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Article

Development and Validation of QuEChERS LC-ESI-MS/MS Method for Determination of Cocaine and Metabolites in Urine

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Abstract: An increase in cocaine abuse has been observed globally since the past decade. Cocaine is among the most commonly abused stimulants used for recreational purposes. In this study, the one-step sample preparation based on quick, easy, cheap, effective, rugged, and safe (QuEChERS)-LC-ESI-MS/MS method was developed and validated. Cocaine was extracted from biological urine samples using QuEChERS extraction followed by liquid chromatography-tandem mass spectrometry analysis. Chromatographic separation was achieved on Agilent Eclipse Plus C18 4.6 × 50 mm, 1.8 µm (p/n 959759-302) using water–acetonitrile in 0.1% formic acid as a mobile phase in gradient elution mode. This study aimed to develop and validate a very simple and reliable QuEChERS -LC-MS/MS method for the quantitative determination of Cocaine in urine samples. Validation of the method was performed using Bias, Linearity, LOD, Selectivity, Specificity, Precision, Robustness, and Intermediate precision. The method showed an excellent linearity with a correlation coefficient (r2 = 0.9993 to 0.9997) observed in the range from 5 to 100 ng/mL of cocaine. The percent recovery value was between 99.58 and 110.51 % for 10 ng/mL; then 107.26 to 111.38 for 100 ng/mL which was with an acceptable percent recovery, The precision (repeatability) was reported as 3.63 % and the intermediate precision of the method resulted in 107.26 % and 111.38 % for two analysts. The limit of detection (LOD) of cocaine was calculated as 1 ng/mL and the selectivity of the method for interferents (coca tea and adulterants) was selective. Generally, the results obtained confirmed that the method is relatively fast, precise, simple, and robust, and can be used in routine forensic analyses for the determination of Cocaine and the metabolites concentration at a concentration level greater than 5 ng/mL.

Keywords: *Cocaine; metabolites; urine, QuEChERS validation; liquid chromatography; mass spectrometry*.

1. Introduction

Cocaine is one of the most frequently consumed as an illegal substance next to Cannabis; opioids and Amphetamines. It is a central nervous system stimulant (CNS) commonly found in clinical or forensic toxicology investigations and is widely abused as a recreational drug because it stimulates high levels of dopamine (a brain chemical associated with pleasure and reward) and appetite suppressant. According to the World Drug Report 2024 released by the United Nations Office on Drugs and Crime (UNODC), an increase in cocaine abuse has been observed in the past decade [1]. Despite the rising trends of new psychoactive substances nowadays, cocaine abuse is still a problematic concern all over the world due to its implications in acute and chronic effects. It is one of the most reinforcing and hepatotoxic drugs, accounting for the majority of illicit drug-related problems [2]. In humans, around 40% of cocaine is hydrolyzed to biologically inactive metabolite

ecgonine methyl ester by plasma enzyme butyrylcholinesterase and liver carboxylesterase-2 (CE-2), but more cocaine is bio-transformed to benzoylecgonine via hydrolysis catalyzed by CE-1, and to norcocaine via oxidization catalyzed by liver microsomal cytochrome P450 (CYP) 3A4 [3, 4].

The toxic concentration of cocaine in urine ranges from, 0.8–13 mg/L [5] One of the key challenges confronted by forensic toxicologists in simultaneous sample extraction and chromatographic separation of cocaine and its metabolites from different biological specimens is their vast polarity difference among them. Despite the fact the positive cutoff value for cocaine is too high; There is high analytical demand for sensitive techniques to detect very low concentrations in several Forensic and clinical laboratories that can improve the analysis of both Anti-mortem and post-mortem investigations [5-7].

Several analytical methods have been used to determine the amount of cocaine and its metabolites in various sample matrices. Among these Immunoassays [8,9]; high-performance Liquid chromatography coupled to an ultraviolet detector (HPLC-UV) [10-13]; Gas chromatography with mass spectrometric detection (GC-MS) [14-19]; liquid chromatography with mass spectrometry (LC-MS) [20] (LC-MS/MS) [21-36] were commonly used. The GC-MS was used combined with derivatization to quantitate cocaine/metabolites in complex sample matrices. While GC-MS had both sensitivity and selectivity, the derivatization process is very expensive and labor intensive and poses a safety risk. To overcome this paucity, LC-MS/MS is still the method of choice for the determination of cocaine and its metabolites for routine Forensic and clinical analysis. However, LC-MS/MS is the most precise and reliable analytical method preferred for the quantitation of cocaine in human blood, urine; hair, and oral fluids in several forensic toxicological analyses [11-14] because it has high sensitivity; specificity; reduced analysis cost; shorter run time and enabled the detection of very small concentration of cocaine and its metabolites, which assumes particular importance when sample volume available is small. For the reliability of the analysis of such complex sample matrices, sample preparation is the most important step. There are several sample preparation techniques available for the determination of cocaine in different biological specimens, including online extraction [23]; solid-phase extraction (SPE) [15, 17, 24,27], and QuEChERS [37].

