

Quantitative Structure-Property Study on Pyrazines with Bell Pepper Flavor

Ch. Th. Klein^a, H. Pircher^b, B. Wailzer^a, G. Buchbauer^b
and P. Wolschann^a

*a - Institut für Theoretische Chemie und Molekulare Strukturbiologie, Universität Wien,
Währinger Straße 17, A-1090 Wien, Austria*

*b - Institut für Pharmazeutische Chemie, Universität Wien, Althanstraße 14,
A-1090 Wien, Austria*

Abstract

A quantitative structure-property (QSPR) study on pyrazines with bell pepper aroma is performed by means of different statistical methods, which correlate appropriate molecular descriptors with the biological activity. The different methods lead to consistent results, indicating which of the molecular properties of the compounds under consideration are significant for bell pepper flavor. These results are compared with other models.

Keywords: Bell pepper flavor, correlation analysis, multiple linear regression, comparative molecular field analysis, cluster analysis

INTRODUCTION

The relationship between the molecular structure of flavor compounds and the intensity and the quality of their aroma impression has received more and more interest in the past years.

Molecular biologists have proven that flavor molecules (and odor molecules in general) bind to specific receptors localized in the olfactory mucosa, which exhibit the typical seven helix bundle motif and are coupled to G-proteins [1]. Based on DNA analysis it is assumed that there exist 100 to 1000 different receptors which can be grouped into subclasses. Compounds of distinct structural classes bind to various subclasses with different affinities. The huge number of possible binding patterns could explain the high diversity of odor impressions [1].

The conformational change, induced by binding the odor or aroma molecule to olfactory receptors, activates the adenylate cyclase cascade, leading to the opening of an unspecific cation channel by cAMP, and thus releasing an action potential [2]. However, it has been shown that only some of the odor molecules simulate the adenylate cyclase cascade. In the mean time, in different species, inositol-1,4,5-triphosphate (IP₃) was found to be a second messenger in the olfactory signal transduction [3]. IP₃ is supposed to open a specific Ca²⁺-channel by binding to this membrane protein [3].

On the other hand, the gap between the knowledge of the primary structure and the three-dimensional geometry of olfactory receptors is large: while the sequence of some receptors is already known, no detailed structural elucidation exists for the moment. This is a strong motivation to study the odor molecule-receptor interaction by molecular modeling approaches. In the present study, structure-flavor relationships on pyrazine-based flavor molecules with bell pepper aroma are analyzed by means of three different methods: multiple linear regression (MLR), cluster analysis and comparative molecular field analysis (CoMFA) [4].

Pyrazine-based aroma compounds show a broad spectrum of flavor impressions, reaching from earthy, nutty, roasted to bell pepper or woody. Their general structure is presented in Figure 1.

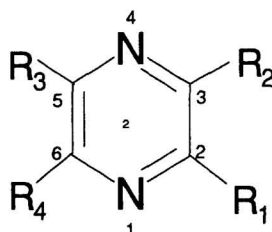


Figure 1. General structure of the pyrazine derivatives

Pyrazines were first identified in heated food as bread [5], different meats [6], baked potatoes [7], or coffee [8], where they are formed during the Maillard reaction from reducing sugars and amino acids [9], but they also occur in fresh vegetables like tomatoes, asparagus, beans, spinach [10], or in bell pepper [11].

From the analysis of the obtained regression and CoMFA [4] models, conclusions on steric and electronic requirements, responsible for the bell pepper flavor are deduced.

