

# Pros and cons of benzodiazepine screening in human saliva by ion mobility spectrometry

S. Armenta · M. Blanco

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**Abstract** The usefulness of ion mobility spectrometry as a screening methodology for the on-site benzodiazepine analysis in saliva samples has been critically evaluated. The procedure involved the injection of clear supernatant extracts after centrifugation and provided limit of detection values ranging from 2.0 to 18  $\mu\text{g L}^{-1}$ , and a precision, expressed as relative standard deviation, from 2.9% to 16%, depending on the different benzodiazepines studied. Those values are appropriate for their positive identification in saliva samples in which benzodiazepine concentration, after a chronic or acute dose, is in the range of 2–30  $\mu\text{g L}^{-1}$ . Problems related with overlapped benzodiazepine signals have been successfully overcome by application of multivariate curve resolution, which is a helpful tool to improve the resolution of the technique, without sacrificing the method simplicity and frequency of analysis. The possibility of false positives caused by the presence of interferents with the same drift time as the benzodiazepines and the possibility of false negatives due to the presence of interferents by competitive ionization have been critically evaluated. The satisfactory results obtained for the analysis of real saliva samples after an acute dose of diazepam through sublingual and oral intakes confirm the capability of the technique to be used as a screening methodology in the analysis of benzodiazepines in oral fluids.

**Keywords** Ion mobility spectrometry · Benzodiazepines · Drug testing · Saliva · Oral fluid

## Introduction

Drug testing has been conducted primarily on blood, plasma, and urine. However, recently, there is a high interest in their replacement by alternative body fluids which can be collected using non-invasive sampling techniques. Among the evaluated biological specimens, oral fluid, sometimes called saliva, can be considered a promising specimen for drug testing [1], with several advantages including (1) its noninvasive and under direct supervision nature, (2) its easy and repeated collection, (3) concentration of drugs in saliva reflects their concentration in blood, (4) their simplicity of analysis over other fluids such as blood and, finally, (5) oral fluids normally contain the parent drug substance rather than drug metabolites.

Recent trends in drug testing point to simple and cheap screening methodologies used on-site to classify samples into two groups (positive or negative) in a fast and reliable way. The primary objectives of those screening systems are [2] (1) to provide a rapid and reliable response about a specific analyte/s for immediate decision making and (2) to avoid the analysis of a large number of negative samples using a high cost and high maintenance instrument. Nowadays, semi-quantitative lateral flow immunoassay-based devices are the most commonly used for the fast and cheap screening of several group of drugs such as cannabinoids, cocaine, opioids, amphetamine and derivatives, and benzodiazepines in saliva with cutoff levels of 4  $\mu\text{g L}^{-1}$  for cannabinoids, 20  $\mu\text{g L}^{-1}$  for cocaine, 50  $\mu\text{g L}^{-1}$  for amphetamines, and 40  $\mu\text{g L}^{-1}$  for opiates [3, 4]. Moreover, typical benzodiazepine cutoff concentrations are between 10 and 90  $\mu\text{g L}^{-1}$ , depending on the device commercial brand and the probability selected to avoid false positive and negative samples. However, those devices do not provide an appropriate sensitivity to

S. Armenta (✉) · M. Blanco  
Department of Chemistry, Faculty of Sciences,  
Universitat Autònoma de Barcelona,  
Bellaterra,  
08193 Barcelona, Spain  
e-mail: sergio.armenta@uab.cat

positively identify benzodiazepines in saliva as their concentration after chronic or acute doses are in the range of 2–30  $\mu\text{g L}^{-1}$  [5, 6], depending on the benzodiazepine administered and their half life.

On the other hand, ion mobility spectrometry (IMS) has been becoming an indispensable tool in drug enforcement departments, for detecting trace amounts of illegal drugs in different objects such as imported articles, clothing, and luggage, in a simple and rapid way [7, 8]. IMS is a highly sensitive gas phase electrophoretic separation of vaporized and ionized analytes in a weak electromagnetic field. Charged analytes migrate through the drift tube with different and characteristic velocities (drift velocities) that are proportional to the electric field strength and the analyte mobility constant ( $K$ ) which depends on the charge, mass, size, and shape of the analytes. Mobility values are generally normalized to standard environmental pressure and temperature conditions (reduced mobility constant,  $K_0$ ), and can be related to the time that a specific ion takes to reach the detection unit (drift times,  $t_d$ ). The main advantages provided by IMS are the high-speed of response and confidence in determinations with samples of relatively complex matrices. Analytical IMS applications vary from drug [9] and chemical warfare agents [10] identification to environmental monitoring [11] and, more recently, it is gaining popularity also in the food area [12].

Benzodiazepines, an important class of sedative hypnotic drugs that are used widely to treat anxiety and insomnia, have been previously characterized by IMS [13–15], obtaining promising results as a qualitative tool for their rapid detection and reliable identification, although partial peak overlapping was presented. Different approaches have been proposed in the literature to increase the resolving capability of IMS procedures for benzodiazepine mixtures based on the coupling of a previous separation system such as gas or supercritical fluid chromatography [16] and coupled to a mass spectrometry (MS) detector [17]. However, the main drawbacks of those methodologies are that related to the acquisition cost of these complex instrumentations, which reduces, at the same time, the possibilities to perform on-site screening. Moreover, digital signal processing algorithms such as second derivative or deconvolution [13] and more complex chemometric treatments such as multivariate curve resolution (MCR) have been also successfully evaluated to improve resolution of IMS overlapping peaks [18]. These mathematical solutions could improve the resolution of benzodiazepine peaks without sacrificing the advantages that the IMS procedure provides as on-site screening methodology.

In summary, the objective of this paper is to critically depict the pros and cons of IMS as screening methodology for the on-site benzodiazepine determination in saliva samples. The main analytical features of the methodology,

the usefulness of chemometric treatments such as MCR to improve the resolution of overlapped signals, the different sample pre-treatment strategies including direct injection and centrifugation of saliva samples, and the effect of different interferents and interference modes will be critically evaluated. Additionally, the capabilities of the IMS methodology for the screening of benzodiazepines in oral fluids will be demonstrated on the analysis of real saliva samples after acute dose of diazepam through sublingual and oral intakes.

## Experimental section

### Reagents

Benzodiazepine drug standards (clorazepate dipotassium salt, medazepam, oxazepam, nitrazepam, diazepam, temazepam, lorazepam, chlordiazepoxide, tetrazepam, and prazepam) were obtained from the Analytical Chemistry Department of the University of Valencia and were used without further purification. Individual benzodiazepine stock solutions (2,000  $\text{mg L}^{-1}$ ) were prepared by dissolving 20 mg drug standard in 10 mL methanol and were stored at room temperature protected from light. Diluted standard solutions from 5 to 200  $\mu\text{g L}^{-1}$  were prepared by suitable dilutions of the stock solution in methanol.

Methanol was supplied by Sigma (Schnelldorf, Germany), and isopropanol, supplied by Acros (Geel, Belgium), was used as spiking solvent due to its spreading properties on the Teflon membrane.

Diazepam Prodes (Kern Pharma, Barcelona, Spain) tablets, Strepsils<sup>®</sup> containing 2 mg lidocaine throat lozenge, and cough syrup containing codeine were acquired in Spanish pharmacies.