The need for developing novel Analytical methods and assessing and validating the developed methodologies for faster and more accurate determination of cocaine in the human-based sample is of great importance. The LC-MS/MS is currently the most commonly used analytical technique for the analysis of these drugs in human specimens because the technique has tremendous capability to detect trace levels of analytes with wider polarity ranges. Moreover, the technique is highly preferred in laboratories dealing with heavily routine laboratory work.

In comparison with others, sample preparation procedures QuEChERS for cocaine and metabolite analysis present several advantages such as ease of sample preparation, free of contamination, reduced interferences, and saving time [35]. Therefore, the main aim of this study was to develop and validate the QuEChERS -LC-MS/MS analytical method for the determination of cocaine and its metabolites in Urine samples.

2. Materials and Methods

Chemicals and reagents

All reagents used throughout the assay were analytical reagent grade and were purchased from Sigma Aldrich, USA. However, ASC acetaldehyde was purchased from Sigma Aldrich (Switzerland), Acetonitrile and Methanol were obtained from Fisher Scientific (Loughborough, UK). Deionized water obtained from a Millipore® Milli-Q gradient system with 18 M Ω cm output was used throughout the analysis.

Cocaine multicomponent mixture-4 containing Cocaine 1000 µg/mL; Norcocaine hydrochloride 1000 µg/mL; Lidocaine 1000 µg/mL; Benzoylecgonine 1000 µg/mL; 2-Diethylaminoethanol 100 µg/mL; Bupivacaine hydrochloride 1000 µg/mL; Procaine hydrochloride 1000 µg/mL; Ropivacaine Hydrochloride Monohydrate 834 µg/mL; deuterated Cocaine-D3 stable labeled internal standard 1000 µg/mL in methanol, LGC standard were used. All stock standard solutions were stored at -18

ºC. A working standard solution was prepared by measuring the appropriate volume of each stock solution into a 5 mL volumetric flask and diluting it with methanol. The obtained concentration of the working standard was 1 µg mL-1. Methanol and acetonitrile were parched from Loba Chemie, Mumbai, India. QuEChERS salts (0.5 g disodium citrate sesquihydrate, 1 g Na-Citrate, 1 g NaCl, 4 g MgSO4) was perched by Sigma Aldrich, USA.

Instrumentation

All experiments were carried out using Agilent 1290 Infinity II HPLC coupled with high-speed pump (G7120A); II multi-sampler with cooler (G7167B); II multicolumn thermostats (G7116B) and Agilent Triple Quadrupole LC/MS system 6470 with Agilent Jet Stream electrospray ionization source. Acquisition parameters Liquid chromatography–triple quadrupole mass spectrometry analysis was done following chromatograph conditions given in Tables 1 and 2.

Table 1. Agilent 1290 Infinity II UHPLC parameters.

Parameters	**Values**	
Column Type	Agilent Eclipse Plus C18 4.6 × 50 mm, 1.8 µm	
Column temperature	40 °C	
Injection volume	2µL	
Mobile phase	A) 0.1% formic acid in Water B) 0.1% formic acid in Acetonitrile	
Flow rate	0.3 mL min-1	
Gradient	**Time (min):**	**% B:**
	0	5
	1	5
	2	15
	2.5	30
	6	45
	7	95
	9	95
	10	5
Stop time	10 min	
Post time	5 min	

Table 2. Agilent Triple Quadrupole 6460 and Agilent Jet Stream source parameters.