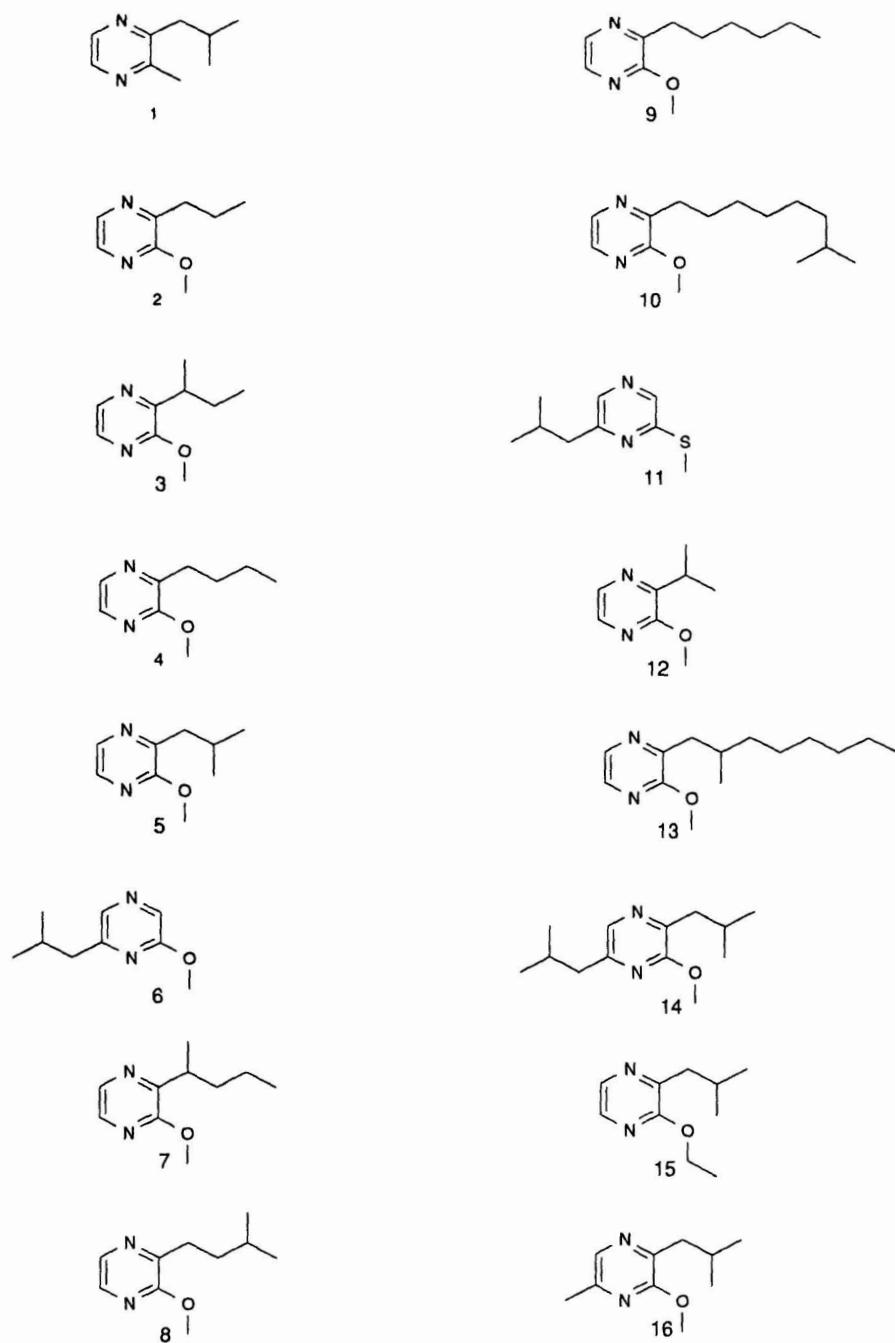
MATERIALS AND METHODS

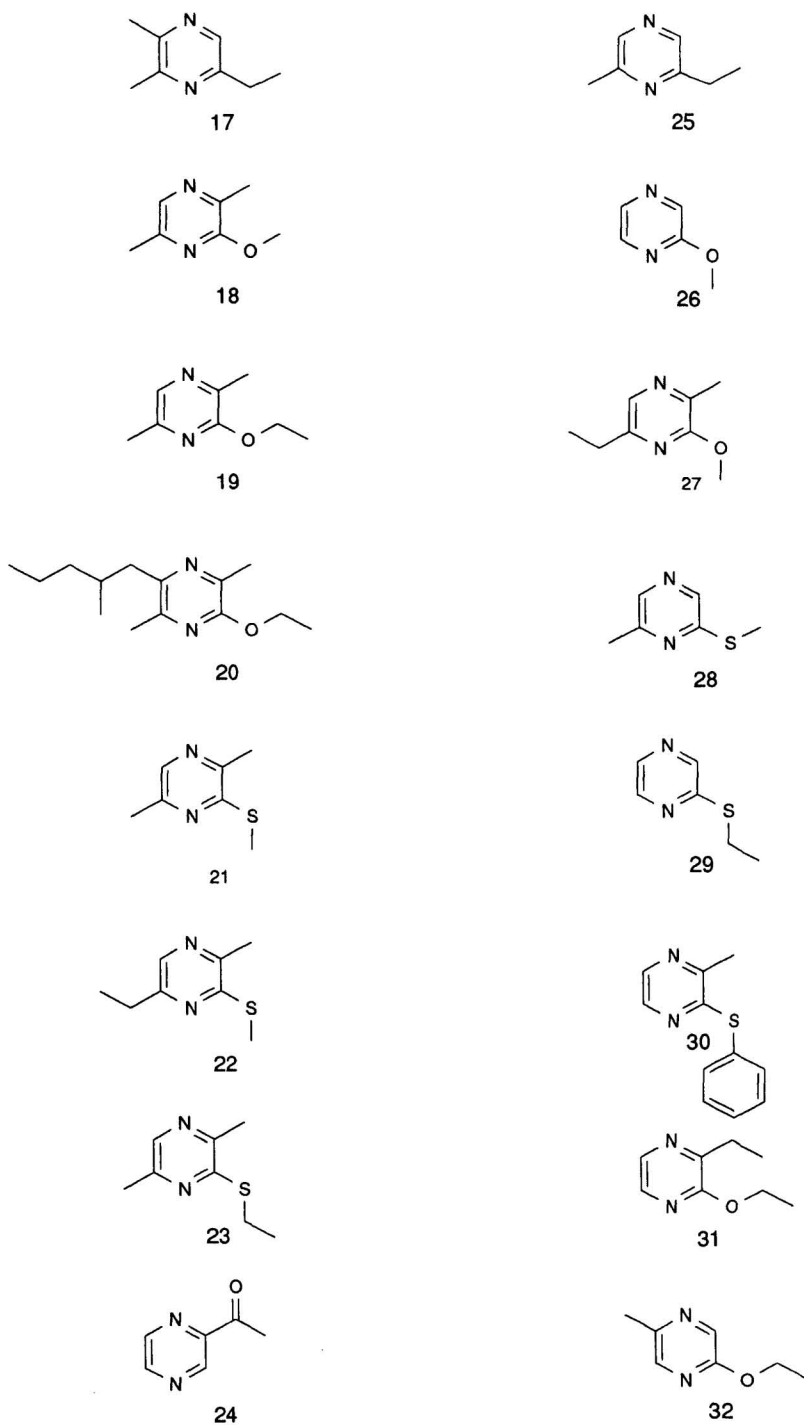
32 pyrazine-based aroma compounds are selected, which belong to two groups with distinct flavor impressions: 16 with bell pepper (1 to 16) and 16 with nutty-roasty (17-32) flavor (Figure 2).

