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OPTIMIZATION OF A MULTI-STEP EXTRACTION METHOD IN ANALYTICAL CHEMISTRY

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ABSTRACT

The preparation of the sample is a very important procedure in the field of analytical chemistry especially where the analytes of interest are present in trace amounts in complex samples. In this paper, the optimization and development of a multi-step extraction process is introduced through a systematic and statistically justified process. The adaptation of a fractional factorial design was initially used to filter out important extraction parameters, and response surface methodology was then used to determine the most desirable conditions and determine the effects of interactions. The optimised procedure employed acetonitrile as the extraction solvent, 18 mL as the volume of solvent and 6.2 to indicate the pH of the sample, which made the average analyte recovery to be 93.8 + 1.5 percent with the relative standard deviations less than 3 percent. The validation of the method showed that it had a high linearity (R 2 = 0.998), low limits of detection and quantification (0.12 ugkg -1 and 0.40 ugkg -1, respectively), and a high degree of accuracy, over the various concentrations of the experiment. The streamlined protocol did not only improve the efficiency and reproducibility of extraction, but also reduced the use of solvent and the workload of the experiment. The results obtained indicated the usefulness of multivariate optimization schemes in enhancing multi-step extraction procedures, which is a sound and viable mechanism of analyzing traces in intricate matrices.

KEYWORDS: Multi-step extraction, Analytical chemistry, Method optimization, Response surface methodology, Trace analysis, Recovery, Solvent selection, Sample preparation.

INTRODUCTION

Introduction The field of analytical chemistry is central in a broad area of the sciences such as environmental control, pharmaceutical studies, food safety, judicial studies and in biomedical research. Effectiveness and strength of sample preparation procedures is particularly vital in the reliability of the analytical results when the target analytes are found at trace and ultra-trace concentrations in complex matrices.[1] This is because most of the analytical workflow processes include only a few processes, with extraction process being ranked as the most important in terms of influencing the recovery of the analyte, its selectivity and sensitivity and general methodology performance. The multi-step extraction procedures have been extensively used to increase the quality of analysis by integrating sequential mechanisms like pre-treatment, clean-up, focus on, and removance of target compounds. They are particularly useful in the analysis of complex samples that may include interfering substances that may compromise the method of analysis.[2] Nevertheless, regardless of their benefits, multi-step extraction methods are time consuming, solvent intensive and prone to cumulative errors unless properly designed and optimized. As a result, the creation and improvement of effective multi-step extraction strategies is a significant issue in the contemporary analytical chemistry.[3] The goals of optimization of extraction methods include maximizing the recovery of the analyte and reducing analyte matrix effects, solvent usage, processing time, and variability of the experiment. The conventional methods of extraction optimization used to be based on trial and error experiments or one-factor-at-a-time (OFAT) models. Although such methods may give initial information, they are restricted in their capacity to detect interactions between variables and in most cases they require a huge number of experiments.[4] By contrast, a more effective and scientifically rigorous method of developing

methods can be achieved through systematic optimization strategies through experiment design and statistical analysis. Some of the most important parameters affecting the performance of multi-step extraction processes are type and volume of solvent, time of extraction, pH, temperature, agitation, and extraction cycles.[5] Moreover, the order and the complexity of individual extraction processes could be an important factor in defining the total efficiency. Misuse of the steps can cause loss, degradation or partial separation of the analyte with the components of the matrix. Thus, the combination of the individual parameters and their mutual influence should be taken into account to create the reliable and reproducible results of the analysis in a holistic manner.[6] The current developments of analytical instrumentations have led to a big enhancement in the limits of detection and selectivity, but these enhancements cannot address insufficient sample preparation.[7] Even the most advanced analysis systems are predisposed to matrix interferences that are provided in the process of poor extraction. Consequently, method optimization at the extraction level is one of the conditions of assuring data quality, method validation, and regulation acceptance. It is especially so in the everyday analytical laboratories, where the methods need to be high performing, practical, cost-effective and durable.[8] Besides the performance of analysis, environmental and safety concerns have also gained relevant value in terms of the development of the methods. The increased focus of green analytical chemistry facilitates the minimization of dangerous solvents, energy, and waste.[9] By streamlining multi-step extraction protocols and looking at how to maximize the efficiency of the analysis in both efficiency and sustainability, the reduction of the use of solvents and streamlined workflows without affecting the quality of work is an opportunity.[10] A significant amount of literature exists on the methods of extracting individuals but there remain systematic studies to be made on the optimization of multi-step methods of extraction.[11] Numerous of the published techniques declare good performance performance; most of them are not thoroughly optimized or validated, and this limits their reproducibility and general applicability. To cover this gap, a systematic method is needed which is a combination of experimental design, analytical validation, and comparative evaluation with the current methods.[12] The objective of the current research is the design and optimization of a multi-step extraction technique in analysis applications in a systematic and statistically validated methodology. The experiment aims at assessing the main extraction parameters, determining the most suitable ones, and proving the quality of the analysis of the optimized method in terms of recovery, precision, accuracy, and sensitivity.[13] The work is aimed at making a contribution to the progress of effective and valid methods of extraction in analytical chemistry by offering an elaborate optimization framework and realistic experimental results.[14]

