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ORIGINAL ARTICLE

# Comparison of the Modified QuEChERS Method and the Conventional Method of Extraction in Forensic Medicine to Detect Methadone in Post-Mortem Urine by GCMS

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## Abstract

*Background:* Extraction of drugs is one of the biggest concerns and the most important part of preparation and determination in forensic medicine. The lack of an easy, efficient and fast extraction method is the most important and most difficult problem despite the development of forensic centers and their being equipped with new diagnostic devices. In the present study, a comparison was conducted between extraction of methadone in post-mortem urine (Autopsy), using modified QuEChERS method and conventional liquid-liquid extraction method in forensic medicine.
*Method:* Methadone extraction from the Post-Mortem urine sample was performed using QuEChERS extraction method, which is a simple and fast micro-extraction method in tube. In this method, the analyte was extracted using MgSO4, NaCl salts and ethyl acetate solvent, and detected by Gas Chromatography-Mass Spectrometry (GCMS).
*Results:* The recovery level of methadone analyte in the urine sample obtained was equal to 67% (N = 15) in QuEChERS method, and 49% (N =1 5) in LLE method. In this center, the LOD and LOQ of the methadone were determined, using GC-MS device, to be equal to 29.1ng/mL and 97ng/mL, respectively. According to the results obtained, there was a significant difference between QuEChERS and LLE methods, in terms of methadone test in post-mortem urine.
*Conclusion:* Cheap, fast, effective, and green QuEChERS, improved with better recovery, could replace the LLE method in detecting methadone in Post-Mortem urine.

**Keywords:** Gas Chromatography-Mass Spectrometry; Methadone; Post-Mortem

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## INTRODUCTION

An important step in the analysis of toxicological samples is extraction. Currently, liquid-liquid extraction(LLE) and solid phase extraction(SPE) are used as pre-test methods for toxicology(1, 2).The toxicological analysis in forensics, the biological samples of blood, urine, contents of the gallbladder, gastric and intestinal contents, such as the liver, are usually using LLE and SPE. The SPE method is costly due to the high price of cartridge and cleaning of homogenizer probe creates a risk of contamination and cross reaction and is laborious. As a result, the SPE method is somewhat tedious and time-consuming and cannot be carried out in many centers. This new method in forensic medicine is QuEChERS. QuEChERS stands for Quick, Easy, Cheap, Effective, Rugged, and Safe. This method has been widely used to determine pesticides and was first developed in 2003(3). In this method, the supernatant is easily separated after centrifugation and ready to be injected into analytical devices such as Gas Chromatography–Mass Spectrometry(GC-MS), Liquid Chromatography–Mass Spectrometry(LC-MS) and High-Performance Liquid Chromatography(HPLC), which can be used as a "green," fast and effective way to detect drugs in clinical and forensic investigations and death cases(3). It was introduced by Anastassiades et al. to extract veterinary drugs from animal tissue in 2003. In QuEChERS extraction, a neat sample of complex matrices is provided for analysis by Gas Chromatography (GC) or Liquid Chromatography (LC). Sample preparation is one of the critical steps to correctly determine a wide range of drugs and toxins by GC-MS and LC-MS devices. In this regard, extraction methods such as Protein Propagation Extraction(4), LLE and SPE have been designed and utilized(5, 6). Clinical and forensic toxicology are heavily dependent on analytical chemistry. Blood, urine, and solid tissue are commonly used in toxicology analysis as alternatives of oral liquid, hair and meconium samples(7, 8). LLE method as well as PPE are simple approaches, however,