### Sample treatment

Human saliva samples taken from different volunteers including different age, ranging from 25 to 35 years old, and sex were collected in a 10-mL glass tube. Prior to IMS analysis, 2-mL saliva and spiked saliva samples were treated for the removal of proteins and insoluble material by centrifugation for 5 min at 5,000 rpm in a Digicen centrifuge (Alresa, Madrid, Spain). The clear supernatant was collected and assayed immediately. When necessary, saliva samples were stored, after centrifugation, at 4 °C and were analyzed as soon as possible to maintain sample integrity.

### Ion mobility spectrometry procedure

An IONSCAN-LS (Smiths Detection, Morristown, NJ, USA) equipped with a <sup>63</sup>Ni foil radioactive ionization

source was used to separate and identify the different compounds involved in this study. IM station software (version 5.389) was used for data acquisition and processing. Plasmagrams were acquired in positive ion mode using nicotinamide, with a reduced mobility,  $K_0$ , of  $1.860 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ , as internal calibrant. The number of segments per analysis was 84, containing every plasmagram 579 data points. The shutter grid width was 0.2 ms, value optimized by the manufacturer, and plasmagrams were collected with a scan period of 30 ms. A counterflow of dry nitrogen, set to  $300 \text{ mL min}^{-1}$ , was introduced as drift gas at the end of the drift region. The electric field strength in the drift region was  $252 \text{ V cm}^{-1}$  with a total drift voltage of 1,763 V and a drift tube length of 7 cm.

Thermal desorption from a Teflon membrane was used for sample introduction. In this strategy, a few microliters of sample are placed onto the Teflon membrane, and it is heated to vaporize the analyte which is transferred to the ionization region. Before analysis, Teflon membranes are introduced two times into the IMS instrument to remove any possible interference. The main disadvantage of this sample introduction method is that non-volatile compounds present in complex samples are not desorbed from the Teflon membrane, forming a solid residue which can cause a decrease of analyte peak intensity due to interferences during the desorption process. Thus, it would be necessary the evaluation of sample treatments to remove proteins and insoluble material present in saliva samples. Desorption, inlet, and drift tube temperatures were adjusted to  $285 \text{ }^\circ\text{C}$ ,  $286 \text{ }^\circ\text{C}$ , and  $232 \text{ }^\circ\text{C}$ , respectively.

Spiking solvents are useful to overcome spreading deficiencies of aqueous solutions which have the tendency to bead on the Teflon membrane. The IONSCAN-LS is equipped with an autosampler which allows precise volume metering and dispensing. Thus,  $1 \text{ } \mu\text{L}$  sample solution is merged with  $4 \text{ } \mu\text{L}$  of isopropanol, as spiking solvent, and is deposited in the Teflon membrane, obtaining a uniform and centered sample spot. Using a 5-s post-dispense delay, the sample tray containing the Teflon membrane is inserted in the heated zone, and the sample is held in this position for 30 s. Two successive injections of a cleaning solution, consisting of deionized water, were analyzed between two consecutive saliva sample runs to avoid carry-over between samples. For benzodiazepine stock solutions, a similar procedure that merges  $1 \text{ } \mu\text{L}$  diluted standard solution with  $4 \text{ } \mu\text{L}$  isopropanol, was used.

#### Acute dosage study

The analysis of diazepam concentration in saliva samples after single-dose administration has been evaluated following two different strategies: sublingual and oral intakes. In the first strategy, a diazepam tablet, Diazepam Prodes (Kern

Pharma, Barcelona, Spain), with a concentration of 5 mg diazepam per tablet, was sublingually administered to a male healthy volunteer on the morning after an overnight fast. The diazepam tablet was placed over the tongue and allowed to dissolve. One-milliliter saliva was sampled at equal time intervals of 10 min during the first hour and every 30 min the second and third hours after taking the drug.

In the second strategy, a male healthy volunteer swallowed and washed down with approximately 10 mL water, on the morning after an overnight fast, a 5-mg tablet of Diazepam Prodes. Saliva samples were obtained at equal time intervals of 30 min after drug administration.

## Results and discussion

### Benzodiazepines plasmagrams

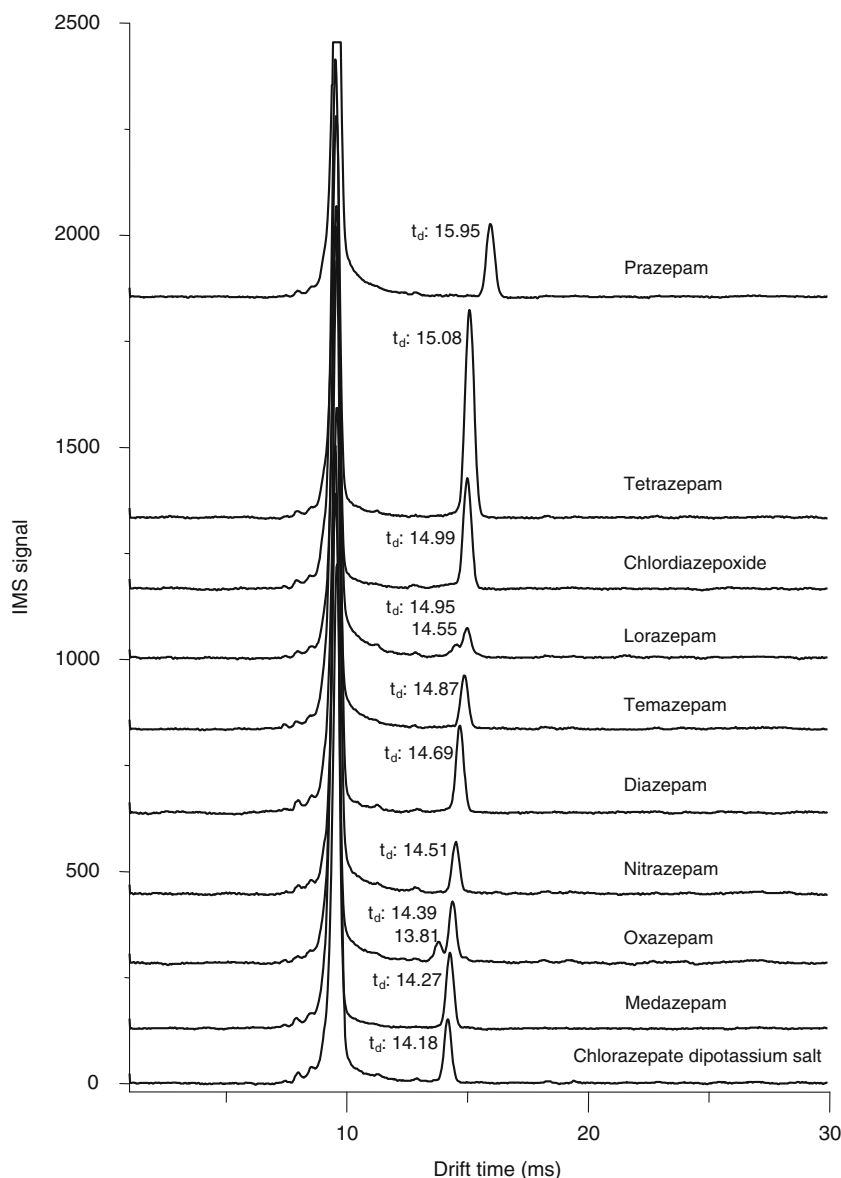
Benzodiazepines are nucleophilic nitrogenous drugs with relatively high gaseous basicity that provide strong response signals in positive IMS [13]. The ion mobility plasmagrams of the selected benzodiazepines are depicted in Fig. 1. The most intense peak in all plasmagrams is due to the internal calibrant, nicotinamide ( $K_0=1.860 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ ), used in the positive ionization mode to correct for variations in temperature, pressure, and drift field. As it can be seen, all the benzodiazepines assayed presented drift times comprised between 13.81 and 15.95 ms. Positive IMS plasmagrams for individual solutions of benzodiazepines were simple with no appreciable fragments under the desorber, inlet, and drift tube temperatures used in this study. On the other hand, oxazepam and lorazepam plasmagrams present a second peak due to  $[\text{M}-\text{H}_2\text{O}]^+$  that is not completely resolved from the  $\text{M}^+$  ion peak. This fact can be explained by the presence of a hydroxyl moiety in their structure (see Table 1), which is a good leaving group and, thus, both analytes exhibit dehydration behavior [13].

