

A mass spectrometry-based method for differentiation of positional isomers of monosubstituted pyrazine *N*-oxides using metal ion complexes

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A series of 11 pairs of substituted pyrazine *N*-oxides, differing in the substituent position, were examined using electrospray ionization mass spectrometry (ESI-MS) in order to use spectra to assess the differentiation of positional isomers. For each compound, mass spectra were recorded with three different metal cations, namely calcium (II), copper (II) and aluminum (III), with characterization of the observed peaks. Differentiation between regioisomeric *N*-oxides has been achieved by comparison of the identity and relative intensities of the peaks originating from the adduct ions formed with the metal ions. Principal component analysis (PCA) has been employed to assist in the interpretation of the results obtained with each metal ion, exploring possible trends according to the nature and position of the substituent in the pyrazine *N*-oxide. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: pyrazine *N*-oxides; positional isomers; electrospray; metal complexation; principal component analysis (PCA)

Introduction

Pyrazines represent an important class of heterocyclic compounds, which are synthetic intermediates for flavors and fragrances, pharmaceuticals and agrochemicals. The analysis and structural elucidation of these compounds are relevant, in the case of pharmaceuticals, for metabolic and mode of action studies, whereas in the case of additives, this analysis is generally performed in order to detect their presence in foods and to regulate their intake.

Determination of the relative position of the substituent in substituted pyrazine *N*-oxides, as well as in other diazines and their metabolic derivatives, is usually a quite difficult task by chemical or spectroscopic methods, and there is a lack of general methods that unambiguously identify regioisomers from a pair of isomeric *N*-oxides. One of the spectroscopic methods employed in identification of the relative position of the substituent is nuclear magnetic resonance (NMR) spectroscopy, by the measurement of coupling constants,^{[1][2]} or the use of multinuclear magnetic resonance.^{[3][4]} However, analysis by this technique is often limited by the low sensitivity and long acquisition times required for these experiments.

Isomer differentiation by means of mass spectrometry (MS) is usually challenging as isomers have identical molecular mass and frequently similar fragmentation pathways. Pyrazine *N*-oxides were systematically analyzed solely by electron ionization mass spectrometry (EI-MS) techniques,^{[5][6]} while most of the methodologies used nowadays employ atmospheric pressure ionization (API) techniques. As a consequence, the study of these isomers with softer ionization techniques which generate less energetic precursor ions becomes an interesting issue. For example, differentiation among *N*-oxides and hydroxylated isomers in mononitrogenated heteroaromatic molecules^[7] has been accomplished by the use of API, and the

fragmentation pathways of two di-*N*-oxides of commercial quinoxalines have been also studied.^[8]

Electrospray ionization (ESI) is a soft ionization technique able to transfer ions from solution to the gas phase. Metal ions of alkali, alkaline earth and transition metals have been extensively used in ESI for the study of many types of compounds, and were also suitable for the analysis of isomeric compounds by means of metal complexation strategies.^{[9][10][11][12]} For instance, two regioisomeric hydroxypyridine *N*-oxides^[13] could be differentiated with various metal cations by ion adduct generation using ESI-MS.

The present work extends the previous investigation on the dissociation behavior^[14] and metal complexation^[13] of two isomeric hydroxypyridine *N*-oxides by examining a larger set of regioisomeric pyrazine *N*-oxides with different substituents on the aromatic ring. Distinction between 2- and 3-substituted pyrazine *N*-oxides was studied employing the ESI mass spectra of each compound in the presence of different metal cations, i.e. calcium (II), copper (II) and aluminum (III), and characterization of the observed species. In order to get a full appreciation of the advantages of this technique, the results obtained by the classical approach are also shown, which is based on the comparison of the fragmentation observed by tandem

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mass spectrometry of the protonated molecules in the case of pairs of positional isomers.

Given the number of compounds analyzed, as well as the different metal ion adducts identified, the variance of the results was difficult to interpret in conventional ways. Exploratory data analysis and pattern recognition techniques^[15] are widely employed for the interpretation of such multivariate data sets, as they reduce the data complexity permitting the elucidation of characteristic features. In this work, principal component analysis (PCA)^[16] was used to explore possible trends for the classification of the behavior of the compounds, as well as rationalizing the differences observed (according to substituent nature and relative position) in the mass spectra obtained with each metal cation.

To the best of our knowledge, there are only a few studies in which PCA was used for the differentiation of isomers by mass spectrometry. In such cases, however, the analysis was applied to the relative abundances of the fragment ion peaks in the mass spectra obtained by electron ionization (EI),^{[17][18]} secondary ion mass spectrometry (SIMS)^[19] or ESI-CID.^[20] In the present work, PCA was applied to the relative abundances of the metal ion adduct peaks observed in the ESI mass spectra obtained in the presence of the different metal cations, instead of the abundance of the fragment ion peaks themselves.