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they suffer from in sufficient sampling, contamination of tools and other disadvantages. Although the SPE method, with a high selectivity performance, may contain less interfering compounds compared to PPE or LLE, this extraction method is relatively troublesome and time-consuming. Therefore, QuEChERS method was recommended for forensic toxicology analysis(9). The QuEChERS method, such as PPE and LLE, is a simple method and, like the SPE method, is a high-performance method. (9, 10).Currently, the QuEChERS method is mainly used for analyzing the residual chemical pesticides in different food products (9, 11). There are two major problems in utilizing the chromatography techniques for drug analysis. One of these problems is involvement of the matrix and the other is repression of the ions (12).In general, QuEChERS extraction is based on extraction with a solvent such as acetonitrile or ethyl acetate and dehydration in the presence of a salt such as magnesium sulfate and sodium chloride. Methadone is mainly prescribed for treatment of heroin addiction and has properties similar to those of morphine (13). Methadone is changed with the cytochrome P450 hepatic enzyme (CYP3A4) and formation of the primary metabolite, 2-ethylidine-1, 5-dimethyl-3, 3-diphenyl-pyrrolidine (EDDP)(14)(Figure 3).The ratio of EDDP to methadone is useful as a parameter for the diagnosis of long-term and short-term treatment (14). EDDP is caused by high temperatures of GC on methadone as an artificial side effect. Reducing the temperature of GC injector port from 260 to 180°C in a methadone sample with an initial concentration of 10,000ng/ml decreased the observed concentration of EDDP from 201ng/ml to 53 ng/ml (Table 2). The results indicate that the temperature of the GC injector port causes methadone to be converted to EDDP as an artificial metabolite. Alternative chromatographic procedures such as HPLC or LC-MS should be taken into consideration, when methadone approval and EDDP are important for determining whether or not individual compliance with addiction treatment programs is appropriate.

