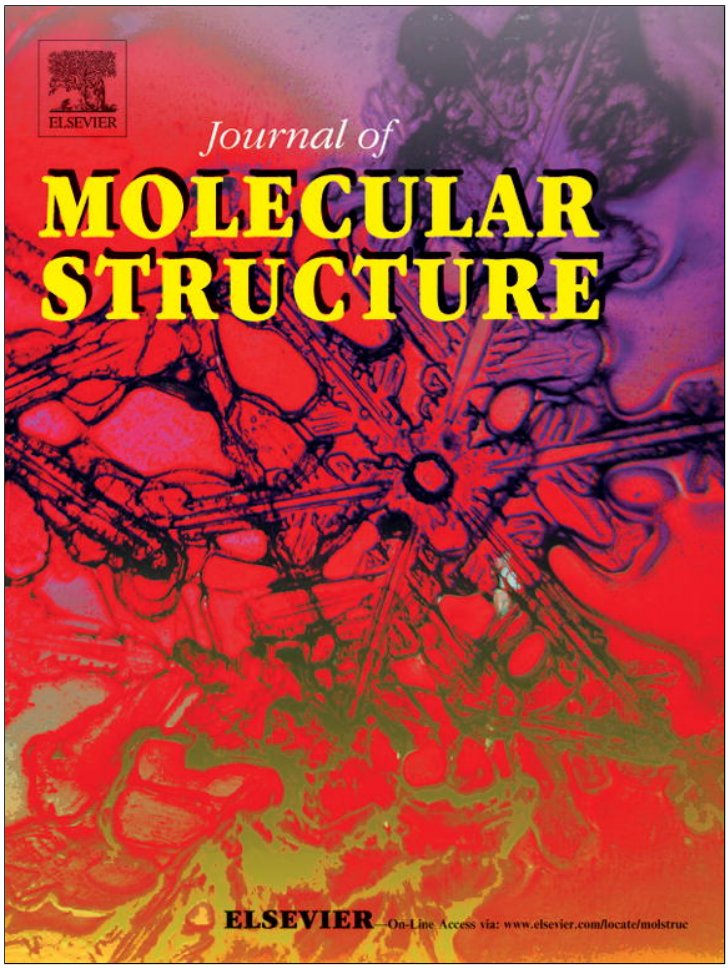

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Determination of the position of the *N*-O function in substituted pyrazine *N*-oxides by chemometric analysis of carbon-13 nuclear magnetic resonance data

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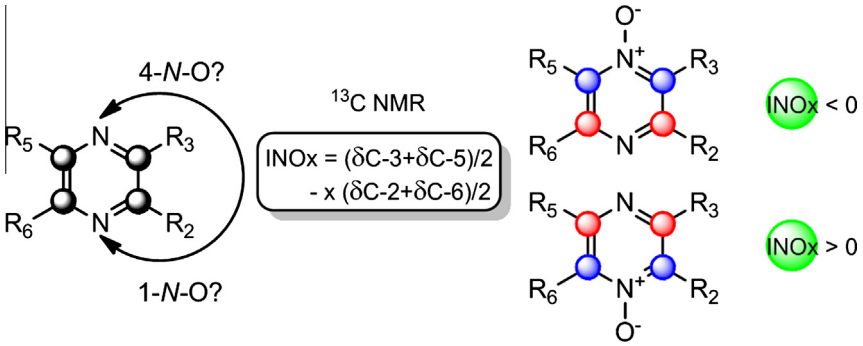
HIGHLIGHTS

- ^{13}C NMR data was used to identify the *N*-oxide position in substituted pyrazines.
- Chemometric techniques were applied to analyze the ^{13}C NMR chemical shifts data set.
- An index of *N*-oxidation whose sign reflects the *N*-oxide position was proposed.
- The index is computed simply contrasting average ^{13}C NMR chemical shifts.
- The substituent effect was pondered through a factor related to Hammett constants.

GRAPHICAL ABSTRACT



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ABSTRACT

Investigations were carried out applying NMR spectroscopy for the unambiguous determination of the position of the *N*-oxide function in a set of 2-substituted pyrazine *N*-oxides synthesized in our group. Applying chemometric techniques of multivariate analysis to the ^{13}C NMR chemical shifts data set, useful relationships for identifying the position of the *N*-oxide group relative to the substituent were unraveled. The relationships obtained were rationalized in terms of the molecular structures and refined. As a result, an index of *N*-oxidation (INO_x) was defined, computed simply contrasting the average ^{13}C NMR chemical shifts of each pair of carbon atoms bonded to a nitrogen atom. The effect of the substituent was included through a factor x (subscript of INO) close to unity, multiplying the average containing the substituted carbon atom. The approach was successful in recognizing the position of the *N*-oxide in all the cases studied, as revealed by the sign of INO_x (positive for 1-*N*-oxides and negative for 4-*N*-oxides). The scope of the methodology was further tested using the ^{13}C NMR chemical shifts of disubstituted pyrazine *N*-oxides from the literature data.