Table 1 lists the structure and molecular weight of the studied benzodiazepines, their  $K_0$  and literature  $K_0$  values, and calculated molecular weight from the  $t_d$ .  $K_0$  values were calculated from experimentally determined  $t_d$  with reference to nicotinamide using Eq. 1:

$$K_{0(\text{analyte})} = \frac{t_{d(\text{standard})}}{t_{d(\text{analyte})}} \times K_{0(\text{standard})} (\text{cm}^2 \text{ V}^{-1} \text{ s}^{-1}) \quad (1)$$

where  $1.860 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$  was the  $K_0$  value used for the nicotinamide standard. It can be shown from Table 1 that the obtained  $K_0$  values for the studied benzodiazepines compare well with those previously reported in the scientific literature. Additionally, it is possible to calculate the molecular weight of the different compounds under

**Fig. 1** IMS plasmagram of the studied benzodiazepines



investigation by using an equation provided by the IMS manufacturer (Eq. 2):

$$\frac{1}{K_0} = 0.001562 \times MW + 0.3837 \quad (2)$$

being  $K_0$  the reduced mobility of the analyte. It should be noted that this formula, applied in the positive mode, provides only an approximate value and could be off by as much as 10–15%. From those calculations and results previously reported in the literature [13], it can be concluded that in almost every benzodiazepine studied, a single major ion peak corresponding to the molecular ion  $M^+$  or  $MH^+$  or  $[M-H]^+$  is produced. Clorazepate dipotassium salt shows a deviation of the calculated molecular weight from the theoretical value. It is probably due to the peak observed in the plasmagram is not due to the

molecular ion but a fragment formed during the thermal desorption or the ionization process. This fragment could be due to the losses of  $CO_2$  to form 7-chloro-5-phenyl-1H-benzo [1, 4]diazepin-2(3H)-one with a molecular mass of  $270 \text{ g mol}^{-1}$ .

#### Resolution of overlapped signals

Although the concomitant administration of benzodiazepines is not useful and could increase the risk to develop pharmacodependency [19], it is easy to imagine taking a look on the  $t_d$  of the benzodiazepines that the analysis of mixtures of benzodiazepines will provide broad and unresolved peaks.

As it has been mentioned in the “Introduction” section, the ability of IMS to separate peaks as required to

**Table 1** List of structure, molecular weight (MW),  $K_0$  and literature  $K_0$  values, and calculated MW of selected benzodiazepines

	Clorazepate dipotassium salt	Medazepam	Oxazepam	Nitrazepam
Structure				
MW	408.9	270.8	286.7	281.3
$K_0$	1.260	1.243	1.234 1.286	1.230
$K_0$ (literature) <sup>a</sup>	-	-	1.23 1.28	1.22 1.23
MW calc	262	269 [M] <sup>+</sup>	273 [M-H] <sup>+</sup> 252 [M-H <sub>2</sub> O] <sup>+</sup>	275 [M] <sup>+</sup>
	Diazepam	Temazepam	Lorazepam	Chlordiazepoxide
Structure				
MW	284.7	300.7	321.2	299.7
$K_0$	1.218	1.194	1.190 1.226	1.184
$K_0$ (literature) <sup>a</sup>	1.21	-	1.19 1.22	1.18 1.19
MW calc	280 [M] <sup>+</sup>	291 [M] <sup>+</sup>	292 [M] <sup>+</sup> 276 [M-H <sub>2</sub> O] <sup>+</sup>	295 [M] <sup>+</sup>
	Tetrazepam	Prazepam		
Structure				
MW	288.8	324.8		
$K_0$	1.182	1.119		
$K_0$ (literature) <sup>a</sup>	-	-		
MW calc	296 [M] <sup>+</sup>	326 [M] <sup>+</sup>		

<sup>a</sup>Lawrence [13]

unequivocally identify ions has been the subject of intensive investigation [20]. In this sense, the application of mathematical algorithms such as the second derivative and more complex chemometric treatments such as MCR have been successfully evaluated for the resolution of overlapped and partially overlapped peaks on complex mixtures [18], and those treatments have been tested for the resolution of benzodiazepine mixtures.

Figure 2 shows the plasmagrams resulting from binary, ternary, and higher order mixtures of benzodiazepines and those corresponding to the injection of individual benzodiazepine standard. As it can be seen, depending on the number and nature of the benzodiazepines present in the mixture, the situation can vary from a simple case, as the binary mixture of clorazepate and diazepam, to a more complicate case, such as the mixture of five benzodiazepines (clorazepate, nitrazepam, diazepam, temazepam, and tetrazepam). The application of the second order-derivative algorithm, available in the software of commercial IMS, is enough to resolve partially overlapped peaks such as those of the simplest case. In Fig. 2, it has been depicted the resolution of overlapped peaks using the positive part of the inverse of the second derivative (PP2D) of the plasmagrams obtained from individual standards of clorazepate and diazepam and a mixture of both benzodiazepines. However, this algorithm has proved to be unsuccessful for more complex situations, perhaps because of the relatively small separation between two or more benzodiazepine peaks.

In such cases, MCR was applied to extract underlying individual benzodiazepine component information from data comprised of a complex mixture. Calculations were performed using the GUIPRO software package written using Matlab [21].

The 3D plasmagram dataset was imported, and sub-ranges for the MCR analysis were selected from 13 to 15 ms  $t_d$  and from 1 to 7 s desorption time. The initial solution was selected by the “needle search” method or by evolving factor analysis (EFA) after manual selection of the number of components of the mixture. For concentration profiles, nonnegativity and unimodality soft constraints were selected, while nonnegativity soft constraints were fixed for spectral profiles using in both cases a penalty weight of 1.0. It should be noted that in complex ternary and higher order mixtures, it is necessary to augment the original data matrix by arrangement of additional datasets obtained from the analysis of mixtures with different benzodiazepine concentration ratios.

As it can be seen in Fig. 2, using soft constraints, more than 97.6% of the variance can be explained, depending on the particular case, and comparing the obtained spectral profile with plasmagrams of individual standards of the mixture, only small deviations in the calculated  $t_d$  can be observed. Additionally, the concentration profile obtained

through MCR from the aforementioned mixtures provided benzodiazepine recoveries ranging from 89% to 116% for benzodiazepine concentrations ranging from 30 to 250  $\mu\text{g L}^{-1}$ .

In summary, MCR is a powerful tool to improve resolution of IMS and can be used to resolve overlapped or partially overlapped peaks without sacrificing the main advantages of the methodology named its simplicity of operation, on-site capabilities, and reduced cost.

