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# Influence of Low Molecular Weight Organic Acids on Transport of Cadmium and Copper Ions across Model Phospholipid Membranes

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The transport of cadmium and of copper ions across the artificially prepared model phospholipid (PL) membranes on the polycarbonate supports was studied in the present manuscript. The processes were investigated in the presence of selected low molecular weight organic acids (LMWOAs) (malic acid (MA), oxalic acid (OA) and citric acid (CA)), which substantially affect the transport conditions in the rhizosphere. It was found that the transport was influenced by presence/absence of ionophores calcimycin and by the value of pH. Electrochemical impedance spectroscopy (EIS) was used to monitor the formation, the stability, and qualitatively the transporting process across the supported phospholipid bilayers (SPLBs), whereas voltammetric methods were applied for quantitative and qualitative characterization of the species (ions and complexes), which are transferred across the SPLBs. The structures of the formed complexes were investigated using mass spectrometry.

**Keywords:** Cadmium; Copper; Low molecular weight organic acid; Phospholipid membranes; Supported phospholipid bilayers; Voltammetry; Electrochemical impedance spectroscopy

## 1. INTRODUCTION

For last decades, extensive research has been applied in estimation of the bioavailability and toxicity of metals in soils [1]. A number of metals, e.g., zinc, iron, copper, are essential micronutrients required for variety of physiological processes, but they can become easily toxic when present in excess and their insufficiency can be very dangerous as well [2]. Therefore in all organisms, a metal homeostasis network is functioning to adjust fluctuations in micronutrient availability. However, some

metals – such as cadmium and lead - have no apparent natural role in organisms and are toxic almost at all concentrations. These metals are prevalent environmental pollutants in industrial countries, and apart from acute exposure in the working environment (inhalation of dusts and fumes, occasionally oral intake), the main entry pathway into human as well as animal food chain is the plants uptake [3].

To start their role in plants or in human body, the elements, the compounds and the other species which are present in polluted environment, must be transported into these organisms, more precisely, into their cells [1]. In other words, each particle which takes part further in metabolic processes, must be transported across the cell membranes [4,5]. Similar processes are realized in the opposite way – out of the cells as well as into and out of any sub cellular structure.

Detail elucidation of membrane transport mechanisms plays a key role and is prerequisite for understanding the distribution of pollutants in real cells of more complex organisms (roots, leaves or the whole plants, animals or men) and for their possible control in the future [6-8]. The biological membrane exists as a surface, at which the hydrophobic parts of phospholipids (PLs) are protected from water, while the hydrophilic ones are in contact with the aqueous medium. Only the ends or edges of the bilayer surface are exposed to unfavorable conditions, however, even these exposed regions can be eliminated by bending them underneath the surface whereby a closed edgeless structure is formed [1]. The closed bilayer is impermeable for most of water soluble molecules, as they would be insoluble in the hydrophobic bilayer core [4,5,9,10].

There are different possibilities, how to form the artificial (model) membranes which can be used for simulation of transporting processes: e.g. the form of vesicules, the form of supported phospholipid bilayers (SPLB) on the surfaces of solid materials, on the metallic substrates (mercury [11], gold), which are flat on atomic level. Therefore, the surfaces of solid amalgam electrodes (e.g., [12-20]) seem to be very suitable for the formation of SPLB [21,22]. Such layers can be prepared in the form of a self-supporting PLBs too (e.g., by filling a small micro-holes in a plate [23], etc.). On the other hand, the polycrystalline composite electrodes (e.g., [24-28]) are too rough and it is not possible to utilize them for these purposes. The application of an electrode, prepared on the base of a polymer or a gel (e.g., agar, agarose), seems to be very promising [29]. Very important condition for formation of such SPLBs is the flat surface on the atomic level. Such type of the formed SPLBs can be applied for construction of biosensors, micro- and nano-structures, blood-compatible surfaces, medical implant devices, and for production of catalytic interfaces [4,30]. However, the most attention has paid our team to the formation and investigation of SPLBs on porous polycarbonate membranes [4,5,10,31-35] in last few years.

The principles, on which the transporting processes are based, have been studied for many years in a many of laboratories all over the world, e.g., passive diffusion, facilitated diffusion, ion pumps and channels (e.g., in cases of $Ca^{2+}$, $K^{+}$, $Na^{+}$), or endocytosis and exocytosis (e.g., larger objects and particles, such as bacteria, viruses) [36]). In spite of a certain progress in this field of research, the transport of some elements or particles (e.g., heavy metals) is still poorly understood and there are many unanswered questions. The rate of diffusion of organic molecules – nonelectrolytes – depends on their lipid-water distribution coefficient. The higher is the molecular solubility in fats, the faster is the diffusion rate across the membrane. Compounds insoluble in fats are transported across amphipathic

proteins and can be dipped into equally oriented lipid bilayer [4,5,37]. The proteins form channels for ions and small molecules and serve for transport of bigger molecules, which would not be otherwise able to pass across the bilayer.

Over the past ten years it was assumed that for $Cd^{2+}$ as non-essential metal ion, there would be no specific uptake mechanism and that $Cd^{2+}$ would enter plant cells via uptake systems for essential cations. Mostly indirect evidences are now available that cadmium is taken up into plant cells by $Fe^{2+}$, $Ca^{2+}$ and $Zn^{2+}$ transporters/channels (LCT1, ZIP family of metal transporters, Nramp family). Only exception is *Thlaspi caerulescens* J. et C. Presl, where evidences for a cadmium specific uptake system were described [38-40]. Serum protein ceruloplasmin is the major copper-carrying protein in the blood, and in addition, it plays a role in iron metabolism. It takes part in its transport of copper into the cells and its liberation at specific intracellular places. 6-8 atoms of copper are bound in one mol of this protein. The mechanism of $Cu^{2+}$ incorporation from z ceruloplasmin into intracellular proteins has been elucidated. On the other hand, the transporting processes from extracellular space have remained mostly unknown [41].

In the present study, instead of real transporters, we used the A23187 ionophore (calcimycin), similarly as in our previous research [2,4,5] where the transport of cadmium ions was confirmed. Calcimycin (also known under the names calcium ionophore, ionophore A23187, antibiotic A23187), is a natural ionophore, highly selective for divalent cations. Ion transport by A23187 is mediated by a dimeric form of the molecule that complexes the metal cation. The relative stabilities of the formed complexes decrease along the series, $Mn^{2+} > Ca^{2+} \approx Mg^{2+} > Sr^{2+} > Ba^{2+}$. A23187 has also been described as a cadmium ionophore [42,43], and several stability constants have been determined for its 1:1 complexes with $Ni^{2+}$, $Fe^{2+}$, $Zn^{2+}$ and some other ions [43,44].

