

SECTION 1. BIOLOGY

1.1 Study of cardiolipin oxidation by mass spectrometry

Apoptosis is recognized as important for normal cellular homeostasis in multicellular organisms. Apoptosis – ingeniously orchestrated cell death – involves a genetically predetermined chain of events that leads to disassembly of intracellular structures and macromolecules and culminates in selective clearance of unwanted or irreparably damaged cells by phagocytes. Mitochondria are key regulators in both intrinsic (initiated by endogenous death signals) and extrinsic (triggered by toxic damaging signals) pathways of apoptosis. Cytochrome *c* (cyt *c*), a relatively simple component of the mitochondrial electron transport system, turns into a dreadful soloist when it finds itself in the new cytosolic environment, where it performs a well characterized initiation of apoptosome formation and activation of caspase-dependent apoptotic processes [1,2].

Perhaps the most complex glycerophospholipid present in eukaryotic cells is cardiolipin (1,3-*bis*-(1',2'-diacyl-3'-phosphoryl-*sn*-glycorol)-*sn*-glycerol). The trivial name “cardiolipin” (CL) was derived because this phospholipid was first found in animal hearts, where it is especially abundant. However, CL is found in the mitochondria of animal species, yeasts, and bacteria. That phospholipid is found almost exclusively on the inner membrane of the mitochondria within cells. It is unusually lipophilic because it contains four fatty-acyl substitutions. The exact role of CL in cellular biochemistry is complex; but, it is clear that CL is necessary for cyt *c* insertion into the mitochondrial membrane, and that it is involved in mitochondrial stability and function [3].

Recently, two other essential functions of cyt *c* in apoptosis, ones that may rely heavily on its redox activity, have been suggested. Both functions are directed towards oxidation of two negatively charged phospholipids – CL in the mitochondria [4] and phosphatidylserine (PS) in the plasma membrane [5]. In both cases, oxidized phospholipids seem to be essential for the transduction of two distinctive apoptotic

signals: one is participation of oxidized CL in the formation of the mitochondrial permeability transition pore (MPTP) that facilitates release of cyt *c* (along with several other pro-apoptotic factors) into the cytosol [6]; the other is the contribution of oxidized PS to the externalization and recognition of PS (and possibly oxidized PS) on the cell surface by specialized receptors of phagocytes [7]. Although association between cyt *c* and these essential apoptotic pathways of phospholipids have been established, specific mechanisms of cyt *c*'s catalysis of their oxidation remain unknown.

CL oxidation is two-fold: first, oxidized CL, in contrast to non-oxidized CL, does not tightly bind cyt *c*; hence, it cannot retain cyt *c* in the inner membrane. Rather the oxidized CL releases the cyt *c* into intermembrane space. Second, CL oxidation is important for the formation of MPTP [6].

In complexes of cyt *c* with unsaturated molecular species of CL function as a peroxidase, this activity should be detectable *in vitro*. We utilized Electrospray Ionization Mass Spectrometry (ESI-MS) analysis to determine whether peroxidase activity of cyt *c*/CL complexes catalyzes oxygenation of CL [8,9].

The majority of mitochondrial CL (70-90%) is represented by only one molecular species – tetra-linoleoyl-cardiolipin (TLCL) containing four, hydrophobic, doubly unsaturated C_{18:2} linoleic acid (LA) residues (Fig. 1). Therefore, as a reactions substrate, we used unsaturated molecular species of CL – TLCL, the most abundant CL molecular species of mitochondria.

