Biomimetic Supramolecular Biophenol−Carbohydrate and Biophenol−Protein Models by NMR Experiments

Armandodoriano Bianco,[†] Ugo Chiacchio,[‡] Antonio Rescifina,[‡] Giovanni Romeo,*[,§] and Nicola Uccella[||]

Dipartimento di Chimica, Università "La Sapienze", Roma, Italy, Dipartimento di Scienze Chimiche, Università di Catania, Italy, Dipartimento Farmaco-Chimico, Università di Messina, Italy, and CIRASAIA−Centro Interdipartimentale Ricerche Applicate Sperimentali in Industria Agroalimentare, Università della Calabria, Arcavacata di Rende, Italy

Biophenols of plant origin, found in traditional Mediterranean foods, show relevant physiological functions and influence the taste sensorial perception. The taste phenomenon involves the establishment of a multiple reversible equilibrium of supramolecular formation among molecular components. The complex taste process has been evaluated by NMR biomimetic experiments performed in abiotic conditions. 3,4-Dihydroxyphenylacetic acid, a model compound, was investigated by NMR titration with caffeine and β-cyclodextrin, respectively. The kinetics and energetics of the process show that the supramolecular interactions, controlled by weak forces occurring at short distance, increase according to the order β-cyclodextrin > caffeine, suggesting that polysaccharides could play a fundamental role in the mechanism that controls the taste perception.

Keywords: *Biophenols; β-cyclodextrin; supramolecular chemistry; taste perception*

INTRODUCTION

Traditional Mediterranean foods (MF) exert several physiological effects in human nutrition (Hackett, 1986) due to the molecular microcomponents; thus, the quality and the authenticity of the food product deserve to be assured and protected (Casuscelli et al., 1994; Romeo and Uccella, 1996).

Olive products, table olive and olive oil, wine, citrus fruits, and aromatic herbs (all of plant origin), are common MF characterized by biophenols (BP) as micro-ingredients. The polident functionality of the natural *o*-diphenolic compounds influences the sensory and nutritional aspects of fresh and processed foods derived from *Olea europaea* L., representing a taste substrate (St) in the sensorial perception (Shallenberger, 1993) and an antioxidant and chelating agent (Castelli et al., 1995). Good relationship between the BP distribution with the organoleptic features relative to the flavor and fragrance of the alimentary product as well as the stability of olive oils has been ascertained (Montedoro et al., 1993).

The taste phenomenon involves the establishment of a multiple reversible equilibrium of supramolecular formation among molecular components (Haslam, 1996). The complex process of the sensorial perception can be evaluated as a supramolecular dynamic interaction between the sensorial receptors, the proteic and glycidic material present in the oral cavity, and BPs. The different sensorial response to the taste stimulus may depend on the dimension and molecular structure of the BP with a different supramolecular reactivity.

The interaction between BPs and protein or carbohydrate models at the molecular level, as regarding the products of *Olea europaea*, can be investigated by sensorial biomimetic experiments performed via NMR methodology in abiotic aqueous solution in order to dissect and discriminate the key parameters of the BP functions in biological systems.

EXPERIMENTAL PROCEDURES

NMR measurements were performed on a Varian VXR-300 spectrometer (Palo Alto, CA) equipped with a temperature control unity (± 0.1 °C). All experiments were conduced in a D_2O solution [0.05 M for caffeine (Caf) and 0.002 M for β-cyclodextrin (β-CD)] containing 0.03% of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard. In a typical experiment, 0.5 mL of Caf or β-CD solution was added with increasing amounts of the 0.5 M solution of BP in order to cover a concentration ratio BP/Caf or BP/β-CD varying from 1:1 to 60:1. The phase-sensitive ROESY spectra were acquired with a spectral width of 2204 Hz in 2 K data points using 32 scans for each of the 512 t_1 increments. The spin lock time was set to 400 ms. The data matrix was zero-filled to 2 K × 1 K, and a Gaussian function was applied for processing in both dimension ($G_f = 0.120$, $G_{f1} = 0.012$).