1. Introduction

Pyrazine *N*-oxides are heteroaromatic rings with two nitrogen atoms in 1- and 4-positions in which one of them may appear oxidized (Fig. 1). Pyrazines are ubiquitous in nature [1] displaying several biological activities [2]. Besides, they have been detected in heated foods as well as in fresh foods. Synthetic pyrazines are used as additives in food manufacture [3] and some of them are important pharmaceuticals such as the antituberculotic pyrazinamide [4]. Pyrazine *N*-oxides such as the antibiotic aspergillic acid [5], and the antimicrobial pigment pulcherrimin [6] are synthesized by several microorganisms including fungi and bacteria whereas acipimox is a niacin analog used as a hypolipidemic synthetic drug [7].

The final structure confirmation of an unknown organic compound is always performed with a set of independent methods

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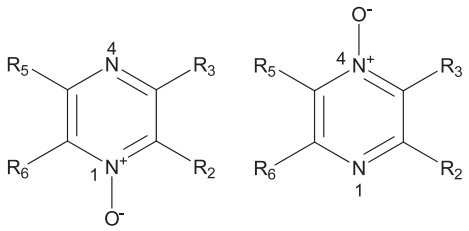

Fig. 1. General structure of pyrazine *N*-oxides.

such as one- (1D) and two-dimensional (2D) nuclear magnetic resonance spectroscopy (NMR) or X-ray crystallography as well as other spectroscopic methods. The term structure elucidation usually refers to full *de novo* structure identification, and it results in a complete molecular connection table with correct stereochemical assignments. Such an identification process without any assumptions or pre-knowledge is commonly the domain of nuclear magnetic resonance spectroscopy.

For instance, the substitution pattern of di- and tri-substituted pyrazines can be elucidated by a combination of NMR methods [8]. Nonetheless, when it comes to the final structure assignment of substituted pyrazine *N*-oxides, an important analytical task is determining which one of the two ring nitrogen atoms is actually oxidized, which is usually quite difficult to solve by chemical or other spectroscopic methods.

In connection with this, the employment of multinuclear magnetic resonance [9] including ^{14}N and ^{15}N NMR spectroscopy may provide unambiguous assignments of the *N*-oxide function [10]. However, the analysis of *N*-containing organic compounds by ^{14}N and ^{15}N NMR techniques is often hindered by the low sensitivity of these nuclei and the signal overlap of chemical shifts which complicate NMR spectral interpretation [11]. Particularly, the differences observed between the ^{15}N chemical shifts of the two nitrogens of methylpyrazines 1- and 4-*N*-oxides were less than 1 ppm in some of the ^{15}N NMR spectra reported [12].

Interestingly, a substituent effect on the ^{15}N chemical shifts of substituted pyrazines and their *N*-oxides has been observed when the substituent is *ortho* to the ring nitrogen [13]. This effect was related to the effect that an identical substituent has on the ^{13}C chemical shifts of the *ortho* carbon in benzene. Moreover, ^{15}N chemical shifts were shown to be among the most sensitive nuclei to the substituent effects in substituted pyridine *N*-oxides [14].

On the other hand, it was noticed that the ^{13}C chemical shifts assigned to the *ortho* carbons to the *N*-oxide group were upfield shifted (~10 ppm) respect to the parent pyrazine compounds in the ^{13}C NMR spectra of alkyl- and phenylpyrazine *N*-oxides [15]. Hence, if this effect of the *N*-oxide group could be employed in other substituted pyrazine *N*-oxides through the proposal of an index not greatly influenced by the nature of the substituents, the need of having both isomeric *N*-oxides and performing nitrogen NMR experiments for the unambiguous determination of the *N*-oxide position would be thus avoided.

Additionally, the measurement of long-range proton carbon coupling constants (^nJ(C,H); $n > 1$) may serve as well to distinguish the position of the *N*-oxide. For example, in the case of 2-methylpyrazine *N*-oxides [15], it has been reported that ^3J(C,H) values across the *N*-oxide group are markedly decreased to ~1.0 Hz while the values across the non-oxygenated nitrogen atom are increased to 12–13 Hz. Nevertheless, many discussions have focused on the accuracy, reliability and simplicity of the data analysis and determination of the ^nJ(C,H) coupling constants [16]. Moreover, long acquisition times in the NMR experiments are imposed by the requirement of high resolution for the measurement of their small values (usually 1–10 Hz). Therefore, taking into account the former drawbacks, a simpler and faster alternative is desirable.

Herein, we present our investigations on ^{13}C NMR spectroscopy of a set of 2-substituted pyrazine 1-oxides and their isomeric 4-oxides (Fig. 1) with 11 different substituents (chloro, amino, methyl, acetyl, carboxyl, hydroxy, methoxy, acetamido, 1-hydroxyethyl, methoxycarbonyl and carbamoyl) synthesized from commercial pyrazines. The resulting study set of 22 molecules differ in the aromatic ring substituent and the identity of the oxidized nitrogen atom. Once unambiguously assigned the carbon atoms and the position of the *N*-oxide group for each compound of the set using 2D NMR techniques, the ^{13}C chemical shifts data set was analyzed by the chemometric technique of Principal Component Analysis (PCA) [17] to explore the substituent effect.

