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Structure influence on biophenols solubility in model biomembranes detected by differential scanning calorimetry

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The protective effects of some foods, in particular fruits and vegetables, against cardiovascular disease and cancer are believed to be due to the presence of antioxidant substances such as hydroxyaromatic compounds. The aim of this work was to study (i) the interaction of three biophenols derived from benzoic acid (*p*-hydroxybenzoic acid, vanillic acid, syringic acid and benzoic acid) with model biomembranes and (ii) their transfer through an aqueous medium to be absorbed into a lipid bilayer, investigating the effect they exert on the thermotropic behaviour of model membranes represented by dimyristoylphosphatidylcholine multilamellar vesicles using differential scanning calorimetry. The compounds, when dispersed in liposomes during their preparation, at pH = 4, were found to modify the gel to liquid crystal phase transition of the lipid vesicles, causing a temperature shift towards lower values. The temperature shift was a function of the concentration of acids in the lipid aqueous dispersions and their lipophilic character. The kinetic experiments of compounds transfer through the aqueous medium and the absorption by the bilayer were performed contacting the antioxidant compounds (at a fixed concentration) and the model membrane at increasing incubation times. These experiments reveal that the transfer of the examined compounds through the aqueous medium and their uptake by bilayer are influenced by the presence of substituents located on the ring, which should consequently modify their lipophilicity.

Keywords: Biophenol / Differential scanning calorimetry / Dimyristoylphosphatidylcholine / Model membranes

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1 Introduction

Diets rich in vegetables and fruit have protective effects against several pathologies, attributed largely to their high antioxidant content. Plant phenolics might have a relevant role in these antioxidant mechanisms [1, 2]. In particular, phenolic antioxidants, present in virgin olive oil and responsible for the high resistance of the oil to oxidation, can protect against LDL oxidation [3 – 5], similar to the phenolic compounds present in red wine and green tea [6 – 8]. These compounds scavenge free radicals and break peroxidative chain reactions. Because of their prolonged exposure to sunlight and pathogens, olives as well as other Mediterranean fruits have developed an array of protective compounds having hydroxyaromatic structures that limit the effects of microbial attack and subsequent oxidative damage.

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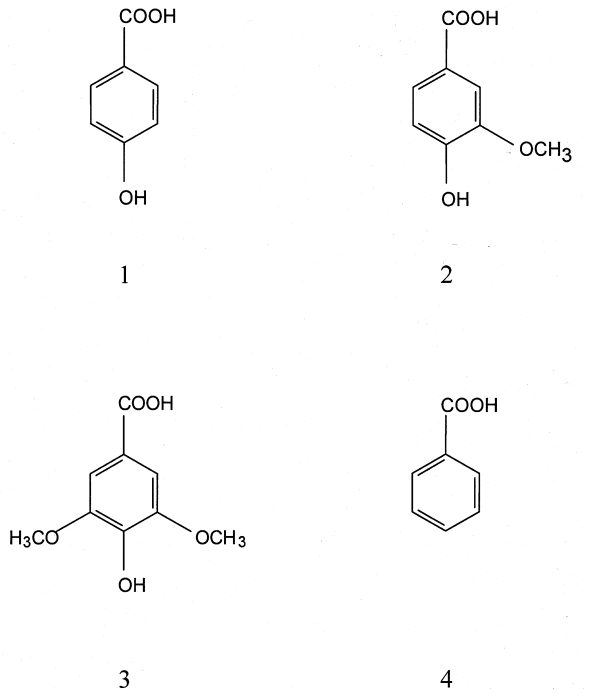
Some epidemiological studies showed a high correlation between a diet particularly rich in biophenols and a lower risk of cardiovascular diseases [9]. Phenolic compounds might play an important role in preventing oxidative stress-linked gastrointestinal diseases which are characterized by inflammatory intestinal injury, such as Chron's disease and ulcerative colitis [10].

Biological activity of biophenols can be better understood by investigating the biophenols/biomembranes interactions to get information about their membrane solubility, a prerequisite of their permeation through the cell wall.

