desorption solvent and volume, and pH were studied and optimized in order to establish the detection limit, linearity, enrichment factor and other analytical figures of merit under optimum operation conditions. In the developed method, FLU and CIT were analyzed by diode array detector at the corresponding maximum wavelengths of 227 and 238 nm, respectively, by using an isocratic elution of 60% pH 3.0 buffer, 30% acetonitrile, and 10% methanol. By using the optimum conditions, limit of detections for FLU and CIT were 1.58 and 1.43 ng mL -1 , respectively, while the limit of quantifications was 4.82 and 4.71 ng mL -1 , respectively. Relative standard deviations (RSD%) for triplicate analyses of model solutions containing 100 ng mL -1 target molecules were found to be less than 5.0%. Finally, the method was successfully applied to urine (both simulated and real healthy human) and wastewater samples, and quantitative results were obtained in recovery experiments.

Dear Editor;

I would like to re-submit the revised manuscript entitled with ‘‘***Sensitive determination of Fluoxetine and Citalopram antidepressants in simulated Urine and wastewater samples by HPLC-DAD”*** by considering reviewer comments in J. Chromatography A.

All comments of reviewer have been replied point by point and detailed new explanations were added as attached file. Some comments of reviewers were about characterization magnetic material. New explanations were added to related section by helping our two colleagues. So, their names were added to revised version of manuscript as 6th and 7th authors.

Thank you for your very valuable contributions .

I am looking forward to hearing from you.

With my best regards,

April, 2021

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Manuscript number: JCA-21-197
Manuscript title: Sensitive determination of Fluoxetine and Citalopram antidepressants in simulated Urine and wastewater samples by HPLC-DAD

Dear Editor;

The authors would like to thank you and the reviewers for valuable comments about the current manuscript.

The authors checked all sections and comments systematically. Corrected or changed sentences were highlighted in red color. All responses to reviewer were submitted in below

Editor's comment

The paper needs a major revision especially considering the report of reviewer #2.
Dear Editor, thank you for your contributions and comments. We carefully checked comments of Reviewer 2 and corrected the paper. All revision recommendations were accepted and reported in the current version. Additionally, due to the addition of real healthy human urine analyses, the title was modified to "Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector".

In addition: abbreviations in the title are not allowed; use the format of JCA, remove tables from the text; Refs., Journal number and pages, missed.
Thank you dear Editor. The abbreviations in the title were removed and the full terms are introduced. Tables were removed from the text and were uploaded separately. Journal number and pages were added to reference list. Additionally, one reference was duplicated in the original version, which is corrected in the revised version; references list was updated and corrected (in the text and tables).

Reviewer #2:

This manuscript presents a study on the development and validation of a pre-concentration and separation method for trace determination of antidepressant drugs, Fluoxetine (FLU) and Citalopram (CIT) in simulated urine and wastewaters, was developed based on HPLC-DAD analysis after magnetic solid phase extraction (MSPE). This manuscript has interesting aspects, such as the development of a magnetic solid-phase extraction, which is a novel format of solid-phase extraction as well as some drawbacks as the determination by HPLC-DAD (nor selective or sensitive enough). Then, this manuscript is in the boundary of studies of interest for the Journal of Chromatography A.
The experimental design of the study is well planned and developed. It has all the elements, interesting optimization, proper validation, comparison with other already published methods and application to a survey of non-spiked samples. Furthermore, the manuscript is well written and presented. Then, the manuscript could be publishable in the Journal of Chromatography A but only if the authors addressed properly the weaknesses of this study.
Thank you very much for your very valuable comments and evaluation of the herein reported work. We have accepted all the Reviewer's comments and the proposed changes are reported in the revised version.

However, it requires to considered the following important aspects:
The comment of the detection system is important and must be considered by the authors. In fact, in section 2.6. Preparation of the urine and wastewater samples, the authors used a

"synthetic prepared urine" to L194-196 "avoid contamination of male and or female hormones and other potential interferences". Then, a question raises what is the real utility of this method? This aspect needs to be very seriously discussed because is the weakest aspect of the manuscript and cannot be addressed.

Thank you for your comments. The developed method was applied to synthetic urine samples by considering some of published articles from literature. The sentence "avoid contamination of male and or female hormones and other potential interferences" is from also published article but in the revised version was deleted. Accordingly, to the Reviewer suggestion, the method was applied to the urine samples taken from healthy volunteers and drugs were spiked to samples. The revised manuscript reports also these data in order to increase the quality of the work.

Additionally, due to the addition of real healthy human urine analyses, the title was modified to "Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector".

The second major aspect of the manuscript is sensitivity; the authors did not define the LOD or the LOQ. This is the first problem. Furthermore, if the instrumental LOD is approx. 1 µg mL^{-1} and the concentration factor is ca. 60, the LODs after SPE must be ca. 30 ng mL^{-1} and not 3. Then, these limits must be carefully checked and better explained. In both cases, sensitivity will be far away of the real concentration in water. The authors apply this to wastewater and urine and this also raises a question: Are the same LODs obtained working with these matrices? Can the authors concentrate 50 mL of urine and see the compounds? At least to the last question, clearly no.

As explained in section 3.7; analytical validation of the developed method was carried out by considering ICH guidelines. Main aim of this paper is to develop and optimize a combination of solid phase extraction and HPLC-PDA. Before MSPE, direct determination of drugs was carried out by HPLC but LOD values of this method was not calculated. 1 ppm was a roughly estimated in order to highlight the minimal approximate concentration to obtain observable peak. Concentration factors are generally described as average. In this type of studies, especially in batch type solid phase extraction studies, final volume of solutions is not known very well because they may include some part of solid. For this reason, average factors are determined. This factor is higher at lower concentrations while lower at higher concentrations in the linear range. Recovery values were added for real urine samples.

Some few minor comments are:

Abstract L30-31, the "newly synthetized magnetic sorbent" needs to be better defined (e.g. Fe_3O_4 NPs coated with graphene oxide-polypyrrole polymer)

Thank you for your comments. Fe_3O_4@PPy-GO was used a code name of NPs.

L240-242 can be deleted because this information is already in the abstract and in the introduction.

This sentence was deleted.

Table 3, the LODs and LOQs of the method reported in Table 3 are not in the same units and then, they are difficult to compared.

Table 3 was rewritten according to the reviewer's suggestion.

It would be better if the authors add a section in the experimental part "Method validation" where they included the information reported in L321-328 and they also explain better how where LODs and LOQs established.

According to the Reviewer suggestion, the paragraph 2.7 was added in the revised version, including also the explanation of LODs and LOQs information.

There are too many figures. Then, the graphic regarding method validation can be presented as supplementary.
As correctly highlighted, in the revised version there are only 5 figures in the manuscript. The others were moved in the supplementary.

Figure 1 shows a very nice chromatogram but without indication of the concentrations used and the type of matrix. It would be very interesting if the authors include chromatogram of the matrices at the LOD or LOQ
A new chromatogram was added as supplementary material for real urine samples.

Reviewer #3:
The authors described a complete study which included the synthesis of a adsorbent material, the optimization of a novel extractive method and its validation for the determination of two antidepressants in synthetic urine and water samples. The article is well written, the analytical method was validated and application of the method gave excellent outcomes. I have just a few question and a recommendation.
Thank you for your very positive evaluation.

In the abstract you mentioned LODs and LOQs but you only described LODs, please include the LOQs values.
Thank you for your comments. Accordingly, the information was added in the abstract as correctly suggested.

Why you did not you use internal standard method in the analytical method? I saw, that even employing external standard approach you got excellent results, but I would like to know if an internal standard could improve your method once applied to real urine samples.
The authors agree with the Reviewer's comment. Generally, the use of an internal standard allows greater control of the analysis process (extraction and instrumental analysis).
This project focuses on the characterization of the MSPE with respect to the two analytes considered and on the analytical performances optimized for this procedure. The use of an internal standard was not considered in order to evaluate the analytical parameters of the entire method in the "worst" conditions, ie in the absence of an element that would allow to correct any variations in the extraction efficiency and by means of the method of 'external matrix-matched calibration.
In addition, this choice opens up the possibility of applying the same procedure to more performing techniques (LC-MS / MS) for which a marked (deuterated) internal standard could be used in order to further improve and increase performances. However, this will inevitably involve a modification of the chromatographic procedure in order to replace the phosphate buffer with another buffer compatible with mass spectrometry.

Maybe you should include the accuracy determination, is the only parameter lacking in your method validation, adding accuracy will round an excellent work.
The information requested by the Reviewer is included in the revised version. The accuracy value is indicated in Table 2 as the recovery value, as permitted by the International Guidelines. These values were determined in all matrices considered in the present work.

If possible, you should apply your method to real urine samples in order to see the method performance under real scenarios.

As correctly suggested also by the Reviewer 1 and accordingly to this comment, in the revised version the method was also successively applied to real urine samples, and the results were added to the Table 2.
Additionally, due to the addition of real healthy human urine analyses, the title was modified to "Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector".

Other than the questions and suggestion described before, I can only recommend to accept this article.
Thanks for your final evaluation. All the suggestions were accepted and reported in the revised version.

Reviewer #4:

Interesting to know how the magnetic solid phase can enhance the trace analysis of SSRIs. Will be more interested to see if presence of other pharmaceutical interferences which also appeared on the same retention time on the chromatogram as FLU and CIT, will overestimate the concentration/peak area of these SSRIs.
As correctly indicated by the Reviewer, in the use of the HPLC-DAD configuration the possible interference by other drugs could lead to an incorrect quantitative analysis.
In this work, the selectivity of the procedure was evaluated by analyzing the blank matrices and the matrices fortified with the analytes of interest and in no case were matrix interferers found. Furthermore, the optimized conditions for the extraction and cleanup of the analytes reduces this possibility. Should there be a minimal instrumental signal linked to other co-administered drugs that interfere with analyte signals, a modification of the HPLC procedure may be required, with consequent re-validation of the method.

Analytical validation results based on urine and wastewater should be performed since the real sample analysis is based on these matrices. Would be nice to investigate how the results will also be affected by considering other matrices, i.e. river water, drinking water, soil, sludge and etc.
In the revised manuscript, real urine samples were added to Table 3 as a new sample. Probably, at an environmental level, the absence of interferents in the water samples analyzed here, and thanks to the selective extraction procedure for the target molecules, also allows the analysis of other aqueous environmental matrices such as those reported by the Reviewer (river water, drinking water). From an analytical point of view, in order to be able to apply this medicament also to solid matrices (soil, sludge, etc.) it is essential to review the entire extraction procedure (for example, a sample "solubilization" step must be envisaged before extraction with the MSPE procedure).
Additionally, due to the addition of real healthy human urine analyses, the title was modified in "Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector".

There are, however, lack of critical discussion in your results and discussions, which I think can be improved by including more supporting evidence along with your claims. Most of the time, only results were reported but not much you have really discussed. There is some missing citation in some paragraphs and minor errors were found. Here are the other comments listed below:

Highlights: Abbreviations should be avoided.
As correctly suggested, the highlights were corrected.

Line 89-94:

* Both LC-MS-MS and HPLC-ESI-MS can be categorised under the LC-MS technique. Please consider combining both references (11, 14) under the same LC-MS method. Similarly, HPLC-UV detector (15), HPLC-fluorescence (18) and UHPLC-PDA (17) can be classed together under the same HPLC techniques. These combinations can avoid unnecessary repetition of the similar techniques and further shorten the sentence.
* Are the spectrofluorimetric determination (13) and spectrofluorimetric method (19) the same technique? If they are, please combine them too.

Thank you for your comments. This section was revised accordingly and the references list was updated and corrected.

Line 108:

What is NPs? Nanoparticles?

Yes, it is nanoparticles. In the revised version, it is specified accordingly.

Line 115-116:

Wastewater not waste water

As correctly suggested, the term is corrected in the revised version.