The application of PCA to spectroscopy in general and to NMR spectroscopy in particular, has increased recently with the aim of providing valuable graphical representations for the analysis of multivariate data [18–21]. PCA is a mathematical method for reducing the dimensionality of a data set consisting of a large number of interrelated variables. The original variables are transformed into a new set of variables, the principal components (PCs), which are linear combinations of the original variables but uncorrelated. The "loadings" represent the contribution (the coefficients of the linear combinations) of each of the original variables (^{13}C chemical shifts here) to the new ones, i.e. PCs.

The first PC corresponds to the direction having the greatest possible variance in the data. Each succeeding PC is orthogonal to the previous ones and usually, the first several PCs explain most of the variance of the data. As the first PCs may be interpreted as axes of maximum variance of the data, it is interesting to visualize the data distribution in these new set of axes. The graphic showing the projection of the data samples on the plane of two different PCs is called a scores plot and is useful for the classification of the samples. Thus, the effects of the substituents and of the *N*-oxide position may be compared by their grouping in the scores plot and from the loading values extract and identify the relationship between the original variables (^{13}C chemical shifts) and the principal components.

2. Experimental

2.1. Synthesis

The studied compounds are 1-*N*-oxides (denoted by **1*NO***) and 4-*N*-oxides (denoted by **4*NO***) of the following pyrazines: 2-chloropyrazine (**1**), 2-aminopyrazine (**2**), 2-methylpyrazine (**3**), 2-acetylpyrazine (**4**), 2-carboxypyrazine (**5**), 2-hydroxypyrazine (**6**), 2-methoxypyrazine (**7**), 2-acetamidopyrazine (**8**), 2-(1-hydroxyethyl)pyrazine (**9**), 2-(methoxycarbonyl)pyrazine (**10**) and 2-carbamoylpyrazine (**11**). The pyrazines **1**, **2**, **3**, **4** and **5** were obtained commercially whereas **8**, **9** and **10** were synthesized from **2** [22], **4** [23] and **5** [24], respectively. Except for compounds **4-1*NO***, **9-1*NO*** and **9-4*NO***, the remaining compounds have been synthesized earlier although in some cases by different methodologies.

Two methods of *N*-oxidation differing in the oxidation agent, i.e. potassium persulphate (Method A) [25,26] and 3-chloroperoxybenzoic acid (Method B) [27] were used affording compounds **1-1*NO***, **10-1*NO*** and **10-4*NO*** by Method A and **1-4*NO***, **2-1*NO***, **3-1*NO***, **3-4*NO***, **4-4*NO***, **8-1*NO***, **8-4*NO***, **9-1*NO*** and **9-4*NO*** by Method B. *N*-oxidations generally produced mixtures of different *N*-oxides which were separated and purified chromatographically. Some of the pure *N*-oxide products were used as precursors for the synthesis of the remaining ones, namely **1-1*NO*** (**6-1*NO*** and **7-1*NO***) [28,29], **1-4*NO*** (**2-4*NO***, **6-4*NO*** and **7-4*NO***) [28,30,31], **9-1*NO*** (**4-1*NO***) [32], **10-1*NO*** (**5-1*NO*** and **11-1*NO***) [30,33] and **10-4*NO*** (**5-4*NO*** and **11-4*NO***) [28,33]. Further details on the synthetic procedures employed and ^{1}H NMR spectroscopic data [25–27,29,34–38] may be found in the Supporting information.

2.2. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II spectrometer operating at 500.13 and 125.77 MHz, respectively. Two-dimensional NMR spectra HSQC-DEPT and HMBC were performed using standard Bruker software. Spectra were acquired from samples as solutions at room temperature in 5 mm tubes. Deuterochloroform was used as solvent unless the compounds were insoluble. If slightly soluble, a few drops of methanol-d_4 (2%) were added and otherwise dimethylsulfoxide-d_6 was used. The concentrations of all solutions were between 0.01 and 0.1 mol dm^{-3}. ^{13}C NMR chemical shifts (δ) are reported in ppm, referenced to TMS as an internal standard in $CDCl_3$ solutions or to the center peak of the solvent DMSO-d_6 (δ = 39.50 ppm from internal TMS).

2.3. Data analysis

PCA was applied using the data set represented by a matrix of 4 variables (^{13}C NMR chemical shifts of C-2, C-3, C-5 and C-6) and 22 samples (synthesized compounds **1-1*NO*** to **11-4*NO***) without preprocessing of the data set by using MATLAB software v. 7.0 (MathWorks Inc., Natick, MA). From the loading values obtained, the scores plot was generated for visualization of data relationships.

3. Results and discussion

^{13}C NMR chemical shifts of compounds **1-1*NO*** to **11-4*NO*** are given in Table 1. Assignment of the carbon resonances was made on the basis of the correlations observed in their HSQC and HMBC spectra. A first assignment of the signals in the ^{1}H NMR spectra of 2-monosubstituted pyrazine *N*-oxides was done considering the known characteristic ^{1}H,^{1}H coupling behavior in pyrazine *N*-oxides [39] and 2-substituted pyrazine *N*-oxides [34] from literature data. The *ortho* constant ^3J(H-5,H-6) is above 4.0 Hz while the *para* constant ^5J(H-3,H-6) is below 1.0 Hz. The constant through the nitrogen atom ^4J(H-3,H-5) ranges from 0 to 0.5 Hz in 1-*N*-oxides whereas it varies between 1.0 and 2.0 Hz in 4-*N*-oxides.