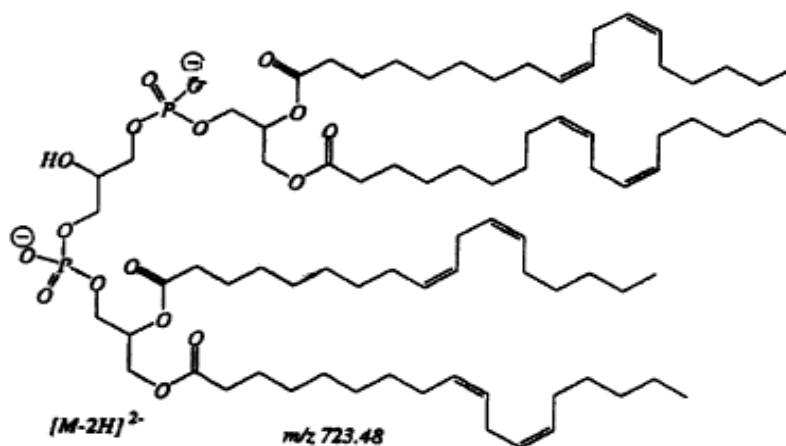


Fig. 1. Scheme of the tetra-linoleoyl-cardiolipin (TLCL). Cardiolipin has two ionizable phosphodiester groups in its structure, therefore, electrospray ionization generates abundant negative ions, including the doubly charged $[M-2H]^{2-}$.

The radical adducts of lipids are usually examined by electron spin resonance (ESR) spectroscopy; however, the ESR spectra give little structural information. More recently, mass spectrometry has been used for the identification adducts of oxidation products of radical-derived reactions, and in particular was applied to the characterization of the adducts of CL [10]. In this study we will present and discuss the results obtained from oxidation of TLCL by cyt *c* and H₂O₂ monitored by ESI-MS. TLCL and phosphatidylcholine (PC) were purchased from Avanti Lipids (USA); cyt *c* (bovine heart), H₂O₂ and all other reagents were of the highest grade available and were purchased from Sigma, Fisher, or VWR Scientific (San Francisco, CA, USA). The recent introduction of "soft" ionization methods has opened completely new vistas in this field. In particular, ESI-MS has been shown to be a very promising technique [11,12,13,14]. However, there are still many issues to be resolved before ESI-MS can become a routine tool for quantitative determination of lipids and lipid compositions.

ESI/MS analyses were performed utilizing a quadrupole mass spectrometer Finnigan TSQ-700 (ThermoFinnigan, Inc., San Jose, CA, USA) equipped with an electrospray ion source, as described previously [15]. The TSQ-700 gave optimal signal in Q3 low mass mode. Typically, a 5-min period of signal averaging in the profile mode was employed for each spectrum of a lipid. All samples of lipids were diluted in chloroform/methanol (1:1, v/v) prior to direct infusion into the ESI chamber using a syringe pump at a flow rate of 2-5 μ L/min in this study. Sheath and auxiliary gases (nitrogen) were set to 30 psi and 5 mL/min, respectively. The capillary voltage was 3,5-5,5 kV, the source temperature was set at 70 °C, and the optimal cone-voltage energy was 30 V. Mass spectra were obtained by scanning the range of 250-1600 m/z every 2 s and summing individual spectra. Anionic phospholipid TLCL (C_{18:2})₄ in the diluted chloroform/methanol samples were analyzed by ESI/MS in the negative-ion mode without an internal standard. TLCL was measured by single-ion monitoring of their one- and double-charged ions. PC can be directly defined only in the positive-ion mode [15] and it doesn't hinder analyses of TLCL.

In order to prepare micelles (liposomes) of TLCL (120 μ L of 16,7 mM solution) we added PC (80 μ L of 25 mM solution) in 1,8 mL of HEPES buffer solution (pH 7,4).

Each reaction mixture was vortexed for 1 min and processed by ultrasound before starting the reaction by adding cyt *c* (200 μ L of 400 μ M solution) and H₂O₂ (100 μ L of 10 mM solution). The reaction mixture was incubated for 60 min at 37°C without shaking. The H₂O₂-cyt *c* system produced hydroxyl radicals ($\bullet$ OH) that converted TLCL into oxidized species of TLCL (TLCLox) [16]. The control was prepared by the TLCL, PC, cyt *c* and H₂O₂ in HEPES buffer without incubation. After the incubation the phospholipid oxidation products were extracted with chloroform/methanol (2:1, v/v), HEPES buffer / chloroform-methanol (1:4, v/v). The organic phase of samples was separated and evaporated under a stream of nitrogen gas. The samples were dissolved hereinafter in 2 mL of chloroform/methanol (1:1, v/v). We used in ESI-MS analysis concentrations 40 pmol/ μ L of TLCL and TLCLox.

