Biomimetic Transport of Simple Olive Biophenol and Analogues through Model Biological Membranes by Differential Scanning Calorimetry

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The different interactions of *p*-hydroxybenzoic acid (**1**), a simple biophenol (BP) found in olives and their food products, and its substitute analogues, benzoic (**2**), anisic (**3**), and toluic (**4**) acids, with a model membrane represented by dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLV) was studied by differential scanning calorimetry (DSC). The influence of their different lipophilic character on transfer and absorption processes through an aqueous medium into a lipid bilayer was also investigated. DSC experiments allowed monitoring of the interaction of BP with biomembranes by considering the effects exerted on the thermotropic behavior of DMPC multilamellar and unilamellar vesicles at different pHs (4 and 7.4). The examined compounds affect the transition temperature (T_m) of phospholipid vesicles, causing a shift toward lower values, which is modulated by the molecular fraction entering into the lipid bilayer, as well as by their molecular interaction with the lipids. Kinetic calorimetric measurements were performed on suspensions of blank liposomes immediately after being added to fixed weighed amounts of powdered compounds and after increasing incubation periods at 37 °C. T_m shifts, due to molecular dissolution and transfer of the compounds into the membrane surface occurring during the incubation time, were compared with those determined by a fixed molar fraction of free compounds directly dispersed in the membrane. The results show that the kinetic process, involved in molecular release, transfer through aqueous medium, and uptake by the model membrane surface, is influenced by lipophilicity as well as by pH, acting on the acid solubility and membrane disorder, allowing us to gather useful information on the BP intake process of olive derived foodstuffs.

Keywords: *Phosphatidylcholine; differential scanning calorimetry; membranes; transport; olive biophenol*

INTRODUCTION

Mediterranean foods play an important role in human physiology and pathology (*1*, *2*). Epidemiological investigations, in fact, show that the incidence of particular diseases is different in geographical areas where populations have been traditionally exposed to somewhat unique diets. In the Mediterranean area, for example, the lower incidence of coronary heart diseases and certain types of cancers (*1*, *3*−*5*) is related to a diet rich in vegetables and fruits (*6*−*9*). Among them, olive foodstuffs have been, at least originally, rather typical of this area and are used for production of high-quality extra virgin olive oil (EVOO) and table olives (TOs).

Because of their prolonged exposure to sunlight and to pathogens, olives as well as other Mediterranean fruits have developed an array of protective compounds having hydroxyaromatic structures, shown as BP **1**, that limit the effects of microbial attack and subsequent oxidative damage (*10*).

Such protective functional groups, BPs (*11*), represent an important factor to evaluate EVOO and TO quality because they are partly responsible for autoxidation stability (*12*) and hedonic-sensory (*13*) and functional characteristics (*14*). Their antioxidant activities exerted in biological systems (*15*−*19*) lead one to suppose their relevant role in human health. Multiple activities of BPs are described as having metal chelator and free radical scavenging activity (*20*), inhibitory effects on low-density lipoprotein oxidation (*21*−*23* and on mutagenesis and carcinogenesis (*24*, *25*), and antibacterial (*26*, *27*) and antiviral (*28*, *29*) activities.

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

The effects exerted by **1** and its analogues **2**−**4**, differing in the presence of hydroxyl (-OH), hydrogen (-H), methoxyl (-OCH_3), and methyl (-CH_3) groups in the para position with respect to the carboxylic group (Scheme 1), on the thermotropic behavior of L-α-dimyristoylphosphatidylcholine (DMPC) vesicles (multilamellar, MLV, and unilamellar, LUV) were investigated. Among these compounds, **1** and **2** are present in olive oil (*30*). DMPC vesicles, used as a synthetic simplified membrane model given that they form a lipid bilayer similar to the lipidic structure of the cell membranes, show temperature-dependent behavior. In fact, by increasing the temperature, the DMPC acyl

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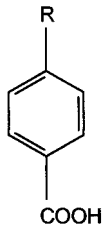
Scheme 1. Structural Formula of *p*-Hydroxybenzoic Acid (1), Benzoic Acid (2), Anisic Acid (3) and Toluic Acid (4)



1. OH
2. H
3. OCH_3
4. CH_3

chains change from an ordered to a disordered configuration. This process, known as the $L_\beta \rightarrow L_\alpha$ or gel-to-liquid crystal phase transition, is characterized by two important thermodynamic parameters: enthalpy change (ΔH) and phase transition temperature (T_m) (*31*−*33*).