Table 1
^{13}C NMR chemical shifts of the set of 2-substituted pyrazine *N*-oxides synthesized.

Compound	Substituent	^{13}C chemical shift (ppm)				Solvent
		δC-2	δC-3	δC-5	δC-6	
1-1*NO*	**Cl**	139.5	147.6	144.8	134.7	$CDCl_3$
1-4*NO*		151.7	133.5	133.1	145.9	$CDCl_3$
2-1*NO*	**NH_2**	147.5	132.5	132.8	131.2	DMSO-d_6
2-4*NO*		158.8	118.0	123.4	145.1	DMSO-d_6
3-1*NO*	**CH_3**	144.4	147.9	145.2	133.6	$CDCl_3$
3-4*NO*		158.1	132.9	131.3	146.7	$CDCl_3$
4-1*NO*	**$COCH_3$**	141.4	149.6	149.0	134.9	$CDCl_3$
4-4*NO*		152.1	132.6	135.7	146.5	$CDCl_3$
5-1*NO*	**COH**	134.3	148.7	149.6	133.9	DMSO-d_6
5-4*NO*		148.2	134.8	135.7	147.5	DMSO-d_6
6-1*NO*	**OH**	152.3	147.3	122.8	128.5	DMSO-d_6
6-4*NO*		158.9	128.4	120.7	130.9	DMSO-d_6
7-1*NO*	**OCH_3**	155.9	132.0	139.9	134.1	$CDCl_3$
		155.3	132.5	139.6	133.8	DMSO-d_6
7-4*NO*		164.0	123.1	129.0	143.3	$CDCl_3$
		163.6	122.2	129.0	143.7	DMSO-d_6
8-1*NO*	**$NHCOCH_3$**	141.4	137.7	140.0	131.2	$CDCl_3$[a]
8-4*NO*		151.4	125.8	130.0	145.0	$CDCl_3$[a]
9-1*NO*	**$CHOHCH_3$**	147.8	145.5	146.6	133.8	$CDCl_3$
9-4*NO*		164.8	131.2	132.4	146.7	$CDCl_3$[a]
10-1*NO*	**$COOCH_3$**	136.5	149.5	148.4	135.3	$CDCl_3$
10-4*NO*		147.5	135.8	135.8	147.3	$CDCl_3$
11-1*NO*	**$CONH_2$**	135.7	149.6	148.7	134.5	DMSO-d_6
11-4*NO*		149.9	132.8	135.6	146.9	DMSO-d_6

[a] Solutions containing 2% methanol-d_4.

As a result, it was possible to identify each proton for 4-*N*-oxides, but this method failed to distinguish between H-5 and H-6 in the case of 1-*N*-oxides, as reliable coupling information was not available from the measured spectra. Effects of quadrupole broadening of the lines due to interaction with the nitrogen atom reduces the accuracy in the measurement of the spin-spin coupling constants of the protons in direct proximity to the nitrogen [34]. This effect is important especially in the case of 1-*N*-oxides where ^4J(H-3,H-5) is not resolved in contrast with 4-*N*-oxides, where the presence of the oxygen atom attached to nitrogen eliminates to a considerable extent this quadrupole broadening.

Moreover, unambiguous distinction between the signals of H-5 and H-6 in 1-*N*-oxides on basis of multiplicity considerations becomes impractical when ^5J(H-3,H-6) appears unresolved. Nevertheless, reliable assignments could be achieved considering correlations observed in the HMBC spectra. In all cases, H-5 correlates with C-3 and C-6 while H-6 correlates generally with C-5. The corresponding ^{13}C resonances were attributed considering the correlations in the HSQC spectrum. Finally, the C-2 peak was easily confirmed with the HMBC spectrum as H-3 and H-6 correlate with this quaternary carbon.

As might be observed in Table 1, ^{13}C NMR chemical shifts were not registered in the same solvent for all compounds. Due to the low solubility of some compounds in $CDCl_3$, a 2% methanol-d_4 in $CDCl_3$ solution or DMSO-d_6 was used. In general, compounds having no capability for hydrogen bonding exhibit only small differences between the concerning ^{13}C chemical shifts in DMSO-d_6 and $CDCl_3$ solution. Investigations with regard to the effect of these common solvents were performed with pyridine *N*-oxide [40], and substituted pyrazines [41], whereas in this work compounds **7-1*NO*** and **7-4*NO*** were dissolved in DMSO-d_6 and $CDCl_3$, showing in all cases deviations not higher than 1 ppm in the chemical shifts between the solvents.

In this work, our goal is to find a relationship using the ^{13}C NMR chemical shifts in substituted pyrazine *N*-oxides for the assignment of the *N*-oxide position irrespective of the nature or position of the substituent. It may be observed that the ^{13}C chemical shift values might be visualized like the coordinates of points in a 4-dimensional space, where each axis would be associated with the chemical shift of a different carbon. Nevertheless, the analysis of such a graph would be rather difficult.