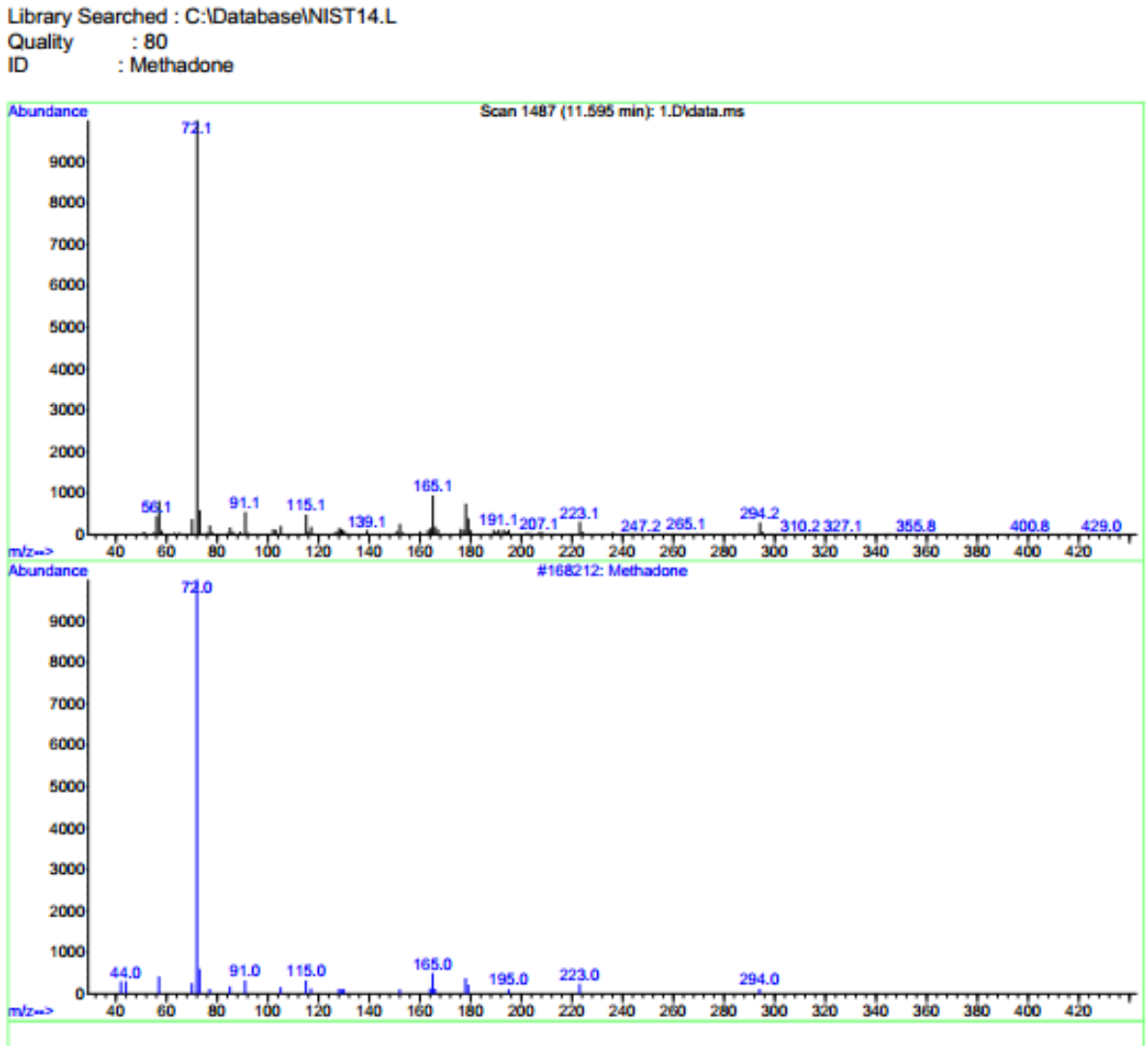


**Figure 1.** Methadone analysis based on index ion in GC-MS

**Table 1.** Specifications of analyte and index ion in GC-MS

| Analyte | Quantifier (m/z) index ion | Qualifier(m/z) subsonic ions | RT |
|---|---|---|---|
| Methadone | 72.1 | 294.1,223.1,165 | 11.6 |
| EDDP | 276.1 | 262.1,220.1.165 | 11.1 |

The main purpose of this paper is to compare the method of extracting QuEChERS with conventional methods in forensic medicine in order to detect methadone in Post-Mortem urine by gas chromatography-mass spectrometry.

## METHODS

*Reagents and Standard*

In the present study, the used materials included Methadone standard with concentration of 100 µg/mL (*Sigma-Aldrich*, Germany), Methanol, chloroform, Isopropanol, Ammonia, Acid hydrochloride, Ethyl acetate, Magnesium sulfate without water, Sodium chloride and distilled water disintegrated with purity (Merck, Germany). Helium (99.99%) was ordered from Maslak (Turkey). Nitrogen was supplied by Air Liquid (Dubai). All the reagents used came from the highest quality available.

*Collecting samples*

Biological samples of corpses' urine were taken at the site of the medical forensic examination based on the data of the deceased's file, containing relatives' testimonies, observations of medical records and examinations as well as observing the ethical and religious conditions, and hiding the individual specifications. Subsequently, the samples were kept at -20° C until carrying out the tests to reduce the post-mortem changes. Among 450 dead bodies referring to the forensic medicine, 20 had drug use history according to the deceased medical records and relatives' statements; among those, 56 had the history of using opium and opium derivatives, such as heroin and crack-Afghan, and 38 of them had a history of drug trafficking and presence in the camp and treatment centers of drug addiction and methadone in the past year. Furthermore, in 18 cases, there was no information about the deceased's condition in the past year. Considering the sampling limits and the permission of the deceased's family, urine samples were taken from 31corpses.

*Method Validation*