The thermotropic behavior is influenced by the presence of molecules dissolved in the lipid layers that act as a solute in a solvent affecting phase transition temperature and enthalpy changes related to the $L_\beta \rightarrow L_\alpha$ transition (*34*, *35*). The variation in temperature is proportional to the amount of compounds dissolved in the lipid layers, and it is related to the compound's lipophilicity. DSC, a nonperturbative technique, permits one to conveniently investigate this thermotropic behavior and the effects exerted on it by (a) molecules directly dispersed in the lipidic layers during MLV preparation or (b) molecules left in contact with empty multilamellar or unilamellar vesicles. Following the second procedure, it is possible to evaluate that lipophilicity, solubility, and effect of substituents in the molecule can modulate the interaction and the molecular penetration into cell membranes, allowing one to better understand the "in vivo" behavior of the investigated molecules. Therefore, the obtained results may be useful to rationalize the effect of different biomolecules contained in the olive foodstuffs on human health.

MATERIALS AND METHODS

Materials. Synthetic L-α-dimyristoylphosphatidylcholine was provided by Fluka Chemical Co. (Buchs, Switzerland). The lipid solution was chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). Lipid concentration was determined by phosphorus analysis (*36*).

Compounds **1**−**4** were provided by Sigma Chemical Co. (St. Louis, MO). Aqueous medium was a 50 mM Tris solution adjusted to pH = 7.4 (buffered solution) or at pH = 4.0 (unbuffered solution) with hydrochloric acid.

Liposomes Preparation. Multilamellar vesicles were prepared in the absence or in the presence of the investigated compounds by the following procedure: chloroform−methanol (1:1, v:v) solutions of lipid and biomolecules were mixed to obtain acids in increasing molar fractions. The solvents were removed under a nitrogen flow, and the resulting film was freeze-dried under a vacuum to remove the residual solvents.

Liposomes were prepared by adding to the film 50 mM Tris buffer (pH = 7.4) or unbuffered (pH = 4.0) solutions, and then heating at 37 °C at a temperature above the DMPC gel−liquid crystalline phase transition, and vortexing three times for 1 min.

The samples were shaken for 1 h in a water bath at 37 °C to homogenize the liposomes. Afterward, aliquots of 120 μL (5 mg of lipid) were transferred in a DSC aluminum pan, hermetically sealed, and submitted to DSC analysis. MLV dispersion was subjected to extrusion through polycarbonate membranes (Nucleopore, Pleasanton, CA) in an extruder system (Liposofast Avenstin) to obtain large unilamellar vesicles (LUV) of defined diameter (100 nm) (*37*).

Differential Scanning Calorimetry. DSC was performed by using a Mettler TA 4000 system equipped with a DSC-30 cell and a TC-11 processor. The scan rate was 2 °C/min and the temperature range was 2−37 °C, with a 1.50 mW sensitivity. Tris buffer solution was employed as a reference.

Temperatures and enthalpies were checked by using indium, palmitic, and stearic acids. Enthalpies and temperatures were evaluated from the DSC peak by using the integration program of the Mettler system.

The samples were cooled and heated at least three times to achieve the reproducibility of results. All samples, after calorimetric scans, were extracted from the pans, and aliquots were used to determine the amount of phospholipid by the phosphorus assay (*36*).

Interaction between Acids and Lipid Vesicles. In Figure 1 (panels A−D) are reported the calorimetric heating curves of DMPC liposomes in the presence of different molar fractions of the studied compounds at pH = 4.0. All the molecules interact with the membrane model causing a shift of the calorimetric peak toward lower temperatures, but they modify the curves in different ways. A higher effect is observed for **4** (Figure 1D), and a lower effect is exerted by **1** (Figure 1A). Decreasing of the transition temperatures (T_m) of the calorimetric peak on increasing the amount of biomolecules dispersed in the aqueous lipidic dispersion is shown in Figure 2A. In this figure, the temperature shifts caused by the presence of the molecules are expressed as $(\Delta T/T_m^\circ) \times 10^3$ (where $\Delta T = T_m^\circ - T_m$, T_m° and T_m are the transition temperatures of pure DMPC multilamellar liposomes and DMPC liposomes loaded with increasing amounts of biomolecules). These values are plotted against the molar fractions of the acids present in the aqueous lipid dispersion.

