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Research Article

Electrophoretic fingerprinting of benzodiazepine tablets in spike drinks

Over the last few years, there has been an increase in the reports of drug-facilitated crimes. The list of drugs associated with these crimes is extensive and benzodiazepines constitute one of the groups of substances more commonly used. The sedative properties, which characterize benzodiazepines, are enhanced when such drugs are combined with alcohol, being more attractive for committing these types of crimes. In this work, a capillary electrophoresis method was applied to the analysis of 63 different samples of club drinks spiked with benzodiazepine tablets. The resulting electropherograms were processed and analyzed with the chemometric multivariate techniques: principal component analysis (PCA) and soft independent modeling of class analogies (SIMCA) classification. The PCA results allowed a clear differentiation of each drug class in a 3D plot. In addition, the SIMCA classification model (5% significance level) showed that eight out of nine test samples were automatically assigned by software to their proper sample class. The conflicting sample was correctly classified in the Coomans' plot (95% confidence). This novel approach based on the comparison of electrophoretic profiles of spiked drinks by chemometric tools allows determining the benzodiazepine used for drink spiking without the use of drug standards. Moreover, it provides an opportunity for the forensic laboratories to incorporate the identification capability provided by the electrophoretic fingerprinting of benzodiazepine solutions in existing or new databases.

Keywords:
Benzodiazepines / Drug-facilitated crimes / Multivariate data analysis / PCA / SIMCA

1 Introduction

Benzodiazepines are psychotropic substances with hypnotic and amnesic properties and muscle relaxant effect. They have been widely prescribed because of their efficacy, slight depen-dence [1], and easy acquisition. When benzodiazepines are used in combination with alcohol, their effects are potenti-ated [2]. This characteristic, together with their high solubility in alcohol [3], has motivated attackers to add benzodiazepines to club drinks while the consumer is distracted. This criminal action is commonly known as "drink spiking" [4]. The potential abuse of benzodiazepines for drug-facilitated crimes is in a steady increase and it is a major public health con-cern [5]. Moreover, the intraindividual variability causes the same amount of a given benzodiazepine to produce different effects from one person to another [6,7]. The short half-life of these compounds, the usual administrations of single doses, and the delay of reporting the abuses by the victims usually makes it difficult to detect of the drug in the organism. There-fore, analysis of the drinks used to perpetrate drug-facilitated crimes becomes necessary, as they might be the only lasting evidence.

Several methods have been devised for the study of drinks spiked with benzodiazepines. Accordingly, a rapid desorption electrospray ionization-MS [3], HPLC [8,9], CE [10,11], micro-chip based CE [12] or multi-pumping flow system with UV detector [13] have been used for the determination of benzo-diazepine standards in spiked alcoholic drinks and nonalco-holic drinks. However, the aim of those works was not the study of drinks spiked with commercially available tablets.

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Abbreviations: DEP-MS, direct electrospray probe-MS; **PC**, principal component; **PCA**, principal component analysis; **SIMCA**, soft independent modeling of class analogy; **TEA**, triethanolamine

Colour Online: See the article online to view Fig. 3 in colour.

Although a set of commercial tablets of benzodiazepines could be separated and quantified by applying HPLC and CE methods [14, 15], the authors prepared samples in chem-ical grade alcohols (methanol or ethanol/water) as solvent instead of beverages. In the current literature there are only two works that investigate drinks spiked with commercially available benzodiazepine tablets. In this circumstance, Chen et al. [16] used direct electrospray probe-MS (DEP-MS) to in-dividually analyze alcoholic and nonalcoholic drinks contain-ing benzodiazepines tablets. The authors needed to perform a liquid extraction in order to pretreat the samples before the analyses because the matrix and the low solubility of the drug hindered its direct introduction into the DEP-MS sys-tem. The advantage of this method was a completed analysis in less than 5 min including sample pretreatment. Regarding Acikkol et al. [17], they developed a GC-MS method for the simultaneous determination of five benzodiazepine tablets from spiked beer and peach juice by extracting them with a chloroform/isopropanol 1:1 v/v mixture. This method needs each of the benzodiazepine standards as reference solutions.