RESULTS AND DISCUSSION

The biomimetic interaction process between BP and proteins or carbohydrates requires subsequent steps of approximation to give a correct interpretation of the event of competitive supramolecular formation between BP proteins and BP carbohydrates. The first step of approximation can be achieved by Caf as a molecular model: this alkaloid easily replaces proteins in the supramolecular association with BP (Spencer et al., 1988). In fact, Caf is characterized by a molecular structure reminiscent of proline-rich peptides, particularly in the functionality -$CONMe_2$- for the presence of the carbonyl group and the tertiary proline-like nitrogen atom; finally, in the aromatic moiety, Caf provides a good site of supramolecular interaction with BP.

The biomimetic model for carbohydrates can be afforded by CDs. The evaluation of the biomimetic

* Corresponding author (e-mail, romeog@imeuniv.unime.it; fax, +39-90-6766562).
[†] Università Tor Vergata.
[‡] Università di Catania.
[§] Università di Messina.
[||] Università della Calabria.

S0021-8561(97)00266-5 CCC: $14.00

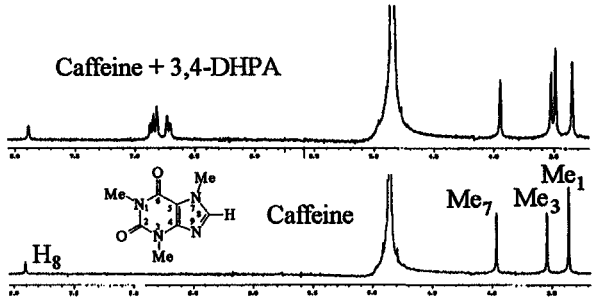


Figure 1. Superimposed NMR spectra of caffeine and caffeine + 3,4-dihydroxyphenylacetic acid (1:1).

phenomenon in NMR experiments, through the comparison of formation constants of the corresponding supermolecule (K_s), has been performed by monitoring the shift of the appropriate resonances in the molecular species of reference.

The titration of 3,4-dihydroxyphenylacetic acid (3,4-DHPA), a model compound, has been carried out with a solution of Caf in D_2O as shown in Figure 1. Addition of the BP results in the modification of the NMR frequencies of the signals of both partners: observation of the NMR signals of Caf supplies informations on the nature of supermolecule formation. Typically, an upfield shift of Caf singlets is evidenced: H_8 is mostly affected, but also the methyl groups show, at a lesser degree, upfield shifts of resonances.

In all experiments, the ^1H signals of Caf follow the fast exchange condition giving average shifts of bonded and free molecules in aqueous solutions. Throughout the titration experiments, in fact, all of the proton resonance signals remained sharp, with no sign of exchange broadening; very similar line shapes are obtained for titrations, carried out at different temperatures. However, a model of fast exchange is justified especially in view of the relatively weak binding constants derived.

On this basis, the value of the chemical shift changes ($\Delta\delta$) observed for increasing amounts of BP reflects the degree of supramolecular proximity as well as the strength of bonding. In particular, the drifting of the H_8 resonance makes possible the assessment of the binding constant K_s.

Usually, NMR methods for determining association constants suffer severe limitations, and curve fitting procedures should always be applied with care. Furthermore, two principal sources of systematic errors are (1) titrations at concentrations inappropriate to the equilibria being measured and (2) confusion of a simple acid−base interaction with supramolecular binding phenomenon (Wilcox, 1991).

The titration method used is based on a simple binding equilibrium model where an acidic receptor P_0 (the BP) and a substrate C_0 (Caf or CD) are assumed to be in equilibrium with the supermolecule PC. The titration process determines the concentration equilibrium constant K_s. Assuming a 1:1 stoichiometry, eq 1 can be derived:

$$K_s = \frac{[PC]}{[P] + [C]} \tag{1}$$

In the case at hand, rates of supermolecule formation and breakdown are rapid; the observed chemical shift of a selected proton on the substrate is therefore a weighted average of the chemical shifts in two possible environments—the chemical shifts in the unbound substrate environment (δ_C) and the chemical shift in the bound substrate environment (δ_{PC}):

$$\delta_{obs} = \frac{[C]\delta_C + [PC]\delta_{PC}}{[C_0]} \tag{2}$$

Substituting [C] with [C_0 − PC] and rearranging eq 2 leads to eq 3:

$$\delta_{obs} = \frac{\delta_C[C_0] + [PC](\delta_{PC} - \delta_C)}{[C_0]} \tag{3}$$

from which it is possible to draw out the values of [C], [P], and [PC] as a function of δ_{obs}, δ_C, and δ_{PC}. The substitution of these values in eq 1 gives

$$K_s = \frac{(\delta_{obs} - \delta_C)}{(\delta_{PC} - \delta_{obs})(P_0 - \dfrac{C_0(\delta_{obs} - \delta_C)}{(\delta_{PC} - \delta_C)}} \tag{4}$$

and by mathematical treatment:

$$\frac{C_0(\delta_{obs} - \delta_C)}{\delta_{PC} - \delta_C} + \frac{\delta_{obs} - \delta_C}{K_s(\delta_{PC} - \delta_{obs})} = P_0 \tag{5}$$

In the titration experiments, a series of measurements, defined by the quantities C_0, P_0, and δ_{obs}, can be obtained. Usually C_0 and P_0 are varied linearly or one of this is varied and the other held constant. The second method has been applied by varying P_0, because this procedure appears more suitable for low values of K_s (Deranleau, 1969; Person, 1965; Weber, 1965); then, the binding of Caf and BP is obtained by a least squares fitting of eq 5 to the experimental chemical shift data (Table 1). Curve fit is shown in Figure 2.

Table 1. Chemical Shift Changes ($\Delta\delta = \delta_{free} - \delta_{obs}$, Hz) of Caf Proton H_8 and Methyl Groups in the Presence of Increasing Amounts of 3,4-DHPA at 25 °C

[P]/[C]	1	2	3	5	10	35	60
H_8	13.8	21.0	26.1	30.9	34.8	37.5	51.0
Me_1	4.5	7.2	8.7	9.6	10.2	10.5	10.5
Me_3	4.8	7.2	9.0	9.9	10.2	10.8	11.1
Me_7	4.5	7.8	9.6	10.5	11.1	11.4	11.7

Furthermore, for weak association, the evaluation of K_s has been performed at a concentration covering saturation range between 20 and 80%, i.e., convenient to operate at equilibrium conditions for supermolecule concentration between 20 and 80%. In fact, beyond these values, all the data fit equally well to calculated curves for association constants that differ by 6 orders of magnitude. The preliminary estimation of K_s overcomes this uncertainty: then, titration has been performed by maintaining C_0 at a concentration value near to 1/10 of K_d ($K_d = 1/K_s$) and by varying concentration of P up to six times the value of K_d.

Finally, the supramolecular formation process can be in competition with acidic receptors binding to substrates through a simple proton transfer, or proton transfer can be followed by binding interaction of the salt. In this case, the appearance of the classical titration curve can be misleading. With a simple experiment, the process involved in the interaction of an acidic receptor with a substrate can be easily discriminated, observing the chemical shifts for a 1:1 mixture of receptor and substrate over a range of initial concentrations. No change of chemical shift has been

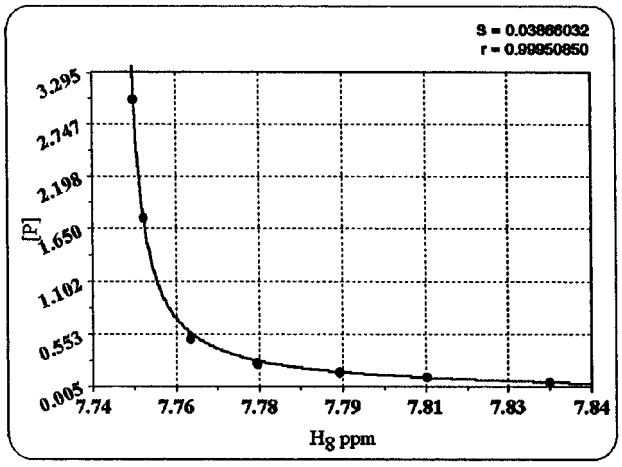


Figure 2. 3,4-DHPA-Caf complexation−binding curve.