The structures are built with the Hyperchem 5.0 software [12] and fully minimized with the MM+ force field implemented therein. The resulted geometries are subsequently optimized with the semiempirical AM1 method [13] implemented in the MOPAC program package [14].

For the obtained structures the following molecular properties are calculated, using the TSAR (Tools for Structure-Activity Relationships) software [15]:

- (i) steric descriptors: molecular volume (V) and surface(S), molecular refractivity (MR), and the Verloop parameters (L , B_1 , B_2 , B_3 , B_4) [16] for the four possible substituents, R_1 to R_4 . (Figure 1). As R_1 always the substituent with the heteroatom is considered, except for the three compounds where the substituents do not contain any heteroatom: compound 3 (R_1 =methyl), compound 59 (R_1 =ethyl) and compound 81 (R_1 =ethyl). The Verloop parameter $L^{(i)}$ represents the maximal length of substituent i along the axis defined by the bond which connects the substituent with the heterocycle. $B_j^{(i)}$ ($j=1, \dots, 4$) denotes the width of a substituent i perpendicular to this axis, and is chosen in such a way that $B_1 < B_2 < B_3 < B_4$; the molecular refractivity, being related to the volume and to the polarizability of a compound, is not only a steric descriptor, but also gives information whether dispersion forces are important in the interaction with the receptor or not.
- (ii) descriptor of lipophilicity: $\log P$ where P is the partition coefficient of the respective compound between octanol and water; the larger P (and thus $\log P$) is, the more hydrophobic is a compound.
- (iii) electronic descriptors: AM1-derived point charges on the atoms of the heterocycle (ipso atoms, C_{N1} , C_{N4} , C_{C2} , C_{C3} , C_{C5} , C_{C6}) and on the first atom of the respective substituent (C_{Ri}).

**Figure 2.** Pyrazines used in the study

**Figure 2** (continued). Pyrazines used in the study

The molecular descriptors are then correlated with the biological activity (1 - bell pepper flavor, 0 - no bell pepper flavor) employing the mentioned statistical methods.

Multiple linear regression (MLR) establishes a linear combination between the molecular properties of the molecules and their biological activities by determining the coefficients such, that the difference of the actual and predicted values are minimized. MLR models are obtained with the TSAR [15] program, using the backward and the two-way stepping algorithm [17]. In backward elimination all variables are included in the initial model. Step by step, less significant variables are then eliminated via their partial *F*-test. Once a variable has been excluded it cannot reenter the model. During two way stepping, reentering of excluded variables is possible; this accounts for the fact, that the *F*-value is model-dependent, i.e. a variable which has lost its significance in a certain model, may become significant in another model, and, vice-versa, a significant variable in one model, may loose its significance in another one. *F*-to-leave and *F*-to-enter are set to 4, as this corresponds roughly to the upper 5 percent point of the *F*-distribution. The quality of a model is judged by the standard error (*s*), the overall *F*-value, the *t*-statistics of individual regression coefficients, the correlation coefficient (*r*) and the cross-validated r^2 (r^2_{cv}), which reflects the predictive ability of the model. Cross-validation is performed by leaving out each compound in turn (unless noted).