The validation of the method was performed accordingly to European Medicines Agency. The modified QuEChERS method was investigated in the following topics.

*Precision*

The precision of the analytic method describes the closeness of individual repeated measurements to an analyte. The exact daily data were measured by analyzing the surface area below the surface of the analyzed analyte graph to the level below the internal standard charts at three different concentrations (low of 62.5, mean 250 and high 2000 ng/ml) at three turns. The values of three concentrations injected for three consecutive days were used to calculate the daily precision, and average CV% was obtained at different concentrations (Table 3).

**Table 2.** The effect of GC injector temperature on EDDP formation of methadone at different concentrations

| | Measured EDDP (µg/ml) | |
|---|---|---|
| Methadone (µg/ml) | GC injector =180°C | GC injector = 260°C |
| 1.0 | 0 | 0 |
| 5.0 | 35 | 126 |
| 10 | 53 | 201 |
| 50 | 134 | 547 |
| 100 | 160 | 665 |

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*Accuracy*

The accuracy of an analytical method has been described as closeness of the determined value obtained from the method to the actual concentration of the analyte in percent (4). The accuracy percentage should be within ±20% of the true value for LOQ and ±15% of the other concentrations along the linear range. The accuracy was obtained through collecting urine samples containing three different concentrations of ANALYTE (low 62.5, mean 250 and high 2000 ng/ml), and with calculating the standard deviation between the calculated value and nominal value obtained from the QC at different concentrations (Table 3).

$$\text{Accuracy (\%)} = \frac{\text{(concentration of test sample / QC concentration)}}{} * 100$$

*Selectivity*

Selectivity is "the ability of an analytical method to detect and measure ANALYTE in the presence of other endogenous components within the sample"(4). Ten Blanc samples were analyzed to investigate the chromatographic interference. No interference peak was identified during the inhibition time (3) of methadone and methadone metabolite (EDDP).Therefore, this analytical method is able to detect ananalyte in the sample.

