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A calorimetric evidence of the interaction and transport of environmentally carcinogenic compounds through biomembranes

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Abstract

The effect exerted by pyrene and nitro-pyrene (N-pyrene), two structurally similar polycyclic aromatic hydrocarbons (PAH_S), possessing mutagenic and carcinogenic activity, on the thermotropic behaviour of model membranes constituted by dimyristoylphosphatidylcholine (DMPC) vesicles, was investigated by differential scanning calorimetry (DSC). Attention was directed to evaluate modifications in mutagen–lipid interaction induced by compound structure and lipophily and evidences in their membrane penetration.

The two examined compounds, when dispersed in liposomes during their preparation, were found to exert a very different action on the L_β to L_α gel-to-liquid crystal phase transition of DMPC multilamellar vesicles (MLV). Pyrene caused a detectable effect on the transition temperature (T_m), shifting it towards lower values with a concomitant decrease of the associated enthalpy (ΔH) changes, while N-pyrene was able to modify the lipid vesicles thermotropic behaviour only for low molar fractions, without deep changes in the ΔH. Modifications induced by pyrene were a function of mutagen concentration while the different behaviour of N-pyrene can be due to different polarity induced by the presence of the nitro-group, which attribute an higher hydrophilic character.

Solid pyrene and N-pyrene and MLV aqueous dispersions (0.12 molar fraction) were left in touch for long incubation times at temperature higher than the transitional temperature of DMPC to detect their spontaneous transfer through the medium. By following this procedure, no interaction was detected for both pyrene and N-pyrene with lipid vesicles, suggesting that their low hydrophilic character avoid their migration through the aqueous layer surrounding the MLV vesicles. Carrying out a kinetic measurement leaving for increasing incubation times charged MLV (0.12 molar fraction) with empty DMPC vesicles the uptake of the PAHs by the empty vesicles and their successive interaction, after several periods of incubations, was monitored. Pyrene and N-pyrene showed a different kinetic behaviour: the N-pyrene rate transfer was faster than that of pyrene but both final transfer and interaction were on the same order that detected by preparation of MLV charged with a 0.06 molar fraction of PAH, carried out in organic solvent. The obtained results suggest that the PAH_S, even if unable to reach and penetrate the biological membranes migrating through an aqueous layer, when dispersed in a lipophilic medium are able to penetrate and diffuse inside a model membrane. The different effects observed could be explained in terms of compound hydrophobicity and a relation between compound structure and membrane interaction can be suggested. This allows the membrane interaction with pyrene for all the tested molar fractions, but the structure of N-pyrene seems to suggest the formation of aggregate on the membrane surface for molar fractions higher than 0.09.

Keywords: Differential scanning calorimetry; Pyrene; Nitro-pyrene; PAH; Phosphatidylcholine; Membranes

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1. Introduction

Polycyclic aromatic hydrocarbons (PAH_S) are benzene derivated molecules possessing condensed rings skeleton. PAH_S are usually classified according to changes they undergo in one or more of their molecule's rings.

PAH_S are partially mutagenic and carcinogenic substances occurring at various concentrations in the atmosphere, soil, water and sediment. These are primarily anthropogenic pollutants that enter nature from industrial emission [1–3].

The main sources of PAH_S consist of household heating by coal, coal fired power station, automobile traffic, cigarette smoke and emission from waste incineration plants. PAH_S, inherited both from natural and anthropogenic processes, are persistent organic pollutants due to their chemical stability and biodegradation resistance. While inhaled PAH_S have long been suspected to induce lung cancer in humans [4]. They are able to pass through the skin even though the percoutaneous penetration is affected by various factors connected to exposure conditions [5]. Pyrene may promote allergic diseases by inducing the production of IL-4 [6].

Actually, PAH_S are not carcinogenic agents by themselves. They have to undergo to several metabolic transformations taking place in the human body to produce carcinogenic molecules. They are generally metabolised to oxygenated products by monooxygenase system composed of a membrane bound cytocrome P-450 and a reductase [7].