The interaction of biophenols with biological membranes plays an important role in their transport, distribution, action, selectivity and toxicity [11]. Small organic acids are compounds sometimes able to interact and penetrate through biological membranes [12], but their ability to exert such an interaction with lipid membranes may be deeply influenced by substituents present on their main structure. As a consequence, structurally similar compounds bearing different groups able to modify their lipophilicity should interact with biological membranes in a different way



Scheme 1. Structural formulae of *p*-hydroxybenzoic acid (1), vanillic acid (2), syringic acid (3) and benzoic acid (4).

which, mainly for acidic compounds, may be modulated by pH variations.

The effects exerted by *p*-hydroxybenzoic acid, vanillic acid, syringic acid and benzoic acid (Scheme 1) on the thermotropic behaviour of L-α-dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLV) were investigated by differential scanning calorimetry (DSC). DMPC vesicles were used as simplified model membranes as they form lipid bilayer similar to the lipid structure of the cell membranes. Such method of investigating the interaction between increasing amount of bioactive compounds and membranes (interaction in organic solvent before MLV preparation) can be considered as only a way to report the maximum interaction between a foreign molecule and lipid bilayer.

To obtain information on the real ability of a compound to permeate the aqueous layer surrounding the lipid membrane or to pass through the lipid bilayer to penetrate into a cell-like model, a comparison of the data obtained through the aforementioned 'classical' method with kinetic experiments of the transfer of compounds to empty membranes through aqueous medium and of the transfer of compounds, dispersed in lipid multilayers, to empty membranes has to be performed. In this way, it is possible to detect differences in the compound's ability to interact and penetrate the lipid bilayer of biomembranes, which causes variations in their packing and fluidity.

2 Materials and methods

2.1 Materials

Synthetic DMPC was obtained from the Fluka Chemical (Buchs, Switzerland). Solutions of the lipid were chromatographically pure as assessed by 2-D TLC. Phospholipid concentrations were determined by phosphorous analysis following the procedure described by Rouser *et al.* [13].

p-Hydroxybenzoic acid, vanillic acid, syringic acid and benzoic acid were obtained from Sigma Chemical (St. Louis, MO, USA) and were >97.0% pure. Tris 50 mM solution, adjusted to pH = 7.4 and pH = 4.0 (unbuffered solution) with hydrochloric acid, was used to prepare liposomes.

2.2 Liposomes preparation

MLV were prepared in the presence or absence of the examined compounds as follows: chloroform–methanol (1 : 1, v : v) solutions of lipid and compounds were mixed to obtain increasing molar fractions of compounds. Solvents were removed under a nitrogen flow and the resulting film was freeze-dried under vacuum to remove residual solvents. Liposomes were prepared by adding 50 mM buffered (pH = 7.4) or unbuffered (pH = 4.0) Tris solutions to the film, and then heated at 37°C (above the gel-liquid crystalline phase transition temperature), and vortexed three times for 1 min. The samples were shaken for 1 h in a 37°C water bath to homogenize the liposomes. Aliquots (120 µL) of aqueous liposome dispersion (5 mg of lipid) were transferred to a DSC aluminium pan, hermetically sealed and submitted to DSC analysis.

2.3 DSC

DSC measurements were performed using a Mettler TA 4000 system equipped with a DSC-30 cell and a TC-11 processor. A scan rate of $2^{\circ}C \cdot min^{-1}$ and temperature range of 2–37°C was used with a 1.5 mW sensitivity. pH = 7.4 and pH = 4 Tris solutions were used as reference. Temperatures and enthalpies were checked using indium, palmitic and stearic acids. Enthalpies and temperatures were evaluated from the DSC peak by using the integration program of the Mettler system. After being sealed in the DSC aluminium pan, the samples were submitted to three subsequent calorimetric cycles of heating and cooling as follows:

(a) a heating scan between 2 and 37°C at the rate of 2°C/min;

(b) a rapid cooling scan between 37 and 2°C at the rate of 4°C/min.

After calorimetric scans, all samples were extracted from the pans and aliquots were used to determine the amount of phospholipids using the phosphorous assay.