Experimental

Chemicals

Synthesis and spectroscopic characterization of the compounds under study (Fig. 1) have been previously reported.^[21] LCMS grade methanol and water were purchased from Carlo Erba (Milan, Italy). The analyte solutions (1a-11a; 1b-11b) were prepared using methanol, each at a concentration of 10 mM. Metal aqueous ion stock solutions (10 mM) were prepared from AlCl₃, CaCl₂ and CuSO₄·5H₂O. A small excess of HCl (0.1 mM) was added to the stock solution of aluminum ion.

Mass spectrometry

Mass spectrometric analyses were performed using a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics, Billerica, MA, USA), equipped with an electrospray ion source. The instrument

was operated at a capillary voltage of 4.5 kV with an end plate offset of -500 V, a dry temperature of 180 °C using N₂ as dry gas at 4.0 l min⁻¹ and a nebulizer pressure of 0.4 bar.

Multi-point mass calibration was carried out using a sodium formate solution from *m/z* 50 to 900 in positive ion mode. Data acquisition and processing were carried out using Bruker Compass Data Analysis version 4.0 software supplied with the instrument.

For experiments involving the protonated molecules, 50 μl of a 10 mM ammonium acetate solution in methanol:water (50:50) was mixed with 50 μl of the solutions of each compound prior to direct infusion. For the experiments with metal ions, the metal solutions were mixed with solutions of each compound (2:1 v/v) prior to infusion into the mass spectrometer. Sample solutions were infused into the source using a KDS 100 syringe pump (KD Scientific, Holliston, MA, USA) at a flow rate of 180 μl h⁻¹.

Each experiment was performed in triplicate on different days in order to ensure reproducibility.

Data analysis

Principal component analysis (PCA) was applied to data matrices using MATLAB v. 7.0 (MathWorks Inc., Natick, MA) without preprocessing of the data set. A matrix was built for each metal ion, placing the peaks observed (variables) as columns and the compounds under study as rows (samples) (Tables S4–S6 in Supporting Information section). The relative intensity of the peaks observed was grouped into four ranks, i.e. 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: 75–100%.

Results and discussion

Fragmentation behavior of the protonated molecules

In an attempt to expand the applicability of the results previously found with a single pair of 2- and 3-hydroxypyridine *N*-oxides^[13] to a larger set consisting of 11 pairs of 2- and 3-substituted pyrazine *N*-oxides, a classical approach was first used to get data to compare with the new methodology for the potential discrimination between each pair of regioisomeric *N*-oxides. For this purpose, tandem mass spectra were recorded from the precursor ions [M+H]⁺ of compounds 1a-11a;1b-11b. Three aspects of the fragmentation behavior were considered, i.e. the main fragmentation pathways, the relative intensities of the product ion peaks and the relative stabilities of the selected precursor ions. The relative stability of the precursor ions [M+H]⁺ was inspected by collision induced dissociation (CID) by the calculation of *E*_{1/2} values,^[22] defined as the amount of the laboratory-frame collision energy required to attenuate the intensity of the precursor ion signal by 50%. This parameter was extracted from the dissociation curves by plotting the relative abundance of the precursor ion peak as a function of the collision energy corresponding to the lab frame. The *E*_{1/2} values obtained for the precursor ions [M+H]⁺ of compounds 1a-11a;1b-11b along with their main fragmentation pathways are summarized in Table 1.

The precursor ions of those compounds substituted with amino (2a;2b), carboxylic acid (5a;5b) and hydroxyl (6a;6b) groups were among the most stable among the whole family of studied substances, with a predominance of the precursor ion signal at all collision energies for compounds 5a, 6a and 6b. Therefore, the *E*_{1/2} values could not be obtained in these cases. As might be observed in Table 1, regioisomeric *N*-oxides of compounds substituted with methyl groups (3a;3b) could be differentiated by the difference in their *E*_{1/2} (8 eV) although both compounds showed the same

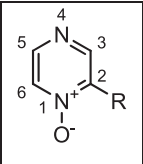
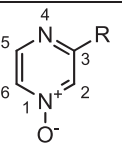
Substituent R		
Cl	1a	1b
NH ₂	2a	2b
CH ₃	3a	3b
COCH ₃	4a	4b
COOH	5a	5b
OH	6a	6b
OCH ₃	7a	7b
NHCOCH ₃	8a	8b
CHOHCH ₃	9a	9b
COOCH ₃	10a	10b
CONH ₂	11a	11b

Figure 1. Structures of the 2-substituted (a) and 3-substituted (b) pyrazine *N*-oxides studied.