A more extensive study of the organization and interaction of the PAH_S in membrane systems is important to assess their potential carcinogenic effect. One of techniques employed to carry out studies on the effect of increasing incorporation of PAH_S on phosphatidylcholine membranes is the differential scanning calorimetry (DSC). This non-perturbative technique permits to study the effect exerted by a substance on the well-known phase transition gel-to-liquid crystal exhibited by phospholipid species [8–10]. Usually a substance dissolved in a lipidic layer can affect the transitional temperature by causing its decrease [11–13]. The magnitude of the destabilising effect is related to the amount of chemicals dissolved in the lipidic structure. By DSC it is also possible to monitor the uptake process of a compound in a model membrane surface. This process is modulated by the compounds lipophilicity and solubility in water, as well as the phospholipid membrane composition and phase separation presence [14–16].

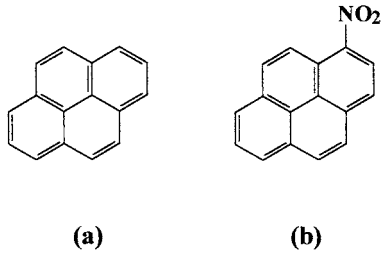

Fig. 1. Structural formula of: (a) pyrene; (b) N-pyrene.

In this work we investigated the effects of the structural difference of two PAH molecules (Fig. 1) on the interaction with DMPC vesicles, similarly as described previously by studying the interaction of structurally similar compounds [15–17] with DMPC MLV. To study this interaction, we have employed the L-α-dimyristoylphosphatidylcholine (DMPC) MLV, as synthetic simplified model membranes which show a change in their thermotropic behaviour if other molecules are dissolved in their ordered structure [12,18–20].

The results obtained by the PAH-membrane "classical" interaction (interaction in organic solvent before multilamellar vesicle (MLV) preparation) were compared with the kinetic experiments of the transfer of compounds to empty membranes (or the transfer between charged to empty membranes). Differences in the compound ability to interact and penetrate the lipid bilayer of biomembranes, which cause variations in their structure and fluidity, so, should be detected. In this way, it is possible to examine the steric hindrance of these compounds which is known to modulate the interaction and/or penetration of foreign molecules into cell membranes, as well as assessing the lipophilicity which should be a factor in such penetration phenomena. It is possible to obtain indication about the role of substituents present on a molecule in the interaction with the microenvironment of the lipid bilayer and to determine the variation in their transport across natural membranes.

2. Experimental

2.1. Materials

Pyrene and nitro-pyrene (N-pyrene) were obtained from Supelco (USA) purity higher than 97%. Synthetic L-α-DMPC was obtained from Fluka Chemical Co. (Buchs, Switzerland). Solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). Lipid concentrations were determined by phosphorous analysis by the method of Bartlett [21]. Buffer solution was 50 mM Tris, adjusted to pH = 7.4 with hydrochloric acid.

2.2. Liposomes preparation

Multilamellar liposomes were prepared in the presence and absence of increasing concentrations of pyrene or N-pyrene by the following procedure. Chloroform stock solutions of lipid and PAH were mixed to obtain the chosen molar fraction of compounds. Solvent was removed under nitrogen flow and the resulting film was freeze–dried to remove the residual solvents.

Liposomes were prepared by adding to the film 50 mM Tris-buffer solution (pH = 7.4), then heating at 37°C, the temperature above the 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 homogenise the liposomes. Then, aliquots of 120 μl (5 mg of lipid) were transferred in a 150 μl DSC aluminium pan, hermetically sealed, and submitted to DSC analysis.

2.3. DSC

DSC was performed by using a Mettler TA 4000 system equipped with a DSC-30 cell and a TC-11 processor. The scan heating rate employed was 2 K/min in the temperature range 5–37°C. The sensitivity was 1.5 mW, and the reference pan was filled with Tris-buffer solution. After the factory-suggested calibration of the calorimetric system, a narrow range of calibration temperatures was determined by using indium, stearic acid, and cyclohexane. Indium was employed to calibrate the transitional enthalpies (ΔH). Temperature and enthalpies were checked also by using palmitic acid. Enthalpies were evaluated from the peak areas using the integration program of the TC-11 processor or by using the software Mettler TA72, permitting the choice of different baselines and ranges of integration. The areas calculated with these different methods lie within the experimental uncertainty (±5%).