Low Molecular Weight Organic Acids (LMWOAs) are encountered mainly as important root exudates, influencing processes in the rhizosphere. They can form different complexes with metals and affect solubility, mobilization, and uptake by plants [45-50]. Apart of this, citrate, malate, and oxalate have been implicated in metal transport through the xylem and vacuolar metal sequestration [3,51,52].

The electrochemical methods seem to be highly suitable for determination of many elements, and of inorganic as well as of organic compounds under physiological conditions (e.g., [13,15,25,26,53-72]). For these purposes, the development of highly sophisticated analytical devices has been realized (e.g., [73-75]). Therefore, in the present work, voltammetry proved to be a very suitable method for elucidation and determination of the formed complexes of cadmium, lead and copper with oxalic acid (OA), malic acid (MA) and citric acid (CA) [31,47,48]. Similarly, electrospray ionization mass spectrometry (ESI-MS) can be successfully applied for elucidation of the structure of such complexes. [8,35,48,76-78].

## 2. EXPERIMENTAL PART

### *2.1. Apparatus*

The electrochemical impedance spectroscopy measurements were realized using CHI 650C Electrochemical Analyzer/Workstation, Software: CHI v 8.1 (IJ Cambria Scientific, UK) and Potentiostat No. 283 and FRA No. 1025, No. 5210 (Princeton Applied Research, USA). The

electrochemical impedances were determined using silver/silver chloride electrodes (silver wire, diameter 1 mm, electroplated by silver chloride). Platinum wire, diameter 1 mm, served as the auxiliary electrode. In our EIS measurement (the dependence of the imaginary part (Z'') on the real part (Z') of impedance recorded in 0.1 M KCl) the system provided satisfactory results in the frequency range of 0.1 – 1000 Hz, amplitude 0.005 V. Because we wanted to investigate the transporting processes under conditions very similar to those which are common in the real biological systems, the voltage -0.1 V has been used in all EIS-measurements described in this paper (this value is relatively close to the plant membrane potential). On the other hand, a shift to negative bias voltages could lead to a significant change of membrane resistance, possibly due to the increasing number of pores or defective structures in the lipid bilayers [10,79].

The voltammetric determinations of cadmium and copper ions or its complexes were carried out by the PC-controlled voltammetric analyzer ECO-TRIBO polarograph (Polaro-Sensors, Prague, Czech Republic), equipped with POLAR.PRO software v. 5.1 and with MultiElchem v. 2.1 software (J. Heyrovský Institute of Physical Chemistry of AS CR, v.v.i., Czech Republic). Pen-type electrode – hanging mercury drop electrode (HMDE) [80,81] was used as the working electrode, Ag/AgCl/KCl(3 mol $L^{-1}$) electrode to which all potentials are referred to and platinum wire served as a counter electrode (both Elektrochemické Detektory, Turnov, Czech Republic).

The measurements were performed at laboratory temperature (23 ± 2 °C).

The values of pH were measured using pH-meter Jenway 3505 (Bibby Scientific Limited, UK).

For determination of $CO_2$, gas ion selective electrode ISE 12-23 (Monokrystaly Turnov, Czech Republic) was used.

### *2.1.1. Voltammetric Determination of Cadmium and Copper Ions*

For the determination of cadmium and copper ions, the sample was diluted with 0.1 M KCl and acidified by addition of $HNO_3$, Suprapur (Merck, Czech Republic), to pH 1. Differential pulse anodic stripping voltammetry (DPASV) was performed at conditions: accumulation potential ($E_{acc}$) -800 mV, accumulation time ($t_{acc}$) was chosen according to the determined concentration level (from 30 to 240 s), initial potential ($E_{in}$) -700 mV, final potential ($E_{fin}$) +150 mV, scan rate 20 $mV.s^{-1}$, pulse amplitude 50 mV. A new drop was used for each record; measurement has been realized in nitrogen atmosphere. Oxygen was removed from the measured solutions by bubbling nitrogen (purity class 4.6; Messer Technogas, Prague, Czech Republic) for 10 minutes. Under described conditions, using accumulation time 240 s, the height of peak for concentration 10 ng/mL was 12.3 nA for $Cd^{2+}$ and 29.5 nA for $Cu^{2+}$. 1.0 % of metal ions transported across PLB would yield concentration 33 ng/mL in polarographic vessel – therefore sufficient conditions for both metal ion determination.

### *2.1.2. Voltammetric Determination of Low Molecular Weight Organic Acids*

The heavy metal complexes with OA were detected in model solution using differential pulse anodic stripping voltammetry and differential pulse cathodic stripping voltammetry. When the pH of

the model solutions were adjusted to pH 7 with sodium hydroxide, a mixed complex consisting of Cd, Pb and OA was found, its peak potential varied from -582 to -542.5 mV (vs. Ag/AgCl/KCl sat.) and depended on the Cd:Pb ratio [7]. According to the calculated stability constants [31], highest values were obtained for mixed complex Cd-OA-Pb, in comparison with "single" complexes of OA with Pb and Cd (Pb-OA and Cd-OA). The existence of all focused metal complexes is confined to neutral or weakly acidic medium. In acidic medium (pH 2) in model and soil solutions do not exist any Pb or Cd complexes, all Cd and Pb were present in free ionic forms [31,47,48]. The formation of mixed Cd-OA-Pb complex was employed for detection of transport of OA across the PL membrane (PLM), for which the adjustment of electrolyte 1 to pH 7.5 was important. Under such condition, transport of oxalic acid has been proved also by ESI-MS. On the other hand, without presence of OA, cadmium ions by themselves at pH 7.5 were not transported [8,35,76].

For the cadmium or copper complexes with CA or MA, pH of the solution was adjusted to the desired value (5.8 – 6.5) with NaOH (Suprapur, Merck, Czech Republic). Adsorptive voltammetry in DP or DC mode was performed using $E_{acc}$ = -100 mV and $t_{acc}$ 30 – 120 s. Scan rate for DP mode was 20 $mV.s^{-1}$, for DC mode values 20, 50 and 100 $mV.s^{-1}$ were employed.

