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**Original article**

# Interactions between β-lactoglobulin and flavour compounds of different chemical classes. Impact of the protein on the odour perception of vanillin and eugenol

Jutta REINERS, Sophie NICKLAUS, Elisabeth GUICHARD*

Laboratoire de Recherches sur les Arômes, INRA, 17 rue Sully, 21034 Dijon Cedex, France

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**Abstract** — Interactions between β-lactoglobulin and flavour compounds were analysed by instrumental and sensory studies. Affinity chromatography was used to determine binding constants of flavour substances belonging to different chemical classes. A systematic study on esters, pyrazines and phenolic compounds revealed that the increase in the hydrophobic chain length increases the affinity for the protein. Concerning the 4 diastereoisomers of 3-oxo-*p*-menthane-8-thiol, only the *cis-trans* isomery led to different binding constants. In general, binding constants increased with increasing log P values, except for the terpenic compounds studied, for which another explanation has to be found, taking into account the geometry of the molecule. Sensory analyses applying a matching test showed that the addition of β-lactoglobulin had no effect on the odour perception of vanillin, but brought about a significant decrease in the odour perception of eugenol.

**β-lactoglobulin / flavour / interaction / affinity chromatography / sensory analysis**

**Résumé — Interactions entre β-lactoglobuline et composés d'arôme de différentes classes chimiques. Impact de la protéine sur la perception de la vanilline et de l'eugénol.** Les interactions entre β-lactoglobuline et composés d'arôme ont été étudiées par des méthodes instrumentales et sensorielles. La chromatographie d'affinité a été utilisée pour déterminer les constantes d'affinité de molécules d'arômes de différentes classes chimiques. Une étude systématique sur des esters, pyrazines et composés phénoliques a montré que l'allongement de la chaîne hydrophobe augmente la constante d'affinité. Concernant les 4 diastéréoisomères du 3-oxo-*p*-menthane-8-thiol, seule l'isomérie

* Correspondence and reprints
Tel.: (33) 3 80 69 32 77; fax: (33) 3 80 69 32 27; e-mail: guichard@arome.dijon.inra.fr
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*cis-trans* conduit à des constantes différentes. De façon générale, l'affinité augmente avec le log P sauf pour les composés terpéniques étudiés, pour lesquels une autre explication doit être trouvée, tenant compte de la géométrie de la molécule. Un test d'appariement en analyse sensorielle a montré que l'ajout de β-lactoglobuline n'a pas d'effet sur la perception de l'odeur de vanilline mais induit une baisse significative de l'odeur de l'eugénol.

**β-lactoglobuline / arôme / interaction / chromatographie d'affinité / analyse sensorielle**

## 1. INTRODUCTION

Factors influencing the release of flavour compounds during eating have been reviewed by several authors [3, 17, 20]. They outlined the importance of proteins on flavour release due to the binding of flavour compounds. Reactions between flavour compounds and proteins have been summarized by Fischer and Widder [6] emphasizing the possibility of reversible and irreversible interactions.

β-Lactoglobulin (BLG) is one of the best characterized milk proteins. Its structure was determined by Papiz et al. [18] by standard crystallographic techniques, showing a similarity to the retinol binding protein. A possible binding site for retinol was identified by model-building. Monaco et al. [12], who analyzed the structure of BLG using X-ray diffraction methods, located the binding site for retinol in a hydrophobic pocket on the surface of BLG. More recently, Wu et al. [27] demonstrated, after co-crystallization of the complex, that palmitate was bound in the central cavity of the protein. Dufour et al. [4] proposed the presence of two binding sites based on their competition studies of retinol and protoporphyrin IX, which was confirmed by the results of Narayan and Berliner [13] on the simultaneous binding of retinol and fatty acids.

Sostmann and Guichard [25] showed that affinity chromatography allowed a rapid screening of flavour compounds (methyl ketones, alcohols and aldehydes) interacting with BLG. The calculated global affinity obtained for methyl ketones was in agreement with the values found by O'Neil and Kinsella [15]. Using the same methodology, Pelletier et al. [19] found no interactions between BLG and short-chain fatty acids or methyl pyrazine and hydrophobic interactions with esters from homologous series. The global affinity calculated was slightly lower than the value obtained by other methods on the same batch of protein, suggesting some hindrance of binding sites due to the immobilization of the protein.

No work has been undertaken until now on key flavour compounds such as ethyl-3-methyl butyrate and furaneol in strawberry aroma [23], or isopropyl-methoxypyrazines and vanillin in chocolate [24]. Therefore, these flavour-producing compounds were analyzed in the present study, together with related molecules in order to assess the influence of structural differences of substances on the binding to BLG.

Moreover, no previous study has investigated the influence of native BLG on flavour perception. BLG is used as an emulsifier or fat replacer by the food industry. For example, in ice creams flavoured with vanillin, Ohmes et al. [16] did not find any change in flavour perception when milk fat was replaced by whey-based fat replacers. Hansen and Heinis [8] found that the addition of a whey protein concentrate to a sucrose solution flavoured with vanillin decreased its flavour perception. In this study, our aim was to assess the real impact of pure BLG on the flavour perception of vanillin and of a structurally similar compound, eugenol.

