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**1H NMR investigation of self-association of vanillin in aqueous solution**

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**Abstract.** A self-association of vanillin have been studied by 1H NMR spectroscopy using the analysis of proton chemical shifts changes in aqueous solution as a function of concentration. The experimental results have been analysed using indefinite non-cooperative and cooperative models of molecular self-association, enabling the determination of equilibrium constants, parameters of cooperativity and the limiting values of vanillin proton chemical shifts in the complex. It was found that the dimer formation creates energetically favourable conditions for subsequent molecular association.

1. Introduction
The process of molecular association induces local and global changes to the molecules involved, which alter their physical and chemical behaviour in solution. The π-π stacked aromatic interactions have bee found to govern diverse supramolecular organizations and aggregates in both the solution and the solid state [1]. In comparison to more conventional interactions, such as hydrophobic interactions and hydrogen bonds, the π-π stacked interaction is not so clear and no commonly used model has been built to interpret the experimental observations. Therefore, the study on the variety of systems involved in the π-π stacked interaction is important to fully understand this type of noncovalent interaction.

Vanillin, on 4-hydroxy-3-methoxybenzaldehyde is the primary component of the extract of the vanilla bean. Natural “vanilla extract” is a mixture of several hundred different compounds in addition to vanillin. Artificial vanilla is a solution of pure vanillin, usually of synthetic origin. Because of the scarcity and expense of natural vanilla extract, there has long been interest in the synthetic preparation of its predominant component. Today, most artificial vanillin is produced from the petrochemical raw material guaiacol [2]. The largest use of vanillin is as flavouring, usually in sweet foods. The ice cream and chocolate industries together comprises 75% of the market of vanillin as flavouring. Vanillin displays antioxidant and antimicrobial properties and may be used as a food preservative [3]. It is also used in the fragrance industry, in perfumes, and the mask unpleasant odours or tastes of drugs. Vanillin has been encapsulated in oxidized starches [4], and in β-cyclodextrin [5] in order to enhance the protection towards oxidation.

In this paper, the concentration dependences of chemical shifts of the non-exchange able protons of vanillin have been investigated, for a quantitative determination of the equilibrium constants of self-association, as well as the limiting values of proton chemical shifts of the molecular complexes.
2. Experimental

1.1. Materials
Vanillin was purchased from Alfa Aesar GmbH&CoKG, Germany and used without any further purification. Deuterium oxide (99.7% D) was obtained from Heavy Water Plant Romag-Prod, Romania.

1.2. Apparatus
The NMR experiments were performed on a Bruker Avance III 500 MHz spectrometer. The $^1$H NMR spectra were recorded in D$_2$O solution at 295 K and all chemical shifts were measured relative to TMS. Typical conditions were as follows: 32 K data point, sweep width 3500 Hz, giving a digital resolution of 0.1 Hz/point. The 90° pulse width was 10 μsec and the spectra were collected by co-addition of maximum 128 scans.

3. Results and discussion
The $^1$H NMR spectrum of vanillin in aqueous solution is shown in figure 1. Assignment of vanillin protons was obtained from 1D and 2D (COSY) experiments and is presented in table 1.

![Figure 1. $^1$H NMR spectrum of 2 mM vanillin in D$_2$O.](image)

<table>
<thead>
<tr>
<th>Proton</th>
<th>$\delta$ (ppm)</th>
<th>$J$ (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1</td>
<td>9.6400</td>
<td></td>
</tr>
<tr>
<td>H-2</td>
<td>7.4644</td>
<td>$J_{2,3} = 2.0$</td>
</tr>
<tr>
<td>H-3</td>
<td>7.4741</td>
<td>$J_{3,4} = 8.2$</td>
</tr>
<tr>
<td>H-4</td>
<td>6.9890</td>
<td></td>
</tr>
<tr>
<td>-CH$_3$</td>
<td>3.8641</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. $^1$H NMR chemical shift values and coupling constants, $J$, of 2 mM vanillin in D$_2$O.

