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Spectrophotometric Multicomponent Analysis of Ternary and Quaternary Drug Mixtures in Human Urine Samples by Analyzing First-order Data

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A new method was developed for the spectral resolution by further determination of three- and four-component mixtures of drugs in urine samples through the complementary application of multivariate curve resolution-alternating least squares with correlation constraint. In the current study, a simple method was proposed to construct a calibration set for the mixture of drugs in the presence of all possible interferents in the human urine samples collected, in duplicate, from volunteers. First, urine samples were collected without any dosage of drugs. Then, urine samples containing a specific brand of drugs were collected. The collected urine samples without any dosage of drugs were spiked with a different concentration of analytes to construct a calibration set; therefore, the proposed method might be successfully used in the presence of matrix effects and unknown calibrated interferences in human urine using first-order data. In this method, a smaller number of calibration samples were used as compared to first-order multivariate calibration methods. Despite intense spectral overlapping and the presence of interferents in the test samples, the results indicated good analytical performance towards the analytes. By calibrating all present components in the unknown samples and imposing the known values in calibration samples during iterations as a correlation constraint, accurate concentrations of the analytes in the unknown set could be predicted. The maximum and minimum band boundaries of feasible solutions corresponding to the species profiles were estimated. The proposed method was used to determine ternary and quaternary mixtures of drugs in urine samples.

Keywords: MCR-ALS, Ternary mixture of drugs, Quaternary mixture of drugs, First order data, Human urine

INTRODUCTION

The appearance of interferences is a common problem in chemical analysis, which needs to be solved by analysts when analyzing complex natural samples, such as environmental specimens, pharmaceuticals and biological matrices [1]. In some cases, the interferents, also called as “expected interferents”, are known to analysts. Therefore, analysts include them in a sufficiently representative training sample set [2]. Fully unknown samples, however, may contain additional, *i.e.* ‘unexpected’ components. These unexpected potential interferences may produce an overlapping signal with that of the analyte of interest that can lead to a systematic error in the analyte determination

using univariate calibration and first-order multivariate calibration methods [3,4]. In other words, a first-order calibration approach may solely compensate for those interferences included in the calibration set.

Multivariate calibration methods such as partial least squares (PLS) and multivariate curve resolution-alternating least squares (MCR-ALS) are the appropriate choices when it comes to interferences and overlapping bands. In PLS regression, interferences can be partly handled when properly represented in the calibration dataset. With this precaution failed to meet, samples containing interferences are likely to be observed as outliers thereby incurring large values of systematic error [5]. However, as an alternative, MCR-ALS approach can be followed in such cases [6-9]. Getting close to a state of maturity in chemometrics, MCR methods have been evolved into a

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powerful tool for the investigation of many types of chemical systems. The underlying principle of MCR is to resolve a mixture's spectra into concentration profiles and weighted pure spectrums of different individual compounds. Although both MCR-ALS and PLS methods are able to analyze first-order data, they work differently, as unexpected interferences are modeled explicitly and separately from the contributions of relevant compounds in MCR. Despite the fact that MCR solutions are of more physical meaning and easier to interpret, rather than those obtained by first-order calibration, they are not generally unique, *i.e.* they are rather associated with an unknown deal of ambiguity. Two types of ambiguities are distinguishable in the course of MCR methods: intensity (or scale) ambiguities and rotation ambiguities [10]. In the presence of rotational ambiguities, rather than a unique solution, one may come to a band of feasible solutions fitting the experimental data. There are different approaches to train a MCR-based method in terms of interference modeling; one of such approaches goes by applying constraints when undertaking ALS optimization. The bands of feasible solutions can be drastically reduced when constraints inherent to and characteristic of the studied chemical system are applied in the course of estimating concentration and spectra profiles. Furthermore, selectivity-related constraints across concentration or spectral regions along with a knowledge of local rank conditions often make it possible to obtain nearly or completely unique solutions [11]. Mohseni *et al.* used standard addition method to convert first-order data to second-order data and attempted to gain second-order advantage, however, their method was not successful to obtain a unique solution using MCR [1]. Correlation constraints represent another type of constraint applicable to concentration profiles when quantification of the compounds is the main goal of analysis [12]. This constraint can be used to correlate known concentrations of components in the calibration set to the concentrations found in the iteration phase. Correlation-constrained MCR-ALS has been used for the analyte quantitation in the presence of unexpected interferences using first-order data [2,12-15].

When the sensitivity of calibration depends on the

matrix composition, quantitative predictions using pure standards might be biased. This problem can be solved by including unknown constituents in the calibration and test samples and following standard addition method [1]. Moreover, the reliability problem of the solutions obtained in first-order calibration can be resolved with the correlation-constrained MCR-ALS method. Ahmadi *et al.* algebraically proved that in analyzing first-order data using MCR, one may achieve a unique solution for the concentration of the analyte of interest, under two principle conditions: first, all components in the unknown set should be contained in the calibration set; and second, known concentration of the analyte in the calibration set should be fixed as a constraint in the course of the iterations [16].