Line 120:

Missing SEM abbreviation

As correctly suggested, the acronym is specified in the revised version.

Line 131:

Please include whether it is the enantiomeric pure or racemic form of the fluoxetine and citalopram been acquired for your study?

In this study, FLU and CIT chemicals were obtained from Sigma with number 34012 and Y0001007, respectively, as Reference Standard.

Line 144:

* What is the wavelength used in the study since this information is missing from Table S1 and Figure 1?

The wavelengths were added to Table S1 and to Figure 1 caption.

* Did you use multiple wavelengths on a single run or just one optimum wavelength?

Accordingly, also to the previous comment, the wavelengths were defined into the text and 284 nm for CIT and 258 nm for FLU were used for all determinations.

Line 149:

* What is the concentration of the conc. H_2SO_4?

It means concentrated solutions at ≥99.9%

* Need to state the full chemical name before the molecular formula.

As correctly suggested, the full chemical name before the molecular formula is added in the revised version.

Line 154 and 156:

* 30% H2O2 as in 30%w/v or 30%v/v? Like the 10% HCl. Please specify them clearly.

As correctly suggested, this information was added in the revised version

* Need to state the full chemical name before the molecular formula.

As correctly suggested, the full chemical name before the molecular formula is added in the revised version.

Line 161:

Need to state the full chemical name before the molecular formula.

As correctly suggested, the full chemical name before the molecular formula is added in the revised version.

Line 192-193:

Need to state the full chemical name before the molecular formula.

As correctly suggested, the full chemical name before the molecular formula is added in the revised version.

Line 210:

* Please specify clearly on IR assignment for the COOH. There are two distinct features here, i.e. C=O and O-H functional groups which should be seen at around 1650-1750 cm^{-1} and 3500 cm^{-1}, respectively.

- The required explanations were added to the related sections.

* How do you ensure the analyzed samples are dried? Sometime, if the sample isn't dry enough, a peak will appear around 3500 cm^{-1} regions due to H_2O too.

- Heating process was continued until the sample reached constant weight.

Line 213-218:

* The peaks appeared on the IR spectrum are due to the stretching or bending instead of stress vibration. Please rectify them accordingly.

* They are corrected.

* Please justify why do you say that "Peaks appeared at 1219 cm-1 and 978 cm-1 are due to the polypyrrole"? Do you have a pure polypyrrole standard IR spectrum to compare from? Else, it is hard to say so since below 1000 cm-1 is a fingerprint region.

* The sentences are corrected.

* You have a nice and clear Raman results with explanation in the following section, not sure if you still wish to include the FT-IR here since there isn't much discussion found in this section and you also did not mention about the FT-IR instrumental details in your instrumentation (2.1)

* FT-IR spectra for the materials were obtained through the Perkin-Elmer Spectrum 400 FT-IR spectrometer (Waltham, MA).

Line 221: Which literature does it (graphene oxide) corroborate with? Citation? Any similar literature SEM results like GO, Mag-GO, and Mag-GO-Ppy have been reported so that you can use them as references to compare with your experimental results? Further discussion can be made from the comparison too.

Line 226-238:

* What are the D and G bands?

The ratio of the intensity of the D / G bands is the measure of the defects found in the graphene structure. While the G band is the result of in-plane vibrations of SP2 carbon atoms, the D band

is due to external vibrations attributed to the presence of defects in the structure. When comparing graphite and graphene oxide spectra, GO will have a higher D band. This is because the SP2 bonds of the carbon are disrupted, as GO has groups in which it is oxidative. while the D band is related to the vibration of sp3 carbon atoms of the disordered GO nano sheets and the G band corresponds to the vibration of sp2 carbon atom domains of graphite

* Which functional group are they (D/G bands) corresponding to?

The ratio of the intensity of the D / G bands is the measure of the defects found in the graphene structure. While the G band is the result of in-plane vibrations of SP2 carbon atoms, the D band is due to external vibrations attributed to the presence of defects in the structure. When comparing graphite and graphene oxide spectra, GO will have a higher D band. This is because the SP2 bonds of the carbon are disrupted, as GO has groups in which it is oxidative.

* Please label D and G band on the Raman spectrum to ease the reading.
* Literature was mentioned but without citation. Need to include reference here.
* Why the D band is higher than the G band in GO?

Wang, L., Zhao, J., Sun, Y. Y., & Zhang, S. B. (2011). Characteristics of Raman spectra for graphene oxide from ab initio simulations. The Journal of chemical physics, 135(18), 184503.

* Why there is a suppression of D and G bands observed on magnetite-GO?

As the surface of the material is coated with Fe3O4 magnetic nanoparticles, the peak intensity of the specific peaks of graphene oxide decreases and the peak ratios change.

* Which functional groups of pyrrole are assigned to 978 cm-1 and 1047 cm-1?

Raman spectrum of the synthesis of pyrrole and magnetite graphene oxide. It was observed that the peaks at wavelengths of 978 cm-1 and 1047 cm-1 belong to N-H nonds in pyrrole

Line 256:

At what concentration of FLU and CIT used in this optimization study?

In all optimization steps, a model solution was used at concentration of 200 ng/mL.

Line 264:

Do you determine the optimum pH of the buffer used for the HPLC mobile phase or buffer to be added during the extraction process? pH 3 buffer was mentioned in the HPLC conditions, but I struggle to find the application of the pH 10 buffer in your sample preparation and magnetic solid phase extraction (Section 2.5).

Thank you for your comments. This study includes two main step. Magnetic solid phase extraction and HPLC determination. Generally, HPLC conditions are determined before extraction step in order to be able to measure the target molecules correctly. The used HPLC method is also original for this study. We did not take this method from reference. As for you question, mechanism of determinations in HPLC and extraction procedure are so different. In MSPE, both surface properties of magnetic particles and molecular structure of drugs are affected by pH. In HPLC, your working conditions can change according to mobile phase and column.

The pH equal to 10 was the optimized condition in the MSPE procedure, while pH 3 was the optimized value for the chromatographic analysis.

Line 278:

Why was 100 rpm used instead of other speed? Is this speed selected based on other established or published method?

This speed was selected by considering our previous studies, where was observed that at higher rpm velocity no further improvements in the extraction efficiency were gained. For this reason, this speed was selected and then the time effect was studied. This parameter is the main responsible and is not related to the rpm value.

Line 279:
What is the concentration of the model solution? Does the different concentration used affect the adsorption and desorption time?
All optimization studies were performed by using model solutions at two concentration level (100 ng/mL and 200 ng/mL). In optimization experiments, all variables were kept constant except the studied ones. The use of two different concentrations do not affect the adsorption and desorption time

Line 280:
How do you perform the recovery study? Will be helpful to give a brief explanation since it is not stated in your experimental.
Recovery values were calculated by using the ratio of the found amount of drugs to their spiked concentrations. This sentence was added to related section in order to better clarify the procedure.

Line 289-292:
What is the concentration of the FLU and CIT used and showed in Figure 5?
The two analytes were at 200 ng/mL as concentration level.

Line 298-300:
Any literature or study can support the statement you made here?
In literature, to our best of knowledge, there are no paper on this topic. This statement is based on our previous experiments, observations, and published articles. This is an experimental observation.

Line 315:
How do you ensure there is no carry over or strong retention of the FLU and CIT on the solid sorbent even after the wash? Perhaps worth looking at any possible FLU and CIT desorption after the wash too?
Thank you for this very good questions. Of course, no desorption process can remove all molecules from the surface of NPs with 100% yield. Some of molecules is retained on surface depending on experimental conditions after every use. We checked this amount a few times. Mostly, the amount of retained molecules was lower than 10% by comparing initial concentration of molecules, even if this amount maybe negligible, magnetic particle were washed with ACN/MeOH before every use (and the chromatograms of blank samples were analyzed before every use).

Line 339:
* What is the superscript (b) of LOQ refers to?
* EF calculation error for fluoxetine. The ratio of fluoxetine's slope after MSPE and before MSPE is not 78. Need to recalculate this.
Thank you for comment. The mistyped values were corrected in the revised version according to the Reviewer suggestion.

* The reported analytical method validation here is based on the model solution (methanol+ deionised water+ buffer matrix). However, in real samples, due to the matrix effect, simulated urine and wastewater matrices used could affect the validation results. Did you validate the analytical method for FLU and CIT in the two different simulated matrices also? It will be good to include these validation results.

As correctly pointed out by the Reviewer, the linearity parameters were obtained by means of an external calibration in solvent.
According to the International Guidelines, in the absence of a blank matrix (or deemed such), it is allowed to proceed with the validation of the linearity in solvent, while the precision and trueness parameters (as well as recovery) can be evaluated (as has been done in the present work) through the use of real samples fortified at known levels of the analytes and interpolated on the linear model.
Additionally, due to the addition of real healthy human urine analyses, the title was modified in "Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector".

* What happen if the urine and wastewater or real samples are spiked with other pharmaceuticals, while they are also appeared on the chromatogram with similar retention time as FLU and CIT? How do you distinguish them accordingly?
As correctly indicated by the Reviewer (and by Reviewer 3), in the use of the HPLC-DAD configuration the possible interference by other drugs could lead to an incorrect quantitative analysis.
In this work, the selectivity of the procedure was evaluated by analyzing the blank matrices and the matrices fortified with the analytes of interest and in no case were matrix interferers found. Furthermore, the optimized conditions for the extraction and cleanup of the analytes reduces this possibility. Should there be a minimal instrumental signal linked to other co-administered drugs that interfere with analyte signals, a modification of the HPLC procedure may be required, with consequent re-validation of the method.

Line 358-368:
Paragraph and Table 3 are better off moving to the introduction or discussion sections since the conclusion should not involve any further discussion and comparison.
Thank you for your comments. As correctly suggested, a new paragraph reporting the comparison with the literature was added before the "Conclusions".

Figures and Tables:
Detailed descriptions and captions not just a short sentence on Tables and Figures are mandatory to ease the reader to understand better without the need of keep looking back to the corresponding paragraphs.
As correctly suggested, the table and figure captions were revised accordingly.

Highlights

- The magnetic solid phase extraction was applied to Fluoxetine and Citalopram molecules for trace determination.
- A new magnetic material (Fe_3O_4@PPy-GO) was synthetized and characterized successively.
- Sensitive and easily applicable method was proposed using MSPE-HPLC-DAD.
- The studied antidepressants drugs were determined successively in urine and wastewater samples.

Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector

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ABSTRACT

A new analyte separation and preconcentration method for the trace determination of antidepressant drugs, Fluoxetine (FLU) and Citalopram (CIT) in urine and wastewaters, was developed based on HPLC-DAD analysis after magnetic solid phase extraction (MSPE). In the proposed method, FLU and CIT were retained on the newly synthetized magnetic sorbent (Fe_3O_4@PPy-GO) in the presence of buffer (pH 10.0) and then were desorbed into a lower volume of acetonitrile prior to the chromatographic determinations. Before HPLC analysis, all samples were filtered through a 0.45 µm PTFE filter. Experimental parameters such as interaction time, desorption solvent and volume, and pH were studied and optimized in order to establish the detection limit, linearity, enrichment factor and other analytical figures of merit under optimum operation conditions. In the developed method, FLU and CIT were analyzed by diode array detector at the corresponding maximum wavelengths of 227 and 238 nm, respectively, by using an isocratic elution of 60% pH 3.0 buffer, 30% acetonitrile, and 10% methanol. By using the optimum conditions, limit of detections for FLU and CIT were 1.58 and 1.43 ng mL^{-1}, respectively, while the limit of quantifications was 4.82 and 4.71 ng mL^{-1}, respectively. Relative standard deviations (RSD%) for triplicate analyses of model solutions containing 100 ng mL^{-1} target molecules were found to be less than 5.0%. Finally, the method was successfully applied to urine (both simulated and real healthy human) and wastewater samples, and quantitative results were obtained in recovery experiments.

