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Study of aroma compound–natural polymer interactions by dynamic coupled column liquid chromatography

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Abstract

Dynamic coupled column liquid chromatography (DCCLC) was used to study the interactions between the model aroma compounds β-ionone and limonene and the model food components dextrin and soya bean trypsin inhibitor. Solutions saturated with aroma compound can be obtained by a dynamic and reversible process. Interactions between the two categories of molecules were evidenced using exponential dilution and DCCLC, and the reversibility of the complex formation between limonene and trypsin inhibitor was established. The association constants K_a calculated for the complexes dextrin–limonene and trypsin inhibitor–limonene were 13 650 ± 250 and 2 944 ± 784 M^{-1}, respectively. The decrease of the calculated K_a values when β-ionone and dextrin are used is indicative of an irreversible adsorption of β-ionone on dextrin whereas a weak interaction, $K_a = 385 \pm 105\ M^{-1}$ is obtained in the presence of trypsin inhibitor.

1. Introduction

Interactions between aroma compounds and macromolecules in aqueous solution are generally studied using equilibrium methods: liquid–liquid partitioning [1–3], gas–liquid partitioning [4] or equilibrium dialysis [5–9]. These methods, however, have several drawbacks: difficulty in determining the equilibrium which is reached after varying lengths of time, non-specific binding and volatization of aroma compounds occurring particularly during equilibrium dialysis [10]. It is, moreover, generally difficult to obtain a quantitative extraction of aroma compounds. The use of dynamic methods based on gas chromatography, such as exponential dilution [11,12] or liquid chromatography have therefore been investigated for the study of these interactions [13–15].

The decrease and increase of the infinite dilution activity coefficient of volatile components in the presence of macromolecules, as a result of the interactions occurring between these categories of molecules [16–18], can be determined by exponential dilution. This method is particularly well adapted for molecule screening.

DCCLC, initially introduced to study the aqueous solubility of polycyclic aromatic hydrocarbons (PAHs) [19,20] is derived from the Hummel and Dreyer gel-filtration techniques [13]. According to this method, a saturated solution of an hydrophobic compound is gener-

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ated by pumping water through a generator column containing glassbeads coated with the hydrophobic compound. The concentration of this compound, which corresponds to the solubility of the compound in water, is determined by HPLC using a reversed phase contained in the analytical column. A short C_{18} reversed-phase column, the extractor column, can be placed between the generator and the analytical columns. When a macromolecule (M) aqueous solution is used instead of water for the generator column flow, an increase in the solubility of the hydrophobic compound (H), resulting from complex formation, is detected by HPLC. The association constant (K_a) corresponding to the equilibrium:

$$M + H \rightleftarrows MH$$

$$K_a = \frac{[MH]}{[M][H]}$$

may be calculated, assuming that the hydrophobic compound concentration in the generator column is not depleted due to extensive complex formation, according to:

$$k_a = \frac{S_t - S_0}{[M]_t S_0}$$

where S_0 is the solubility of the hydrophobic compound in water, S_t its solubility in macromolecular solution and $[M]_t$ the initial macromolecular concentration.

The objective of the present study is to adapt DCCLC in order to determine the intensity of the interactions between food constituents such as aroma compounds and natural polymers previously detected by exponential dilution. Limonene and β-ionone and dextrin and soya bean trypsin inhibitor were chosen as models compounds for these two categories of components.

2. Experimental

2.1. Material

Corn starch dextrin Tackidex J060K was kindly donated by Roquette Frères (62136 Lestrem). Soya bean trypsin inhibitor, typeII-S, limonene and β-ionone were from Sigma (St. Louis, MO, USA).

2.2. Dynamic coupled column liquid chromatography (DCCLC)

DCCLC equipment, similar to that used by May et al. [19,20] was used. The stainless steel generator column (25 × 0.46 cm I.D.) was fitted with glass beads 80–120 mesh coated with aroma compounds (1% w/w).

For the determination of aroma compound solubility this column was eluted with distilled water at 25°C at a flow-rate varying from 2.0 to 4.7 ml/min using a Milton Roy pump. The saturated aqueous solutions were introduced through the sample loop (20 μl) of a 6-port switching valve (Rheodyne) onto the HPLC column.

For the determination of aroma compound solubility in the presence of macromolecules the distilled water was replaced by an aqueous solution of the macromolecule under study.

The extraction column, when used, located between the generator and analytical column in the place of the sample loop, was a Bondapack C_{18}, 300 Å, 37–55 μm, 6 × 0.46 cm I.D. column (Waters).

