Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Analytical Chemistry

CHARACTERISATION OF ROOT EXUDATES BY HPLC

(diploma thesis)

In cooperation with

Université de Limoges

Faculté de Pharmacie

Departement de Chimie analytique

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Limoges, Hradec Králové 2007/2008 Kateřina Flutková

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra analytické chemie

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Ve spolupráci s

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I declare that I worked up this diploma thesis individually and all the literature and data sources are mentioned in the list of literature.

15th May 2008, in Hradec Králové ……

1. CONTENTS

2. INTRODUCTION

Nowadays all the world is forced to solve a heavy pollution of an environment and so also a problem with polluted soils. Also this work is here for research on the solution.

Most of the contaminants are structurally and chemically foreign to the organisms and pathways that transform or degrade molecules in the environment, and are thus potentially persistent pollutants.[1] The toxicity of heavy metals to plants is well documented at various levels, from reduced yield, through effects on leaf and root growth, to inhibition on enzymatic activities. Moreover, decrease in root growth is a most common effect due to the toxicity of heavy metals in trees and crops.[2] Contaminants and their degradation products may be hazardous to the flora and fauna of soil and water. If transferred into agricultural crops and animals, they may have the potential to adversely impact human health.[1]

There are several possibilities to clean soils. Plants can be valid means for development of processes for the rehabilitation of soils, because plants are capable to remove contaminants from the environment. This action is called phytoremediation.

The phytoremediation is possible because some of plants uptake of pollutants (heavy metals) which are then immobilized and/or degraded within the plant, or indirectly by supporting root-associated microorganisms.[1] Uptake causes organic acids and amino acids exudation by these plants and it is possible to determine which plants are applicable for this purpose by finding out a character and quantity of exuded acids. Understanding organic acid exudation and its mechanism with plants exposed to abiotic stress may provide an effective approach for illustrating fundamental aspects of plant physiology, making new strategies of developing crop varieties with high tolerance to environmental stress, including heavy metal stress,[2] and phytoremediation.

The character of organic acids and amino acids exuded by plant roots and its dependence on presence of the heavy metals in a soil was investigated in this work through the use of high performance liquid chromatography.

3. AIM OF THE THESIS

This experimental work is a part of the doctorate thesis called „Eco-dynamics and determination of metal elements in continuum soil – plant“. This doctorate thesis makes effort to two objectives:

- to specify methods for determination of amount of exuded organic acids and amino acids by HPLC and ion chromatography
- characterization of impact of metals on this exudation by means of measurement of exuded acids, anions, cations, metals and metalloids

This diploma thesis deals with determination of organic acids in roots exudates by HPLC.

4. THEORETICAL PART

4.1. Chromatography

4.1.1. The basic principles of chromatographic methods

Chromatographic methods are high effective separation methods that are used to separate analysed substances from mixture and to make their qualitative and quantitative analysis at the same time.

Chromatography utilizes separation of analyzed substances between two phases. One is a stationary phase,[3] which retains components of the analyzed mixture.[4] The second is a mobile phase. When the mobile and stationary phases are in contact with separated substances, there are interactions that are the base for separation.[3] The mobile phase elutes components of the analyzed mixture from the stationary phase and carries them away in the direction of flow with different rapidity.[4] Separation depends on different affinities of separated substances to the stationary and to the mobile phase[3] and on their retention in the column. The mobile phase, which is either liquid (eluent) or gaseous (inert gas), is the moving power of the chromatographic system. Stationary phase can be solid (sorbent) or liquid placed on an inert support.

Nowadays there is a considerable quantity of chromatographic methods. It can be divided according to:

- state of mobile phase
    - liquid chromatography (LC)
    - gas chromatography (GC)
- form of stationary phase
    - column chromatography
    - paper chromatography (PC)
    - thin layer chromatography (TLC)
- principle of separating process
    - ion-exchange chromatography
    - gel chromatography (size exclusion chromatography)
    - adsorption chromatography
    - partition chromatography

- bio-affinity chromatography

Among the most used separation methods in analysis of substances belongs gas and liquid chromatography.

Gas chromatography is used mainly for analysis of gas substances and also for analysis of volatile substances, which evaporate at higher temperature, but don't decompose. Most of substances are substances thermally stable, therefore the liquid chromatography is used more frequently. It enables separation of all less volatile and solid substances soluble in water, organic solvents or diluted acids. Today a column form is mainly realized.[4]

4.1.2. High performance liquid chromatography

High performance liquid chromatography is a widely used separation method with several advantages:

1) There is a possibility of qualitative and quantitative valuation of separated mixture components.
2) This process is fast and has good sensitivity of determination.
3) Minimal quantity of samples is sufficient.
4) There is possibility of automation.[3]

Separation of substances proceeds between the stationary phase packed in a column and the mobile phase flowing through the column under high pressure (40 MPa).

Solvents or their mixtures are common as a mobile phase.[4] Mobile phases used for HPLC have to be very pure and deaerated. Solvents are arranged to the eluotropic series in consonance with increasing elution efficiency: heptane < cyklohexane < tetrachloromethane < toluene < diethylether < chloroform < acetone < acetonitile < ethanol < methanol < acetic acid. If separation on reversed-phases is used, the eluotropic series applies in the opposite order.[5]

There is possibility to realize HPLC analysis with a constant composition of mobile phase during all the analysis. It is so-called isocratic

elution. If the composition of the mobile phase is changing, it is called gradient elution.[3] The solution of a sample is dosed to the column where it is carried away by flow of the mobile phase. They are adsorbed on the stationary phase and then desorbed by the mobile phase. These substances are going to a detector, where their presence is noted, and a peak is drawn in a chromatogram as the answer to this signal.