Databases are usually employed for data comparison in forensic sciences. These comparisons may provide an iden-tification of the analyzed sample and speed up the genera-tion of reliable information. However, databases usually con-tain spectral information. Data from separation techniques (e.g. chromatographic and electrophoretic techniques) usu-ally contain a huge amount of information, which may be partly veiled because data may be too complex to be easily in-terpreted. For such data, multivariate analysis techniques be-come a powerful mathematical tool to unwrap complex prob-lems. Moreover, the use of chemometric tools may accelerate the study of multiple samples. For example, at least duplicate analyses are needed for the analysis of a given sample in CE: the first one, for the analysis of the sample itself; and the sec-ond one, to confirm the presence of the substance of interest by fortifying the sample with standards. However, the use of chemometric tools and the creation of databases might sub-stantially reduce the number of electrophoretic analyses since a single run would provide a characteristic electrophoretic profile, which could be introduced in the database and read-ily classified according to the constituents of the sample. So far, literature collects the application of multivariate calibra-tion methods for the identification of benzodiazepine drugs in human plasma using partial least squares and multivari-ate linear regression [18] and for the study of the relation-ship between chromatographic parameters (in HPLC analy-sis) and the chemical structure or biological activity of the benzodiazepines [19]. Recently, Roggo et al. [20] performed a study with the aim of creating a Raman spectral library for different pharmaceutical tablets, including benzodiazepines. It permits their classification with a nonlinear classification method, the Support Vector Machines, in different families according to the active pharmaceutical ingredient.

This work aims to detect seven benzodiazepine tablets mixed in nine spiked club drinks using their electrophoretic profiles as fingerprints, by means of chemometric tools. The novel approach of this work is to focus and use commercially available benzodiazepine tablets, thus avoiding the need of using drug standards for obtaining reliable information.

2 Materials and methods

2.1 Reagents

All chemicals used were analytical grade. Triethanolamine (TEA), acetic acid, and sodium hydroxide (NaOH), were ob-tained from Sigma-Aldrich (St. Louis, MO, USA). Benzodi-azepine tablets were kindly gifted by the “Príncipe de As-turias” University Hospital (Alcalá de Henares, Spain) and the dosage of active ingredient was considered as the dosage claimed by the laboratories. Ultrapure water was obtained from a Millipore Milli-Q water system (Bedford, MA, USA). The running buffer consisting of 0.5 M acetic acid (pH 2.5) was prepared fresh daily. The pH-value of the buffer was determined with a pH-meter Crison GPL-21 (Crison Instru-ments, Barcelona, Spain).

2.2 Sample preparation

Soft and alcoholic drinks were purchased in a local super-market. The alcoholic beverages prepared for the study were chosen for being commonly consumed or because they are increasing in popularity. The proportion of 1/4 v/v of the alcoholic drink with 3/4 v/v of the corresponding soft drink was used for the preparation of the mixed drinks. That ratio is similar to that used in a previous study [3]. The nine drinks prepared were: cola, lemon, whisky with cola, whisky with lemon, rum with cola, rum with lemon, gin with tonic water, vodka with tonic water, and vodka with lemon.

An amount of 33±1 mg of each crushed commercial drug were weighed and then individually added to 1 mL of each of the selected drinks. TEA was added to the drinks as internal standard, to a concentration of 50 M. Then, the solutions were centrifuged at 2500 rpm for 5 min in order to ensure that there were no particles in suspension. Afterwards, the supernatants were injected in the CE system. To avoid potential changes during the storage of the spiked drinks, all the samples were daily prepared and injected while they were fresh.

2.3 CE-C^{4}D analysis and instrumentation

The CE experiments were performed on a Beckman Coulter PA-800 (Beckman-Coulter, Fullerton, CA). The instrument was fitted with a contactless conductivity detector (eDAQ, Deninstone East, NSW, Australia). The detector excitation frequency was set to 1200 kHz and the amplitude to 100%. The signals were inverted in order to show positive-going peaks.