## 2. MATERIALS AND METHODS

### 2.1. Materials

PEEK columns (5 cm × 4.6 mm i.d.) and supplements were purchased from Touzart & Matignon (Courtabœuf, France). β-Lactoglobulin (variants AB, purity 90%) was obtained from Besnier (Chateaulin, France), silica diol 'LiChroprep Diol' (25–40 μm) was from Merck (Darmstadt, Germany) and 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) was obtained from Sigma-Aldrich (Deisenhofen, Germany). Flavour compounds were from IFF (Longvic, France), Givaudan-Roure (Dübendorf, Switzerland), Sigma-Aldrich or Fluka (Neu-Ulm, Germany). Their purity was determined by high resolution gas chromatography. Purity of flavour compounds was ≥ 95% except for 5-ethyl-3-hydroxy-4-methyl-2(5*H*)furanone (93%), (1*R*, 4*R*)-*trans*- and (1*S*, 4*R*)-*cis*-3-oxo-*p*-menthane-8-thiol, 4-vinylguaiacol and nerol (90%), 3-ethyl-2-methoxypyrazine (87%), ethyl isobutyrate and 4-vinylphenol (83%), pulegone, (1*S*, 4*S*)-*trans*- and (1*R*, 4*S*)-*cis*-3-oxo-*p*-menthane-8-thiol (80%), (–)-carveol (98%; a mixture of isomers, 52% and 46%). The qualitative purity of vanillin and eugenol was checked by gas chromatography/olfactometry, applying the on-column injection technique. All other chemicals used were of p.a. quality. For sensory analysis, Evian mineral water was used.

### 2.2. Affinity chromatography

#### *2.2.1. Preparation of the columns*

The method of immobilizing the protein developed by Sostmann and Guichard [25] was modified with reference to Felix and Liu [5]. Silica diol was activated with tresyl chloride. The dried activated diol was then filled into a PEEK column under vacuum. A BLG solution was prepared by suspending 1.5 g of the protein in 150 mL buffer (0.3 mol·L$^{-1}$ sodium phosphate containing 0.3 mol·L$^{-1}$ sodium chloride, pH 7.2), and then filtered (0.45 μm). Immobilization of the protein was performed by connecting the PEEK column to a high-performance liquid chromatography (HPLC) pump and forcing 67.5 mL of the BLG solution (9.3 mg·mL$^{-1}$) to circulate at a flow rate of 0.5 mL·min$^{-1}$ for 18 h at room temperature. Then the column was rinsed successively at a flow rate of 1 mL·min$^{-1}$ with the following buffers: 0.2 mol·L$^{-1}$ Tris–HCl containing 1 mmol·L$^{-1}$ dithioerythriol, pH 8.0 (180 mL), 0.1 mol·L$^{-1}$ sodium phosphate containing 0.5 mol·L$^{-1}$ sodium chloride and 1 mmol·L$^{-1}$ dithioerythriol, pH 7.5 (90 mL), 0.1 mol·L$^{-1}$ sodium phosphate containing 1 mmol·L$^{-1}$ dithioerythriol, pH 7.5 (90 mL). The column was then rinsed with water containing 25 mmol·L$^{-1}$ NaCl, pH 3.0 for equilibration before use. No variation in pH was observed during the experiment. The amount of bound protein was calculated from the difference in BLG concentrations before and after immobilization (determined spectrophotometrically at 278 nm). The 4 columns produced contained 8.0, 8.2, 8.3 and 8.5 mmol·L$^{-1}$ BLG. Column production was thus repeatable under the conditions described above. Maximal immobilization of protein on the activated silica diol was indicated by the fact that BLG concentration in the solution after coupling did not decrease when increasing the time of circulation.

#### *2.2.2. High performance liquid chromatography*

The HPLC system consisted of a Waters 600 pump coupled to a Waters LC spectrophotometer model 481. Flavour compounds were detected at the following wavelengths, determined by recording UV spectra of their solutions in water (25 mmol·L$^{-1}$ NaCl, pH 3.0): ester (208 nm), pyrazines (214 nm), phenols (200 nm), 2-phenylethanol (206 nm), vanillin and ethylvanillin (204 nm), norfuraneol, furaneol and ethylfuraneol (286 nm), mesifurane (278 nm),

sotolon and abhexone (232 nm), pulegone (259 nm), (–)-carvone (242 nm), other terpenes (200 nm). Aqueous solutions of flavour compounds were injected using a Rheodyne injector with a 50-µL loop. The concentrations of flavour solutions were between 0.05 and 1 mmol·L$^{-1}$, depending on water solubility and sensitivity of UV absorption of the substances. Elution of the compounds was performed at room temperature at a flow rate of 1 mL·min$^{-1}$. A multichannel chromatography workstation (Almanza and Mielle, INRA, Dijon, France) was used for data acquisition and treatment of data. Retention times of flavour substances, at 2 different concentrations, and void times of the columns (injection of pure water) were determined for 2 columns containing BLG and for 1 column without BLG.

#### *2.2.3. Calculation of binding constants*

Binding constants ($K_B$) were calculated with reference to Nilsson and Larsson [14] basing on the retention times determined by affinity chromatography and on the protein concentration of the columns:

$$K_B = \frac{t_R - t}{c_P \times t_0} \ (L \cdot mol^{-1})$$

with:

$t_R$: retention time of the compound on the column containing protein,
t: retention time of the compound on the column without protein,
$c_p$: protein concentration (mol·L$^{-1}$),
$t_0$: void time.