2.3. High-performance liquid chromatography

A Shimadzu LC 9A pumping system fitted with a Spheri 5 RP_{18}, 5 μm, 25 × 0.46 cm I.D. (Applied Biosystem, San José, CA, USA), a Varian 2550 UV detector and a Shimadzu CR 6A integrator was used. The column was eluted with acetonitrile–water (80:20, v/v) at a flow rate of 1 ml/min. Detection was performed at 294 nm for β-ionone and 210 nm for limonene, the aroma compound concentrations were calculated from the peak areas using response coefficients determined for each compound from solutions of known concentration.

2.4. Exponential dilution

The exponential dilution equipment as previously described [17] was used. Dilute solutions of

volatile compounds contained in an equilibrium cell, maintained at 25 ± 0.1°C, were stripped by nitrogen at a flow-rate between 30 and 100 ml/min according to the nature of the volatile compound under study. The gas leaving the cell was automatically sampled every 1 to 3 min through a 6-way electropneumatic valve VICI thermostated at 150°C. This valve was operated by the chromatograph relay.

A Varian Model 3300 chromatograph, fitted with an FID detector and a CP-Sil 5 CB 5 (methyl silicone) (Chrompack, Middelburg, Netherlands) silica capillary column (50 m × 0.32 mm I.D.) and a Shimadzu CR 3A integrator, isothermally operated at 150°C, was used for dynamic head space analysis.

An all-glass closed diffusion cell as described by Duhem and Vidal [12] for non-foaming materials was used, the gas flow was dispersed into small-diameter bubbles through a glass frit disk (No. 4).

A 0.5-μl volume of limonene or 50 μl of β-ionone were added with a chromatographic syringe fitted with a long needle to 30 g of water contained in the diffusion cell. The infinite dilution activity coefficient in water, γ^{∞}_{iw}, was calculated from the exponential decrease of the volatile compound concentration determined by GLC.

For the determination of the infinite dilution activity coefficient in the presence of a macromolecular system, γ^{∞}_{im}, the same quantities of aroma compounds were introduced in 30 g of water containing 0–5% (w/w) dextrin, 0–2% (w/w) of trypsin inhibitor. The mixture was incubated for 2 h at 25°C under magnetic stirring prior to measurement.

The reduced activity coefficient γ^{∞}_{ir}, defined by the ratio $\gamma^{\infty}_{im}/\gamma^{\infty}_{iw}$, gives a good indication of the extent of these interactions [16,17].

2.5. Statistical treatment of data

The population standard deviation indicated in Table 3 has been calculated with the equation:

$$\sqrt{\frac{n\Sigma x^2 - (\Sigma x)^2}{n^2}}$$

The mean difference test (two-sided) accounts for a significant difference between two means μ_1 and μ_2 in two normal distributions $N(\mu_1,\sigma^2)$ and $N(\mu_2,\sigma^2)$.

From a n_1-size sample $(x_{11}, x_{12}, \cdots, x_{1n1})$ and a n_2-size sample $(x_{21}, x_{22}, \cdots, x_{2n2})$, the hypothesis, to be tested is $H_o:\mu_1 = \mu_2$ against the alternative hypothesis $H_1:\mu_1 \neq \mu_2$. The test is performed using the t-distribution of degree of freedom $(n_1 + n_2 - 2)$:

$$\frac{|\bar{X}_1 - \bar{X}_2|}{\sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)\left(\frac{S_1 + S_2}{n_1 + n_2 - 2}\right)}} > t\left(\frac{\alpha}{2}, n_1 + n_2 - 2\right)$$

μ_1: population mean 1; μ_2: population mean 2
$\bar{X}_1$: sample mean 1; $\bar{X}_2$: sample mean 2
S_1: sum of square 1; S_2: sun of square 2
σ^2: population variance; α: significance level (5%)

$$S = \Sigma(x - \overline{X})^2$$

3. Results and discussion

3.1. Preliminary study

When DCCLC is used, the first step in the calculation of the association constant is the determination of the solubility of the aroma compound in water. It was necessary to check the constancy of the aroma compound concentration at the exit of the generator column for sufficiently large eluent volumes.

The results given in Figs. 1 and 2 for β-ionone and limonene show that solutions with a constant concentration may be obtained after a short purge period. It was also shown, using limonene, that a modification (increase or decrease) of the flow-rate of water through the generator column when the equilibrium is reached slightly alters the concentration. The fact that the equilibrium is rapidly restored is indicative of the dynamic and reversible nature of the transfer process.