When cyt *c*/TLCL complex was incubated with H₂O₂ for 1 h, several oxygenated species of TLCL were detected. TLCL has two ionizable phosphodiester groups in its structure, therefore, electrospray ionization generates abundant negative ions. The results obtained by the use of ESI-MS for the identification of radical-derived products of phospholipid oxidized show the presence of several products formed. Among the products thus formed, adducts of oxidized TLCL and free fatty acid (linoleic acid oxidized) were identified. The TLCL and LA oxidation products identified, observed as singly [M-H]⁻ and doubly charged [M-2H]²⁻ ions, were characterized as adducts of mono-, di- and tri- hydroxy, hydroperoxides and epoxy-derivatives. Mass weight of TLCL is 1450 Da, mass weight of free LA is 280 Da.

The negative-ion ESI mass spectra of TLCL (control), the range of 250-1600 m/z: the peak of m/z 1448.9 is singly charged TLCL [TLCL – H]⁻, m/z 723.8 is doubly charged TLCL [TLCL – 2H]²⁻ (Fig. 2).

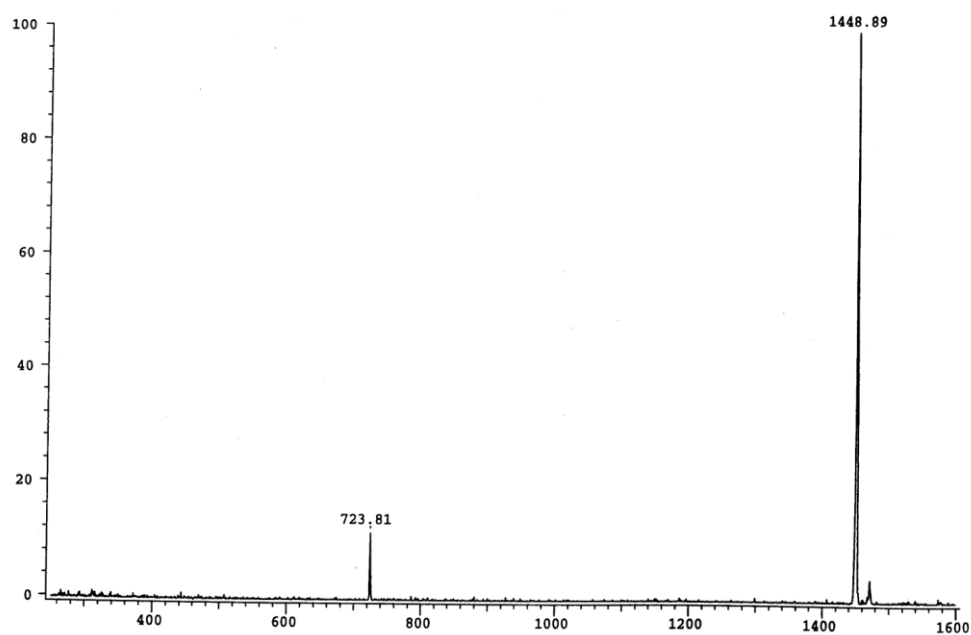


Fig. 2. Negative-ion ESI mass spectra of TLCL (control), the range of 250-1600 m/z.