Atenolol (Fig. 1A), Carvedilol (Fig. 1B), and Propranolol (Fig. 1C) are β -blocker group forming antihypertensive drugs which are widely used to treat hypertension and some other disorders. β -blockers affect the heart and blood circulation and decrease systemic blood pressure, pulmonary artery pressure, and pulmonary capillary wedge pressure due to the vasodilatation occurring through blocking of 1-receptors. Blocking of β -receptors reduces the heart rate while increasing diastolic filling time [17]. As a consequence of the widespread use of β -blockers, several analytical β -blocker determination techniques have been developed in recent years [18-22].

Paracetamol (PAR) (Fig. 1D), Ibuprofen (IBU) (Fig. 1E), Aspirin (ASA) (Fig. 1F), and Caffeine (CAF) (Fig. 1G) are active principles widely used and frequently combined in pharmaceutical preparations. These drugs represent popular antipyretic and analgesic agents. Antipyretics cause the hypothalamus to override an interleukin-induced increase in temperature. The body then works to lower the temperature, resulting in a reduction in fever. Most antipyretic medications have other purposes. The most common antipyretics are used primarily as pain relievers. Non-steroidal anti-inflammatory drugs (NSAIDs) are antipyretic, anti-inflammatory, and pain relievers [23]. Various methods have been proposed for the determination of the above compounds [24-28], either alone or in combination with other drugs. Multivariate calibration and curve resolution methods have been used to

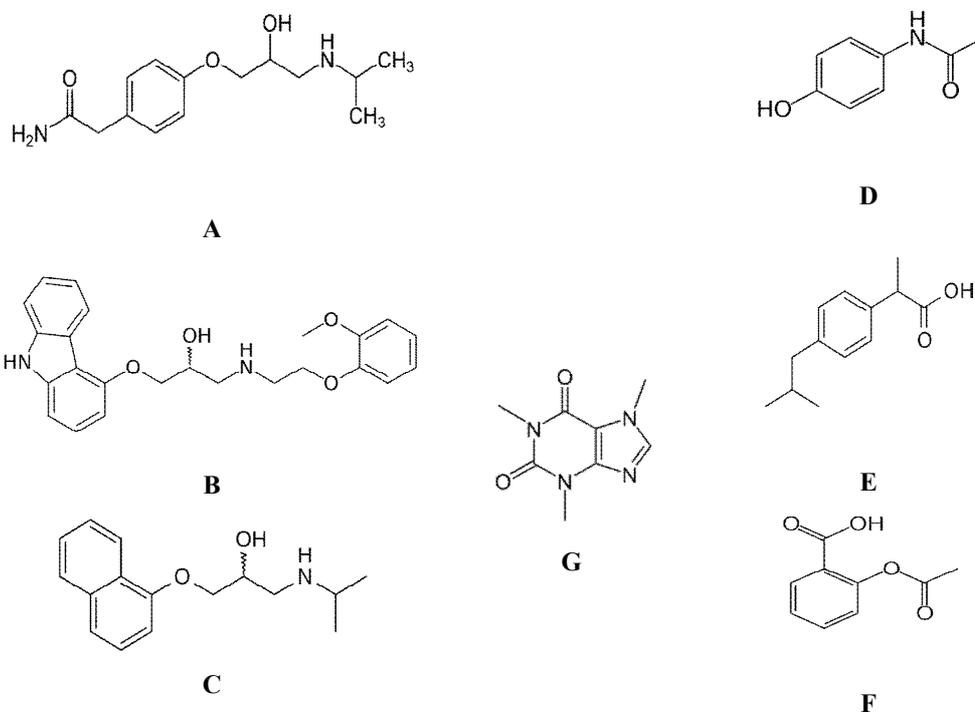


Fig. 1. Chemical structure of the studied compounds: (A) Atenolol, (B) Carvedilol, (C) Propranolol, (D) Paracetamol, (E) Ibuprofen, (F) Aspirin and (G) Caffeine.

determine drug simultaneously and to study chemical processes [29,30].

The aim of this work is to show that applying the correlation constraint in the MCR-ALS algorithm can result in an accurate analyte prediction in first-order data sets containing interferences. This work makes a good usage of this fact in the spectrophotometric determination of some pharmaceutical mixtures. The proposed methodology is developed for the simultaneous spectrophotometric determination of three- and four-component mixture of drugs in urine samples (Atenolol, Carvedilol, and Propranolol in a three-component mixture and Paracetamol, Ibuprofen, Aspirin, and Caffeine in a four-component mixture). Finally, the MCR-BANDS algorithm is applied to evaluate the extent of rotational ambiguity

in the obtained solutions.