Within cluster analysis a distance matrix is calculated from the molecular properties, which is then used to classify samples into clusters of similar members. Cluster analysis is performed with the TSAR [15] program, using Ward clustering [18] with Euclidean distances.

CoMFA analysis [4] is performed with the SYBYL software [19]. The molecules are superimposed by fitting the atoms of the heterocycle and the first atom of substituent 1 (*R*₁). Grid sizes of 1, 2 and 3 Å and different probe atoms [*sp*³C(+1), *sp*³O(-1) and H(+1)] are employed for the evaluation of the molecular field. For the calculation of the electrostatic field the same AM1 charges as in MLR are used. The SAMPLS [20] variant of PLS is applied, with the cross-validation option of leaving out one compound in turn. The quality of the models is estimated by the same statistical indicators as in MLR.

RESULTS

Multiple Linear Regression

The best regression model found by two-way stepping reads as:

$$\begin{aligned}
 A &= 0.024 \cdot MR - 6.400 \cdot C^{(C3)} - 0.804 \cdot C^{(C6)} - 0.165 \cdot L^{(R1)} + 0.233 \cdot L^{(R2)} \\
 &\quad - 0.164 \cdot B_3^{(R3)} - 1.187 \\
 A &= 0.243 \cdot MR^* - 0.263 \cdot C^{(C3)*} - 0.118 \cdot C^{(C6)*} - 0.115 \cdot L^{(R1)*} + 0.331 \cdot L^{(R2)*} \\
 &\quad - 0.102 \cdot B_3^{(R3)*} + 0.5
 \end{aligned}
 \tag{1}$$

$$s = 0.198, \quad F = 29.763, \quad r = 0.936, \quad r_{cv}^2 = 0.825$$

where A denotes the activity (1 for bell pepper flavor, 0 for no bell pepper flavor). The second equation (*-variables) is obtained by using data standardized to zero mean and unity variance, and thus the regression coefficients reflect the weights of individual contributions to the activity.

As can be seen from Table 1, the variables from Eqn. (1) show no significant correlation among each other ($r^2 < 0.40$ indicates that no correlation exists among the variables).

	S	MR	$C^{(C3)}$	$C^{(C6)}$	$C^{(R1)}$	$L^{(R1)}$	$L^{(R2)}$	$B_3^{(R3)}$	$\log P^{(R4)}$
S	1								
MR	0.979	1							
$C^{(C3)}$	0.455	0.479	1						
$C^{(C6)}$	0.024	0.088	-0.159	1					
$C^{(R1)}$	-0.264	-0.118	-0.032	0.099	1				
$L^{(R1)}$	0.005	0.108	0.104	0.073	0.569	1			
$L^{(R2)}$	0.526	0.499	0.566	-0.102	-0.519	-0.366	1		
$B_3^{(R3)}$	0.256	0.223	-0.005	0.021	-0.149	0.188	-0.197	1	
$\log P^{(R4)}$	0.163	0.166	-0.261	0.192	0.179	0.054	-0.241	-0.077	1

Table 1. Correlation coefficients of descriptors from Eqn. (1)

A regression model can be considered to be significant if the overall F -value exceeds at least four times the percentage point $F_{\alpha, k, n-k-1}$ [21]. Because the latter, for Eqn. (1), is $F_{0.05, 6, 25} = 2.49 \ll F$, the regression model is significant at 95 % level. A similar analysis of the individual regression coefficients reveals their statistical significance. The statistics of the coefficients of Eqn. (1) is shown in Table 2.

The t -values of the coefficients are all larger than the percentage point $t_{\alpha/2, n-k-1}$ ($t_{0.025, 25} = 2.06$), i.e. the significance, at 95 % level, of the individual regression coefficients is also given.

The predictive power of the model is high, since r_{cv}^2 is high and fairly close to r^2 (0.893).

Variable	Coefficient	Coefficient*	Stand.Error	Stand.Error*	t-value
MR	0.024	0.243	0.007	0.077	5.017
C^(C3)	-6.400	-0.263	1.548	0.063	-5.412
C^(C6)	-0.804	-0.118	0.107	0.016	-3.201
L^(R1)	-0.165	-0.115	0.046	0.032	-2.641
L^(R2)	0.233	0.331	0.034	0.048	5.688
B3^(R3)	-0.164	-0.102	0.047	0.029	-2.598

Table 2. Statistics of the coefficients from Eqn. (1)

