

## Research Paper

# Development of a GC-MS Method for the Analysis of Selected Opioids in Human Hair Samples



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## ABSTRACT

**Background:** Hair samples are recognized as alternative biological specimens in forensic and clinical toxicology for detecting drug abuse and poisoning. Forensic testing for opioids in hair has become a useful diagnostic measure for assessing chronic drug usage through segmental analysis. However, accurate, sensitive and specific analytical methods are needed. The aim of this study was to introduce a simple, sensitive and specific GC-MS method for the identification and quantitation of selected and commonly abused opioids (tramadol, methadone, morphine, and codeine) in hair samples.

**Methods:** After external decontamination, a 50 mg portion of powdered hair sample was combined with hydrochloric acid (0.1 M) and incubated on a magnetic stirrer at 56°C for 16 hours. Then, 1 mL of sodium hydroxide (0.1 M) and 2 mL of phosphate buffer (1 M, pH=8.4) were added. Chloroform-isopropanol (ratio: 80:20 V/V) was utilized as the extracting solvent and the sample was homogenized and centrifuged for 5 minutes (at 3500 rpm). After centrifugation, the organic phase was dried using dry nitrogen gas. The sample was derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). The blank, standards, and real samples were subsequently analyzed by GC-MS.

**Results:** The limits of detection in the linearity experiments ranged from 0.12 to 0.21 ng/mg. According to the validation results, the method exhibited linearity in the concentration range of 0.1-2.5 ng/mg for all analytes, with calibration curve slopes ranging from  $R^2=0.98$  to 0.99. Good inter and intra-day precision relative standard deviations (RSDs) were observed to be <3.5% for all compounds. Extraction efficiency varied from 91.8 to 102.4%.

**Conclusion:** The validation and analysis of actual samples indicate that this method is straightforward, sensitive, and specific for the analysis of opioids in routine hair analysis.

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## Introduction

**A**buse and addiction to medicinal or illegal natural, semisynthetic, and synthetic opioids represent a growing serious concern that endangers human health in Iran and worldwide [1, 2]. Opioid abuse continues to be the leading cause of fatal overdoses globally [2, 3]. Harms associated with addiction resulting from both medical and non-medical opioid use are significant issues worldwide, considering their clinical and legal implications. For example, the [United Nations Office on Drugs and Crime \(UNODC\)](#) estimated that 60 million people engaged in non-medical opioid use in 2021, with 31.5 million individuals using opiates (primarily heroin) [4]. Also, opioid use leads to indirect mortality through suicide, HIV, hepatitis C virus, car accidents and other injuries. The application of drug screening tests plays a vital role in detecting opioid abuse in various settings, including drug abuse rehabilitation centers, anti-doping control, clinical and forensic toxicology screening, workplace, and traffic medicine [5]. Analytical methods have been primarily based on urine and blood screening in clinical and forensic laboratories. However, methods for analyzing alternative biological samples, such as oral fluid, sweat, hair, and nails have been used to perform drug screening testing with different levels of sensitivity and specificity [6, 7]. Urine is the most preferred biological sample due to its ease of collection and the high concentrations of drugs and metabolites it contains [7]. However, because of the limitations of urine as a biological sample for drug screening, alternative samples such as hair have been utilized for laboratory detection of drug abuse. Hair analysis for opioids is an accurate and specific alternative to urine drug testing (UDT). It offers increased sensitivity, is less susceptible to adulteration, reduces the risk of infection to staff, provides ease of storage, allows for non-invasive collection and ensures high sample stability. Also, retrospective hair analysis provides a significantly broader detection window (from weeks to several months) than urine and saliva, depending on hair length [8]. Hair can be utilized as a sample for investigating past drug exposure. Segmental hair analysis can distinguish between a single exposure from chronic use, making it a valuable method for discerning a one-time drug ingestion from repeated exposures [9]. While UDT and blood analysis provide short-term information regarding opioid addiction, hair analysis enables the detection of long-term drug history [10].

Basic drugs, as cationic molecules, ideally bind to melanin in pigmented hair, especially at physiological pH. Weak bases, like codeine may be more incorporated into

hair than weak acids, like phenobarbital at physiological pH [10, 11]. In addition, hair pigmentation is proposed as a major factor affecting drug incorporation into hair [12]. Melanin pigments determine hair color and are considered the primary sites for drug binding. Melanin polymers have carboxylic acid groups with negative charges in their structure, which attract positively charged drug molecules under physiological conditions, indicating a preferential affinity of melanin for basic drugs. Various hair colors and types may reveal different levels of drug exposure under similar conditions. Studies have shown that coarse and dark hair may potentially harbor higher drug concentrations compared to light brown or blonde hair [11-13].

Complex methods for drug extraction, along with the high cost and time-consuming nature of the analysis, represent some drawbacks of hair analysis [10]. Today, hair analysis for drug detection is one of the widely accepted prominent techniques in the field of forensic toxicology. Furthermore, it has now evolved into a common tool in clinical toxicology, and occupational and environmental medicine for detecting xenobiotics (drugs, medications, environmental pollutants, doping substances) [13].

Today, gas or liquid chromatographic techniques coupled with mass spectrometry (GC-MS or LC-MS) or tandem mass spectrometry (GC-MS-MS or LC-MS-MS), are regarded as the gold standard for the identification and quantification of drugs, including opioids in hair, due to their higher sensitivity and specificity [10, 14]. Recently, several analytical methods for drug detection through hair analysis have emerged, including high-performance liquid chromatography with high-resolution mass spectrometry (HPLC-HRMS) [15], chiral separation [16], direct analysis in real-time high-resolution MS [17] and matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) for intact hair analysis [18]. Nevertheless, these advanced methods are primarily employed in academic research, whereas most laboratories utilize GC-MS and LC-MS-MS for routine hair analysis.

The aim of this study was to introduce an optimized and simple GC-MS method for the identification and quantification of selected opioids (morphine, codeine, tramadol and methadone) in the hair of forensic cases, marking the first instance in Iran for potential application in forensic toxicology laboratories.

## Materials and Methods

### Chemicals and reagents

HPLC-grade chloroform, isopropanol, phosphate buffer, ethyl acetate, dichloromethane, distilled water, ethanol, methanol, sodium dodecyl sulfate and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) were obtained from the Merck Company (Darmstadt, Germany). Methadone, morphine, cocaine, and codeine (analytical grade) were obtained from Tolid Daru Pharmaceutical Company (Tehran, Iran). Also, tramadol hydrochloride was obtained from Sigma Aldrich, Missouri, USA and hydrochloric acid, ethyl acetate and sodium hydroxide were from Azhang Chemistry, Tehran, Iran.

### Equipment

A PerkinElmer gas chromatography-mass spectrometry (GC-MS) system equipped with a PerkinElmer Clarus 680 SQT mass spectrometer (Waltham, Winter490, USA) was used. Substances were separated in this system using a Rxi-5MS column (30 m length, 0.25 mm inner diameter, and 0.25  $\mu\text{m}$  thickness; Crossbond 5% diphenyl/95% dimethyl polysiloxane). The injection volume was 1  $\mu\text{L}$  in splitless mode, and the ionization energy in the mass spectrometry was set to 70 electron volts (eV). The thermal program involved an initial temperature of 60  $^{\circ}\text{C}$  for 1 minute, followed by a 20  $^{\circ}\text{C}/\text{minute}$  increase to 280  $^{\circ}\text{C}$  (at a rate of 20  $^{\circ}\text{C}$  per minute with a 5-minute hold at this temperature), and an injection port temperature of 250  $^{\circ}\text{C}$ . Helium gas (99.99% purity) served as the carrier gas at a flow rate of 1 mL/minute, with an ionization source temperature of 300  $^{\circ}\text{C}$  [19]. The selected ion mass for each analyte in the selected ion monitoring (SIM) mode is presented in Table 1. Equipment, such as a homogenizer (Polytron, Indonesia), magnetic stirrer (Pars Azmoon, Tehran, Iran), vortex (IKA, Staufen, Germany), centrifuge (Hettich, Westphalia, Germany), pH meter (Hanna Instruments, Switzerland) and oven (Tajhizat Toos, Tehran, Iran) were used in this study.