Bare-fused silica capillaries of 50 m id and 365 m od (Polymicro Technologies, Phoenix, AZ, USA) with a total

length of 80 cm and an effective length of 60 cm were em-ployed. The capillaries were conditioned at 20 psi by flush-ing with 1 M NaOH for 40 min, then water for 5 min, and finally running buffer for 30 min. Injections were carried out at 0.5 psi for 10 s. After each analysis run, the capillary was rinsed with running buffer for 4 min to maintain the reproducibility of the analysis. The temperature in the CE sample storage was set to 10°C, to avoid the degradation of the samples, while the capillary temperature during the elec-trophoretic separation was kept at 25°C. The applied voltage was 25 kV and a voltage ramp of 0.17 min was set at the beginning of the separation.

2.4 Data analysis by PCA and SIMCA models

For the data analysis, data were exported from the software with a sampling rate of five samplings per second (data points recorded per second) instead of the raw signal recorded by the detector (1000 samplings per second, in this case). This sampling rate was good enough for data processing: elec-trophoretic fingerprinting of benzodiazepines neither did nor changed and it has the advantage of treating lower density of points. A combination of a variety of software was used for data treatment. The baseline of data between the 6.9–28.3 min was manually subtracted by using the Adjacent-Averaging smoothing method in Origin (OriginLab, USA). The base-line adjustment was needed for further data treatment in order to avoid baseline drifts that may hinder correct normal-ization and further classification. The resulting CE data were imported into SpecAlign (University of Oxford, UK) [21] for their alignment. Data need to be aligned in order to avoid smooth peak deviations and to provide similar groups of data that can be analyzed. Each family of drug class was indepen-dently aligned taking the internal standard peak as reference. The electropherogram showing the closest migration time of the internal standard to the average in each family was chosen as the reference sample for each alignment.

Once the data were pretreated, they were organized in Excel 2010 (Microsoft, USA) into a transposed matrix con-taining all the preprocessed samples (as rows). The matrix was then imported into The Unscrambler X 10.3 (Camo, Nor-way, http://www.camo.com/) for the normalization by range and for further testing. The normalization by range implies that each row (each sample) was divided by its range (max value–min value). Normalization was needed in order to pro-vide a set of values that can be compared. The sample classes (groups or row groups) consisted of the drug spiked drinks grouped by drug and named with its specific categorical vari-able. On the other hand, the variables comprised every data point collected by time. All the sample classes together con-stituted the calibration (training) set.

In this work principal component analysis (PCA) were calculated for every sample class and for the training set. The chosen weighting and validation methods for the PCA analysis were 1/(StdDev) and cross-validation, respectively. No auto-pretreatment for further samples classification was used. Moreover, in order to test if the PCA models were adequate to perform a classification of randomly chosen un-known samples, the soft independent modeling of class anal-ogy (SIMCA) classification [22] and its probabilities were cal-culated by means of the SIMCA 13.02 software (Umeå, Swe-den, http://www.umetrics.com/).

3 Results and discussion

3.1 Sample selection criteria

Different benzodiazepines have different therapeutic and toxic doses. For example, Alprazolam causes greater toxic-ity in overdose compared with other benzodiazepines [23] and Flunitrazepam is about ten times more potent than Di-azepam [3]. Therefore, the range of doses administered to the patients is wide. On the other hand, higher doses than those administered in a single dose are needed to produce the symptoms of deep sedation and severe amnesia sought by the attackers. Since the effects for the same amount of active in-gredient are different among different benzodiazepines and, because the dose of active ingredients was different among the different tablets used in this study, it was not possible to state a common dosage for benzodiazepines. Therefore, doses consumed with recreational purposes (Table 1) were deemed relevant because these amounts are close to the toxic doses [24]. Considering these doses, a concentration of 33 ± 1 mg in 1 mL (representing an average of five capsules/tablets per 150 mL of drink) was used to estimate a concentration equivalent to doses taken for recreational purposes.