### *2.2. Reagents and Materials*

The 0.1 M KCl base electrolyte solutions were prepared from KCl Suprapur, purchased from Merck, Prague, Czech Republic. The p.a. solvents were obtained from Penta-Švec, Prague, Czech Republic. All the other chemicals used were of analytical grade. For all the measurements, deionized water from Milli-Q-Gradient, Millipore, Prague, Czech Republic (conductivity < 0.05 $\mu S.cm^{-1}$) was used. The AAS standard solution of $Cd^{2+}$, $Cu^{2+}$, and $Pb^{2+}$ (1000 $mg.L^{-1}$ in 2 % $HNO_3$) were purchased from Analytica, Prague, Czech Republic and they were diluted as necessary.

pH of the solutions was adjusted to desired value with NaOH (Suprapur, Merck, Czech Republic).

Two types of PLs were used for the preparation of SPL membranes: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (lecithin, DPPC, GPCho (16:0/16:0)) (Avanti Polar Lipids, Alabaster, USA) and Asolectin from soya beans (Sigma-Aldrich, Prague, Czech Republic) (a mixture, which comprises roughly equal proportions of lecithin (25 %), cephalin (phosphatidylethanolamine) and phosphatidylinositol along with minor amounts of other PLs and polar lipids; about 24 % saturated fatty acids, 14 % mono-unsaturated and 62 % poly-unsaturated fatty acids).

LMWOAs (CA, MA, and OA) were purchased from Sigma Aldrich, Prague, Czech Republic, and their 0.01 M standard solutions were prepared by dissolving appropriate amounts in deionized water.

The used calcimycin was > 98 % (TLC) (Calcium Ionophore, Antibiotic A23187).

The SPLBs were formed by self-assembling in the holes of the Isopore™ Membrane Filters (Millipore, USA) polycarbonate, hydrophilic 8.0 μm, and the supporting membrane thickness amounted to 7-22 μm. The area of one pore amounted to 50 $\mu m^2$, the experimentally found porosity of the membranes was about 25-45 %.

*2.3. Electrochemical Cells for EIS Measurements*

Two types of the electrochemical cells were utilized:

a) "Insert" cell, which was described, e.g., in [10]. In this arrangement, the polycarbonate membrane was glued (epoxy resin CAS 25068-38-6 (UHU, Bühl, Germany)) onto the plastic cup with a small hole in the center (0.12 $cm^2$), prior to the application of the PL solution. This cup subsequently formed the bottom of the upper part of the polypropylene electrochemical cell. Such arrangement proved more convenient, due to the same areas provided for the bilayer formation. To prevent contamination in the experiments with calcimycin and cadmium ions, all the parts (cup, upper and lower part) were exchanged for the new ones prior to each experiment. The volume of the upper part of the cell with "Electrolyte 1" amounted to 3 mL, the compartment with "Electrolyte 2" contained 17 mL of the solutions. This type of cell was employed for experiments concerning cadmium ions transport.

b) "Glass" cell, which was newly developed for experiments with copper ions transport (Fig. 1). The cell was composed of two glass columns in which 2 mL of "Electrolyte 1" and 2 mL of "Electrolyte 2", respectively, were inserted. The compartments were separated by two Teflon parts with a hole (0.07 $cm^2$), where the polycarbonate porous membrane was inserted. The electrodes were placed into the holes in the top of glass compartments.

In both compartments, the same supporting electrolyte (0.1 M KCl) was inserted. The transported heavy metal ions were placed in "compartment 1" always. If necessary, the solutions of LMWOAs were inserted into this compartment (final concentration of LMWOAs amounted to 2 mM). pH adjustment was realized either by direct addition of 0.1 M NaOH into the "Compartment 1" (pH 7.5) or by exchange of aliquot of "Electrolyte 1". The added amounts of heavy metals (final concentration amounted to 0.1 mM of $Cd^{2+}$ ions and 0.16 mM of $Cu^{2+}$ ions, respectively) and/or of LMWOAs were too low to change the osmotic pressure difference between both compartments.

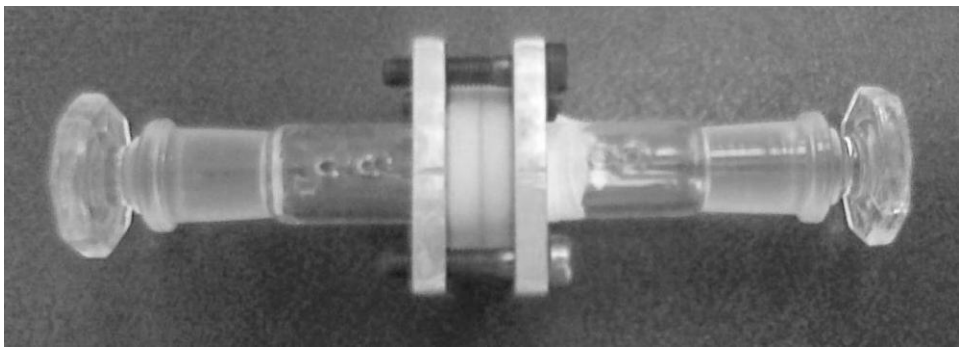

**Figure 1**. Electrochemical "Glass" cell, "Compartment 1" on the right.

*2.4. Electrical Equivalent Circuits*

Several types of electrical equivalent circuits (EEC) were tested and utilized for characterization of the formed SPL membranes in the pores of polycarbonate membrane.

The two simplest EECs (Fig. 2A and Fig. 2B), composed of one resistor ($R_s$) in serial combination with parallel combination of a resistor ($R_p$) and a capacitance ($C_p$), can be successfully applied for characterization of free polycarbonate membranes [2,10] and gel electrodes without covering by PLs [79].

The two EECs depicted in Fig. 2C and Fig. 2D proved to be applicable for characterization of SPL membranes formed in the pores of polycarbonate membranes [4,10], of metal electroplated surfaces and of the surface of polymer electrode too. These circuits are similar to the simpler ones, but additionally, a parallel combination of one capacitor and one resistor was added to the first capacitor (series-connected) [4]. As in the case of the first two circuits, they differ mutually by a capacitance ($C_s$) included serially to the first resistor ($R_1$).

The pairs of EECs 2A and 2B; 2C and 2D differ mutually by a capacitance ($C_s$) included serially to the first resistor. Each member (resistor, capacitance) of the circuits can be used for characterization of the system. Serial resistors ($R_s$) correspond to the resistance of the electrolyte, connectors, etc. Similarly, the serial capacitances ($C_s$) (Fig. 2B, Fig. 2D) represent the capacitances of these parts of the tested cells. However, importance of these capacitances for characterization of the formed SPL membranes proved to be negligible. The EECs 2B and 2D were not therefore used in this manuscript.