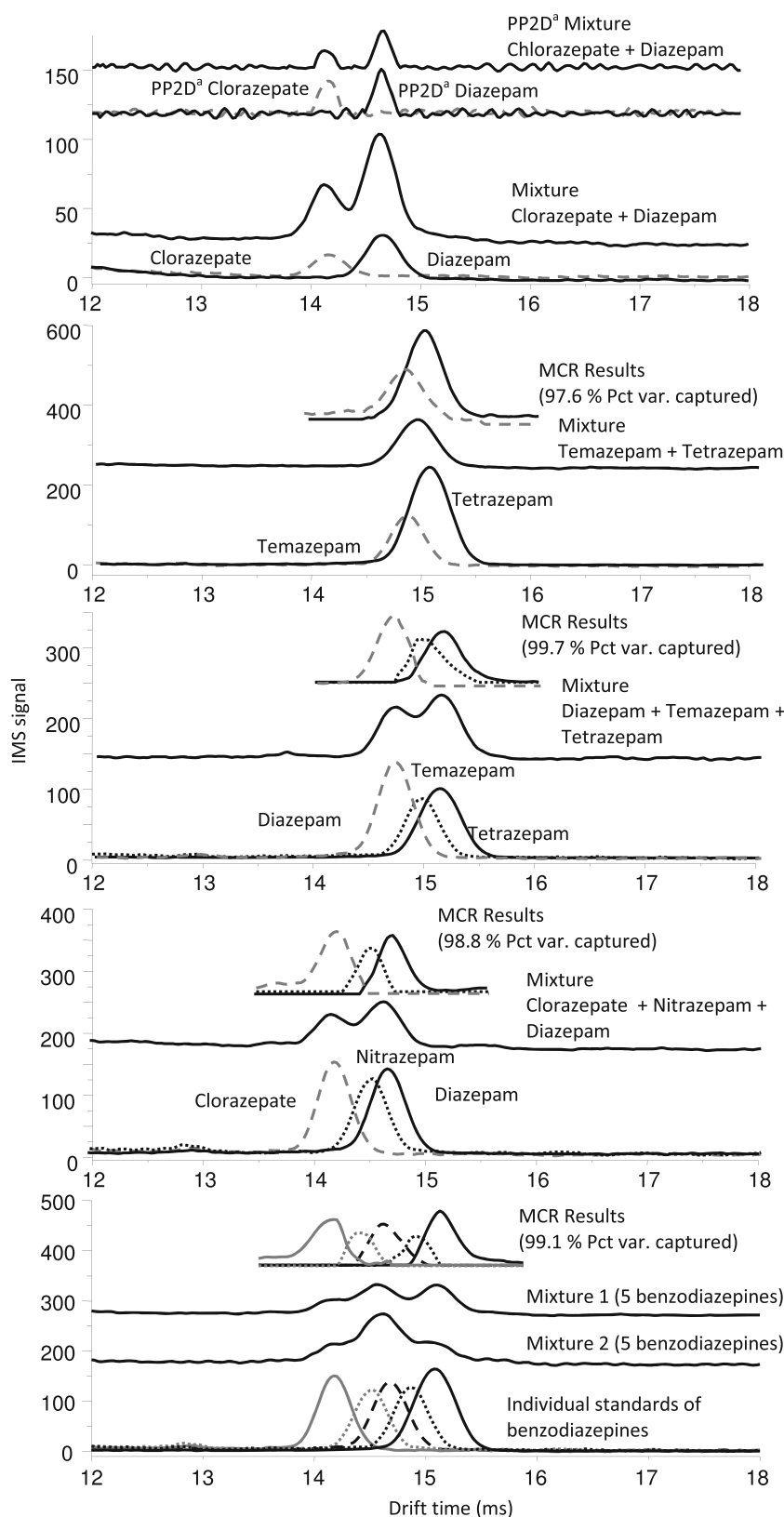
#### Analytical features of the method

The analytical features of the methodology, in terms of linearity, linear range, precision, and limit of detection (LOD) and quantification (LOQ) values, were studied (see Table 2). The analytical response used for the analysis of each benzodiazepine corresponds to the cumulated maximum amplitude of the corresponding peak. The IMS instrument software automatically calculates the cumulative amplitude based on the sum of the maximum amplitude or peak height of all segments of the plasmagram in which the different analytes have been positively detected. It should be mentioned that oxazepam and lorazepam plasmagrams show two peaks, and the analytical response used for quantitative purposes has been (1) cumulated amplitude of the most intense peak and (2) the sum of the cumulated amplitudes of the two peaks.

The linearity and the linear range of the procedure was checked through the examination of the correlation coefficient of the calibration curve obtained by representation of the cumulated amplitude of the plasmagram obtained from individual benzodiazepine standard solutions versus its concentration from 5 to 200  $\mu\text{g L}^{-1}$ , using nine levels of concentration. Correlation coefficients higher than 0.993 were obtained, indicating a linear correspondence between the cumulated amplitude and analyte concentration in the studied range. In the case of oxazepam and lorazepam, the relationship between cumulated amplitude of the most intense peak versus benzodiazepine concentration is not linear, with correlation coefficients of 0.92 and 0.95, respectively. However, by plotting the sum of the cumulated amplitude of both peaks versus the concentration of benzodiazepine, a linear relationship with correlation coefficient of 0.995 and 0.997 were obtained, respectively, and thus, the sum of the cumulated amplitude of both peaks were selected as analytical response. Probably, the better linearity of the cumulated amplitude of the double peak is a consequence of the reversible character of the hydration/dehydration equilibrium responsible of the two peaks of the plasmagrams.

The precision of the methodology, expressed as the relative standard deviation (RSD), was established from five independent measurements of a 20- $\mu\text{g L}^{-1}$  benzodiazepine individual standard solution, except in the case of

**Fig. 2** IMS plasmagrams of individual benzodiazepine standards, binary, ternary, and higher order benzodiazepines mixtures and the spectral profiles obtained by application of the positive part of the inverse of the second derivative (PP2D) and multivariate curve resolution (MCR). Mixtures 1 and 2 are the plasmagrams resulting from mixtures of five benzodiazepines (clorazepate, nitrazepam, diazepam, temazepam, and tetrazepam) at different concentration ratios



oxazepam and lorazepam that a 40- $\mu\text{g L}^{-1}$  individual standard solution was employed due to their LODs. As it

can be seen in Table 2, those values varied between 2.9% and 16%, for tetrazepam and oxazepam, respectively.

