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Determination of Dextromethorphan and Dextrorphan in Urine Using Bar Adsorptive Microextraction Followed by Gas Chromatography–Mass Spectrometry Analysis

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Abstract

Over the past few years, the misuse of medications has progressively increased, posing a significant public health concern. This study proposed the development and validation of an alternative and greener analytical method for the determination of dextromethorphan (DXM) and its major metabolite, dextrorphan (DXO), in urine matrices using bar adsorptive microextraction (BAμE), followed by gas chromatography–mass spectrometry (GC-MS) analysis. Under optimized experimental conditions, average recoveries of 96.3% and 80.4% were achieved for DXM and DXO, respectively. The analytical limits obtained were 0.016 μg/mL for the limit of detection and 0.054 μg/mL for the limit of quantification. The working range was from 0.06 μg/mL to 2.0 μg/mL, with linearity for both compounds by determination coefficients ($r^2 > 0.99$) and the goodness-of-fit and lack-of-fit tests. Intra-day precision and trueness yielded values below 8.77% and 16.28%, respectively, for both compounds. Inter-day precision and trueness values were below 7.67% and 9.73%, respectively. The application to 26 urine samples allowed the quantification of both compounds, with concentrations ranging from 0.06 to 3.21 μg/mL for DXM and 0.06 to 8.88 μg/mL for DXO. The method proved to be effective, selective, sensitive, simple, and cost-effective in the detection and quantification of DXM and DXO, reinforcing its applicability and feasibility in various laboratory contexts.

Keywords: dextromethorphan; dextrorphan; BAμE; GC-MS; urine

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1. Introduction

The abuse of medicines has increased globally, driven both by ease of access and the misconception that medicines are completely safe [1]. This includes self-medication and intentionally exceeding the recommended dose [2,3]. This trend is particularly concerning in the younger populations, and it is becoming a public health issue. Data reported by the National Center for Drug Abuse Statistics show that 12% of prescription drug users are addicted [4]. A report from SAMHSA 2022 [5] further highlights that 14.2 million people in the U.S. aged 12 or older misused prescription drugs in 2022. Painkillers were the most misused medicines, followed by tranquilizers or sedatives and stimulants.

Non-prescribed medications that many adolescents and adults commonly abuse are antihistamines and cough suppressants, such as dextromethorphan (DXM) [3]. DXM is an antitussive agent found in most medications prescribed for the symptomatic treatment of dry coughs. Originally synthesized as a non-narcotic alternative to codeine and other narcotic cough suppressants. Despite its purpose, DXM has been subject to abuse through the consumption of excessive doses since the 1960s and 1970s [3]. More recently, DXM has also become available in powder form, sold on the internet, and marketed as a more potent compound for recreational use, with 5 reported deaths connected to DXM [6,7]. These drugs can be found in syrup, oral solution, or soft tablet form. The antitussive action begins 15 to 30 min after administration of 15 mg and lasts 5 to 6 h, depending on the activity of the CYP2D6 enzyme in each individual.

DXM is primarily metabolized into dextrorphan (DXO), its active metabolite, through O-demethylation by CYP2D6 [8–10]. The half-life of DXM is 1.4–3.9 h, and the half-life of DXO is between 3.4 and 5.6 h. After enzymatic hydrolysis, urinary concentrations of DXO typically fall within the μg/mL range in extensive metabolizers, while DXM concentrations are usually in the low ng/mL range [11]. Although structurally similar to opioids, DXM does not bind to opioid receptors. Its mechanism of action involves antagonizing the N-methyl-D-aspartate (NMDA) receptor and agonizing sigma-1 receptors [12]. As an NMDA receptor antagonist, it provides cough relief. It also induces hallucinogenic and dissociative effects, often sought for recreational use [13]. Both compounds can induce psychoactive effects; however, DXO has a higher affinity for NMDA receptors, resulting in stronger effects. At therapeutic doses, DXM is considered a safe drug, but at doses between 100 and 200 mg, it may cause restlessness and euphoria [12,14].

Recreational use of DXM is rising due to its wide availability on the market, low cost, and the abundance of information on "how to abuse" the substance. Young people believe that this compound is a "safe" alternative to other recreational drugs, such as ecstasy, methamphetamine, and ketamine. However, there is a lack of knowledge on the part of health professionals regarding the toxicological capacity and addictive potential of DXM when taken in high doses [3]. This highlights the importance of developing analytical methodologies that are effective in monitoring this compound in biological matrices. Various methods have been reported for both qualitative and quantitative analysis of DXM and DXO in biological matrices, including high-performance liquid chromatography (HPLC), gas chromatography (GC), chromatography coupled to mass spectrometry (GC-MS and LC-MS), and capillary electrophoresis [9,10,15,16]. Although GC-MS methods often require derivatization of DXO, which adds steps and increases the variability [8,9,17]. In this study, the analytical technique used is GC-MS. However, prior to analysis, it is necessary to carry out a sample preparation stage to extract and concentrate the target analytes while removing interferents. Additional challenges include the variability of the urinary pH and the stability of the compounds during storage and pre-analytical preparation [18]. Furthermore, the large difference in physiological concentration between DXM, typically lower than DXO, complicates simultaneous quantification and ratio determination [11]. Traditionally, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are sample preparation techniques commonly used. These approaches, particularly LLE, although widely used, have the disadvantages of requiring a large sample volume and toxic organic solvents and therefore are not considered environmentally friendly approaches.