Table 1. $E_{1/2}$ values obtained for the precursor ion signals $[M+H]^+$ of compounds 1a-11a;1b-11b along with their main fragmentation channels

N	Substituent R	2-Substituted <i>N</i> -oxide (a)		3-Substituted <i>N</i> -oxide (b)	
		$E_{1/2}$ (eV)	Main fragment. channel	$E_{1/2}$ (eV)	Main fragment. channel
1	Cl	14.5	-OH [•]	14.5	-OH [•]
2	NH ₂	22.0	-OH [•] /-H ₂ O	20.0	-OH [•] /-H ₂ O
3	CH ₃	15.0	-OH [•] /-H ₂ O	23.0	-OH [•] /-H ₂ O
4	COCH ₃	14.0	-CH ₂ CO	17.0	-CH ₂ CO
5	COOH	^a	-H ₂ O	25.0	-H ₂ O-CO
6	OH	^a	-OH [•]	^a	-OH [•]
7	OCH ₃	14.0	-OH [•] /-H ₂ O	14.0	-OH [•] /-H ₂ O
8	NHCOCH ₃	7.5	-CH ₂ CO	9.0	-CH ₂ CO
9	CHOHCH ₃	12.0	-OH [•] /-H ₂ O	9.0	-H ₂ O/-2H ₂ O
10	COOCH ₃	11.5	-CH ₃ OH + H ₂ O	11.0	-CH ₃ OH + H ₂ O
11	CONH ₂	10.5	-NH ₃ + H ₂ O	10.5	-NH ₃ + H ₂ O

^aThe relative intensity of the selected precursor ion signal remained unchanged.

dissociation pathways. Likewise, regioisomeric *N*-oxides of compounds substituted with carboxyl (5a;5b) or 1-hydroxyethyl (9a;9b) groups could be differentiated since each isomer displayed a distinctive fragmentation pathway. In the remaining cases, each pair of positional isomers exhibited the same main fragmentation pathway as well as very similar $E_{1/2}$ values (Table 1), thus preventing their differentiation by this approach.

As an example, the MS/MS spectra obtained by CID at 15 eV for the precursor ions $[M+H]^+$ of compounds 4a;4b, 7a;7b and 10a;10b are presented in Fig. 2. Generally, CID spectra of $[M+H]^+$ ions of the compounds under study shared some common features showing peaks corresponding to the loss of hydroxyl radical or water, as observed for compounds 7a;7b (Fig. 2). This behavior had been previously reported in CID studies of hydroxypyridine

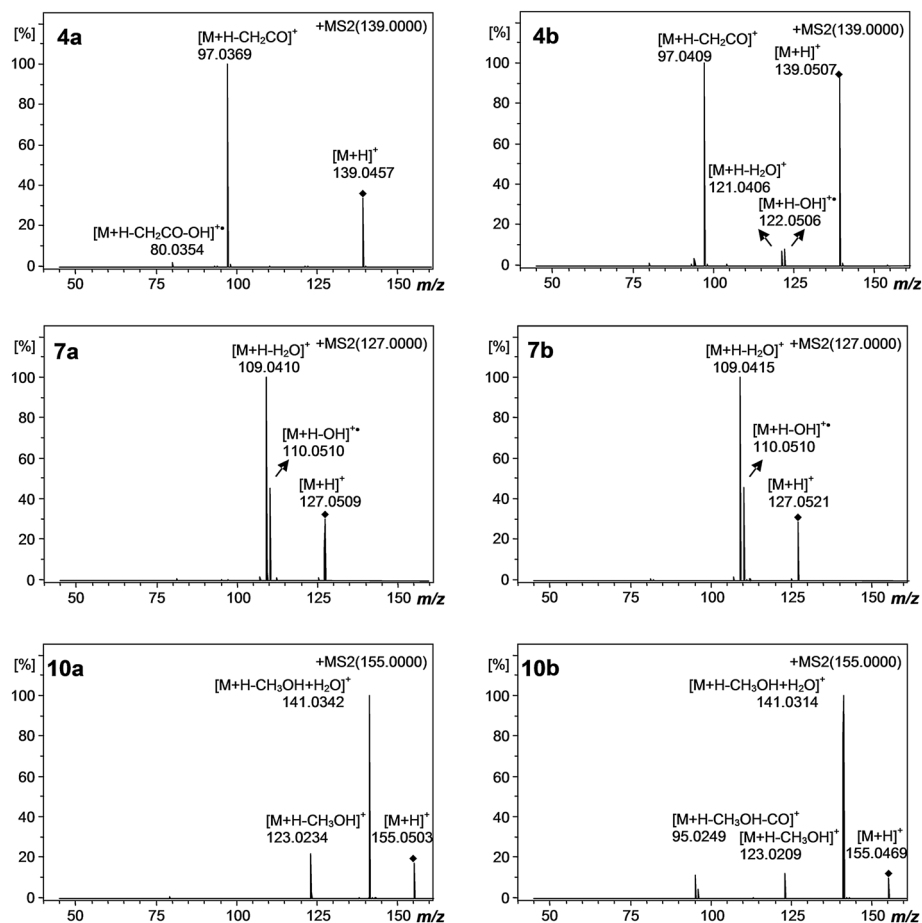


Figure 2. ESI-MS/MS spectra of protonated precursor molecules $[M+H]^+$ from the *N*-oxides of 2-acetylpyrazine (4a) and 3-acetylpyrazine (4b); 2-methoxypyrazine (7a) and 3-methoxypyrazine (7b); 2-methoxycarbonylpyrazine (10a) and 3-methoxycarbonylpyrazine (10b) with collision energy of 15 eV.