This equation supposed that the concentration of ligand was small in comparison to the number of binding sites, which was the case. However, we verified that the value obtained was the same for 2 different concentrations of ligands.

### 2.3. Calculation of log P values

As BLG-flavour compound interactions are considered to be hydrophobic, log P values of the substances were calculated (Tab. I). If experimental values of related molecules were available, the π-method was applied [21]. The log P values for the terpenes and the furanones were calculated using the fragment method [7].

### 2.4. Sensory analysis

The test panel consisted of 15 experienced and inexperienced assessors (11 males and 4 females, aged from 25 to 70 years). Sensory evaluations were performed in a testing room equipped with computerized booths. Data acquisition and data treatment were conducted with FIZZ software (Biosystèmes, Dijon, France).

Samples (20 mL) were presented in brown glass bottles (60 mL) closed with screw caps. After an equilibration time of 4 h at 21 ± 1 °C, their odour intensities were evaluated.

In two training sessions, panelists were asked to classify 8 concentrations of vanillin and of eugenol. Additionally, they were familiarized with the method described in the following.

Odour intensities of aqueous solutions (Evian water, 25 mmol·L$^{-1}$ NaCl, pH 3.0) of vanillin and eugenol in the presence of β-lactoglobulin were estimated by applying the matching test according to Rousseau et al. [22]. The concentration ranges of BLG and of the flavour compounds had been determined in preliminary tests.

During an experimental session, panelists were first provided with a set of 8 samples of the flavour compound analyzed, which served as a reference. Concentrations ranged from 1.60 to 447 mg·L$^{-1}$ (vanillin) and from 0.16 to 45 µL·L$^{-1}$ (eugenol), respectively, differing by a concentration step of √5. The samples coded from 1 to 8 were spotted on a continuous line scale overlapping the 8 reference points, ranging from 0 to over 8. Subjects were asked to sniff the 8 samples in order of increasing concentration, and to

**Table I.** Binding constants $K_B$ determined by affinity chromatography and log P values of analyzed substances (SD = standard variation on 2 columns).

**Tableau I.** Constantes d'affinité $K_B$ déterminées par chromatographie d'affinité et valeurs de log P pour les composés analysés (SD = coefficients de variation sur 2 colonnes).

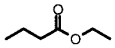
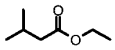
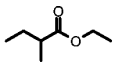
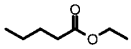
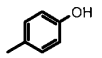
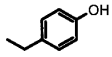
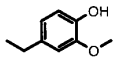
| Flavour compound | | $K_B$ (SD %) | log P |
|---|---|---|---|
| Ethyl isobutyrate |  | 132 (5) | 1.51[a] |
| Ethyl butyrate |  | 136 (1) | 1.71[a] |
| Ethyl 3-methylbutyrate |  | 284 (7) | 2.01[a] |
| Ethyl 2-methylbutyrate |  | 288 (7) | 2.01[a] |
| Ethyl pentanoate |  | 366 (7) | 2.21[a] |
| 2-Methoxypyrazine |  | 47 (16) | –0.24[b] |
| 3-Methyl-2-methoxypyrazine |  | 62 (10) | 0.32[b] |
| 3-Ethyl-2-methoxypyrazine |  | 171 (3) | 0.82[b] |
| 3-Isopropyl-2-methoxypyrazine |  | 452 (8) | 1.12[b] |
| 3-Isobutyl-2-methoxypyrazine |  | 795 (9) | 1.62[b] |
| 3-*sec*-Butyl-2-methoxypyrazine |  | 912 (9) | 1.62[b] |
| 2-Phenylethanol |  | 132 (5) | 1.36[c] |
| 4-Methylphenol (*p*-cresol) |  | 440 (3) | 1.95[c] |
| 4-Ethylphenol |  | 888 (5) | 2.26[c] |
| 2-Methoxyphenol (guaiacol) |  | 245 (2) | 1.33[c] |
| 4-Ethyl-2-methoxyphenol (4-ethylguaiacol) |  | 830 (5) | 2.38 [d] |

**Table I/Tableau I.** (Continued/Suite).

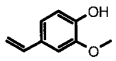
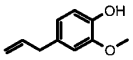
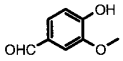
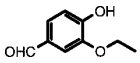
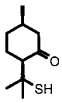
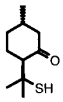
| Flavour compound | | $K_B$ | log P |
|---|---|---|---|
| 4-Ethenyl-2-methoxyphenol (4-vinylguaiacol) |  | 1 165 (1) | 2.08[d] |
| 2-Methoxy-4-(2-propenyl)phenol (eugenol) |  | 1 360 (1) | 2.58[d] |
| 4-Hydroxy-3-methoxybenzaldehyd (vanillin) |  | 319 (3) | 1.26[c] |
| 3-Ethoxy-4-hydroxybenzaldehyd (ethylvanillin) |  | 475 (2) | 1.76[e] |
| Linalool |  | 565 (9) | 2.91[f] |
| Nerol |  | 1 134 (10) | 3.00[f] |
| α-Terpineol |  | 483 (9) | 3.15[f] |
| (-)-Carveol |  | 542 (14) | 2.60[f] |
| (-)-Carvone |  | 748 (7) | 1.91[f] |
| Pulegone |  | 857 (5) | 2.46[f] |
| α-Menthone |  | 1 138 (4) | 3.01[f] |
| *cis*-3-oxo-*p*-Menthane-8-thiol 1 |  | 208 (1) | 3.10[f] |
| *rans*-3-oxo-*p*-Menthane-8-thiol |  | 1 461 (2) | 3.10[f] |