Because the proposed approach uses the electrophoretic profile for the classification of the drugs, an internal standard was included as reference. The internal standard was used for the alignment of the electropherograms in each family of spiked drinks, which were organized according to the drug used. TEA was chosen as the internal standard due to its cationic nature and because it did not interfere with any signal in the sample's electropherograms.

3.2 Electrophoretic analysis

An acetic acid buffer of low pH (0.5 M acetic acid at pH 2.5) was selected in order to protonate, at least partially, all the basic compounds in the sample and to obtain complex elec-trophoretic signals that can be compared. Taking into account the pK_a-(base) values of the benzodiazepines (Table 2), at this pH they are partially mono-protonated in the imine N4 group, with a percentage of ionization given by Eq. (1). This equation gives the percentage of ionization in equilibrium of the imine group N4 for a specific benzodiazepine on base on the buffer pH and the specific pK_a of the benzodiazepine (Table 2). Con-versely, benzodiazepines having high pK_a-(acid) values, given by the tertiary amine in position N1, are not charged in that position at pH 2.5, as is calculated by Eq. (2). This equation gives similar information than Eq. (1) based on the pH value

Table 1. Benzodiazepines and the reported number of pharmaceutical forms [24]

Laboratory	Product	Active ingredient	Dosage active ingredient (mg)	Number of pharmaceutical forms consumed
Sanofi Aventis	Tranxilium® capsules	Clorazepate dipotassium	10	1–10
Kern Pharma	Diazepam tablets	Diazepam	5	2–8
Roche	Lexatin® capsules	Bromazepam	1′5	5–10
Roche	Rohypnol® tablets	Flunitrazepam	1	0.25–6
Normon	Alprazolam tablets	Alprazolam	2	0.5–5
Pensa	Lorazepam tablets	Lorazepam	2	1–7
Bayer	Noctamid® tablets	Lormetazepam	1	Data not found

Table 2. Molecular mass, pK_a-values and % ionized at pH = 2.5 of the studied benzodiazepines, given by Eq. (1)

Benzodiazepine	Molecular mass	pK_a(base)	pK_a (acid)	%Ionized at pH = 2.5
Diazepam	284.7	3.3 [25, 26]	Data not found	86
Alprazolam	308.7	2.5 [27]	Data not found	50
Flunitrazepam	313.3	1.8 [15, 25, 26]	Data not found	17
		1.6 [27]		11
Clorazepate dipotassium	314.7	3.5 [15]	12.5 [15]	91
Bromazepam	316.2	2.9 [25, 26]	11.0 [25, 26]	72
		2.0 [27]		24
Lorazepam	321.2	1.3 [25, 26]	11.5 [25, 26]	6
		1.6 [27]		11
Lormetazepam	335.2	1 ± 0.5*	13.6 ± 1.0*	3

*Calculated using advanced chemistry development (ACD/Labs) Software V11.02 (© 1994–2013 ACD/Labs).

of the buffer and the pK_a value of the benzodiazepine for the tertiary amine in position N1. Under such experimental conditions, the positively charged benzodiazepines migrated ahead of the EOF after applying a positive potential.