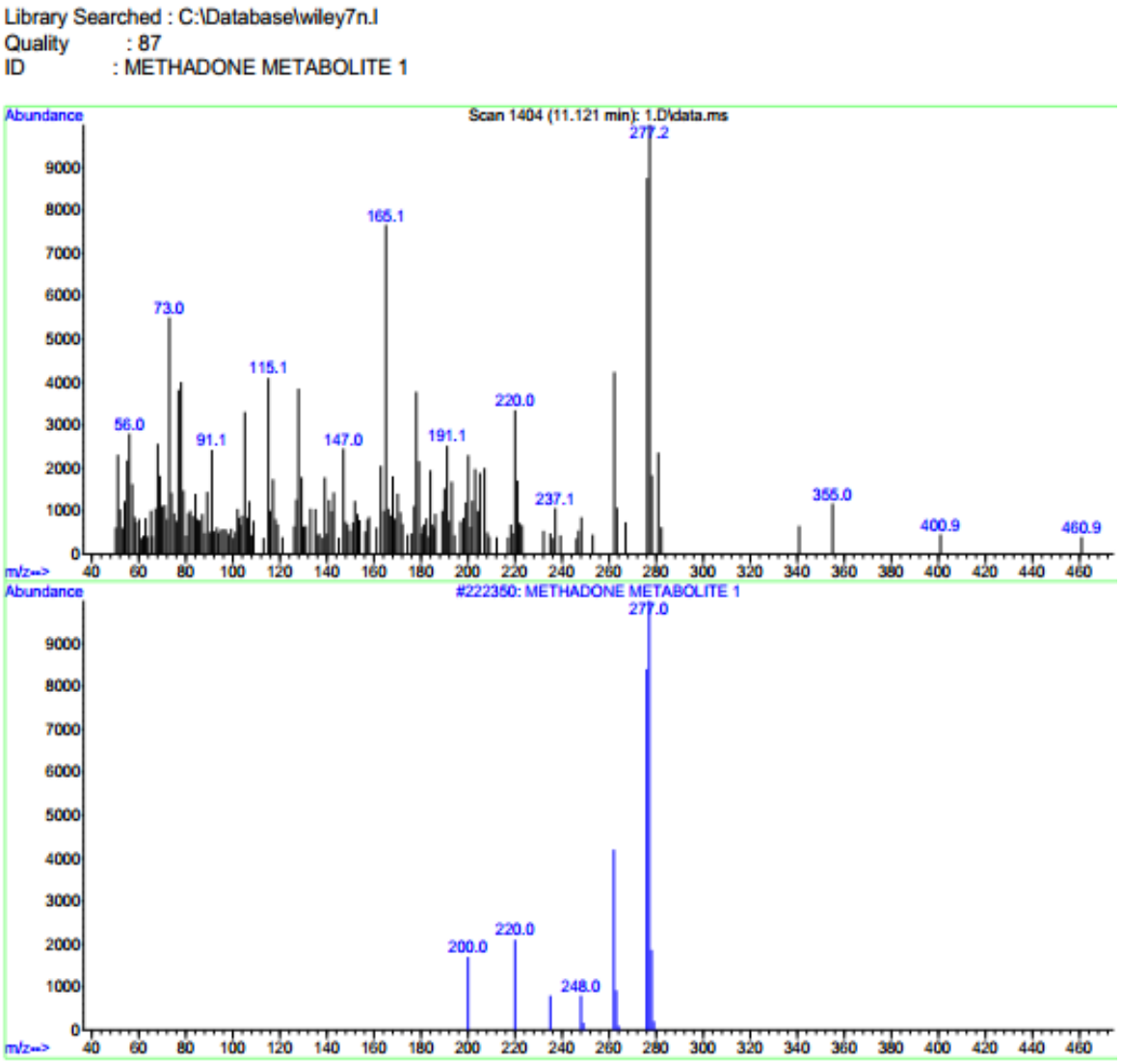


**Figure 2.** Methadone metabolite analysis (EDDP) based on index ion in GC-MS

*Carry-over*

During the validation process, a methadone containing 100ng/mL Ethyl acetatesolvent or methanol-free methadone was injected followed by the injection of a standardsample. The results revealed the lack of methadone detection in the solvent.

*Preparation of Stock, Quality Control and Working Solutions*

Methadone standard of 100µg/ml at a concentration of 2000 ng/ml in methanol was prepared as a working solution. Then, working solution was used for standard calibration of dilution series of 2000, 1000, 500, 250, 125 and 62.25ng/ml. They were prepared and stored at -20ºC until use. For Quality Control (QC) spiking solutions (low, 62.25; medium, 250; high, 2000 ng/ml), serial dilutions from the work solutions were also used. No dilution integrity was performed as part of the method validation since the selected standard

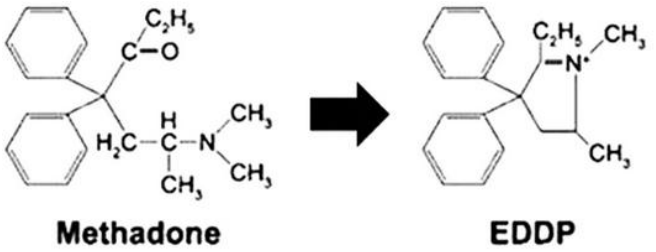


**Figure 3.** Chemical structure of methadone and EDDP

concentrations cover most of the concentrations found in clinical and forensic settings as demonstrated previously (15).

*Samples Extraction Using QuEChERS*

It is necessary to first break down the glucose compound

**Table 3.** Methadone validation

| | N=3 | Y= m x +b | Concentration Range (ng/ml) | LOD (ng/ml) | LOQ (ng/ml) | QualityControl Concentration (ng/ml) | Precision (n=3, %) | Accuracy (n=3 ,%) |
|---|---|---|---|---|---|---|---|---|
| Methadone | Day=1 | Y=0.00006x+0.0018 | 62.5-2000 | 29.1 | 97 | 62.5 | 13.5 | 89 |
| | Day=2 | Y=0.00004x+0.0071 | 62.5-2000 | | | 250 | 8 | 95 |
| | Day=3 | Y=0.00005x+0.0038 | 62.5-2000 | | | 2000 | 11 | 101 |

**Table 4.** Comparing the two extraction methods after recovery

| Index | Method | Average | Standard deviation | T statistics | Sig Level |
|---|---|---|---|---|---|
| Recovery | LLE | 0.49 | 0.006 | -2.644 | 0.047 |
| | QuEChERS | 0.67 | 0.101 | | |

before extracting it by QuEChERS method. The urine samples were initially decanted with 50% acid hydrochloride. In order to break down the conjugate bond, 1 ml of hydrochloric acid was added to 1 ml urine sample in a 15-ml Falcon tube, mixed with the vortex for 20 seconds and placed at 60° C for 30 minutes. After straightening it with a filter paper, 500 µl of it was transferred to a clean flask tube and mixed with 500µl of distilled water. It then became alkaline with 200µl of ammonia 2 molar (pH=8-9) and vortex for 10 seconds and centrifuged for 3 minutes with round of 8000g and placed in the ultrasonic bath for 5 minutes. The extracted QuEChERS powder containing 400 mg of magnesium sulfate and 200 mg of sodium chloride (weighing 2 to 1), and 1000µl of ethyl acetate, were added to the tube and severely shook for 10 second; It was then centrifuged at 7500g for 3 minutes. Subsequently, the supernatant was removed in a glass tube and dried with nitrogen flow. After cooling at room temperature, 1µl of the extract was injected into GC / MS (3).