A regression equation with similar quality is found by backward elimination:

$$\begin{aligned}
 A &= 0.0043(0.001) \cdot S - 6.596(1.347) \cdot C^{(C3)} - 0.795(0.610) \cdot C^{(C6)} + 0.601(0.256) \cdot C^{(R1)} \\
 &\quad - 0.189(0.064) \cdot L^{(R1)} + 0.333(0.041) \cdot L^{(R2)} + 0.191(0.142) \cdot \log P^{(R4)} - 1.417(0.399) \\
 A &= 0.159(0.049) \cdot S^* - 0.271(0.055) \cdot C^{(C3)*} - 0.117(0.089) \cdot C^{(C6)*} + 0.126(0.054) \cdot C^{(R1)*} \\
 &\quad - 0.133(0.045) \cdot L^{(R1)*} + 0.473(0.059) \cdot L^{(R2)*} + 0.083(0.062) \cdot \log P^{(R4)*} + 0.5(0.040)
 \end{aligned} \tag{2}$$

$$s = 0.188, \quad F = 28.930, \quad r = 0.945, \quad r_{cv}^2 = 0.826$$

The values in brackets denote the standard errors of the coefficients [which for Eqn. (1) are given in Table 2]. As in the previous case, the used variables do not correlate with each other. The overall regression and the individual regression coefficients again are statistically significant, as judged by the F - and the t -values. The predictions of the biological activity with the MLR equations is shown in Table 3.

The high cross-validation r^2 (r_{cv}^2) values suggest that the remarkable statistical qualities of the models should not stem from chance correlation. Nevertheless, in order to exclude chance correlation, the effects of randomization on the dependent variables are analyzed: the 32 dependent variables are redistributed by a random number generator, and subsequently models are generated as previously by F-stepping variable selection. Table 4 shows that randomization causes, in all cases, the loss of correlation and statistical significance. $r^2 < 0.40$ (i.e. $r < 0.63$) indicates that no significant correlation exists among the independent variables. This is the case in five out of the ten situations. However, the other five cases have values only slightly above 0.63, attesting very poor correlation. Moreover, diminished significance of the regression equations is indicated by the F -values, since they should exceed the percentage point at least four times [21], which is not the case in any of the randomized models. Last but not least, the r_{cv}^2 s decrease drastically, to values between 0.143 and 0.372.

Structure	Actual	Biological Effect		
		Calculated		
		Equation 1	Equation 2	CoMFA
1	1	0.906	0.895	0.780
2	1	0.930	1.007	0.800
3	1	1.036	1.062	0.920
4	1	0.996	1.118	0.850
5	1	0.748	0.774	1.000
6	1	0.799	0.835	0.800
7	1	1.074	1.187	1.040
8	1	0.630	0.546	1.020
9	1	0.822	0.739	0.960
10	1	1.255	1.116	0.960
11	1	0.608	0.799	0.740
12	1	1.002	1.011	0.470
13	1	1.293	1.046	1.130
14	1	1.178	1.286	1.480
15	1	0.873	0.844	0.970
16	1	0.864	0.885	0.910
17	0	0.085	0.113	0.002
18	0	0.058	-0.005	0.270
19	0	-0.006	-0.169	-0.050
20	0	0.005	0.198	-0.130
21	0	-0.023	0.036	0.140
22	0	-0.006	0.093	0.070
23	0	-0.031	0.008	-0.070
24	0	-0.144	-0.025	0.010
25	0	0.814	0.079	-0.003
26	0	0.365	0.178	0.340
27	0	0.160	0.166	0.300
28	0	0.086	0.130	0.160
29	0	0.080	0.077	-0.040
30	0	0.007	-0.099	-0.080
31	0	0.261	0.212	0.190
32	0	-0.101	-0.146	0.070

Table 3. Actual and predicted biological effects. CoMFA prediction stems from the best model (3, Table 6)

Another test which confirms the good statistical qualities of the regression models (1) and (2) is the relatively high stability of the r^2_{cv} to different sizes of leave out groups, shown in Table 5

	Equations									
	1	2	3	4	5	6	7	8	9	10
s	0.426	0.467	0.445	0.392	0.407	0.466	0.411	0.427	0.403	0.473
F	7.453	6.569	5.654	8.006	9.550	6.746	6.443	7.347	5.539	5.734
r	0.582	0.424	0.529	0.679	0.630	0.428	0.638	0.579	0.671	0.401
r^2_{cv}	0.190	0.143	0.157	0.372	0.347	0.163	0.328	0.287	0.327	0.148
$F_{0.05,k_0-k-1}^a$	3.330	4.080	3.330	2.950	3.330	4.080	2.950	3.330	2.730	4.080

Table 4. Statistical parameters of ten regression models obtained by randomization of the dependent variables. ^a percentage point at 95 % confidence level

Finally, cluster analysis excellently supports the regression models. Performing the analysis with the molecular descriptors included in Eqn. (1) leads to 2 clusters, in which 30 out of 32 compounds are classified well. The dendrogram of this situation is presented in Fig. 3.