From this perspective, the development of cleaner, more sustainable alternatives has emerged, focusing on analytical miniaturization and reduced solvent use [19,20]. Single-drop microextraction (SDME) and sorptive extraction techniques such as solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE), among others, stand out in this context [20,21]. These sorptive techniques provide significant sensitivity in the recovery

of the target analytes, high reproducibility, speed, low cost, and ease of automation [21]. Sorptive extraction relies on equilibrium, where analytes partition between a sample matrix and an extracting phase (e.g., a polymer), thereby concentrating the compounds for later analysis [22]. A notable advancement in this field is bar adsorptive microextraction (BAμE), which is effective for trace analysis of polar and non-polar compounds in various matrices [23,24]. This technique uses a polypropylene (PP) device coated with a sorbent material, which can be selected based on the target analytes and specific application. It is therefore essential to know the physical and chemical characteristics of the sorbent materials to understand how they work. Sorbent materials that are commonly used are activated carbons and polymers, which allow the sorption of both polar and non-polar molecules. Their large surface areas and porous structures promote electrostatic and dispersive interactions [23,25]. Another advantage of using BAμE is the lower volume of solvent required for desorption when compared to other techniques such as solid-phase extraction (SPE) [26]. As mentioned above, BAμE combines sampling, isolation, and enrichment in a single step. The device is cost-effective and easy to prepare and handle. It demonstrates excellent performance, doubles the enrichment factor, and reduces analytical limits [20,24].

The objective of this study was to develop, optimize, and validate an alternative analytical method based on bar adsorptive microextraction (BAμE) followed by gas chromatography–mass spectrometry (GC-MS) for the trace determination of dextromethorphan (DXM) and its main metabolite, dextrorphan (DXO), in urine samples. This approach introduces a simplified, low-cost, and solvent-efficient extraction alternative compared with traditional sample preparation techniques.

2. Materials and Methods

2.1. Chemicals, Standards, and Materials

Methanol (MeOH) HPLC grade (99.9%) was acquired from Honeywell (Saint Germain En Laye, France). Acetonitrile (ACN) HPLC grade (99.9%) was obtained from Carlo Erba (Val-de-Reuil, France). Ultrapure water was obtained from the Direct-Q® water purification system from Merck Millipore (Burlington, MA, USA). The standard solution of DXM (99%) and DXO (99%) was purchased from Sigma-Aldrich (Steinheim, Germany). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, 95–100% from Macherey-Nagel (Düren, Germany)) was used as the derivatizing agent. The external standard (ES) was bromopentafluorobenzene (BPFB, 99% from Sigma-Aldrich (Steinheim, Germany)). Bisoltussin® oral solution was produced by SANOFI (Porto Salvo, Portugal).

Stock solutions of each of the analytes, DXM and DXO, were prepared with a final concentration of 1000 μg/mL in MeOH. A mixed standard solution of DXM and DXO was prepared from the stock solutions at a concentration of 10 μg/mL in MeOH. The working solutions were prepared daily from the appropriate dilution of the 10 μg/mL mixed solution. ES was prepared at a concentration of 100 μg/mL in ACN. All the solutions were stored at −20 °C.

The Oasis® HLB co-polymer (HLB; reverse phase *N*-vinylpyrrolidone-divinylbenzene co-polymer; particle size of 30 μm, pore size of 80 Å, and surface area of 800 m^2/g) was purchased from Waters (Milford, MA, USA).

2.2. Urine

Urine samples were provided from healthy volunteers (n = 5) following oral administration of a single therapeutic dose (15 mL) of Bisoltussin®, corresponding to 30 mg of DXM. Samples were collected at defined time intervals post-administration: 1, 3, 6, 12 (for one volunteer), and 24 h for all participants. Urine samples were collected from all

the volunteers before consuming the drug, resulting in a total of 26 samples. For method validation purposes, drug-free urine was collected from a 24-year-old female volunteer with no reported use of DXM or related compounds. All samples were stored at −80 °C until analysis. Prior to instrumental analysis, urine specimens were filtered using 0.22 µm PTFE syringe filters (13 mm diameter) to remove particulate matter.

2.3. Experimental Set-Up

2.3.1. Preparation of the BAµE

The preparation of the BAµE devices was conducted in-house following previously established protocols [24,25]. Each device consisted of a hollow cylindrical tube (∼7.5 mm in length and 3 mm in diameter), externally wrapped with a layer of adhesive tape coated with powdered polymer HLB Oasis (1.5 ± 0.1 mg). After preparation, the devices were subjected to a cleaning step in ultrapure water under constant agitation for 10 min.

2.3.2. Optimization Assays

For method optimization, preliminary microextraction assays were performed using 1.0 mL of ultrapure water placed in microtubes of 2 mL and spiked with 100 µL of a standard working solution containing DXM and DXO at a concentration of 10 µg/mL. Then, the BAµE devices were inserted into the spiked aqueous media. Microextraction was carried out at room temperature under agitation using a thermoshaker (Biosan, Riga, Latvia) set to defined time and speed parameters. After the microextraction, the devices were retrieved with tweezers, dried on absorbent paper, and transferred to glass vials with an insert containing 100 µL of an organic desorption solvent. Back-extraction was performed under ultrasonic agitation for a specific period. Upon completion, the devices were removed, and the extracts were evaporated to dryness at 75 °C using a heated evaporator.

The dry residue was then derivatized with 50 µL of MSTFA, followed by microwave-assisted derivatization at 1200 W for 3 min. After cooling, 2 µL of the external standard solution was added. Vials were immediately sealed and placed in the GC-MS autosampler for analysis. Blank controls were prepared in parallel using 1.0 mL of ultrapure water and 100 µL of methanol.

To achieve the highest extraction efficiency, various parameters were optimized using a one-variable-at-a-time (OVAT) approach. First, the best sorbent material was found among 9 different materials evaluated (including 5 types of activated carbons (ACs) and 4 polymers). Then, the following tests included desorption solvent (MeOH, ACN, and MeOH/ACN (50:50, v/v)), desorption time (15, 30, 45, and 60 min), speed (600, 1000, and 1400 rpm), extraction time (30, 60, and 90 min), matrix pH (2.0, 5.5, 10, and 12), ionic strength (0, 5, 10, and 15% in NaCl, m/v), and organic modifier (0, 5, 10, and 15% in MeOH, v/v).