Therefore, PCA was applied with the aim of reducing the dimensionality of the data. The first two principal components described 99.94% of the variance in the data contained in Table 1. In Fig. 2 is

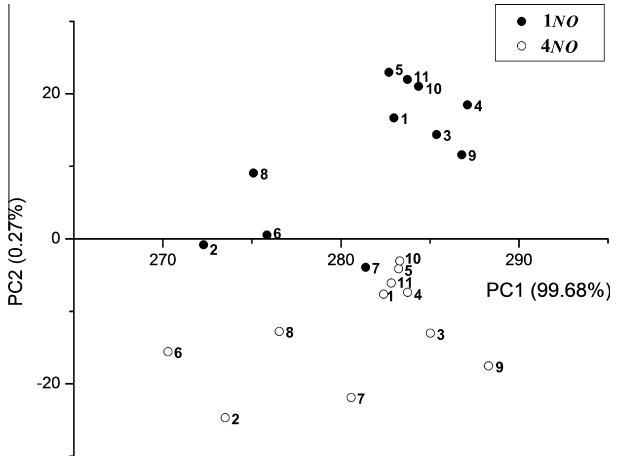

Fig. 2. Scores plot of the first two principal components (PC1 and PC2) obtained applying PCA to the ^{13}C NMR chemical shifts data for the set of 2-substituted pyrazine *N*-oxides **1-1*NO*:11-4*NO***. The percentage of the variance in the data explained by each PC is shown in the axes titles.

shown the scores plot of PC1-PC2. The first principal component, PC1 represents essentially the sum of the ^{13}C chemical shifts with all the carbons nearly equally weighted. On the other hand, the second principal component, PC2 represents a contrast between the ^{13}C chemical shifts of each pair of carbon atoms bonded to a nitrogen atom (C-3 and C-5 vs. C-2 and C-6). This is derived from the "loadings" values, i.e. the coefficients of the variables which define each PC in Eq. (1) and (2)

$$PC1 = 0.530\delta C\text{-}2 + 0.488\delta C\text{-}3 + 0.487\delta C\text{-}5 + 0.494\delta C\text{-}6 \quad (1)$$

$$PC2 = -0.614\delta C\text{-}2 + 0.543\delta C\text{-}3 + 0.465\delta C\text{-}5 - 0.335\delta C\text{-}6 \quad (2)$$

From the inspection of Fig. 2, it might be noted that PC1 seems to separate the compounds according to the substituent. Moreover, the position of the *N*-oxide does not seem to exert a significant effect on PC1 scores as both isomers have similar values appearing more or less aligned vertically in most cases (differences < 1.5 ppm approx.).

On the other hand, PC2 seems to separate the compounds into two big groups, one with positive scores which includes most of the 1-*N*-oxides and other with negative scores including all the 4-*N*-oxides and a few of the 1-*N*-oxides. Thus, most of the 2-substituted pyrazine *N*-oxides are separated and differentiated according to the position of the oxidized nitrogen atom. The separation (difference between the scores of PC2) between each pair of isomeric *N*-oxides ranges from 16 ppm for **6-1*NO*:6-4*NO*** to 29 ppm for **9-1*NO*:9-4*NO***.

Regarding the Eq. (2) defining PC2, it is interesting to note that the positive coefficients (loadings of δC-3 and δC-5) are very similar while the negative ones (loadings of δC-2 and δC-6) are more polarized probably due to the variable effect of the substituent at C-2. Interestingly, a similar trend had been found in the ^{13}C NMR chemical shifts of substituted pyridine *N*-oxides [42,43], observing a shielding effect of about the same magnitude at C-2, C-4 and C-6 and an opposite and much smaller effect at C-3 and C-5, not greatly influenced by the nature or position of the substituent. A similar effect was also observed in the ^{13}C NMR spectra of alkyl- and phenylpyrazine *N*-oxides [15], and was rationalized suggesting the increase of the electronic density (shielding) at C-2 and C-6 in pyrazine 1-oxides by electron back-donation from the *N*-oxide group.

The above observations suggest an index of *N*-oxidation (INO) might be proposed to identify the *N*-oxide position in a more general, reliable and simpler way, in analogy to PC2 scores obtained by PCA which proved to be successful identifying the position of the oxidized nitrogen in most of the 2-substituted pyrazine *N*-oxides studied in this work. A simplification for the calculus of the index might be introduced by considering only two coefficients, one for the sum of the ^{13}C chemical shifts of each pair of carbon atoms bonded to a nitrogen atom, i.e. a positive coefficient (C_{35}) for δC-3 + δC-5 and a negative one (C_{26}) for δC-2 + δC-6:

$$INO = C_{26}(\delta C\text{-}2 + \delta C\text{-}6) + C_{35}(\delta C\text{-}3 + \delta C\text{-}5) \quad (3)$$

A positive value of the index INO would indicate the presence of a 1-*N*-oxide while a negative value that of a 4-*N*-oxide in resemblance to PC2 scores, as long as C-2 identifies the substituted carbon atom. Values close to zero might lead to uncertainty or errors and should thus be avoided. In Fig. 2, it may be observed that PC2 scores for the 1-*N*-oxides of strong electron donor substituents (**2-1*NO***, **6-1*NO*** and **7-1*NO***) are close to zero as well as for the 4-*N*-oxides of electron withdrawing substituents (**1-4*NO***, **4-4*NO***, **5-4*NO***, **10-4*NO*** and **11-4*NO***). In fact, some of the discrepancies recognized from the PCA analysis, especially noted in compounds **2-1*NO*** and **7-1*NO*** which have negative PC2 scores despite being 1-*N*-oxides, might be related with the scattering of the data not evenly distributed due to the different nature of the substituents.