MATERIALS and METHODS

Instruments and Software

A double beam UV-Vis spectrophotometer (SHIMADZU UV-1601 PC, Kyoto, Japan) with a quartz cell of 1 cm path length, coupled with UV-PC v. 3.7 software was used to record UV-Vis absorption spectra. A 2 nm spectral band width was scanned at a wavelength-scanning speed of 280 nm min⁻¹. The obtained data was fed into and analyzed in MATLAB 2013a software where the respective analysis were undertaken. MCR-ALS and rotational ambiguities calculations were implemented using MCR-ALS GUI 2.0 [31].

Chemicals and Solvents

All experiments were performed with analytical grade chemicals and double distilled water. With >98% purity, working standards of Carvedilol, Propranolol, and Atenolol were obtained from Sigma-Aldrich (Denmark). With >99% purity, Ibuprofen, Paracetamol, Aspirin, and Caffeine were obtained from Merck (Germany). Ba(OH)₂ and ZnSO₄ were kindly supplied by Sigma-Aldrich (Denmark) at certified purities of 98% and 99%, respectively. Methanol and hydrochloric acid were of analytical grade.

Solutions

Stock standard solutions of Ibuprofen, Paracetamol, Aspirin, Caffeine, Carvedilol, Propranolol, and Atenolol in methanol were prepared, on a daily basis, at the concentration of 1000 mg l⁻¹. Working standard solutions and more diluted solutions of the seven components were prepared by frequent diluting with double distilled water each at a different concentration of the analyte. These solutions were kept in dark of a refrigerator at 4 °C. The Ba(OH)₂ and ZnSO₄ solutions (0.1 M) were prepared by dissolving certain amounts of Ba(OH)₂ and ZnSO₄ in ethanol (96%).

Urine Sample Preparation

In order to train the MCR-ALS model towards resolving and quantifying mixtures of drugs with matrix effects and unknown calibrated interferences in human urine, the urine samples were twice collected from volunteers, with the first time collecting urine samples of no dose of drugs, and the other time collecting urine samples containing drugs of a specific brand. The two volunteers (one for ternary mixture and the other for quaternary mixtures) had their urine samples collected in the same way as described elsewhere [32]. Both volunteers were instructed not to use any medication since 10 days before the urine collection date. In the morning of the first sample collection date, the urine samples were collected from the volunteers' full bladders of no dose of drugs, into thoroughly washed and clean plastic bottles. Continuing with the research, the volunteers were instructed to have the same as yesterday diets, while each of them was managed to take tablets of a specific

brand (containing drugs) 12 h past the first urine collection. Finally, 12 h later, *i.e.* in the morning of the second sample collection date, the second set of samples were taken in the same way as of the first sample set without drugs.

The volunteers were provided with the following specific brands of drugs: AXAR (ACA) tablets (a ternary mixture of Aspirin (32 mg), Caffeine (32.5 mg), and Paracetamol (162.5 mg)), Deltafen capsules (a ternary mixture of Ibuprofen (345 mg), Caffeine (40 mg), and Paracetamol (200 mg)), Carvedilol tablets (12.5 mg), Propranolol tablets (20 mg), and Atenolol tablets (50 mg). Once finished with collecting urine samples, 1 ml of each sample was gently vortex-mixed together with 2 ml of 0.1 M Ba(OH)₂ and 0.1 M ZnSO₄ solution; the resultant mixture was left at room temperature for 10 min before being centrifuged at 4,000 rpm for 10 min. The clear supernatant (a 250 times diluted sample of urine) was diluted to the desired concentration before being analyzed according to the general procedure.

To predict drug concentrations within urine samples, calibration, validation and unknown sets were randomly prepared with spiking different concentrations of analytes to the first theme collected and pretreated urine samples.

PROCEDURE

Single-component Calibration

To find the corresponding linear dynamic concentration range to each drug, single-component calibration was performed. Different volumes of 1000 mg l⁻¹ solutions of each drug were added into different 10 ml volumetric flasks and diluted to the mark with double distilled water. The absorbance spectra were recorded over the spectral range of 200-400 nm. The linear dynamic range for each drug was determined by plotting the absorbance at its λ_{max} versus the sample concentration (2-50 $\mu\text{g ml}^{-1}$ for Atenolol, 2-17.5 $\mu\text{g ml}^{-1}$ for Carvedilol, 1-14 $\mu\text{g ml}^{-1}$ for Propranolol, 2-40 $\mu\text{g ml}^{-1}$ for Ibuprofen, 1-40 $\mu\text{g ml}^{-1}$ for Aspirin, 1-28 $\mu\text{g ml}^{-1}$ for Paracetamol and 1-13 $\mu\text{g ml}^{-1}$ for Caffeine).