2.3.3. Instrumental Set-Up

GC-MS analysis was carried out using an Agilent Technologies system (Agilent, Santa Clara, CA, USA), comprising a 6890N Series gas chromatograph coupled to a 5973 Network mass-selective detector. Sample injection was performed via an Agilent 7683 Series automatic liquid sampler equipped with a split/splitless inlet. Chromatographic separation was achieved using an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies) operated at a constant flow rate of 1.0 mL/min, using helium as the carrier gas. The injection volume was 1.0 µL, introduced in splitless mode with a viscosity delay of 3 s. The injector temperature was set at 280 °C. The GC oven temperature program was as follows: initial temperature of 50 °C (held for 2 min), increased at 2.5 °C/min to 60 °C, followed by a ramp of 40 °C/min to 300 °C, held for 2 min, resulting in a total runtime of 14 min.

The temperatures of the transfer line, ion source, and quadrupole were set at 300 °C, 250 °C, and 200 °C, respectively. The mass spectrometer operated in electron impact ionization mode (70 eV), with data acquisition performed in selected ion monitoring (SIM) mode. A solvent delay of 5 min was applied to prevent detector saturation. Instrument control and data processing were conducted using MS ChemStation software (version G1701EA E.02.02.1431, Agilent Technologies).

2.3.4. Validation Assays

To validate the method in urine, tests were carried out under optimized conditions (microextraction: 1 h (1000 rpm), pH 5.5; 0% MeOH; 5% NaCl; back-extraction: 100 µL of MeOH/ACN (50:50, *v*/*v*), 45 min under ultrasonic treatment). All the validation tests were conducted and evaluated according to UNODC standards, and the following parameters were assessed: linearity, sensitivity, selectivity and specificity, trueness and precision [27]. Calibration standards were prepared between 0.06 and 2.0 µg/mL, with 8 concentration levels, to assess the linearity (estimated with the lack-of-fit and goodness-of-fit tests) and the coefficients of determination (r^2). To determine intra- and inter-day precision and trueness, blank urine samples were fortified with the analytes at three concentration levels. Each level was analyzed in triplicate, and the inter-day assays were evaluated on three different days. Precision and fairness were assessed according to the UNODC guideline. Precision was expressed as the relative standard deviation (RSD%), with acceptance criteria set at ≤15% for all concentration levels, except for the lowest, where ≤20% was accepted. Trueness was determined by calculating the bias parameter (%) with acceptance values between ±15% and ±20% for the lowest concentration, as per UNODC standards [27]. To assess the method's selectivity and specificity, blank urine samples were analyzed to verify the absence of interfering compounds at the retention times of the target analytes. The sensitivity of the methodology was assessed by determining the limits of detection (LOD) and quantification (LOQ), which were determined using the signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively.

3. Results and Discussion

3.1. GC-MS(SIM) Optimization

The first step in this work was to establish instrumental conditions; a mixed solution with an equal concentration of DXM and DXO (10 µg/mL) was analyzed in full scan mode. Chromatographic conditions were optimized to ensure peak resolution with a reduced run time (14 min), yielding retention times of 12.232 min for DXM and 12.323 min for DXO. Subsequently, the mass spectra of each compound were analyzed, and the molecular ions and main fragments for each compound were selected to achieve better selectivity and sensitivity. Table 1 shows the retention times and ions selected for each compound.

Table 1. Retention time and selected ions in SIM mode for each analyte.

	t_R (min)	Selected Ions (*m*/*z*)
DXM	12.232	59; 150; 171; 207; 271 [1]
DXO	12.323	59; 150; 207; 272; 329 [1]

[1] Molecular ions, base peaks, and quantification ions.

After selecting the ions to be monitored, the method was adapted for SIM mode. Instrumental LOD and LOQ were determined based on S/N ratios of 3:1 and 10:1, resulting in 0.16 µg/mL and 0.54 µg/mL, respectively. Calibration curves were constructed using eight concentration levels (0.63–10.0 µg/mL), showing good linearity: r^2 = 0.9921 (DXM) and 0.9962 (DXO). Linearity was confirmed by residual analysis and statistical fit tests, the

goodness-of-fit test (GoF), and the lack-of-fit test (LoF). Intra- and inter-day precision and trueness were also determined at three concentration levels: 1.5 μg/mL, 4.0 μg/mL, and 8 μg/mL. Intra-day and inter-day RSDs ranged from 1.0 to 5.6% and trueness from 1.7 to 19.5%, all within UNODC-accepted limits.

3.2. Optimization of Derivatization Time

Due to the low volatility of the DXO compound, it was necessary to derivatize this compound before GC-MS analysis. For the derivatization method, silylation with 50 μL of MSTFA was used to improve the instrumental response. The derivatization times studied were 1, 2, 3, 4, and 5 min (microwave at 1200 W). The results obtained (Supplementary Materials, Figure S1) show the variation in the compound response with different derivatization times. For the DXO compound, the derivatization time of 3 min reached the maximum of the reaction, presenting a significantly larger area, after which it declined, likely due to degradation or evaporation. DXM showed a consistent response across all times, as it was not derivatized. Thus, a 3 min derivatization time was selected as optimal for DXO.

3.3. BAμE Method Optimization

3.3.1. Sorbent Selection

With the various fixed initial parameters described above, five ACs (SX plus, SX1, R1, CN1, and CA1) and four polymers (HLB, Strata X, ENVI 18, and Strata DVB) were tested. Figure 1 shows the average recoveries obtained for the different sorbent materials tested. Initial observations showed that the polymers provided a better analytical response, with significantly higher recoveries between 10.7% and 70.4%. In contrast, the ACs showed average recoveries between 4.5% and 36.2% for the analytes under study. The AC's performance varied due to their pH_{PZC}, surface area, and pore dimensions, conditioning the interactions based on the acidic or basic characteristics of the surface of the materials involved. Since the tests were carried out at pH 5.5, the surface charge of the ACs varies from negative to positive and almost neutral, which influenced their interaction with the molecules. The polymers, especially HLB and Strata X, showed higher recoveries (61–70.4%) due to their larger surface areas (830 and 800 m^2/g) and chemical structures favoring π–π and hydrophobic interactions with the semi-polar analytes (log $K_{o/w}$: 2.9 for DXO, 3.49 for DXM). Based on these results, Strata X and HLB were selected for further optimization.