**Table I/Tableau I.** (Continued/Suite).

| Flavour compound | | $K_B$ | log P |
|---|---|---|---|
| 4-Hydroxy-5-methyl-3(2*H*) furanone (norfuraneol) |  | 4 (100) | 0.24 [f] |
| 4-Hydroxy-2,5-dimethyl-3(2*H*) furanone (furaneol) |  | 16 (2) | 0.78 [f] |
| 2-Ethyl-4-hydroxy-5-methyl-3(2*H*) furanone (ethylfuraneol) |  | 39 (3) | 1.32[f] |
| 4-Methoxy-2,5-dimethyl-3(2*H*) furanone (mesifurane) |  | 19 (7) | 1.61[f] |
| 3-Hydroxy-4,5-dimethyl-2(5*H*) furanone (sotolone) |  | 31 (14) | –0.22[f] |
| 5-Ethyl-3-hydroxy-4-methyl-2(5*H*) furanone (abhexone) |  | 82 (4) | 0.32[f] |

[a] Calculated based on the experimental value of ethyl propionate (1.21, [6]) by applying the π-method [15].
[b] Calculated basing on the experimental value of pyrazine (–0.22, [6]) by applying the π-method [15].
[c] Mean of the experimental values cited by Hansch and Leo [6].
[d] Calculated based on the experimental value of guaiacol by applying the π-method [15].
[e] Calculated based on the experimental value of vanillin by applying the π-method [15].
[f] Calculated by applying the fragment method [6].

[a] Calculé à partir de la valeur expérimentale du propionate d'éthyle (1,21, [6]) en appliquant la π-méthode [15].
[b] Calculé à partir de la valeur expérimentale de la pyrazine (–0,22, [6]) en appliquant la π-méthode [15].
[c] Moyenne des valeurs expérimentales citées par Hansch et Leo [6].
[d] Calculé à partir de la valeur expérimentale du guaiacol en appliquant la π-méthode [15].
[e] Calculé à partir de la valeur expérimentale de la vanilline en appliquant la π-méthode [15].
[f] Calculé en appliquant la méthode des fragments [6].

memorize the intensity of the odours perceived. After this calibration exercise, panelists had to estimate the odour intensity of 9 samples coded with 3-digit random numbers, following a presentation order based on a Latin square. The samples were prepared according to a complete factorial design with the flavour compound factor at 3 levels (8, 40 and 200 $mg \cdot L^{-1}$ vanillin or 0.8, 4.0 and 20.0 $\mu L \cdot L^{-1}$ eugenol, respectively) and the BLG factor at 3 levels (0, 0.3 and 1.0%). Their odour intensities were evaluated by comparing them with the references and by marking their position on the line scale. Sessions were duplicated.

Sensory data were subjected to a 3-way analysis of variance (3-way ANOVA) testing the 3 following factors (concentration of BLG, concentration of flavour compound, assessor) and all interactions, considering the assessor factor as random.

Means and confidence limits ($P = 0.05$) were calculated. Sample means were compared by the multiple range test of Newman-Keuls and of Dunnett ($P = 0.05$).

## 3. RESULTS

### 3.1. Affinity chromatography

After stocking the HPLC columns for several weeks, retention times decreased independently on the use of the columns. The $K_B$ values obtained with a freshly prepared and an aged column differed for most of the substances by 25 to 35%. This was also observed by Vidal-Madjar et al. [26], and may be due to protein degradation, contamination by organic substances or microbial agents. In order to minimize this phenomenon, Aubel and Rogers [1] added sodium azide. As we noticed interactions between sodium azide and BLG, we did not add this antimicrobial agent. Binding constants were calculated from the retention times obtained on 2 freshly prepared columns (Tab. I). In these conditions, the obtained values were highly reproducible (CV < 10%). Log P values of the substances were calculated as mentioned in Table I.

For the esters, a significant increase in the binding constants by increasing the number of C-atoms of the acid chain from 3 (ethyl butyrate) to 4 (ethyl pentanoate) was found, thus confirming previous results obtained by Pelletier et al. [19]. The effect of branching on a hydrophobic chain was studied for 2 series of esters. Branching on a chain length of 3 C-atoms had practically no influence on the binding constants of the esters (ethyl butyrate and ethyl isobutyrate had the same $K_B$), whereas the 2- and 3-branched esters of pentanoic acid showed lower $K_B$ values and lower log P values than the corresponding linear ester. Obviously, the position of the methyl group was not important, as ethyl 2-methylbutyrate and ethyl 3-methylbutyrate had the same binding constants.

2-Methoxypyrazine had a rather low binding constant, corresponding to its negative log P value. $K_B$ and log P increased with increasing chain length of the substituent in position 3. Here, differing from the esters, the position of branching influenced $K_B$. The binding constant of 3-isobutyl-2-methoxypyrazine was lower than that of 3-*sec*-butyl-2-methoxypyrazine, although the 2 substances have the same theoretical hydrophobicity. The difference between the constants of the structural isomers was smaller than the difference between $K_B$ of the pyrazines differing by 1 C-atom in the substituent chain.