**Table 2** Analytical features of merit of benzodiazepines determination by IMS

	Linear range ( $\mu\text{g L}^{-1}$ )	Sensitivity (arbitrary units)	$R^2$	%RSD <sup>a</sup>	LOD <sup>c</sup> ( $\mu\text{g L}^{-1}$ )	LOQ <sup>c</sup> ( $\mu\text{g L}^{-1}$ )
Clorazepate dipotassium salt	7–145	6.00	0.993	7.2	6.2	21
Medazepam	5–112	10.65	0.993	5.8	5.7	19
Oxazepam	29–293	2.66	0.95	16 <sup>b</sup>	49	164
– <sup>d</sup>	29–293	4.11	0.995	12 <sup>b</sup>	18	60
Nitrazepam	6–96	5.79	0.994	14	6.2	20
Diazepam	4–80	13.95	0.992	3.6	3.6	12
Temazepam	5–150	5.49	0.999	10	3.4	11
Lorazepam	27–266	1.51	0.92	11 <sup>b</sup>	59	198
– <sup>d</sup>	27–266	1.57	0.997	10 <sup>b</sup>	15	49
Chlordiazepoxide	5–74	11.35	0.997	5.2	2.0	6.7
Tetrazeepam	8–166	11.71	0.996	2.9	6.8	26
Prazepam	5–96	9.08	0.999	3.6	3.4	11

<sup>a</sup>%RSD established from five independent measurements of a 20- $\mu\text{g L}^{-1}$  individual benzodiazepine standard solution

<sup>b</sup>%RSD established from five independent measurements of a 40- $\mu\text{g L}^{-1}$  individual standard solution

<sup>c</sup>LOD and LOQ calculated as three and ten times, respectively, the standard deviation of the intercept divided by the slope of the respective calibration lines

<sup>d</sup>Analytical features using the sum of the cumulated amplitudes of the two peaks as analytical response

The LOD and LOQ were calculated as three and ten times, respectively, the standard deviation of the intercept divided by the slope of the respective calibration lines. LOD and LOQ values ranging from 2.0 to 18  $\mu\text{g L}^{-1}$  and from 6.7 to 60  $\mu\text{g L}^{-1}$  for chlordiazepoxide and oxazepam, respectively, were found.

The provided analytical features demonstrate that the developed IMS procedure is a sensitive, rapid, and reliable methodology for benzodiazepine screening in human saliva samples. Comparing the LOD and LOQ values of the IMS procedure with those of other methods used for benzodiazepines screening in human fluids, it can be concluded that the proposed method presents superior features with respect to immunoassays, which provides a yes/no response with a proposed cutoff criterion between 10 and 90  $\mu\text{g L}^{-1}$  [2, 3], depending on the commercial brand. Taking into consideration the benzodiazepine blood levels obtained from therapeutic or recreational uses [22] and their saliva blood ratios, positive saliva samples using immunoassays are probably due to detection of residual benzodiazepine pill fragments in the mouth indicating very recent oral administration.

#### Human saliva analysis

##### *Sample pre-treatment: direct injection versus centrifugation*

Although saliva can be considered a simple matrix, it contains plasma electrolytes and many other constituents including enzymes, proteins, glucose, amino acids, lipids, and DNA [23]. Different sample pretreatments have been

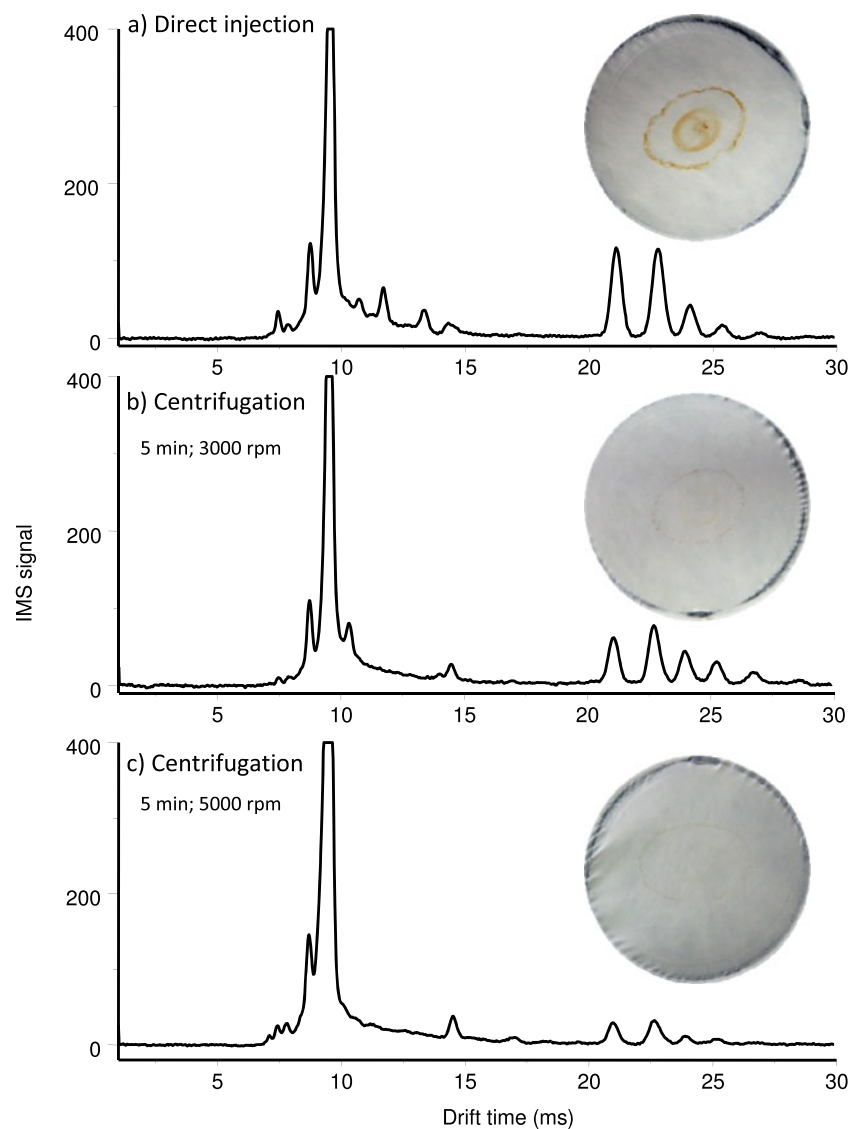
evaluated to improve the sensitivity and selectivity of the methodology, taking also into consideration not sacrificing the speed and easiness of analysis.

Direct injection of diazepam spiked saliva samples at a concentration level of 50  $\mu\text{g L}^{-1}$  was performed by IMS, and the obtained plasmagram is shown in Fig. 3a. As it can be seen, diazepam IMS signal, at 14.69 ms, is partially overlapped by saliva matrix constituents, and its determination is difficult. Moreover, after the injection of 1  $\mu\text{L}$  untreated human saliva, the Teflon membrane presents solid residues (inset of Fig. 3a), being the analysis of up to three blank solutions between sample runs not enough to eliminate this residue. The presence of those solid residues that do not generate an IMS response could cause losses in benzodiazepine peak intensity due to interferences during the desorption process [7].

Alternatively, the effect of centrifugation of the sample at 3,000 rpm for 5 min was evaluated on the determination of diazepam in spiked saliva samples. The plasmagram of the centrifuged sample shows no peaks in the region between 10 and 14 ms, probably by the precipitation of proteins and insoluble amino acid residues. Moreover, the intensity of the peaks appearing from 20 to 25 ms has been substantially reduced compared to the direct injection of saliva samples. Another advantage of the centrifugation as sample treatment is that the solid residue which remains in the Teflon membrane after sample injection is lower compared to the previous situation. The inset of Fig. 3b shows the Teflon membrane resulting from the injection of five saliva samples centrifuged at 3,000 rpm for 5 min and two blank solutions between sample runs to avoid carry-over. How-



**Fig. 3** IMS plasmagrams of diazepam spiked saliva samples at a concentration level of  $50 \mu\text{g L}^{-1}$  after different sample treatments: (a) direct injection, (b) centrifugation at 3,000 rpm for 5 min, and (c) centrifugation at 5,000 rpm for 5 min. *Insets:* Picture of the Teflon membrane used for sample introduction after (a) injection of  $1 \mu\text{L}$  untreated human saliva, (b) injection of five saliva samples centrifuged at 3,000 rpm for 5 min, and (c) injection of ten saliva samples centrifuged at 5,000 rpm for 5 min



ever, the analysis of saliva samples centrifuged at 3,000 rpm required a preventative maintenance such as PTFE membrane replacement every five sample injections, indicating a possible lack of automation in routine applications.