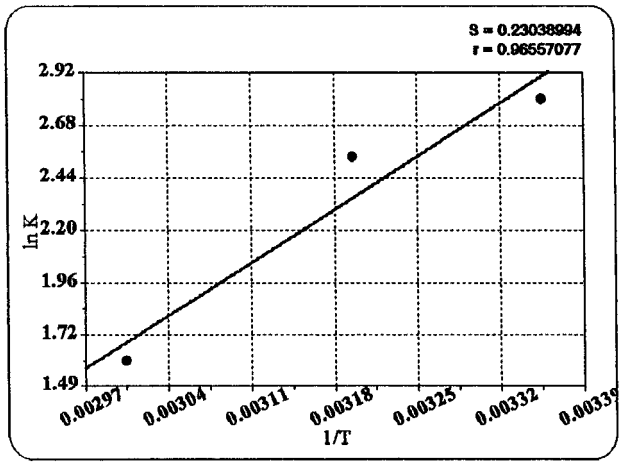


Figure 3. van't Hoff plot for the supramolecular complex 3,4-DHPA-Caf.

Table 2. Thermodynamic Parameters in the BP-Caf Supermolecule[a]

$K_{25\,°C}$	$K_{40\,°C}$	$K_{60\,°C}$	$\Delta G_{25\,°C}$	$\Delta G_{40\,°C}$	$\Delta G_{60\,°C}$	$\Delta H°$	$\Delta S°$
16	12	5	−6.9	−6.5	−4.4	−28.4	−71

[a] $K = M^{-1}$; ΔG = KJ/mol; ΔH = KJ/mol; ΔS = J/mol·deg.

observed during the dilution, so the proton transfer can be surely ruled out.

As a further confirmation, K_s values for 3,4-DHPA and its methyl ester appear to be very similar. $\Delta\delta$ measurements with respect to the increasing of temperature provide the evaluation of thermodynamic parameters ΔH and ΔS: according to the classical van't Hoff relationship $\ln K = -(\Delta H°/RT) + (\Delta S°/R)$, the slope and the intercept of the linear plot ln K vs $1/T$ furnish values for the standard enthalpy and entropy changes, respectively (Figure 3).

The enthalpy and entropy values are typical of charge transfer complexes (Foster, 1979). ΔH is about 1 order of magnitude stronger than for a van der Waal's interaction, about 1 order of magnitude weaker than for a hydrogen bond interaction, and almost 2 orders of magnitude weaker than for a σ-bond. This data characterize the BP-Caf supermolecule as a weak π-bonded molecular complex. Table 2 summarizes the calculated values (from the experimental results).

The supramolecular formation can be interpreted in terms of frontier orbital interactions: the novel molecule BP-Caf originates from the approach of nucleophile BP-HOMO to electrophile Caf-LUMO (Russo et al., in preparation). The formation of a supermolecule between the *o*-diphenolic functionality of BP and the aromatic group of Caf (the peptide analogue) depends, as outlined above, on a charge transfer interaction: the observed shielding indicates the electronic density increase around H_8 of Caf, compatible with a supramolecular interaction process, which involves the electron donor activity of BP MO toward Caf.

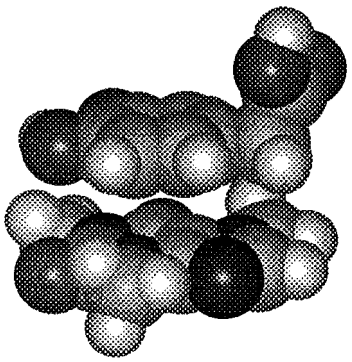


Figure 4. Side view representation of 3,4-DHPA-Caf supermolecule.

In the taste process, the molecular St behaves as good donor of an electron pair through π orbitals of the aromatic system of *o*-diphenolic functionality, activated by the presence of two electron donor OH groups, which gives to the substrate a high π HOMO. The model Caf represents a good electron acceptor, through the extended π unsaturation, increased by the presence of *N* heteroatoms and carbonyl groups, which determine a low π LUMO.

The NOE experiments show a through space interaction between the N_3 and N_7 Caf methyl groups and the aromatic protons of BP with a separation distance of 3.5 Å; the quantification of the NOE effect suggests the indicated stereochemistry (Figure 4) as an interpretative model for the supramolecular structure of BP-Caf.