3.3.2. Optimization of the BAμE-μLD

The back-extraction step was optimized by evaluating two main parameters: desorption time and the type of solvent used (Figure 2a,b). The solvent must have sufficient elution strength and allow the analytes to fully desorb from the sorbent, a process that can be improved by sonication. The back-extraction time was studied under ultrasonic treatment for 30, 45, and 60 min using both HLB and Strata X polymers. The highest recoveries were obtained for both polymers at 45 min. However, the HLB polymers showed higher average recoveries of 85% for DXM. Subsequently, HLB with a 45 min desorption time was selected for solvent optimization. The solvents tested were MeOH, ACN, and the MeOH/ACN mixture (50:50, *v*/*v*). The results showed that the back-extraction capacity using the MeOH/ACN (50:50, *v*/*v*) mixture was clearly superior for both analytes (Figure 2b). In this sense, the MeOH/ACN (50:50, *v*/*v*) mixture and an ultrasonic treatment of 45 min were established as the optimized conditions for the back-extraction step for the following tests.

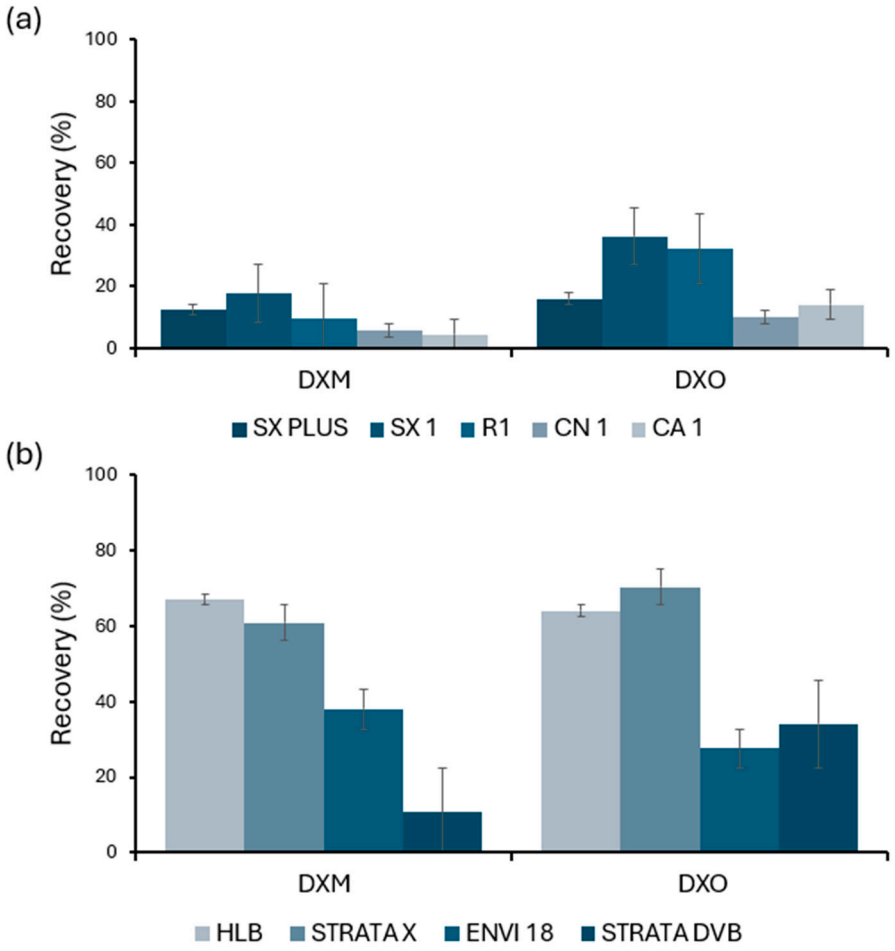

Figure 1. Effect of the selectivity of the different sorbent phases tested, (**a**) ACs and (**b**) polymers, on the recovery of the analytes under study, obtained by BAμE-μLD/GC-MS(SIM). Test conditions: 1 mL of ultrapure water fortified with 100 μL of working solution.

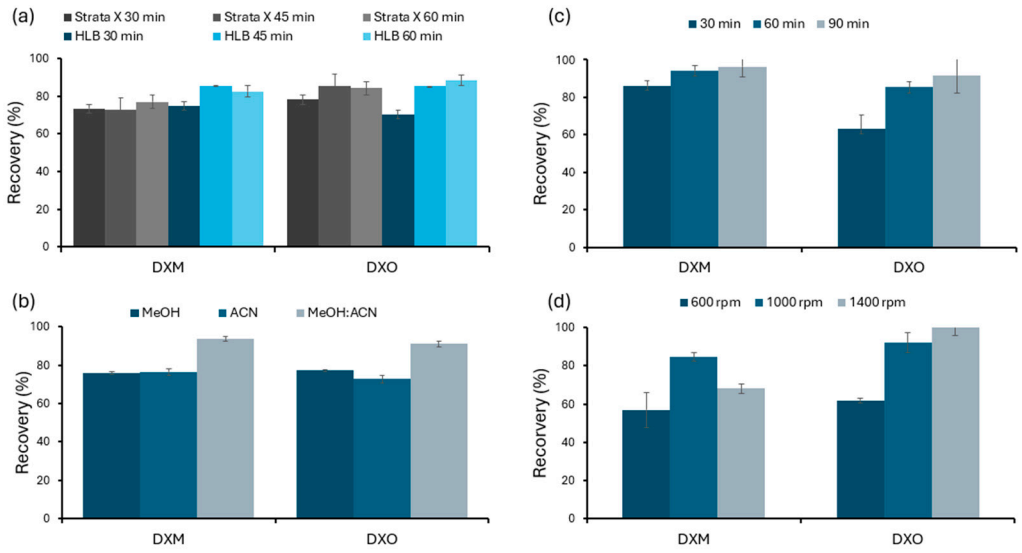

Figure 2. Effect of retroextraction time (**a**), desorption solvent (**b**), equilibrium time (**c**), and speed (**d**).