### Hair sample collection

In this study, hair samples were obtained from two groups. The first group included 11 hair samples from cadavers referred to the Forensic Medicine Center of Kermanshah Province (Kermanshah City, West of Iran) from January 2022 to August 30, 2023. Postmortem biological samples (blood, urine and liver tissue) for selected opioids were analyzed using routine forensic

toxicological methods (GC-MS, HPLC-DAD) during forensic autopsies.

The second group as control consisted of hair samples from healthy volunteer individuals, including five hair samples from women and five samples from men (without a history of drug abuse or recent medicine use). The control group was selected from the staff of the Forensic Toxicology Laboratory of Kermanshah. All hair samples were collected according to the UNODC guidelines [19]. Head hair samples (50-100 mg as a pen-size bundle, from the posterior vertex of the scalp) were obtained. The samples were wrapped in a paper bag and stored at room temperature until analysis.

### Preparation of hair samples and extraction of opioids from hair

The hair samples were prepared as follows [19]: Every 100 mg of hair samples underwent two washes with 5 mL of dichloromethane for 2 minutes and then were dried with absorbent paper. The remaining hair was divided into smaller pieces with scissors and uniformly ground using a mortar. Fifty mg of the powdered hair sample was combined with 1 mL of hydrochloric acid (0.1 M) and incubated on a magnetic stirrer at a speed of 35 rounds per minute at a temperature of 56  $^{\circ}\text{C}$  for 16 hours. Subsequently, 1 mL of 0.1 M sodium hydroxide and 2 mL of phosphate buffer (1 M) at pH=8.4 were added. Then, 2 mL of chloroform-isopropanol (ratio: 80:20 V/V), was added and the sample was homogenized. The sample was then shaken horizontally for 20 minutes (with the tube containing the sample moving up and down) and then centrifuged for 5 minutes (at 3500 rpm). After centrifugation, the aqueous phase was decanted, and the remaining organic phase in the tube was dried using dry nitrogen gas. Finally, 50  $\mu\text{L}$  of 1% MSTFA as the derivatizing agent was added to the extracted sample and dried. The derivatized sample was incubated at 70  $^{\circ}\text{C}$  for 20 minutes and finally, 50  $\mu\text{L}$  of ethyl acetate containing 1.0 ng/ $\mu\text{L}$  of cocaine was added to the sample, and after shaking for one minute, 1.0  $\mu\text{L}$  was injected into the GC-MS instrument using a Hamilton syringe.

### Optimization of hair sample preparation

Three critical factors were investigated: The type of the extracting solvent, pH, and temperature, in the preparation of hair samples.

### Validation of the method

Validation experiments were performed for the assay according to the guidelines of the scientific working group for forensic toxicology (SWGTOX) guidelines [20].

### Linearity

Calibration of the assay was evaluated using drug-free hair fortified with morphine, codeine, tramadol and methadone at seven concentrations ranging from 0.0 to 2.5 ng/mg, with cocaine as the internal standard at a concentration of 1.0 ng/mg. Each concentration was analyzed three times and a graph of the resulting signals (relative peak area) against concentration was plotted (internal standard method). The regression equation (Equation 1)

$$1. y = ax + b$$

and the correlation coefficient ( $R^2$ ) were calculated using Microsoft Excel. An  $R^2 > 0.99$  indicates the linearity of the regression line.

### Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were calculated based on criteria of 3.3 for LOD and 10 for LOQ, with  $S$  representing the standard deviation of blanks and  $m$  representing the slope of the calibration graph.

### Intra and inter-day precision

Experiments were conducted for four different drug concentrations at three intervals within a day (intraday assay) and repeated on three different days (interday as-

say) under constant conditions. Precision was assessed as the percentage of relative standard deviation (RSD%).

### Accuracy

Three specified concentrations of control samples were prepared within the range of concentration, and each was analyzed three times. The average signals ( $y$ ) were calculated and concentrations were determined using the regression equation (Equation 1) derived from the initial standard curve. By dividing the known value of  $y$  by the obtained concentration, the slope of the line ( $a$ ) and the intercept ( $b$ ) were used to calculate the accuracy of the method against the actual concentration (Equation 2).

2.

$$\text{Recovery\%} = \frac{\text{obtained concentration}}{\text{Real concentration}} \times 100$$

### Selectivity

Ten control hair samples, with and without a history of substance abuse, were used with and without standards for injection into the GC/MS instrument. The assessment focused on determining the presence or absence of interfering peaks (endogenous substances) near the retention times of the analytes.

### Application of the method in real samples

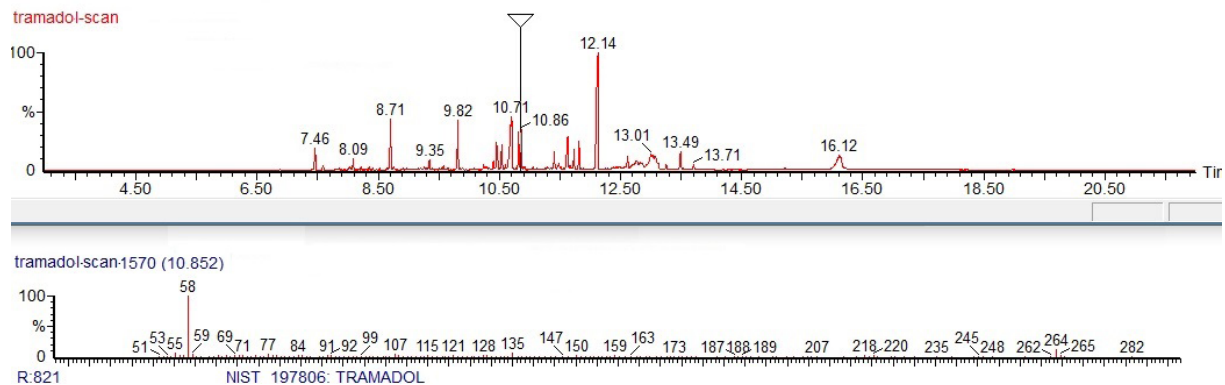
Eleven head hair samples consisting of 10 males and one female obtained during forensic autopsy were analyzed. Simultaneously, routine postmortem toxicological analyses were conducted on conventional biological samples such as stomach content, urine, blood and liver tissue.