N-oxides.^[14] Nevertheless, other fragmentation pathways were observed in structures containing a carbonyl moiety. Similarly, other 'prevailing' fragmentation pathways were observed in tandem mass spectrometry experiments involving the protonated molecule of quinoline derived *N*-oxides^[8] by electrospray, when the heterocyclic ring had substituents containing a carbonyl group. In such cases, hydroxyl radical loss, although characteristic of the *N*-oxide group, was not the only the primary fragmentation process.

For compounds having an acetyl group (4a;4b and 8a;8b), ketene ($\text{H}_2\text{C}=\text{C}=\text{O}$) loss was the predominant fragmentation pathway. This neutral loss has also been reported as the only fragmentation process in CID experiments of *N*-alkylacetyl-4-(dimethylamino)pyridinium.^[23] On the other hand, for carboxylic acid derivatives (10a;10b and 11a;11b), species obtained by hydrolysis of the carboxyl derivative (loss of methanol or ammonia and gain of water) were observed in the tandem mass spectra (Fig. 2). Under ESI conditions employing QIT or FT-ICR analyzers, it was found that acylium fragment cations may associate with residual solvent molecules (like water or methanol), giving rise to unexpected artifacts (acid derived fragment ions).^[24] For example, in the CID studies of protonated isoquinoline-3-carboxamide,^[25] product ions eliminating nominal fragments of 11 u were observed, which were attributed to a neutral loss of CH_2NH (-29 u) and water addition ($+18$ u) to the resulting acylium cation to form the respective protonated carboxylic acid product ion. Water addition to fragment ions was also inferred in Q-TOF hybrid analyzers; although in this case the origin was attributed to adsorption of atmospheric water to the stainless steel tubing connected to the collision cell.^[26]

When considering the lower intensity fragment ion peaks of the compounds whose main fragmentation channel was not the loss of hydroxyl radical or water, the presence of some useful species to differentiate both isomers was noticed. For example, 3-substituted pyrazine *N*-oxides could be differentiated by the characteristic fragment ion peaks only observed for these positional isomers, involving water loss $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$ for acetyl (4b), and methanol and CO loss $[\text{M} + \text{H} - \text{CH}_3\text{OH} - \text{CO}]^+$ for methoxycarbonyl (10b) substituents (Fig. 2). Likewise, the product ion peak involving water loss was only observed in the 3-substituted regioisomer for *N*-acetyl (8b). Besides, the product ion peak $[\text{M} + \text{H} - \text{NH}_3 - \text{CO}]^+$ was observed in the 3-substituted positional isomer of the compound with a carbamoyl group (11b). Similarly, in the 3-substituted compound with a carboxyl group (5b), the fragment ion for the CO loss from the acylium ion $[\text{M} + \text{H} - \text{H}_2\text{O} - \text{CO}]^+$ was the main fragmentation channel too, besides being useful for the differentiation between isomers.

The loss of the oxygen atom from the molecular ion of 2- and 3-substituted pyrazine *N*-oxides under EI-MS conditions has been noted previously,^[5] and the comparison of the relative intensity of this fragment ion peak and the one involving hydroxyl radical loss showed differences between some of the regioisomeric *N*-oxides. However, in the present work, only small differences were found in the relative intensities of the fragment ion peaks (hydroxyl radical and water losses) from the precursor protonated molecules between the regioisomeric *N*-oxides of the compounds substituted with amino (2a;2b) or hydroxyl (6a;6b) groups, whereas the compounds substituted with chloro (1a;1b) or methoxyl (7a;7b) groups were undistinguishable by this method.

Taking into account the aforementioned results, differentiation was achieved for seven pairs of regioisomeric *N*-oxides, but not for the other four pairs. The analysis of the fragmentations by ESI-CID included the protonated molecule stability upon dissociation measured by the value of the parameter $E_{1/2}$, useful for the compounds

with methyl (3a;3b) and carboxyl (5a;5b) groups; the presence of a distinctive main fragmentation pathway for compounds (5a;5b) and (9a;9b) and of characteristic fragment ions in the 3-substituted positional isomers of compounds with a carbonyl group (4b, 5b, 8b, 10b and 11b).

However, it becomes evident that a more effective strategy is necessary to improve the differentiation of the pair of isomeric hydroxypyridine *N*-oxides. For this reason, metal complexation strategies, which are the main objective of this work, were subsequently tested for the same set of compounds.

Metal complexation MS

Strategies based on the formation of adduct ions with metal cations were assessed to differentiate the regioisomeric pyrazine *N*-oxides by the analysis of ESI mass spectra. Mass spectra of freshly prepared mixtures of each compound (Fig. 1) with different metal cation solutions (calcium(II), copper(II) and aluminum(III)) were registered using ESI. The number and identity of characteristic species as well as the relative intensity of the peaks were examined. The peaks observed included the protonated molecules of the isomeric substituted pyrazine *N*-oxides 1a-11a; 1b-11b, as well as singly or doubly charged ion adducts, formed from the divalent or trivalent metal cation and the ligands in their neutral or deprotonated forms.