Spectral Characterization

The absorption spectra for each of analytes in

ternary and quaternary mixtures were recorded over the range of 200-400 nm.

MCR-ALS METHOD

Building the Calibration Models

To train MCR-ALS model for ternary and quaternary mixtures of drugs, a calibration set was prepared with spiking four different concentrations of analytes to the first theme collected and pretreated urine samples of no dose of drugs (in the concentration ranges of 10-36 $\mu\text{g ml}^{-1}$ for Atenolol, 4-15 $\mu\text{g ml}^{-1}$ for Carvedilol, and 1-8 $\mu\text{g ml}^{-1}$ for Propranolol in ternary mixtures, and 8-20 $\mu\text{g ml}^{-1}$ for Ibuprofen, 8-20 $\mu\text{g ml}^{-1}$ for Aspirin, 4-16 $\mu\text{g ml}^{-1}$ for Paracetamol and 7-19 $\mu\text{g ml}^{-1}$ for Caffeine in quaternary mixtures).

Assay of Validation Set

The validation set consisting of 9 and 17 different ternary and quaternary mixtures of drugs, respectively, was prepared.

Assay of Unknown Set

The unknown set consisting of 21 and 30 different ternary and quaternary mixtures of specific brand drugs, respectively, was prepared *via* spiking different concentrations of analytes to the second theme collected and pretreated urine samples.

MCR-ALS Algorithm

MCR method is based on a bilinear model like the one given in Eq. (1).

$$D = CS^T + E \quad (1)$$

MCR-ALS is aimed at bi-linearly decomposing the data matrix 'D' into 'true' pure response profiles associated with variations in each contribution along the rows and columns represented by matrices 'C' and ' S^T ', respectively; such variations are supposed to be responsible for the variance within the observed data. The rows in matrix 'D' are the spectra measured during the experiment; furthermore, the columns in matrix 'C' and the rows in matrix ' S^T ' contain the concentration profiles and pure

spectra profiles of the resolved components, respectively. The superscript 'T' refers to the transpose of matrix. Being the matrix of residuals not explained by the model, 'E', ideally, should be close to experimental error.

Equation (1) is the multi-wavelength extension of Beer-Lambert law in a matrix form. MCR-ALS solves iteratively Eq. (1) by an ALS algorithm which calculates concentration, 'C', and pure spectra, ' S^T ', matrices optimally fitting the experimental data matrix 'D'. This optimization is carried out for a proposed number of components and using initial estimates of either 'C' or ' S^T '.

When undertaking ALS optimization, several constraints can be applied to model the shapes of 'C' and ' S^T ' profiles, such as non-negativity, selectivity, correspondence among species, correlation or/and other shape or hard-modeling constraints [11,33].

Non-negativity constraint ensures that concentration and/or spectral profile will be equal to or larger than zero [34]. In quantitative analysis, a correlation constraint can be applied to the concentration profiles to establish calibration models for the quantitative determination of analytes in the presence of unknown interferences[2]. The correlation constraint builds internal univariate calibration models between the concentration values calculated by the MCR models and reference values from calibration samples. As a consequence, the concentration profiles are expressed in real concentration units. Implementing a correlation constraint, a local univariate regression is established between the concentrations estimated by ALS for the calibration set (C) and known values of the reference concentrations (C^*), as expressed in Eq. (2).

$$C = bC^* + b_0 \quad (2)$$

Since C and C^* are known, the model parameters, *b* and b_0 , can be obtained by a simple least square method, and are used to predict unknown concentrations in the testing or unknown sets.

Just like other constraints, this constraint can be applied to one or more chemical components (concentration profiles) in the dataset. The correlation constraint can be applied in a flexible way when the

dataset contains sample subsets of different behaviors[35]. Correlation constraint can be implemented in single or local regression models. A single regression model fits to situations where all the calibration samples in the dataset are simultaneously used to build a single calibration model per analyte. A local regression model, however, presents separate models for different sample subsets or groups of sample subsets; as such, it can be used to overcome matrix effect problems among samples of different subsets.

Set to recover unique solutions, the main conditions subject to correlation constraint can be found in the literatures discussing on such topics as: I) the presence of interferences in calibration set; and II) the implementation of correlation constraints[16].