After the optimization of the back-extraction conditions, the parameters influencing the analytical microextraction process were studied. These included the equilibrium time, agitation speed, pH, organic modifier, and ionic strength of the aqueous matrix.

The extraction times evaluated were 30, 60, and 90 min (Figure 2c). Similar recoveries were observed at 60 and 90 min, suggesting a stabilization of the extraction after 60 min. In addition, extending the extraction time beyond 60 min contributed to an increase in the relative standard deviation (RSD), indicating greater variability in the analyte's recovery. Therefore, 60 min of analytical microextraction was selected as the optimal extraction time. The following parameter was the influence of agitation speed (Figure 2d). The tests were carried out at three different speeds: 600, 1000, and 1400 rpm. The highest recoveries were obtained at 1000 rpm for both analytes. However, at 1400 rpm, the performance declined due to instability and equipment limitations. Thus, 1000 rpm was chosen for further experiments.

The next tests were related to the matrix, where the effect of pH was tested first (2, 5.5, 10, and 12) (Figure 3a). The results show that higher recoveries were obtained at pH 5.5 for both analytes under study. Next, the polarity and ionic strength of the compounds were evaluated. The addition of an organic modifier (e.g., MeOH) to the sample helps prevent the "wall-effect", where non-polar analytes tend to adhere to the walls of the glass vials, thereby reducing recovery [23,28,29]. The organic modifier used on this test was MeOH at 0, 5, 10, and 15% (Figure 3b). The highest recovery values were obtained when no amount of MeOH was added, which would be expected given that the log $K_{O/W}$ of the compounds under study is <5 (DXM-3.49; DXO-2.9). The work was then continued without the addition of an organic modifier. Then, the effect of adding a salt (NaCl) was investigated, a phenomenon known as the "salting-out" effect, which can enhance the migration of organic compounds from the matrix to the sorbent [25,30,31]. This test evaluated the addition of 0, 5, 10, and 15% NaCl (Figure 3c). The results showed an improvement when 5% NaCl was added, with a gradual decrease in recovery as the NaCl concentration increased, likely due to decreasing the solubility of the analytes and facilitating their extraction onto the sorbent phase. The microextraction was therefore optimized with the addition of 5% NaCl.

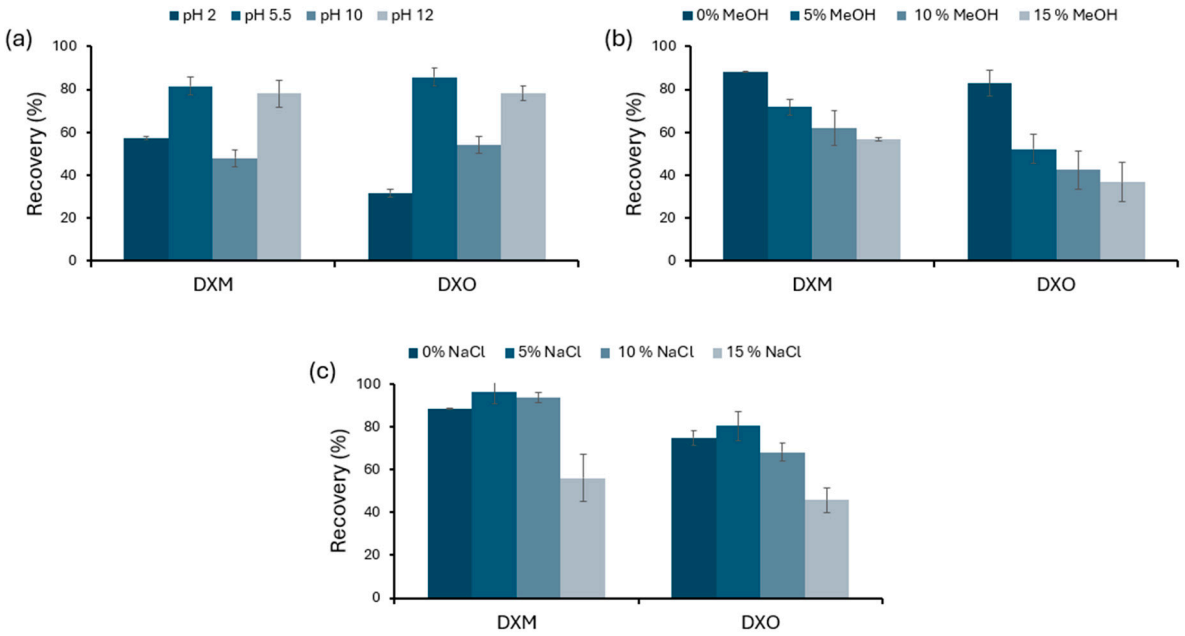

Figure 3. Effect of the pH (**a**), organic modifier (**b**), and ionic strength (**c**).

The optimal microextraction conditions consisted of a 60 min extraction time at 1000 rpm, pH 5.5, no organic modifier, and 5% NaCl in the aqueous phase, with recoveries of 96% and 80% for DXM and DXO, respectively.

3.4. Method Validation

Following the optimization of the experimental parameters, the developed BAμE(HLB)-μLD/GC-MS(SIM) methodology was subjected to validation under the optimized conditions. The method validation was performed using urine matrices. The following parameters were assessed: sensitivity, linearity, selectivity and specificity, trueness, accuracy, and precision, and were evaluated in accordance with the requirements of the UNODC guideline for quantitative analysis.