**Table 1.** The selected ratio of mass-to-charge ( $m/z$ ) values of identifier ions with and without derivatization by MSTFA for each selected opioid in the GC-MS

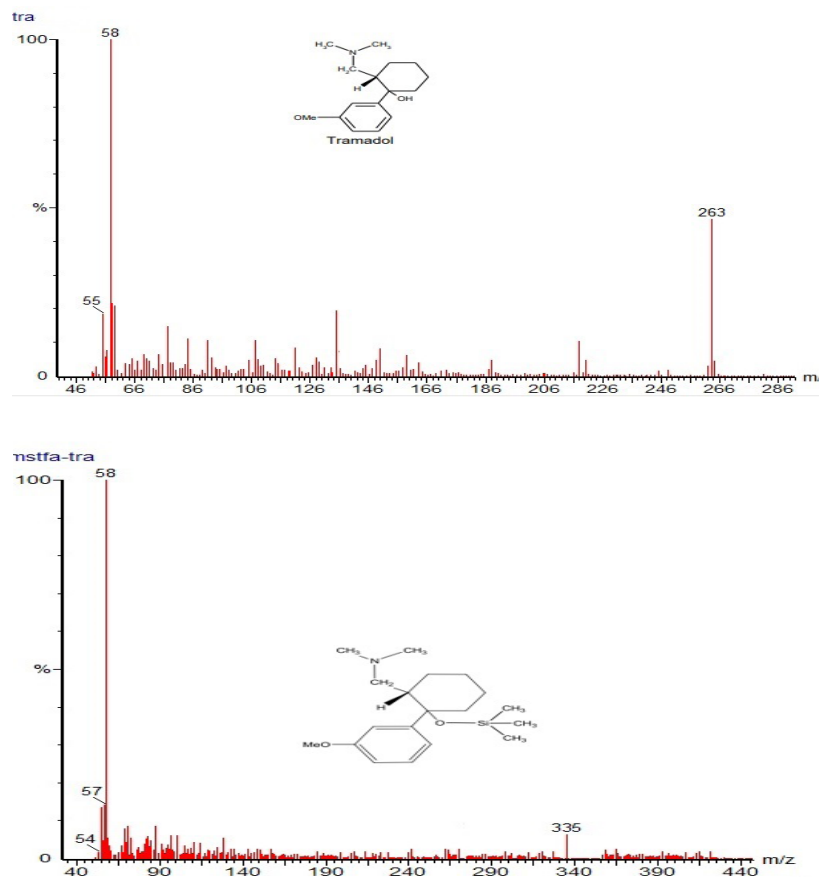
Analyte	$m/z$ Values of the Identifier Ions	
	Before Derivatization	Derivatization With MSTFA
Tramadol	58, 263	58, 335
Methadone	72, 309	72, 309
Codeine	162, 299	73, 178, 196, 371
Morphine	42, 162, 285	73, 146, 236, 429

MSTFA: N-methyl-N-trimethylsilyl-trifluoroacetamide.

A



B



**Figure 1.** The extraction and reaction of tramadol with MSTFA when spiked in hair

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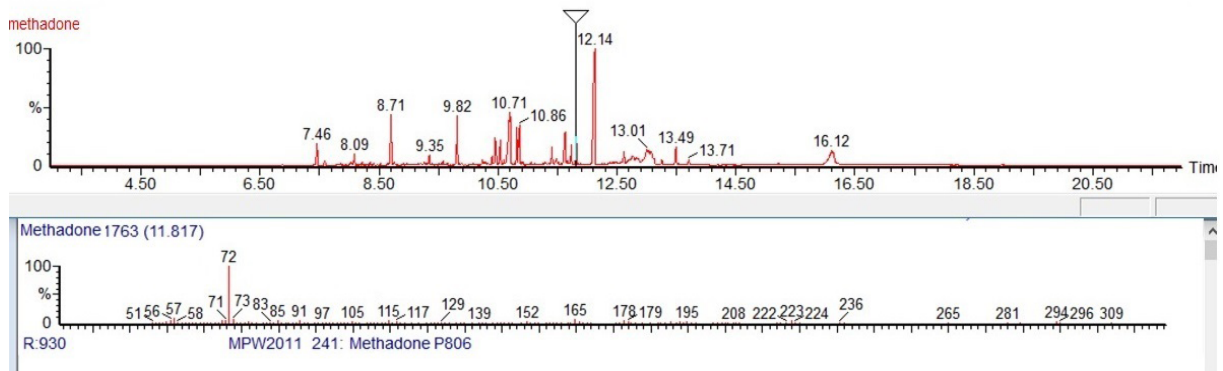
Notes: The GC chromatogram of the extracted hair sample is as follows: A) Chromatogram of tramadol ( $R_t=10.85$  min) for hair spike concentration (50 ng/mg); B) Mass spectral data of tramadol (selected ions  $m/z=58, 263$ ) and tramadol-TMS derivative (selected ions  $m/z=58, 335$ ) when spiked at 50 ng/mg.

## Results

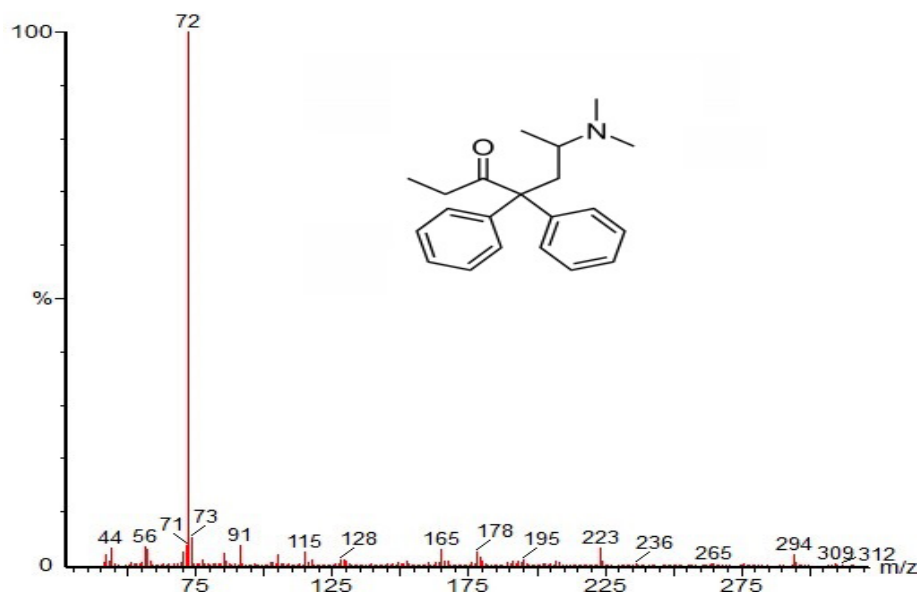
Table 1 shows the retention times and mass-to-charge ratio ( $m/z$ ) of identifier ion fragments of tramadol, methadone, codeine, and morphine both before and after

derivatization with MSTFA. Each compound was converted to its trimethylsilyl (TMS) derivative (Figures 1, 2, 3 and 4).

A



B



**Figure 2.** The extraction and reaction of methadone when spiked in hair

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A) Chromatogram of methadone ( $R_t=11.83$  min) for hair spike concentration (50 ng/mg); B) Mass spectral data of methadone (selected ion  $m/z=72, 309$ ) when spiked at 50 ng/mg.

Table 2 shows the peak area of compounds in blank hair samples with concentrations of 0.1, 0.2, 0.3, 0.5, 1, and 2.5 ng/mg for each compound.