$$\%\ ionized = {}^{100}/_{1 + anti\log(\mathrm{pH} - pk_a)} \quad (1)$$

$$\%\ ionized = {}^{100}/_{1 + anti\log(pk_a - pH)} \quad (2)$$

The acetic acid buffer at pH 2.5 produced good baseline separations between peaks allowing the differentiation of several zones in the electropherograms. Figure 1 shows the electropherogram for the sample, whisky with cola spiked with (a) Tranxilium®, which shows a different profile regarding the drugs studied, and (b) Lorazepam, which present a similar electrophoretic profile for the rest of the analyzed drugs. Three different zones were clearly identified in all electropherograms. Zone A (0–6.9 min) consisted of the system peaks and the initial shift curve caused by the ramp voltage set at the beginning of the experiment. This zone also included some fast peaks that were proven to belong exclusively to the soft drinks and thus were common to all of them. Peaks appearing in zone B (6.9–18.7 min) belonged to the added internal standard (TEA) and to the soft drinks. However, in this zone, the observed peaks depended on the kind of soft drink used in the beverages. The last peaks that appeared along zone C (18.7–28.3 min) were very characteristic and depended on the drug used. Only when Tranxilium® was added to the drinks, the major peaks appeared in zones A and B. Moreover, no peaks were detected after zone C. The electropherograms obtained for all the drinks spiked with benzodiazepines were later used for the creation of the PCA models.

It is known that benzodiazepines must be preserved at low temperature, however, the stability of these drugs in alcoholic beverages has been rarely described in the litera-

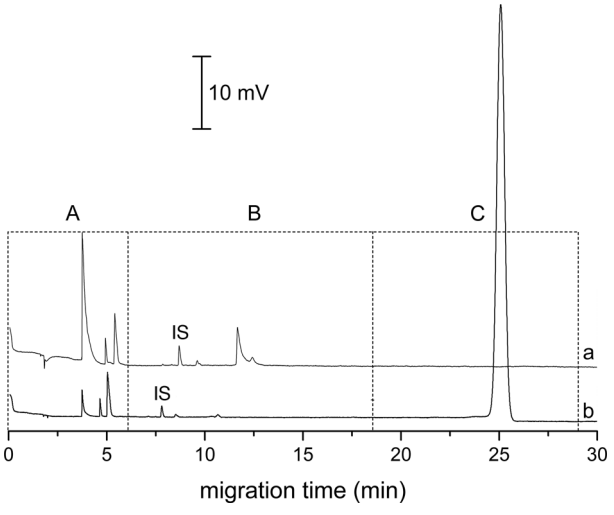

Figure 1. Electropherogram for a sample of whisky with cola spiked with: (A) Tranxilium® (Clorazepate dipotassium) and (B) Lorazepam. Zones A–C found in all the electropherograms are indicated. CE conditions: buffer, 0.5 M acetic acid at pH 2.5; applied voltage, 25 kV; hydrodynamic injection at 0.5 psi for 5 s.

ture and unclear information have been provided. During this study we observed certain deviation in the data when samples were analyzed after a period of storage of 24 h at 10°C. For this reason, samples were analyzed immediately after preparation in order to avoid sample degradation. Further experiments would be required in order to know the dynamics of degradation, however, this was out of the scope of this work. For this reason, we suggest to analyze samples as soon as possible when they are collected until more specific information about the preservation of these drugs in alcoholic beverages is obtained.

3.3 Data treatment

In order to extract the relevant chemical information from the pretreated CE data previously ordered in a transposed table (see Section 2), a PCA was performed. This is a bilinear modeling method that reduces the original variables to a smaller number of latent variables called principal components (PC) [28, 29]. Each PC clarifies a portion of all the information contained in the original data, where PC1 explains most of the information in the dataset, PC2 contains less information than the previous one and so on. In addition, plotting the PCs reveals significant sample and variable interrelationships, which leads to the interpretation of some sample similarities, differences, or groupings [28,29]. In turn, the meaning of the scores is given by the loadings that provide information about the relationship of the original variables and the samples [28, 29]. The loadings set the electropherogram area with more discriminating power.

The PCA model was better explained when considering only zones B and C. Consequently, in order to get an easier and better classification, zone A (Fig. 1) was not considered in the data analysis. Considering this, all the individual groups of the PCA model were generated and no outliers were identified. This guaranteed that none of the individual samples in a class badly influenced or attracted its entire PCA model due to a nonidentified data defect. In addition, only the scores for PC-1, PC-2, and PC-3 were used for the study of the class separation since they summarize more variation in the data than any other group of components. PC-1, PC-2, and PC-3 explained 50, 23, and 13% of the model, respectively.