For successful HPLC analysis is necessary to optimize chromatographic conditions to obtain sharp, symmetric and separated peaks.

Basic qualitative characterization of HPLC is time of retention t_R or retention volume.[4] Time of retention is a period from injection of a sample on the column to the maximum of a chromatographic peak. The most often confirmation of identity is corresponding time of retention of the sample peaks and the peaks of standard. Quantitative characterization is the value of a peak surface and the height of a peak.[3] It is compared the peak surface of a sample with the surface of a standard peak which is analyzed under the same conditions. More exact is so-called method of the inner standard, where is analyzed the substance and the standard in one sample under absolutely identical conditions.[4]

Some modern UV detectors make possible to take UV spectrum in the maximum of the peak; identity of UV spectrum of sample and standard is other identification characteristics.[3]

4.1.3. Scheme of a liquid chromatograph

A liquid chromatograph consists of components for dosing of sample mixtures, flowing of a mobile phase, separation of substances and detection with noting of peaks and data-station (mostly PC) to collect and to analyse data. Some newer instruments have vacuum degasser for deaeration of mobile phase.[4]

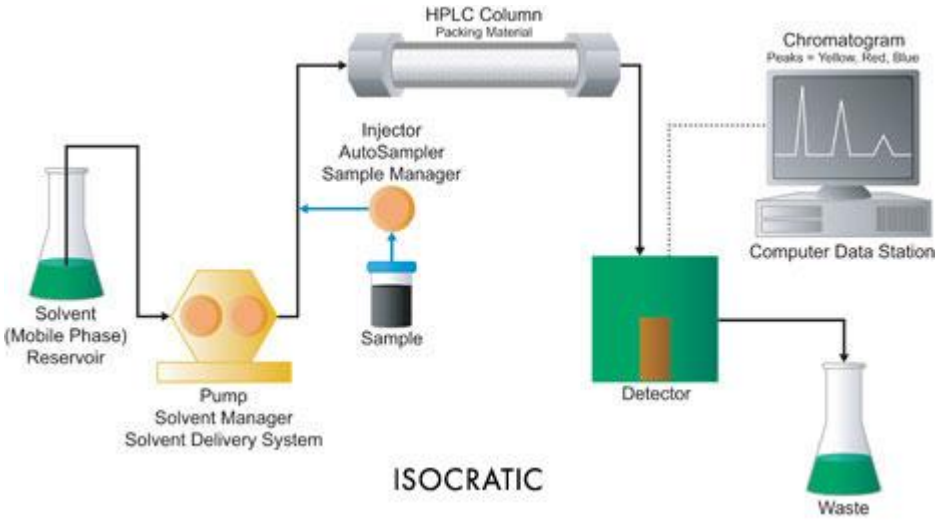

Fig. 1: Scheme of a liquid chromatograph[6]

4.1.4. Chromatographic columns

A column has critical effect for quality of chromatography and time of analysis.[4]

HPLC columns for analytical purposes are the most often long 10 - 25 cm (mainly 10, 15 or 25 cm) with inside diameter 3 - 5 mm (mainly 4.6 or 5 mm), they are generally made from stainless steal or glass. Common flow rate of the eluent is from 1 to 2 ml/min. Short columns (3 – 5 cm) allow to achieve fast separations, to reduce quantity of the used mobile phase and improve weigh sensitivity that is important for low volumes analyses. Columns with very small inner diameter, so-called micro-columns, have length 25 – 50 mm and inner diameter 1 – 2 mm. They exceed by high efficiency and low usage of solvent (100 – 1000 μl).[3,4]

Often there are used pre-columns to protect a column against impurities and insoluble materials. They are installed either directly in front of the column or between the pump and the sample manager.[4]

The columns are packed with suitable sorbents. Material in the columns has diameter 1.7 – 10 μm. The most often sorbents are so-called chemical bonded stationary phases. Silicagel is a polar adsorbent with amorphous structure. To the hydroxyl groups on the surface of silicagel granules there are chemically bonded radicals like:

- hydrocarbons chains with 18 or 8 carbon atoms. It is non-polar chemical bonded phase, so-called reversed phases.
- three-carbons chain radicals with amino- or cyano- group on the termination. It is medium-polar phase.[3,4]

These stationary phases are possible to use only for analyses made in pH rate 2 – 8. Silicagel and aluminium oxide (polar phases) are used as HPLC sorbents too, but less than chemical bonded stationary phases. Silicagel has acid properties and it causes holding of alkaline substances. Recently there is also zirconium oxide as a stationary phase or other macromolecular materials. Suitable ionexes are used for ion-exchange chromatography. Importance of stability of columns under high temperature rises recently because there is possibility to achieve higher efficiency and faster analysis. [3,4]

4.1.5. Detectors in HPLC

The basic function of a detector is to convert results of separation in column to some form that is suitable to note. The detector reacts to presence of the analyzed compound and sends signal that is noted depending on time.[4]

Sensitivity and selectivity of chromatographic analysis depend on used detector.[3] Detectors should be high selective for determined components of mixture, have to have sufficient sensitivity – low detection limit – and his response should be linear function of measured substance volume. It is necessary that the detector is universal, it has to be possible to detect all separated components of a sample and it is also important independence of the detector response from change of mobile phase during gradient elution.[4]

List of the most common detectors:

- spectrophotometric (mainly UV/VIS region) – the most often used, measures absorbance of electromagnetic radiation by components of the eluate flowing in the detector. Mainly UV region of spectrum is used, sometimes also VIS, exceptionally IR region.