When comparing the phenols with different functional groups (4 methyl with 4-ethylphenol, guaiacol with 4-ethylguaiacol or 4-vinylguaiacol with eugenol, vanillin with ethylvanillin), a prolongation of the substituent chain length always enhanced the hydrophobicity and hence the interactions of the compound with the protein. 2-Phenylethanol had a rather low binding constant, especially when compared with 4-ethylphenol which has a similar structure and the same number of C-atoms in the side-chain, but with a lower hydrophobicity. Introduction of a methoxy function in position 2 of 4-ethylphenol had very little influence on $K_B$ or on log P. Only a slight decrease in the binding constant of 4-ethylguaiacol was observed. The double bond of 4-vinylguaiacol caused an increase in $K_B$ compared to the same structure with a single bond (4-ethylguaiacol) with lower hydrophobicity. The vanillin aldehyde group was responsible for only a slight but significant increase in $K_B$ of guaiacol.

In general, the terpenes analyzed had high binding constants compared with the other classes of substances, indicating strong interactions with β-lactoglobulin. The terpenic compounds are rather apolar, in spite of their carbonyl or alcohol functions. For ketones with a similar structure, $K_B$ values are related to log P values. For the alcohols, the differing binding constants cannot be explained by the log P values.

Steric effects have been studied by application of affinity chromatography to a chiral substance. Four enantiomerically pure thiols were analyzed: *cis*-(1*R*, 4*S*)- and (1*S*, 4*R*)- as well as *trans*-(1*R*, 4*R*)- and

(1*S*, 4*S*)-3-oxo-*p*-menthane-8-thiol. While retention times on the HPLC column without protein were exactly the same for all 4 enantiomers, they were different on the column containing BLG for *cis*- and *trans*-isomers. $K_B$ of the 2 *trans*-isomers were similar, and significantly higher than $K_B$ of the 2 *cis*-compounds.

The furanones showed very low or almost no interactions with β-lactoglobulin expressed by the lowest binding constants of all analyzed substances. Log P values were also rather low. No interaction was found for norfuraneol (4-hydroxy-5-methyl-3(2*H*)-furanone), having only 1 methyl group in the furanone ring. Increasing the proportion of the apolar substituents led to an increase in hydrophobicity and $K_B$ (norfuraneol, furaneol, ethylfuraneol). Replacement of the hydroxyl group in furaneol by a methoxy group (mesifurane) doubled log P, but did not change $K_B$. When comparing sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone) with furaneol (4-hydroxy-2,5-dimethyl-3(2*H*)-furanone) and abhexone (5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone) with ethylfuraneol (2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone), $K_B$ of the 2(5*H*)furanones was 2-fold the $K_B$ of the corresponding 3(2*H*)furanones, though log P values behaved in the reverse manner. Sotolon and ethyl furaneol showed the same behaviour to BLG in spite of the ethyl group of the 3(2*H*)-furanone. The relation between binding constant $K_B$ and the hydrophobicity of the analyzed substances is depicted in Figure 1.

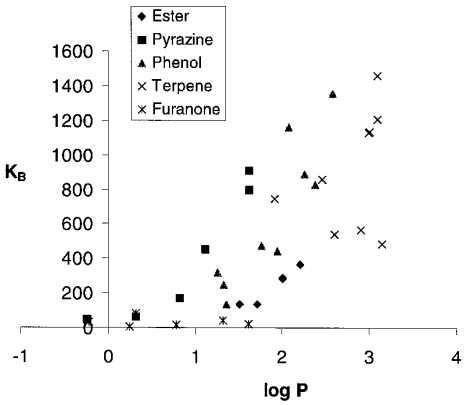



**Figure 1.** Binding constants $K_B$ in relation to hydrophobicity of the analyzed substances.

**Figure 1.** Relation entre hydrophobicité et constante d'affinité $K_B$ pour les composés analysés.

### 3.2. Sensory analysis

Vanillin was selected because it is commonly used in the aromatization of dairy products and eugenol because of its similar structure, different chemical properties and higher binding constants. The interactions between BLG and vanillin can be of reversible and irreversible nature, due to the possibility of the aldehydic function to form Schiff bases with free $NH_2$-groups of the protein. However, affinity chromatography only measures reversible interactions [25].

BLG concentrations were chosen based on their natural content (0.3%) in milk. A 3-way ANOVA was performed in order to establish the effect of each variable (concentration of vanillin or eugenol, concentration of β-lactoglobulin, assessor) and their interactions on perceived odour intensities.

Results are shown in Table II. For the analysis of the vanillin odour intensity, significant effects of the vanillin concentration, of the assessors and of the β-lactoglobulin-assessor interaction were found. For the analysis of the eugenol odour intensity, significant effects of the eugenol concentration, of the assessor and of the interactions between eugenol and BLG were observed.