The samples were cooled and heated four times to check the reproducibility of results. All samples, after calorimetric scans, were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorous assay [21].

2.4. Permeation experiments

To study the capacity of the two examined PAH_S to permeate the model membrane a kinetic experiment was carried out. DMPC aqueous suspension (MLV) was left in contact with a fixed amount of finely powdered PAH (to obtain a 0.12 molar fraction with respect to the phospholipid) placed in the bottom of the DSC crucible. The samples, after having been hermetically sealed in the pans, were gently shaken for 10 s, then submitted to subsequent calorimetric cycles by using the following step procedure.

1. A scan between 5 and 37°C, to detect the interaction between the compounds, if able to dissolve in the aqueous medium and to transfer to membrane surface, and the model membrane during the heating of the sample.
2. An isothermal period (1 h) at 37°C to permit to the PAH to eventually permeate the lipid layers staying in a disordered state at a temperature over the lipid transitional temperature.
3. A cooling scan between 37 and 5°C, at the rate of 4 K/min, before restarting the heating program (step 1).

This procedure was run at least six times to detect variations in the temperature of the calorimetric peak and repeated for a long incubation time (12 h), to observe eventual further shifts in the peak temperature.

2.5. Transmembrane transfer kinetics

The ability of the two examined PAHs to be transferred from charged MLV (0.12 molar fraction) to

empty MLV inducing an effect on the membrane similar to that observed for the organic preparation at 0.06 molar fraction of PAH was determined. These kinetic experiments were carried out by transferring an empty (60 μl) DMPC aqueous suspension (MLV) in a 140 μl calorimetric pan where 60 μl of MLV charged with a 0.12 molar fraction of PAH had previously been placed. The samples, after have been hermetically sealed in the pans, were gently shaken for 10 s, then submitted to subsequent calorimetric cycles by using the same step procedure reported in the previous section. In this case during the first step (the scan between 5 and 37°C) we detect the interaction between charged liposomes and empty model membrane during the heating of the sample, due to the compounds transfer from charged membrane to surface of empty liposomes.

3. Results

The interaction between the two similar PAHs with model membranes was favoured by complete dissolution in organic solvents of their increasing molar fractions in presence of a fixed amount of DMPC to obtain (after drying the organic solutions and resuspending in buffer aqueous solution) a very close contact PAH/lipidic molecules. The results of this complete dispersion inside the MLV system are reported in Fig. 2A and B. The calorimetric heating curves, here reported, of DMPC MLV in presence of increasing pyrene and N-pyrene molar fractions put in evidence as the examined compounds are able to exert interesting interactions with DMPC vesicles: the disappearance of the typical pretransitional peak exhibited by DMPC liposomes as well as the shift of the calorimetric peak towards lower values. By increasing