Parallel capacitors of the circuits (denoted as $C_p$ and $C_1$) correspond to the parasitic capacitance of the supporting membrane; parallel resistors to its resistance ($R_p$ and $R_2$). The parallel combination of capacitances (denoted $C_2$) and resistors (denoted $R_3$) in the second pair of the circuits describe the electrical properties of the SPL membranes formed in the pores of the supporting membranes (including ionophores and transport of metal cations) [4,33,34,76].

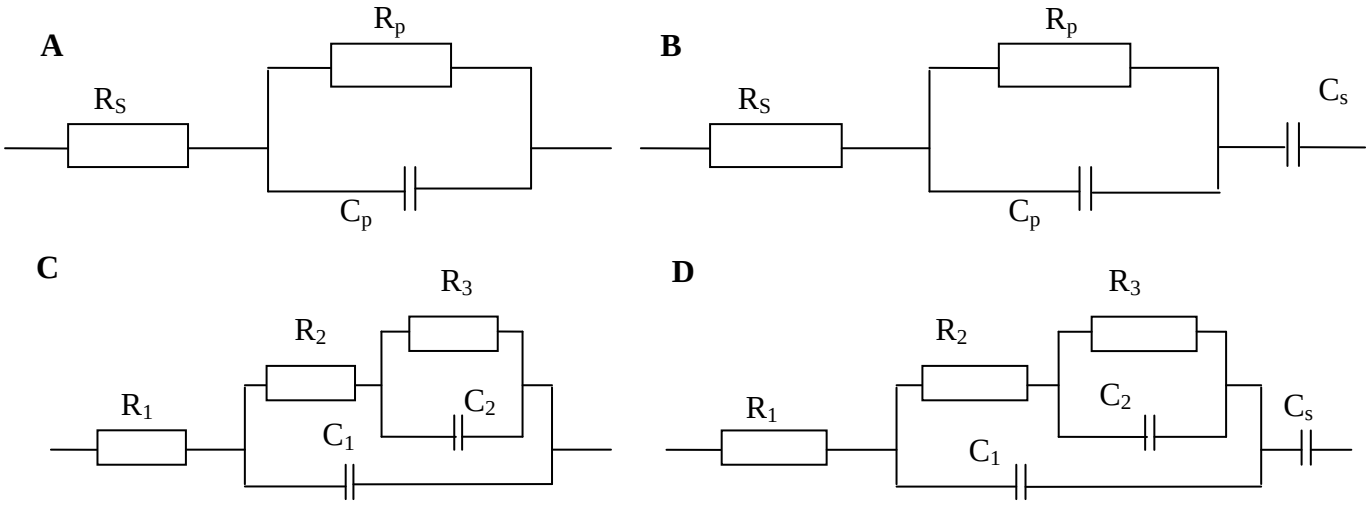


**Figure 2**. Electrical equivalent circuits used for characterization of SPL membranes formed on polymer surfaces.

*2.5. Preparation of Supported Phospholipid Bilayers*

The SPL membranes were prepared in way as it was described, e.g., in [10,34]: A volume of 10 μL of a PL solution (20 mg.mL$^{-1}$, in n-heptane (p.a., Penta-Švec, Prague, Czech Republic)) was applied to one side of the polycarbonate membrane, the solvent was let to evaporate,, and then another volume of 10 μL was applied to the other side of the membrane. After 30 minutes, both sides were simultaneously exposed to aqueous electrolyte. For this procedure and lipid concentration, formation and thinning of PLB in the holes of polycarbonate membrane was demonstrated [82,83].

*2.6. ESI-MS measurements*

The ESI-MS experiments were performed with a Finnigan LCQ Advantage ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) fitted with an electrospray ionization source operated in positive and negative-ion mode [84]. The sample solutions of OA and $CuCl_2$ or KCl as well as the transported species were introduced into the ESI source via a fused-silica capillary at a flow rate of 0.6 mL h$^{-1}$ maintained using a syringe pump (kdScientific, USA). Nitrogen was used as the nebulizer gas. The operating conditions were set as follows: spray voltage 5.0 kV, capillary voltage 20 V and tube lens offset 10 V, heated capillary temperature 120°C, sheath gas flow rate, and auxiliary gas flow rate 10–50 arbitrary units. The sample solutions were prepared from a $10^{-2}$ mol L$^{-1}$ stock solution of OA and $CuCl_2$ (Sigma-Aldrich) in pure water.

## 3. RESULTS AND DISCUSSION

As it was found by us earlier, the SPL membrane is completely formed and stabilized approximately one hour after insertion of PL solution on the porous material [10]. If the porous support was free of any PL, almost one third of the total content of heavy metal was transported from one compartment to the other one. On the other hand, if the supporting membrane is completely covered by the formed PLs and none LMWOA is present, insignificant amounts of metals are transported between compartments. The very small amounts transported in such cases were transported via the imperfectness in the formed SPL membranes. Between two cells described, there was no difference in cadmium ions transport, but only glass cell was appropriate for study with copper ions (glue interferences).

*3.1. Transport of copper and cadmium ions in the absence of an ionophore across the SPL membranes*

Transport of copper and cadmium ions can be realized using calcimycin [7,10], because this ionophore is suitable for transport of divalent cations [42,43]. Nevertheless, its application for transport of these cations has not been used yet. However, its activity can be influenced by the presence of LMWOAs in Compartment 1. It was proved that pH of the electrolyte plays very important

role. In the acidic area of pH (about 2.4 – 2.6), small amounts of heavy metals were transported only (in the case of CA 0.05 % of $Cu^{2+}$, in the case of MA 0.8 % of $Cu^{2+}$ and in the case of OA 0.9 % of $Cu^{2+}$ was transported to the "Compartment 2"). As it was stated above, this transport was realized though the holes (disturbances) in the formed PL layers. Simultaneously, the LMWOAs were not transported across the SPL membranes. In this pH range, neither CA ($pK_{a1}$ = 3.15; $pK_{a2}$ = 4.77; $pKa_3$ = 6.40 [85]) nor MA ($pK_{a1}$ = 3.46; $pK_{a2}$ = 5.13 [85]) does form any stable complex with neither copper nor cadmium. On the contrary, due to low dissociation constants of OA ($pK_{a1}$ = 1.25; $pK_{a2}$ = 4.26 [85]), the complexes with $Cd^{2+}$ and $Cu^{2+}$ ions are formed. Nevertheless, transport of complexes was not proved at this pH values in absence of ionophores. The absence of OA in "Electrolyte 2" was proved using differential pulse voltammetry (DPV).

If pH value of "Electrolyte 1" was adjusted to the value of about 7.5 and none ionophore was present, the transported amounts of metal ions substantially increased.