The most used UV detectors are:

- UV detector with fixed wavelength (254 or 280 nm) – they are simple and cheap
- UV-VIS detector with variable wavelength
- scanning UV detector – it records absorption spectrum in maximum of a peak in several seconds
- diode array detector – controlled by computer, three-dimensional projection, it records absorption spectrum, it is capable to use several wavelengths together and to compare absorbance of individual substances

Spectrophotometric detectors are used the most often, because they have high sensitivity (10^{-9} – 10^{-10} g/ml) and it is possible to use them at gradient elution.

- fluorimetric – advantageous if the substance (product or metabolite) is fluorescent or if the possibility to convert it to some fluorescing derivate exists. They are less universal than UV detectors but they have better sensitivity (10^{-9} – 10^{-12} g/ml), selectivity and also it is possible to use them at gradient elution.

- electrochemical – they are used for the analysis of substances that undergo an electrochemical reaction on the boundary electrode/eluent. It measures electrochemical quantities that depend on the concentration of the analytes. These detectors are sensitive (10^{-9} – 10^{-12} g/ml), but commonly there is not possibility to use them at gradient elution.

- refractometric – measures different refractive index between pure mobile phase and the eluate flowing from the column, containing analyzed substance. They are almost universal, but they are used sporadically because of disadvantages – low sensitivity (10^{-6} g/ml), necessity of keeping constant temperature and no possibility to use them at gradient elution.

- connection of HPLC with mass spectrometry (MS) - it is necessary to separate the mobile phase from the eluate after outflow from the column and then molecules of the substance in gas state are ionized by hits of electrons, thermo-ionisation or electro-ionisation. Charged particles (ions) are separated according to weigh and charge in magnetic or high-frequency field. After that, the mass spectrum is noted – quantity of ions in relationship to weigh/number of charges ratio. HPLC-MS is high selective and high sensitive, but expensive method.[3]

4.1.6. Liquid chromatography types by separation process

4.1.6.1. Ion-exchange chromatography

Ion exchangers use mainly strong electrostatic forces between ionized functional groups of ion exchangers and ions in surroundings. Individual ion interactions are responsible for retention in ion-exchange chromatography. The analyte with stronger charge is more strongly attracted to ion surface. Separation at ion-exchange chromatography is influenced by basic exchange of ions and also by other bond interaction, for example by physical adsorption and so on. The mobile phase is a water buffer where is used both pH and ion force. The biggest influence of pH is close to pK_A of the substance. Ion-exchange chromatography is possible to use also for non-ionized substances in water solutions with organic solvents where balance is established between organic solvent in the eluent and the liquid in inner pores of the exchanger, which has the character of a stationary phase. The exchanger has the function of a stationary phase support and is more polar than the eluent. Very polar substances with neutral character have higher affinity to the stationary phase than to the mobile phase and are possible to analyze by chromatography. Retention of analyzed substances increases with increasing concentration of the organic solvent. Exchangers used in liquid chromatography can be divided:

- micro-particular exchangers with organic matrix
- chemically bonded exchange phases on a porous micro-particle silicagel support

- surface porous - layer of the exchanger is chemically bonded on the surface of a porous support

Ion-exchanger can be divided also by ionogenic groups:

- cation exchanger – changes and liberates cations (e.g. aromatic sulfonic acid)
- anion exchanger – changes and liberates anions (e.g. quaternary amines)

4.1.6.2. Size exclusion chromatography

The gel chromatography is a method where molecules are divided by molecular weight and shape. The stationary phase is made from gel with non-soluble polymer net that is saturated with liquid. The same liquid is used as the mobile phase. The biggest molecules are quickly flowing out of the gel column, smaller molecules are penetrating to the gel pores and they are leaving later.

4.1.6.3. Adsorption chromatography

Adsorption during this type of chromatography is caused by difference in forces interacting between the surface of adsorbent and the analyzed substance and forces interacting between the adsorbent surface and the mobile phase. Molecules of a sample and the mobile phase compete for the sorption centre on the surface of the adsorbent. Adsorption chromatography can be divided:

- normal phase chromatography – mainly interactions between induced or permanent dipoles and electric field of the adsorption surface are participating
- reversed phase chromatography – analyzed substances interact with the surface of the adsorbent by small hydrophobic forces

4.1.6.4. Partition chromatography

Partition chromatography is based on distribution of analyzed substances between two liquid phases where the liquid stationary phase is fixed on a suitable support. When distributing, the difference in distribution between

both immiscible liquids is used. Reversible physical interactions in consequent of disperse, induction and orientation forces, e.g. interaction dipole-dipole, ion-dipole and interactions based on hydrogen bonds, are important for separation process.[7]