### Optimization of the hair sample extraction

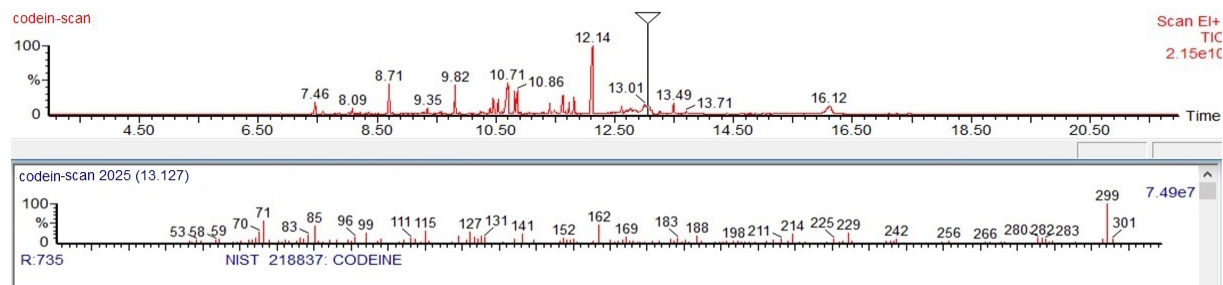
The impact of incubation time on the extraction of analytes from hair was examined at temperatures of 30, 40, 56, 70 and 80°C. Optimal results for all standards were achieved at a temperature of 56 °C (Figure 5). To determine the pH effect on extraction efficacy, each analyte was triplicate-injected into the GC-MS at various pH levels ranging from 6.4, 7.4, 8.4, 9.4 to 10.4. The most

favorable outcomes for all analytes were observed at pH 8.4 (Figure 6). The effect of the type of extraction solvent on the recovery of morphine, codeine, tramadol, and methadone in hair samples was investigated using different solvents, including chloroform: Isopropanol (80:20 V/V), chloroform, and dichloromethane. The best results for all analytes were obtained with a mixture of chloroform /isopropanol (80:20 V/V) (Figure 7).

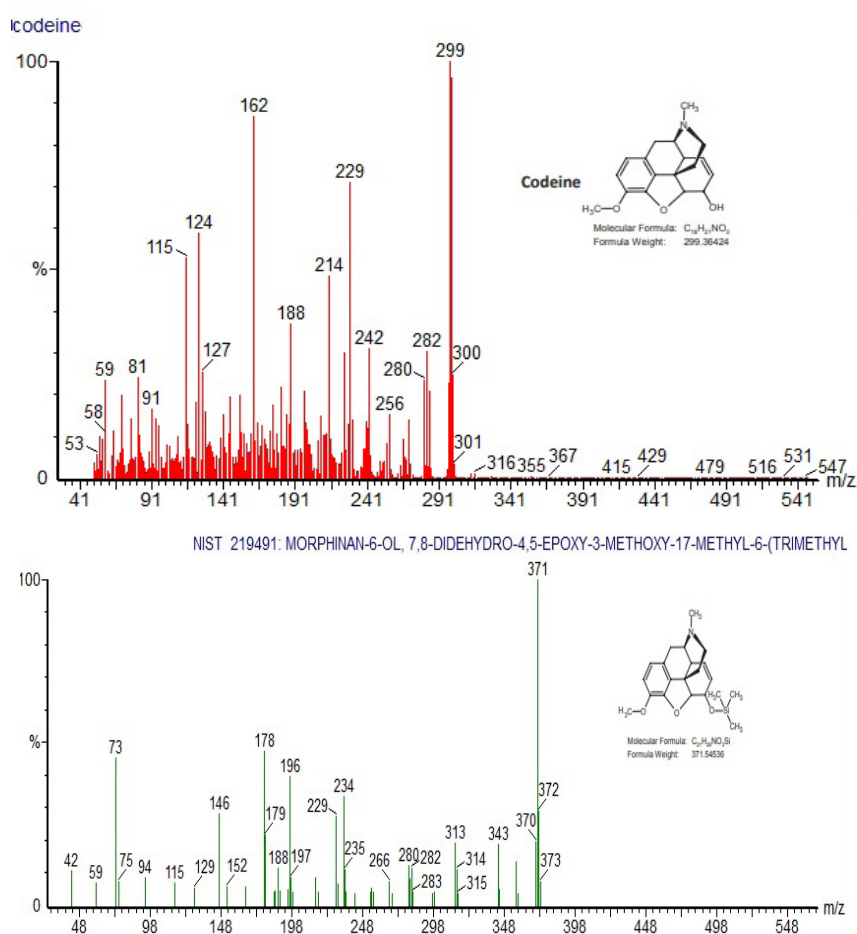
### Results of method validation

To assess the linearity of calibration curves, regression analysis was conducted. Linear evaluation across con-

A



B



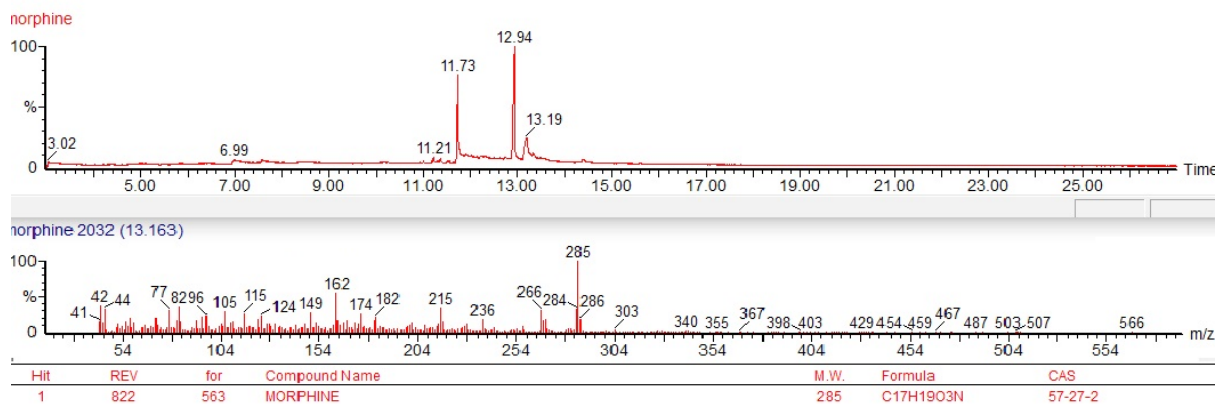
**Figure 3.** The extraction and reaction of codeine with MSTFA when spiked in hair

Notes: The GC chromatogram of the extracted hair sample is as follows: A) Chromatogram of codeine (Rt=13.1 min) for hair spike concentration (50 ng/mg); B) Mass spectral data of codeine (selected ions m/z=162, 299) and codeine-TMS derivative (selected ions m/z=73, 178, 196, 371) when spiked at 50 ng/mg.

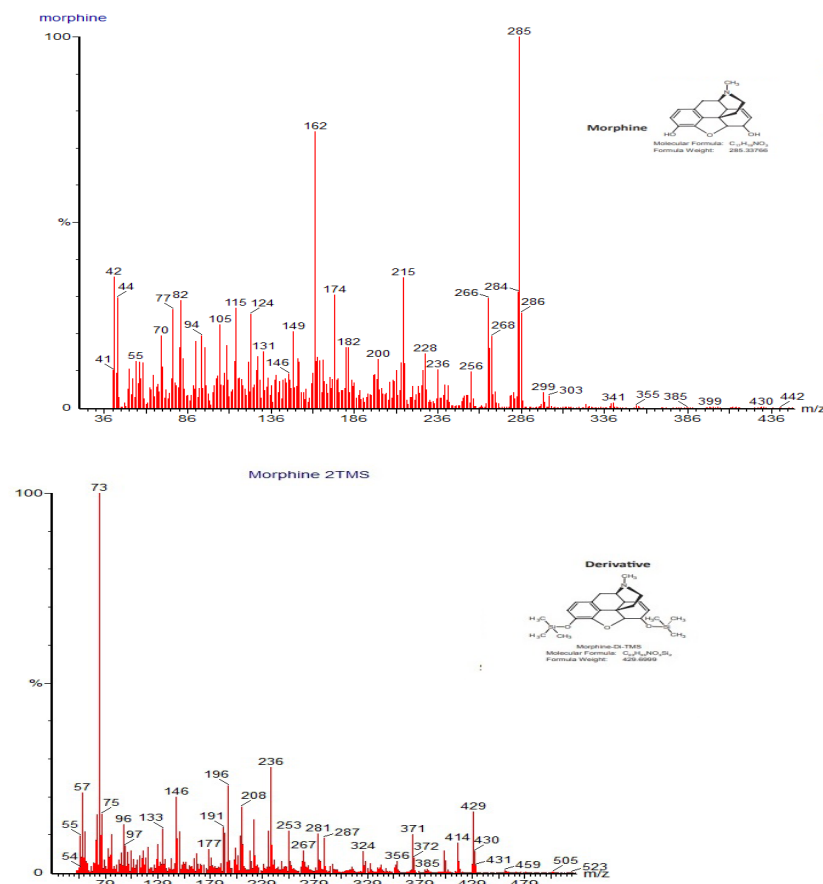
centrations ranging from 0.1 to 2.5 ng/mg of hair sample, considering the slope of the calibration curve, yielded R<sup>2</sup> values between 0.98 and 0.99 for all analytes (Table 3, Figure 8). Method precision, expressed as the percentage of relative standard deviation over five repetitions at four different concentrations in blank hair samples (0.3, 0.5,