Keywords: Fluoxetine, Citalopram, HPLC, Magnetic Solid Phase Extraction, Urine samples, Environmental water samples

1. Introduction

Therapeutic drug monitoring (TDM) is one of the most important research areas in drug discovery and development process used in pharmaceutical research and development. TDM of antidepressants is necessary for an optimal supervision of patient drug regimen to avoid medical non-responsiveness, intoxication, complications or noncompliance [1,2]. Antidepressants have seen exponential growth in their use during the last couple of decades. Many antidepressants act by blocking the reuptake of norepinephrine and serotine substances in the brain [3]. When their structures are examined, it is seen that most of them have tricyclic or tetracyclic nuclei. These drugs are generally used for the treatment of mental depression which has become a health problem in many parts of the society today, causes loss of productivity and workforce in many areas of life. In some cases, it imposes substantial economic losses with the treatment process. If both drugs are used before the recommended time, it causes muscle stiffness, heart rhythm, sudden changes in blood pressure, fainting, and clouding of the mind called serotonin syndrome. If it is used under the clinical supervision, the liver interacts less with the toxin enzyme [4].

Fluoxetine {*N*- methyl- 3- phenyl- 3- [4- (trifluoromethyl)phenoxy]propane- 1- amine} was the first selective serotonin reuptake inhibitor (SSRI), synthesized and marketed under the name Prozac®, produced by the pharmaceutical company Eli Lilly [5,6]. Its pharmacologically active metabolite, norfluoxetine, derived from the biological *N*- demethylation of fluoxetine. This active metabolite has prolonged action with clinical activity of inhibition of the reuptake of 5-HT and inhibition of cytochrome P450 isoenzymes in the liver. Fluoxetine is metabolized by the CYP2D6 enzyme, such as neuroleptics and tricyclic antidepressants [7]. The drugs is generally used in the treatment of diseases with similar effects belong to the antidepressant drugs group known as SSRIs.

Citalopram is a bicyclic phthalate and belongs to the SSRI family. It is a racemic drug used for the treatment of depression with the *S*-enantiomer being the pharmacologically active compound [8]. Citalopram (1-[3-(dimethyl amino) propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile is a "second generation" antidepressant drug, whose pharmacological activity is based on the selective serotonin reuptake inhibition. Its efficacy is comparable to tricyclic antidepressants, but it is better tolerated and is characterized by a lower risk of adverse effects [9].

In many drug formulations, active principle is one of the components, and in this scenario, after the drug assumption it is important to analyze in complex matrices (biological and/or environmental) the concentrations in terms of both monitoring the therapeutic dose and

monitoring the excretion products after use. Two main problems encountered in these analyses are, in most cases, the complexity of the sample matrix and the concentration of the target molecules below the detection limits of the chromatographic system. In order to overcome these problems, it is preferred to use separation and enrichment methods with a suitable carrier system.

Several methods have been published for the determination of one or more antidepressants in complex matrices (biological fluids and environmental samples) for therapeutic drug monitoring, for toxicological purposes, or for environmental pollution evaluation. The methods available in the literature for trace determination of Fluoxetine and Citalopram are based on ion transfer stripping voltammetry [10], liquid chromatography–mass spectrometry LC–MS methods [11,12] and gas chromatography–mass spectrometry (GC–MS) [13], spectrofluorometric determination [14, 15], liquid chromatography techniques [16-18], adsorptive square wave voltammetry (ASWV) [19]. However, due to the trace levels of antidepressants in complex matrices and the disruptive effects of the matrix components, clean up and preconcentration techniques have become an inevitable stage prior to the analysis of these drugs [20].

Magnetic solid phase extraction (MSPE), as a versatile approach of SPE, is carried out based on adsorption and desorption of the target molecules on a magnetic material. The used external magnetic field without some tedious steps (centrifugation or filtration) facilitates extraction steps. Sorbent particles can be easily isolated and collected during adsorption and desorption, making the sample pretreatment procedure more convenient, time-saving and cost-effective [21,22]. When magnetic solid phase extraction methods in the literature are examined, it is seen that carbon-based nanoparticles such as carbon nanotubes (CNTs), graphene oxide grafted nanostructures, nano diamond and carbon nanofibers (C-NFs), which have high surface area and adsorption capacity and can be used repeatedly due to their inertness in the working solution environments, are frequently preferred as adsorbents [22–27].

In this study, Fe_3O_4 nanoparticles (NPs) were coated with graphene oxide-polypyrrole polymer (PPy-GO) and characterized by instrumental methods. The graphene oxide was preferred as a supporting material in order to provide multi imprinting sites, large surface area, and easy separation of magnetic nanocomposites. Then, the capability of these new sorbent (Fe_3O_4@PPy-GO) for simultaneous preconcentration and determination of two widely used antidepressant drugs (Fluoxetine and Citalopram) as model compounds were studied and examined by using magnetic solid phase extraction and HPLC-DAD system. Finally, the

applicability of the proposed method was successively investigated for the extraction and determination of CIT and FLU in simulated urine, urine from healthy volunteers, and wastewater samples.

2. Materials and Methods

2.1. Instrumentation

Characterization of synthesized magnetic nanoparticles was carried out using Raman spectroscopy, X-ray diffraction spectroscopy and scanning electron microscopy techniques. The Raman spectra of the nanomaterials were obtained using a Raman Spectrophotometer (WITEC alpha 300M + micro-Raman system, Germany) with a 532 nm laser source. X-ray diffraction spectrum of magnetic nanoparticles was taken with a Bruker AXS D8 brand X-ray diffractometer. Scanning electron microscopes (SEM) and SEM Mapping analyses were performed using scanning electron microscopy (Zeiss Gemini 500 Field Emission Scanning Electron Microscope) to elucidate the morphological structures of magnetic nanoparticles. FT-IR spectra for the materials were obtained through the Perkin-Elmer Spectrum 400 FT-IR spectrometer (Waltham, MA). Chromatographic analysis of Fluoxetine and Citalopram were performed by the Shimadzu (Prominence) HPLC (Kyoto, Japan) system. All separations and determinations were performed on a phenyl hexyl column (Luna® 5 µm Phenyl-Hexyl 100 Å, 250mm × 4.6mm) under isocratic conditions.

2.2. Chemicals and reagents

In this study, all chemicals used are at the rate of 99.5% purity. Deionized water system had 18.2 MΩ cm resistivity was used to obtain deionized water (MES, MP Minipure Dest Up, Turkey). The HPLC grade acetonitrile (ACN) and methanol were used for HPLC-DAD analysis (Sigma Aldrich, St. Louis, MO, USA) without further purification steps. For HPLC analysis, were used a mixture of phosphate buffer solution (pH 3.0, 50mM), methanol and acetonitrile (60:10:30) as mobile phase under isocratic elution conditions. Stock solutions of Fluoxetine (FLU) and Citalopram (CIT) (Sigma Aldrich, St. Louis, MO, USA) were prepared in methanol and calibration mix standards were prepared by serial dilutions. FLU was racemic standard, while CIT is a pure standard.

2.3. HPLC determination conditions

A phenyl-hexyl column was used as the most suitable stationary phase in this analysis**.** In order to determine the better mobile phase compositions, the mobile phases containing buffers

at different pH values and various organic phase compositions were tested in order to obtain the most suitable conditions in terms of peak resolutions and symmetry. Optimized HPLC conditions in this work were given in **Table S1**, while in **Figure 1** was reported the chromatogram showing the peaks profile gained by increasing calibration standards concentration under the described conditions. All quantitative determinations were performed at 238 nm for CIT and 227 nm for FLU.

2.4. Synthesis of magnetic nanoparticles

Synthesis of graphene oxide by the Hummer method

3.0 g of graphite powder was added to the flask, which was cooled to 0°C in an ice bath, and 70 mL of concentrated sulfuric acid (≥ 99.9%) was slowly transferred on graphite. Under vigorous stirring, 9.0 g of potassium permanganate, $KMnO_4$, was added to this reaction mixture and the reaction temperature was kept around 20°C for 30 minutes. Then, the reaction mixture was stirred at 40°C for 30 minutes more. 150 mL of deionized water was added to the mixture and the reaction temperature was raised to 95°C on a magnetic stirrer. After refluxing the reaction mixture at 95°C for 15 minutes, 500 mL of deionized water and 15 mL of 30% (*w/v*) H_2O_2, hydrogen peroxide, were added to the reaction mixture, and the reaction was allowed to continue for 10 minutes. After this step, the reaction mass was cooled to room temperature. The brown-yellow reaction mixture was filtered and washed with 10% (*w/v)* hydrochloride acid, to remove unreacted reagents. The product obtained was then dried in an oven at 50°C for 24 hours.

Synthesis of magnetite graphene oxide

0.5 g of graphene oxide, which was synthesized in the previous step, was pulverized and weighed carefully. A mixture of 0.5 g of Iron (III) chloride and 2.0 g of sodium acetate, previously homogenized in 20 mL ethylene glycol, was added to the graphene oxide particles. After being kept in an ultrasonic bath for 10 minutes, it was transferred to an autoclave for hydrothermal synthesis. The hydrothermal synthesis unit was allowed to react at 180°C for 12 hours. After the reaction, the product was washed twice with ethanol and once with deionized water and allowed to dry in an oven at 70°C.

Synthesis of magnetite graphene oxide-poly pyrrole (PPy) nano composite material (Magnetic PPy/GO)

0.5 g of the synthesized magnetite graphene oxide was weighed and dispersed in 200 mL of deionized water. Later, 500 µL of pyrrole was added to the mixture in an ice bath and 1.6 g of ammonium persulfate solution dissolved in 10 mL of water. This solution was added dropwise to the reaction medium. Stirring was continued until the reaction was complete. The synthesis product formed on the surface of the solution was separated from the mixture by filtration. It was washed 2 times with deionized water during the filtration process. It was left to dry in an oven at 70°C.

2.5. Magnetic solid phase extraction

50 mg of Fe_3O_4@PPy-GO was weighed and transferred to 50 mL of falcon tubes. Then, 20 mL of sample solution including FLU and CIT in the range of 5.0-500.0 ng mL^{-1} and the volume of the tube was completed to 50 mL with distilled water. Falcon tubes were tightly closed and placed in orbital shaker device by setting 100 rpm for 20 minutes. After the time was over, magnetic particles were separated by using an external magnet, then 800 µL of acetonitrile was added and the tubes were vortexed for 40 seconds for the target molecules desorption. The ACN phase were taken into an injector, the 0.45 µm injector tip was passed through the filter and transferred to the vials and placed in the HPLC device. The contents of samples for Fluoxetine and Citalopram enriched were determined by HPLC-DAD system.

2.6. Preparation of simulated urine samples and wastewater samples

The application of proposed method was carried out by simulated urine, healthy human urine, and wastewater samples. Content of simulated urine samples was prepared as mentioned in literature [28–30]. 25.00 g of urea, 1.08 g of calcium chloride, 1.00 g of ammonium chloride, 1.60 g of potassium chloride, 1.40 g of sodium sulfate, 1.40 g of potassium dihydrogen phosphate, and 2.92 g of sodium chloride were dissolved in 1 L of ultra-pure water. The pH of simulated urine solution was adjusted to 6.0 using sodium hydroxide (0.1 M) or hydrochloride acid (0.1 M). The mixture was stirred on a magnetic stirrer for 15 min and kept in an ultrasonic water-bath. Then, the solution was diluted at 1:2 and 1:4 ratios. The obtained solutions were stored in amber glass bottles until analysis.

The healthy human urine samples were collected in a capped sterile test tube from volunteers free from any kind of medication who had been informed about the experimental procedure and the nature of the study. All samples were left at room temperature for 20 min and then centrifuged for 10 min at 4000 rpm [31].