Under such conditions, the presence of OA as well as metallic cations in "Electrolyte 2", transported from "Electrolyte 1", were proved by voltammetric measurement.

This transport can be explained by decarboxylation of LMWOAs in "Electrolyte 1", which is realized in neutral and alkaline solutions. Due to this process the bubbles of $CO_2$ are released [1] which partly destroy the compact SPL membranes and through these pores the cations of heavy metals are transported. The intensity of decarboxylation process increases: CA < MA< OA at pH 7.5. It was found that the amount of transported cadmium ions to "Electrolyte 2" increases linearly with concentration of OA in "Electrolyte 1" [1]. Because the molecules of MA and CA are larger than OA, they cannot pass through the formed pores in SPL membranes. The evolution of $CO_2$ was proved using gas ion selective electrode.

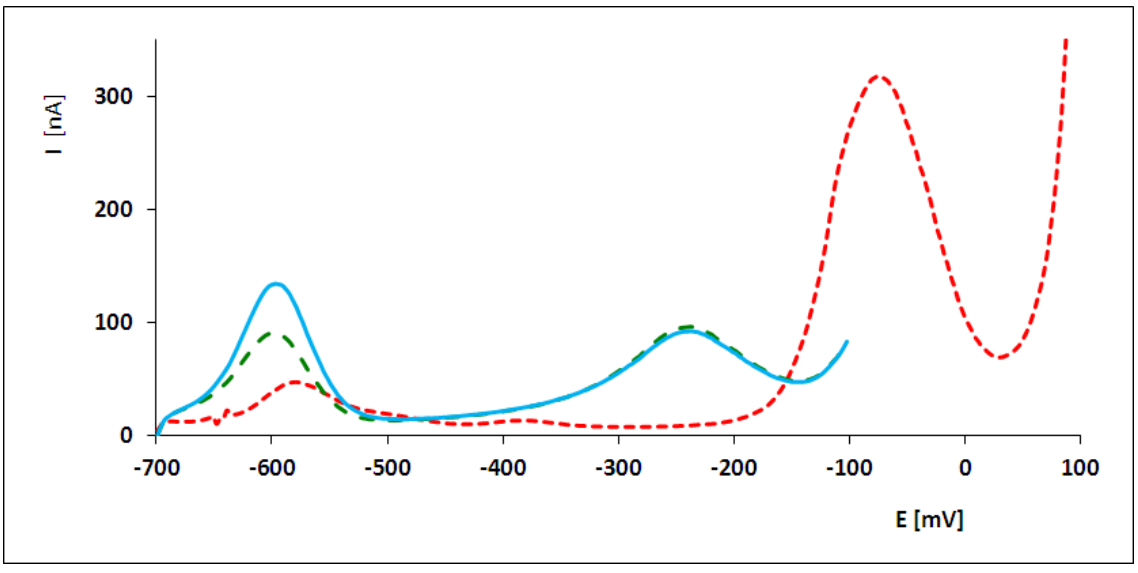


**Figure 3.** Evidence of OA transport across PLB, voltammetric detection of its mixed complex Cd-OA-Pb in the "Electrolyte 2" (red curve) realized by additions of $Cd^{2+}$ and $Pb^{2+}$ ions to the "Electrolyte 2" and by adjustment of pH to 7.5 (green and blue curves).

The presence of OA in "Electrolyte 2" was proved voltammetrically using formation of mixed complex Cd-OA-Pb. In the experiments with transport of copper ions, $Cd^{2+}$ and $Pb^{2+}$ had to be added to the solution of "Electrolyte 2" in polarographic cell. This illustrates the red curve (Fig. 3), where the peak at about -100 mV corresponds to the presence of free $Cu^{2+}$ ions. The peak at about -380 mV corresponds to the presence of $Pb^{2+}$ ions and at about -550 mV to the presence of $Cd^{2+}$ ions. When pH was adjusted to the value of about 7.5, a new peak arose at about -600 mV (green curve). This peak corresponded to the mixed complex Cd-OA-Pb and its height increased with further addition of $Pb^{2+}$ (blue curve). Furthermore, the peak of Cu-OA complex was recorded at about -250 mV.

Similarly, the presence of OA in "Electrolyte 2" can be proved using ESI-MS. This technique can detect the patterns corresponding to the mixed complex, as it was described in detail in [48]. Free OA cannot be detected using ESI-MS. However, it was proved that the presence of OA in "Electrolyte 2" can be detected in the form of negatively charged complex $[K(COO_2)]^-$. The registered calibration line ESI-MS signal intensity (corresponding to the complex concentration) vs. OA concentration is depicted in Fig. 4. Its signal is directly proportional to the OA concentration from 0 to 30 μg $L^{-1}$. The application of this technique is rather complicated due to the presence of relatively high concentration of KCl in supporting electrolyte. In all our experiments, the concentration of supporting electrolyte 0.1 M KCl was used, because it is similar to that which is present in real soils or in real cells (the concentration of NaCl in physiological saline amounts to 0.15 M). However, by injection of so concentrated solution into the MS device, the liquid gets evaporated and the formed crystals of KCl can foul the device. Due to the solution for ESI-MS measurements was diluted to obtain 25 mM concentration of KCl.