In order to obtain information about the solution behaviour of vanillin, the dependence of proton chemical shift on concentration was analysed. The dependence of proton chemical shift on concentration of vanillin is reported in figure 2. A general decrease in proton chemical shift values was observed with increasing concentration, suggesting that vanillin is involved in a self-association process through stacking interactions [6]. This interaction causes a shielding term modulation due to
change in intensity of the anisotropic magnetic field arising from electron motion in the aromatic rings. The experimental results have been analysed using indefinite non-cooperative and cooperative models of molecular self-association [7], enabling the determination of equilibrium constants, parameter of proton chemical shifts in the complex.

![Figure 2](image)

**Figure 2.** Experimental concentration dependences of chemical shifts of vanillin non-exchangeable protons.

1.3. Analysis by indefinite non-cooperative (isodesmic) model

The isodesmic model of solute self-association is based on the assumption that solute molecules associate to from stacks (dimers, trimers etc.) where the equilibrium constant $K$ for each step is the same.

Thus

$$A_j + A \rightleftharpoons K A_{j+1}$$

(1)

It can be shown that:

$$[A_h] = \frac{[A]}{(1 - K[A])^2}$$

(2)

where $[A_h]$ is the total concentration of solute $A$ and $[A]$ is the concentration of the monomer in solution. Further it is assumed that the proton chemical shifts changes observed for a solute molecule binding at the end of a stack are half those observed for a molecule associating in the middle of a stack [8]:

$$\delta_j = \frac{(j-1)\delta_i + \delta_m}{j}$$

(3)

where $\delta_i$ is the proton chemical shift of the j-mer, $\delta_m$ is the proton chemical shift of the monomer, i.e. at infinite dilution and $\delta_j$ is the maximal proton chemical shift of solute $A$ present in a stack.

Owing to rapid exchange in self-association reactions, the observed chemical shift of all species present in solution is given by:
\[
\delta_{\text{obs}} = \frac{[A] \delta_m + 2[A_j] \delta_d + \ldots + [A_i] \delta_i + \ldots}{[A_n]} = \left[ \frac{[A]}{[A_n]} \left\{ \frac{\delta_m}{1 - K[A]} + \frac{\delta_i [A]}{(1 - K[A])^2} \right\} \right] \tag{4}
\]

Substituting equation (2) into equation (4) gives:
\[
\delta_{\text{obs}} = \left( \frac{\gamma + 4 K[A_n] - 1}{2 K[A_n]} \right) \delta_m + \left( 1 + \frac{1 - \sqrt{1 + 4 K[A_n]}}{2 K[A_n]} \right) \delta_i \tag{5}
\]

We note that a dimerization model assuming only the shift values \(\delta_m\) and \(\delta_{\text{dimer}}\), with an association constant \(K_{\text{dimer}}\), gives an identical equation the only differences being that \(K\) is replaced by \(2 K_{\text{dimer}}\) and \((\delta_i - \delta_m)\) is replaced by \((\delta_{\text{dimer}} - \delta_m)\) which is equivalent to \((\delta_i - \delta_m)/2\), since a molecule in a dimer is effectively at the end of a stack of two.

Therefore, NMR chemical shift data cannot be used to distinguish dimerization from stacking, if the isodesmic model is applicable. The parameters \(\delta_m\), \(\delta_i\), and \(K\) in equation (5) were calculated from a least-squares fitting of the data to the isodesmic model. Table 2 presents the obtained results. In order to estimate the probability of formation a vanillin complexes of higher order than dimers, the experimental results have also been analysed using the indefinite cooperative model of molecular self-association.

1.4. Analysis by indefinite cooperative (semi-isodesmic) model

This model allow for the possibility that the equilibrium constant for the first step in a self-association dimer formation differs from the remaining constants. In this model the equilibrium constants of reactions (1) are assumed to be equal for all \(j \geq 2\) (\(K = K_3 = K_4 = \cdots = K_i\)) and \(K_{\text{dimer}} = \sigma K\). If the dimer forms more easily than subsequent additions, \(\sigma > 1\), while if the dimer forms with more difficulty, \(\sigma < 1\). The case \(\sigma = 1\) corresponds to the isodesmic model considered above.