The negative-ion ESI mass spectra of TLCLox, the range of 250-1600 m/z: the peak of m/z 1448.9 is singly charged TLCL [$\text{TLCL} - \text{H}$] $^-$, m/z 723.8 is doubly charged TLCL [$\text{TLCL} - 2\text{H}$] $^{2-}$, m/z 311.4 is hydroperoxide of linoleic acid oxidized [$\text{LA} - \text{H} + 2\text{O}$] $^-$, m/z 325.3 is epoxy-derivative and hydroperoxide of LA [$\text{LA} - 3\text{H} + 3\text{O}$] $^-$, m/z 339.4 is di-epoxy-derivative and hydroperoxide of LA [$\text{LA} - 5\text{H} + 4\text{O}$] $^-$ (Fig. 3).

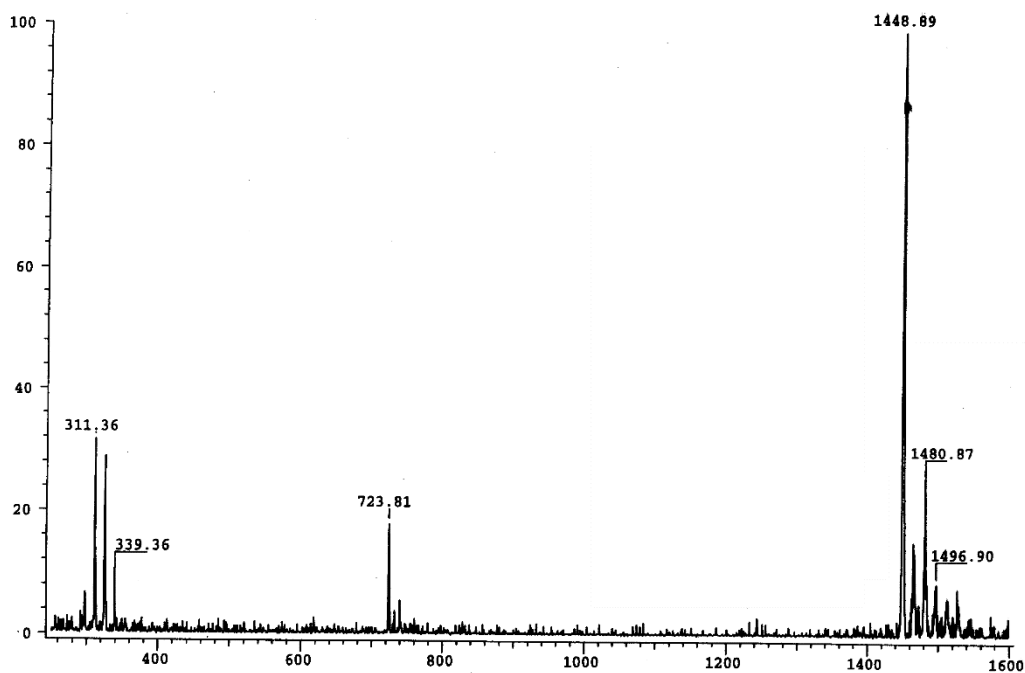


Fig. 3. Negative-ion ESI mass spectra of TLCLox, the range of 250-1600 m/z.

The negative-ion ESI mass spectra of TLCL (control), the range of 1400-1580 m/z: the peak of m/z 1448.9 is singly charged TLCL $[\text{TLCL} - \text{H}]^-$ (Fig. 4).