Table 2
Substituent optimized factor x[a] of the set of 2-substituted pyrazine *N*-oxides synthesized and Hammett substituent constants σ_p[b].

Substituent	Factor *x*[a]	σ_p[b]
Cl	0.978	0.23
NH_2	0.870	−0.66
CH_3	0.956	−0.17
$COCH_3$	0.986	0.50
COOH	1.009	0.45
OH	0.910	−0.37
OCH_3	0.877	−0.27
$NHCOCH_3$	0.938	0.00
$CHOHCH_3$	0.937	−0.07
$COOCH_3$	1.005	0.45
$CONH_2$	1.000	0.36

$x = [(\delta C\text{-}3 + \delta C\text{-}5)_{1NO} + (\delta C\text{-}3 + \delta C\text{-}5)_{4NO}]/[(\delta C\text{-}2 + \delta C\text{-}6)_{1NO} + (\delta C\text{-}2 + \delta C\text{-}6)_{4NO}]$, using the ^{13}C NMR data from Table 1.

[a] Optimum values of the factor x calculated under the constraint INO_x(**1*NO***) + INO_x(**4*NO***) = 0.

[b] Data for σ_p taken from Ref. [44].

To avoid values of the index close to zero, the coefficients C_{35} and C_{26} may be optimized in order to maximize the value of the index INO for both *N*-oxides of each pair of isomers, so that the values are as far apart from zero as possible. This might be accomplished fixing the value of one coefficient (for example, taking C_{35} = 0.5, which is the average of the PC2 "loadings" of δC-3 and δC-5) and calculating the value of the other (C_{26}), requesting the indices INO for both *N*-oxides of each pair to be centered respect to zero, i.e., under the constraint INO(**1*NO***) + INO(**4*NO***) = 0. Moreover, the index may be defined as a difference between the average ^{13}C chemical shifts of each pair of carbon atoms bonded to a nitrogen atom, including the substituent effect through a factor *x*, multiplying the average containing the substituted carbon atom. Then,

$$INO_x = -x(\delta C\text{-}2 + \delta C\text{-}6)/2 + (\delta C\text{-}3 + \delta C\text{-}5)/2 \quad (4)$$

In Table 2 are presented the values of the optimized factors for each pair of *N*-oxides of the set of 2-substituted pyrazine *N*-oxides synthesized. From the values obtained, it may be noted that strong electron donor substituents (NH_2, OH and OCH_3) have values around 0.9 while electron withdrawing substituents (Cl, $COCH_3$, COOH, $COOCH_3$ and $CONH_2$) adopt values close to 1. In order to further analyze if a relationship with the substituent electronic effect actually exists, the Hammett substituent constants [44] were

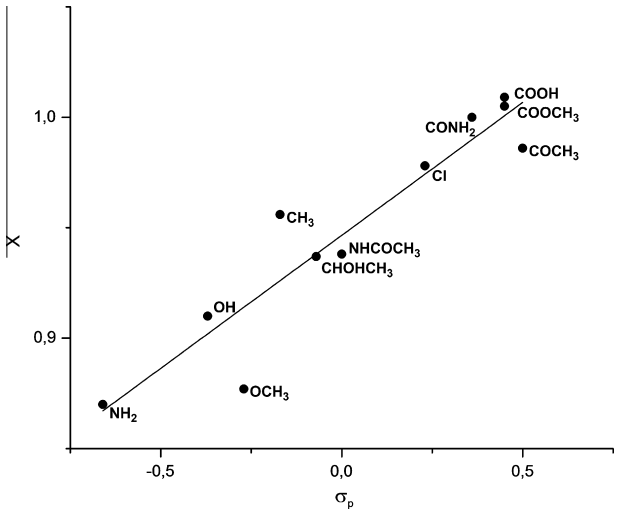

Fig. 3. Plot of the optimized substituent factors (*x*) calculated for the set of 2-substituted pyrazine *N*-oxides vs. Hammett substituent constants (σ_p).

Table 3
Indices of *N*-oxidation of the set of 2-substituted pyrazine *N*-oxides synthesized.