1 and 2.5 ng/mg), was calculated. The precision of the method exhibited acceptable ranges of RSD within a day and between days for all compounds (<3.5%) based on the obtained results (Table 4).

A



B



**Figure 4.** The extraction and reaction of morphine with MSTFA when spiked in hair

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Notes: The GC chromatogram of the extracted hair sample is as follows: A) Chromatogram of morphine (Rt=13.19 min) for hair spike concentration (50 ng/mg); B) Mass spectral data of morphine (selected ions m/z=42, 162, 285) and morphine-TMS derivative (selected ions m/z=73, 146, 236, 429) when spiked at 50 ng/mg.

Accuracy, represented as the percentage of recovery (R%), fell within an acceptable range of deviation from 102.4% to 91.8%. The proposed method defined a detection range for selected opioids in hair samples from 0.12 to 0.22 ng/mg (Tables 3, 4 and 5).

Given that the analysis of drug-free hair samples revealed no interference from matrix components, it can be inferred that the method operates selectively (Figure 9).

**Table 2.** The peak area of chromatograms for different concentrations of standards and the blank sample

Concentration (ng/mg)	Peak Area				
	Cocaine (IS)	Tramadol	Methadone	Morphine	Codeine
0.00	5438303	214341	182459	203635	194892
0.10	5326096	538299	869392	490406	311930
0.20	5317405	1166445	1293643	1468312	1106665
0.30	5232603	1488464	1355475	1601707	1453767
0.50	5097315	2793358	2180794	2413195	2556753
1.00	5623883	4143013	5624121	4557698	4434643
2.50	5540098	10989863	11365840	11072987	10814765
CV (%)	3.4	0.9-2.5	0.4-2.2	0.9-2.4	0.7-2.3

IS: Internal standard.

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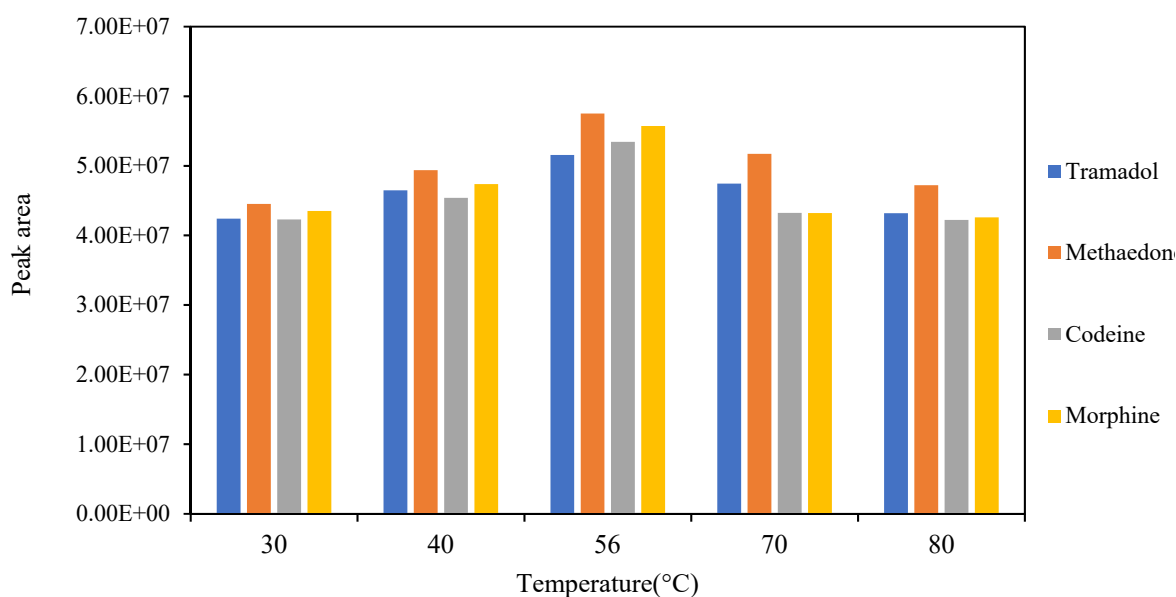
Notes: Cocaine concentration as an internal standard is 1.0 ng/ $\mu$ g.

### Application of the method in real forensic hair samples

Table 6 summarizes the results of the analysis of the opioids in real hair samples from cadavers that underwent forensic autopsies and postmortem toxicological analyses.

### Discussion

In this study, we developed a GC-MS method in SIM mode for the simple and reliable identification and quantitation of the selected opioids (tramadol, methadone, morphine, and codeine) in the human hair samples in forensic cases. Optimization of derivatization parameters with MSTFA containing pH, temperature and solvent type, indicated that the best results for all selected drugs were achieved at a temperature of 56 °C, pH 8.4 and an isopropanol/chloroform mixture as extracting solvents.



**Figure 5.** The effect of temperature on the extraction efficacy of opioids in hair samples

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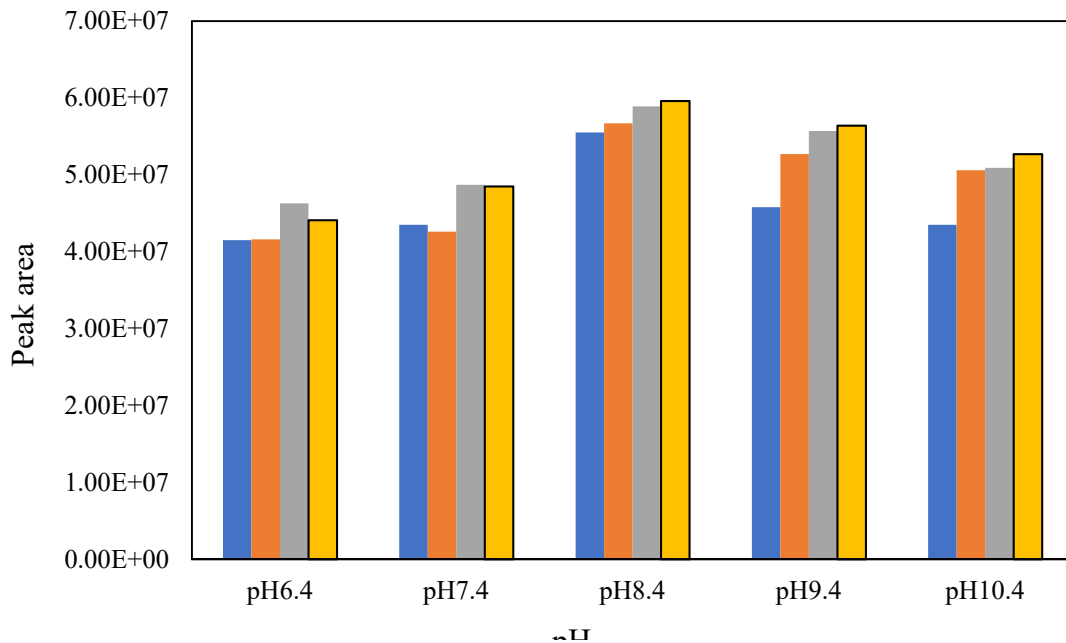