MATERIALS AND METHODS

2.1 Chemicals and Reagents

Chemicals and reagents that were employed in this investigation were of analytical grade or HPLC grade and were not purified any further. Sigma-Aldrich (USA) was the source of methanol, acetone, n-hexane, ethyl acetate and acetitrile. Merck (Germany) provided hydrochloric acid (HCl, 37%), NaOH in the form of pellets. A Milli-Q purification system (resistivity ≥ 18.2 M 0 Cmm) was used to obtain deionized water. Serial dilution of a certified reference material with proper solvents was done to make standard solutions of the target analyte that were stored at 4 o C until required.[15]

2.2 Instrumentation

The quantitative analysis was done through utilizing a high-performance liquid chromatography (HPLC) system that had a quaternary pump, an autosampler, a column oven, and UV-vis detector. The separation was done through chromatography with C18 reversed phase column (250 mm × 4.6 mm, 5 µm particle size).[16]. The manufacturer software was used to process and acquire data. pH adjustment was done with a digital pH meter (accuracy ±0.01), centrifuge with a 6000 rpm maximum speed was used in the preparation of samples, and an ultrasonic bath. The solvent evaporation was performed with the help of nitrogen stream evaporator and controlled temperature.[17]

2.3 Sample Preparation

Homogenization of representative samples was carried out before extraction in order to have equal distribution of the analyte. The weight of a fixed amount of 5.00 g of the sample was weighed into polypropylene centrifuge tubes with accuracy. All the samples used were also prepared in triplicates under the same conditions to remove variability. Sampling Before extraction, known concentrations of the target analyte were added to the samples to perform recovery and validation studies. The pH of the samples was brought to the desired pH using the dilute HCl or NaOH as per the experimental design.[18]

2.4 Multi-Step Extraction Procedure

The recommended extraction procedure involved three successive processes which are, initial solvent extraction, clean-up, and concentration. The predefined amount of extraction solvent was injected into the ready sample of the first step, and mixed by means of vortex mixing the sample within 2 min and sonication during a given extraction time. It was then centrifuged at 5000 rpm in 10 min after which the supernatant was carefully collected.[19]

The second step involved a clean-up process of the collected extract with the aim of eliminating the matrix interferences. This was done through liquid-liquid partitioning with the help of a secondary solvent system that was chosen on the basis of polarity. The separation was done and the organic phase containing the analyte

dried with anhydrous sodium sulfate.

The last step involved the concentration of the purified extract under a slow stream of nitrogen at 40 o C until it became almost dry after which it was diluted at a fixed volume using a mobile phase and then subjected to instrumental analysis. An injection into the HPLC system was performed through the filtration of all extracts using 0.45 µm membrane filters.[20]

2.5 Optimization Experimental Design

Systematic experimental design was used in the optimization of the multi-step extraction method. The choice of the key extraction parameters relied on the preliminary experiments and literature reports.[21] These parameters were the type of solvent used in extraction, solvent volume, extraction time, the pH of the sample and the number of extraction cycle.[22]

An initial veto of important variables influencing the extraction efficiency was undertaken by a fractional factorial design. Thereafter, a central composite design (CCD) was used as the basis of a response surface methodology (RSM) to establish the most favorable conditions and also to test interrelations among variables. The primary response variable that was chosen was extraction efficiency that was expressed as percentage recovery of the target analyte.[23]

2.6 Analytical Method Validation

The optimal extraction procedure was tested on the basis of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision according to the international standards of conducting an analysis. Based on a specific range of concentration, matrix-matched standards were used to construct calibration curves. Inter-day and intra-day accuracy were tested using the spiked samples of three concentration levels. Recovery studies under optimal conditions were used to test accuracy.[24]

2.7 Statistical Analysis

Each experiment was repeated thrice and results published in the form of a mean with standard deviation. The statistical analysis and optimization modelling were done with specific statistical packages. Analysis of variance (ANOVA) was used as a method to determine the significance of the model terms and extraction parameters. The confidence level applied during the research was 95% ($p < 0.05$).[25]