Using the descriptors from Eqn. (2)

in the cluster analysis, yields a slightly worse, but nevertheless good classification (28 out of 32 molecules are well classified).

Number of leave outs	r^2_{cv}	
	Eqn. (1)	Eqn. (2)
2	0.845	0.850
3	0.734	0.860
4	0.830	0.830
5	0.771	0.759
6	0.773	0.842

Table 5. Cross validated r^2 at different number of left out compounds

CoMFA Study

The results of CoMFA are summarized in Table 6. The models obtained with the three different probes [$sp^3C(+1)$, $sp^3O(-1)$ and $H(+1)$] and different grid spacing have comparable qualities, as reflected by the statistical parameters. The best model (no. 3, Table 6) has the highest predictive r^2 (r^2_{cv}) and the lowest standard error of prediction (*SEP*). Favorable and non favorable steric and electrostatic components of the molecular field are shown in Figure 4.

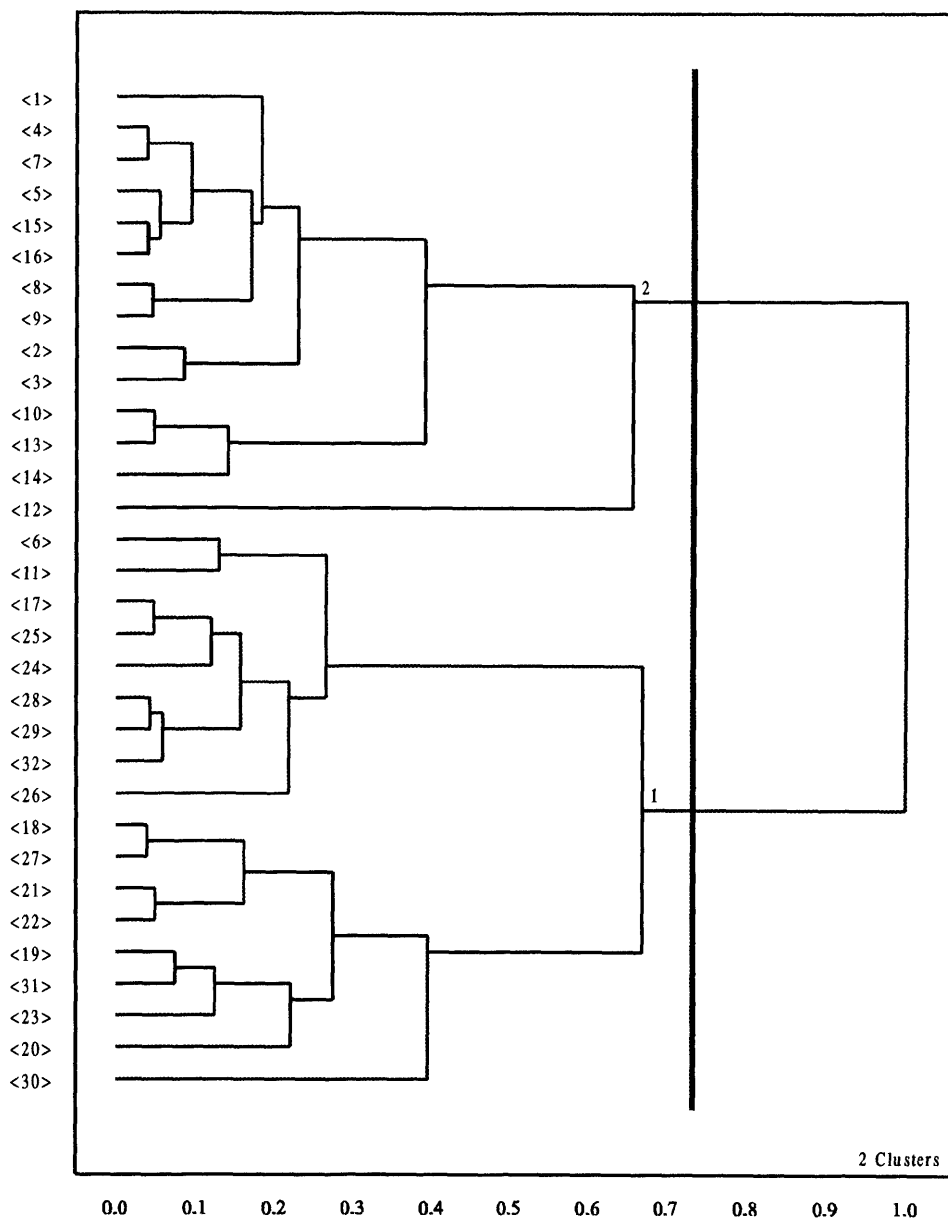


Figure 3. Dendrogram of the cluster analysis, performed with the molecular descriptors from Eqn. (1)

In Figure 4 light grey indicates unfavourable, dark grey favourable steric regions, i.e. bulky substituents in the light grey *zone* will diminish, in the dark grey region will increase the biological activity (bell pepper flavor). A corresponding picture for electrostatic interactions indicates the region(s) where a stronger negative field (light grey), or a stronger positive field (dark grey) increases the biological activity.