		Index of *N*-oxidation[a] (ppm)			PC2 Scores[b]
Compound	Substituent	$INO_{0.90}$	$INO_{0.95}$	$INO_{1.00}$	(ppm)
1-1*NO*	**Cl**	22.8	16.0	**9.1**	16.7
1-4*NO*		−0.62	−8.06	**−15.5**	−7.64
2-1*NO*	**NH_2**	**7.24**	0.27	−6.7	−0.82
2-4*NO*		**−16.1**	−23.7	−31.3	−24.7
3-1*NO*	**CH_3**	21.5	**14.5**	7.55	14.4
3-4*NO*		−5.06	**−12.7**	−20.3	−13.0
4-1*NO*	**$COCH_3$**	25.0	18.1	**11.2**	18.5
4-4*NO*		−0.22	−7.69	**−15.2**	−7.37
5-1*NO*	**COOH**	28.5	21.8	**15.1**	23.0
5-4*NO*		2.19	−5.21	**−12.6**	−4.11
6-1*NO*	**OH**	**8.69**	1.67	−5.35	0.53
6-4*NO*		**−5.86**	−13.1	−20.4	−15.6
7-1*NO*	**OCH_3**	**6.00**	−1.27	−9.05	−3.92
7-4*NO*		**−12.7**	−20.4	−27.6	−21.9
8-1*NO*	**$NHCOCH_3$**	16.2	**9.37**	2.55	9.10
8-4*NO*		−5.48	**−12.9**	−20.3	−12.8
9-1*NO*	**$CHOHCH_3$**	19.3	**12.3**	5.25	11.6
9-4*NO*		−8.38	**−16.2**	−24.0	−17.5
10-1*NO*	**$COOCH_3$**	26.6	19.8	**13.1**	21.0
10-4*NO*		3.14	−4.23	**−11.6**	−3.02
11-1*NO*	**$CONH_2$**	27.6	20.8	**14.1**	22.0
11-4*NO*		0.64	−6.78	**−14.2**	−6.09

[a] Index of *N*-oxidation calculated from Eq. (4) using the values of $x = (0.90; 0.95; 1.00)$ and the ^{13}C NMR data from Table 1. The best values for isomer differentiation are shown in bold.
[b] Values of the PC2 scores obtained by Eq. (2) and the ^{13}C NMR data from Table 1.

considered as shown in Table 2. Fig. 3 shows the quite satisfactory correlation obtained with these substituent constants, which take only the electronic effect of the substituent into account. Moreover, the deviations observed are not unexpected considering the constants for the first eight functional groups of Table 2 were defined on the basis of ionization constants of *para* substituted benzoic acids in water [45].

As may be observed in Fig. 3, the optimized values of x are distributed more or less evenly forming three clusters according to the substituent effect as previously commented. As a result, general indices might be employed according to the nature of the substituent, thus avoiding specific substituent factors. For instance, considering three different factor values in accordance with the clusters observed, three indices of *N*-oxidation might be proposed, i.e. $INO_{0.90}$ for strong electron donor substituents, $INO_{0.95}$ for weak electron donor substituents and $INO_{1.00}$ for electron withdrawing substituents. The results of this approach were more successful than those previously obtained from PC2 scores for the whole set of compounds as shown in Table 3.

It can be seen that if no assumptions are made about the nature of the substituent, that is, if a "mean value" factor is considered (i.e. $x = 0.95$, which is close to the value for zero substituent effect, see Fig. 3), in general the sign of the index $INO_{0.95}$ is consistent with the position of the *N*-oxide (positive for 1-*N*-oxides and negative for 4-*N*-oxides) as shown in Table 3. However, the results obtained from the calculus of the index $INO_{0.95}$ are not conclusive for some compounds whose value is close to zero or below a threshold value.

The definition of a threshold value although arbitrary should be consistent with the uncertainty associated to the calculus of the index. Considering the index INO_x is calculated as an almost evenly weighted difference between two ^{13}C chemical shifts (see Eq. (4)), the expected uncertainty for the index should be about twice the uncertainty of the ^{13}C chemical shifts, which in this case, is mostly influenced not by the accuracy of the measurement but by deviations caused by different experimental conditions (solvent, temperature). Our former observations regarding the effect of the solvent, neglecting the effect of the temperature, indicate that deviations in the chemical shift values as much as 1 ppm might be expected. Therefore, even in the worst case, the expected uncertainty of the index INO_x would be around 2 ppm (~1% of the average ^{13}C chemical shifts). In consequence, a threshold value above 5–6 ppm may be used to avoid misleading values.

It is interesting to examine in further detail if the indices applied to the study set of monosubstituted pyrazine *N*-oxides, are still useful to identify the *N*-oxide position in disubstituted pyrazine *N*-oxides. It is worth mentioning that in the case of disubstituted pyrazine *N*-oxides only three substitution patterns are possible, i.e. 2,3-; 2,5- and 2,6-. In these cases, the sign of the index would reflect the position of the *N*-oxide function relative to the substituent located in C-2, which can be chosen arbitrarily.

When substituents are placed in *meta* (2,6-), as both substituted carbon atoms belong to the same average (see Eq. (4)), there is no ambiguity about the location of the factor accounting for the substituent effect, making clear that the index INO_x would be the first approach in a similar fashion to that employed earlier. However, in other substitution patterns (2,3- and 2,5-), as the substituents are located in carbon atoms bonded to opposite nitrogen atoms, the substituent effect should be equally included by a factor multiplying each average as in Eq. (3). Then,

$$INO_{x,y} = -x(\delta C\text{-}2 + \delta C\text{-}6)/2 + y(\delta C\text{-}3 + \delta C\text{-}5)/2 \quad (5)$$

As long as the effect exerted by both substituents is similar, it seems reasonable to use the same factor value multiplying both averages, i.e. $x = y$ in Eq. (5).