Parameters	**Value**
Drying gas temperature	300 °C
Drying gas flow	10 L min^{-1}
Sheath gas temperature	300 °C
Sheath gas flow	11 L min^{-1}
Nebulizer pressure	40 psi
Capillary voltage	4000 V (+)
Nozzle voltage	1500 V (+)
Cycle time	500 ms

Q1 scan of the mass spectra was recorded to select the most abundant mass-to-charge ratio (m/z) ion using continuous infusion of each cannabinoid directly into the MS using a syringe pump at a flow rate of 0.1 mL min-1. In this study, the proton adduct [H+] of the molecular ion was chosen as the precursor ion for all analytes. Then, an enhanced product ion scan was conducted to obtain the product mass spectra of the precursor ion. The first transition, which corresponds to the most abundant production was used for identification and quantification, while the second one for confirmation purposes [37]. To obtain maximum sensitivity for the identification and quantification

of the analytes, collision energy (CE), cell Acc energy (CA), and fragmentation energy (Frag.) were performed for each analyte using 1 µg mL-1 solution of individual compounds in methanol. Finally, the presence of precursor and product ions was investigated using multiple reaction monitoring (MRM) experiments with a cycle time of 500 ms. The optimized LC-MS/MS parameters are summarized in Table 3.

Table 3. Transitions for Cocaine and its metabolites detection in dMRM mode.

Compound	Precursor Ion *(m/z)*	Product Ion *(m/z)*	Frag. *(V)*	CE *(V)*	Cell Acc *(V)*	RT *(min)*	Polarity
2-Diethylaminoethanol	118.1	72	98	16	4	0.6	Positive
2-Diethylaminoethanol	118.1	58	98	28	4	0.6	Positive
Bupivacaine	289.2	140.1	98	20	4	3.9	Positive
Bupivacaine	289.2	84.1	98	44	4	3.9	Positive
Cocaine	304.16	182.27	86	20	4	3.7	Positive
Cocaine	304.16	82.1	86	32	4	3.7	Positive
Cocaine	304.16	77	86	68	4	3.7	Positive
Cocaine-d3	307.18	185.2	96	20	4	3.7	Positive
Cocaine-d3	307.18	77	96	68	4	3.7	Positive
Lidocaine	235.2	86.1	98	20	4	3.4	Positive
Lidocaine	235.2	58.1	98	40	4	3.4	Positive
Norcocaine	290.1	77.1	98	68	4	3.8	Positive
Norcocaine	290.1	68.1	98	40	4	3.8	Positive
Procaine	237.2	120.1	98	28	4	2.5	Positive
Procaine	237.2	65	98	64	4	2.5	Positive
Ropivacaine	275.2	126.1	106	20	4	3.7	Positive
Ropivacaine	275.2	84.1	106	48	4	3.7	Positive
Tetracaine	265.2	176.1	78	12	4	4.2	Positive
Tetracaine	265.2	72.1	78	28	4	4.2	Positive

Preparation of samples, internal standards, calibrators, controls and validation parameters

Two different working solutions containing 100 and 1000 ng/mL of multi-component analyte (Cocaine; Norcocaine; Lidocaine; Benzoylecgonine; 2-Diethylaminoethanol; Bupivacaine hydrochloride; Procaine hydrochloride; Ropivacaine Hydrochloride Monohydrate) were prepared by spiking 101.1 and 443.4 µL pure Methanol 99.99% (v/v) into 10 mL volumetric flask and diluting the content to the final volume with a urine sample.

Besides, 8 calibration solutions with three replicates (n=7) 1, 5, 10, and 20 ng/mL were prepared by transferring 10; 50; 100; and 200 µL of 100 ng/mL working solution respectively into 2000 µL vials. Similarly, 50, 100, 150, and 200 ng/mL were also prepared by transferring 50, 100, 150, and 200 µL 1000 ng/mL working solution respectively into 2000 µL vials after the addition of 100 ng/mL Cocaine-d3 (internal standard) in each calibration solution. Sample preparation Spiking of the blank urine samples as follows: 500 µL of blank urine sample was spiked with 100 µL of Internal standard (IS) (conc. 1 ng mL-1), with 250 mg of salt (0.5 g disodium citrate sesquihydrate, 1 g Na-Citrate, 1 g NaCl, 4 g MgSO4) and working standard solution 10; 50; 100; and 200µL (conc. 100 ng mL-1) and 50, 100, 150, 200 µL (conc. 1000 ng mL-1), resulting in final concentrations of 1,5,10, 20, 50, 100, 150 and 200 ng mL-1 and it was shaken on vortex for 2 min. The sample prepared in this way was added with1 mL of acetonitrile and vortex again for 1 min, then centrifuged the sample for 10 minutes at 12000 RPM and filtered the supernatant using a micropipette, transferred 100 µL of the supernatant to the autosampler vial and added with 200 µL of ammonia formate and Shaked and analyzed it on LC-MS/MS.