RESULTS AND DISCUSSION

After collecting the urine samples, $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ solution was used to minimize non-specific interactions of the analytes with other proteins and chemicals in the samples. This pretreatment procedure apparently increases the recovery of added analytes in the proposed assay by eliminating interfering substances, such as ascorbic acid and dopamine [36]. After the pretreatment of the first collected urine samples, calibration and validation sets were prepared by spiking the different concentrations of analytes into these samples. Twenty-two samples were randomly prepared as the calibration and validation sets for the ternary mixtures of drugs, while thirteen samples were used in building the model and nine samples were left for the external validation set. Additionally, calibration and validation sets were randomly prepared for the quaternary mixtures of drugs with thirty-nine samples. More specifically, twenty-two samples were used to build the model, while seventeen samples were left for the external validation set. The corresponding absorption spectra to the dataset showed serious spectral overlapping within the region corresponding to 200-400 nm (Figs. 2 and 3). Therefore, our primary aim was to develop a simple, sensitive, selective, and efficient spectrophotometric method to determine the compounds of interest simultaneously without prior separation. As explained earlier, MCR-ALS is an

alternative method, which can be applied to a first-order data set to reach resolution and quantitation of multicomponent samples. By calibrating all the present components in the unknown samples and imposing the known values in the calibration samples during iterations as a correlation constraint, accurate concentrations of the analytes in unknown set can be predicted. In the present research, after augmentation of the first-order data set, the spectral data matrix was analyzed by the MCR-ALS algorithm, using the spectral matrix as an initial estimate. Initial matrix consisted of a spectra of analytes and urine sample after pretreatment. To determine the correct number of components, and chemical rank, singular value decomposition (SVD) is typically applied to data matrices. Table 1 reports the results of the rank analysis of individual experimental data matrices using SVD, for the D_1 and D_2 data matrices. For D_1 , the first four singular values are much larger than the following ones. Furthermore, looking at ratios between consecutive singular values, the fourth singular value was observed to be very larger than the fifth one (see S_4/S_5 ratio). In line with the obtained results, it can be argued that only four species within D_1 are absorbent, of which three singular values were observed to be related to the analytes, with the remaining one corresponding to an unknown, but calibrated interferent. Concerning D_2 , the first five singular values were much larger than the following ones. In addition, the set of ratios between consecutive singular values indicated that, the fifth singular value was very larger than the sixth one (see S_5/S_6 ratio). Hence, the obtained results showed that, only five species within D_2 were absorbent, of which four singular values were related to the analytes, with the remaining one related to an unknown, but calibrated interferent. For optimization, different constraints, namely non-negativity and correlation constraints, were applied to drive the final solution towards a chemical meaning. The non-negativity constraint ensured that the components concentrations and spectra would be positive. Another constraint was the correlation constraint, which helped in removing the rotational ambiguity of the analytes' concentration profile completely by calibrating all the present components in the unknown samples and incorporating

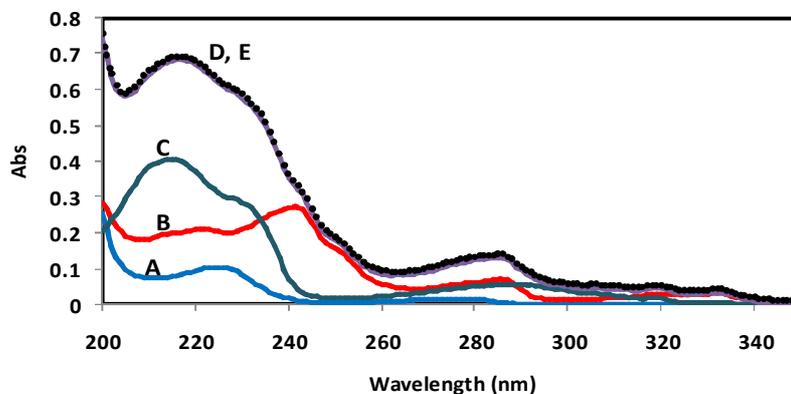


Fig. 2. The corresponding absorption spectra to $3 \mu\text{g ml}^{-1}$ for each of (A) Atenolol, (B) Carvedilol, (C) Propranolol, (D) their ternary mixture with same concentrations and (E) mathematical sum of three analytes.