Wastewater sample was obtained from main wastewater discharge line of University in Sivas, Turkey. Wastewater samples were collected in amber glass bottles and immediately filtered through 0.45 µm cellulose nitrate membrane. Subsequently, pH of samples were adjusted to 3 to reduce biological activity [32] and were stored in the dark at +4°C until analysis.

2.7. Method validation

The method validation was carried out according to International Conference on Harmonization guidelines [33-34]. Analytical figures of merit such as enhancement factor (EF), preconcentration factor (PF), relative standard deviation (RSD), limit of detection (LOD), limit of quantification (LOQ), linear range and correlation coefficient were calculated by considering correctness and sensitivity of the method.

Preconcentration factors (PF) were calculated by using the ratio of the initial solution volume (50 mL) to the last elution solvent volume (0.8 mL). The enhancement factors (EF) were obtained from the ratio of the slope of calibration curve of the analytes after MSPE application to that of prior MSPE application. The relative standard deviations (RSD%) were found by applying the MSPE method for seven repetition analysis, which includes 100 ng mL^{-1} of CIT and FLU. The LODs and LOQs values herein reported were obtained by means of the signal-to-noise ratio. Specifically, LOD was defined by a signal-to-noise ratio of 3:1, while the LOQ was defined by a signal-to-noise ratio of 10:1, accordingly to International Guidelines [34].

3. Results and Discussions

3.1.Characterization of the magnetic nanoparticles

The results of FTIR analysis for the graphene oxide, magnetic graphene oxide and polypyrrole magnetic graphene oxide components used in the synthesis of the magnetite graphene oxide-polypyrrole nanomaterial material were given in the **Figure S1**. The FTIR spectrum of graphene oxide is compatible with other studies currently available in the literature. Characteristic peaks of graphene oxide were detected: (C-O-C) (1230-1320 cm^{-1}), sp^2-hybrid C=C (1500-1600 cm^{-1}, in-plane vibrations), (COOH) (1650-1750 cm^{-1}, 3530 cm^{-1} carboxyl vibration modes.

Magnetic graphene oxide peaks are 588 cm^{-1}, known as the Fe-O characteristic peak, 1651 cm^{-1} (C=O) symmetrical stretching vibration peaks at 1085 cm^{-1} (C-O). In FTIR analysis of magnetic graphene oxide-polypyrrole nanomaterial as the end product of the synthesis, NH symmetric stretching vibration at 3271 cm^{-1} wavelengths, 3123 cm^{-1} (OH), 1714 cm^{-1}, 1614 cm^{-}

[1] C=O stretching peaks with bending vibration of 1219 cm^{-1} (C-N)and 978 cm^{-1} (C-N) can be attributed to the presence of polypyrrole in the composite material.

In the SEM images given in **Figure 2** of the magnetic graphene oxide-polypyrrole nanomaterial, the layer in the form of a web cover, which is seen to corroborate with the studies in the literature, is known as "graphene oxide". It can be seen from the transparent SEM images that low-layer graphene oxide is successfully produced from graphite (**Figure 2a**). The formation of Fe_3O_4 magnetic particles has been proven by SEM-Mapping analysis of Fe (**Figure 2b**). As a result of the modification of magnetic graphene oxide with polypyrrole, the formation of polypyrrole particles is observed (**Figure 2c-f**).

Figure S2a shows the characteristic peaks of the synthesized graphene oxide (GO) in the D and G bands, which are compatible with the literature. It is easily understood from the fact that the D band of GO is more dominant than the G band where graphene oxide is successfully synthesized from graphite. **Figure S2b** contains the Raman spectrum of magnetic graphene oxide. In the spectrum, it was observed that magnetite graphene oxide was synthesized from graphene oxide, and the suppression in the D and G bands can be clearly seen from the change in the ratio of the peaks to each other. **Figure S2c** shows the Raman spectrum of the synthesis of pyrrole and magnetite graphene oxide. It was observed that the peaks at wavelengths of 978 cm^{-1} and 1047 cm^{-1} belong N-H bonds in pyrrole in the literature and that these peaks originated from pyrrole were formed in this spectrum and the desired structure was obtained as a result of the synthesis reaction. In **Figure S2d**, it is seen that the spectra of magnetite graphene oxide and magnetite graphene oxide-pyrrole synthesis are overlapped, and the Raman shifts and the change in D and G band ratios can be easily noticed.

3.2. Extraction optimization experiments

The objective is to keep the analyte type in the solid phase at the highest possible level and to separate it from other substances in the environment, and after the separation process is achieved, all of the analytes in the solid phase pass into the solvent. Preliminary trials were made to determine the necessary parameters to achieve this. It was aimed to obtain a fast and easy separation process and to obtain the highest concentration of analyte by using as little amount of organic solvent as possible. Thus, it was brought to the concentration range that the HPLC device can read. Accordingly, a chromatographic method was developed by optimizing all parameters.

3.3. pH effect

Ambient pH is an important factor as it affects the adhesion of the analyte to the solid phase and the reactions between species. Model solutions containing both antidepressants respectively were interacted with a series solution in the range of 2.0-12.0. All experiments were studied with 50 mg of Fe_3O_4@PPy-GO. Following these processes, FLU and CIT molecules were retained on solid phase and separated with an external magnet. After desorption of target molecules, eluent solvent phase was transferred by a syringe and filtered through a 0.45 μm PTFE membrane filter, transferred into HPLC vials and subsequently injected into the HPLC system.

As can be illustrated in **Figure 3**, the optimum pH value for the enrichment steps was observed at a pH value of 10.0. A literature review revealed that, the pKa value for Fluoxetine is 8.70, while the pKa value for citalopram is 9.50 [35]. As can be seen in the Figure 3, the efficiency of extraction reaches to maximum beyond these values. Therefore, pH 10.0 was selected as the optimum for subsequent experiments.

3.4. desorption solvent and time

Adsorption and desorption processes of drug molecules on the surface of magnetic nanoparticles are carried out by means of an equilibrium process, guided by the partition coefficient of the analyte between the aqueous sample matrix and the magnetic nanoparticle-based sorbent. Generally, binding of the target molecules to solid sorbent needs longer time due to the slow diffusion of the analytes into the solid sorbent and the sluggish rate of the mass transfer of analytes from the bulk to the sorbent in absence of any external energetic stimuli. To increase the mass transfer kinetics, this process was facilitated with an orbital shaker or a rotator by means of increasing interactions between sorbent and molecules. Desorption process is faster than adsorption because a pure and clean solvent is used to remove molecules directly from the sorbent surface. Rate and efficiency of desorption are increased by using vortex at high speed (up to 100 rpm). Time for both the processes should be optimized in order to find optimal extraction conditions. The first step of extraction (adsorption) was carried out by using an orbital shaker with 100 rpm. Model solutions including both molecules and adsorption time was studied in the range of 0-90 minutes. As can be seen in **Figure 4a**, 20 min is enough for high recovery. Desorption time on the vortex was also studied in the range of 0-90 seconds as can be seen in **Figure 4b**. The results show that 20 min for adsorption and 40 s for desorption are acceptable for the optimal extraction and desorption, respectively.

3.5. Eluent type and volume

After liquid sample matrix was removed by using a syringe, retained molecules on sorbent should be removed by using an ideal solvent. The optimal solvent must be suitable with the chromatographic system and will not decompose molecular structure of the drugs. Various solvents were tried to find out the best solvent for both the molecules. Experimental procedure with all steps was repeated by using 1 mL of different solvent in the last step. Methanol, ethanol, acetonitrile, water, isopropanol, acetone, 50% MeOH, *n*-hexane, and mixture of acetonitrile: methanol (1:1, *v:v*) were used as desorption solvent. As can be seen in **Figure 5a**, the best signals were obtained with acetonitrile for both the molecules. After the ideal solvent was determined as acetonitrile, the next optimization procedure was volume of the solvent. The applied magnetic solid phase extraction procedure is based on the preconcentration of target molecules by means of decreasing volume of solution with extraction. The final volume of desorption solvent directly effects on the success of extraction procedure. In an ideal situation, the volume of desorption solvent should be at a minimum level for the maximum preconcentration factor which is evaluated by the first and last volume of solution, even if the recovery of drug molecules from the sorbent surface will be low due to weak interactions between solid and liquid phases. Moreover, the filtration process is not easy with volumes lower than 200 uL. Consequently, this optimization is also important for an ideal method. Volume of acetonitrile was studied in the range of 200-1500 µL. As can be seen in **Figure 5b**, the highest signals were obtained with 800 µL of acetonitrile and this volume was selected for desorption process.

3.6. Reusability of magnetic nanoparticles

As known, development of new sorbents for drug residues in various medium is challenging for new studies. The developed sorbents can be used in many different areas such as drug delivery systems, adsorption studies, and solid phase material for commercial columns, etc. One of the most important indicators for a new sorbent is its robustness and reusability. As explained in the synthesis of magnetic particles section, the used NPs was prepared step by step in order to increase its durability. All experiments were carried out by using 50 mg of sorbent. In order to test reusability of the magnetic particles, all experimental steps in the optimized conditions were repeated by using model solutions including 100 ng mL^{-1} of both drug molecules. After every use, the NPs was washed 2 mL of acetonitrile: methanol mix and 1 mL of water. 50 mg of Fe_3O_4@PPy-GO was weighed again after it was dried in 40°C. The

evaluation of reusability was carried out by comparing peak areas after every use. After 20-cycles, the change of peak area for FLU and CIT molecules was lower than 10% of RSD%.

3.7. Analytical Performances

Analytical validation of the MSPE-HPLC-DAD method was carried out after the developed magnetic solid phase extraction procedure was optimized systematically. The developed MSPE based methodology was applied to model solutions containing increasing concentrations of CIT and FLU antidepressants to determine the linear working range. The linear calibration curves for both molecules were found in the range of 5.0-500.0 ng mL^{-1}. Linearity of method describes the direct proportionality between the concentration of CIT and FLU molecules in model solutions and peak areas. 10 calibration standards in the linear range were tested for 3 replicate analysis. All the analytical figures of merit (LOD, LOQ, RSD (%), Slope of Calibration, R^2, Preconcentration Factor, and Enhancement Factor) were reported in **Table 1**. In **Table 2** were also reported the values of precision and trueness (recovery) observed for the real sample analyses, as reported in the next paragraph.

3.8. Analysis of Real Samples

Simulated urine, healthy human urine, and wastewater samples were analyzed in order to investigate the applicability of the proposed method by means of recovery tests. CIT and FLU contents of the studied samples were shown in **Table 2**. In none of the samples were detected both drug molecules. The recoveries of target molecules in the spiked samples were in the range of 96.2-104.8. Recovery values were calculated by using the ratio of the found amount of drugs to their spiked concentrations. These satisfactory results demonstrate that the prosed MSPE based HPLC-DAD method is suitable for trace determination of both drug molecules in the real samples.

3.9. Comparison of analytical figures of merit with the methods published in the literature

A comparison table with existing literature was given **Table 3**. The developed method has comparable merits with more complex approaches mass spectrometer (MS) based methods. As known, analysis cost is higher in MS based methods. The applicable linear range and convenience of simultaneous analysis of two antidepressants with the developed method are among the major advantageous features of this study. The reproducibility of the procedure highlights how this method can be effectively applied in different types of samples, both biological and environmental.

4. Conclusions

In the present work, the combined magnetic solid phase extraction procedure and HPLC-DAD method was examined as the extraction sorbent for the MSPE for CIT and FLU molecules. The proposed approach showed good sensitivity, wide linearity, simple operation, and excellent recovery for the selected antidepressant molecules. This proposed approach was successfully employed for analyzing simulated urine, healthy human urine, and wastewater samples. Briefly, polypyrrole coated NPs (Fe_3O_4@PPy-GO) was synthesized as a magnetic sorbent in SPE experiments for CIT and FLU in real samples before HPLC-DAD analysis, and several parameters were optimized to achieve optimum extraction conditions.