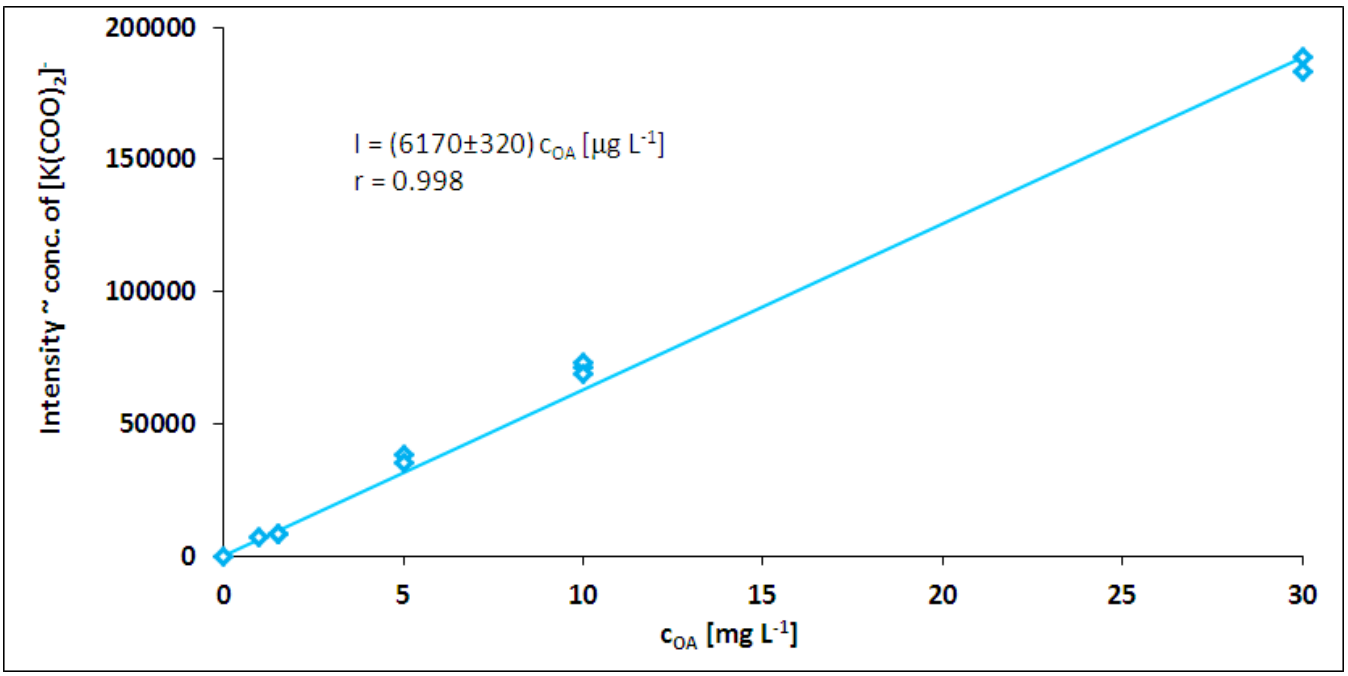


**Figure 4.** Calibration dependence of determination of OA using ESI-MS. Negative mode ESI-MS *m/z* 127. Registered signal intensity corresponds to the concentration of $[K(COO)_2]^-$ complex.

The fact that the ions of $Cu^{2+}$ are transported between electrolytes across the SPL membrane can be seen from the time dependence of capacitance $C_2$ in EEC according to Fig. 2C. Such dependences of capacitances $C_1$ and $C_2$ are depicted in Fig. 5. As it was stated in [10], the capacitances $C_2$ and $C_1$ increase in time. It is connected with compacting of the formed SPL membranes. If transport of some charged particle is started, the capacitance $C_2$ decreases or its increase is stopped (Fig. 5). This phenomenon is caused by transport of charged particles across the capacitor.

It was found that 3.0 % of total content of $Cu^{2+}$ was transported across the SPL membrane into the "Electrolyte 2" in the presence of OA at pH 7.5. The amounts of $Cu^{2+}$ ions transported under similar conditions in presence of MA or CA were smaller. Similar results were achieved in the case of $Cd^{2+}$ ions.

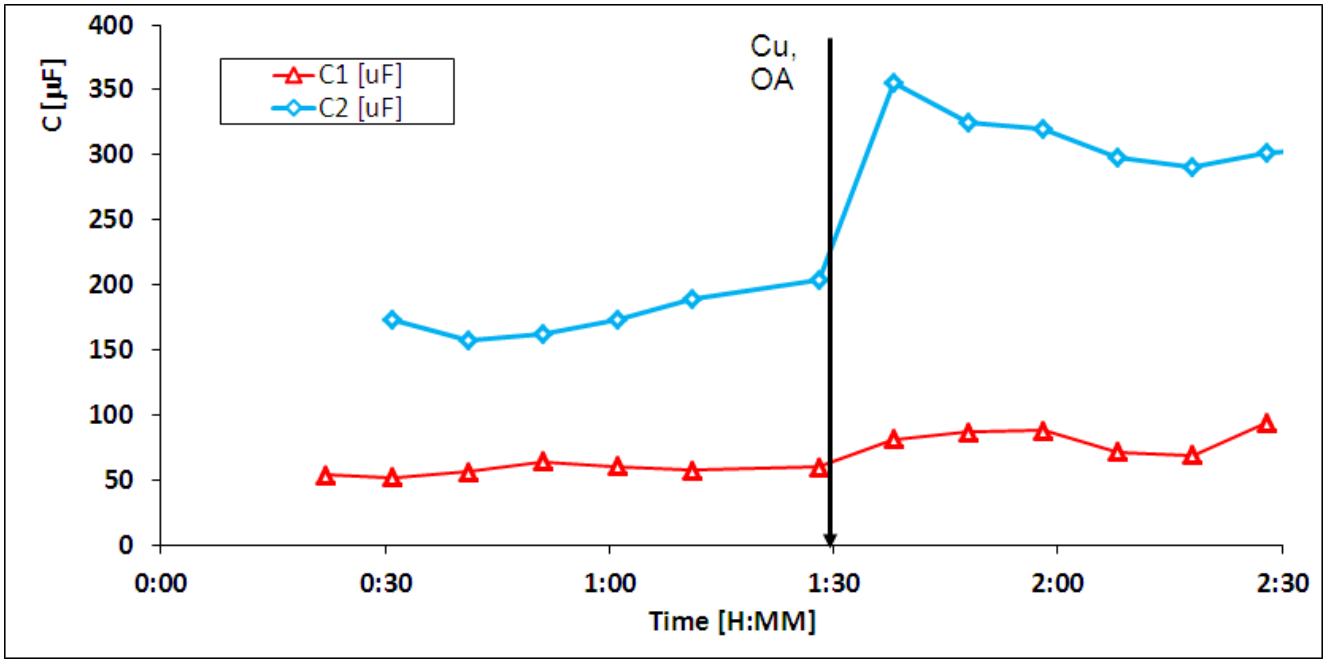


**Figure 5.** Time dependence of capacitances $C_1$ and $C_2$ in EECs according to Fig. 2C. The arrow denotes addition of $Cu^{2+}$ ions and OA into the "Compartment 1". Supporting electrolyte 0.1 M KCl, pH 7.5, in ionophore absence.

### *3.2. Transport of $Cu^{2+}$ and $Cd^{2+}$ ions in the presence of the calcimycin ionophore across PLBs*

If the ionophore calcimycin was incorporated into the SPLB, the transported amounts were substantially higher than in its absence, i.e., 3.8 % of $Cu^{2+}$ and 1.6 % of $Cd^{2+}$ ions. pH value of the system does not play much important role and therefore, the cations were transported in the whole investigated range (pH from 2 to 6.5). Similarly as in the case of ionophore absence, if the transport was realized, the capacitance $C_2$ of the system decreased (Fig. 6). The lower transported amounts of $Cd^{2+}$ ions in comparison with $Cu^{2+}$ can be explained by the structure and properties of dimeric

calcimycin complex. $Cd^{2+}$ ions exhibit smaller ionic diameter than the ions of $Cu^{2+}$ (0.103 nm, 0.073 nm, respectively), lower relative molecular mass (112.4, 63.5 nm, respectively), but higher electronegativity (1.69, 1.9, respectively). On the basis of these parameters it is possible to suppose that the dimeric complex of Cd-ionophore is larger than the Cu-complex and therefore its transport across the SPLB is more complicated. Nevertheless the principle of the transport is similar, i.e., the hydrophobicity of the complex is increased by complexation of the cation and the complex is better built in the SPLBs.