β-CD has been exploited as carbohydrate models in the biomimesis of the sensorial process in order to ascertain the BP competitive aptitude in the formation of supermolecules with carbohydrate or proteic food ingredients.

β-CDs provide loose bonding with BP substrates, offering a suitable model for carbohydrates contained in MF and in the oral cavity; the cavity width of β-CD gives a unique opportunity to investigate the nature of the binding mechanism in aqueous solutions and to elucidate the energetics and the geometries of the supramolecular complexes formed.

A tight fit is expected to lead to predominating van der Waals interactions, of a dispersive type, and should increase with substrate polarizability; at the same time one expects the equilibria to be driven mostly by enthalpic contributions. In contrast, the classical hydrophobic effect, which is more likely with loosely bound substrates, should be seen as entropic gain.

The previously mentioned NMR titration methods were used to determine the supermolecular formation constant K_s and the thermodynamic parameters ΔH and ΔS.

The insertion of a guest molecule into the hydrophobic cavity of a CD results in the modification of the NMR frequencies of the signal of both the host and the guest. Then, observation of the NMR signals in the β-CD region in the presence of 3,4-DHPA supplied information on the nature of inclusion. Methine protons at positions 3 and 5 (Figure 5) in β-CD glucose units are

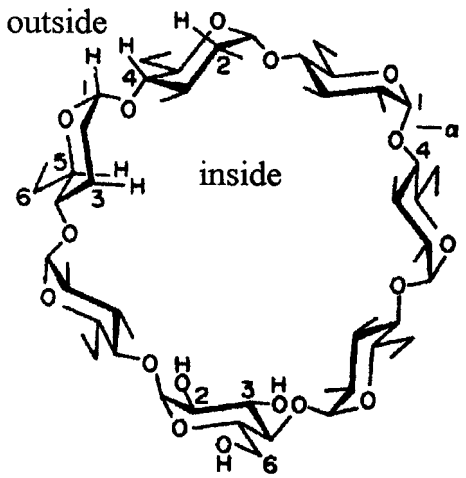


Figure 5. Structure and numeration of β-cyclodextrin molecule.

directed toward the inside of the hydrophobic bag, while methine protons at C_2, C_4, and C_6 are turned outside. In the ^{1}H NMR titration of CD with BP, the H_3 and H_5 are revealed to be mostly affected.

The ^{1}H NMR signals of the carbohydrate follow the fast exchange condition, giving average shifts of complexed and free host in aqueous solutions. Therefore, the values of the chemical shifts changes ($\Delta\delta$) observed upon inclusion of the guest molecule reflect the degree of intermolecular proximity as well as the strength of the novel moiety.

NOE measurements have been used to elucidate the supramolecular geometry. The unfavorable correlation times of complexes with molecular weights around 10^3 required the application of spin-lock techniques such as ROESY for obtaining sizable NOEs. The relative values obtained (++ for strong, + for medium, and − for no cross peaks in the ROESY matrix) and a partial contour plot of ROESY spectrum are reported in Figure 6.

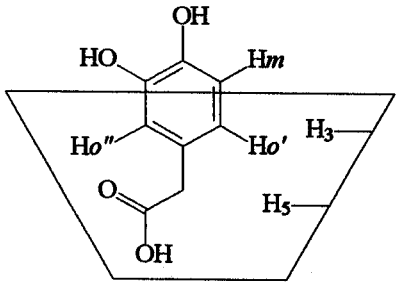
Intermolecular NOEs on the substrates are only observed upon irradiation of the protons H_3 and H_5. Figure 6 describes also the inclusion geometry determined for the BP-β-CD supermolecule, the observed fit showing the most solvated hydroxyl groups at the rim of the secondary site where no or little desolvation is needed upon supermolecule formation.

The same picture emerges from the complexation-induced shifts (CIS) on the CD protons H_1−H_6. As expected, protons H_1, H_2, H_4, and H_6, being remote from the supramolecular binding site, show almost negligible CIS. Only proton H_5 shows substantial shielding effects of up to 12.3 Hz (Table 3), which not only proves intracavity inclusion but is in full accord with the proposed geometry (Mucci et al., 1996). In fact, as shown in Figure 6, only H_3 rests on the center of the benzene ring, which has been estimated to lie 0.7 Å above the H_3 plane.