In an analysis, it is aimed to use less amount of organic solvent, to be fast and economical, to prepare samples easily and to take less time, to obtain efficient results, and to be environmentally friendly. The main purpose of the solid phase extraction method in accordance with these parameters is to selectively extract the components that are dissolved in the solvent medium and desired to be analyzed into a solid phase and to enrich by transferring to a lower volume of solvent phase. Thus, components that are lower than the level that the devices can determine are concentrated to the measurable levels.

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.

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593 **Figure Captions**

594 **Figure 1**. Chromatogram of antidepressant drugs reported at 227 nm (wavelength where both
595 the two molecules can be observed)

596 **Figure 2**. SEM images of the developed magnetic material (a) Low-layer graphene oxide; (b)
597 Formation of Fe_3O_4 magnetic particles by SEM-Mapping analysis; (c-f) The modification of magnetic graphene
598 oxide with polypyrrole and the formation of polypyrrole particles

599 **Figure 3.** pH effect on the developed method on analytes peak area (200 ng/mL)

600 **Figure 4**. Optimization of adsorption (A) and desorption (B) time on analytes peak area (200
601 ng/mL)

602 **Figure 5.** Optimization of desorption solvent (A) and its volume (B) on analytes peak area
603 (200 ng/mL)

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Sensitive determination of Fluoxetine and Citalopram antidepressants in urine and wastewater samples by liquid chromatography coupled with photodiode array detector

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ABSTRACT

A new analyte separation and preconcentration method for the trace determination of antidepressant drugs, Fluoxetine (FLU) and Citalopram (CIT) in urine and wastewaters, was developed based on HPLC-DAD analysis after magnetic solid phase extraction (MSPE). In the proposed method, FLU and CIT were retained on the newly synthetized magnetic sorbent (Fe_3O_4@PPy-GO) in the presence of buffer (pH 10.0) and then were desorbed into a lower volume of acetonitrile prior to the chromatographic determinations. Before HPLC analysis, all samples were filtered through a 0.45 µm PTFE filter. Experimental parameters such as interaction time, desorption solvent and volume, and pH were studied and optimized in order to establish the detection limit, linearity, enrichment factor and other analytical figures of merit under optimum operation conditions. In the developed method, FLU and CIT were analyzed by diode array detector at the corresponding maximum wavelengths of 227 and 238 nm, respectively, by using an isocratic elution of 60% pH 3.0 buffer, 30% acetonitrile, and 10% methanol. By using the optimum conditions, limit of detections for FLU and CIT were 1.58 and 1.43 ng mL^{-1}, respectively, while the limit of quantifications was 4.82 and 4.71 ng mL^{-1}, respectively. Relative standard deviations (RSD%) for triplicate analyses of model solutions containing 100 ng mL^{-1} target molecules were found to be less than 5.0%. Finally, the method was successfully applied to urine (both simulated and real healthy human) and wastewater samples, and quantitative results were obtained in recovery experiments.

Keywords: Fluoxetine, Citalopram, HPLC, Magnetic Solid Phase Extraction, Urine samples, Environmental water samples

1. Introduction

Therapeutic drug monitoring (TDM) is one of the most important research areas in drug discovery and development process used in pharmaceutical research and development. TDM of antidepressants is necessary for an optimal supervision of patient drug regimen to avoid medical non-responsiveness, intoxication, complications or noncompliance [1,2]. Antidepressants have seen exponential growth in their use during the last couple of decades. Many antidepressants act by blocking the reuptake of norepinephrine and serotine substances in the brain [3]. When their structures are examined, it is seen that most of them have tricyclic or tetracyclic nuclei. These drugs are generally used for the treatment of mental depression which has become a health problem in many parts of the society today, causes loss of productivity and workforce in many areas of life. In some cases, it imposes substantial economic losses with the treatment process. If both drugs are used before the recommended time, it causes muscle stiffness, heart rhythm, sudden changes in blood pressure, fainting, and clouding of the mind called serotonin syndrome. If it is used under the clinical supervision, the liver interacts less with the toxin enzyme [4].

Fluoxetine {*N*- methyl- 3- phenyl- 3- [4- (trifluoromethyl)phenoxy]propane- 1- amine} was the first selective serotonin reuptake inhibitor (SSRI), synthesized and marketed under the name Prozac®, produced by the pharmaceutical company Eli Lilly [5,6]. Its pharmacologically active metabolite, norfluoxetine, derived from the biological *N*- demethylation of fluoxetine. This active metabolite has prolonged action with clinical activity of inhibition of the reuptake of 5-HT and inhibition of cytochrome P450 isoenzymes in the liver. Fluoxetine is metabolized by the CYP2D6 enzyme, such as neuroleptics and tricyclic antidepressants [7]. The drugs is generally used in the treatment of diseases with similar effects belong to the antidepressant drugs group known as SSRIs.

Citalopram is a bicyclic phthalate and belongs to the SSRI family. It is a racemic drug used for the treatment of depression with the *S*-enantiomer being the pharmacologically active compound [8]. Citalopram (1-[3-(dimethyl amino) propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile is a "second generation" antidepressant drug, whose pharmacological activity is based on the selective serotonin reuptake inhibition. Its efficacy is comparable to tricyclic antidepressants, but it is better tolerated and is characterized by a lower risk of adverse effects [9].

In many drug formulations, active principle is one of the components, and in this scenario, after the drug assumption it is important to analyze in complex matrices (biological and/or environmental) the concentrations in terms of both monitoring the therapeutic dose and

monitoring the excretion products after use. Two main problems encountered in these analyses are, in most cases, the complexity of the sample matrix and the concentration of the target molecules below the detection limits of the chromatographic system. In order to overcome these problems, it is preferred to use separation and enrichment methods with a suitable carrier system.

Several methods have been published for the determination of one or more antidepressants in complex matrices (biological fluids and environmental samples) for therapeutic drug monitoring, for toxicological purposes, or for environmental pollution evaluation. The methods available in the literature for trace determination of Fluoxetine and Citalopram are based on ion transfer stripping voltammetry [10], liquid chromatography–mass spectrometry LC–MS methods [11,12] and gas chromatography–mass spectrometry (GC–MS) [13], spectrofluorometric determination [14, 15], liquid chromatography techniques [16-18], adsorptive square wave voltammetry (ASWV) [19]. However, due to the trace levels of antidepressants in complex matrices and the disruptive effects of the matrix components, clean up and preconcentration techniques have become an inevitable stage prior to the analysis of these drugs [20].

Magnetic solid phase extraction (MSPE), as a versatile approach of SPE, is carried out based on adsorption and desorption of the target molecules on a magnetic material. The used external magnetic field without some tedious steps (centrifugation or filtration) facilitates extraction steps. Sorbent particles can be easily isolated and collected during adsorption and desorption, making the sample pretreatment procedure more convenient, time-saving and cost-effective [21,22]. When magnetic solid phase extraction methods in the literature are examined, it is seen that carbon-based nanoparticles such as carbon nanotubes (CNTs), graphene oxide grafted nanostructures, nano diamond and carbon nanofibers (C-NFs), which have high surface area and adsorption capacity and can be used repeatedly due to their inertness in the working solution environments, are frequently preferred as adsorbents [22–27].

In this study, Fe_3O_4 nanoparticles (NPs) were coated with graphene oxide-polypyrrole polymer (PPy-GO) and characterized by instrumental methods. The graphene oxide was preferred as a supporting material in order to provide multi imprinting sites, large surface area, and easy separation of magnetic nanocomposites. Then, the capability of these new sorbent (Fe_3O_4@PPy-GO) for simultaneous preconcentration and determination of two widely used antidepressant drugs (Fluoxetine and Citalopram) as model compounds were studied and examined by using magnetic solid phase extraction and HPLC-DAD system. Finally, the

applicability of the proposed method was successively investigated for the extraction and determination of CIT and FLU in simulated urine, urine from healthy volunteers, and wastewater samples.

2. Materials and Methods

2.1. Instrumentation

Characterization of synthesized magnetic nanoparticles was carried out using Raman spectroscopy, X-ray diffraction spectroscopy and scanning electron microscopy techniques. The Raman spectra of the nanomaterials were obtained using a Raman Spectrophotometer (WITEC alpha 300M + micro-Raman system, Germany) with a 532 nm laser source. X-ray diffraction spectrum of magnetic nanoparticles was taken with a Bruker AXS D8 brand X-ray diffractometer. Scanning electron microscopes (SEM) and SEM Mapping analyses were performed using scanning electron microscopy (Zeiss Gemini 500 Field Emission Scanning Electron Microscope) to elucidate the morphological structures of magnetic nanoparticles. FT-IR spectra for the materials were obtained through the Perkin-Elmer Spectrum 400 FT-IR spectrometer (Waltham, MA). Chromatographic analysis of Fluoxetine and Citalopram were performed by the Shimadzu (Prominence) HPLC (Kyoto, Japan) system. All separations and determinations were performed on a phenyl hexyl column (Luna® 5 µm Phenyl-Hexyl 100 Å, 250mm × 4.6mm) under isocratic conditions.

2.2. Chemicals and reagents

In this study, all chemicals used are at the rate of 99.5% purity. Deionized water system had 18.2 MΩ cm resistivity was used to obtain deionized water (MES, MP Minipure Dest Up, Turkey). The HPLC grade acetonitrile (ACN) and methanol were used for HPLC-DAD analysis (Sigma Aldrich, St. Louis, MO, USA) without further purification steps. For HPLC analysis, were used a mixture of phosphate buffer solution (pH 3.0, 50mM), methanol and acetonitrile (60:10:30) as mobile phase under isocratic elution conditions. Stock solutions of Fluoxetine (FLU) and Citalopram (CIT) (Sigma Aldrich, St. Louis, MO, USA) were prepared in methanol and calibration mix standards were prepared by serial dilutions. FLU was racemic standard, while CIT is a pure standard.

2.3. HPLC determination conditions

A phenyl-hexyl column was used as the most suitable stationary phase in this analysis**.** In order to determine the better mobile phase compositions, the mobile phases containing buffers

at different pH values and various organic phase compositions were tested in order to obtain the most suitable conditions in terms of peak resolutions and symmetry. Optimized HPLC conditions in this work were given in **Table S1**, while in **Figure 1** was reported the chromatogram showing the peaks profile gained by increasing calibration standards concentration under the described conditions. All quantitative determinations were performed at 238 nm for CIT and 227 nm for FLU.

2.4. Synthesis of magnetic nanoparticles

Synthesis of graphene oxide by the Hummer method

3.0 g of graphite powder was added to the flask, which was cooled to 0°C in an ice bath, and 70 mL of concentrated sulfuric acid (≥ 99.9%) was slowly transferred on graphite. Under vigorous stirring, 9.0 g of potassium permanganate, $KMnO_4$, was added to this reaction mixture and the reaction temperature was kept around 20°C for 30 minutes. Then, the reaction mixture was stirred at 40°C for 30 minutes more. 150 mL of deionized water was added to the mixture and the reaction temperature was raised to 95°C on a magnetic stirrer. After refluxing the reaction mixture at 95°C for 15 minutes, 500 mL of deionized water and 15 mL of 30% (*w/v*) H_2O_2, hydrogen peroxide, were added to the reaction mixture, and the reaction was allowed to continue for 10 minutes. After this step, the reaction mass was cooled to room temperature. The brown-yellow reaction mixture was filtered and washed with 10% (*w/v)* hydrochloride acid, to remove unreacted reagents. The product obtained was then dried in an oven at 50°C for 24 hours.