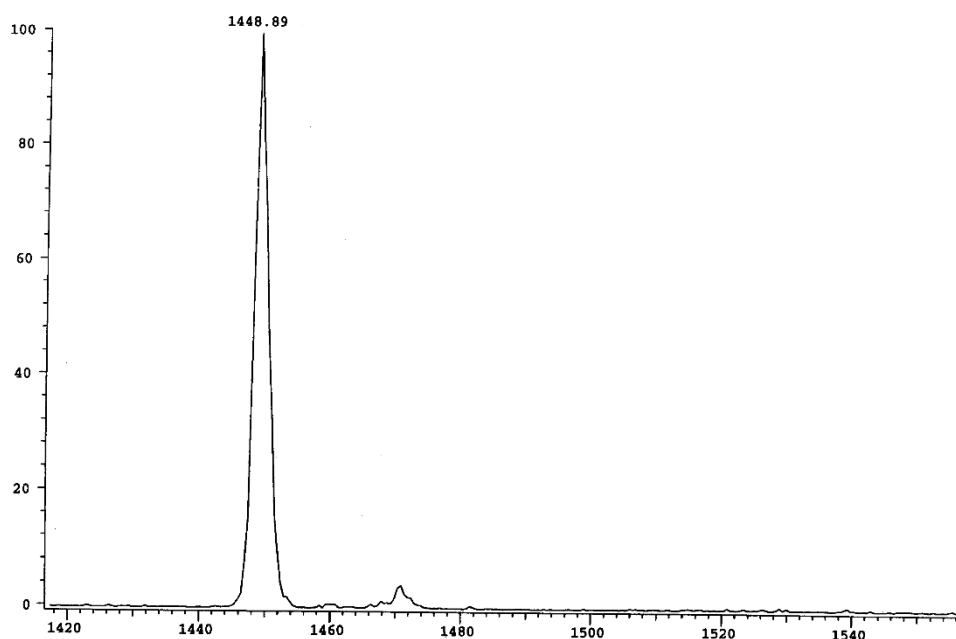


Fig. 4. Negative-ion ESI mass spectra of TLCL (control), the range of 1400-1580 m/z.

The negative-ion ESI mass spectra of TLCLox, the range of 1400-1580 m/z: the peak of m/z 1448.9 is singly charged TLCL $[\text{TLCL} - \text{H}]^-$, m/z 1463.8 most likely is oxide of singly charged TLCLox $[\text{TLCL} - \text{H} + \text{O}^\bullet]^-$, m/z 1464.9 is hydroxide of singly charged TLCLox $[\text{TLCL} - \text{H} + \text{O}]^-$, m/z 1480.9 is hydroperoxide of TLCLox $[\text{TLCL} - \text{H} + 2\text{O}]^-$, m/z 1496.9 is hydroxide and hydroperoxide of singly charged TLCLox $[\text{TLCL} - \text{H} + 3\text{O}]^-$, m/z 1512.9 is di-hydroperoxide of singly charged TLCLox $[\text{TLCL} - \text{H} + 4\text{O}]^-$, m/z 1526.4 most likely is epoxy-derivative and di-hydroperoxide of singly charged TLCLox $[\text{TLCL} - 3\text{H} + 5\text{O}]^-$ (Fig. 5).