The training PCA results and scores plot (Fig. 2 and Supporting Information Video 1) allowed a clear differentiation of each drug class in nonconfounded or entangled classes. Alprazolam and Lorazepam sample classes appeared rather close in the training PCA plot although the model could still differentiate them as they form nonentangled groups (see inset). Lexatin® was clearly in a different position in the Y-axis than Alprazolam and Lorazepam. Even though it was difficult to attribute the location of the drug classes in the PCA plot to a chemical aspect of the drug or differentiate among the drinks used in the mixtures, this data processing model was very successful for the classification of a drug added to different drinks. Furthermore, this is a relevant result because it is possible to discriminate the drug independently of the drink where the drugs were added.

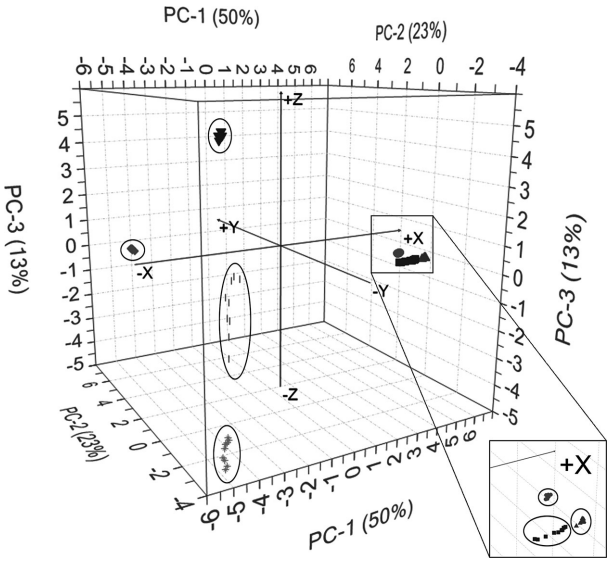

Figure 2. 3D scores plot of the training PCA: ♦, Noctamid® (Lormetazepam); ■, Alprazolam (Alprazolam); ●, Lexatin® (Bromazepam); |, Rohypnol® (Flunitrazepam); +, Diazepam (Diazepam); ▼, Tranxilium® (Clorazepate dipotassium); ▲, Lorazepam (Lorazepam).

In order to explain the meaning of the different scores, a PCA for Tranxilium® and Lorazepam samples was made. Tranxilium® and Lorazepam are quite apart from each other in the PCA-training plot, and they differ in the zone of the electropherogram in which the largest peak appeared (Fig. 1). In this PCA for Tranxilium® and Lorazepam PC-1 explained 95% of the model. Figure 3 shows the scores and loadings plots of PC-1 for both drugs. The score of a sample and the loading of a variable on a particular PC have the same sign. The larger are the scores and loadings, the stronger their relationship [28, 29]. The scores-plot of PC-1 (Fig. 3) shows that Lorazepam have positive scores and Tranxilium® negatives scores. Therefore in the loadings-plot, the four negative-going peaks correspond to Tranxilium® while the positive-going peak corresponds to Lorazepam. The peak that appeared at 25.3 min in the X-axis corresponds to the biggest peak that comes out in zone C for the sample of whisky with cola spiked with Lorazepam. At the same time, the peaks that appeared at 12.0, 11.4, 8.6, and 6.9 min in the X-axis correspond to peaks emerging in zone B for the sample of whisky with cola spiked with Tranxilium® (Fig. 1). Hence, in the case of Tranxilium® and Lorazepam, a quick view of the loadings serves to distinguish two differentiated zones in which two predominant peaks appeared, i.e. one belonging to Lorazepam in zone C and another belonging to Tranxilium® in zone B. There were also other minor peaks belonging to the internal standard and the soft drink. These analyses corroborate the importance of zones B and C of the electropherograms for the samples discrimination.