4.2. Effect of metals on root exudation

4.2.1. Function and mechanism of root exudation

The rhizosphere is the zone of soil immediately surrounding plant roots that is modified by root activity. In this critical zone, plants perceive and respond to their environment. As a consequence of normal growth and development, a large range of organic and inorganic substances are exchanged between the root and soil, which inevitably leads to changes in the biochemical and physical properties of the rhizosphere. Plants also modify their rhizosphere in response to certain environmental signals and stresses. Organic anions are commonly detected in this region, and their exudation from plant roots has now been associated with nutrient deficiencies and inorganic ion stresses.[8]

Seedlings of a range of Australian *Banksia*, *Hakea a*nd *Dryandra* species (Proteaceae) were assayed for their exudation of carboxylates. The sample solution was used for further analyses without being concentrated or diluted and for collection of exudates did not contain phosphate or nitrate, because these compounds may interfere with the high-performance liquid chromatography (HPLC) analysis of carboxylates. In addition, we also collected exudates from cluster and non-cluster roots separately. Then roots had been washed for 1 min in two different batches of deionized water. Analysis was performed on a reversed phase C-18 column with 4.5 μm particle size (Alltech Associates, Inc., Deerfield, IL, USA). For identification of the carboxylates in the exudate, their retention times were compared with standards, which were prepared in double-deionized water from free acids. Standards were also used to spike the samples. Standards of the following organic acids were prepared (in the order of increasing retention time): tartaric, formic, L-malic, malonic, lactic, acetic, maleic, citric, succinic, fumaric, *cis*-aconitic and *trans*-aconitic acid.

The largest proportions of carboxylates exuded by entire root systems of most species were accounted for by L-malate, malonate, lactate, acetate, citrate, succinate and *trans*-aconitate. Fumarate (2% of total carboxylates) was

exuded by all species, with exudation by *B. grandis* significantly different from the other four species. Trace amounts of formate were found only in exudates of *H. prostrata*. Compared with the other species, *B. grandis* exuded the largest proportions of acetate, *D. sessilis* of malonate and citrate, *H. prostrata* of lactate, and *B. occidentalis* of *trans*-aconitate. The exudate spectrum of *B. grandis,* with a small proportion of citrate, no malonate, and a high proportion of acetate, differed from the spectra for the other species tested. For all the species tested, succinate was found in exudates from the entire root system, whereas it only occurred in the exudates of cluster roots of *H. undulate* and *D. sessili.* Lactate was a common component of the exudates, both from clusters roots and non-cluster roots. The exudation of lactate appears to be species dependent and to reflect the developmental stage of the cluster, rather than a lack of oxygen.

Cluster roots of these Proteaceae exuded malate, malonate, lactate, acetate, maleate, citrate, fumarate, *cis*- and *trans*-aconitate. The relative contributions of each of these carboxylates differed between species. Malate, malonate, lactate, citrate and *trans* -aconitate, however, were invariably present in large proportions of total carboxylate exudation. Non-cluster roots of *H. prostrate* exuded a spectrum of carboxylates (mainly malonate, lactate and citrate), which differed somewhat from the exudation pattern of cluster roots (mainly malate, malonate, lactate and citrate).

Many carboxylates, most notably malate and citrate, are quite common compounds in exudates from both Proteaceae and other plant families. *Trans*-aconitate, however, is not common outside the Proteaceae, but has been identified in the exudates of other species, e.g. *Holcus mollis*, *Melica celiata* and *Fagus grandifolia*. A second carboxylate which is not common outside the Proteaceae is malonate, although it has been detected in root exudates of *Acer saccharum, Cicer arietinum*, *Sorghum bicolor*, *Glycine max* and *Cajanus cajan*. However, only in exudates of chickpea (*C. arietinum*) and pigeon pea (*C. cajan*) and the Proteaceae species tested in the present study does malonate form a substantial proportion of the total carboxylate exudation.

The rate of exudation is higher in the Proteaceae tested in the present study than in any other reported species. Rates are up to 1000-fold higher than in northern European native species, and also higher than in a range of crop species. Even in comparison with *L. albus* (based solely on citrate), the rate of exudation by Proteaceae is still twice as high.[9]

An HPLC method employing an ion exclusion column was developed for the determination of low molecular weight organic acids in soil solution. Low molecular weight (LMW) organic acids are produced during degradation of organic material in soils, e.g. example litter and dead roots by fungi and bacteria. The HPLC system (Shimadzu LC-10, Shimadzu, Osaka, Japan) featured a diode array detector. Detection was performed at 210 nm. Ion exclusion column (300 x 7.8 mm) was employed for separation of the small organic acids.[10]

In ion-chromatographic analysis of low molecular weight organic acids in Spodosol forest floor solutions was found that low molecular weight organic acids (LMWOA) from root exudates, decomposing organic matter, and other sources are important ligands involved in solution complexation reactions as well as ligand exchange reactions at mineral surfaces.[11] A number of different aliphatic acids have been found in forest soils, e.g. oxalic, citric, formic, acetic, malic, lactic, and fumaric acids. The total content of these acids is estimated to account for 2–10% of the total dissolved organic carbon (DOC) in spodosols (e.g. podzols).[10] Dissolved organic C from the forest floor contained a maximum concentration of 2.0% acetic acid, 0.01% formic acid, and 0.15% oxalic acid.[11] Succinic acid was also tested and it was found that this acid had very similar retention times to shikimic acid, both at 30 and 60°C. Good recovery values were obtained for all acids. The poorer value for oxalic acid can partly be explained by the larger variance and the fact that this acid elutes close to the void peak.[10]