Most of the compounds studied showed no evidence of decomposition products in their mass spectra in presence of the metal ions. Nevertheless, in certain cases the product ion peaks previously observed in the MS/MS spectra were noticed. For example, for the *N*-oxides of 2- and 3-acetamidopyrazine 8a and 8b, peaks associated with ketene loss were noticed in coincidence with the less energetic fragmentation pathway of the protonated molecules. On the other hand, the mass spectra of the *N*-oxides of 2- and 3-carboxypyrazine 5a and 5b showed peaks where the ion adducts with calcium and copper contained the decarboxylated ligand. The decarboxylation of copper(II) and calcium(II) pyrazincarboxylates has been reported under pyrolysis conditions.^[27]

The most abundant ion peaks observed in the mass spectra are summarized in Table 2 whereas the whole number of ion peaks characterized in the mass spectra are summarized in Tables S1–S3 in Supporting Information, containing the identified ion adducts with each metal ion along with their observed m/z values and errors in ppm. The confirmation of the assignments was made on the basis of the accurate m/z ratios, by tandem mass spectra obtained by CID and by the analysis of the characteristic isotopic patterns, in the case of copper and the chloride counter-ion. The representative mass spectra registered for each compound with the different metal ions can be found in Figures S1–S10 in the Supporting Information section. A few examples are shown in Fig. 3 to illustrate some of the main features.

As can be seen in Table 2, the protonated molecule was frequently the most abundant ion peak observed in the ESI mass spectra of the studied compounds with metal ions, more often for the 3- than the 2-substituted *N*-oxides. The main metal ion adducts formed with calcium(II) were singly charged ions, either containing a deprotonated ligand like $[\text{M} + \text{Ca} - \text{H}]^+$ or with chloride attached, $[\text{2M} + \text{Ca} + \text{Cl}]^+$ (mostly observed for 2-substituted *N*-oxides) as well as doubly charged ions, such as $[\text{2M} + \text{Ca} + n\text{H}_2\text{O}]^{2+}$ observed for both regioisomeric *N*-oxides.

Unlike calcium(II), in the case of copper(II) a lower number of doubly charged species (mainly $[\text{2M} + \text{Cu}]^{2+}$) were detected, and singly charged cations resulted not only from a deprotonated ligand, such as $[\text{M} + \text{Cu} + \text{H}_2\text{O} - \text{H}]^+$ which was observed for some

Table 2. Summary of the most abundant ion peaks (relative intensities higher than 75%) observed in the ESI mass spectra of compounds 1a-11a; 1b-11b with the metal cations studied

N	Substituent R	Calcium(II)			Copper(II)			Aluminum(III)		
		2-Substituted N-oxide a	3-Substituted N-oxide b	2-Substituted N-oxide a	3-Substituted N-oxide b	2-Substituted N-oxide a	3-Substituted N-oxide b			
1	Cl	$[2M + Ca + 2H_2O]^{2+}$	$[M + H]^+$ $[2M + Ca + 2H_2O]^{2+}$ $[2M + Ca + 2H_2O]^{2+}$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	
2	NH ₂	$[M + Ca - H]^+$	$[2M + Ca + 2H_2O]^{2+}$ $[2M + Ca + 2H_2O]^{2+}$	$[M + Cu + H_2O - H]^+$	$[M + H]^+$	$[M + H]^+$	$[2M + Al - 2H]^+$	$[M + H]^+$ $[2M + Al + 2Cl]^+$		
3	CH ₃	$[2M + Ca + 2H_2O]^{2+}$	$[2M + Ca + 2H_2O]^{2+}$	$[M + Cu + H_2O - H]^+$	$[M + Cu + H_2O]^{2+}$	$[M + Cu + H_2O]^{2+}$	$[M + H]^+$	$[M + H]^+$ $[2M + Al + 2Cl]^+$		
4	COCH ₃	$[2M + Ca + H_2O]^{2+}$	$[M + H]^+$ $[2M + Ca + 2H_2O]^{2+}$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$		
5	COOH	$[M + Ca + H_2O - H - CO_2]^+$	$[M + H]^+$ $[M + Ca + H_2O - H - CO_2]^+$	$[M + Cu + H_2O - H - CO_2]^+$	$[M + Cu + H_2O - H - CO_2]^+$	$[M + Cu + H_2O - H - CO_2]^+$	$[M + H]^+$	$[M + H]^+$		
6	OH	$[M + Ca - H]^+$	$[M + H]^+$	$[M + Cu + H_2O - H]^+$	$[M + Cu + H_2O - H]^+$	$[M + Cu + 2H_2O - H]^+$	$[2M + Al - 2H]^+$ $[2M + Al + H_2O - 2H]^+$ $[2M + Al + 2Cl]^+$	$[M + H]^+$		
7	OCH ₃	$[2M + Ca + 2H_2O]^{2+}$	$[2M + Ca + 2H_2O]^{2+}$ $[3M + Ca + H_2O]^{2+}$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H - COCH_3]^+$	$[M + H]^+$		
8	NHCOCH ₃	$[M + Ca - H]^+$	$[M + H]^+$	$[M + Cu + H_2O - H]^+$	$[M + Cu + H_2O - H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$		
9	CHOHCH ₃	$[2M + Ca + Cl]^+$	$[M + H]^+$	$[M + Cu + H_2O]^{2+}$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$		
10	COOCH ₃	$[2M + Ca + H_2O]^{2+}$	$[2M + Ca + 2H_2O]^{2+}$	$[2M + Cu]^{2+}$	$[2M + Cu]^{2+}$	$[2M + Cu]^{2+}$	$[M + H]^+$	$[M + H]^+$		
11	CONH ₂	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$	$[M + H]^+$		