Perceived odour intensities of the flavour compounds in aqueous solutions with or without BLG are depicted in Figures 2 and 3. A significant difference between samples was observed in only 1 case: at a concentration of 20 $\mu L \cdot L^{-1}$, eugenol odour intensity was significantly ($P = 0.05$) diminished by 1% BLG. Performing the Newman-Keuls

**Table II.** Matching test of vanillin and eugenol. Results of 3-way ANOVA.
**Tableau II.** Test d'appariement pour la vanilline et l'eugénol. Résultats de l'ANOVA à 3 facteurs.

| | Vanillin | | Eugenol | |
|---|---|---|---|---|
| Factor | *F*-value | *P*-value | *F*-value | *P*-value |
| Concentration of flavour (F) | 131.41 | < 0.0001* | 238.43 | < 0.0001* |
| Concentration of β-lactoglobulin (BLG) | 1.75 | 0.1929 | 2.31 | 0.1175 |
| Assessor (A) | 2.35 | 0.0061* | 4.85 | < 0.0001* |
| F × BLG | 0.19 | 0.9432 | 3.01 | 0.0254* |
| F × A | 1.25 | 0.2023 | 0.96 | 0.5234 |
| BLG × A | 1.57 | 0.0466* | 1.14 | 0.3074 |
| F × BLG × A | 1.33 | 0.0916 | 0.98 | 0.5221 |

* Significant at $P \leq 0.05$.

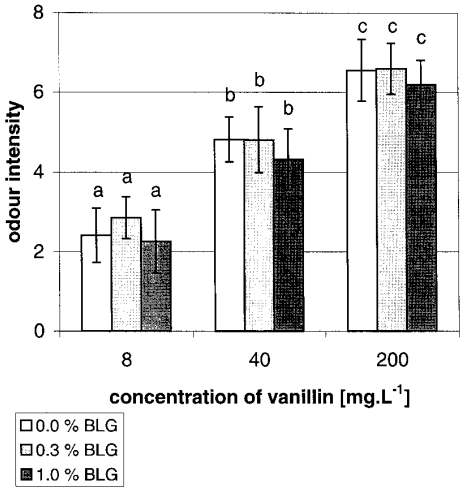



**Figure 2.** Results of the matching test for vanillin; **a**, **b**, **c** are the groups from the Newman-Keuls test ($P = 0.05$).

**Figure 2.** Résultats du test d'appariement pour la vanilline. **a**, **b**, **c** sont les groupes du test de Newman-Keuls ($P = 0,05$).

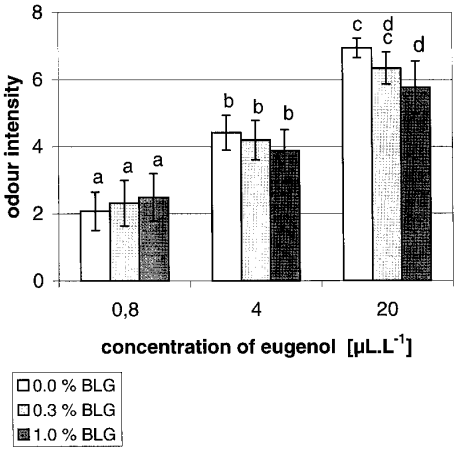



**Figure 3.** Results of the matching test for eugenol. **a**, **b**, **c**, **d** are the groups from the Newman-Keuls test ($P = 0.05$).

**Figure 3.** Résultats du test d'appariement pour l'eugénol. **a**, **b**, **c**, **d** sont les groupes du test de Newman-Keuls ($P = 0,05$).

test at $P = 0.1$ revealed that the 3 samples containing 20 $\mu L \cdot L^{-1}$ eugenol and 0, 0.3 and 1% BLG were significantly different. No significant effect was observed for the vanillin samples.

## 4. DISCUSSION

### 4.1. Affinity chromatography

As interactions between BLG and small molecules are considered to be of hydrophobic

nature, binding constants $K_B$ were related to log P values of the substances (Fig. 1). Regarding especially the pyrazines, an increase in the chain length of the substituent caused an increase in the hydrophobicity of the molecules and therefore of their interactions with BLG. Pelletier et al. [19] did not observe any significant interaction between different alkylpyrazines and BLG. On the other hand, Boudaud and Dumont [2] observed quenching of the fluorescence emission of BLG by 3-isobutyl-2-methoxypyrazine and also by 2,3,5-trimethylpyrazine, indicating complex formation. They concluded that the ring structure and not the methoxy group was important for the interactions between BLG and the pyrazines. In the present study, 3-isobutyl-2-methoxypyrazine and 3-*sec*-butyl-2-methoxypyrazine with the same theoretical hydrophobicity differ in their binding constants (Tab. I), indicating a steric influence. We suppose that the 3-isobutyl-2-methoxypyrazine enters less easily into the hydrophobic pocket of BLG because of the ramification at the end of the chain resulting in weaker interactions with the protein. This steric effect was less important than the hydrophobicity effect, because the difference in $K_B$ values of the 2 stereoisomers was only 13%, while the difference between $K_B$ of 3-isopropyl- and of 3-isobutylmethoxypyrazine was 43%.

The interactions of an alkyl chain with BLG can also be reduced by a polar group, as in the case of 2-phenylethanol. The OH function at the end of the hydrophobic substituent is responsible for weaker interactions when compared with the $K_B$ of 4-ethylphenol. The $K_B$ of 4-vinylguaiacol is higher than that of 4-ethylguaiacol despite its lower hydrophobicity. This may be explained by a stabilization of the 4-vinylguaiacol–BLG-complex resulting from a structural analogy between the planar flavour compound and the planar side-chain of an amino acid in the hydrophobic pocket of BLG. Monaco et al. [12] suggested that the planar ring of β-ionone was fixed by phenylalanine. The same effect was discussed for tryptophan by Papiz et al. [18] for the other binding site of BLG.