A clearer supernatant solution is expected by increasing the rotation speed of the centrifuge to 5,000 rpm for 5 min, which means a reduction of the solid residue in the Teflon membrane. In the inset of Fig. 3c, the Teflon membrane, resulting from the injection of ten saliva samples centrifuged at 5,000 rpm for 5 min and two blank solutions between sample runs to avoid carry-over, is depicted.

In summary, after a centrifugation step using the most appropriate conditions, the solid residue that saliva samples leave in the Teflon membrane is insignificant, and diazepam analysis can be performed without interferences from sample matrix constituents and which is more important

without sacrificing the simplicity and fastness of the screening procedure.

#### *Benzodiazepines recoveries*

As it has been aforementioned, the probability to find several benzodiazepines in the same saliva sample would be very low due to the uselessness of the concomitant administration of several benzodiazepines. Consequently, recovery studies of benzodiazepines in saliva were performed by spiking blank samples with known amounts of individual benzodiazepine solutions at two concentration levels: 20 and  $40 \mu\text{g L}^{-1}$  (see Table 3). For oxazepam and lorazepam, recoveries were calculated at 100–150 and 200–220  $\mu\text{g L}^{-1}$  concentration level due to their low LOD values.

After benzodiazepine oral intake, the drug must be mobilized from the intestinal mucosa to the plasma and

**Table 3** Recovery values for benzodiazepine determination in human saliva by IMS after centrifugation

	Concentration added ( $\mu\text{g L}^{-1}$ )	Concentration found ( $\mu\text{g L}^{-1}$ )	% Recovery
Chlorazepate dipotassium salt	20	19 $\pm$ 3	97 $\pm$ 14
	40	33.2 $\pm$ 1.2	83 $\pm$ 3
Medazepam	20	18 $\pm$ 2	92 $\pm$ 10
	40	37 $\pm$ 4	92 $\pm$ 11
Oxazepam <sup>a</sup>	100	84 $\pm$ 10	77 $\pm$ 9
	150	142 $\pm$ 20	94 $\pm$ 16
Nitrazepam	20	–	–
	40	7 $\pm$ 2	18 $\pm$ 5
	60	20 $\pm$ 4	38 $\pm$ 13
	90	30 $\pm$ 2	33 $\pm$ 2
Diazepam	20	20.8 $\pm$ 1.0	104 $\pm$ 5
	40	39 $\pm$ 2	98 $\pm$ 6
Temazepam	30	28.3 $\pm$ 1.1	94 $\pm$ 4
	50	43 $\pm$ 2	86 $\pm$ 5
Lorazepam <sup>a</sup>	200	191 $\pm$ 14	96 $\pm$ 7
	220	198 $\pm$ 11	90 $\pm$ 5
Chlordiazepoxide	20	15.4 $\pm$ 1.0	77 $\pm$ 4
	40	21.0 $\pm$ 0.8	53 $\pm$ 2
Tetrazepam	20	18.2 $\pm$ 1.2	91 $\pm$ 6
	40	32 $\pm$ 4	81 $\pm$ 10
Prazepam	20	16.0 $\pm$ 1.8	80 $\pm$ 9
	40	25.7 $\pm$ 0.6	64.2 $\pm$ 1.5

<sup>a</sup> Only one peak is detected

later to the saliva. It is known that benzodiazepine protein binding is as high as 95% in plasma because benzodiazepines drive along the bloodstream bound to plasmatic proteins. However, various reports have shown that only the unbound (free) drug diffuses into the saliva [24, 25]. Moreover, it should be noted that for this study, different blank saliva samples taken from different volunteers including different age, ranging from 25 to 35 years old, and sex were used to include variability of saliva characteristics. Acceptable recovery values were obtained for most of the studied benzodiazepines ranging from 53% to 104%.

It should be highlighted that additional recovery studies were performed for nitrazepam, at concentration levels of 60 and 90  $\mu\text{g L}^{-1}$  due to the poor recovery values obtained at 20 and 40  $\mu\text{g L}^{-1}$ . As it can be seen in Table 3, in all the cases, recovery values of nitrazepam are lower than 40%. This fact could be partially explained by the low stability of nitrazepam in saliva samples [26]. As it has been previously reported, nitrazepam was found to be unstable in saliva at room temperature, being converted into 7-aminonitrazepam, and this conversion rate is strongly dependent on the composition of the subject saliva. 7-Aminonitrazepam (molecular weight of 251  $\text{g mol}^{-1}$ ) would have a calculated

$K_0$  of 1.289  $\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$ , corresponding with a drift time of 13.46 ms. In the plasmagram of the spiked saliva sample with nitrazepam, it can be seen a peak with a  $K_0$  of 1.265  $\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$ , corresponding with a drift time of 13.72 ms and a calculated molecular weight of 260  $\text{g mol}^{-1}$ , which could be due to 7-aminonitrazepam, although a more accurate identification would be necessary.

#### Interferents by drift time

The effect of potential interferences to provide a false positive response, from an ion associated with a molecule with a similar  $t_d$  than that of the target analyte, in the IMS analysis of benzodiazepines in saliva samples has been evaluated. The IMS response of several drugs and molecules, which can be present in human saliva such as vitamins and sweeteners, were evaluated, and the  $t_d$  was compared to that of the studied benzodiazepines.

As shown in Table 4, the main interferences were caused by diphenhydramine, doxylamine, amitriptyline, and codeine, molecules with a tertiary amino group in their structure, which possess a relative volatility and a molecular weight similar to those of benzodiazepines. The presence of those interfering substances in the saliva sample would provide a false positive response demanding the application of a confirmatory analytical methodology with a selective detection mode as MS and/or a sample treatment cleanup.

On the other hand, common active principles in pharmaceuticals such as acetylsalicylic acid, ibuprofen, and ketoprofen have no ionizable groups in positive mode, and thus do not represent interference in the determination of benzodiazepines by IMS in saliva samples. Moreover, other compounds, such as saccharin and nimesulide containing sulfonamide groups ( $\text{R}_1\text{-SO}_2\text{-NHR}_2$ ) and paracetamol containing an amide group do not provide any appreciable signal under the IMS conditions employed in these experiments, probably due to their low vaporization and/or ionization capabilities in positive mode. Moreover, xanthine derivatives including caffeine, teophylline, theobromine, hypoxanthine, and xanthine, provided a weak IMS signal with lower  $t_d$  than those of the benzodiazepines, probably due to their low volatilities, and thus, those compounds should be considered as no interferences.

It is clear that this is only an example of the capabilities and limitations of the IMS methodology, and interferences of other drugs and/or drug metabolites not tested here should be carefully evaluated.

#### Interferents by competitive ionization

The principle of ionization in positive mode IMS detection is based on proton exchange mediated by a dopant gas.