4.2.2. Root exudation under the effect of phosphorus and aluminum

Organic acids exuded from roots in phosphorus uptake and aluminum tolerance of plants in acid soils were studied in Hocking, P. J.[12] Acid soils comprise about 30% of the world's arable land, and aluminum (Al) toxicity and phosphorus (P) fixation to soil minerals are major problems for crop production on these soils. Plants that can access fixed P and are Al tolerant have an important role in sustaining crop production on these soils. It is now clear that organic acids exuded from roots can benefit the P nutrition of plants and protect roots by detoxifying Al in the rhizosphere. Organic acid anions such as citrate, malonate, oxalate, and tartrate are commonly implicated in enhancing access to fixed soil P, and citrate, malate, and oxalate are implicated in enhancing aluminum tolerance.

Genetic engineering has the potential to increase organic acid efflux from roots. Mechanisms by which P deficiency and Al activate or induce the efflux of specific organic acids from roots need to be identified. Anion channels in the plasma membrane are likely to have a major regulatory role in the transport of organic acids from roots, and genes that encode for these channels will be key targets for genetic engineering.[12]

In white lupin, both phosphorus (P) deficiency and aluminum (Al) toxicity induce root exudation of carboxylates, but the relationship between these two effects is not fully understood. P deficiency triggered citrate release from mature cluster roots, whereas Al stimulated citrate exudation. Al-induced citrate exudation was inhibited by P limitation at the seedling stage, but was stimulated at later growth stages. Citrate exudation was sensitive to anion-channel blockers.[13]

Neumann, G., Römheld, V.[14] compared root excretion of carboxylic acids and protons in phosphorus-deficient plants. Phosphorus deficiency-induced metabolic changes related to exudation of carboxylic acids and protons were compared in roots of wheat (Triticum aestivum L. cv Haro), tomato (Lycopersicon esculentum L., cv. Moneymaker), chickpea (Cicer arietinum)

and white lupin (Lupinus albus L. cv. Amiga), grown in a hydroponic culture system. P deficiency strongly increased the net release of protons from roots of tomato, chickpea and white lupin, but only small effects were observed in wheat. Release of protons coincided with increased exudation of carboxylic acids in roots of chickpea and white lupin, but not in those of tomato and wheat. P deficiency-induced exudation of carboxylic acids in chickpea and white lupin was associated with a larger increase of carboxylic acid concentrations in the roots and lower accumulation of carboxylates in the shoot tissue compared to that in wheat and tomato.

Citric acid was one of the major organic acids accumulated in the roots of all investigated species in response to P deficiency, and this was associated with increased activity and enzyme protein levels of PEP carboxylase, which is required for biosynthesis of citrate. Accumulation of citric acid was the most pronounced in the roots of P-deficient white lupin, chickpea and tomato. Increased PEP carboxylase activity in the roots of these plants coincided with decreased activity of aconitase, which is involved in the breakdown of citric acid in the TCA cycle. In the roots of P-deficient wheat plants, however, the activities of both PEP carboxylase and aconitase were enhanced, which was associated with little accumulation of citric acid. In some plant species such as white lupin, there are indications for a specific transport mechanism (anion channel), involved in root exudation of extraordinary high amounts of citric acid.[14]

Citrate is the dominant organic anion released by cluster roots of white lupin representing as much as 11% and up to 23% of the total plant dry weight, depending on plant age and severity of P deficiency. Exudates from white lupin were concentrated by evaporating to dryness in a freeze-drier. The solutions were analyzed for carboxylates using reversed phase-HPLC. Separation of 10 organic acids (malic, malonic, lactic, acetic, maleic, citric, *cis*-aconitic, succinic, fumaric, and *trans*-aconitic) was achieved on an Alltima C18 column (250 x 4.6 mm).

Citrate was the dominant carboxylate in root exudates, although small amounts of fumaric, *trans-/cis*-aconitic, acetic and maleic acid anions were

detected in some samples. Total amount of citrate exuded in the Ca-P or Fe-P supplemented compartments was about 10-fold higher than those in the corresponding no-P-applied compartments, but no significant difference was observed in the Phy-P or K-P treatments. The highest amount of citrate exudation was observed in the Fe-P supplemented compartment, followed by the Ca-P compartment. The citrate exudation rate in the Fe- P-supplemented compartment was more than twice that of the corresponding no-P-applied compartment, and was highest amongst all the root compartments. There was no significant difference in citrate exudation rates between other treatments.
Citrate exudation rate was enhanced in the roots grown in the Fe-P supplemented compartment, but did not respond to other P sources, indicating the existence of specific mechanisms regulating root exudation in plants supplemented with different P sources.[15]

Root exudates from +P-treated plants had very low concentrations of organic acids, frequently below the detection limit of the detector and/or the fraction collector, resulting in few data points for citrate and malate concentration. The individual organic acids from plant organ extracts and root exudates in white lupin were separated by HPLC equipped with an Aminex HPX - 300 x 7.8 mm column. Root exudate was concentrated in an auto evaporator.