Introduction of a methoxy group in the phenol ring led to only a slight decrease in $K_B$ (when comparing 4-ethylguaiacol with 4-ethylphenol), while the introduction of an aldehyde function led to a slight increase in $K_B$ (when comparing vanillin with guaiacol). The observed effect was only small but significant, as was the difference in the log P values.

That hydrophobicity cannot be the only factor influencing BLG-flavour compound interaction is also evident when regarding the results obtained for the terpenic alcohols (Tab. I). Linalool and nerol, with a similar polarity, differ in $K_B$ by a factor of 2. The higher $K_B$ of nerol can be explained by the position of the OH function at the end of the carbon chain, allowing the rest of the molecule to enter into the hydrophobic pocket of BLG. In linalool, the hydroxyl group is positioned within the hydrophobic chain. Therefore, only a part of the molecule interacts with the protein. The cyclic terpenic alcohols are supposed to enter by the apolar end of the molecule opposite the OH function into the hydrophobic pocket of BLG. Other parameters taking into account the topological characteristics of the molecule may be investigated using QSAR (quantitative structure-activity-relationship).

Apart from the described steric effects of different origin, the influence of the stereochemistry of flavour compounds on their interactions with BLG was shown by the analysis of the 4 3-oxo-*p*-menthane-8-thiol enantiomers. As the only difference between the molecules was the arrangement of the substituents in positions 1 and 4, and as each substituent is once in the axial and once in the equatorial position for *cis*- and *trans*-isomers respectively, the difference in $K_B$ for *cis*- and *trans*-3-oxo-*p*-menthane-thiol must be due to the relative position of the 2 substituents. Lamiot et al. [9] also found a greater affinity for *trans* isomers of dodecadienols

in comparison to the *cis* isomers. However, we observed no stereoselectivity of BLG for these compounds, which confirms the results obtained by Massolini et al. [11]. The importance of the geometry of the molecule has also been pointed out by Lübke et al. [10], who showed, by infrared spectroscopy, that the fixation of retinol and β-ionone into the hydrophobic cavity induced no significant conformational change in BLG, whereas the fixation of α-ionone did. Both isomers only differ by the position of the cyclohexene double bond, which is in the 'retinol-like' position for β-ionone.

The weak interactions between BLG and the furanones are supposed to be due to the polarity of the furanone ring combined with only a few short hydrophobic side-chains. The higher $K_B$ values of the 2(5*H*)furanones, sotolone and abhexone, compared to the $K_B$ values of the corresponding 3(2*H*)furanones, furaneol and ethylfuraneol, may be due to the proximity of the apolar substituents.

Looking at Figure 1, a general tendency toward increased interactions indicated by increasing $K_B$ with increasing hydrophobicity (expressed by log P values) was observed. The $K_B$ rose when the number of C-atoms in the side-chain of the ligand (pyrazines) was increased. For less homogeneous classes, for example when functional groups change (phenols and related compounds like 2-phenylethanol, vanillin and ethylvanillin; terpenes), the relation between $K_B$ and log P is not evident. Obviously, the topological characteristics of flavour molecules play an important role in their interactions with BLG, which are not taken into account by calculated log *P*-values.

### 4.2. Sensory analysis

The assessor effect was significant for the sample sets of vanillin and eugenol, meaning that the assessors estimated the intensity of the odorants in a different way. A different use of the scale may be due to the interindividual variations in the perception of BLG odour. The odour of BLG could also be the reason for the BLG–assessor interaction observed for the vanillin sample set. The significance of this factor results from the different tendencies of the assessors in evaluating the samples containing 0, 0.3 and 1% BLG.

The vanillin and the eugenol effects were significant, as samples were grouped according to their flavour compound concentration. The *F*-value for the concentrations of vanillin and eugenol was much higher than the *F*-value for the assessor effect, reflecting the greater importance of the flavour compounds. The higher *F*-value for eugenol compared with that for vanillin indicated a greater difference between the 9 samples when they contained eugenol as the odorous substance.

There was a significant effect for the eugenol-BLG interaction, corresponding to the fact that a significant influence of BLG on the perception of the odour intensity of eugenol was observed for only a certain concentration of eugenol. This means that the influence of BLG on the odour intensity of eugenol is dependent on the eugenol concentration. Headspace analyses performed on the same solutions showed a retention of around 15% eugenol by BLG, which is in agreement with the binding constants calculated and the concentrations used.

No significant effect was found for vanillin, in agreement with the relatively low binding constant, indicating weaker interactions with BLG. It was not possible to detect vanillin by headspace analysis at the concentrations studied.

## 5. CONCLUSION

Affinity chromatography is a rapid, repeatable method to measure global affinity between BLG and flavour compounds. Even if the reversible interactions measured are closed related to the hydrophobicity of the

molecules, topological characteristics have to be taken into account for a better interpretation. The influence of binding on odour intensity was demonstrated for 2 phenolic compounds in water solution, showing a significant effect only for the compound with the higher binding constant. Further experiments are in progress to study the incidence of these interactions on flavour perception in real food systems.

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## REFERENCES

[1] Aubel M., Rogers L.B., Effects of pretreatment on the enantioselectivity of silica-bound bovine serum albumin used as high-performance liquid chromatographic stationary phases, J. Chromatogr. 392 (1987) 415–420.