2.4 Permeation experiments

The ability of examined compounds to dissolve in the aqueous phase, migrate through it to be absorbed on the external lipid layer of MLV, and successively be transferred into the deeper layers was investigated by carrying out the following 'kinetic' experiment. Fixed amount (to obtain a 0.15 M fraction with respect to the phospholipid) of finely powdered compound was placed in the bottom of 160 µL DSC aluminium crucible, and then a fixed amount (120 µL, 5 mg of lipid) of DMPC aqueous dispersion (MLV) was added. The samples, hermetically sealed in the pans, were gently shaken for 10 s and then submitted to subsequent calorimetric cycles by using the following procedure: (1) a scan between 2 and 37°C to detect the interaction between the compounds and the model membrane during the sample heating; (2) an isothermal period (1 h) at 37°C to allow the compounds to dissolve in the aqueous medium and possibly interact and permeate the lipid layers, which at 37°C exist in a disordered state as they are over the lipid transitional temperature; and (3) a cooling scan between 37 and 2°C, at the rate of 4°C/min, to bring back the lipid layers in an ordered state before restarting the heating program (step 1).

This procedure was run at least six times in order to follow eventual variations of the transitional temperature of the DMPC calorimetric peak due to the release of the compound by the solid to the model membrane. If an interaction or a full lipid layer penetration did occur then an effect similar to that detected when the preparation (0.15 molar fraction) carried out in organic solvent was examined should be observed.

3 Results and discussion

The effect of three biophenols (*p*-hydroxybenzoic acid, vanillic acid and syringic acid) and of benzoic acid (considered as reference compound which does not carry any substituents on the aromatic ring) on the thermotropic behaviour of DMPC MLV was studied, by DSC technique, in order to have information on their biological activity. DSC technique gives information on the thermotropic parameters, transition temperature (T_m) and enthalpy variation (ΔH), related to the melting process of phospholipid bilayers subjected to a heating scan. These thermotropic parameters are affected by the presence of foreign substances in the bilayers.

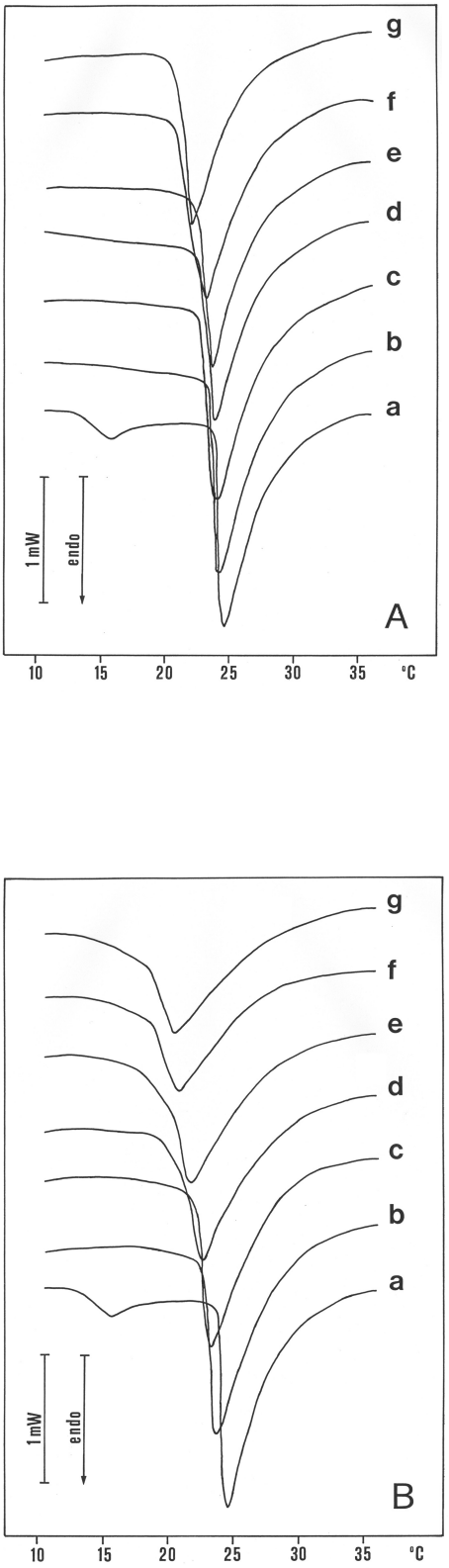


Figure 1. DSC heating curves of hydrated DMPC MLV containing (A) *p*-hydroxybenzoic acid, (B) benzoic acid at different molar fractions: a = 0.00; b = 0.03; c = 0.06; d = 0.09; e = 0.12; f = 0.15; g = 0.18.