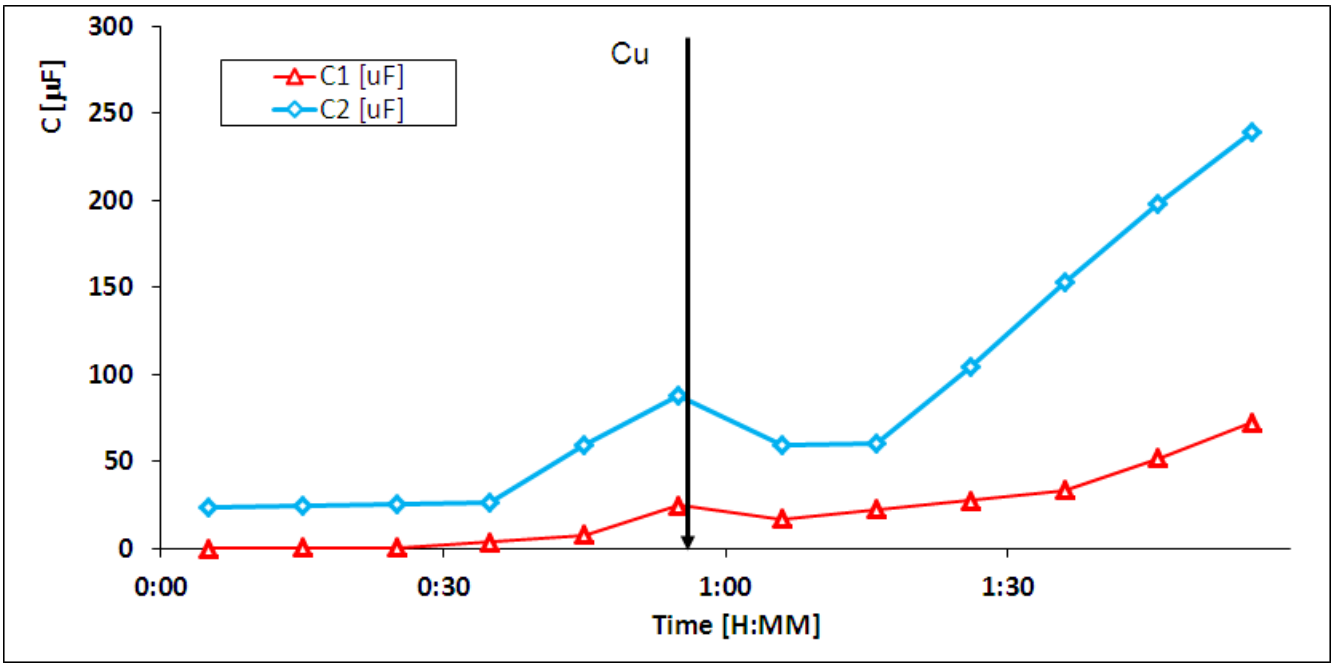


**Figure 6.** Time dependences of capacitances $C_1$ and $C_2$.in EECs according to Fig. 2C. The arrow denotes addition of $Cu^{2+}$ ions into the "Compartment 1". Supporting electrolyte 0.1 M KCl, pH 2.4, ionophore calcimycin present.

In the following, the effect of simultaneous presence of LMWOAs and calcimycin ionophore was studied. In general, the behavior of $Cd^{2+}$ ions was similar to that of $Cu^{2+}$ ions. Nevertheless, there is a great variety of copper complexes formed with MA or CA with lowest stability constant for Cu:MA:H equal 1.96 [86]. In our experiment with presence of MA and of pH about 2.5, the amount of transported $Cu^{2+}$ ions was increased in comparison with other tested LMWOAs (8.3 %). This phenomenon was confirmed by EIS by sharp decrease of $C_2$ capacitance after $Cu^{2+}$ addition. Probably, the labile behavior of the formed complex, which leads to the increase of ion transport, could be the explanation [87].

With increasing pH value of the "Electrolyte 1", the part of $Cu^{2+}$ cations, which were complexed with LMWOAs, increased. Due to some portion of cations bound in complexes, smaller amount of cations could be transported using calcimycin. At the same time the formed complexes of

MA and CA with metals increase the capacitance $C_2$ in EEC (Fig. 7). The absence of MA and CA in "Electrolyte 2" was proved voltammetrically. This finding confirmed that these LMWOAs cannot be transported using the calcimycin ionophore. A partly different situation was observed in the case of OA. As it was mentioned above, OA can complex the $Cu^{2+}$ ions in acidic pH values (about 2.5). Moreover, the molecules of complexes are relatively small and can be (on the contrary to MA and CA complexes) transported across the PLB with calcimycin.