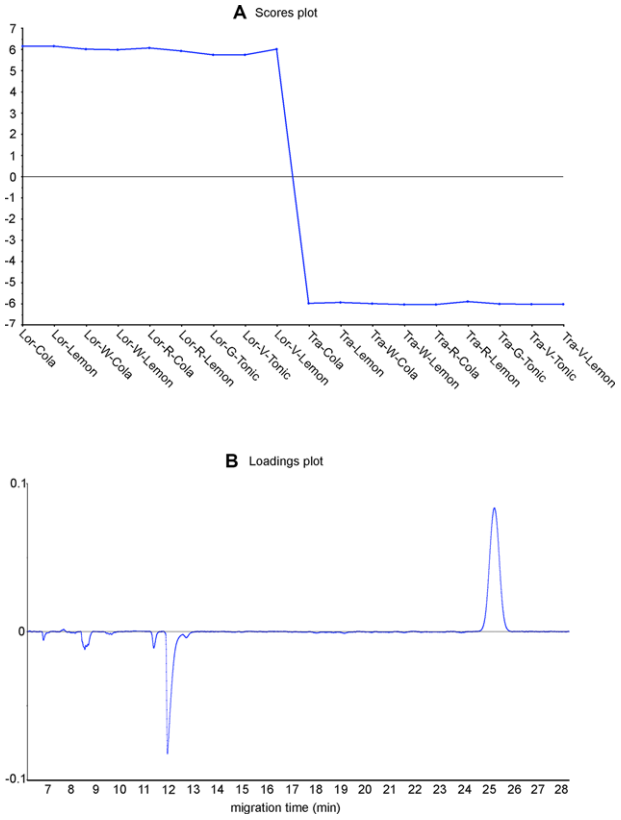

Figure 3. PC-1 scores plot of the PCA for Tranxilium® and Lorazepam (A) and loadings plot of the PCA for Tranxilium® and Lorazepam (B). Lor, Lorazepam, W, Whisky; R, Rum, G, Gin, V, Vodka, Tra, Tranxilium.

Finally, a SIMCA classification model (95% of confidence) was performed in order to test the classification power of the calculated PCA models. SIMCA is a supervised pattern recognition method based on making a PCA model for each class in the training set, where the classification rules are defined by the individual PCA models. Unknown samples are then compared to those class models and assigned to classes according to their proximity to the training samples. All values were mean centered, and a 95% confidence level was set. As a result, all but one sample were automatically assigned by software to their rightful class model (Table 3). These results confirm what was found with the PCA training model, i.e. it is possible to distinguish the type of drug added to the drinks. The anomalous sample arranged as belonging to two classes was whisky with cola spiked with Diazepam, which was classified both as Diazepam and as Rohypnol® (Flunitrazepam) classes (Table 3). Nonetheless, the probability of this sample to pertain to the Diazepam class was much larger than the probability of it to belong to the Rohypnol® class (99.3%>>>13.1%). In addition, the Coomans' plot (sample-to-model distances for two models, plotted against each other) for both classes clearly showed that this anomalous sample was closer to the center of the Diazepam model rather than to the Rohypnol® model (Fig. 4A). The same way, although the sample of cola spiked with Tranxilium® had a low probability (41.4%) of belonging to the Tranxilium® class, it had an even lower probability of belonging to the Rohypnol® class (0.0002%) (Table 3). The Coomans' plot for Tranxilium® and Rohypnol® classes visually indicated that the sample of cola spiked with Tranxilium® was far closer to the Tranxilium® model rather than to the Rohypnol® model (Fig. 4B).

PCA and SIMCA results showed that the different commercial benzodiazepine drugs in solution were correctly differentiated and classified. Additionally, the recreational drinks in which the tablets were dissolved did not influence the drugs classification. These results are of great interest because, once the models were generated, a single analysis was needed to classify the samples. Besides, since no drug standards were needed for the benzodiazepines determination, this procedure allowed expediting the analytical process. Moreover, the proposed approach permits the creation of databases, which can be extended with new benzodiazepines and drinks if they fit the model. Therefore, the proposed method can be used for a rapid screening of samples in forensic laboratories in which the determination of the benzodiazepine tablet is intended.