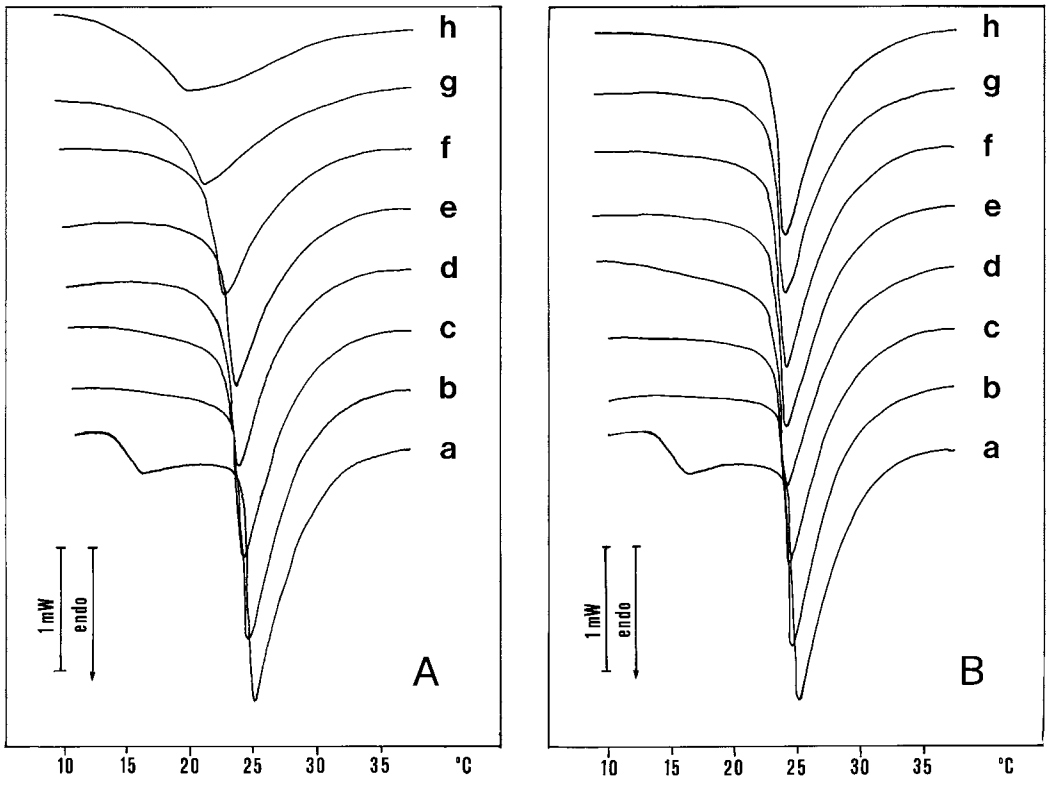

Fig. 2. Differential scanning calorimetry heating curves of hydrated DMPC containing: (A) pyrene; (B) N-pyrene; obtained starting from organic solvent solutions, at a PAH molar fraction: $a = 0.0$; $b = 0.015$; $c = 0.030$; $d = 0.045$; $e = 0.06$; $f = 0.09$; $g = 0.12$; $h = 0.15$.

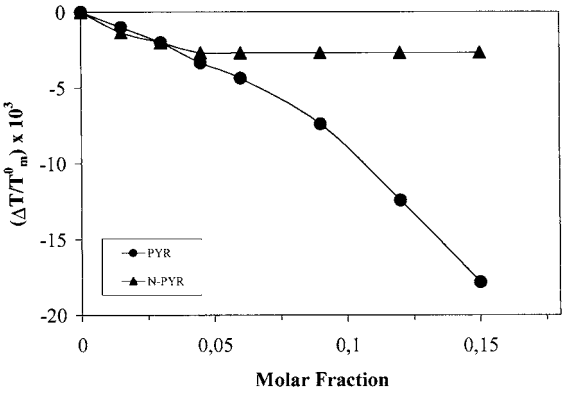

Fig. 3. Transitional temperature variations (as $\Delta T/T^0_m$) values (average of at least three runs), in heating mode, as a function of pyrene and N-pyrene molar fractions.

the amount of pyrene and N-pyrene, the transitional temperature (T_m) was differently shifted towards lower values. In fact, while pyrene decreases the transitional temperature for all the tested molar fractions, the N-pyrene exerts such fluidifying effect up to a 0.06 molar fraction. This effect is showed in the Fig. 3, where the temperature shifts are reported as $\Delta T \times 10^3/T^0_m$, ($\Delta T = T_m - T^0_m$, where T_m is the transition temperature obtained for increasing pyrene and N-pyrene molar fractions and T^0_m is the transition temperature of the pure DMPC).

The data were obtained from experiments carried out in triplicate and for each value reported the standard deviation was less than 1.5%; thus, no statistical treatment of the data was reported.

The enthalpy change (ΔH), calculated from the calorimetric peak area, was negligibly affected (data not reported) by N-pyrene, instead a decrease in the ΔH was observed when pyrene was used, as it is possible to see looking at the calorimetric curves shape (Fig. 2A and B).