3.4.1. Linearity, LOD, and LOQ

For the linearity study, the following parameters were evaluated: coefficient of determination (r^2), residue graph and statistical tests, the goodness-of-fit test (GoF), and the lack-of-fit test (LoF) calculated using Excel.

Looking at the coefficients of determination obtained ($r^2 > 0.9975$) demonstrated good linear regressions for both compounds (see Figure 4). The validity of the linear regression model used in the method was confirmed through GoF and LoF tests. In both cases, the calculated F values (F_{calc}) were lower than the corresponding tabulated F values (F_{tab}) at a 95% confidence level, as shown in Table 2. These results demonstrate that the experimental data were consistent with the linear model, with no significant systematic deviation or lack of fit, confirming the model's adequacy for quantitative analysis.

The sensitivity of the method was evaluated using the LOD and LOQ, calculated from the signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively. The LOD obtained was 0.016 μg/mL, and the LOQ was 0.054 μg/mL for both compounds.

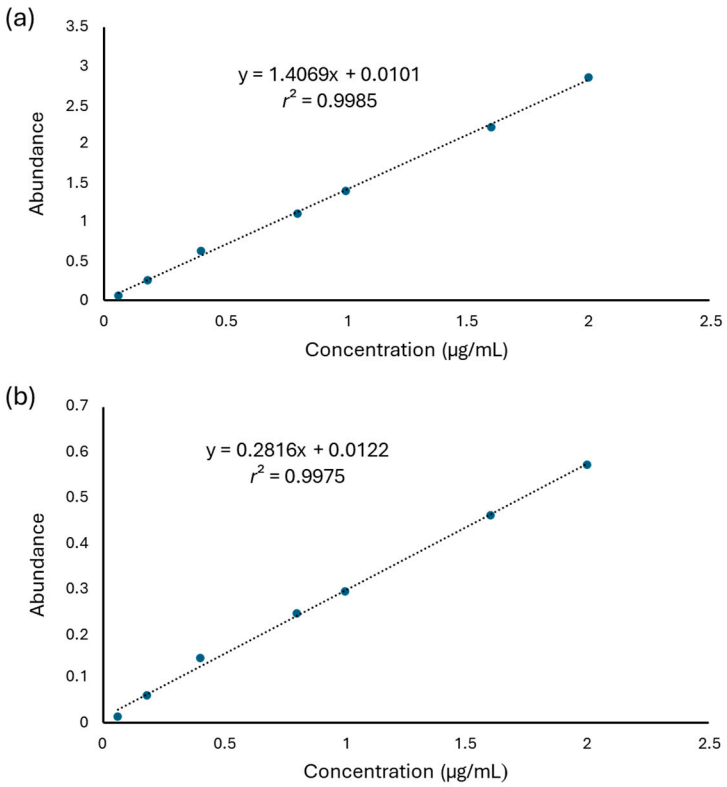

Figure 4. Calibration curve DXM (**a**) and DXO (**b**).

Table 2. Goodness-of-fit and lack-of-fit statistical tests to check the linearity of the two compounds.

Goodness-of-Fit Test			Lack-of-Fit Test		
	DXM	**DXO**		**DXM**	**DXO**
MSS_{Factor}	0.004584	0.000314	MSS_{LoF}	0.001834	0.000126
$MSS_{Residuals}$	0.033779	0.003171	MSS_{Error}	0.007687	0.000771
F_{calc}	0.136	0.099	F_{calc}	0.239	0.163
F_{tab} (95% significance level)	6.944	6.944	F_{tab} (95% significance level)	2.958	2.958

3.4.2. Precision, Trueness, and Accuracy

As mentioned in the experimental part, the determination of precision and trueness was carried out intra- and inter-day at three concentration levels: 0.18 µg/mL, 1.0 µg/mL, and 1.6 µg/mL. The acceptance criteria for precision and trueness were assessed according to UNODC standards. The guidelines define that the RSD (%) values should be $\leq$15%, except for the lowest concentration, where values $\leq$20% are accepted. The bias (%) values should be within the $\pm$ 15% range, except for the lowest concentrations, where values within the $\pm$ 20% range are accepted [27].

The results obtained can be seen in Table 3. The intra-day precision and trueness values ranged from 3.89 to 8.77% and 8.12 to 16.28%, respectively, while the inter-day values ranged from 2.38 to 5.67% and 5.77 to 9.73%, respectively. All these results are in line with the acceptance criteria established by the UNODC, proving the accuracy of the method [27].

Table 3. Precision and trueness values for the different concentrations of both compounds, both for intra-day and inter-day studies.

		Intra-Day		**Inter-Day**	
	Conc. (µg/mL)	**Precision (RSD %)**	**Trueness (Bias %)**	**Precision (RSD %)**	**Trueness (Bias %)**
DXM	0.18	6.98	8.12	5.47	5.77
	1.0	8.08	14.54	5.24	8.47
	1.6	8.54	13.63	4.25	6.47
DXO	0.18	8.77	16.28	5.67	9.73
	1.0	3.89	14.84	2.38	7.67
	1.6	8.47	13.82	4.68	7.87

3.4.3. Selectivity

The selectivity and specificity of the method were tested for the analytes under study. These parameters were evaluated by checking the presence of possible interfering compounds from endogenous materials in the biological matrix studied. The study was carried out by applying the optimized methodology to blank urine samples and checking the resulting chromatograms for any interfering compounds, especially the retention times of each compound. The acceptance criteria were that no interfering compounds should coincide with the retention times of the target compounds. Figure 5 shows a comparison between a blank urine sample containing only ES and a urine sample fortified with the two analytes and the ES. This comparison verifies the selectivity and specificity of the method developed, since the analytes are clearly identifiable and there are no interfering peaks observed in the retention times of the target compounds.