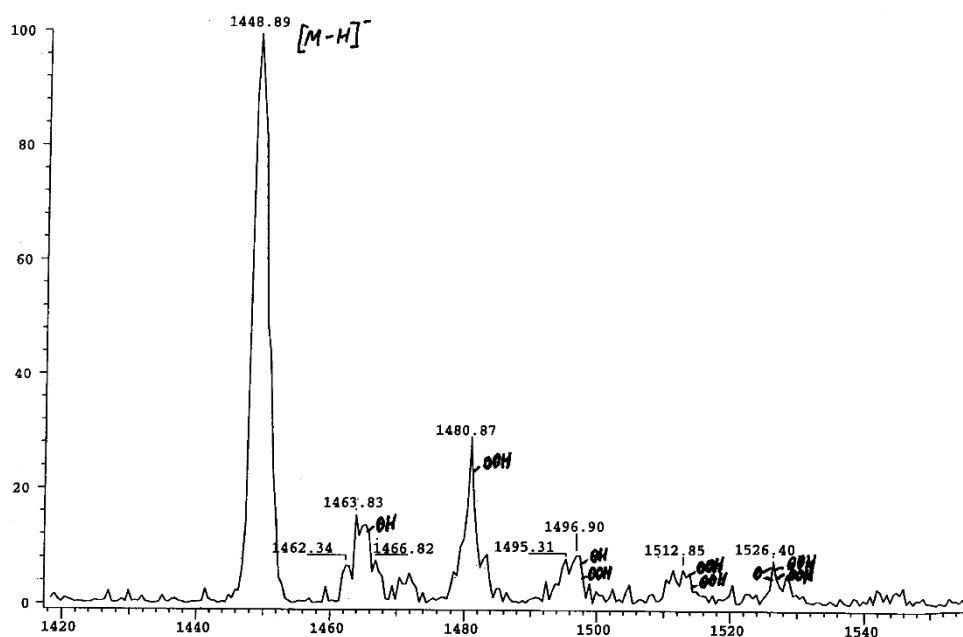


Fig. 5. Negative-ion ESI mass spectra of TLCLox, the range of 1400-1580 m/z.

But we can see better TLCLox in doubly charged ions. The negative-ion ESI mass spectra of TLCLox, the range of 650-800 m/z: the peak of m/z 723.8 is doubly charged TLCL $[TLCL - 2H]^{2-}$, m/z 731.8 is hydroxide of doubly charged TLCLox $[TLCL - 2H + O]^{2-}$, m/z 739.9 is hydroperoxide of TLCLox $[TLCL - 2H + 2O]^{2-}$, m/z 747.4 is hydroxide and hydroperoxide of TLCLox - $[TLCL - 2H + 3O]^{2-}$, m/z 755.9 is di-hydroperoxide of TLCLox - $[TLCL - 2H + 4O]^{2-}$, m/z 763.5 is hydroxide and di-hydroperoxide of TLCLox $[TLCL - 2H + 5O]^{2-}$, m/z 771.5 is tri-hydroperoxide of TLCLox $[TLCL - 2H + 6O]^{2-}$, m/z 779.5 is hydroxide and tri-hydroperoxide of TLCLox $[TLCL - 2H + 7O]^{2-}$, m/z 787.5 most likely is tetra- hydroperoxide of TLCLox $[TLCL - 2H + 8O]^{2-}$ (Fig. 6).

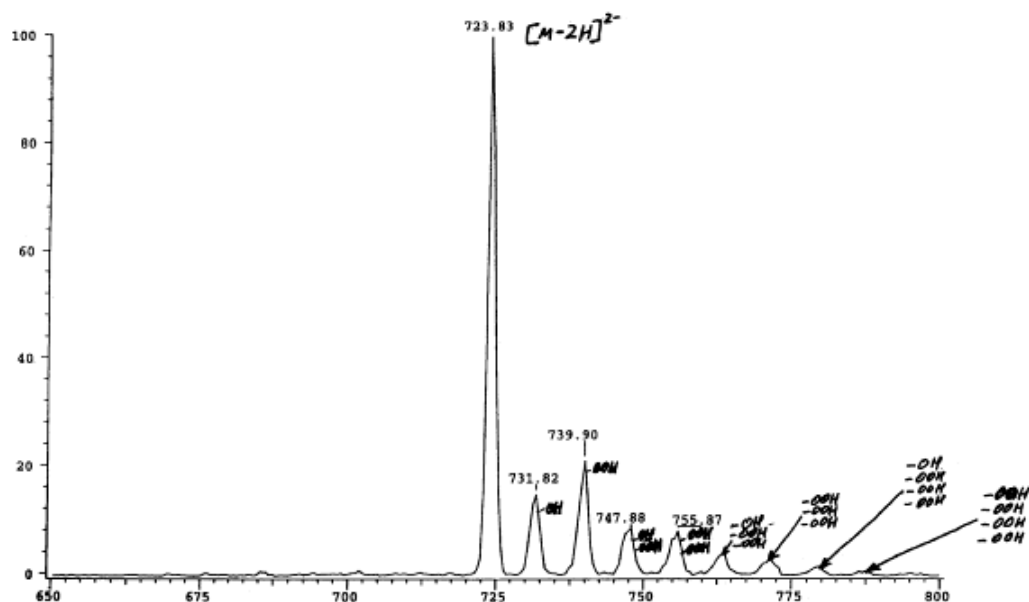


Fig. 6. Negative-ion ESI mass spectra of TLCLox, the range of 650-800 m/z.