[2] Boudaud N., Dumont J.-P., Interaction between flavor components and β-lactoglobulin, in: McGorrin R.J., Leland J.V. (Eds.), Flavor-Food Interactions, ACS Symp. Ser. 633, Am. Chem. Soc., Washington, DC, 1996, pp. 90–97.

[3] Delahunty C.M., Piggott J.R., Current methods to evaluate contribution and interactions of components to flavour of solid foods using hard cheese as an example, Int. J. Food Sci. Technol. 30 (1995) 555–570.

[4] Dufour E., Marden M.C., Haertlé T., β-Lactoglobulin binds retinol and protoporphyrin IX at two different binding sites, FEBS Lett. 277 (1990) 223–226.

[5] Felix G., Liu M., New method for grafting proteins on silica gel, Biol. Sci. 8 (1989) 2–6.

[6] Fischer N., Widder S., How proteins influence food flavor, Food Technol. 51 (1997) 68–70.

[7] Hansch C., Leo A., Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley & Sons, New York, USA, 1979.

[8] Hansen A.P., Heinis J.J., Decrease of vanillin flavor perception in the presence of casein and whey proteins, J. Dairy Sci. 74 (1991) 2936–2940.

[9] Lamiot E., Dufour E., Haertlé T., Insect sex pheromone binding by bovine β-lactoglobulin, J. Agric. Food Chem. 42 (1994) 695–699.

[10] Lübke, M., Guichard, E., Le Quéré, J.L., Infrared spectroscopic study of β-lactoglobulin interactions with flavour compounds, in: Roberts D., Taylor A. (Eds.), Flavour Release, ACS Symp. Ser., Am. Chem. Soc., Washington, DC, 2000 (in press).

[11] Massolini G., De Lorenzi E., Lloyd D.K., McGann A.M., Caccialanza G., Evaluation of β-lactoglobulin as a stationary phase in high-performance liquid chromatography and as buffer additive in capillary electrophoresis: observation of a surprising lack of stereoselectivity, J. Chromatogr. 712 (1998) 83–94.

[12] Monaco H.L., Zanotti G., Spandon P., Bolognesi M., Sawyer L., Eliopoulos E.E., Crystal structure of the trigonal form of bovine β-lactoglobulin and of its complex with retinol at 2.5 Å resolution, J. Mol. Biol. 197 (1987) 695–706.

[13] Narayan M., Berliner L.J., Fatty acids and retinoids bind independently and simultaneously to β-lactoglobulin, Biochemistry 36 (1997) 1906–1911.

[14] Nilsson K., Larsson P.-O., High performance liquid affinity chromatography on silica-bound alcohol dehydrogenase, Anal. Biochem. 134 (1983) 60–72.

[15] O'Neil T.E., Kinsella E., Binding of alkanone flavors to β-lactoglobulin: effects of conformational and chemical modification, J. Agric. Food Chem. 35 (1987) 770–774.

[16] Ohmes R.L., Marshall R.T., Heymann H., Sensory and physical properties of ice creams containing milk fat or fat replacers, J. Dairy Sci. 81 (1998) 1222–1228.

[17] Overbosch P., Afterof W.G.M., Haring P.G.M., Flavor release in the mouth, Food Rev. Int. 7 (1991) 137–184.

[18] Papiz M.Z., Sawyer L., Eliopoulos E.E., North A.C.T., Findlay J.B.C., Sivaprasadarao R., Jones T.A., Newcomer M.E., Kraulis P.J., The structure of β-lactoglobulin and its similarity to plasma retinol–binding protein, Nature 324 (1986) 383–385.

[19] Pelletier E., Sostmann K., Guichard E., Measurement of interactions between β-lactoglobulin and flavor compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography, J. Agric. Food Chem. 46 (1998) 1506–1509.

[20] Plug H., Haring P., The influence of flavour-ingredient interactions on flavour perception, Food Qual. Pref. 5 (1994) 95–102.

[21] Rekker R. F., The Hydrophobic Fragmental Constant: Pharmacochemistry Library, in: Nauta W.T., Rekker R.F. (Eds.), Elsevier Sci. Publ. Co., Amsterdam, Vol. 1, 1977.

[22] Rousseau F., Castelain C., Dumont J.P., Oil-water partition of odorant: discrepancy between sensory and instrumental data, Food Qual. Pref. 7 (1996) 299–303.

[23] Schieberle P., Hofmann T., Evaluation of the character impact odorants in fresh strawberry juice by quantitative measurement and sensory studies on model mixtures, J. Agric. Food Chem. 45 (1997) 227–232.

[24] Schnermann P., Schieberle P., Evaluation of key odorants in milk chocolate and cocoa mass by aroma extract dilution analyses, J. Agric. Food Chem. 45 (1997) 867–872.

[25] Sostmann K., Guichard E., Immobilized β-lactoglobulin on a HPLC-column: a rapid way to determine protein–flavour interactions. Food Chem. 62 (1998) 509–513.

[26] Vidal-Madjar C., Jaulmes A., Racine M., Sebille B., Determination of binding equilibrium constants by numerical simulation in zonal high-performance affinity chromatography, J. Chromatogr. 458 (1988) 13–25.

[27] Wu S.Y., Perez M.D., Puyols P., Sawyer L., β-Lactoglobulin binds palmitate within its central cavity, J. Biol. Chem. 274 (1999) 170–174.