Thermodynamic data (summarized in Table 4) show consistently that the complex is dominated by ΔH with opposing entropy contribution $T\Delta S$.

The intermolecular interaction responsible for the cyclodextrin inclusion complexes have been largely discussed (Blokzjil and Engberts, 1993; Saenger, 1980; Smith, 1994). Several forces can act simultaneously: the extent to which each of them is involved is related to the substrate concerned.

In the variable-temperature ^{1}H NMR experiments, the investigation of the thermodynamic characteristics



NOE of	H_3	H_5
on H*m*	+	-
on H*o*	++	++

++ strong; + medium; - absent

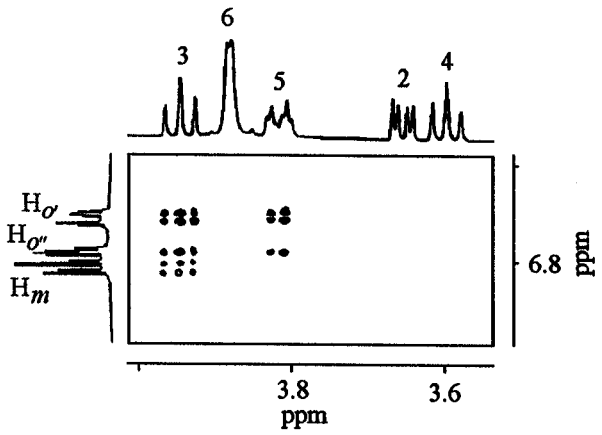


Figure 6. Ggraphical representation of the 3,4-DHPA-β-CD supermolecule with intermolecular NOEs and a partial contour plot of ROESY spectrum.

Table 3. Chemical Shift Changes ($\Delta\delta = \delta_{free} - \delta_{obs}$, Hz) of β-CD Protons H_3 and H_5 in the Presence of Increasing Amounts of 3,4-DHPA at 25 °C

[P]/[C]	1	2	3	5	10	35	60
H_3	2.1	4.5	5.7	7.5	8.4	9.0	9.3
H_5	4.8	7.5	8.7	10.2	11.4	12.0	12.3

Table 4. Thermodynamic Parameters in the BP-β-CD Supermolecule[a]

$K_{25\,°C}$	$K_{40\,°C}$	$K_{60\,°C}$	$\Delta G_{25\,°C}$	$\Delta G_{40\,°C}$	$\Delta G_{60\,°C}$	$\Delta H°$	$\Delta S°$
528	358	188	−15.5	−15.3	−14.5	−24.2	−29

[a] $K = M^{-1}$; ΔG = KJ/mol; ΔH = KJ/mol; ΔS = J/mol·deg.

for the formation of supermolecule among 3,4-DHPA and β-CD has shown a consistent enthalpic driving force, while the entropic term resulted unfavorable for the binding process.

The thermodynamic characteristics measured for the BP model and CD in water, i.e., negative $\Delta H°$ and $\Delta S°$, are in sharp contrast to those measured for processes driven by the classical hydrophobic effect, characterized by $\Delta H° \approx 0$ and $T\Delta S° > 0$. This effect, controlled by the entropic contribution, should most likely to be operative with loosely bound substrates, such as with BP and β-CD, while the experiments reveal the consistent contribution of ΔH with opposing entropy domination $T\Delta S$ to the formation of the supermolecule under investigation.

When the two molecules BP and β-CD bind into the supermolecule BP-β-CD, the reduction of the degrees of freedom for the interacting partners occurs with a consistent entropy loss, i.e., $T\Delta S$ results−8.6 KJ/mol, partially compensating the enthalpic driving force and obscuring the favorable entropic desolvation effect.

CONCLUSION

The systematic investigation of supramolecular complexation between 3,4-dihydroxyphenylacetic acid and peptidic and glycidic models points out that the 3,4-DHPA interacts more strongly with β-CD than with caffeine, suggesting that polysaccharides could play a fundamental role in the mechanism that controls the taste perception.