Model	Probe	Grid (Å)	No	r^2	s	F	r^2_{cv}	SEP ^b	StCb ^c
1	sp ³ C(+1)	1	2	0.866	0.193	93.441	0.689	0.293	79.1
2	sp ³ C(+1)	2	2	0.860	0.197	89.006	0.667	0.299	77.9
3	sp ³ C(+1)	3	2	0.855	0.200	85.443	0.696	0.290	78.4
4	sp ³ O(-1)	1	2	0.868	0.191	95.646	0.691	0.292	80.5
5	sp ³ O(-1)	2	2	0.866	0.193	93.337	0.680	0.297	78.6
6	sp ³ O(-1)	3	3	0.887	0.180	72.995	0.696	0.295	79.1
7	sp ³ H(+1)	1	2	0.868	0.191	95.600	0.689	0.293	80.3
8	sp ³ H(+1)	2	2	0.866	0.192	93.477	0.678	0.298	78.7
9	sp ³ H(+1)	3	3	0.890	0.177	75.822	0.696	0.295	80.6

^a SEP: standard error of prediction; ^b StCb: steric contribution

Table 6. Results of the different CoMFA models

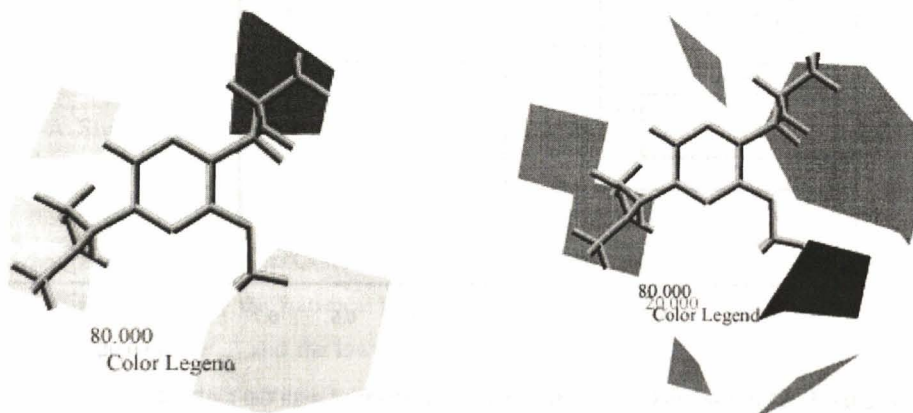


Figure 4 Left: light grey indicates unfavorable, dark grey favorable steric regions; Right: a stronger negative field (light grey), or a stronger positive field (dark grey) increases the biological activity. Molecules are oriented as in Figure 1.

DISCUSSION

The obtained correlation models [MLR and PLS (within CoMFA)] give consistent results, although the two mentioned methods are used in different approaches: While the 2D-QSAR MLR models calculate different properties of certain atoms (or substituents) of the molecule, CoMFA generates (a much higher) number of properties (sterical and electrostatical) in predefined grid points.

The two MLR models [Eqns. (1) and (2)] have nearly the same predictive power, and are somehow complementary to the CoMFA results: Eqn. (1) is in better agreement with the steric picture of CoMFA (Fig. 4), while Eqn. (2) is in better concordance with CoMFA concerning the electrostatical situation (Fig. 4).

Three of the four steric regions, which appear to be important according to CoMFA (Fig. 4), are reproduced by Eqn. (1). Increased favorable steric contribution to the biological activity (bell pepper flavor) in the region of substituent R_2 is reflected by the positive sign of the Verloop parameter $L^{(R2)}$. According to the absolute values of the regression coefficients obtained with scaled variables, the favorable steric contribution of R_2 is the most important parameter for the biological activity [in both, Eqn. (1) and (2)]. The decrease of the activity by bulky groups in substituent R_1 and in substituent R_3 is also modeled in agreement with CoMFA by the negative signs of the Verloops $L^{(R1)}$ and $B_3^{(R3)}$. Only the unfavorable contribution of bulky groups in the region of substituent R_4 as predicted by CoMFA, is not reproduced by the MLR equations.