The calorimetric curves of DMPC MLV pure or containing increasing molar fraction of *p*-hydroxybenzoic acid are shown in Fig. 1 A. This compound causes the shift of the main calorimetric peak towards lower temperature values, the broadening of the same peak and the disappearance of the pretransition peak even at low molar fractions.

The calorimetric curves of DMPC MLV prepared in absence and in the presence of vanillic acid are similar to those of *p*-hydroxybenzoic acid (not shown). The curves and transition temperatures of MLV containing vanillic acid are shifted towards lower values with respect to the pure DMPC MLV. The increase of vanillic acid molar fraction also causes a slight broadening of the main peak. The pretransition peak disappears with the lowest molar fraction present in the MLV. The calorimetric curves of pure DMPC MLV and syringic acid containing MLV (not reported) clearly evidence that, as the molar fraction of syringic acid present in the liposomal suspension increases, the main peak is shifted towards lower temperatures; however, the shape of the peak remains almost constant. Higher molar fractions cause the disappearance of the pretransitional peak.

Figure 1 B reports the calorimetric curves of DMPC liposomes prepared in absence and in the presence of benzoic acid. A shift of the calorimetric curves towards lower values of temperature, their broadening and a concomitant decrease of the area of the main peak are evident by increasing the amount of compound inserted in the lipid sea. The pretransition peak disappears at the lowest molar fraction used.

The effects on DMPC MLV transition temperature of the four examined compounds have been compared; the results are shown in Fig. 2 and plotted as $\Delta T/T^{\circ}_{m}$ ($\Delta T = T^{\circ}_{m} - T_{m}$; where T°_{m} is the transition temperature of pure DMPC liposomes and T_{m} is the transition temperature of DMPC liposomes prepared in the presence of compounds at different molar fractions) in function of the molar fraction of compounds present in the aqueous lipid dispersion. If we consider the compound as an impurity dissolved in an ideal 2-D solution, the lowering of the lipid bilayer transition temperature can be easily rationalized in terms of Vant'Hoff decrease of freezing temperature [14, 15]. In this hypothesis an almost linear decrease of the bilayer transition temperature with the concentration of the foreign molecule within the bilayer should be observed, while the associated enthalpy should remain constant. With regard to the temperature variation this behaviour has been observed in Fig. 2; in fact, all the used compounds cause a decrease of the transition temperature, and the decrease is proportional to the increase of the molar fraction used. However, the effect of benzoic acid (which does not carry any hydrophobic substituents on the aromatic ring) is the most pronounced, followed by vanillic acid, *p*-hydroxybenzoic acid and syringic acid. A really small difference in the effect exerted by both vanillic acid and *p*-hydroxybenzoic acid is also evident.

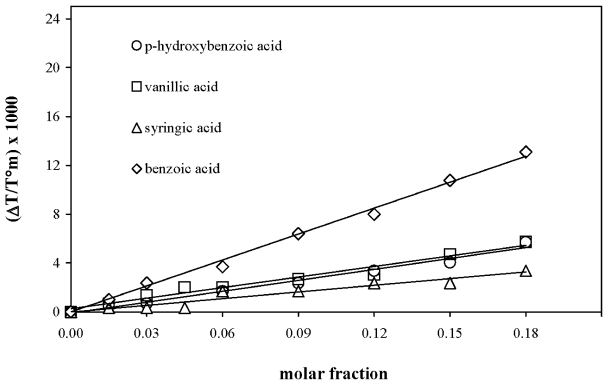


Figure 2. Calibration curves relating the depression of DMPC MLV transition temperature ($\Delta T/T^{\circ}_{m}$) to the molar fractions of acid (average of at least four runs) present in the aqueous phospholipid dispersion.