Table 3. SIMCA classification table

Sample-class membership 5%	Alprazolam	Lexatin®	Lorazepam	Noctamid®	Tranxilium®	Diazepam	Rohypnol®
Alp-R-Lemon	0.999						
Dia-W-Cola						0.993	0.131
Tra-Cola					0.414		
Lor-G-Tonic			0.994				
Noc-W-Lemon				1.000			
Lex-R-Cola		0.942					
Lor Lemon			0.994				
Roh-V-Lemon							0.967
Roh-V-Tonic							0.615

Empty cells imply 0.000 values. Alp, Alprazolam; R, Rum; Dia, Diazepam; W, Whisky; Tra, Tranxilium; Lor, Lorazepam; G, Gin; Noc, Noctamid, Lex, Lexatin; Roh, Rohypnol; V, Vodka.

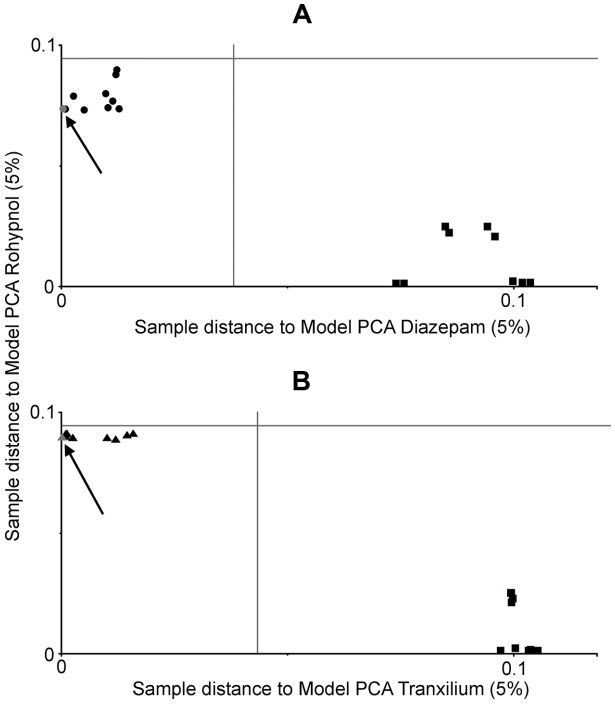

Figure 4. Coomans plot for Tranxilium® vs. Rohypnol® model distances (A) and Diazepam vs. Rohypnol® model distances (B). ▲, samples with Tranxilium®; ■, Samples with Rohypnol®; •, samples with Diazepam. The arrow in A indicates the sample of whisky with cola spiked with Diazepam. The arrow in B indicates the sample of cola spiked with Rohypnol®.

4 Concluding remarks

For the first time, the electropherograms of drinks spiked with benzodiazepine tablets have been used as fingerprints for the classification of different groups according to the drugs used. The electrophoretic method using an acetic acid buffer (pH 2.5) and C^4D detection resulted in complex electropherograms, which were subsequently compared. The PCA analysis of the electropherograms between 6.9 and 28.3 min allowed to visually classify the samples in groups using a 3D scores plot. A further examination of the plot confirmed that the drinks used for the sample preparation did not influence the classification, so it can be stated that the classification depended only on the drug, regardless of the drink used. The performed SIMCA classification model (95% of confidence), mathematically proved the results, showing that all but one sample were automatically assigned by software to their rightful class model. The incorrectly classified sample was then correctly classified in the Coomans' plot.

In conclusion, this novel approach based on the comparison of electrophoretic profiles of spiked drinks by chemometric tools allows determining the benzodiazepine used for drink spiking without the use of drug standards. It opens a new horizon for forensic laboratories because they will be able to incorporate the identification capability provided by the electrophoretic fingerprinting of benzodiazepine solutions in existing or new databases. However, before adopting this method by any forensic laboratory, the method should be tested with more benzodiazepines, other drugs and more blind tests.

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