Figure 6. The effect of pH on the extraction efficacy of opioids in hair samples

Based on the results of the method validation, linearity assessment at concentrations ranging from 0.1 to 2.5 ng/mg of hair, considering the slope of the calibration curve, yielded R<sup>2</sup> values exceeding 0.99 for all analytes. Additionally, in terms of accuracy and precision, the repeat-

ability index (RSD) within a day and between days for all drugs was <3.5%. Concerning accuracy, the recovery values ranged from 91.8-102.4% for all compounds, indicating a deviation within an acceptable standard. The

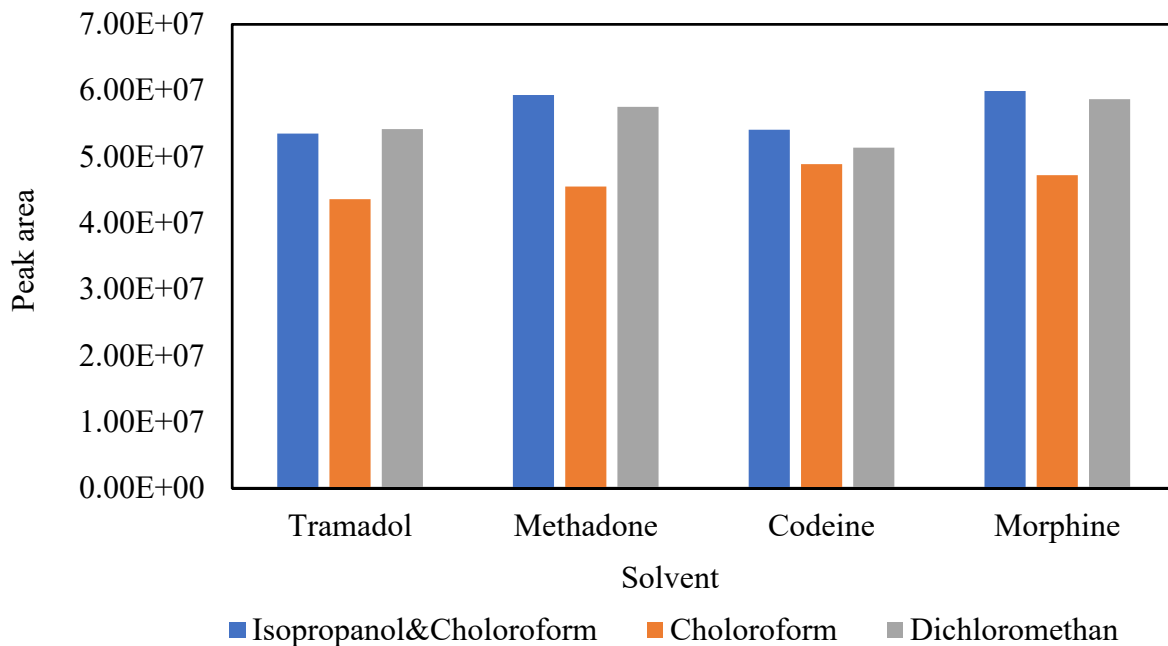


Figure 7. The effect of type of solvent on the extraction efficacy of opioids in hair samples

**Table 3.** Summary of the merits of the proposed method for the extraction and determination of opioids from hair samples

Analyte	Regression Equation	DLR (ng/mg)	R <sup>2</sup>	LOD (ng/mg)	LOQ (ng/mg)
Tramadol	RPA=0.7633C-0.0554	0.6-2.5	0.9924	0.17	0.58
Methadone	RPA=0.8099C-0.0647	0.7-2.5	0.9925	0.19	0.65
Morphine	RPA=0.7744C-0.0618	0.4-2.5	0.9972	0.12	0.39
Codeine	RPA=0.7664C-0.0425	0.7-2.5	0.9963	0.21	0.69

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Abbreviations: RPA: Relative peak area; LOD: Limit of detection; LOQ: Limit of quantitation; R<sup>2</sup>: The coefficient of determination.

**Table 4.** Results of sample validation in terms of inter- and intra-day precision

Analyte	Inter-day Precision (RSD%)			Intra-day Precision (RSD%)								
				1 <sup>st</sup> day			2 <sup>nd</sup> day			3 <sup>rd</sup> day		
	0.5*	1.0*	2.5*	0.5*	1.0*	2.5*	0.5*	1.0*	2.5*	0.5*	1.0*	2.5*
Tramadol	2.2	2.3	1.4	1.7	0.4	1.9	2.5	1.4	2.1	1.6	0.9	1.9
Methadone	2.5	1.4	2.7	1.8	2.0	2.2	1.6	2.0	1.5	1.5	0.4	0.5
Codeine	2.3	1.6	2.6	3.1	0.7	2.3	1.0	0.9	1.2	0.9	0.8	2.5
Morphine	2.5	3.6	1.7	1.6	0.8	2.4	1.1	1.6	0.9	3.5	1.9	0.9

RSD: Relative standard deviation. \*Concentration (ng/mg).

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proposed method set a detection range for selected drugs in hair samples from 0.12 to 0.21 ng/mg.

The developed method successfully identified morphine tramadol, methadone, and codeine in hair samples from deceased individuals. Based on the results, the proposed GC-MS method is potentially applicable for the identification of selected opioids in hair samples in forensic investigations.

Today, in forensic toxicological analyses, hair samples are recognized as an important biological specimen

alongside urine and blood. In forensic or clinical toxicology, detecting drugs in human hair is considered a complementary approach to analyzing drugs in urine or blood, particularly for assessing long-term exposure.