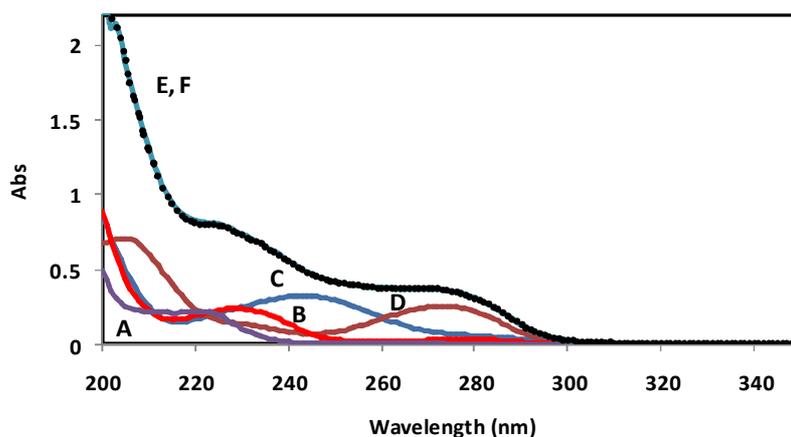


Fig. 3. The corresponding absorption spectra to $5 \mu\text{g ml}^{-1}$ for each of (A) Ibuprofen, (B) Aspirin, (C) Paracetamol, (D) Caffeine, (E) their quaternary mixtures with same concentrations and (F) mathematical sum of four analytes.

known values in the calibration samples during iterations. This constraint consisted of a series of steps performed during each iteration step of the ALS optimization. Accordingly, concentrations of a given analyte in the calibration samples at each ALS iteration were correlated with the previously known reference concentration values of the analyte in the samples. Continuing with the research, a local linear model was generated, wherein the ALS estimated values were related to nominal concentrations.

Concentration values were then updated according to the predicted values, using the estimated parameters via the local model. The best model was supposed to be converged wherever either of a minimum number of iterations or minimum values of lack of fit% was achieved.

With MCR-ALS decomposed, the extent of remaining rotational ambiguity in the retrieved profiles was investigated. Spectral profiles were submitted, as the

Table 1. Rank Analysis of Data Matrices by SVD

Matrix ^a	Singular values							Singular value ratios					
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₇	S ₁ /S ₂	S ₂ /S ₃	S ₃ /S ₄	S ₄ /S ₅	S ₅ /S ₆	S ₆ /S ₇
D ₁	277.01	26.40	4.10	0.73	0.14	0.11	0.09	8.59	6.43	5.58	5.03	1.23	1.15
D ₂	296.63	39.87	5.59	0.89	0.15	0.03	0.02	7.43	7.12	6.27	5.80	4.53	1.18

^aD₁ is a data matrix containing three analytes and an interferent mixture and D₂ is a data matrix containing four analytes and an interferent mixture.

Table 2. Analysis results of Carvedilol (CAR), Propranolol (PRO) and Atenolol (ATE) in the Validation Sets

Experiment No.	Taken ($\mu\text{g ml}^{-1}$)			Found ($\mu\text{g ml}^{-1}$)			Recovery (%)		
	ATE	CAR	PRO	ATE	CAR	PRO	ATE	CAR	PRO
1	0.00	7.30	16.40	0.00	7.22	16.44	-	98	100
2	0.00	10.50	11.60	0.00	10.60	11.66	-	100	100
3	4.50	0.00	26.70	4.48	0.00	26.67	99	-	99
4	10.00	0.00	3.30	9.96	0.00	3.22	99	-	97
5	3.80	12.40	0.00	3.82	12.33	0.00	100	99	-
6	9.30	3.80	0.00	9.33	3.73	0.00	100	98	--
7	2.80	15.50	2.20	2.77	15.46	2.17	98	99	98
8	5.70	12.30	6.30	5.67	12.23	6.27	99	99	99
9	7.30	4.90	15.00	7.30	4.80	15.15	100	97	101
Mean recovery (%)							99	99	99
R.S.D (%)							1.1	1.0	0.5
R.S.E (%)							0.5	0.7	0.4

initial input values, to the MCR-BANDS program [31]. Implementing non-negativity and correlation constraints, the optimization was conducted to obtain the maximum and minimum band boundaries of concentration and spectral profiles for the ternary and quaternary mixtures of drugs within the urine samples. The difference between the maximum and minimum components of the relative

contribution optimization function ($f_n^{\max} - f_n^{\min}$) was calculated as a proxy for the associated rotational ambiguity with the analytes' concentration profiles. As Fig. 4 shows, unique solutions were obtained for the analytes in both ternary and quaternary mixtures, indicating that the obtained results for MCR-ALS were free from any