Citrate and malate were the only organic acids recovered in root exudates after the 22-h chase. Sixteen-fold more citrate and malate was recovered in root exudates from -P-treated plants compared with +P-treated plants. The exudation rate was estimated from the amount of citrate and malate recovered in the root exudates. The exudation rate of citrate and malate per unit weight of root was about 20-fold higher from -P-treated plants compared with +P-treated plants. The increased exudation rate per gram fresh weight indicates that differences in total citrate and malate exuded are not simply due to differences in total plant mass. When root exudates were allowed to accumulate for 48 h (collected just before labelling), a small amount of succinate was detected in some -P-treated exudate samples. Citrate acts via severa1 mechanisms to mobilize soil P, affecting both inorganic and organic P

fractions by (a) anion exchange; (b) chelation of bridging metal cations from organic matter in the soil; and (c) suppressing readsorption and precipitation of inorganic.[16]

Citrate exudation is known to be one of the most important mechanisms used by P-efficient species (e.g., L. albus) to mobilize sparingly available inorganic P. Findings of Wang, Z., Shen, J., Zhang, F.[17] suggest that L. pilosus, in comparison to L. albus, may use a similar strategy to acquire P from the P-deficient calcareous soils. Phosphorus deficiency in L. pilosus was associated with significantly higher rates of citrate exudation. Carboxylates in root exudates were directly analysed by reverse phase HPLC without concentrating the solution of exudates based on a preliminary experiment on citrate exudation in L. pilosus. Separation was conducted on a 250 x 4.6 mm reversed phase column (Alltima C-18). The mobile phase was 25 mM KH_2PO_4 (pH 2.5) with the flow rate of 1 $ml.min^{-1}$ at 28°C and UV detection was set at 214 nm.[17]

Lipton, D. S., Blanchar, R. W. and Blevins, D. G.[18] deal with the effect of low P upon the exudation of organic acids by roots of alfalfa (Medicago sativa L.) seedlings. Citric, malic, and succinic acids were detected in the root exudates of 24-day-old alfafa seedlings. Citrate exudation from the roots of P-stressed alfalfa was 182% that of plants receiving a complete nutrient solution. In both groups of plants, succinate constituted the smallest proportion of the organic acids in the root exudates. The increased release of citrate may provide a mechanism by which P-stressed plants enhance the availability of P in the rhizosphere.[18]

Responses of eucalypt species to aluminium and the possible involvement of low molecular weight organic acids in the Al tolerance mechanism was investigated in Silva, I. R.[19] Aluminum (Al) tolerance mechanisms in crop plants have been extensively researched, but our understanding of the physiological mechanisms underlying Al tolerance in trees is still limited. To investigate Al tolerance in eucalypts, seedlings of six species and seedlings of six clones of *Eucalyptus* species were grown for 10 days in nutrient solutions containing Al concentrations varying from 0 to 2.5 μM (0 to 648 μM Al^{3+} activities). *E. globulus* seedlings were tolerant to Al

toxicity, they were highly sensitive to lanthanum (La), indicating that the tolerance mechanism is specific for Al. Fine roots accumulated more Al and their elongation was inhibited more than that of thick roots. In *E. globulus*, accumulation of Al in root tips increased linearly with increasing Al concentration in the nutrient solution.

Gas chromatography/mass spectrometry and ion chromatography analyses indicated that root exposure to Al led to a greater than 200% increase in malic acid concentration in the root tips of all eucalypt species. The increase in malate concentration in response to Al treatment correlated with the degree of Al tolerance of the species. A small increase in citric acid concentration was also observed in all species, but there were no consistent changes in the concentrations of other organic acids in response to Al treatment. In all eucalypt species, Al treatment induced the secretion of citric and malic acid in root exudates, but no trend with respect to Al tolerance was observed. Thus, although malate and citrate exudation by roots may partially account for the overall high Al tolerance of these eucalypt species, it appears that tolerance is mainly derived from the internal detoxification of Al by complexation with malic acid.[19]

4.2.3. Root exudation under the effect of aluminum, copper and zinc

Qin, R., Hirano, Y., Brunner, I.[20] were dealing with exudation of organic acid anions from poplar roots after exposure to Al, Cu and Zn. Aluminum induced root exudation of oxalate and citrate, copper induced root exudation of oxalate, malate and formate, and zinc induced root exudation of formate. The threshold treatment concentrations were 100 µM for Cu and 500 µM for Al and Zn, corresponding to about 30 µM Cu^{2+}, 140 µM Al^{3+} and 290 µM Zn^{2+}. Simultaneous with the increase in organic acid anion exudation, sulphate and the nutrient cations K^{+}, Ca^{2+} and Mg^{2+} were released into the solutions. Significant positive correlations between the organic acid anions and the cations indicate possible co-release. Toxicity symptoms of the poplar roots included browning of the root apices, which occurred at Cu concentrations of

50 μM and above, at Zn concentrations of 500 μM and above, and at an Al concentration of 1000 μM, and callose formation, which was observed solely in response to Al concentrations of 500 μM or higher. The results indicate that the composition of the exuded organic acid anions from poplar roots and the toxicity symptoms are specific to each of the applied heavy metals.[20]