Synthesis of magnetite graphene oxide

0.5 g of graphene oxide, which was synthesized in the previous step, was pulverized and weighed carefully. A mixture of 0.5 g of Iron (III) chloride and 2.0 g of sodium acetate, previously homogenized in 20 mL ethylene glycol, was added to the graphene oxide particles. After being kept in an ultrasonic bath for 10 minutes, it was transferred to an autoclave for hydrothermal synthesis. The hydrothermal synthesis unit was allowed to react at 180°C for 12 hours. After the reaction, the product was washed twice with ethanol and once with deionized water and allowed to dry in an oven at 70°C.

Synthesis of magnetite graphene oxide-poly pyrrole (PPy) nano composite material (Magnetic PPy/GO)

0.5 g of the synthesized magnetite graphene oxide was weighed and dispersed in 200 mL of deionized water. Later, 500 µL of pyrrole was added to the mixture in an ice bath and 1.6 g of ammonium persulfate solution dissolved in 10 mL of water. This solution was added dropwise to the reaction medium. Stirring was continued until the reaction was complete. The synthesis product formed on the surface of the solution was separated from the mixture by filtration. It was washed 2 times with deionized water during the filtration process. It was left to dry in an oven at 70°C.

2.5. Magnetic solid phase extraction

50 mg of Fe_3O_4@PPy-GO was weighed and transferred to 50 mL of falcon tubes. Then, 20 mL of sample solution including FLU and CIT in the range of 5.0-500.0 ng mL^{-1} and the volume of the tube was completed to 50 mL with distilled water. Falcon tubes were tightly closed and placed in orbital shaker device by setting 100 rpm for 20 minutes. After the time was over, magnetic particles were separated by using an external magnet, then 800 µL of acetonitrile was added and the tubes were vortexed for 40 seconds for the target molecules desorption. The ACN phase were taken into an injector, the 0.45 µm injector tip was passed through the filter and transferred to the vials and placed in the HPLC device. The contents of samples for Fluoxetine and Citalopram enriched were determined by HPLC-DAD system.

2.6. Preparation of simulated urine samples and wastewater samples

The application of proposed method was carried out by simulated urine, healthy human urine, and wastewater samples. Content of simulated urine samples was prepared as mentioned in literature [28–30]. 25.00 g of urea, 1.08 g of calcium chloride, 1.00 g of ammonium chloride, 1.60 g of potassium chloride, 1.40 g of sodium sulfate, 1.40 g of potassium dihydrogen phosphate, and 2.92 g of sodium chloride were dissolved in 1 L of ultra-pure water. The pH of simulated urine solution was adjusted to 6.0 using sodium hydroxide (0.1 M) or hydrochloride acid (0.1 M). The mixture was stirred on a magnetic stirrer for 15 min and kept in an ultrasonic water-bath. Then, the solution was diluted at 1:2 and 1:4 ratios. The obtained solutions were stored in amber glass bottles until analysis.

The healthy human urine samples were collected in a capped sterile test tube from volunteers free from any kind of medication who had been informed about the experimental procedure and the nature of the study. All samples were left at room temperature for 20 min and then centrifuged for 10 min at 4000 rpm [31].

Wastewater sample was obtained from main wastewater discharge line of University in Sivas, Turkey. Wastewater samples were collected in amber glass bottles and immediately filtered through 0.45 µm cellulose nitrate membrane. Subsequently, pH of samples were adjusted to 3 to reduce biological activity [32] and were stored in the dark at +4°C until analysis.

2.7. Method validation

The method validation was carried out according to International Conference on Harmonization guidelines [33-34]. Analytical figures of merit such as enhancement factor (EF), preconcentration factor (PF), relative standard deviation (RSD), limit of detection (LOD), limit of quantification (LOQ), linear range and correlation coefficient were calculated by considering correctness and sensitivity of the method.

Preconcentration factors (PF) were calculated by using the ratio of the initial solution volume (50 mL) to the last elution solvent volume (0.8 mL). The enhancement factors (EF) were obtained from the ratio of the slope of calibration curve of the analytes after MSPE application to that of prior MSPE application. The relative standard deviations (RSD%) were found by applying the MSPE method for seven repetition analysis, which includes 100 ng mL^{-1} of CIT and FLU. The LODs and LOQs values herein reported were obtained by means of the signal-to-noise ratio. Specifically, LOD was defined by a signal-to-noise ratio of 3:1, while the LOQ was defined by a signal-to-noise ratio of 10:1, accordingly to International Guidelines [34].

3. Results and Discussions

3.1.Characterization of the magnetic nanoparticles

The results of FTIR analysis for the graphene oxide, magnetic graphene oxide and polypyrrole magnetic graphene oxide components used in the synthesis of the magnetite graphene oxide-polypyrrole nanomaterial material were given in the **Figure S1**. The FTIR spectrum of graphene oxide is compatible with other studies currently available in the literature. Characteristic peaks of graphene oxide were detected: (C-O-C) (1230-1320 cm^{-1}), sp^2-hybrid C=C (1500-1600 cm^{-1}, in-plane vibrations), (COOH) (1650-1750 cm^{-1}, 3530 cm^{-1} carboxyl vibration modes.

Magnetic graphene oxide peaks are 588 cm^{-1}, known as the Fe-O characteristic peak, 1651 cm^{-1} (C=O) symmetrical stretching vibration peaks at 1085 cm^{-1} (C-O). In FTIR analysis of magnetic graphene oxide-polypyrrole nanomaterial as the end product of the synthesis, NH symmetric stretching vibration at 3271 cm^{-1} wavelengths, 3123 cm^{-1} (OH), 1714 cm^{-1}, 1614 cm^{-}

[1] C=O stretching peaks with bending vibration of 1219 cm^{-1} (C-N)and 978 cm^{-1} (C-N) can be attributed to the presence of polypyrrole in the composite material.

In the SEM images given in **Figure 2** of the magnetic graphene oxide-polypyrrole nanomaterial, the layer in the form of a web cover, which is seen to corroborate with the studies in the literature, is known as "graphene oxide". It can be seen from the transparent SEM images that low-layer graphene oxide is successfully produced from graphite (**Figure 2a**). The formation of Fe_3O_4 magnetic particles has been proven by SEM-Mapping analysis of Fe (**Figure 2b**). As a result of the modification of magnetic graphene oxide with polypyrrole, the formation of polypyrrole particles is observed (**Figure 2c-f**).

Figure S2a shows the characteristic peaks of the synthesized graphene oxide (GO) in the D and G bands, which are compatible with the literature. It is easily understood from the fact that the D band of GO is more dominant than the G band where graphene oxide is successfully synthesized from graphite. **Figure S2b** contains the Raman spectrum of magnetic graphene oxide. In the spectrum, it was observed that magnetite graphene oxide was synthesized from graphene oxide, and the suppression in the D and G bands can be clearly seen from the change in the ratio of the peaks to each other. **Figure S2c** shows the Raman spectrum of the synthesis of pyrrole and magnetite graphene oxide. It was observed that the peaks at wavelengths of 978 cm^{-1} and 1047 cm^{-1} belong N-H bonds in pyrrole in the literature and that these peaks originated from pyrrole were formed in this spectrum and the desired structure was obtained as a result of the synthesis reaction. In **Figure S2d**, it is seen that the spectra of magnetite graphene oxide and magnetite graphene oxide-pyrrole synthesis are overlapped, and the Raman shifts and the change in D and G band ratios can be easily noticed.

3.2. Extraction optimization experiments

The objective is to keep the analyte type in the solid phase at the highest possible level and to separate it from other substances in the environment, and after the separation process is achieved, all of the analytes in the solid phase pass into the solvent. Preliminary trials were made to determine the necessary parameters to achieve this. It was aimed to obtain a fast and easy separation process and to obtain the highest concentration of analyte by using as little amount of organic solvent as possible. Thus, it was brought to the concentration range that the HPLC device can read. Accordingly, a chromatographic method was developed by optimizing all parameters.

3.3. pH effect

Ambient pH is an important factor as it affects the adhesion of the analyte to the solid phase and the reactions between species. Model solutions containing both antidepressants respectively were interacted with a series solution in the range of 2.0-12.0. All experiments were studied with 50 mg of Fe_3O_4@PPy-GO. Following these processes, FLU and CIT molecules were retained on solid phase and separated with an external magnet. After desorption of target molecules, eluent solvent phase was transferred by a syringe and filtered through a 0.45 μm PTFE membrane filter, transferred into HPLC vials and subsequently injected into the HPLC system.

As can be illustrated in **Figure 3**, the optimum pH value for the enrichment steps was observed at a pH value of 10.0. A literature review revealed that, the pKa value for Fluoxetine is 8.70, while the pKa value for citalopram is 9.50 [35]. As can be seen in the Figure 3, the efficiency of extraction reaches to maximum beyond these values. Therefore, pH 10.0 was selected as the optimum for subsequent experiments.

3.4. desorption solvent and time

Adsorption and desorption processes of drug molecules on the surface of magnetic nanoparticles are carried out by means of an equilibrium process, guided by the partition coefficient of the analyte between the aqueous sample matrix and the magnetic nanoparticle-based sorbent. Generally, binding of the target molecules to solid sorbent needs longer time due to the slow diffusion of the analytes into the solid sorbent and the sluggish rate of the mass transfer of analytes from the bulk to the sorbent in absence of any external energetic stimuli. To increase the mass transfer kinetics, this process was facilitated with an orbital shaker or a rotator by means of increasing interactions between sorbent and molecules. Desorption process is faster than adsorption because a pure and clean solvent is used to remove molecules directly from the sorbent surface. Rate and efficiency of desorption are increased by using vortex at high speed (up to 100 rpm). Time for both the processes should be optimized in order to find optimal extraction conditions. The first step of extraction (adsorption) was carried out by using an orbital shaker with 100 rpm. Model solutions including both molecules and adsorption time was studied in the range of 0-90 minutes. As can be seen in **Figure 4a**, 20 min is enough for high recovery. Desorption time on the vortex was also studied in the range of 0-90 seconds as can be seen in **Figure 4b**. The results show that 20 min for adsorption and 40 s for desorption are acceptable for the optimal extraction and desorption, respectively.

3.5. Eluent type and volume

After liquid sample matrix was removed by using a syringe, retained molecules on sorbent should be removed by using an ideal solvent. The optimal solvent must be suitable with the chromatographic system and will not decompose molecular structure of the drugs. Various solvents were tried to find out the best solvent for both the molecules. Experimental procedure with all steps was repeated by using 1 mL of different solvent in the last step. Methanol, ethanol, acetonitrile, water, isopropanol, acetone, 50% MeOH, *n*-hexane, and mixture of acetonitrile: methanol (1:1, *v:v*) were used as desorption solvent. As can be seen in **Figure 5a**, the best signals were obtained with acetonitrile for both the molecules. After the ideal solvent was determined as acetonitrile, the next optimization procedure was volume of the solvent. The applied magnetic solid phase extraction procedure is based on the preconcentration of target molecules by means of decreasing volume of solution with extraction. The final volume of desorption solvent directly effects on the success of extraction procedure. In an ideal situation, the volume of desorption solvent should be at a minimum level for the maximum preconcentration factor which is evaluated by the first and last volume of solution, even if the recovery of drug molecules from the sorbent surface will be low due to weak interactions between solid and liquid phases. Moreover, the filtration process is not easy with volumes lower than 200 uL. Consequently, this optimization is also important for an ideal method. Volume of acetonitrile was studied in the range of 200-1500 µL. As can be seen in **Figure 5b**, the highest signals were obtained with 800 µL of acetonitrile and this volume was selected for desorption process.