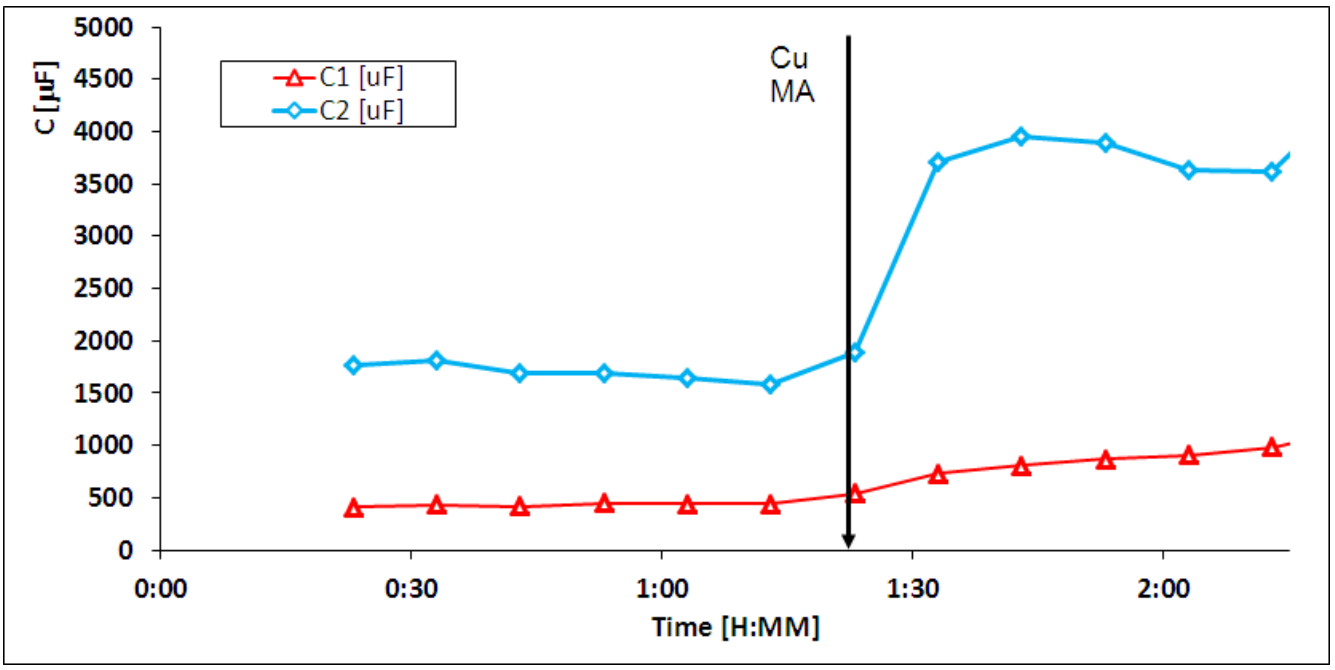


**Figure 7.** Time dependences of capacitances $C_1$ and $C_2$.in EECs according to Fig. 2C. The arrow denotes addition of $Cu^{2+}$ ions and MA into the "Compartment 1". Supporting electrolyte 0.1 M KCl, pH 6.4, ionophore calcimycin present.

The stoichiometry of copper/oxalate complexes were elucidated under soft ionization conditions using ESI-MS. It is expected that many of possible copper/oxalate complexes carry no charge; however, two of them are visible in negative ESI-MS spectrum (Fig. 8). On the contrary to other experiments with Cu(II) under ESI conditions (e.g., [77]), copper in visible complexes carries only 2+ charge (Cu(I) can be captured in non-charged (non-visible) complexes). Two negatively charged copper/oxalate complexes, $[Cu(COO)_2H(COO)_2]^-$ and $[Cu((COO)_2H)_3]^-$, contain mono-deprotonated oxalic acid $[(COO)_2H]^-$ as well as double-deprotonated oxalic acid $[(COO)_2]^{2-}$. Other dominant signals belong to $[(COO)_2H]^-$ and to oxalic acid $[(COOH)_2]$ adducts $[(COO)_2H(COOH)_2]^-$. Additional minor signal appertain to carbonic acids adducts with water and or with oxalic acid.

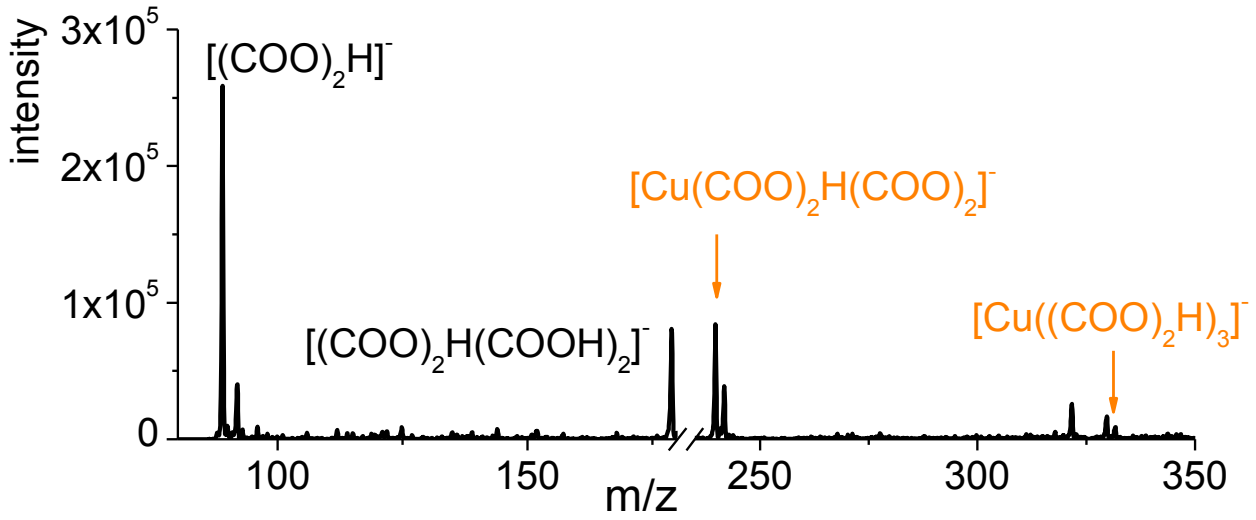


**Figure 8.** ESI-MS spectrum in negative mode of $CuCl_2$ and oxalic acid (both $5 \cdot 10^{-4}$ mol $L^{-1}$) in pure water as a solvent.

## 4. CONCLUSIONS

It is possible to conclude that simulation of transporting processes across PL membranes can be realized using a two-electrolyte arrangement with artificial SPL membrane which is formed in direct pores of a polycarbonate membrane. Calcimycin proved to be a suitable transporter for transport of divalent heavy (hazardous) metals ($Cd^{2+}$, $Cu^{2+}$). Similarly, transport of $Pb^{2+}$ ions can be mediated using this ionophore; however contamination with this metal is too high to follow the transport of its low concentrations reliably. To realize the transport ionophores calcimycin must be incorporated in the SPLB.

Transport of heavy metal ions is substantially affected by presence/absence of LMWOA(s). It was found that pH plays a very important role in transporting processes in presence of LMWOAs. Adjustment of pH to about 7.5 and higher causes decarboxylation of LMWOAs and the formed bubbles of $CO_2$ partly destroy the model membrane across which the particles can be transported. This transport is the highest in case of OA in comparison with MA and CA which are too large to be passed through the SPL membranes. The transport is reflected in EIS measurement and the transported species are determined subsequently by voltammetry. Using ionophore calcimycin, apart from cadmium or copper ions, OA is also transported, whereas CA or MA is not.

It is possible to conclude that combination of an electrochemical method (voltammetry, EIS, ISE) with non-electrochemical methods (ESI-MS) proved to be successful and suitable in detection and elucidation of transporting processes.

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