The negative-ion ESI mass spectra of TLCLox, the range of fatty acid, 250-500 m/z: the peak of m/z 311.4 is singly charged hydroperoxide of linoleic acid oxidized $[LA - H + 2O]^-$, m/z 325.3 is epoxy-derivative and hydroperoxide of LAox $[LA - 3H + 3O]^-$, m/z 339.4 is di-epoxy-derivative and hydroperoxide of LAox $[LA - 5H + 4O]^-$ (Fig. 7).

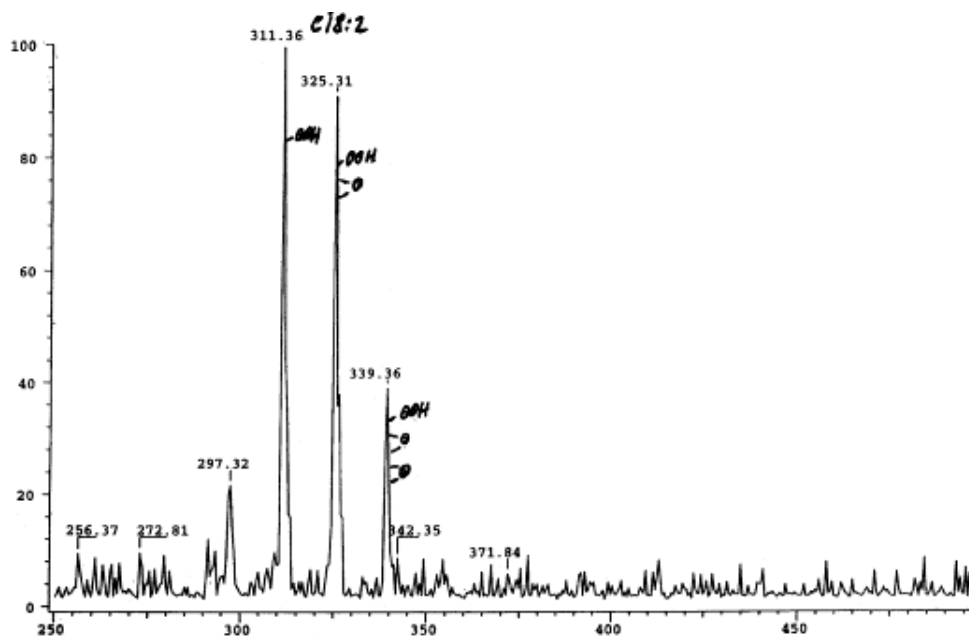


Fig. 7. Negative-ion ESI mass spectra of TLCLox, free LAox, the range of fatty acid, 250-500 m/z.

The peak of m/z 279 (does not oxidized free linoleic acid) is missing. In the light of our results (Fig. 3,7), we concluded that *cyt c* in *cyt c*/CL system may works as phospholipase by oxidized unsaturated molecular species of fatty acids of CL.

We also confirmed that *cyt c* in the presence of CL (*cyt c*/CL system) and H_2O_2 could produce some radical species. The *cyt c*/CL system may play a role in lipid peroxidation.

Thus peroxidase activity of *cyt c*/CL complex acts as CL oxygenase and catalyzes peroxidation of CL during apoptosis. The *cyt c*/CL complexes are important in cardiolipin oxidation and release of pro-apoptotic factors from mitochondria. CL-bound *cyt c* acts as a mitochondrial “death receptor” giving pro-apoptotic signals into executing oxidative cascades that produce an overload of oxidized polyunsaturated CL species – which, in turn, are required for both detachment of *cyt c* from the membrane and for the formation of MPTP through which *cyt c* is released into the cytosol.