Solubility, which is the mean concentration calculated for a flow-rate of 2 ml/min in the generator column, was determined from the data of Figs. 1 and 2 for β-ionone and limonene, the

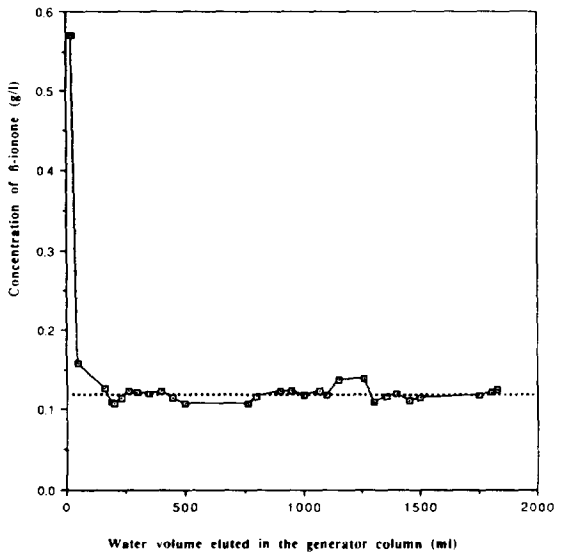

Fig. 1. Variation of the concentration of β-ionone aqueous solution (g/l) eluted from the generator column as a function of the water volume (ml) passed through the generator column. (□) β-Ionone concentration. (· · · · ·) mean β-ionone concentration.

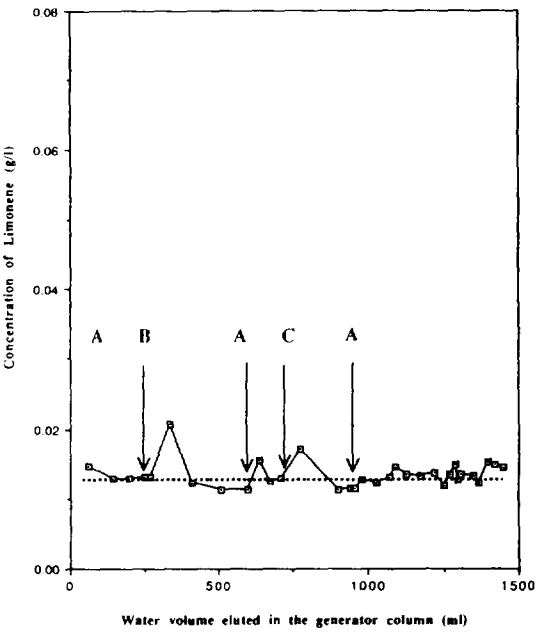

Fig. 2. Variation of the concentration of limonene aqueous solution (g/l) eluted from the generator column as a function of the water volume (ml) passed through the generator column at several flow-rates; (A) 2.0 ml/min, (B) 3.7 ml/min, (C) 4.7 ml/min. (□) Limonene concentration, (· · · · ·) mean limonene concentration.

values obtained being 119.6 ± 7.9 mg/l and 12.7 ± 1.9 mg/l, respectively.

A major problem in determining the solubility of hydrophobic compounds is that adsorption on the vessel walls generally leads to underestimated values. This problem is largely avoided using DCCLC, although adsorption may occur in the sample loop which alternately contains saturated solutions of aroma compound and solvent used for elution of the HPLC column.

The adsorption of limonene, the most hydrophobic compound used in this study, was evaluated with different volumes of the saturated solution pumped through the sample loop before injection. The increase in limonene concentration detected by HPLC when the volume of the limonene saturated solution passed through the sample loop was more than 2 ml (Table 1) is indicative of adsorption of limonene on the sample loop walls. According to this result a volume of saturated solution less than 2 ml should have been used for the determination of the solubility of limonene or β-ionone.

It was found that the concentration column used for PAH studies [19,20] was not needed when β-ionone and limonene were used as the hydrophobic compounds.