4.2.4. Root exudation under the effect of cadmium

Effects of Cadmium Amendments on Low-Molecular-Weight Organic Acid Exudates in Rhizosphere Soils of Tobacco and Sunflower were studied in Chiang P.-N. et al.[21] The aims of this study were to assess the effect of LMWOAs on uptake of Cd by tobacco and sunflower under pot experiments, thus comparing the ability of tobacco and sunflower for phytoremediation. LMWOAs were detected by gas chromatograph in bulk soils, and low amounts of LMWOAs were found in uncontaminated rhizosphere soils. Acetic, lactic, glycolic, maleic, and succinic acids were present in the tobacco and sunflower rhizosphere soils. Glycolic and maleic acids were dominant in tobacco rhizosphere soils. On the other hand, acetic, malic, maleic, and succinic acids were identified and dominated by acetic acids in the sunflower rhizosphere soils. The citric acid was not determined in the rhizosphere soils of sunflower and tobacco. Concentrations of LMWOAs increase with increasing amendment of Cd concentrations in tobacco and sunflower rhizosphere soils. The increase in LMWOAs release in the presence of Cd can be explained based on the complexation of the soil particulate-bound Cd with LMWOAs, which is reflected in the LMWOAs release from the two plants with the increase in the concentration of Cd.[21]

4.2.5. Root exudation under the effect of lead

Many kinds of low-molecular-weight organic acids (LMWOAs) have been found in rhizosphere soils of agricultural and forest lands, including acetic acid, citric acid, formic acid, fumaric acid, maleic acid, malic acid, malonic acid, lactic acid, oxalic acid, and succinic acid. The composition and quantities

of these LMWOAs were determined by GC analysis. One volatile acid (propionic acid) and nine nonvolatile acids (lactic, glycolic, oxalic, succinic, fumaric, oxalacetic, D-tartaric, *trans*-aconitic, and citric acids) were identified in the rhizosphere quartz sand mixed with anion-exchange resin. D-tartaric and citric acids were found to be major among the nonvolatile acids. However, glycolic, fumaric, and *trans*-aconitic acids were not detected in the anion-exchange resin and rhizosphere quartz sand of blanks (without $Pb(NO_3)_2$ solution) under high and low transpiration on the three days.

The amount of the water-soluble LMWOAs present in the resin and rhizosphere quartz sand increased with increasing amount of Pb uptake, which was again dependent on the concentration of Pb present in the resin and rhizosphere quartz sand.[22]

4.2.6. Root exudation under the effect of chromium

The effect of chromium (Cr) stress on the changes of rhizosphere pH, organic acid exudation, and Cr accumulation in plants was studied using two rice genotypes differing in grain Cr accumulation. The roots of 5 rice plants were used and the collected root exudates were diluted to 500 ml with deionized water. The sample solution was passed through a cation-exchange column (16 x 14 mM), followed by an anion-exchange column (16 x 14 mM). The organic acids retained on anion-exchange resin were eluted by 1M HCl, and the eluate was concentrated to 10 ml. Concentrated solutions (20 ml) were injected into a Symmetry C18 column (3.9 mm i.d. x 150 mm, 5 μm). The quantitative determination of organic acids was carried out using a high-performance liquid chromatography.

Among organic acids measured in this study, oxalic and malic acid contents were much higher than the other four organic acids. Cr stress markedly increased oxalic acid exudation, irrespective of rice genotypes and the time of Cr exposure. The malic acid exudation in rice roots varied greatly with the time of Cr exposure and rice genotype. At the 8th day of Cr exposure, malic acid exudation showed a decrease in the treatment of 10mM Cr relative to that of the control (without Cr addition), and an increase in the treatment of

50mM Cr; and then again showed decline in the treatment of 100mM Cr. Both Xiushui 113 and Dan K5 had the maximum malic acid exudation in the treatment of 50mM Cr. When the time of Cr exposure was prolonged to 16 days, malic acid exudation in Dan K5 showed a massive increase under 100mM Cr, while that in Xiushui 113 still decreased under 100mM Cr compared to other Cr concentrations. There was little exudation for both lactic and acetic acids, irrespective of Cr level and exposure time. Similar to malic acid, citric acid exudation was dependent on the time of Cr exposure and rice genotypes. At the 8th day of Cr exposure, citric acid exudation of the two genotypes showed an increase with increased Cr level up to 50mM, and declined when the level of Cr increased to 100mM. In contrast, at the 16th day of Cr exposure, both genotypes had the maximum citric acid exudation under 100mM Cr. Significant differences were found for succinic acid exudation among Cr levels and between two genotypes. At the 8th day of Cr exposure, Dan K5 showed the highest succinic acid exudation under 50mM and as the exposure time extended, Dan K5 under 100mM stress had the highest succinic acid exudation. Cr accumulation showed significantly synergistic relationships with oxalic, malic and citric acid exudation, no significant correlations were found between Cr accumulation and lactic, acetic or succinic acid.