3. Results and Discussion

The method for determination of cocaine in Urine using QuEChERS-LC-ESI-MS/MS was validated according to the guidelines established by the International Conference on Harmonization (ICH), such as Bias; Precision; Linearity; LOD; LOQ; Carryover.

Bias and precision

For biological samples, to check the accuracy and precision of the method, a spiked sample representative of the area of application of the method and the nature of the samples being analyzed was used. To determine the precision of the method, statistical processing of the obtained experimental results was carried out (a total of n=6 tests) and evaluated in terms of relative standard deviation (RSD). The RSD was calculated by dividing the standard deviation of six replicate analyses by the mean and presented in Table 4.

Table 4. Results for method precision - sample urine.

Component	Mean value, X_{sr} [ng/mL]	Standard deviation, s_r	Relative standard deviation, RSD_R%	Criteria RSD <20%
Procaine	10.63	0.806	7.58	Yes
Lidocaine	10.26	0.498	4.85	Yes
Ropivacaine	10.25	0.784	7.65	Yes
Cocaine	11.05	0.401	3.63	Yes
Norcocaine	10.10	1.036	10.3	Yes
Bupivacaine	10.06	0.920	9.14	Yes
Tetracaine	9.96	0.966	9.70	Yes

Based on the presented results for the precision of the method, it can be concluded that all the criteria for the precision of the method have been met because it is consistent with the guidelines that establish the maximum acceptable bias should be < 20%. Moreover, to determine the accuracy of the method, based on the obtained experimental results (a total of 3 tests n=3 for each of the 2 concentration levels), the yield (recovery) for individual components was calculated and the obtained values are by the defined Rec % criteria: 80 – 120%. Results are presented in Table 5 and Table 6.

Table 5. Results for testing the accuracy of the method - sample urine at lower limit (10 ng/mL).

Component	Mean value, X_{sr} [ng/mL]	The actual concentration of the spiked sample, [ng/mL]	Recovery, Rec%
Procaine	10.63	10.00	**106.30**
Lidocaine	10.26	10.00	**102.62**
Ropivacaine	10.25	10.00	**102.48**
Cocaine	11.05	10.00	**110.51**
Norcocaine	10.10	10.00	**100.96**
Bupivacaine	10.06	10.00	**100.58**
Tetracaine	9.96	10.00	**99.58**

Table 6. Results for testing the accuracy of the method - sample urine at upper limit (100 ng/mL).

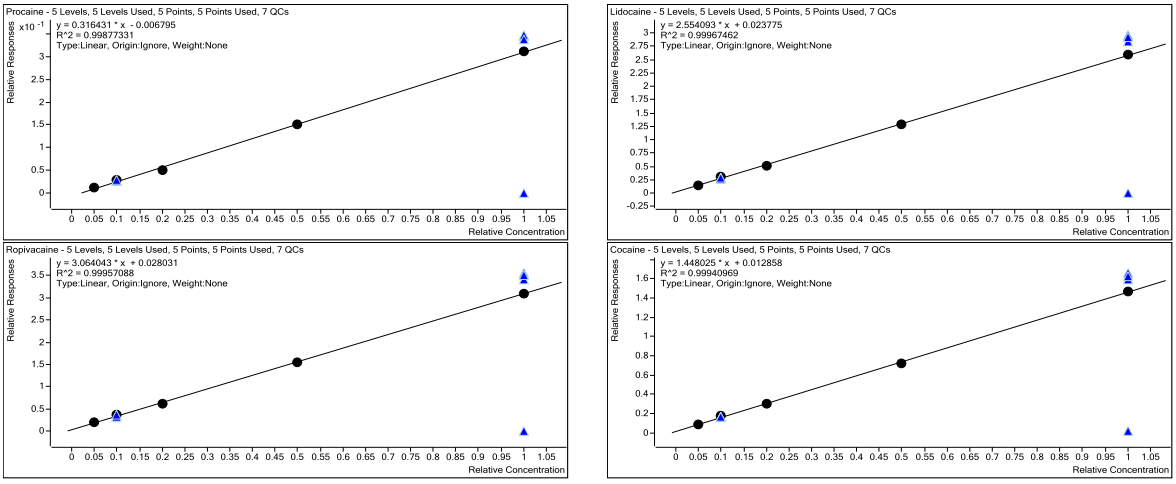
Component	Mean value, X_{sr} [ng/mL]	The actual concentration of the spiked sample, [ng/mL]	Recovery, Rec%
Procaine	107.26	100.00	**107.26**
Lidocaine	110.35	100.00	**110.35**
Ropivacaine	111.38	100.00	**111.38**
Cocaine	110.17	100.00	**110.17**
Norcocaine	110.96	100.00	**110.96**
Bupivacaine	109.43	100.00	**109.43**
Tetracaine	109.93	100.00	**109.93**