3.6. Reusability of magnetic nanoparticles

As known, development of new sorbents for drug residues in various medium is challenging for new studies. The developed sorbents can be used in many different areas such as drug delivery systems, adsorption studies, and solid phase material for commercial columns, etc. One of the most important indicators for a new sorbent is its robustness and reusability. As explained in the synthesis of magnetic particles section, the used NPs was prepared step by step in order to increase its durability. All experiments were carried out by using 50 mg of sorbent. In order to test reusability of the magnetic particles, all experimental steps in the optimized conditions were repeated by using model solutions including 100 ng mL^{-1} of both drug molecules. After every use, the NPs was washed 2 mL of acetonitrile: methanol mix and 1 mL of water. 50 mg of Fe_3O_4@PPy-GO was weighed again after it was dried in 40°C. The

evaluation of reusability was carried out by comparing peak areas after every use. After 20-cycles, the change of peak area for FLU and CIT molecules was lower than 10% of RSD%.

3.7. Analytical Performances

Analytical validation of the MSPE-HPLC-DAD method was carried out after the developed magnetic solid phase extraction procedure was optimized systematically. The developed MSPE based methodology was applied to model solutions containing increasing concentrations of CIT and FLU antidepressants to determine the linear working range. The linear calibration curves for both molecules were found in the range of 5.0-500.0 ng mL^{-1}. Linearity of method describes the direct proportionality between the concentration of CIT and FLU molecules in model solutions and peak areas. 10 calibration standards in the linear range were tested for 3 replicate analysis. All the analytical figures of merit (LOD, LOQ, RSD (%), Slope of Calibration, R^2, Preconcentration Factor, and Enhancement Factor) were reported in **Table 1**. In **Table 2** were also reported the values of precision and trueness (recovery) observed for the real sample analyses, as reported in the next paragraph.

3.8. Analysis of Real Samples

Simulated urine, healthy human urine, and wastewater samples were analyzed in order to investigate the applicability of the proposed method by means of recovery tests. CIT and FLU contents of the studied samples were shown in **Table 2**. In none of the samples were detected both drug molecules. The recoveries of target molecules in the spiked samples were in the range of 96.2-104.8. Recovery values were calculated by using the ratio of the found amount of drugs to their spiked concentrations. These satisfactory results demonstrate that the prosed MSPE based HPLC-DAD method is suitable for trace determination of both drug molecules in the real samples.

3.9. Comparison of analytical figures of merit with the methods published in the literature

A comparison table with existing literature was given **Table 3**. The developed method has comparable merits with more complex approaches mass spectrometer (MS) based methods. As known, analysis cost is higher in MS based methods. The applicable linear range and convenience of simultaneous analysis of two antidepressants with the developed method are among the major advantageous features of this study. The reproducibility of the procedure highlights how this method can be effectively applied in different types of samples, both biological and environmental.

4. Conclusions

In the present work, the combined magnetic solid phase extraction procedure and HPLC-DAD method was examined as the extraction sorbent for the MSPE for CIT and FLU molecules. The proposed approach showed good sensitivity, wide linearity, simple operation, and excellent recovery for the selected antidepressant molecules. This proposed approach was successfully employed for analyzing simulated urine, healthy human urine, and wastewater samples. Briefly, polypyrrole coated NPs (Fe_3O_4@PPy-GO) was synthesized as a magnetic sorbent in SPE experiments for CIT and FLU in real samples before HPLC-DAD analysis, and several parameters were optimized to achieve optimum extraction conditions.

In an analysis, it is aimed to use less amount of organic solvent, to be fast and economical, to prepare samples easily and to take less time, to obtain efficient results, and to be environmentally friendly. The main purpose of the solid phase extraction method in accordance with these parameters is to selectively extract the components that are dissolved in the solvent medium and desired to be analyzed into a solid phase and to enrich by transferring to a lower volume of solvent phase. Thus, components that are lower than the level that the devices can determine are concentrated to the measurable levels.

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.

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593 **Figure Captions**

594 **Figure 1**. Chromatogram of antidepressant drugs reported at 227 nm (wavelength where both
595 the two molecules can be observed)

596 **Figure 2**. SEM images of the developed magnetic material (a) Low-layer graphene oxide; (b)
597 Formation of Fe_3O_4 magnetic particles by SEM-Mapping analysis; (c-f) The modification of magnetic graphene
598 oxide with polypyrrole and the formation of polypyrrole particles

599 **Figure 3.** pH effect on the developed method on analytes peak area (200 ng/mL)

600 **Figure 4**. Optimization of adsorption (A) and desorption (B) time on analytes peak area (200
601 ng/mL)

602 **Figure 5.** Optimization of desorption solvent (A) and its volume (B) on analytes peak area
603 (200 ng/mL)

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Figure 1

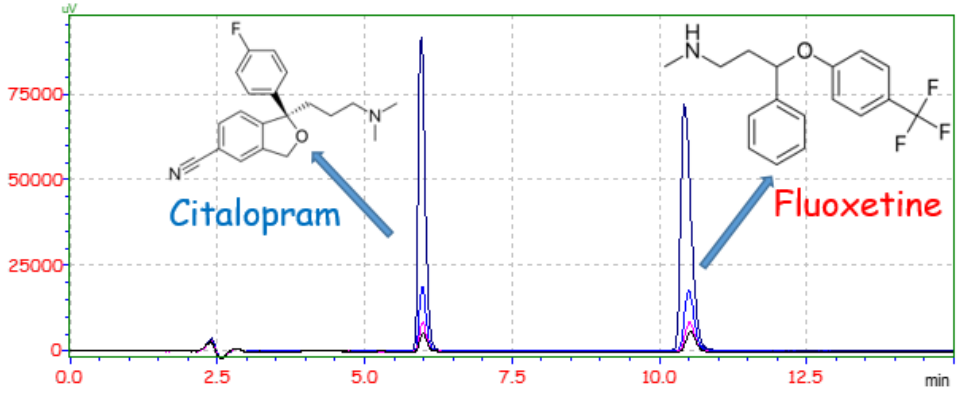


Figure 2

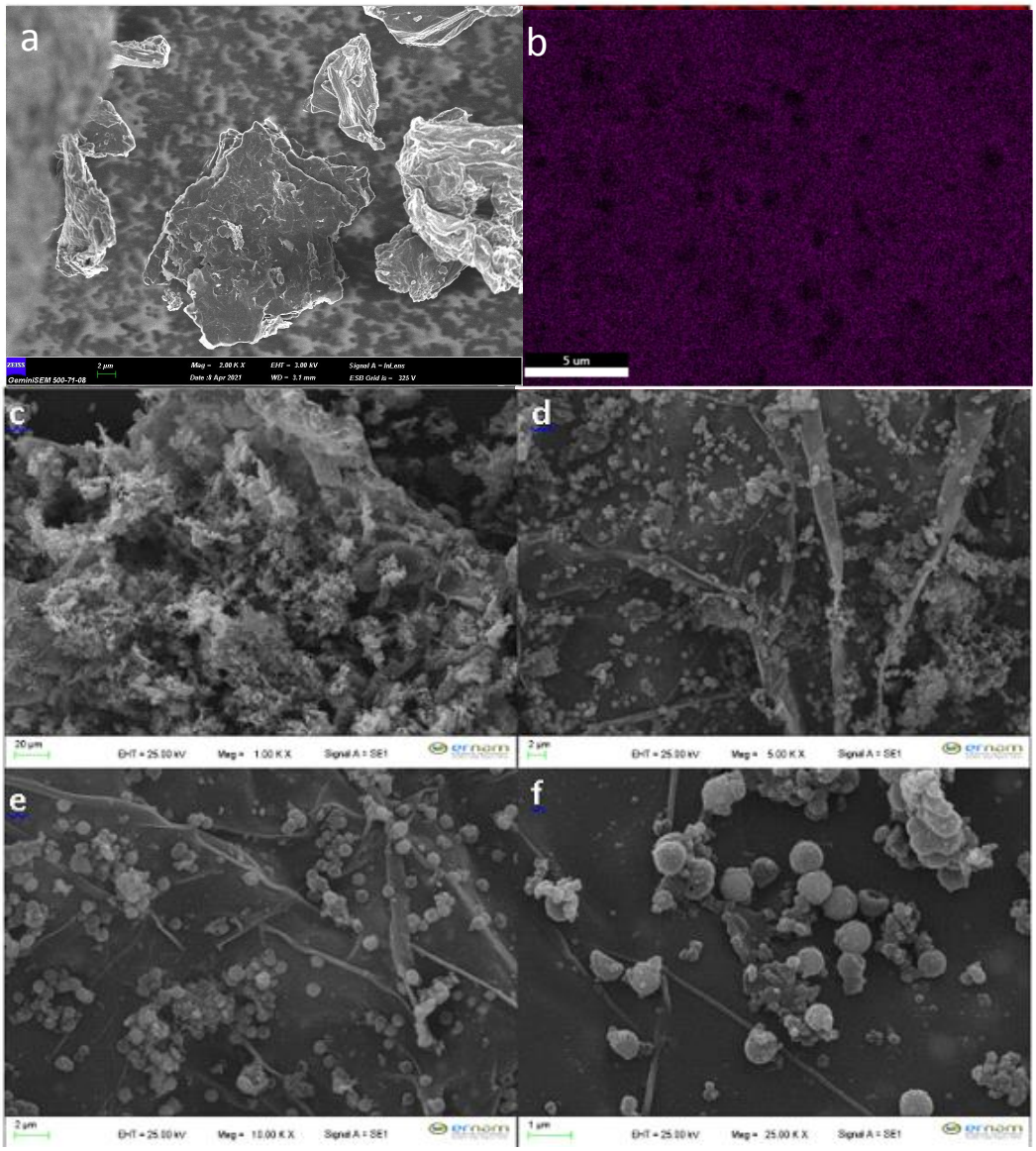


Figure 2. SEM images of the developed magnetic material

a) Low-layer graphene oxide

b) Formation of Fe_3O_4 magnetic particles by SEM-Mapping analysis

c-f) The modification of magnetic graphene oxide with polypyrrole and the formation of polypyrrole particles