3.2. Evidence for interactions between aroma compounds and polymers

A decrease in γ_{ir}^{∞} is observed when limonene is added to an aqueous solution of dextrin or of soya bean trypsin inhibitor (Fig. 3). As a consequence of the interactions occurring between the aroma compounds and the macromolecular systems in aqueous solutions, the reduced infinite dilution activity coefficient γ_{ir}^{∞} varies with the polysaccharide or peptide concentration. A decrease indicates the retention of the aroma compound by the macromolecule whereas an increase indicates an increase of the vapor pressure of the volatile compound in the vapor phase known as "salting-out effect" [3,16–18]. The

Table 1
Adsorption from a saturated solution of limonene on the sample loop walls

Volume of limonene saturated solution passed through the sample loop (ml)	Limonene concentration (mg/l)	Adsorption (%)
1	13.00	0
2	12.72	0
3	14.32	11
5	16.26	22
10	19.54	35

Water flow-rate (generator column): 2 ml/min. Limonene concentration is determined by HPLC, Spheri 5 RP 18 (5 μm, 25 × 0.46 cm I.D.) column, eluted with acetonitrile–water (80:20, v/v) at a flow-rate of 1 ml/min. Detection at 210 nm.

observed decrease of the limonene γ_{ir}^{∞} is the result of the retention of this compound by the two polymers.

This retention is confirmed using DCCLC. When the generator column coated with β-ionone or limonene is eluted with a solvent containing dextrin or soya bean trypsin inhibitor, an increase of the peak area of the compound–limonene or β-ionone–in the HPLC chromatogram is observed (Fig. 4). This increase, corresponding to an increase in the solubility of the aroma compound, is the result of the formation of a complex between the aroma compound and the polysaccharide or the peptide [21].

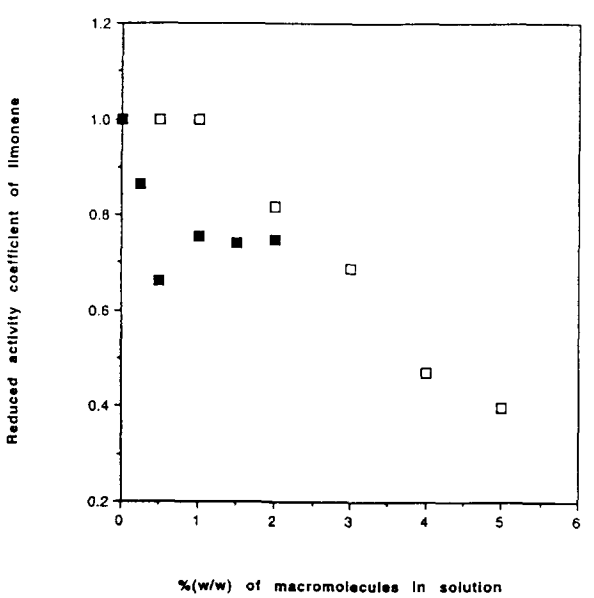

Fig. 3. Variations of limonene reduced infinite dilution activity coefficient as a function of: (□) dextrin Tackidex J060K, (■) soya bean trypsin inhibitor percent (w/w) in water.

3.3. Complex reversibility

The reversibility of the complex formation between limonene and dextrin or soya bean trypsin inhibitor was established from the results obtained with alternate elution with water and with the macromolecular system solutions from the generator column coated with limonene. As indicated in Table 2 the limonene concentration rapidly increased from ca. 13 mg/l, corresponding to the water solubility of this compound, to ca. 21 mg/l when 0.5% (w/w) of trypsin inhitor is substituted for water. When this solvent is used again, the limonene solubility again decreases to its original value. Analogous results were obtained in the presence of dextrin. The rate of the increase and decrease of the limonene concentration is a strong argument for the reversibility of the complex formation [22].

3.4. Determination of association constants

The values of the association constants for limonene and dextrin and soya bean trypsin inhibitor, calculated according to the equation