Since the above plots represent the correlation between the molar fractions of acids and the shift of the lipid transition temperature, they were used to follow the transfer kinetics from the external medium to the lipid membrane. In other words, the amount of transferred acid to the model membrane was obtained through the T_m depression exerted by the foreign molecules in the lipid bilayers. These numbers, however, must be corrected to consider the water−membrane partition coefficient of the studied compounds. This issue is investigated in the following section.

Acids Partition between Lipid and Aqueous Phases. By using the curve reported in Figure 2A, it is possible to determine the thermotropic effect exerted by different amounts of acids present in the lipidic aqueous dispersion. The acid, however, is partitioned between the membrane and the aqueous phase. To evaluate the real amount of drug present in the membrane alone, three samples of multilamellar vesicles loaded with different molar fractions (0.06, 0.12, and 0.18) of acids have been prepared. The samples were centrifuged by a Beckman model J2-21 centrifuge at 37800*g* for 25 min at controlled temperature. Afterward, the supernatant was separated from the lipidic pellets, which were dried and lyophilized. The amount of drug present in the aqueous and lipidic fractions was detected by UV spectroscopy at respectively 254 nm for **1**; 229 nm for **2**; 255 nm for **3**; and 239 nm for **4**. The amounts of acids found in the lipid phase were respectively 59 ± 1% for **1**; 78 ± 1% for **2**; 86 ± 1% for **3**; and 93 ± 1% for **4**. These values were employed to correct the nominal molar fractions (plots in the Figure 2A), obtaining the plots of Figure 2B, which represent the effect exerted by the real molar fraction of drug transferred to the membrane. These plots can be employed to calculate the acid's uptake by void liposomes.

Permeation Experiments Monitored by DSC. To explore the capacity of the examined biomolecules to migrate through the aqueous phase and permeate a model membrane, kinetic experiments were carried out by putting DMPC aqueous suspension (MLV or LUV) in contact with a finely