The presence of different substituents in the backbone structure of benzoic acid biophenol derivatives should modulate their characteristic and incorporation into the bilayer. If we look at the partition coefficient Log $P_{o/w}$ of the compounds, interesting considerations can be made. In fact, a higher partition coefficient is associated with a higher effect on the transition temperature, the partition coefficients of the compounds being 1.04 (syringic acid), 1.43 (vanillic acid), 1.58 (*p*-hydroxybenzoic acid) and 1.87 (benzoic acid). As the Log $P_{o/w}$ can be well correlated with the compounds lipophilicity, we can suppose that benzoic acid affects, more than the other acids, the transition temperature due to its higher lipophilicity that permits it to better solubilize inside the phospholipid bilayer, and that syringic acid is the least effective on the thermotropic behaviour of MLVs, it being the least lipophilic compound; vanillic acid and *p*-hydroxybenzoic acid have an intermediate effect as well as intermediate Log $P_{o/w}$ values. Many authors [16, 17] suggest that the interaction between a molecule and the model membranes and, consequently, the shift of lipid phase transition temperature is modulated by structural changes in the molecular backbone of the molecule. Molecules interacting with lipids in liposomes can take the place of lipid molecules as 'substitutional impurities' causing T_{m} variations and ΔH decrease, or can intercalate between the flexible acyl chains of lipids as 'interstitial impurities' causing T_{m} variations without ΔH change [15], in accord with the temperature depression of melting point for ideal solutions [18, 19].

The enthalpy data in Table 1 show that the values relative to vanillic acid and *p*-hydroxybenzoic acid are almost constant

Table 1. Enthalpic changes calculated for increasing molar fraction of acid present in the lipid aqueous dispersion

Molar fraction	ΔH, kJ/m			
	p-Hydroxy-benzoic acid	Vanillic acid	Syringic acid	Benzoic acid
0.00	25.3	25.3	25.3	25.3
0.015	25.1	28.5	29.5	23.1
0.03	25.0	26.9	30.4	20.7
0.06	23.2	24.5	32.0	22.0
0.09	23.8	26.9	30.4	21.3
0.12	23.3	25.3	33.7	21.6
0.15	22.2	30.8	29.5	17.2
0.18	21.7	29.3	33.7	16.8

for the entire molar fraction used, those relative to syringic acid undergo a slight increase with the molar fraction used whereas those relative to benzoic acid decrease as the molar fraction increases. The negligible variation in ΔH can be explained as an interaction which occurs only between the molecules and the phospholipids without deeply interacting with the acyl chains [15], whereas the ΔH decrease can be explained with a strong interaction of the compound with the lipid chain which prevents the highly cooperative melting of the lipid tails, making the gel-to-fluid phase transition of the bilayer less endothermic and less cooperative. As a result, the intensity of the calorimetric peak decreases and the peak shape broadens.

Therefore, we may conclude that benzoic acid is the most soluble inside the lipid membrane among the closely related studied acids (Fig. 2), and that it exerts a greater perturbation of the lipid structure while other molecules mainly behave as interstitial impurities.

The effect of the change in lipophilicity due to compound dissociation was also investigated and calorimetric analyses on samples prepared at pH = 7.4 were carried out: a very negligible interaction between compounds and MLVs was detected (data not reported).

The intake of biomolecules is mainly determined by the uptake process from the aqueous medium to the membranes; in this point of view it is interesting to investigate and compare the ability of the used compounds to be *adsorbed* and *transported* inside the membranes. The method to analyze these processes has been reported by us in previous papers [20–23]. It consists of leaving in contact empty MLVs with a fixed amount (0.15 molar fraction with respect to the phospholipids) of the examined compound and determining, over time, the effects exerted by the adsorption of such molecules on the transition temperatures of the lipid vesicles by DSC. From the comparison of the effect on the T_m shift with the values obtained by the preparation in organic phase, it is possible to obtain the amount of the effect exerted on the lipid vesicles by compounds