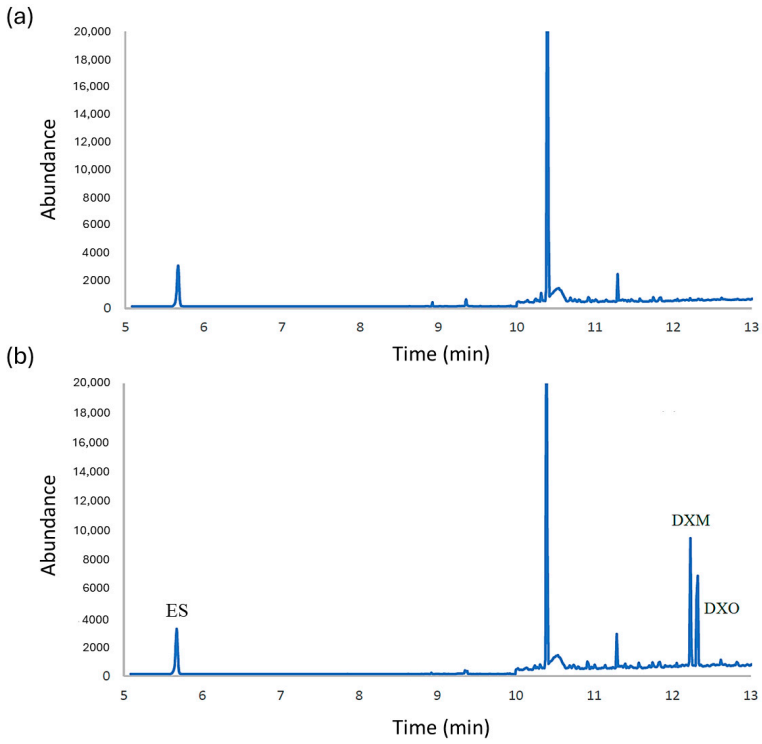

Figure 5. Chromatogram obtained from the analysis of a blank urine sample (**a**) without fortification and (**b**) fortified with 1 μg/mL standards, carried out according to the optimized method.

3.4.4. Real Samples

To assess the analytical capacity of the developed BAμE(HLB)-μLD/GC-MS(SIM) method, 26 urine samples were analyzed. The samples were taken from the five volunteers (aged 23–33) after ingesting 15 mL of Bisoltussin containing 30 mg of DXM. Urine was collected at intervals of 1, 3, 6, 12 (only for one volunteer), and 24 h after administration of the drug, and an additional blank sample was taken from each volunteer. The concentrations of the analytes were calculated from the validated calibration curves. To ensure that the samples were within the range of the calibration curve, the urine samples were diluted at a ratio of 1:10 before analysis.

Initially, by comparing retention times, it was possible to confirm the presence of the two analytes, DXM and DXO, in the 26 urine samples analyzed. It was also confirmed that the blank samples did not contain the compounds under study. The samples were then quantified, and the concentrations of DXM and DXO were determined in the majority of the samples. However, the sample from volunteer 5, collected after 24 h, presented a concentration below LOQ for DXO. The concentrations of DXM and DXO in the samples ranged between 0.06 and 3.21 μg/mL for DXM and between 0.06 and 8.88 μg/mL for DXO (Table 4).

The method also allowed for direct analysis of DXM and DXO without requiring enzymatic hydrolysis of the urine. The chromatograms obtained from the urine samples of volunteer 2 (Figure 6) demonstrated that the proposed methodology is effective in detecting and quantifying DXM and DXO in urine over a period of 24 h after administration. This shows the ability to quantify these substances at different time intervals, which is crucial for assessing the pharmacokinetics of DXM, as well as for detecting their use in clinical and forensic contexts. The decrease in concentration over time reflects the metabolism and excretion of DXM and DXO. The presence of measurable concentrations up to 24 h after administration demonstrates the sensitivity of the method, which is particularly useful in cases of monitoring use or intoxication.

Table 4. DXM and DXO concentrations of urine samples from the 5 volunteers determined by BAμE-μLD/GC-MS.

	Volunteer 1		Volunteer 2		Volunteer 3		Volunteer 4		Volunteer 5	
	Concentration (μg/mL)									
Time (h)	DXM	DXO	DXM	DXO	DXM	DXO	DXM	DXO	DXM	DXO
Before Dose	0	0	0	0	0	0	0	0	0	0
1	0.62	3.71	0.30	0.30	0.65	1.41	0.17	0.45	0.09	0.42
3	0.38	1.14	0.75	0.78	3.21	8.88	0.10	0.31	0.07	0.39
6	0.29	0.80	1.72	1.63	1.11	5.64	0.07	0.20	0.09	0.08
12	0.26	0.50	-	-	-	-	-	-	-	-
24	0.07	0.08	1.03	0.31	0.14	0.34	0.06	0.09	0.06	<LOQ