Figure 3

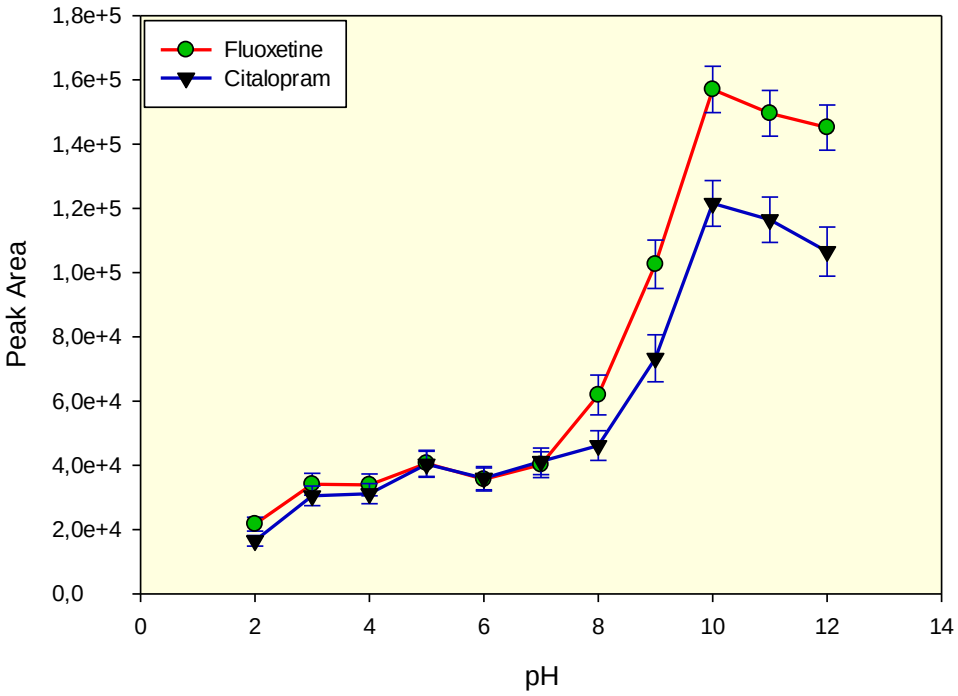


Figure 4

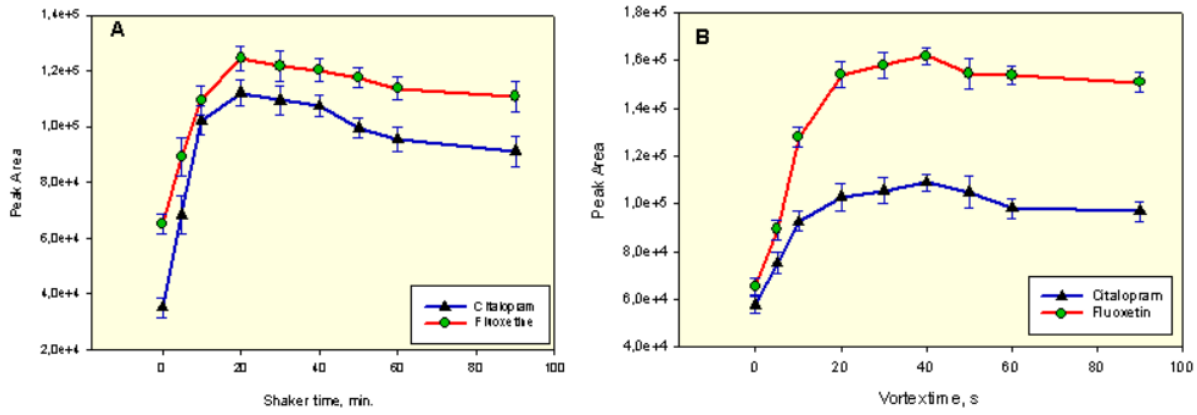


Figure 5

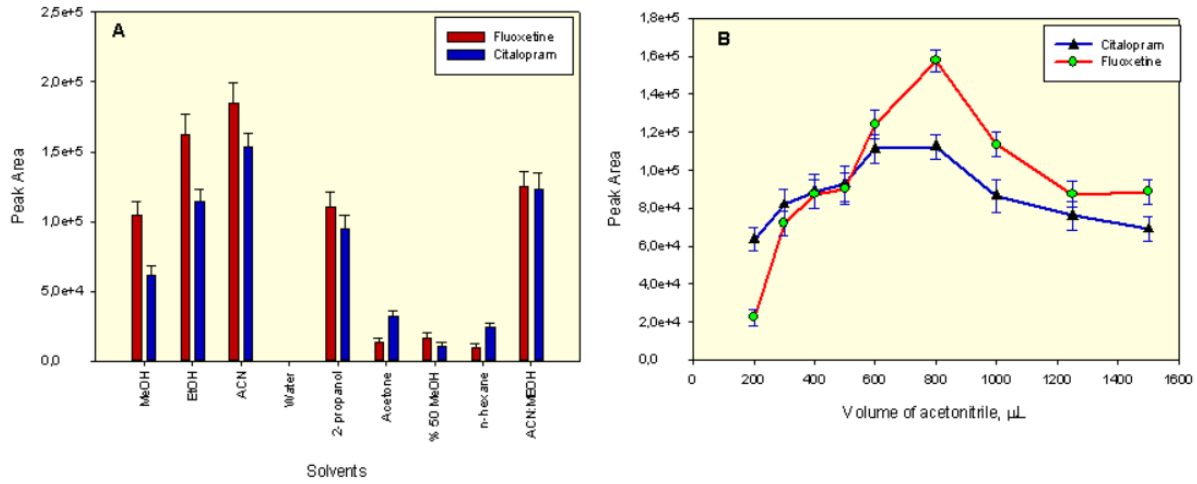


Table 1. Analytical figures of merit of the new method

Parameter	Before MSPE		After MSPE	
	Fluoxetine	**Citalopram**	**Fluoxetine**	**Citalopram**
Linearity	1.0-20.0 μg mL^{-1}	1.0-20.0 μg mL^{-1}	5.0-500.0 ng mL^{-1}	5.0-500.0 ng mL^{-1}
LOD	0.38 μg mL^{-1}	0.32 μg mL^{-1}	1.58 ng mL^{-1}	1.43 ng mL^{-1}
LOQ	1.88 μg mL^{-1}	1.90 μg mL^{-1}	4.82 ng mL^{-1}	4.71 ng mL^{-1}
RSD (%)	4.7	3.8	3.2	3.5
Slope of Calibration	18.27	15.27	1425.14	1266.99
(R^2)	0.9975	0.9986	0.9954	0.9873
Preconcentration Factor	-	-	62.5	62.5
Enhancement Factor	-	-	78	83

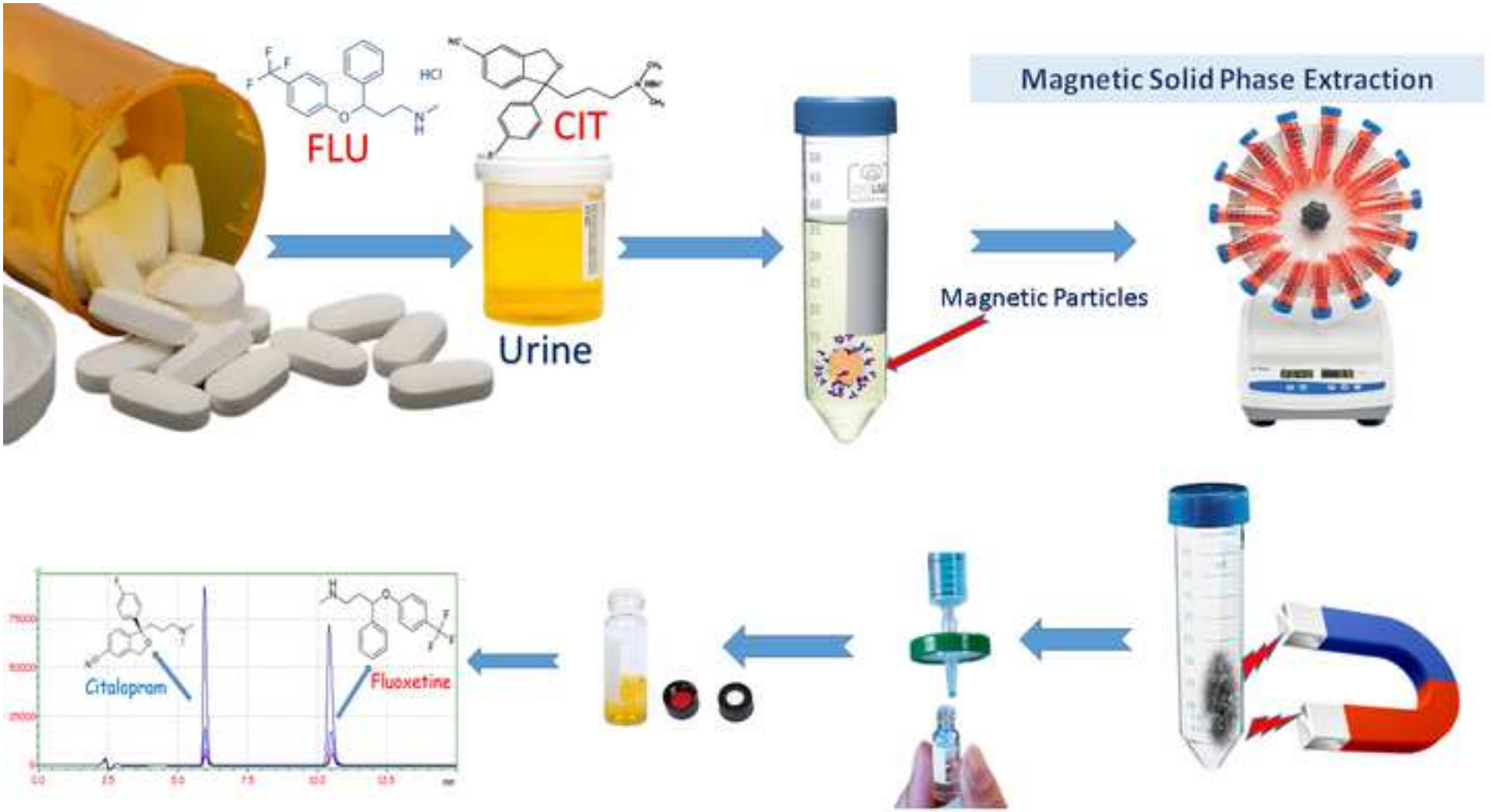
Table 2. Analytical results obtained from real sample analyses using the developed method

Samples	Added ng mL^{-1}	Found [a] ng mL^{-1}		RSD%		Recovery %	
		Fluoxetine	Citalopram	Fluoxetine	Citalopram	Fluoxetine	Citalopram
Simulated Urine	0.0	<LOD	<LOD	-	-	-	-
	100.0	98.7±4.1	104.8±4.5	4.2	4.3	98.7	104.8
	250.0	255.1±12.5	240.5±11.5	4.9	4.8	102.0	96.2
Urine 1	0.0	<LOD	<LOD	-	-	-	-
	100.0	95.4±3.8	98.7±3.5	3.9	3.5	95.4	98.7
	250.0	242.7±10.5	246.8±9.7	4.3	4.8	97.1	98.7
Urine 2	0.0	<LOD	<LOD	-	-	-	-
	100.0	106.7±5.0	95.8±3.7	4.2	3.9	106.7	95.8
	250.0	239.4±11.1	253.2±10.7	4.6	4.2	95.7	101.3
Wastewater 1	0.0	<LOD	<LOD	-	-	-	-
	100.0	99.8±4.8	98.8±3.5	4.8	3.5	99.8	98.8
	250.0	242.5±11.2	255.9±12.8	4.6	5.0	97.0	102.4
Wastewater 2	0.0	<LOD	<LOD	-	-	-	-
	100.0	103.5 ±3.6	104.5±3.7	3.5	3.5	103.5	104.5
	250.0	259.5±12.7	260.1±12.5	4.9	4.8	103.8	104.0

[a]The average value of five replicates ± standard deviation (N=5)

Table 3. Comparison of the analytical figures of merit of the new method with other methods published in the literature

Target Molecules	Pre-treatment Procedure	Determination Method	LOD	Linearity	Samples	References
FLU	SPE	HPLC	25 ng mL^{-1}	25-500 ng mL^{-1}	Human Plasma	[36]
CIT FLU	SPE	Capillary Chromatography	8.1 ng L^{-1} 9.3 ng L^{-1}	48.6-243.0 ng mL^{-1} 49.4-463.5 ng mL^{-1}	Human Plasma	[37]
CIT FLU	SPE	LC–MS/MS	25 ng L^{-1}	2–346 ng L^{-1}	Spring and waste water	[33]
FLU	Liquid-liquid microextraction	GC–MS	3 ng mL^{-1}	10 - 500 ng mL^{-1}	Plasma	[38]
FLU	Stir Bar Sorptive Extraction	LC-MS	3 ng mL^{-1}	10–500 ng mL^{-1}	Human Plasma	[39]
CIT	Micro Solid phase extraction	HPLC-UV	0.2–1.0 ng mL^{-1}	2-800 ng mL^{-1}	Biologic samples	[40]
CIT	SPE	Chiral HPLC Method	10 ng mL^{-1}	100-500 ng mL^{-1}	Human Plasma	[41]
CIT	Molecular imprinting polymer/SPE	HPLC	0.5 ng L^{-1}	2–120 μg L^{-1}	Human Plasma and urine	[42]
CIT FLU	Micro solid phase extraction	HPLC	10 ng mL^{-1}	50-2000 ng mL^{-1}	Human urine	[43]
CIT FLU	MSPE	HPLC	1.58 ng mL^{-1} 1.43 ng mL^{-1}	5-500 ng mL^{-1} 5-500 ng mL^{-1}	Urine and waste water	This method



Authors credit Statement

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Declaration of interests

☒ 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.

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