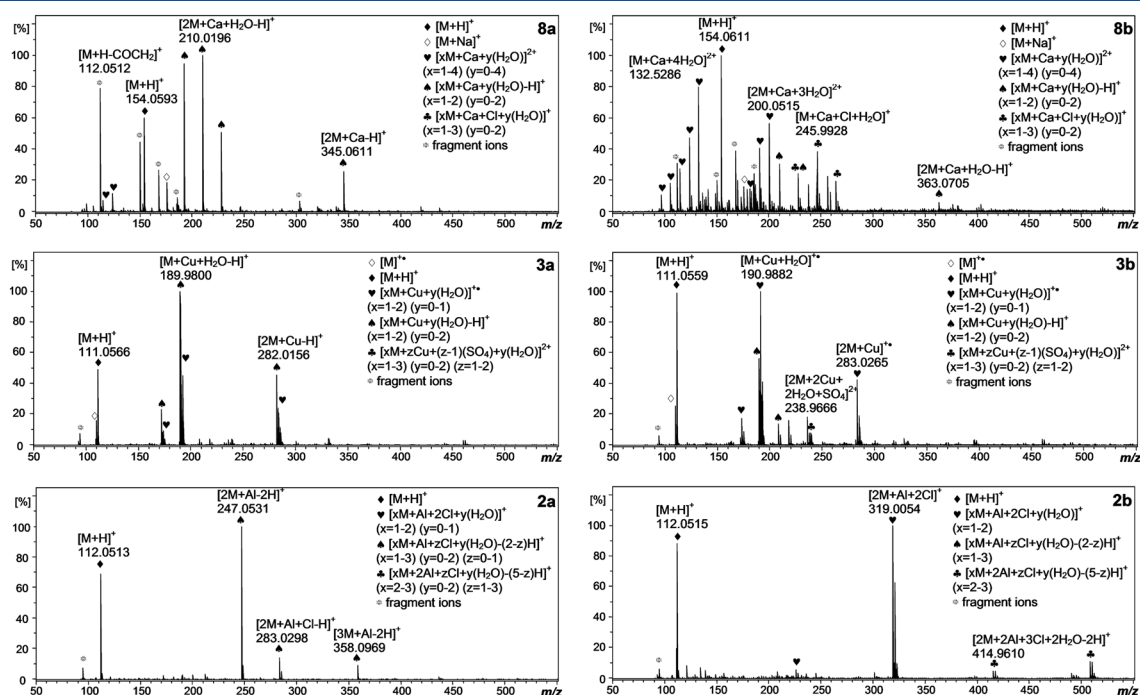


Figure 3. ESI mass spectra recorded for produced metal ion adducts of the regioisomeric *N*-oxides of a) 2-acetamidopyrazine 8a and 3-acetamidopyrazine 8b with calcium (II); b) 2-methylpyrazine 3a and 3-methylpyrazine 3b with copper (II); c) 2-aminopyrazine 2a and 3-aminopyrazine 2b with aluminum (III).

2-substituted *N*-oxides, but also from reduction of Cu(II) to Cu(I), which is known to occur easily during the ESI process.^[28] It has been reported^[29] that these species may be produced by dissociation reactions of adduct ions of Cu(II) generating as well the molecular radical cation (M^+), which was also identified for some of the studied compounds.

The key feature of the mass spectra obtained with aluminum(III) was the presence of only one or two predominant species for each compound, unlike with the other metals. These species were the protonated molecule and/or singly charged adducts made up of two ligands attached to the metal cation, either deprotonated as $[2M + Al - 2H]^+$ (only observed with certain 2-substituted *N*-oxides) or with chloride attached, like $[2M + Al + 2Cl]^+$ (observed for both regioisomers).

Adduct formation with metal ions allowed the clear differentiation between positional isomers by the comparison of either the different species observed in their mass spectra or the differences in the relative intensities of common peaks. Among the studied metal ions, calcium(II) afforded the best global results because of the large number of characteristic species observed. Copper(II) and aluminum(III) produced simpler mass spectra, showing a decrease in the number of characteristic adduct ion peaks for a given isomer in most of the cases.

PCA was used to identify the most relevant species related to the structural differences for each of the metal cations among the 22 compounds analyzed within this study. From the set of mass spectra obtained with calcium(II), copper(II) and aluminum(III), the ion peaks with abundances (relative intensities) larger than 5% were extracted, hence considering 26, 17 and 19 variables, respectively. The relative intensities were ranked into four groups so as to simplify the variability of the different replicates. The first two principal components obtained upon applying PCA to the data matrices built by this procedure described at least 80% of the variance in the data obtained with copper(II) and aluminum(III), whereas in the case of calcium(II) a variance of 75% was reached.