Table 4
Indices of *N*-oxidation calculated for a series of disubstituted pyrazine *N*-oxides.[a]

Compound	Substituents				^{13}C chemical shift (ppm)				Index of *N*-oxidation (ppm)	
	R2	R3	R5	R6	δC-2	δC-3	δC-5	δC-6	$INO_{0.95}$[b]	$INO_{0.95,0.95}$[c]
12-4*NO*	**Ph**	**H**	**H**	**Ph**	155.9	128.7	128.7	155.9	−19.4	−25.8
13-1*NO*	**CH_3**	**CH_3**	**H**	**H**	142.9	156.2	142.9	131.9	19.0	**11.5**
13-4*NO*	**CH_3**	**CH_3**	**H**	**H**	156.2	142.9	131.9	142.9	−4.7	**−11.5**
14-1*NO*	**CH_3**	**H**	**CH_3**	**H**	141.1	146.7	154.9	132.3	20.9	**13.4**
14-4*NO*	**CH_3**	**H**	**CH_3**	**H**	154.9	132.3	141.1	146.7	−6.6	**−13.4**
15-1*NO*	**CH_3**	**H**	**H**	**CH_3**	145.3	143.7	143.7	145.3	**5.67**	−1.50
15-4*NO*	**CH_3**	**H**	**H**	**CH_3**	156.7	130.2	130.2	156.7	**−18.7**	−25.2
16-1*NO*	**CH_3**	**Ph**	**H**	**H**	143.7	158.1	143.5	132.1	19.8	**12.3**
16-4*NO*	**CH_3**	**Ph**	**H**	**H**	157.1	144.5	132.2	144.9	−5.10	**−12.0**
17-1*NO*	**CH_3**	**H**	**Ph**	**H**	142.1	147.2	154.6	130.5	21.4	13.9
18-1*NO*	**CH_3**	**H**	**H**	**Ph**	144.5	146.0	146.0	144.5	**8.73**	1.43
18-4*NO*	**CH_3**	**H**	**H**	**Ph**	157.0	130.7	128.2	155.8	**−19.3**	−25.6

[a] ^{13}C NMR chemical shifts values taken from Ref. [15].
[b] Index of *N*-oxidation calculated from Eq. (4) using the values: $x = 0.95$ and the ^{13}C NMR data from literature.
[c] Index of *N*-oxidation calculated from Eq. (5) using the values: $x,y = 0.95$ and the ^{13}C NMR data from literature.

In Table 4 are shown some examples taken from the literature [15] along with the indices used in accordance to the substitution patterns. The Hammett constants for the studied substituents, i.e. phenyl and methyl are −0.01 and −0.17, respectively [45] and thus may be considered as weak electron donor groups. Consequently, the factor accounting for the substituent effect should have the same value for both of them (x, $y = 0.95$). As shown in Table 4, the index $INO_{0.95,0.95}$ seems better to identify the position of the *N*-oxide for the *ortho* and *para* substituted compounds (**13-1*NO***, **13-4*NO*, 14-1*NO***, **14-4*NO*, 16-1*NO*** and **16-4*NO***) while the index $INO_{0.95}$ seems more appropriate for the *meta* substituted ones (**15-1*NO***, **15-4*NO***, **18-1*NO*** and **18-4*NO***) as expected.

4. Conclusions

^{13}C NMR spectroscopy data was used to identify unambiguously the position of the *N*-oxide of a series of 2-substituted pyrazine *N*-oxides. Analysis by the chemometric technique of PCA allowed recognizing relationships among the carbon chemical shifts which prove to be useful identifying the position of the oxidized nitrogen for most of the compounds studied. These relationships could be rationalized in terms of the *N*-oxide known effect of increasing the electronic density at its neighboring carbon atoms. Thus, by further refinement, a general index of *N*-oxidation (INO_x), whose sign reflects the position of the *N*-oxide relative to the substituent at C-2 (positive for 1-*N*-oxides and negative for 4-*N*-oxides), was proposed for the first time.

The main advantage of this index is its simplicity as it is easily computed contrasting the average ^{13}C NMR chemical shifts of each pair of carbon atoms bonded to a nitrogen atom. The methodology proved to be successful in identifying the position of the *N*-oxide in mono- as well as di-substituted pyrazine *N*-oxides, and in principle, might be used for other substituted pyrazine *N*-oxides as long as the substituents effects to be considered could be well represented by a single factor multiplying each average.

As a concluding remark for the extension of the former methodology to other substituted pyrazine *N*-oxides, the only caution to be noticed is that the value to be used of the factor should be chosen adequately taking into account the different substituent effects present in the molecule. For example, the Hammett substituent constants might be used as a first approach to estimate the value of the factor.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molstruc.2013.03.058.

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