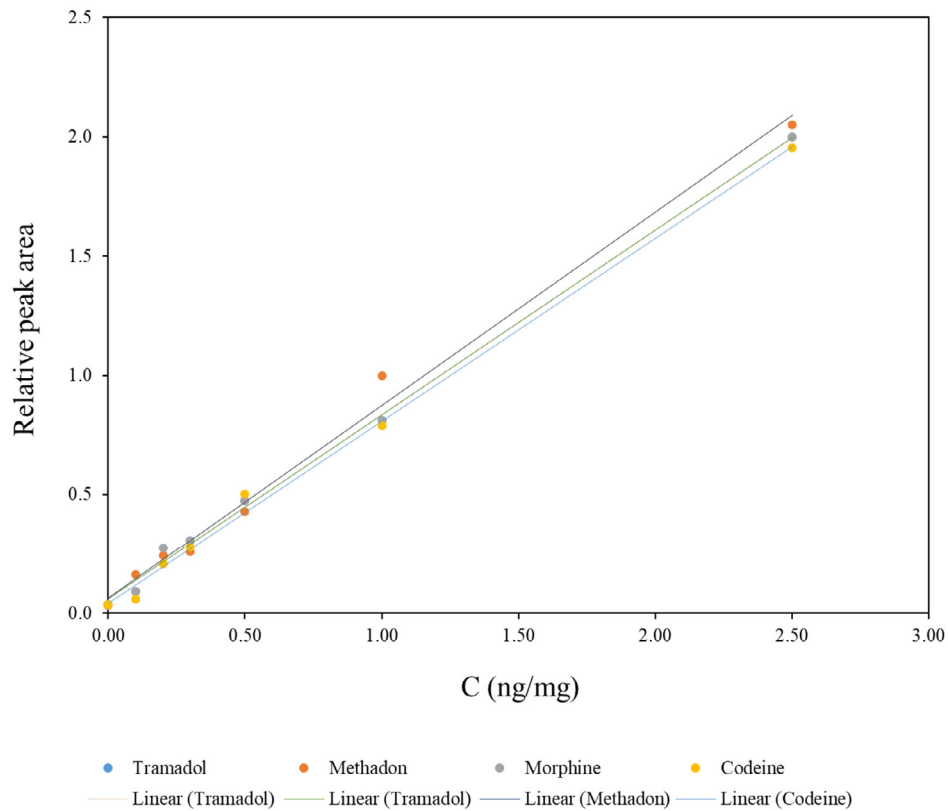
GC-MS is the most common used analytical method in hair analysis, surpassing other routine methods in terms of selectivity, sensitivity, and specificity. The quantification of drugs in hair is conducted using SIM mode due to the minute amounts of drugs present. GC-MS has been used for analyzing opiates, cocaine and related metabolites (benzoylecgonine, ecgonine methyl ester and coca-

**Table 5.** Accuracy as presented extraction recovery at different concentrations of selected opioids in hair samples

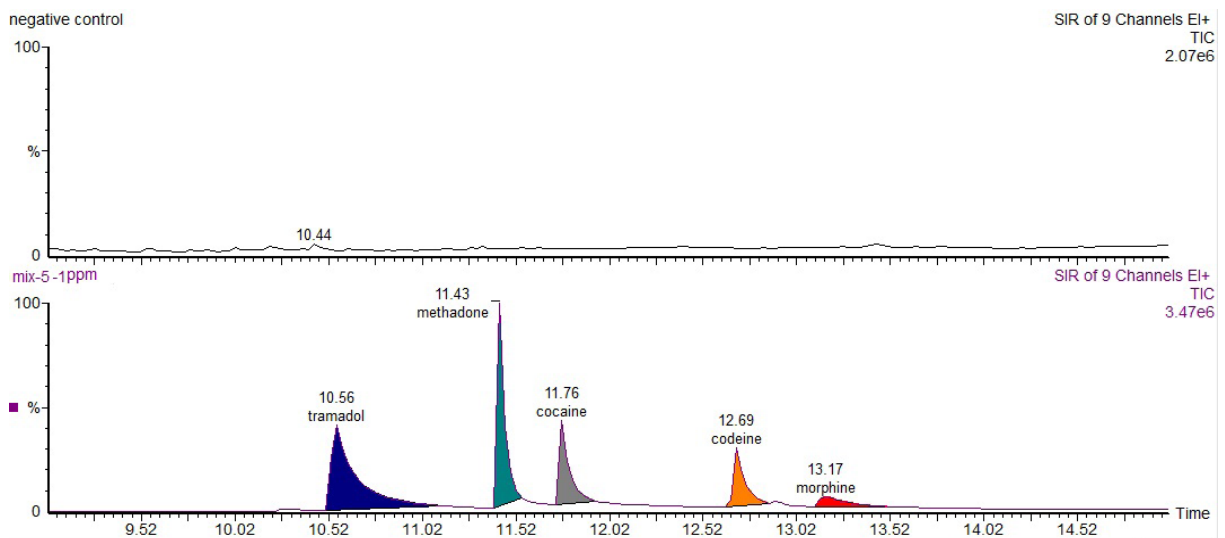
Analyte	Recovery (%)		
	0.5*	1.0*	2.5*
Tramadol	91.8	95.7	98.2
Methadone	92.3	96.3	102.4
Codeine	94	98	98
Morphine	94	97	99

\*Concentration (ng/mg).

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**Figure 8.** The calibration curve and linear equation of tramadol, methadone, codeine, and morphine in spiked hair samples by GC-MS analysis



**Figure 9.** The selectivity of the proposed method for the analysis of tramadol, methadone, codeine, and morphine in spiked human hair samples

The chromatogram above shows a blank (drug-free) hair sample and the chromatogram below shows the spiked sample at 5 ng/mg (cocaine was added as an internal standard with a concentration of 1.0 ng/ $\mu$ L).

**Table 6.** Analysis of hair samples for the determination of opioids in cadavers referred to the forensic toxicology laboratory of Kermanshah Province (Kermanshah City, Iran) (n=11 cases)

Case No.	Gender	Age (y)	Hair Color	Length of hair (cm)	The Result of Hair Analysis (Concentration, ng/mg)	Postmortem Toxicological Finding (Type of Sample)
1	Female	27	Brown (colored)	4	Negative	Phosphine (stomach content)
2	Male	42	Dark brown	3	Tramadol (1.3)	Methamphetamine (urine)
3	Male	36	Black	3	Methadone (0.2)	Methadone (stomach content)
4	Male	34	Dark brown	3	Morphine (2.1) Codeine (1.5)	Morphine and Codeine (stomach content and urine)
5	Male	36	Dark brown	3	Methadone (1.2)	Methadone (stomach content and urine)
6	Male	40	Dark brown	4	Negative	Phosphine (stomach content)
7	Male	20	Black	5	Codeine (2.1) Morphine (1.3)	Methadone (stomach content and urine)
8	Male	38	Dark brown	3	Methadone (2.3) Codeine (0.8) Morphine(1.6)	Methadone (stomach content and urine)
9	Male	28	Black	3	Negative	Negative
10	Male	59	White	3	Negative	Negative
11	Male	30	Dark brown	3	Methadone (4.4) Codeine (0.9)	Methadone (stomach content and urine)

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**Table 7.** Comparing GC-MS with other analytical methods for the determination of opioids and other abused drugs in hair samples

Method	Analytes	LOD (ng/mg)	Correlation Coefficient (R <sup>2</sup> )	Recovery (%)	Reference No.
LC-MS-MS	Codeine, Morphine, 6-acetylmorphine, Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone	0.05	>0.99	54-81	[30]
LC-MS-MS	Codeine-6-glucuronide, Dihydrocodeine-6-glucuronide, Hydromorphone-3-glucuronide, Morphine-3-glucuronide, Morphine-6-glucuronide, Oxymorphone-3-glucuronide, Codeine, Dihydrocodeine, Dihydromorphone, Hydrocodone, Hydromorphone, Morphine, Oxycodone, Oxymorphone and 6-acetylmorphine	0.0002-0.001	>0.99	92.6-107.8	[31]
UHPLC-MS/MS	33 opioids, opiates, cocaine and amphetamines	0.006-0.063	0.99	37-107	[32]
ESI-MS/MS	Morphine, 6-Methylacetylmorphine (6-MAM), Codeine, Cocaine, Benzoylcegonine (BZE)	0.0044-0.039	0.98	96-106	[33]
GC-MS	Morphine	0.03	0.98	96.97-97.63	[34]
GC-MS	Morphine, Cocaine	0.01-0.02	0.998 to 0.999	82.2-119.4	[35]
GC-MS	Morphine, Codeine, Tramadol, Methadone	0.12-0.22	0.98-0.99	98-102.4	Present study

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ethylene), amphetamine-type stimulants (STA), cannabinoids, ethanol chronic use biomarkers (such as ethyl glucuronide [EtG] and fatty acid ethyl esters [FAEE]), benzodiazepines and other therapeutic drugs (including antidepressants and antipsychotics), pesticides and other environmental pollutants and doping substances [21]. GC-MS with electron ionization (EI), similar to many other ionization methods, like tandem MS or hyphenated with time-of-flight-mass spectrometry (TOF-MS) has been used.