The obtained data allow a rationalization of the important noncovalent interactions that operate in the supramolecular recognition process. The extension of this study to a large variety of BPs appears to be especially promising in developing and understanding the sensory features exerted by these important microcomponents of MFs.

LITERATURE CITED

Blokzjil, W.; Engberts, J. B. F. N. Hydrophobic Effects. Opinions and Facts. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1545−1579.

Castelli, F.; Romeo, G.; Saia, A.; Uccella, N. Phenolic Antioxidant in Mediterranean Foods and their Activities in Biological System. In *Alpac-QSFNE*; Kumpulainen, J. T., Ed.; Helsinki, Finland, 1995; LAQE6.

Casuscelli, F.; De Nino, A.; Gallo, F.; Procopio, A.; Romeo, G.; Uccella, N. I Polifenoli nell'Olea Europaea: Applicazioni delle Moderne Tecnologie Analitiche. In *Ricerche ed Innovazioni nell'Industria Alimentare*; Porretta, S. Ed.; Chiriotti: Pinerolo, Italia, 1994.

Deranleau, D. A. Theory of the Measurement of Weak Molecular Complexes *J. Am. Chem. Soc.* **1969**, *91*, 4044−4049.

Foster, R. In *Organic Charge Transfer Complexes*; Academic Press: London, UK, 1979; Table 7.13, p 210.

Hackett, A. M. In *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationships*; Cody, V., Middleton, E., Jr., Harborne, J. B., Eds.; Alan R. Liss, Inc.: New York, 1986; p 177.

Haslam, E. Natural Polyphenols (Vegetable Tannins) as Drugs: Possible Modes of Action. *J. Nat. Prod.* **1996**, *59*, 205−215.

Montedoro, G. F.; Servili, M.; Baldioli, M.; Magnarini, C.; Cossignani, L.; Damiani, P. I Potenziali Modelli che Definiscono la Tipicità degli Oli Extra Vergini di Oliva. *Ind. Alimen.* **1993**, *32*, 618−631.

Mucci, A.; Schenetti, L.; Vandelli, M. A.; Forni, F.; Ventura, P.; Salvioli, G. One- and Two-Dimensional NMR Study of Complexation of Ursodeoxycholic Acid with β-Cyclodextrin. *J. Chem. Soc., Perkin Trans. 2* **1996**, 2347−2349.

Person, W. B. A Criterion for Reliability of Formation Constants of Weak Complexes. *J. Am. Chem. Soc.* **1965**, *87*, 167−170.

Romeo, G.; Uccella, N. SRM Sensorial Biomimetic Experiments with Mediterranean Food Biophenols. In *Ricerche ed Innovazioni nell'Industria Alimentare*, Vol. 2; Porretta, S., Ed.; Chiriotti: Pinerolo, Italia, 1996.

Saenger, W. Cyclodextrin Inclusion Compounds in Research and Industry. *Angew. Chem. Int. Ed. Engl.* **1980**, *19*, 344−362.

Shallenberger, R. S. In *Taste Chemistry*; Chapman-Hall: London, UK, 1993.

Smith, D. R. Supramolecular Chemistry. *Chem. Ind.* **1994**, 14−17.

Spencer, C. M.; Cai, Y.; Martin, R.; Gaffney, S. H.; Goulding, P. N.; Magnolato, D.; Lilley, T. H.; Haslam, E. Polyphenol Complexation−Some Thoughts and Observations. *Phytochemistry* **1988**, *27*, 2397−2409.

Weber, G. In *Molecular Biophysics*; Pullman, B., Weissbluth, M., Eds.; Academic Press: New York, 1965; pp 369−397.

Wilcox, C. S. Design, Synthesis, and Evaluation of an Efficacious Functional Group Dyad. Methods and Limitations in the Use of NMR for Measuring Host-Guest Interactions. In *Frontiers in Supramolecular Organic Chemistry and Photochemistry*; Schneider, H. J., Dürr, H., Eds.; VCH: New York, 1991.

Received for review April 1, 1997. Revised manuscript received July 15, 1997. Accepted July 16, 1997.[⊗] Research supported by Bromatheia Project, POP Calabria 1990−1993.

JF970266Y

[⊗] Abstract published in *Advance ACS Abstracts*, September 15, 1997.