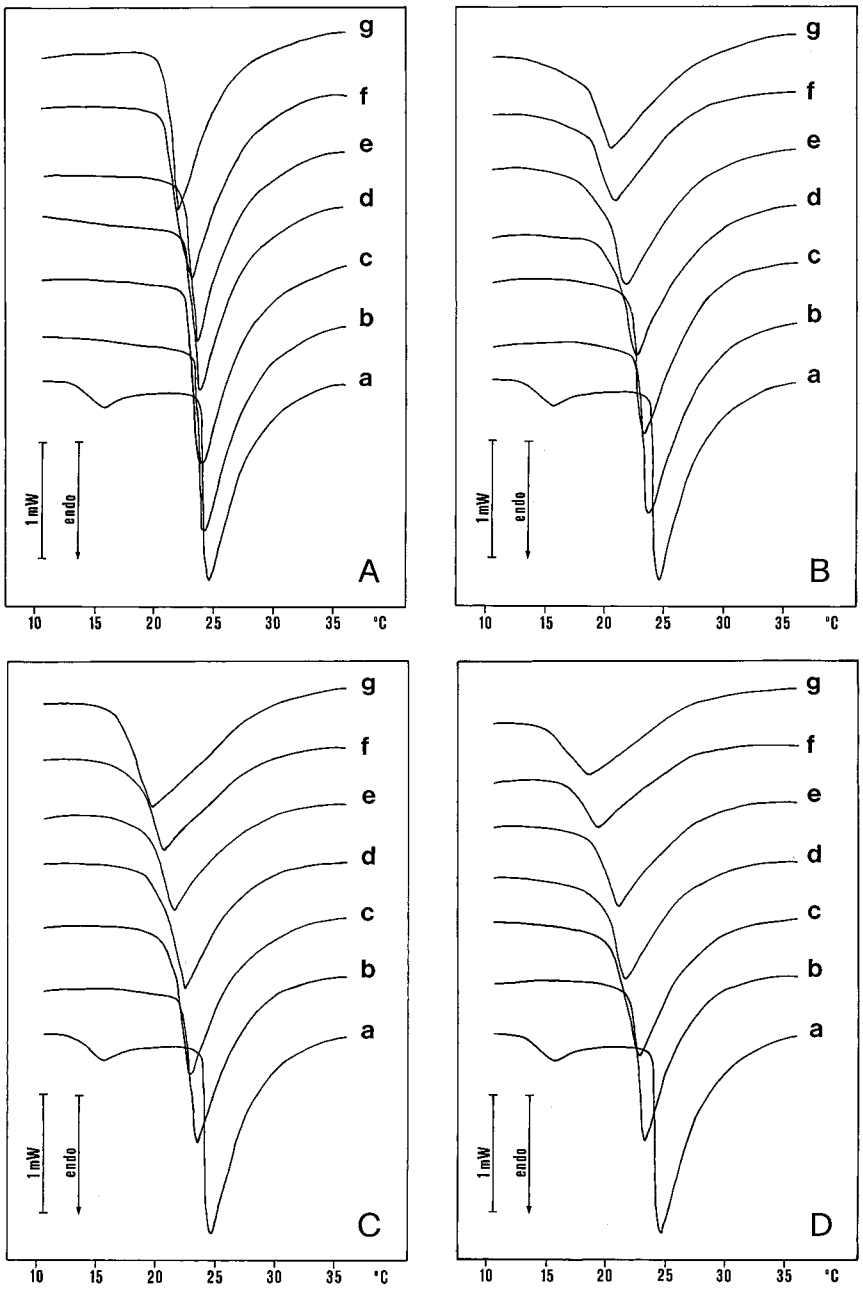


Figure 1. Differential scanning calorimetry heating curves of hydrated DMPC MLV containing (A) *p*-hydroxybenzoic acid, (B) benzoic acid, (C) anisic acid, (D) toluic acid at the follow molar fractions: $a = 0.0$; $b = 0.03$; $c = 0.06$; $d = 0.09$; $e = 0.12$; $f = 0.15$; $g = 0.18$.

powdered substance (a 0.15 molar fraction with respect to the whole lipid) placed in the bottom of the DSC crucible. The samples, after they were sealed in the DSC aluminum pan and gently shaken for 10 s, were submitted to subsequent calorimetric cycles of heating, isothermal, and cooling modes by using the following procedure:

(i) a first rapid scan between 2 and 37 °C, to detect the eventual interaction consequent to the transfer of the biomolecules from the solid phase to the model membrane in its gel state;

(ii) a long isothermal period (1 h) at 37 °C (above the lipid transitional temperature) to permit the substance to permeate (if able) the lipid layer/s held in a fluid state;

(iii) a rapid cooling scan between 37 and 2 °C (7 min) before restarting the heating program (i).

This procedure was run, in triplicate, at least six times to detect the variations caused by the interaction of acids with model membranes.

RESULTS AND DISCUSSION

Interactions, at equilibrium conditions, between the acids **1**−**4** and DMPC vesicles, chosen as a membrane model, were investigated to obtain information about their capability to interact with and penetrate into biological membranes.

It appears evident that a great difference exists between **1** and the other analogues' behavior (Figure 1, panels A−D). In fact, the shape of the curves is different on increasing the amount of molecules dissolved inside the multilamellar system. While **2**−**4** broaden the DMPC calorimetric peak and decrease the area of the peak (Table 1), **1** does not show either broadening or area variation. The differences in these effects, mainly enthalpy changes (related to the peak area) and temperature shifts, are related to the substituents' polarity