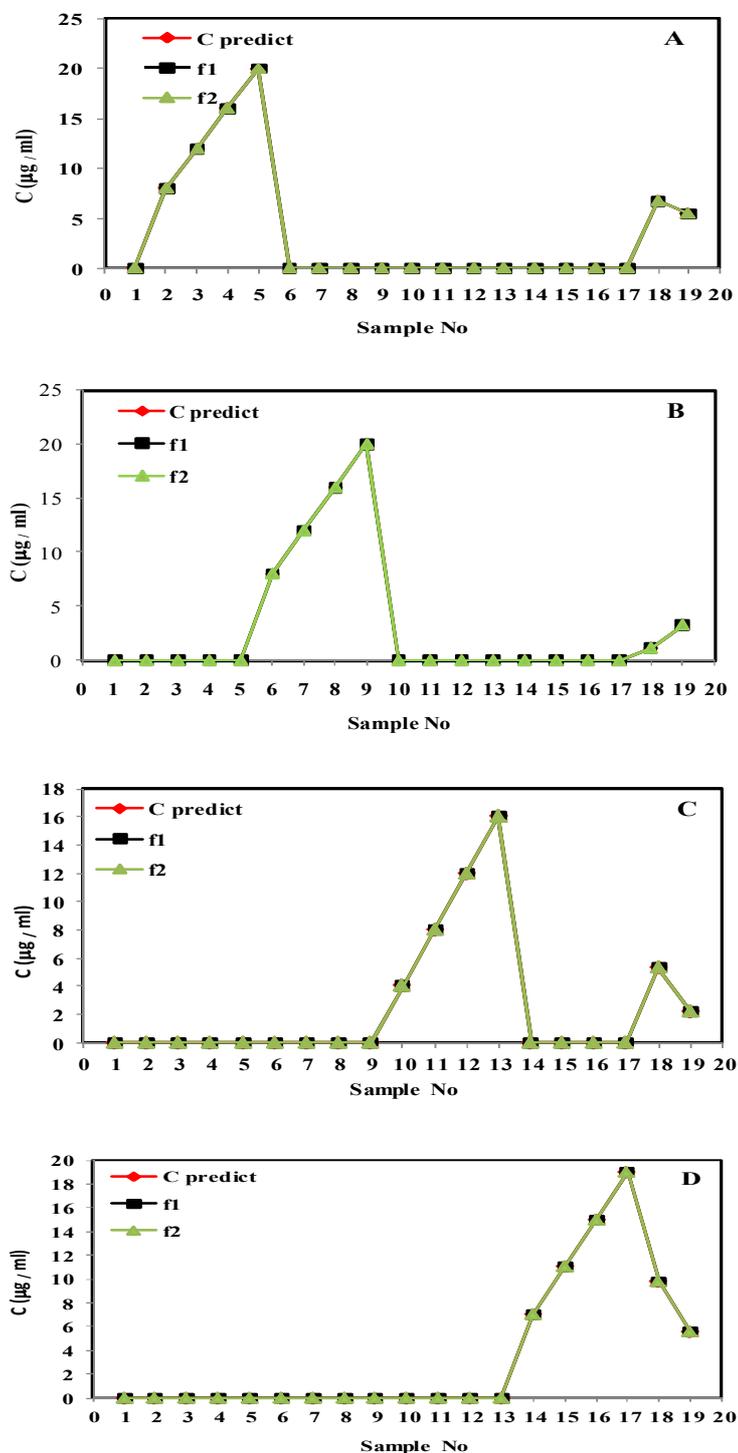


Fig. 4. The results of MCR-BANDS for the concentration profiles of (A) Ibuprofen, (B) Paracetamol, (C) Aspirin and (D) Caffeine (the first seventeen samples are the calibration ones and the remaining ones is the test samples containing of quaternary mixtures of drugs). Predict: result of resolution, f_1 : minimum of feasible band and f_2 : maximum of feasible band.

Table 3. Analysis Results of Ibuprofen (IBU), Paracetamol (PAR), Aspirin (ASA) and Caffeine (CAF) in the Validation Sets

Experiment No.	Taken ($\mu\text{g ml}^{-1}$)				Found ($\mu\text{g ml}^{-1}$)				Recovery (%)			
	IBU	ASA	PAR	CAF	IBU	ASA	PAR	CAF	IBU	ASA	PAR	CAF
1	0.00	0.00	9.50	9.00	0.00	0.00	9.36	8.86	-	-	98	98
2	0.00	7.00	0.00	8.50	0.00	7.00	0.00	8.62	-	100	-	101
3	10.50	0.00	0.00	8.30	10.52	0.00	0.00	8.34	100	-	-	100
4	0.00	7.70	9.00	0.00	0.00	7.76	9.22	0.00	-	100	102	-
5	10.00	9.50	0.00	0.00	9.91	9.41	0.00	0.00	99	99	-	-
6	10.00	7.30	0.00	0.00	9.93	7.29	0.00	0.00	99	99	-	-
7	0.00	9.00	10.30	6.80	0.00	9.05	10.30	6.77	-	100	100	99
8	4.30	5.50	0.00	7.30	4.27	5.37	0.00	7.23	99	97	-	99
9	4.80	0.00	6.80	8.00	4.79	0.00	6.75	8.00	99	-	99	100
10	4.20	6.00	7.00	0.00	4.11	6.16	7.10	0.00	97	102	101	-
11	9.80	5.30	1.20	6.80	9.77	5.31	1.14	6.77	99	100	95	99
Mean recovery (%)									98	99	99	99
R.S.D (%)									3.9	2.2	1.1	0.8
R.S.E (%)									1.0	1.1	0.9	0.9

rotational ambiguity in the ternary and quaternary mixtures of drugs.