*Samples Extraction Using LLE*

In the LLE method (liquid-liquid extraction), 25-50 ml of 50% hydrochloric acid was added to 25-50 ml of urine sample in a 250 ml arranger. The samples were placed at room temperature for 24 hours so that they are hydrolyzed (samples can be placed in Ben-Marie Welding for 10-20 minutes) and pH of samples were adjusted between 8.5 and 9 using ammonia and utilizing a pH meter. Afterwards, about 250 ml of chloroform and isopropanol with ratio of 4:1was added to the arranger and after styling it with a rotator for 10-20 minutes, its organic portion was isolated from blue portion in a test tube using filter paper containing sodium sulfate powder. After drying the solvent by a hot water bath and smoothing it by a stainless filter paper, it was injected to the GC-MS device for detection.

*GC-MS Conditions*

The GC-MS, Agilent is a US model of 5975C, which has been combined with a 7890 A gas chromatography device. In this Agilent model, isolation takes place at 5 milliseconds. Column Specifications include film thickness of GC 30m * 0.25mm ID 0.25µm which has been coated with 5% phenylmethyl silicon as a constant phase. The splitless injection mode was selected with a 3-minute valve-off time. The conditions of GC-MS device are as follow: the initial temperature of oven is 60° C (kept for 1 minute) for 20 minutes, the temperature reaches to 280° C, the total run time is 30 minutes; and the injection port and heat transfer line are 250 and 280°C, respectively. The carrier gas was helium with a purity of 99,999 with a constant pressure of 1 ml/min. The temperature of the MS source was 230° C, MS method and MS Quad temperature was 150°C. The method used to identify the methadone was put into the GC-MS, SIM system.

The results were evaluated based on ion index (m/z) and Wiley7n.1 software(Table 1and Figure 1 and 2).

The ultrasonic processor UP50H (50 watts, 30 kHz) of Ultrasonic device (Heilscher, Germany) was used.

The centrifugal model PIT320 (Universal, Iran) with a centrifugal adjustment of up to 15000 rpm is adjustable.

## RESULTS

LOD and LOQ were measured based on the signal-to-noise ratio and comparing the noise signals. A standard sample with a specific concentration was used in order to determine the minimum level of concentration at which the analysis should be taken place. Signal-to-noise ratios of 3:1 and 1:10 were considered acceptable for LOD and LOQ, respectively.LOD and LOQ values were determined equal to 29.1ng/mL and 97ng/ ml, respectively (Table 3).

*Extraction recovery*

Extraction recovery was calculated by comparing the concentration of extracted analyte from the matrix to the concentration of the analyte not extracted. Urine samples with three different concentrations of analyte (low 125, mean 500 and high 1000 ng/ml) were evaluated through calculating the standard deviation percentage between extracted and not-extracted amounts.

$$\text{Recovery} = \frac{\%\ \text{extracted standard}}{\text{not-extracted standard}} * 100$$

In order to assess the recovery rate of methadone, 3 concentrations of 500, 125 and 1000 ng/mL were added to the urine samples which had been previously detected as negative in terms of methadone. After the extraction by both of the methods, the recovery rate of methadone was obtained 67% using QuEChERS method, which was an acceptable value compared to 49% in the LLE method.

The results were analyzed using SPSS Software version 21; Wiley7n.1 Software was also used for GC-MS searching.

According to the results obtained from Table (4), methadone test results of QuEChERS (0.67) are significantly higher than those of LLE (0.49) (P = 0.047).

In the QuEChERS extraction process, the clean-up process is used in biological samples to detect the drugs(16, 17). QuEChERS is sometimes used to purify the sorbents in order to improve the removal of proteins and lipids, the most commonly used interferers in biological specimens(16, 18). To create a quick and reliable method for clinical and legal applications, our goal was to reduce the sampling process to minimize the mistakes and costs(3).

*Clean-up*

Cleaning the sample is a very important step in the analysis of toxicity. It aims to isolate the target analysis from interfering components such as proteins and lipids, and the concentration of analytes in the sample. This stage is used by centrifuges and ultrasonics to separate the protein-bound compounds. In our modified method, the use of 50% hydrochloric acid and the use of heat used to break down conjugated grafts in the urine can play an important role in cleaning up and eliminating the intervention. Ethyl acetate was then used as an alternate acetonitrile extraction solvent in the classic QuEChERS method.