The interaction between PAHs and MLV vesicles can be explained in terms of a "fluidifying" effect due to the introduction of lipophilic molecules into the ordered structure of the lipid bilayer. These molecules acting as spacers in such a structure cause a destabilisation of the lipid mosaic with a decrease in the T_m of the gel to liquid crystal phase transition. The slight variation in the ΔH, which occurs when N-pyrene is used, can be explained as a superficial interaction between molecules and lipids. In fact, molecules can interact with lipids in liposomes as "substitutional impurities" of a membrane, taking the place of lipid molecules and such an effect can cause T_m variation and ΔH change because of their deep interaction (pyrene). Instead when they interact as "interstitial impurities", by intercalating among the flexible acyl chain of lipids cause T_m variations without ΔH change (N-pyrene), according to the temperature depression of melting point for ideal solution [18,22–27]. As previously reported, using DSC technique it is possible to investigate the potential of molecules to interact with a model membrane's surface and to permeate lipid liposomes passing through bilayers [13,15,16,28]. PAHs are able to react with membrane model and to be transferred, under certain experimental conditions, to the lipidic layers (MLV) causing a shift of the gel-to-liquid crystal phase transition.

Temperature shifts versus different pyrene and N-pyrene molar fractions are compared with those relatives to effects of solid PAHs (0.12 molar fraction) left in contact with DMPC MLV aqueous dispersion. This comparison is essential to understand PAH's ability to pass through the medium reaching and reacting with membrane models and, successively, to permeate the lipidic layer [14–16]. From the calorimetric curves (data not reported) it is evident as both compounds are not able to pass through the aqueous medium even if left to incubate at temperature higher than the transitional temperature of DMPC for several hours. In fact, not reaching the liposomes surface and not interacting with the lipidic layer, no decrease in the transitional temperature of DMPC MLV was observable.

Different conclusions can be drawn by considering the experiments carried out by leaving in touch equimolar amounts of charged liposomes (0.12 molar fraction) with empty liposomes, for increasing incubation times. In Fig. 4A and B, the effects of increasing incubation times on the mixed charged and empty vesicles are reported and compared with the curves of pure DMPC as well as 0.06 PAH molar fraction. By comparing the values obtained, as calorimetric shift, with the data obtained for the liposomal suspension in presence of a 0.06 molar fraction of both compounds (Fig. 5), it is evident as the data obtained from the transfer and interaction of pyrene and N-pyrene present in the liposomal suspension with empty MLV