The data matrices used as inputs for this analysis can be found in Tables S4–S6 in Supporting Information.

Plots of the first two principal components were employed to assist in the interpretation of the results from PCA. The grouping (or separation) between pairs of regioisomeric compounds as well as among compounds containing different substituents may be visualized with the help of the scores plot. The position of the points in this plot can provide information about similarities or differences among the samples. The identification of the variables responsible for discrimination among the samples can be accomplished by analysis of the loadings plot, where it is possible to estimate how much a single variable contributes to each principal component.

With the aim of simplifying the visual interpretation of the PCA loading plots, a numerical code was used to identify the ion species (variables). The first digit denotes the ion charge (either 1 or 2) whereas the following ones refer to the stoichiometric ratios in the adduct ion of the ligand compound, metal ion, water molecules and metal counter-ion, respectively. This may be illustrated in the case of a generic adduct ion $[(M)_b(Z)_c(H_2O)_d(X)_e]^{j+}$, whose code would be *abcde*, where M represents the compound (1a-11a; 1b-11b); Z, the metal cation (calcium(II), copper(I/II) or aluminum(III)) and X, the metal counter-ion (chloride or sulfate). The presence of deprotonated species may be recognized through the numerical code by balancing the ion charge (first digit) against the metal and counter-ion charges. In the particular case of copper, where two oxidation states were actually observed, a * was added in the numerical code of Cu(I) species to avoid ambiguity and to differentiate adduct ions containing Cu(I) from those with Cu(II) and a deprotonated ligand.

The scores and loadings plots obtained upon applying PCA to the data set of mass spectra obtained with calcium(II) are shown in Fig. 4. The analogous plots obtained with copper(II) and aluminum(III) are shown in Figures S11 and S12, respectively, in the Supporting Information section. As can be seen in Fig. 4a), most

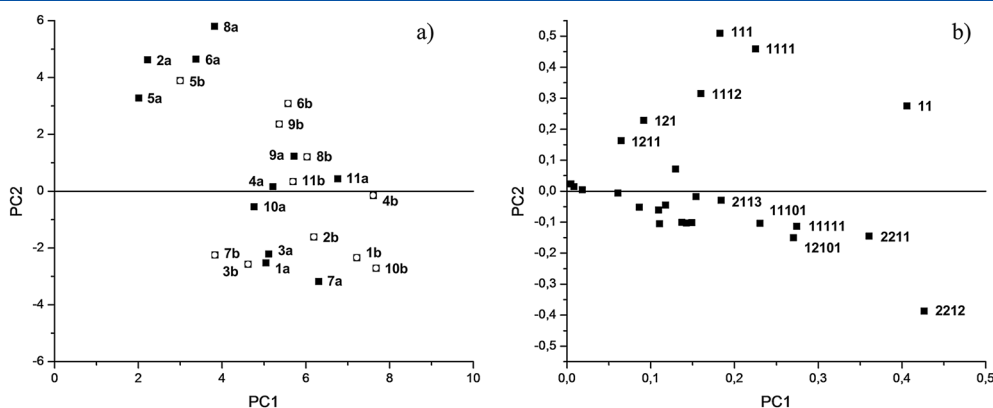


Figure 4. PCA analysis of the data obtained for produced metal ion adducts of the regioisomeric pyrazine *N*-oxides (1a-11a;1b-11b) with calcium (II). a) scores plot; b) loadings plot (see the text for the numerical code interpretation).

of the pairs of regioisomeric compounds appear separated in the plot suggesting they can be differentiated. The examination of the loadings plot of Fig. 4b) allows the study of the origin of these differences. The first principal component (PC1) has all its loadings of the same sign, with the frequently most abundant variables having the highest values, as is suggested by the comparison between the identity of these variables and their relative intensities (Table 2). On the other hand, the second principal component (PC2) contrasts the occurrence of deprotonated ligands among the variables, having this feature the variables with positive loadings (except the protonated molecule). The same interpretation of the principal components meaning remained valid with copper(II) and aluminum(III) data sets.

The samples distributed in the upper half of the scores plot show a predominance of singly charged ion adducts containing a deprotonated ligand whereas for those located in the lower half of the plot, the neutral ligand prevails in the adduct ions with the metal. Taking the *N*-oxides of 2- and 3-hydroxypyrazine 6a and 6b

as an example (Fig. 5), analog results were observed with the three metal cations studied. A predominance of singly charged ion adducts was found, with a deprotonated ligand for the 2-substituted *N*-oxide whereas the protonated molecule and adduct ions with ligands in their neutral form predominated in the case of the 3-substituted *N*-oxide. Other regioisomeric pyrazine *N*-oxides including substituents with acidic protons like the hydroxyl group, amino group (*ortho* to a nitrogen heteroaromatic atom), carboxyl and *N*-acetyl shared this same behavior.