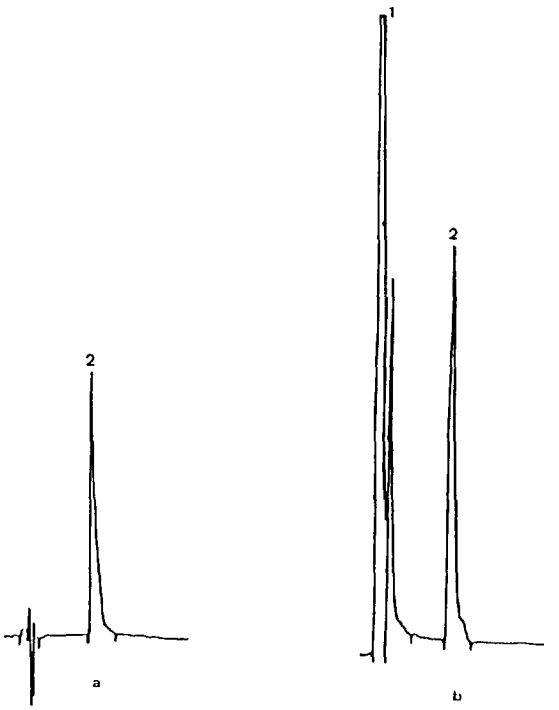

Fig. 4. HPLC chromatogram of limonene eluted at a flow-rate of 2 ml/min from the generator column, (a) with water, (b) with a soya bean trypsin inhibitor aqueous solution (10 g/l). Spheri 5 RP 18 (5 μm, 25 × 0.46 cm I.D.) column; elution solvent, acetonitrile–water (80:20, v/v) at a flow-rate of 1 ml/min; detection at 210 nm. Peaks: 1 = trypsin inhitor, 2 = limonene.

given in the Introduction, are given in Table 3. A 1:1 stoichiometry for the complexes is assumed, and the values reported are means of the values obtained for different generator column eluent volumes. A mean difference test reveals no significant differences at the 95% confidence level for the values obtained for different macromolecule concentrations in the eluent, 0.5–5% for dextrin and 0.25–1% for soya bean trypsin inhibitor. Since the association constants of the complexes formed between limonene and dextrin and soya bean trypsin inhibitor are independent of the macromolecule concentration it can be concluded that neither the macromolecule concentration nor the limonene concentration are limiting.

Under the same experimental conditions, when elution of the generator column coated with β-ionone is performed with dextrin solutions (1 and 2.5% w/w), a steady decrease of the calculated value for the association constant is observed when the elution volume is increased (Table 4). According to Blyshak et al. [21] this phenomenon is indicative of an irreversible adsorption of β-ionone to dextrin.

When a β-ionone generator column is eluted using a 0.5% soya bean trypsin inhibitor solution the β-ionone solubility is unchanged relative to pure water. In contrast, the β-ionone solubility is increased and the association constant $K_a = 385 \pm 105\ M^{-1}$ can be calculated when the trypsin inhibitor concentration is increased to 1% (w/w). The K_a value obtained is indicative of a weak interaction between the two components.

Table 2
Reversibility of limonene–soya bean trypsin inhibitor complex: solubility of limonene as a function of the nature and the volume of eluent used

Eluent	Eluent volume (ml)	Limonene concentration (mg/l)
Water	60	14.68
	200	12.99
	600	15.53
	1000	12.41
Soya bean trypsin inhibitor (0.5% w/w)	50	21.86
	100	31.04
	150	20.74
	175	20.80
Water	20	14.53
	100	13.26
	200	13.51

Solvent flow-rate (generator column): 2 ml/min. Limonene concentration is determined by HPLC, Spheri 5 RP 18 (5 μm, 25 × 0.46 cm I.D.) column, eluted with acetonitrile–water (80:20, v/v) at a flow-rate of 1 ml/min. Detection at 210 nm.

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Table 3
Association constants (M^{-1}) for dextrin–limonene and soya bean trypsin inhibitor–limonene complexes as a function of the macromolecule percent

Macromolecule (%)	K_a (M^{-1})	
	Dextrin	Soya bean trypsin inhibitor
0.25		2929 ± 207
0.50	15 607 ± 2094	2932 ± 1124
1.00	16 122 ± 2573	2978 ± 220
2.50	12 801 ± 1008	-
5.00	11 963 ± 603	-
Mean	13 650 ± 250	2944 ± 784

Solvent flow-rate (generator column): 2 ml/min. Limonene concentration is determined by HPLC, Spheri 5 RP 18 (5 μm, 25 × 0.46 cm I.D.) column, eluted with acetonitrile–water (80:20, v/v) at a flow-rate of 1 ml/min. Detection at 210 nm.

Table 4
Variation of the calculated values for the association constant of the dextrin–β-ionone complex as a function of the dextrin solution elution volume for two dextrin concentrations

Elution volume	K_a (M^{-1})	
(ml)	Dextrin 1%	Dextrin 2.5%
40	3960	3580
60	3360	3247
120	3028	3207
160	2762	3114
200	2728	3021
250	2495	2875
280	2395	2821

Solvent flow-rate (generator column): 2 ml/min. β-Ionone concentration is determined by HPLC, Spheri 5 RP 18 (5 μm, 25 × 0.46 cm I.D.) column, eluted with acetonitrile–water (80:20, v/v) at a flow-rate of 1 ml/min. Detection at 294 nm.

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