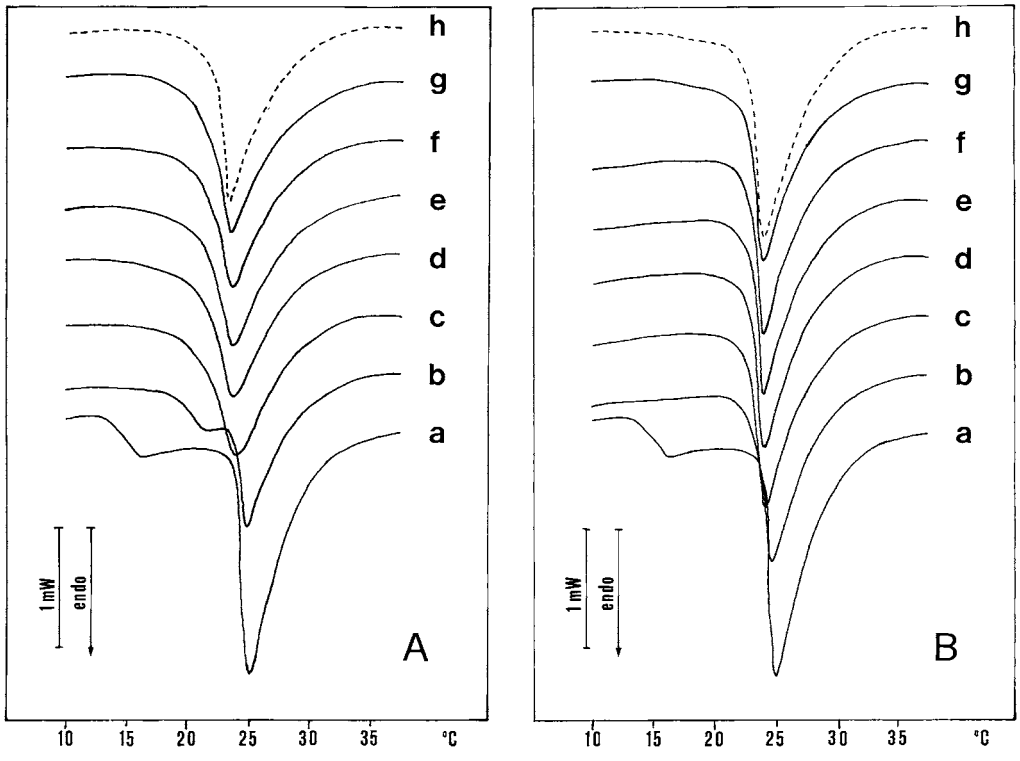

Fig. 4. Differential scanning calorimetry heating curves of: empty DMPC multilamellar vesicles alone (curve a) or left in the presence of DMPC multilamellar vesicles charged with (A) pyrene or (B) N-pyrene at a fixed molar fraction (0.12) for increasing incubation times (curves b–g). Curve h represents the effect of the 0.06 molar fraction of PAH on the MLV, obtained starting from organic solvent solutions, to be considered as the effect to be reached if the PAH was transferred from charged to empty vesicles.

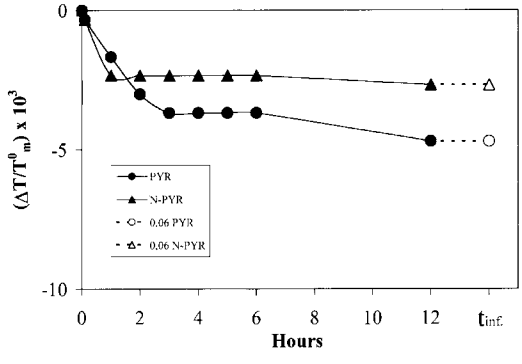

Fig. 5. Transitional temperature variations (as $\Delta T/T_m^0$) of empty DMPC multilamellar vesicles in the presence of DMPC multilamellar vesicles charged with pyrene or N-pyrene at a fixed molar fraction (0.12) for increasing incubation times. The t_{inf} values represent the effect exerted of the 0.06 molar fraction of PAH on the MLV, obtained starting from organic solvent solutions, to be considered as the maximum interaction between compound and vesicles.

dispersion are trending to values representing the maximal interaction between compounds and DMPC vesicles (data are obtained from the interaction of a fixed molar fraction prepared in organic phase, in which pyrene and N-pyrene are compelled to stay inside the MLV). The data obtained for PAH molar fraction (0.06) are reported in Fig. 5 by empty signs (as values at t_{inf}).

The transfer rate depends on the kind of examined molecule, in fact N-pyrene seems to be transferred faster than pyrene even if after 3 h the nearly saturated kinetic can hamper to differentiate the interaction. The reported data show that transition temperature shift increases with time when both the compounds are in contact with MLV (Fig. 5). This augmentation continues, even if with a different trend depending on the liposome specie, until it reaches a constant value close to that obtained by direct mixing of N-pyrene with the lipid component during the vesicle preparation.

We can conclude that transition temperature shift tends to reach the same values observed for direct mixing of pyrene and N-pyrene and DMPC.

4. Conclusions

The analysis of this behaviour permits us to make some considerations about PAHs permeation and distribution into membranes.

1. PAHs compounds were found unable to migrate through an aqueous medium to reach biological membranes.
2. PAHs compounds are able to interact with model membranes by shifting the transitional temperature.
3. PAHs are able to be transferred from charged vesicles to empty vesicles, suggesting as their absorption is favoured by lipophilic agents being others mechanisms not to invoke, for instance a drug transfer mediated by fusion of MLV which was demonstrated impossible to happen [29].

The obtained results confirm the influence of structural changes on the molecular backbone in modifying the interaction with model membranes by affecting the shift of lipid phase transition temperature and the enthalpy change [13,17,18].

The results obtained suggest that PAH carcinogenic and mutagenic properties can be due to the interaction with biological membrane even if such interaction is not caused by a simple passive transport through biological membranes but could be correlated with other mechanism of action caused by the transfer from a lipophilic medium to a biological membrane. Finally, we have shown the importance of the calorimetric technique to indirectly detect transport process through biological membranes, trying to clarify the factors influencing the possibility of a biological membrane to absorb a substance.