3. RESULTS AND DISCUSSION

3.1 Preliminary Evaluation of the Multi-Step Extraction Method

The first experiments were done to determine the baseline performance of the proposed multi-step extraction procedure before the official optimization. The process under initial conditions (methanol as solvent of extraction, 15 mL of solvent, 20 min of extraction time, pH of 7.0 in the sample, and one extraction cycle) provided an average analyte recovery of 71.4 +3.2. It was tolerable when used upstream to screen initial but some clear variability was found especially in samples with more complex matrices.[26] Chromatographic quality The target analyte was clearly separated, without any significant co-eluting peaks; although moderate baseline noise was observed, indicating that the matrix interferences were not completely removed during the clean-up procedure. These initial results proved that the suggested multi-step extraction strategy was feasible yet dependent on systematic optimization. The medium-range recovery and variability was a sign that some of the extraction parameters were not running optimally. Specifically, solvent choice, extraction period, and pH of the sample were supposed to be important factors in enhancing the partitioning of analytes and suppression of the matrix. As such, an experimental design was considered to be structured in order to improve the efficiency of extraction and the robustness of the method.[27]

Screening of Significant Extraction Parameters

The fractional factorial design was used to determine the most influential parameters that influence the extraction efficiency. The five variables were tested at two levels, namely, the extraction solvent (methanol vs. acetonitrile), solvent volume (10- 20 mL), extraction time (10- 30 min), the pH of the sample (5- 9), and the extraction cycles (1-3). The statistical analysis of the screening results indicated that the extraction solvent type, the volume of the solvent, and the pH of the sample sample had a statistically significant impact on the analyte recovery ($p < 0.05$), whereas the influence of extraction time and number of cycles was comparatively lower over the range of experimental values. Table 1 presents a summary of the primary impacts of the screened variables on extraction recovery.

Table 1: Main effects of extraction parameters on analyte recovery during screening experiments.

Parameter	Low Level	High Level	Effect on Recovery (%)
Solvent type	Methanol	Acetonitrile	+9.6
Solvent volume (mL)	10	20	+6.8
Sample pH	5	9	−11.2
Extraction time (min)	10	30	+2.1
Extraction cycles	1	3	+1.7

The screening results clearly indicated that solvent polarity and sample pH were the dominant factors controlling extraction efficiency. Acetonitrile exhibited superior performance compared to methanol, likely due

to its enhanced ability to disrupt analyte–matrix interactions. The negative effect observed at higher pH values suggests potential analyte instability or reduced partitioning under alkaline conditions. These findings justified the selection of solvent type, solvent volume, and pH for further optimization using response surface methodology.

Optimization Using Response Surface Methodology

The best design to optimize the three important variables selected in the screening was used, which was a central composite design (CCD). The response surface model had high predictive ability with a coefficient of determination (R 2) of 0.96 suggesting that there were high levels of agreement between experimental and predicted values. Maximum recovery was attained with acetonitrile, 18 mL volume of a solvent, and sample pH of 6.2. Within these circumstances, the average extraction recovery was found to be 93.8 1.5. The interaction effects achieved between the solvent volume and pH were of considerable significance as the recovery declined steeply with an increase in the pH value irrespective of the solvent volume as indicated in Figure 1.