Intermediate precision

The intermediate precision was examined by performing analyses by two different analysts with the same number of replicate analyses (n=6), concentration (10 ng/mL and 100 ng/mL), instrument, and on the same day. As a result, a comparison of the two analysts was performed by comparing their precision using a simple F-test, as the target value for precision was not decided, thus the F-experimental was calculated as 1.48, and F critical was (α =0.05, 2,2) is 5.05, therefore, F-theoretical is larger than F experimental the null hypothesis is accepted and confirmed that there is no significant difference between the two analysts.

Linearity

The calibration curve was plotted by running a series of standard solutions containing cocaine at six concentration levels and internal standards of the mixture of tested components. The highest and lowest concentration ranges tested were (5 ng/mL 10 ng/mL 25 ng/mL 50 ng/mL 75 ng/mL - 100 ng/mL) by a series of three injections. After an outlier has been removed from the data, the correlation coefficient (r2) of (0.9986-0.9997) was obtained as can be seen from Figure 1, and all of them meet the set criterion of linearity (r2 0.999) from point 7 of this Protocol indicating the proposed analytical method is appropriate for the determination of Cocaine and its metabolites. (The conducted validation study established a linear range for tested analytes with results presented in Table 7) To assess the acceptance criteria for linearity, visual evaluation, and residual plots are useful in clear-cut situations.



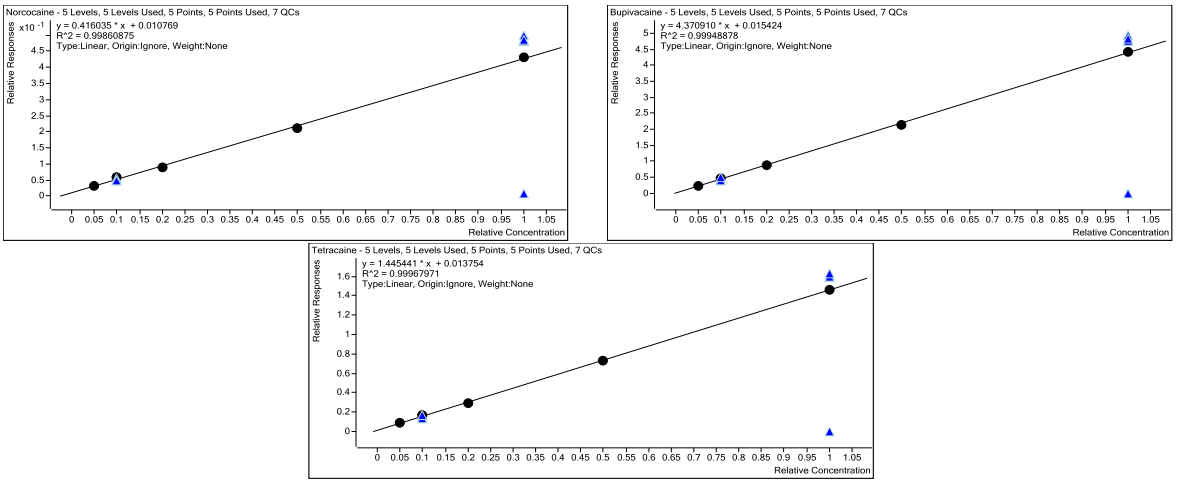


Figure 1. Calibration curve for seven components investigated.

Table 7. Calibration curve equations and correlation coefficients.

	Component	$y= ax^2+bx+c$ $y= ax+b$	R^2	*Concentration range ng/ml*
1	Procaine	y = 0.316431 * x – 0.006795	0.9988	5 – 100
2	Lidocaine	y = 2.544903 * x + 0.023775	0.9997	5 – 100
3	Ropivacaine	y = 3.064043 * x + 0.028031	0.9996	5 – 100
4	Cocaine	y = 1.448025* x + 0.012858	0.9994	5 – 100
5	Norcocaine	y = 0.416035 * x + 0.010769	0.9986	5 – 100
6	Bupivacaine	y = 4.370910 * x + 0.015424	0.9995	5 – 100
7	Tetracaine	y = 1.445441 * x + 0.013754	0.9997	5 – 100

y –peak area, x – standard concentration, a – the slope, b – segment on the ordinate, r – correlation coefficient.