A similar complexation behavior has been reported previously for analogous ligands, namely 2- and 3-hydroxypyridine *N*-oxides^[13] with the same metal cations. Hence, differences between the two regioisomers had been established, considering the ability of the isomer substituted in position 2- to form stable chelates acting as a bidentate ligand upon deprotonation, thereby forming mainly singly charged ion adducts. In contrast, its positional isomer substituted in position 3- showed no evidence of deprotonated forms in its ion adducts and exhibited doubly charged or singly charged ion peaks

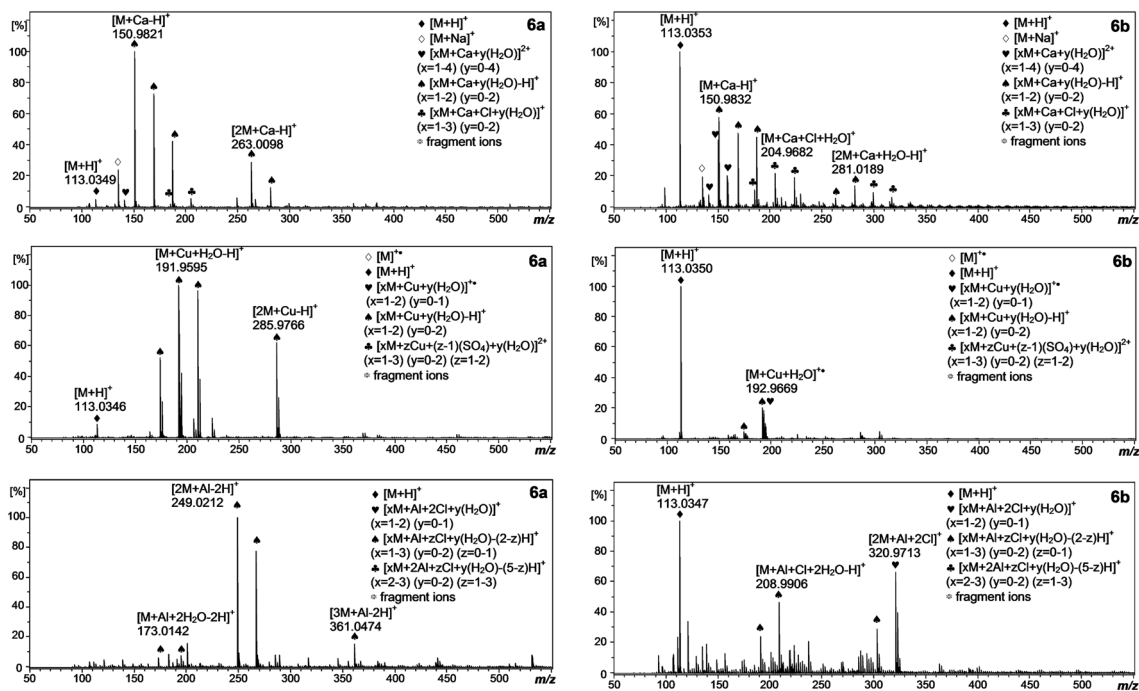


Figure 5. ESI mass spectra recorded for produced metal ion adducts of the regioisomeric *N*-oxides of 2-hydroxypyrazine 6a and 3-hydroxypyrazine 6b with the metal ions, top: calcium (II), middle: copper (II) and bottom: aluminum (III).

with the metal counter-ion attached, depending on the metal ion, besides a higher preponderance of the protonated molecule.

The above mentioned argument might be used to explain the differences observed in the mass spectra obtained with metal cations for the pairs of regioisomeric pyrazine N-oxides 2a;2b, 5a;5b, 6a;6b and 8a;8b. For the remaining compounds, the differences were not easily explained as substituent effects. Nevertheless, for each pair of regioisomers of the study set of compounds, a good differentiation was obtained for at least one metal ion.

Conclusions

This work aimed to distinguish between positional isomers in substituted pyrazine N-oxides on the basis of the complexation of these ligands with calcium(II), copper(II) and aluminum(III) employing electrospray mass spectrometry. Prior to this work there were no available methodologies that allow the differentiation of isomeric 2- and 3-substituted N-oxides by mass spectrometry.

The classic approach involving the fragmentation behavior of the protonated molecules within the set of isomeric 2- and 3-substituted pyrazine N-oxides showed fragmentation pathways which were dependent on the substituent nature. The regioisomeric compounds could be distinguished only in certain cases, on the basis of the main fragmentation channels, the intensity ratio between the precursor and primary fragment ion peaks and the relative stabilities of the selected precursor ions.

However, upon electrospray generation of adduct ions with the metal cations, differences are found between each pair of regioisomeric compounds highlighting the usefulness of metal complexation strategies. The differences arise mainly from the nature of the substituent, and also to a lesser extent, from its position relative to the N-oxide; the latter is the result of the interaction between the substituent and the metal cation. The application of PCA assisted in the detection of some of the differences observed through graphical representations, clustering the compounds on the basis of the observed ion species. Hence, some useful relationships between the state of the ligand in the metal ion adduct peaks observed in the mass spectra and the positional isomer of the N-oxide moiety can be established when the substituent contains a functional group capable of being deprotonated.

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