**Table 4** Possible interferents by drift time of benzodiazepines determination in human saliva by IMS

Drug	Therapeutic uses	Concentration tested (mg L <sup>-1</sup> )	MW (g mol <sup>-1</sup> )	$K_0$	$t_d$ (ms)
Diphenhydramine	Anxiolytic, sleep aid	1	255	1.235	14.39
Doxylamine	Sedative	1	270	1.226	14.35
Amitriptyline	Antidepressant	1	314	1.193	14.93
Mirtazapine	Antidepressant	1	265	1.250	14.28
Paroxetine	Antidepressant	1	375	1.076	16.38
Lidocaine	Anesthetic	1	234	1.308	13.68
Isoproterenol	Bradycardia, heart block	1	211	1.451	12.14
				1.391	12.68
Epinephrine	Cardiac arrest	1	183	1.640	10.88
				1.585	11.28
				1.505	11.87
Phenylephrine	Decongestant, blood pressure	1	167	1.563	11.28
Norephedrine	Decongestant, stimulant, anorectic agent	1	151	1.624	10.88
Etafedrine	Bronchodilator	1	193	1.478	11.92
Labetalol	High blood pressure	1	328	1.124	15.69
				1.098	16.05
Nadolol	Migraine, high blood pressure, chest pain	1	309	1.133	15.69
Codeine	Analgesic, antitusive	1	299	1.216	14.72
				1.188	15.10
Meloxicam	Analgesic, anti-inflammatory	1	351	1.110	16.06
Nimesulide	Analgesic, anti-inflammatory	10	308	–	–
Paracetamol	Analgesic	10	151	–	–
Acetylsalicylic acid	Analgesic	10	180	–	–
Ibuprofen	Analgesic	10	206	–	–
Ketoprofen	Analgesic	10	254	–	–
Hypoxanthine	–	10	136	1.716	10.40
Xanthine	Respiratory diseases	100	152	1.717	10.41
Teophylline	Respiratory diseases	100	180	–	–
Theobromine	Respiratory diseases	100	180	1.712	10.42
Caffeine	Stimulant	100	194	1.595	11.20
Saccharin	Sweetener	100	183	–	–
Aspartame	Sweetener	10	294	1.570	11.38
				1.450	12.31
				1.149	15.50
Vitamin B1	Vitamin	1	337	1.315	13.57
				1.269	14.07
				1.202	14.85
Vitamin B6	Vitamin	1	247	1.639	10.90

Briefly, the ionization cascade is initiated by the radioactive  $\beta$ -emitter  $^{63}\text{Ni}$ , and the ionization of compounds whose proton affinities are greater than that of the dopant gas is obtained. Luckily, most drug compounds exhibit proton affinities greater than the common dopants and are readily ionized. However, charge exchange reactions and competitive

ionization may result in complete or largely signal suppression of selected analytes when a mixture of components is present, especially when interferent concentrations become larger than that of the analytes [27].

The effect of different drugs as lidocaine and codeine, with  $t_d$  and proton affinities similar to those of the analytes,

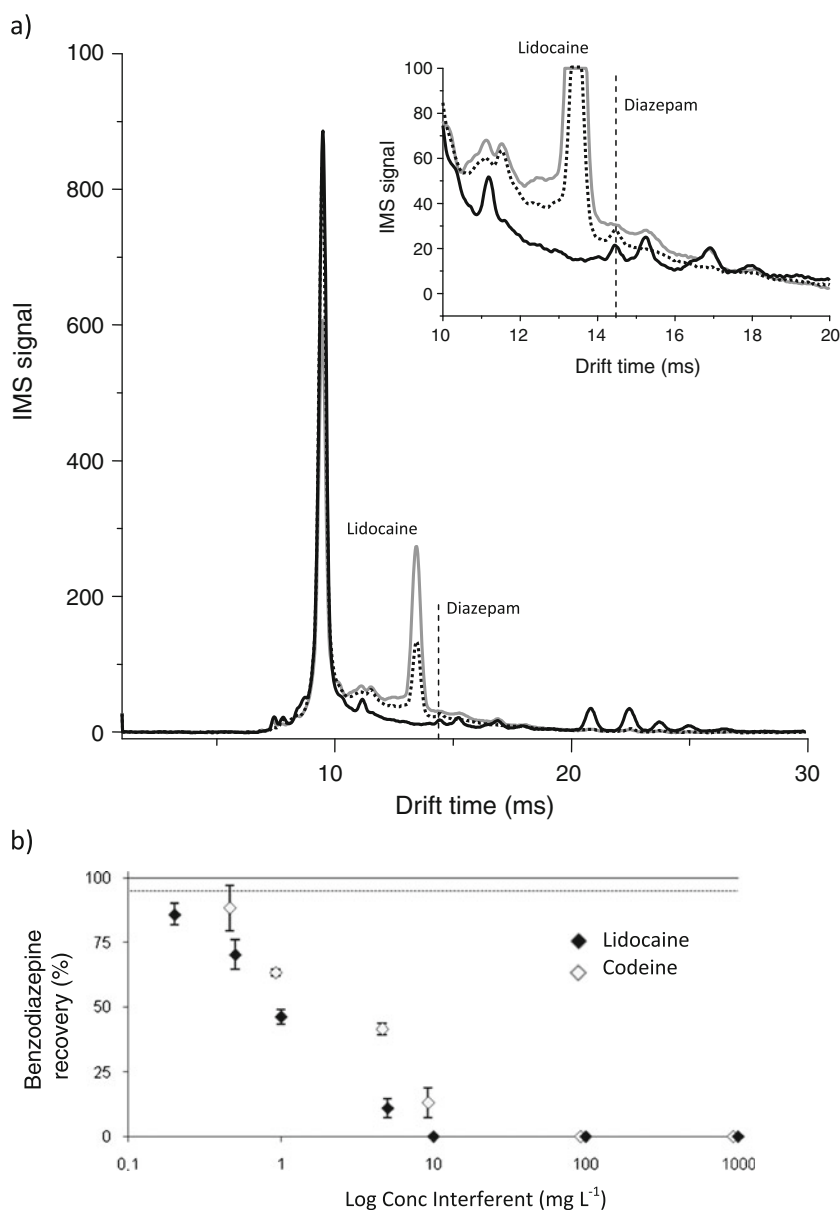
on the ionization of benzodiazepines has been evaluated. Those molecules have been selected for the interference study because they are present in common oral administered formulations such as syrup or throat lozenges where drug remain at high concentrations in the oral cavity during an acceptable period of time.

A single dose Strepsils<sup>®</sup>, containing 2 mg lidocaine, throat lozenge was administered to a male healthy volunteer, and two samples were taken for diazepam spiking, after 5 and 30 min drug administration, respectively. Diazepam was spiked at  $50 \mu\text{g L}^{-1}$  concentration level, and saliva samples were centrifuged for 5 min at 5,000 rpm. Figure 4a shows the plasmagrams obtained from the IMS analysis of the supernatant solution. As it can be seen, after 5 min lidocaine administration, its concentration in saliva is

extremely high, and diazepam signal is completely missing. However, lidocaine signal substantially decreased after 30 min, and benzodiazepine peak can be easily identified obtaining a recovery value of approximately 60%.