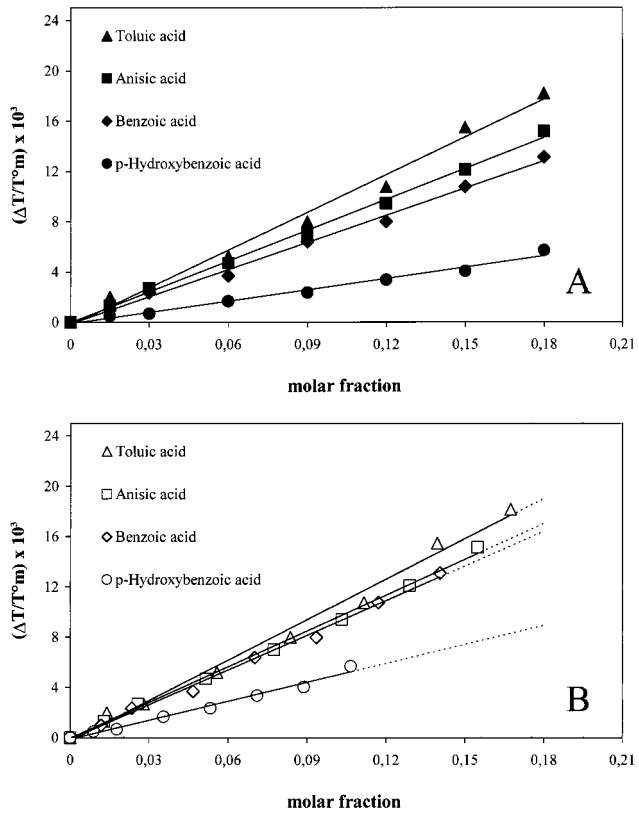


Figure 2. Calibration curves relating the depression of DMPC multilamellar vesicles transition temperature ($\Delta T/T^{\circ}_{m}$) to the concentration of acids (average of at least four runs): (A) present in the phospholipid aqueous dispersion; (B) corrected for the partition coefficients to obtain the real amounts of acids dispersed in the lipid phase.

Table 1. Enthalpic Changes Calculated for Increasing Molar Fraction of Acids Present in the Lipid Aqueous Dispersion

	ΔH (kJ/m)			
molar fraction	**1**	**2**	**3**	**4**
0	25.3	25.3	25.3	25.3
0.015	25.1	23.1	19.5	17.2
0.03	25.0	20.7	19.5	17.6
0.06	23.2	22.0	20.6	19.1
0.09	23.8	21.3	20.2	15.0
0.12	23.3	21.6	18.1	13.5
0.15	22.2	17.2	17.5	9.6
0.18	21.7	16.8	17.6	7.1

present in the para position with respect to the carboxylic group. By increasing the polarity, in the order $-CH_3 < -OCH_3 < -H < -OH$, the capability of the studied compounds to interact with the liposomes and to shift the transition temperature decreases.

The interaction between lipophilic substances and lipid bilayers can be explained in terms of a "fluidifying" effect due to the introduction of lipophilic molecules into the ordered structure of the lipidic bilayer (*31*, *34*, *38*−*43*). Biomolecules act as spacers in such structures, causing a destabilization of the lipid mosaic with a decrease in the T_m of the gel-to-liquid crystal phase transition. Differences in the enthalpy changes can be justified in the compound localization inside the lipid bilayer. In fact, while a negligible variation in ΔH can be explained as an interaction occurring only between the molecules and lipid surface without deeply interacting with the acyl chains (*44*), a perturbing effect on the enthalpy can be explained as due to a deep interaction

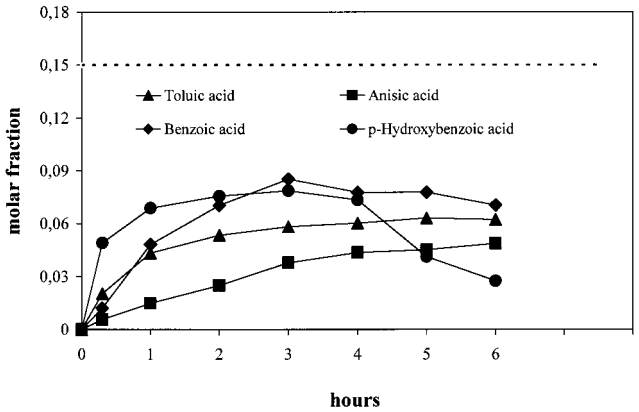


Figure 3. Acids transfer from free solid biocompounds (0.15 molar fraction with respect to the lipid aqueous dispersion) to DMPC multilamellar vesicles for increasing incubation time.

with the lipid chain related to the high lipophilicity of the penetrating molecule. This occurs for compounds **2**−**4**, while the effect is negligible for **1**.