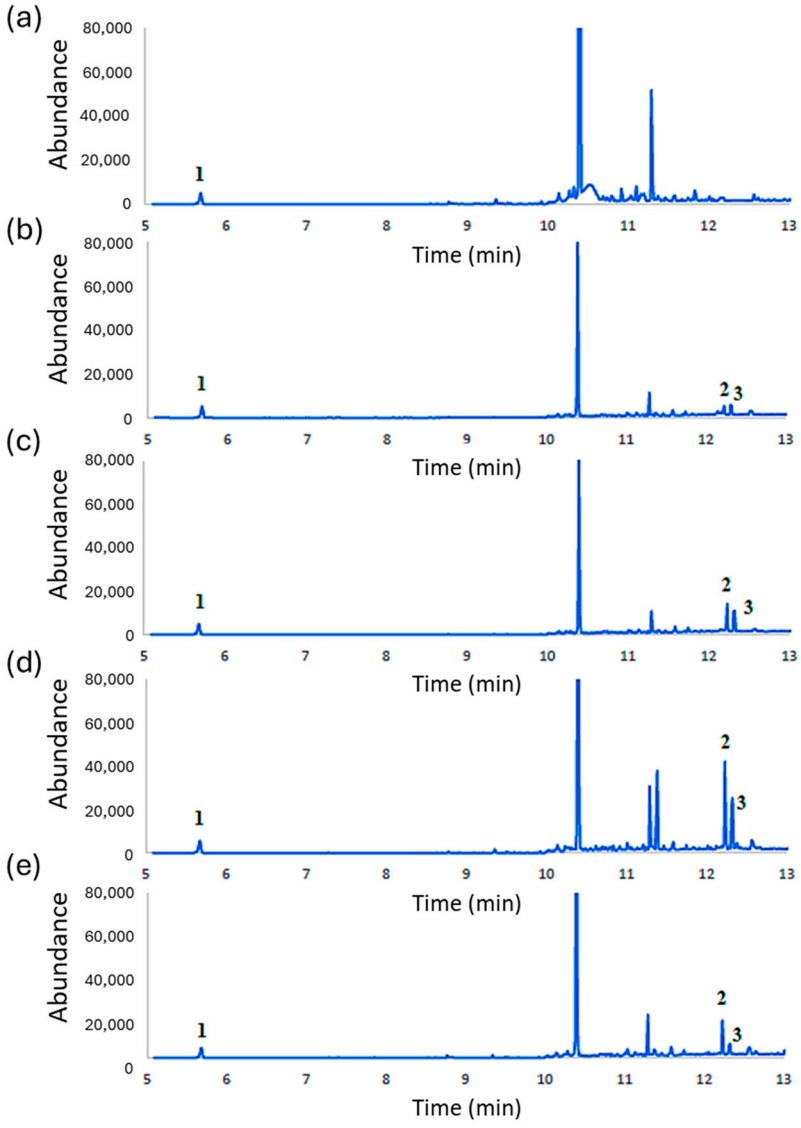

Figure 6. Chromatograms obtained from the urine samples of volunteer 2, taken before ingestion of the drug (**a**) and 1 h (**b**), 3 h (**c**), 6 h (**d**), and 24 h (**e**) after administration of DXM, carried out by BAμE(HLB)-μLD/GC-MS, under optimized conditions (legend: 1—ES; 2—DXM; 3—DXO).

3.5. Performance Comparison with Other Microextraction Techniques

The comparison of analytical methodologies with previously published studies is essential to contextualize the relevance and performance of the proposed method. Table 5 summarizes the main analytical parameters reported for the determination of dextromethor-

phan (DXM) and dextrorphan (DXO) in urine samples, including recovery values, limits of detection (LOD), limits of quantification (LOQ), and linear ranges. As observed, the recovery results obtained in the present study are generally superior or comparable to those described in the literature, which demonstrates the high efficiency of the developed microextraction methodology. Moreover, the linear range, as well as the LOD and LOQ, are of the same order of magnitude as those reported in other studies, indicating that the sensitivity of the proposed method is consistent with established analytical standards. Although comparable results are seen, the literature shows few microextraction studies for DXM and DXO in biological matrices, with most methods relying on SPE or LLE. The lack of studies prevents direct comparison with similar techniques and highlights the novelty of our microextraction-based protocol for quantifying DXM and DXO in urine.

Table 5. Comparison of the proposed methodology with other extraction techniques.

Methodology	Compounds	Recov. (%)	LOD (µg/mL)	LOQ (µg/mL)	Linear Range (µg/mL)	Ref.
BAµEµLD/GC-MS(SIM)	DXM DXO	96.3 80.4	0.016 0.05	0.054 0.1	0.06–2.0	-
LLE-GC-MS(SIM)	DXM DXO	85 84	0.05	0.1	0.1–2	[30]
DSPE-GC-MS(SIM)	DXM	>80	0.01	0.01	0.01–1.5	[31]
SPE-GC-MS(SIM)	DXM DXO	93.1 93.9	0.001	0.01	0–0.1	[8]
LLE-LCMS/MS	DXM DXO	59.2 90.8	-	0.001 0.06	0.005–0.5 0.2–3	[32]
SPE-HPLC-FLD	DXM DXO	93.3 84.8	-	0.025	0.005–2	[33]
SPE-HPLC-MS/MS	DXM DXO	102 86	-	0.002 0.25	0.002–0.2 0.25–20	[11]
LLE-HPLC-FLD	DXM DXO	95 86	0.003 0.24	0.015 1	0.015–10 1–10	[34]
LLE-RPHPLC-UV	DXM DXO	84.8–90.4 90.6–104.8	0.02	0.05	0.2–5.0	[35]
SPE-HPLC-DAD	DXM	96–101	0.5	1.6	10–30	[36]

4. Conclusions

In this study, a BAµE-µLD/GC-MS(SIM) method was successfully developed, optimized, validated, and applied for the determination of DXM and its major metabolite, DXO, in urine matrices. The optimization of these parameters led to average recoveries of 96.3% and 80.4% for DXM and DXO, respectively. To assess the analytical capacity of the developed methodology, it was applied to real samples. Of the 26 samples, it was possible to quantify both compounds in 20 samples, obtaining concentrations between 0.06 and 3.21 µg/mL for DXM and 0.06–8.88 µg/mL for DXO, highlighting the robustness of the methodology in detecting these analytes. The proposed method proved to be effective in detecting and quantifying DXM and DXO in urine over a period of 24 h after administration, highlighting the sensitivity of the method, which is particularly useful in clinical and forensic contexts. In addition, the method is simple, easy to perform, and low-cost, which reinforces its applicability and viability in different laboratory contexts.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/separations12120341/s1, Figure S1: Results obtained in the derivatization time optimization test.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Laboratório de Ciências Forenses e Psicológica Egas Moniz (Project identification code 0023) on 16 October 2023.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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