Derivatization is necessary to enhance the chromatographic performance and detection response of analytes, with alkylations, acylations, and silylation being common methods. Silylation is widely used for various types of analytes, including opioids and amphetamines [22]. Therefore, in the present study, we used silylation for the derivatization of the selected opioids. Given that morphine, codeine (as indicators of opium and opiate abuse), tramadol, and methadone abuse or addiction are the most common types of opioids in Iran [2, 22, 23], they were chosen as the key analytes in this study.

Khajuria and Nayak developed a GC-MS method for the quantification of morphine in hair samples from 40 chronic heroin abusers at different time intervals. Hair samples were collected on the day of admission, as well as 45 and 90 days post-admission. All hair samples showed detectable levels of morphine, with concentrations ranging from 0.26 to 2.2 ng/mg (average 1.33 ng/mg) and a detection limit of 0.1 ng/mg [24]. Our results align with this finding regarding the detection limit of morphine.

In another study, a sensitive GC-MS method was used for the identification and quantification of abused drugs, including morphine, codeine and 6-monoacetylmorphine. The hair samples were prepared with methanol under ultrasonication at a temperature of 50 °C following a three-stage rinsing process to eliminate external contaminants and hair contamination. Subsequently, derivatization with BSTFA was carried out and optimization of derivatization parameters, including time, temperature and derivatization agent volume, was performed. The method exhibited a linear range of 2-350 ng/mg ( $R^2=0.99$ ). LOD ranged from 0.05 to 0.46 ng/mg of hair with an RSD of 14.94% to 7.88% and accuracy ranging from 96% to 118% [25]. Although the detection range of our proposed method for morphine was higher than that of this study, our method exhibited an RSD for inter- and intra-day precision not exceeding 3.7%, indicating an advantage of this method.

Moller et al. presented a sensitive analytical method for the separation of morphine, codeine, and 6-monoacetylmorphine from hair samples using headspace solid-phase microextraction (HS-SPME) coupled with GC-MS. The LOQ was 0.01 ng/mg for morphine and 6-acetylmorphine and 0.005 ng/mg for codeine, with  $R^2$  values exceeding 0.992. The robustness of the method was demonstrated to be acceptable as inter-day and intra-day precision fell below 15% for each opiate analyzed. Linearity was observed between 0.01 ng/mg and 5 ng/mg for each opiate. Precision within a day ranged from 3.44% to 11.46% and between days from 3.4% to 5.17 ng/mg. Our study results utilizing the proposed method exhibited improved precision within days and accuracy in a day compared to the method [26].

In a study, the researcher used a GC-MS method coupled with solid-phase extraction (SPE) for the simultaneous determination of tramadol and its main metabolite, O-desmethyl tramadol (ODMT), in human hair samples. Hair samples (60 mg) were decontaminated with dichloromethane, water and acetone and then extracted with methanol in an ultrasonic bath. Subsequently, the samples underwent SPE for purification. Derivatization was carried out using 1% BSTFA and TMCS. Then, the compounds were analyzed by GC-MS. Regression analysis for tramadol and ODMT in the concentration range of 0.20-1 ng/mg was linear, with LOD and LOQ values of 0.0003 and 0.0013 ng/mg for tramadol, and 0.0001 and 0.0006 ng/mg for ODMT, respectively [27]. The obtained results showed a superior detection limit for tramadol compared to our study (0.17 ng/mg).

In this study, we successfully detected methadone in hair samples with linearity in the calibration curve within the range of 0.1 to 2.5 ng/mg ( $R^2=0.99$ ). The LOD was 0.19 ng/mg in hair samples, with accuracy ranging from 92% to 102%. In a previous study, a simple and sensitive method for the analysis of methadone and its main metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in hair samples was developed and validated. After a short alkaline incubation, the analytes were extracted from the hair and the extracts were cleaned up by SPE. Linearity was obtained from 0.1 to 30 ng/mg for both analytes with correlation coefficients  $>0.99$  [28]. These results were consistent with our developed method for methadone analysis in hair.

Selectivity is expressed as the ability of a method to differentiate the analyte from all potentially interfering substances. The method selectivity was evaluated by analyzing blank hair samples to detect any possible interferences in the retention times of the studied analytes. In

our study, the blank hair samples showed no interference when selected opioids were added. The separation of the opioids was successfully achieved under optimized conditions.

Our developed method was successfully applied to real forensic samples, yielding appropriate findings that aligned with the case history. The concentrations of detected opioids in hair samples of our cases ranged from 1.3 to 2.1 ng/mg for morphine, 0.8 to 2.1 ng/mg for codeine, 1.3 ng/mg for tramadol, and 0.2-4.4 ng/mg for methadone. These results are consistent with previous studies on the analysis of opioids in hair samples. For instance, Khajuria and Nayak [24] reported a range of 0.26 to 2.2 ng/mg (mean, 1.33 ng/mg) for morphine concentration [24].

In a previous study, a GC-MS method was developed for the simultaneous analysis of morphine, codeine, 6-monoacetylmorphine (6-MAM), dihydrocodeine, EDDP (methadone metabolite), methadone, amphetamine, BZE and cocaine in hair samples. Following enzymatic digestion of the hair samples using beta-glucuronidase/aryl-sulfatase, a SPE procedure and derivatization with pentafluoropropionic anhydride/pentafluoropropanol, the drugs were analyzed using GC-MS. Fifteen hair samples of a methadone treatment program were collected, of which 95% were positive for methadone (mean concentration=10.9 ng/mg), 76% for the metabolite EDDP (mean concentration=1.2 ng/mg) and 69% for opiates (mean concentration, 6-MAM, 7.3 ng/mg; morphine, 2.9 ng/mg; codeine, 1.0 ng/mg) [29].

Table 7 summarizes the comparison of the proposed method for the determination of opioids in hair samples using GC-MS with previous methods. One of the significant limitations of the current study is the absence of investigation into drug metabolites, attributed to the lack of access to analytical samples of metabolites due to financial constraints. It is recommended that future studies focus on developing and validating methods for the simultaneous determination of drugs along with their main metabolites.

## Conclusion

The present study aimed to develop a selective, accurate, and specific method for the analysis of selected opioids (morphine, codeine, tramadol and methadone as the most frequently abused opioids in Iran) in human hair samples. The developed and validated method was successfully applied to the real forensic samples. The proposed GC-MS method, characterized by accuracy, preci-

sion, sensitivity and reasonable linearity, can be utilized for determining the history of chronic opioid exposure in hair samples within forensic and clinical toxicology settings.

## Ethical Considerations

### Compliance with ethical guidelines

This study was approved by the Ethics Committee of the [University of Tehran](#), Tehran, Iran (Code: IR.UT.VETMED.REC.1401.014). All volunteers participating in the project were provided with verbal and written information and signed an informed consent form.

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This study was extracted from PhD dissertation of Vida Shiri-Ghaleh, approved by the Department of Comparative Bioscience, [University of Tehran](#), Tehran, Iran.

### Authors' contributions

Conceptualization and study design: Kambiz Soltaninejad, Jalal Hassan and Mohammad Kazem Kohi; Experiments performing, data interpretation, statistical analysis and writing the original draft: Vida Shiri-Ghaleh; Review and editing: Kambiz Soltaninejad; Final approval: All authors.

### Conflict of interest

The authors declared no conflicts of interest.

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