In a similar fashion, Eqns. (1) and (2) suggest that increased negative charges on atoms C3 and C6 are of advantage for the bell pepper flavor, because the values of $C^{(C3)}$ and $C^{(C6)}$ are negative for all compounds and have negative regression coefficients. This situation is more or less in agreement with the CoMFA picture (Fig. 4). However, the favorable negative electrostatic field in this regions seems to result from both, the ipso *and* the substituents atoms. The favorable effect of a positive electrostatic field in the region of substituent R_1 resulting from CoMFA, is also in agreement with Eqn. (2): since the values of $C^{(R1)}$ can be positive and negative; the more positive $C^{(R1)}$ will be, the larger its contribution to “bell pepper flavor” will be. However, one has to keep in mind that the MLR models consider only the first atom of the substituent, while CoMFA takes into account the whole substituent. The differences of the two methods, 2D-QSAR and CoMFA, stem obviously from the differences in the approaches used. Considering the convergent results of the two approaches, these can be summarized as follows:

- a bulky, rather long shaped [correlation with $L^{(R2)}$] substituent R_2 is favorable for bell pepper flavor; this suggests the existence of a binding pocket for this substituent.
- an increased electrostatic field in the regions of atoms C3 and C6 (and the substituents R_2 and R_4 , respectively) is advantageous for bell pepper aroma impression.
- the substituent R_1 should not be too bulky; larger substituents than the methoxy group appear to be unfavorable for the biological activity.
- substitution at the other positions (C5 and C6) counteracts the biological activity.

However, one has to recognize that the bell pepper flavor impression is the result of synergistic interactions of the different contributions. The most general condition is, that the favorable binding energy of the flavor molecule to the receptor exceeds a certain value to induce a conformational change. Thus, not all of the above mentioned (positive and negative) contributions are absolute requirements for bell pepper flavor. Structures 11 or 22, for example, are not substituted at C3 (the favorable R_2 is missing) and yet the compounds have bell pepper flavor, structures 11, 22 and 14 are substituted at C6 (R_4) (unfavorable contribution) but still have bell pepper flavor.

The results are mostly in agreement with other models for structural requirements of the bell pepper flavor.

Parliment and Epstein [22] point out the importance of the magnitude of the alkyl substituents R_2 for bell pepper aroma, suggesting that larger substituents (up to 6-9 C-atoms) favor the aroma impression. This is in agreement with both approaches (MLR and CoMFA).

Masuda and Mihara [23] propose that besides the hydrophobic interaction stemming from the alkyl group R_2 , hydrogen bonding between the nitrogen atoms of the pyrazine nucleus and the heteroatom as donors on one hand, and acceptors from the receptor-pocket on the other hand, should be important for bell pepper flavor. Although we have no direct evidence for hydrogen bonding in our models, a favorable negative electrostatic field in the vicinity of the pyrazine nitrogen and the heteroatom of R_1 is in agreement with the hydrogen bonding hypothesis.

A more general model (including pyrazines, pyridines and thiazoles) has been proposed by Rognon and Chastrette [24] . It is presented in Figure 5 and will be briefly discussed.

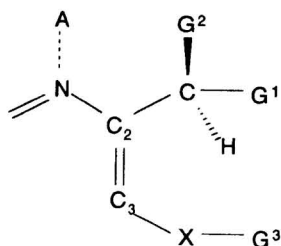


Figure 5. Structural requirements for the bell pepper flavor (following [24])

The bulky group at C2, with a volume between $34 \text{ \AA}^3$ and $85 \text{ \AA}^3$, is R_2 in our notation. However, R_2 is supposed to consist of two substructures, G^1 and G^2 , with G^1 lying in the N-C2-C3-plane. G^2 is preferentially a branched alkyl group. The sp^2 -nitrogen is assumed to form a hydrogen bond with a donor from the receptor. The substituent at C3, $-X-G^3$, is R_1 in our notation. It is supposed to be smaller (volume between $13 \text{ \AA}^3$ and $34 \text{ \AA}^3$) than R_2 . Dimensions and positions of G^1 , G^2 and G^3 are postulated to be relevant parameters for bell pepper flavor. The steric requirements of the model are, more or less, in agreement with our results (bulky R_2 , less bulky R_1). However, in our models no substructures, G^1 and G^2 , in R_2 are identified, since they do not exist in the pyrazine derivatives used.

CONCLUSIONS

The MLR models developed on the basis of 16 pyrazines with bell pepper and 16 pyrazines with no bell pepper flavor, have high predictive power, as reflected by the cross-validation r^2 (r^2_{cv}). The dependent variables are uncorrelated, and thus permit conclusion on the parameters important for bell pepper flavor. The results from MLR models are in good agreement with CoMFA and identify steric and electronic requirements for bell pepper aroma impression of pyrazine molecules. Moreover, the results are in good agreement with other models.

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