Calorimetric analysis also were carried out on samples prepared at pH = 7.4 (data not reported) to investigate the effect of the changes in lipophilicity due to the compound's dissociation. But because of the water solubility related to the carboxylic group ionization, only a small interaction of all compounds with the membrane model was detected.

Because the intake of biomolecules is mainly determined by the uptake process from the aqueous medium by the membranes, it is interesting to investigate and compare the ability of the simple BP **1** and its analogue derivatives not only in their interaction with model membranes but also in their capacity to be *adsorbed* and *transported* inside them. The method to analyze these processes was largely reported by us in previous papers (*45*−*49*).

It consists of leaving in contact empty MLV or LUV with a fixed amount of solid compounds (molar fraction = 0.15 in the aqueous lipid dispersion) and determining, over time, the effects exerted by the adsorption of such molecules on the transition temperatures of the lipid vesicles by DSC. Then, by comparing the effect on the T_m shift with the values obtained by the calibration plots reported in Figure 2B, it is possible to obtain the amount of acids transferred inside the lipid vesicles. The results of the transfer kinetics of the acids **1**−**4** from the solid through the aqueous medium to the MLV and LUV surface are reported in Figures 3 and 4, where they are compared with the theoretical value (dotted line representing the effective 0.15 molar fraction) obtainable assuming that the total amount of acids reach and penetrate the lipid vesicle. The transfer of all the studied molecules through the aqueous medium and the successive binding with lipids appears to be faster and larger when LUVs (Figure 4) are considered with respect to the MLV (Figure 3). These results can be easily explained because for the same lipid concentration, LUVs expose a greater surface for the molecule's uptake process as compared to the multilamellar vesicles.

Among the examined molecules, **1**, even if it interacts with the biomembranes less than the other acids because of its hydrophilic characteristics (Figure 2B), appears to reach the lipid bilayer more rapidly than the other more lipophilic compounds (Figure 3).

The unusual behavior observed for some molecules at high incubation times and consisting of a decrease

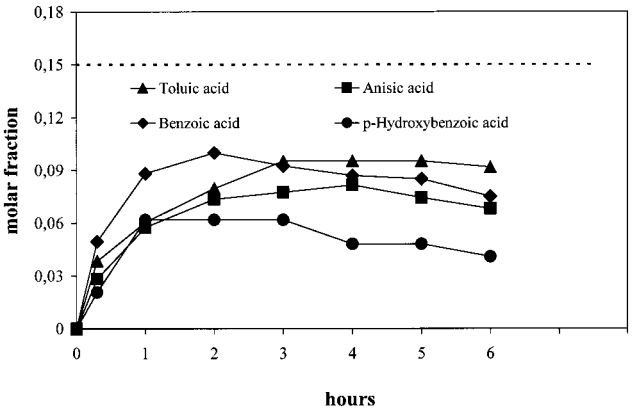


Figure 4. Acids transfer from free solid biocompounds (0.15 molar fraction with respect to the lipid aqueous dispersion) to DMPC large unilamellar vesicles for increasing incubation time.

of the temperature shift following the initial increase, means that a part of the substance is withdrawn from the interaction with the lipids. This effect could be explained as 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 (*50*).

By comparing Figures 3 and 4, it appears evident that the amounts of acids transferred into the lipid layer of MLVs and LUVs, incubated for 6 h at a temperature over the DMPC transitional temperature, are quite different. The uptake and transport processes are influenced by the lipid layer numbers allowing the compounds to better dissolve in LUVs as compared to MLV vesicles, hindering compounds to reach the same concentration in both lipid systems but permitting one to affirm that all compounds are able to be absorbed by model membranes in a differentiated way as a function of their structure.

The comparative biomimetic experiments, performed on **1** and its analogues **2**−**4** with different para substitutions, on the transition temperature of DMPC multilamellar vesicles, permit a deeper understanding of the interaction and penetration of biological membranes by simple BPs and biomolecules found in olive drupes and their Mediterranean food derivatives. The relevance of the results thus determined concerns the kinetic processes, involving the dissolution of the molecules in an aqueous medium and the transfer and mainly the potential absorption through a biological membrane, evaluated by in vitro measurements.

The explanation of the results, obtained by DSC techniques in evaluating both *static* and *dynamic* interaction processes among biomembrane models and olive BPs, may provide new insights into the health effects of Mediterranean foods on human well being.

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