*Solvent Selection*

Ethyl acetate is a simpler and safer solvent than acetonitrile, and a "solvent selected" has a wide range of multivariate experiments for different types of analytes(19). Salting out steps: In the QUeChERS method, $MgSO_4$ and NaCl salts are used for dehydration and increasing extraction efficiency. NaCl reduces the solubility of organic matter in the aqueous phase and increases the concentration of the analyte in organic solvents(19).

31 urine samples were extracted from 31 deceased using LLE and QuEChERS methods. According to the results of the analysis, methadone was detected in 9 samples of urine samples through extracting by 2 methods.

According to the results obtained from Table (5), there is no significant difference between the two methods of QuEChERS and LLE in terms of methadone test result in the urine sample.

According to the fact that the samples extracted by QuEChERS method were transparent and free of contamination, this matter is the advantage of QuEChERS method compared to the liquid-liquid extraction method.

## DISCUSSION

The most commonly used extraction method in most of the forensic centers is LLE which needs a lot of time and effort due to the large volume of required sample and high amount of solvents. The matter is one of the main disadvantages of LLE method beside its other problems such as inadequate sample extraction and contamination of tools and the environment (9).Potential carcinogenesis and mutagenesis properties of compounds such as chloroform and ammonia are a threat to the health of the staff and experts in forensic centers. In addition, utilizing large amounts of solvent and environmental chemicals seriously threatens the human community. As a result, finding an efficient method with fewer risks, along with a greener environment and free from chemical materials force us to make use of new techniques such as SPE and QuEChERS. In our work, the use of alkaline PH during the extraction process was an important correction to compensate for the absence of a second phase, the primary amine (PSA)(18).

According to the results obtained from Table (4), recovery of methadone of by QuEChERS method is %67 that significantly higher than LLE %49 .In general, the recovery of extraction methods should be between 80% and 120%.According to the study of Anzillotti et al, recovery of 60-70%to non-moderate and polar compounds is acceptable for QuEChERS method (17).

QuEChERS was approved as a sensitive, renewable and relatively simple method for qualitative and quantitative analysis of drugs and medicine. Since this method does not require specific equipment and requires less time, it has a great potential for analyzing clinical and legal samples(3). In the case of samples such as the liver, kidney, lung, muscle tissue and adipose tissue, more endeavors must be made for the preparation process in order to increase the use of developed methods in clinical and legal toxicology (20, 21). A review of the results from previous studies in QuEChERS extraction has proved its ability to detect drugs in biological samples. In a study by Emanuele Amorim Alves and colleagues in 2016 aimed at refining and developing the QuEChERS method, he was able to detect 13 opioid, methadone, and cocaine and their metabolites in total blood with the GC/MS device(3).It showed that this method has a high operational capability in various forensic cases. However, most of these studies have been conducted to introduce and implement the QuEChERS extraction method on non-corpuscular samples, and the results can be different from actual examples due to post-mortem corruption, post-mortem redistribution, and metabolism. The reason why the

**Table 5.** Comparison of the two extraction methods in urine sample

| Index | Method | Standard deviation | T statistics | Sig Level |
|---|---|---|---|---|
| Urine samples | LLE | 0.475 | 0.000 | 1.00 |
| | QuEChERS | 0.475 | | |

LOQ values obtained from legal data are higher than published articles is that most of the data are reported by High-Pressure Liquid Chromatography (HPLC) cobbled to MS (LC-MS), which are very sensitive to the analysis of drugs (16, 17). However, GC-MS has been described as a useful method for analyzing legal and clinical cases due to its high repeatability in the diagnosis of opioids (3, 22). According to the results obtained from Table (5), there is no significant difference between the two methods of QuEChERS and LLE. According to the fact that the samples extracted by QuEChERS method were transparent and free of contamination, this matter is the advantage of QuEChERS method compared to the liquid-liquid extraction method.

## CONCLUSION

The results of this study did not show a significant difference between the "results" of the costly and time-consuming LLE method with a cheap, easy and fast QuEChERS method. Due to higher methadone recovery in urine specimen in the QuEChERS method, this method can be a better alternative than the LLE in post-mortem urine.

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**Conflict of interest**: None to be declared.

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