Taking into account that the mass conservation law for reactions (1) is given by:
\[
[A_n] = \sum_{j=1}^{\infty} j [A_j] \tag{6}
\]
and:
\[
[A_j] = \sigma K [A]^j ; [A_j] = \sigma K^{j-1} [A] ; \ j \geq 2 \tag{7}
\]
the summation in equation (6) leads to:
\[
[A_n] = (1 - \sigma) [A] + \frac{\sigma [A]}{(1 - K[A])^2} \tag{8}
\]

The observed proton chemical shift, \(\delta_{\text{obs}}\) is given by:
\[
\delta_{\text{obs}} = \frac{1}{[A_n]} \sum_{j=1}^{\infty} j [A_j] \delta_j \tag{9}
\]

Substituting equations (3) and (7) in equation (9) we obtain:
\[
\delta_{\text{obs}} = \frac{[A]}{[A_n]} \left( \frac{\sigma K [A]}{1 - K[A]} \right) \delta_m + \frac{\sigma K [A]}{(1 - K[A])^2} \delta_i \tag{10}
\]

Equation (8) is cubic in \([A]\), and was solved analytically. Equation (10) contains there unknown parameters \(\delta_m\), \(\sigma\) and \(K\) to by determined from experimental concentration dependences of vanillin proton chemical shifts. We developed a computer programme based on an iteration procedure, in order
to fit the experimental values of $\delta_{\text{obs}}$ to the approximate equation. Each iteration sets up a quadratic programme to determine the direction of search and the loss function:

$$E = \sum_{i=1}^{N} \left( \delta_{\text{exp}}^{i} - \delta_{\text{calc}}^{i} \right)^{2}$$

(11)

until the search converges. $N$ is the number of experimental points. The values of $E$ obtained in the present calculations are in all cases centred around $6 \cdot 10^{-5}$ indicating a very good fit to the experimental data. The mean values of the calculated parameters of equation (10) are presented in table 2.

<table>
<thead>
<tr>
<th>Table 2. Calculated self-association parameters of vanillin.</th>
<th>Isodesmic model</th>
<th>Semi-isodesmic model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proton</td>
<td>$\delta_{\text{m}}$ (ppm)</td>
<td>$\delta_{i}$ (ppm)</td>
</tr>
<tr>
<td>H-1</td>
<td>9.6466</td>
<td>8.0686</td>
</tr>
<tr>
<td>H-2</td>
<td>7.4756</td>
<td>5.6040</td>
</tr>
<tr>
<td>H-3</td>
<td>7.4801</td>
<td>6.2404</td>
</tr>
<tr>
<td>H-4</td>
<td>6.9960</td>
<td>5.3486</td>
</tr>
<tr>
<td>$-\text{CH}_3$</td>
<td>3.8682</td>
<td>2.9745</td>
</tr>
<tr>
<td>Dimerization</td>
<td>$\delta_{\text{dimer}} = \delta_{i}$</td>
<td>$K_{\text{dimer}} = 5.31±0.86$ M$^{-1}$</td>
</tr>
<tr>
<td>$K_{\text{dimer}} = 1.07±0.23$ M$^{-1}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Conclusion

In the present study, experimentally measured chemical shifts of vanillin up to a concentration of 50 mM, provide clear evidence that vanillin undergoes self-association in solution as the concentration increases.

Two modes which account for the self-associative properties of vanillin were tested. The model which gave the most satisfactory fit to the experimental data was one in which self-association occurs according to an indefinite cooperative scheme. The value of $\sigma \approx 0.5$ for vanillin self-associative proves that this complexation process is cooperative. Hence one may assume that formation of j-mers ($j > 2$) complexes of vanillin in aqueous solution is favourable from an energetic point of view and the cooperative model of molecular association is realized in the conditions of the experiment.

References