Tables 2 and 3 present the specific prediction results of some examples of the ternary and quaternary mixtures in the validation set, respectively. As it can be observed, not only good statistical indicators were obtained, but also all three repetitions per sample exhibited recoveries greater than 97%. This implies that the proposed methodology represents a feasible approach to spectrophotometric multicomponent analysis of the sample components of intense spectral overlapping with matrix effects in the presence of interferents in test samples.

Case Study

To assess the applicability of the proposed method

in the analysis of ternary and quaternary mixtures, unknown urine samples containing drugs of a specific brand were collected from volunteers. For this purpose, AXAR tablets, Deltafen capsules, Carvedilol tablets, Propranolol tablets, and Atenolol tablets were taken by the volunteers. Tables 4 and 5 present the prediction results of analytes in the unknown urine samples. As Table 4 shows, Atenolol, Carvedilol and Propranolol were detected in the unknown urine samples at $5.35 \mu\text{g ml}^{-1}$, $0.91 \mu\text{g ml}^{-1}$ and $1.26 \mu\text{g ml}^{-1}$, respectively. The accuracy of predictions was tested by spiking the analytes. Thus, the urine samples were spiked with the standards of Atenolol, Carvedilol and Propranolol at different concentration levels within their linear dynamic ranges; all experiments were performed in triplicates ($n = 3$). Table 4 summarizes the corresponding relative recoveries.

Table 4. Analysis Results of Carvedilol (CAR), Propranolol (PRO) and Atenolol (ATE) in the Unknown Set

Sample	No	Taken ($\mu\text{g ml}^{-1}$)			Found ($\mu\text{g ml}^{-1}$)			Recovery (%)			RSD (%)		
		ATE	CAR	PRO	ATE	CAR	PRO	ATE	CAR	PRO	ATE	CAR	PRO
Atenolol tablet	1	0.00	0.00	0.00	5.35	ND	ND	-	-	-	0.5	-	-
	2	17.00	0.00	0.00	22.44	ND	ND	100	-	-	0.3	-	-
	3	6.00	10.00	0.00	11.44	9.95	ND	101	99	-	0.1	0.7	-
	4	2.00	15.00	2.50	7.40	14.64	2.46	102	97	98	0.9	0.6	1.7
				± 0.05	± 0.03	± 0.03							
Carvedilol tablet	1	0.00	0.00	0.00	ND	ND	ND	-	-	-	-	4.6	-
	2	0.00	14.50	0.00	ND	15.51	ND	-	103	-	-	0.4	-
	3	0.00	5.00	5.00	ND	20.32	5.04	-	96	100	0.4	0.8	-
	4	13.50	3.00	11.00	13.40	18.47	10.92	99	98	99	0.4	0.5	0.7
				± 0.04	± 0.07	± 0.06							
Propranolol tablet	1	0.00	0.00	0.00	ND	ND	1.26	-	-	-	-	-	3.3
	2	0.00	0.00	5.00	ND	ND	6.20	-	-	98	-	-	0.9
	3	0.00	7.50	9.00	ND	7.40	10.03	-	98	97	0.5	0.7	-
	4	8.80	4.00	7.00	8.61	4.08	8.26	97	101	99	0.9	0.6	0.6
				± 0.06	± 0.03	± 0.04							

As observed, the relative recoveries for Atenolol, Carvedilol, and Propranolol in spiked human urine sample were within 97-103%. With RSD values below 2%, the samples exhibited satisfactory determination precision. As Table 5 shows, in the unknown urine sample containing AXAR tablets, IBU, ASA, PAR and CAF were detected at $0.00 \mu\text{g ml}^{-1}$, $0.0 \mu\text{g ml}^{-1}$, $4.11 \mu\text{g ml}^{-1}$ and $1.17 \mu\text{g ml}^{-1}$, respectively. Moreover, in the unknown urine sample containing Deltafen capsules, IBU, ASA,

PAR and CAF were detected at $0.69 \mu\text{g ml}^{-1}$, $0.00 \mu\text{g ml}^{-1}$, $2.29 \mu\text{g ml}^{-1}$ and $0.74 \mu\text{g ml}^{-1}$, respectively. Table 5 presents the results of the recoveries and concentrations obtained from studies on spiked unknown urine samples. The obtained relative recoveries (>97%) and RSD (<4%) values clearly demonstrated quite satisfactory precision of the developed method for the analysis of analytes in real samples.

providing all of the support.

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