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References

[1] V. Librando, A. Liberatori, D.A. Fazzino, J. Polycyclic Arom. Comp. 3 (1992) 587.
[2] V. Librando, D.S. Fazzino, Chemosphere 27 (1993) 1649.
[3] V. Librando, G. D'Arrigo, D. Spampinato, Analysis 22 (1994) 340.
[4] P. Gerde, B.A. Huggenburg, G.G. Scott, J.L. Lewis, K.H. Pyon, A.R. Dahl, Carcinogenesis 19 (1998) 483.
[5] P. Sartorelli, A. Cenni, G. Matteucci, L. Montomoli, M.T. Novelli, S. Palmi, Int. Arch. Occup. Environ. Health 72 (1999) 528.
[6] H. Bommel, M. Li-Weber, E. Serfling, A. Duschl, J. Allergy Clin. Immunol. 105 (2000) 796.
[7] G.J. Zylstra, X.P. Wang, E. Kim, V.A. Didolkar, Ann. NY Acad. Sci. 721 (1994) 386.
[8] S. Mabrey-Gaud, Differential scanning calorimetry of liposomes, in: C.G Knight (Ed.), Liposomes: From Physical Structure to Therapeutic Applications, Elsevier, North Holland, 1981, pp. 105–138.
[9] J.R. Silvius, Chem. Phys. Lipids 57 (1991) 241.
[10] D. Bach, Calorimetric studies of model and natural biomembranes, in: D. Chapman (Ed.), Biomembrane Structure and Function, Macmillan, London, 1994, pp. 1–41.
[11] M.K. Jain, N.M. Wu, J. Membr. Biol. 34 (1977) 151.
[12] M.K. Jain, in: M.K Jain (Ed.), Introduction to Biological Membranes, Wiley, New York, 1988, p. 122, and references therein.
[13] F. Castelli, G. Valencia, Thermochim. Acta 154 (1989) 323.
[14] A. Raudino, F. Castelli, J. Colloid Interf. Sci. 200 (1998) 52.
[15] F. Castelli, S. Caruso, N. Giuffrida, Thermochim. Acta 327 (1999) 125.
[16] F. Castelli, N. Uccella, A. Saija, D. Trombetta, J. Agric. Food Chem. 47 (1999) 991.
[17] F.Castelli.N. Giuffrida, C. Tringali, G. Ruberto, J. Agric. Food Chem. 48 (2000) 4123.
[18] B.R. Cater, D. Chapman, S.M. Hawes, J. Saville, Biochem. Biophys. Acta 363 (1974) 54.
[19] D. Papahadjopoulos, K. Jacobson, G. Poste, G. Shepherd, Biochim. Biophys. Acta 394 (1975) 504.
[20] T.J. O'Learly, P.D. Ross, I.W. Levin, Biophys. J. 50 (1986) 1053.
[21] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466.
[22] A.G. Lee, Biochim. Biophys. Acta 472 (1977) 285.
[23] T.N. Estep, D.B. Mountcastle, R.L. Biltonen, T.E. Thompson, Biochemistry 17 (1978) 1984.
[24] O.G. Mouritsen, M. Bloom, Biophys. J. 46 (1984) 141.
[25] G. Cevc, D. Marsh, in: E.E. Bittar (Ed.), Phospholipid Bilayers Physical Principles and Models, Wiley, New York, 1987, p. 1.
[26] K. Jorgensen, J.H. Ipsen, O.G. Mouritsen, D. Bennet, M.J. Zuckermann, Biochim. Biophys. Acta 1062 (1991) 227.

[27] F. Castelli, G. Puglisi, G. Giammona, C.A. Ventura, Int. J. Pharm. 88 (1992) 1.
[28] F. Castelli, B. Conti, D.E. Maccarrone, U. Conte, G. Puglisi, Int. J. Pharm. 176 (1998) 85.
[29] N. Duzgunes, J. Wilschut, D. Papahadjopoulos, in: F. Conti, W.E. Blumberg, J. De Gier, F. Pocchiari (Eds.), Physical Methods on Biological Membranes and their Model Systems, Plenum Press, NY, USA, 1985, pp. 193–218.