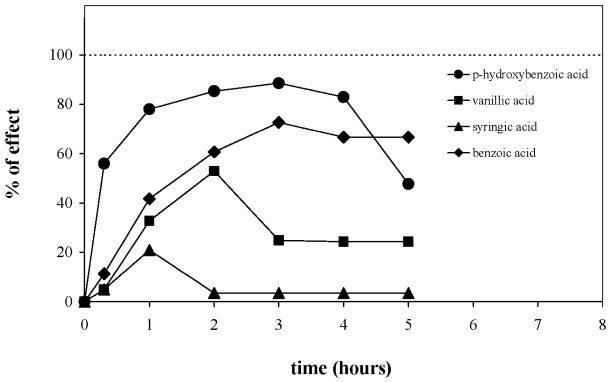


Figure 3. Acids transfer from free solid biocompounds (0.15 M fraction with respect to the lipid aqueous dispersion) to DMPC MLV for increasing incubation time reported as percent of the effect exerted on the DMPC calorimetric peak. Dotted line represents the maximum effect that can be exerted obtained by the interaction in the organic phase preparation employing a molar fraction of 0.15 for all the compounds.

transferred inside the lipid vesicles. The results of the transfer kinetics of the compounds from the solid through the aqueous medium to the MLVs are shown in Fig. 3, where they are compared with the theoretical value (dotted line representing the effect obtained when a 0.15 molar fraction of compound was dispersed in the aqueous lipid dispersion) obtainable assuming that the compounds reach and penetrate the lipid vesicle.

By looking at Fig. 3, among the examined molecules, *p*-hydroxybenzoic acid is the fastest in reaching the lipid bilayer, and the effect being related to the amount reaching the lipid vesicle surface it is possible, also, to affirm that the highest amount reaching the lipid bilayer is achieved by *p*-hydroxybenzoic acid followed by benzoic acid, vanillic acid and syringic acid. The amount of the last two compounds, distributed between aqueous phase and lipid multilamellar phase and migrating into the vesicles is really smaller than that incorporated into the vesicles during the direct compound–lipid vesicles preparation. This unusual behaviour, observed for some molecules at high incubation times and consisting of a decrease of the effect (related to the temperature shift and amount of compound dissolved in the lipid multilayer) following an initial increase, means that a part of the substance is withdrawn from the interaction with the lipids. This effect could be explained due to the formation inside the lipid phase of clusters of two or more molecules due to the increase in the entering molecule concentration in the lipid bilayer [24].

4 Concluding remarks

Phenols are a major group of natural products that occur in higher plants. Of the various plants, olive has been recog-

nized as a source of biophenols. Its possible benefit to human health has only recently been considered. Epidemiological studies have correlated the low incidence of coronary heart disease, atherosclerosis and some types of cancer (colorectal and breast cancer) with olive oil consumption in the Mediterranean diet. A number of reports have linked the health benefits of olive oil with its phenolic content.

Demonstration of their mechanism of action also implies the elucidation of the steps of bioavailability and bioabsorption in cells and tissues. In order to estimate the relationships between the amounts of biophenols taken up by food intake and the several possible benefits illustrated from *in vitro*/*in vivo* experiments and from epidemiological studies, it is essential to demonstrate the absorption of biophenols by biomembranes. We have applied DSC to have information on the absorption process. We compared the effects of the various phenols to determine whether structural features influence the interaction and absorption process by biomembrane.

From our study it can be concluded that environment plays an important role on the absorption of the examined compounds by biomembranes. In fact, it occurs at acidic pH when the compounds are present in an unionized form. On the other hand, the compound dissolution process is hindered by gastric pH. Moreover, our experiments demonstrate that the compounds uptake by biomembranes depends on the compounds structure.

The comparative biomimetic experiments permit a deeper understanding of the interaction and penetration of biological membranes by biophenols and biomolecules found in olive drupes and their Mediterranean food derivatives. The explanation of the results, obtained by DSC techniques in evaluating both *static* and *dynamic* interaction processes among biomembrane models and biophenols, may provide new insights into the health effects of Mediterranean foods on human well-being.

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