In order to evaluate the effect of the concentration of lidocaine on the IMS signal of diazepam, different binary mixtures of both analytes were prepared. The concentration of diazepam was fixed at  $100 \mu\text{g L}^{-1}$ , and lidocaine concentration was varied from 0.2 to  $1,000 \text{ mg L}^{-1}$ . As it can be seen in Fig. 4b, the IMS signal of diazepam is completely missing for concentrations of lidocaine higher than  $10 \text{ mg L}^{-1}$ , increasing diazepam recovery as the concentration of lidocaine decreases. The achieved recovery values for diazepam:lidocaine ratios of 1:2, 1:5, and 1:10 were  $86 \pm 4\%$ ,  $70 \pm 6\%$ , and  $46 \pm 3\%$ , respectively.

**Fig. 4** (a) IMS plasmagrams of diazepam spiked saliva at a concentration level of  $50 \mu\text{g L}^{-1}$  (black line), 5 min after administration of single dose throat lozenge containing 2 mg lidocaine (grey line), and 30 min after the administration of the drug (dashed line). (b) Effect of increasing concentration of lidocaine and codeine on benzodiazepine recoveries from spiked saliva at a concentration level of  $100 \mu\text{g L}^{-1}$



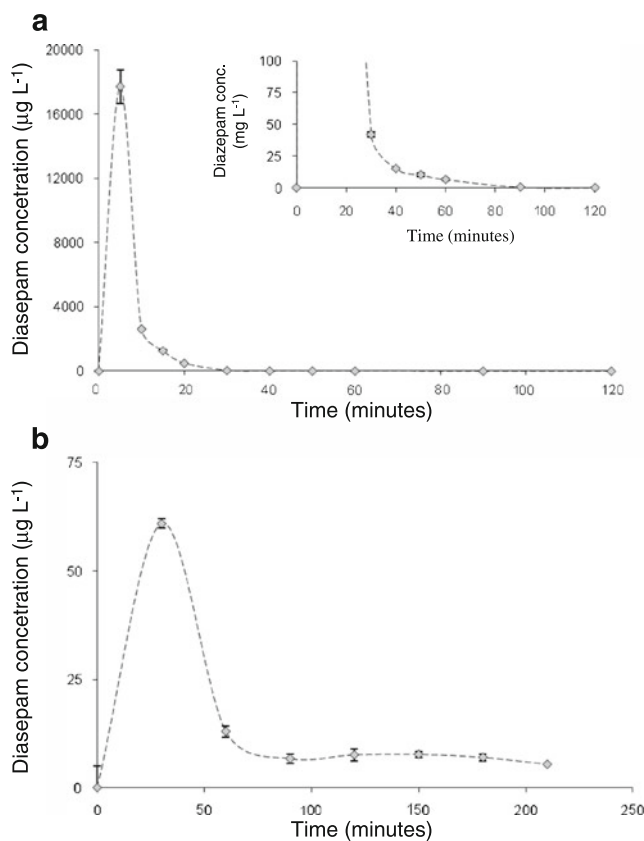
A similar interference study was performed with codeine, an active principle ingredient of cough syrups, and the results were depicted in Fig. 4b. As it has been aforementioned, codeine is an interferent by  $t_d$  of diazepam, and thus, to evaluate only the effect of competitive ionization of codeine, clorazepate dipotassium salt presenting a different  $t_d$  was selected. Once again, the IMS signal of clorazepate dipotassium salt is completely missing for concentrations of codeine higher than  $100 \text{ mg L}^{-1}$ , increasing benzodiazepine recovery as the concentration of codeine decreases. The achieved recovery values for clorazepate: codeine ratios of 1:5, 1:10, and 1:50 were  $88 \pm 9\%$ ,  $63 \pm 2\%$ , and  $42 \pm 2\%$ , respectively.

In summary, IMS spectrometers can saturate, depending on the drug and the operating conditions, at amounts ranging from a few nanograms to hundreds of nanograms, and centrifuged saliva samples with abnormally higher IMS signals different than those of the matrix and coming from analytes with different  $t_d$  than those reported for the benzodiazepines should be carefully analyzed. Probably, the best choice to avoid false negatives is to consider those samples as possible positives due to the aforementioned interference by competitive ionization and, in such cases, an alternative chromatographic analysis methodology must be used.

#### Analysis of single-dose diazepam intake

Diazepam concentration in saliva samples over time after acute dosage by sublingual administration has been depicted in Fig. 5a. In this strategy, the diazepam tablet placed over the tongue was allowed to dissolve in the mouth. Immediately after drug dissolution, high benzodiazepine concentrations have been found in saliva, till  $18 \text{ mg L}^{-1}$ , probably due to residual drug remaining in the oral cavity. Under these circumstances, results from benzodiazepine concentration in saliva cannot be correlated with drug concentration in blood. However, these transiently elevated concentrations improve the likelihood of detecting the drug and are helpful in some specific situations like driving tests. Moreover, the concentration of diazepam found in saliva after 90 min is practically negligible, probably due to diazepam has been removed from saliva in the two–three firsts samples, and the amount adsorbed by the body is significantly lower than the normal amount after 5 mg diazepam dose.

The time course of the drug in the saliva fluid after acute dose oral ingestion is depicted in Fig. 5b, obtaining a salivary drug concentration from 60 to  $5 \text{ } \mu\text{g L}^{-1}$  in the evaluated time range. Values obtained are comparable with those previously reported in the literature [25, 28] providing a similar time concentration profile, demonstrating the usefulness of the IMS-based methodology for the analysis



**Fig. 5** Mean±SD diazepam concentration in saliva over time after acute intake of 5 mg diazepam by (a) sublingual and (b) oral administration

of benzodiazepines in saliva samples with minimal sample preparation.

Salivary and plasma diazepam concentration levels have been correlated previously obtaining a time-dependent ratio varying between 0.017 and 0.035. It implies that the concentration of diazepam in plasma is substantially higher than that in saliva, being approximately 99% of diazepam in plasma protein bounded. Since the unbound fraction of the drug is in equilibrium with the site of action, salivary diazepam could be directly related to “active” diazepam in plasma.

In summary, the time range in which benzodiazepines could be detected in saliva by IMS is, of course, dependent on the dose administered, but taking into consideration the normal benzodiazepine levels in saliva after normal acute or chronic benzodiazepine administration, it could be detected during 0.5–10 h.

## Conclusions

The capability of IMS for the screening of drugs in human saliva has been demonstrated on the example of benzodiazepines. The methodology is simple, fast, and provides

appropriate precision and LOD values for the determination of benzodiazepines in saliva after single or chronic dose. Moreover, the resolution of overlapped peaks, such as those obtained in benzodiazepine mixtures, has been afforded by chemometric methods such as MCR. The obtained spectral profile match perfectly with the theoretical response of individual benzodiazepines, and concentration profiles agree well with the concentration of those benzodiazepines in the mixtures. The sample preparation is reduced to a centrifugation step which significantly removes the protein and solid in suspension content. The interferences caused by the sample matrix are reduced after the centrifugation step reducing at the same time the system maintenance regarding Teflon membrane replacement.

However, the IMS procedure also presents some drawbacks as it has been demonstrated by the possibility of false positives, due to the presence of interferences with the same  $t_R$ , and false negatives due to competitive ionization. Both types of interferences should be considered as positive samples and analyzed by a reference methodology based on a chromatographic separation. Those IMS interferences would be easily reduced by coupling of a short GC column prior to IMS detection or by using in-line a MS detector to provide structural information.

Finally, the application of the methodology has been successfully demonstrated on the example of the determination of diazepam in saliva as a function of time after single dose. The calculated concentration levels are comparable with those previously reported in the scientific literature.

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