Limit of detection (LOD) and limit of quantification (LOQ)

The instrumental quantification (LOQ) and instrumental detection (LOD) limits were calculated from blank determinations by using a signal-to-noise ratio of 10 and 3, respectively, and they ranged in intervals of 5 and 1 ng/mL, respectively. Therefore, the developed method can be used for the determination of cocaine and its metabolites in urine samples at LoD is 1 ng/mL.

Selectivity/specificity

A selectivity study of the method was carried out by injecting a sample matrix spiked with possible interferents. Accordingly, interferents that could be obtained in the matrix components such as coca leaf tea or adulterated natural products, that can co-elute with cocaine were evaluated. The method confirmed excellent chromatographic selectivity with no interferents from the spiked matrix component at the retention times of cocaine, interferants, methanol, and IS. As the acceptance criteria are the existent compounds that must not interfere with the analysis of the targeted analyte, none of the interferents affects the cocaine determination. Therefore, the absence of the interfering signal with the analyte of interest agreed with the recommendations of both ICH (ICH, 2005) guidelines. Therefore, matrix components were not expected to interfere with the determination of cocaine in urine samples.

Robustness

The robustness of the method was examined by changing the flow rate (FR) within ± 0.2 mL/min as a result no effect on the peak area of cocaine and the metabolites was observed.

Carryover

To evaluate carry-over as part of method validation, blank matrix samples are analyzed immediately after a high-concentration sample or reference material. The highest analyte concentration at which no analyte carryover is observed (above the method's LOD) in the blank matrix sample is determined to be the concentration at which the method is free from carryover.

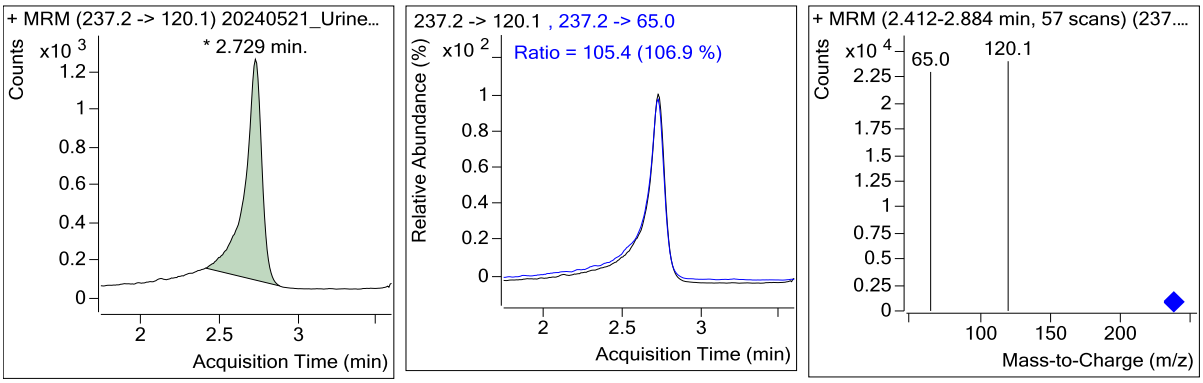


Figure 2. Carryover investigation of a blank matrix samples is analysed immediately after a high concentration sample or reference material.

4. Conclusions

A simple sample treatment workflow, based on a QuEChER, has been developed for the multi-determination of cocaine and its metabolites in human urine before LC-MS-MS determination. This sample treatment has valuable assets that render it well-suited for the intended purpose. Unlike other sample preparation methods, this sample pre-treatment is very fast and the developed analytical method is reliable and robust for the determination of cocaine and its metabolites, it aligned with criteria stated in ICH and FDA guidelines by using Bias, Precision, LOD, Selectivity, and Linearity. Moreover, with this method, several samples can be analyzed in a very short period. Hence, the developed method is applicable in routine forensic toxicology laboratories. Besides, compared with the analytical methods reported in several kinds of literature this method is very fast and reliable and it offers excellent selectivity, bias, and robustness.

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Conflicts of Interest: The authors declare no conflict of interest.

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