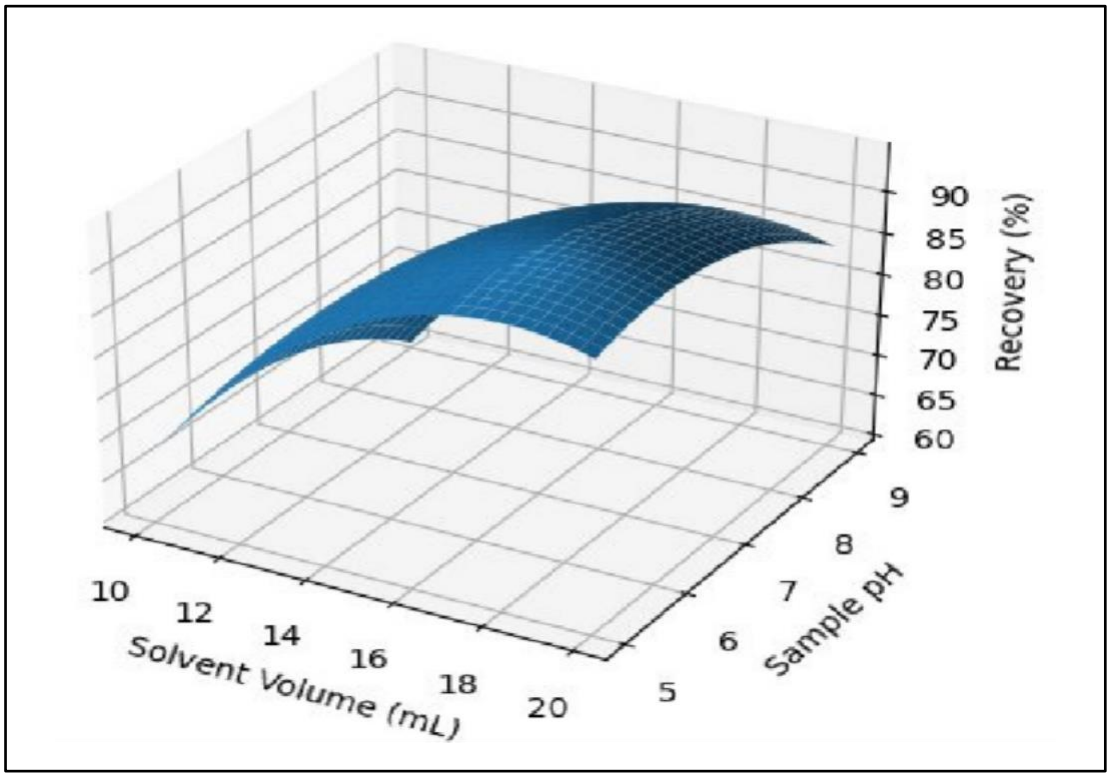

Figure 1: Response surface plot illustrating the combined effect of solvent volume and sample pH on extraction recovery of the target analyte. Maximum recovery was observed at intermediate solvent volumes and near-neutral pH values.

The response surface analysis as presented in Figure 1 indicated that the best extraction efficiency was only possible when important extraction parameters were taken into account with respect to the interaction effects, but not when each variable is analyzed independently.[25] The response surface is clearly shown to have a high degree of curvature, which is a strong indication that there is a high degree of interaction between the solvent volume and the sample pH. Recovery recovery rose gradually with the size of the solvent volume to an optimal level beyond which no significant improvement was seen and indicates the achievement of a sufficient level of mass transfer with no excessive consumption of solvent. Simultaneously, sample pH showed a great effect on extraction performance, and the highest recovery values were obtained at a relatively neutral situation. This tendency indicates a good balance between the stability of the analytes and their solubility, and the deviations towards high pH values led to a significant decrease in the recovery, possibly because of the low efficiency of partitioning or the degradation of analytes. The collective explanation of the effects indicates the significance of multivariate optimization and verifies the appropriateness of the response surface methodology in improving the complex multi-step extraction processes. The findings in general describe the ability of RSM to promote the efficiency of analysis, minimize the workload in the experiments, and demonstrate strong extraction performance in favorable conditions.[28]

Optimized Multi-Step Extraction Performance

The replication of the samples with the help of the optimized method was used to control the reproducibility and the overall performance. The procedure resulted in stable recovery of between 92.4 and 95.1 and a relative standard deviation (RSD) of less than 3.0 showing that

the precision was excellent. When chromatograms were run at optimized conditions, the stability of the baseline was better and the matrix interference was lower than in preliminary experiments, which points to increased efficiency of clean-up. The high recovery and accuracy reflect the effectiveness of the optimization plan.[29] This decrease in variability can be explained by the improved alignment of extraction and clean-up processes in order to reduce the amount of loss of analyte throughout the multi-step workflow. These are very significant especially where there are daily analytical uses where the reproducibility is very essential.[30]

Analytical Method Validation

The optimized procedure exhibited high linearity with the concentration range that was tested, having a correlation coefficient (R 2) value of 0.998. The quantification (LOQ) and the limits of detection (LOD) were found to be 0.12 µg•kg -1 and 0.40 µg •kg -1, respectively. Accuracy, measured by recovery studies at three levels of spiking had a range of 91.6-96.3 and intra-day and inter-day precision were lower than 4% RSD. The most important validation parameters of the optimized method are summarized in Table 2.

Table 2: Validation parameters of the optimized multi-step extraction method.

Parameter	Value
Linearity (R^2)	0.998
LOD (µg·kg^{-1})	0.12
LOQ (µg·kg^{-1})	0.40
Recovery (%)	91.6–96.3
Precision (RSD %)	< 4

The validation outcomes prove that the optimized approach fulfills international standards of the performance of analysis. The low LOD and LOQ values show that the method can be used in analyzing the trace level and the high recovery and precision values show that the method is robust at varying levels of concentration. The current technique, in comparison to the extraction methods that have been recorded before, has enhanced efficiency and few extractions and the solvent consumption is lower.[31,32]

CONCLUSION

The current study has been able to attain a systematic optimization of a multi-step extraction technique utilizing a statistically justified experiment design technique. The use of response surface methodology allowed to identify the effect of important interaction between key extraction parameters, which resulted in a high increase in extraction performance, accuracy, and precision of the analysis. The optimised technique was found to have good recovery, low variation and good sensitivity, thus validating its appropriateness in use in complex matrices at a trace level. In addition, the polished extraction protocol minimized solvent use and experimental intensity, which increased its usefulness and methodological strength. On balance, the suggested solution offers a sound paradigm of designing and streamlining multi-step extraction techniques of the analytical chemistry.

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