Among the six organic acids detected in the experiment, oxalic acid and malic acid played the most important role in the variation of organic acid exudation in the rhizosphere. These organic acids could be important in alleviating phytotoxicity induced by Cr stress. There is little or no conclusive evidence about whether these acids play a protective role and what level of organic acid content is sufficient for providing direct protection against Cr toxicity in the rhizosphere.[23]

4.2.7. Root exudation under the effect of cobalt

The exposure to cobalt ions resulted in the increase of citrate and cysteine in cells.[24]

4.2.8. Effect of the type of added phosphate on root exudation

Lambers, H. et al.[25] showed that the pattern of carboxylate exudation in *Banksia grandis* (Proteaceae) is affected by the form of phosphate added to the soil. *B. grandis* roots exude a range of carboxylates. Results suggest that plants modify the exudate spectrum in response to soil chemical factors, e.g. the presence of Al or Fe. Carboxylates can form stable complexes with cations in the soil, such as Ca, Fe and Al and thus make phosphate available by complexing the cation. Malate, citrate and aconitate have been shown to form relatively stable complexes with Al. Lactate and acetate on the other hand form less stable Al complexes and are probably also less effective at releasing complexed phosphate. Citrate increases Fe availability from mineral surfaces from limestone and also increases phosphate availability in limestone soil. Phosphate is mobilised from calcium-phosphate as a result of Ca chelation by formate and citrate.

Separation was in an Alltima C-18 reverse phase column (250 × 4.6 mm). The mobile phase was 125mM KH_2PO_4, pH 2.5, with a methanol gradient to flush the column of more polar analytes between sample injections. Roots of a wide range of plant species exude carboxylates, e.g. citrate, into the rhizosphere, to mobilise sparingly available phosphate. Sand-grown plants supplied with Al-phosphate exuded three main tri- and dicarboxylates: citrate (60%), malate (25%) and trans-aconitate (14%), with trace amount of cis-aconitate and fumarate. Plants supplied with Fe-phosphate exuded the same five as the Al-phosphate treatment plus two additional monocarboxylates. The relative amounts were lactate (30%), citrate (31%), malate (14%), trans-aconitate (12%) and acetate (12%), plus traces of cis-aconitate and fumarate.

Roots of plants grown with K-phosphate exuded small and variable amounts of citrate, maleate, succinate, cis-aconitate, trans-aconitate and lactate. Only trans-aconitate was common to all replicates. The exudates of plants grown with glycerol phosphate were, again, variable, with maleate, fumarate,

cis-aconitate, trans-aconitate and lactate being exuded. Only *cis*- and *trans*-aconitate were common to all replicates. The exudation of di- and tricarboxylates by *B. grandis* grown with Al-phosphate might be due to Al activation of an anion channel, as observed for malate exudation in wheat. To explain the different exudation spectrum of plants grown with Fe-phosphate, we hypothesise that there is another channel, responsible for the exudation of monocarboxylates. Fe would activate both channels, whereas Al would only activate the di- and tricarboxylate channel. Alternatively, Al might inhibit the monocarboxylate channel.

The difference in the carboxylates recovered from the rhizosphere, comparing the Fe-phosphate and the Al-phosphate treatments of the sand-grown plants, indicates that the plants perceived a difference in the chemistry of their rhizosphere environment and exuded different carboxylates in response. The variability in the exudates collected from the plants fertilised with either glycerol-phosphate or K-phosphate treatments probably reflects the stress these plants appeared to be under.[25]

The relationship between carboxylate release from roots and the ability of the species to utilize phosphorus from sparingly soluble forms was studied by comparing *Triticum aestivum*, *Brassica napus*, *Cicer arietinum*, *Pisum sativum*, *Lupinus albus*, *Lupinus angustifolius* and *Lupinus cosentinii*. Analysis of samples of rhizosphere extracts was performed by HPLC using an Alltima C-18 reverse-phase column. The working standards included malic, malonic, lactic, acetic, maleic, citric, *cis*-aconitic, succinic, fumaric and *trans*-aconitic acids.

Total carboxylates accumulated in the rhizosphere varied among species, and significantly among forms of P supplied for *T. aestivum*, *B. napus*, *L. albus* and *L. cosentinii*. *Triticum aestivum* had the lowest carboxylate concentrations in its rhizosphere for all treatments. *Triticum aestivum* had the most carboxylates present in its rhizosphere when P was supplied as $Ca_5OH(PO_4)_3$, while for *B. napus* this occurred when P was supplied as either $Ca_5OH(PO_4)_3$ or KH_2PO_4. KH_2PO_4 significantly suppressed the release of

carboxylates from all *Lupinus* species. *Lupinus cosentinii*, followed by *L. albus*, accumulated the most carboxylates in the rhizosphere.

Carboxylate composition varied among species. *Cicer arietinum* was the only species to release malonate, which comprised, on average, between 50% and 77% of all carboxylates. *Pisum sativum* released only citrate in appreciable amounts. *Lupinus cosentinii* released the greatest percentage of citrate for all P sources (79–94%), compared with *L. albus* (9–67%) and *L. angustifolius* (44–76%). Change in percentage composition with KH_2PO_4 supply was greatest for *L. albus* of all species in the present study. All *Lupinus* species had a larger percentage of malate in their rhizosphere when P was supplied as KH_2PO_4. *Brassica napus* tended to release the greatest percentage of malate (78–85%) and fumarate (5–7%) of all species in the present study.

Pisum sativum and *C. arietinum* are especially intriguing species with regard to P acquisition, because they were completely unable to use P from either $AlPO_4$ or $FePO_4$, compared with the control receiving no P for both forms. *Pisum sativum* released exclusively citrate, therefore it was expected that *P. sativum* was capable of using some P from $AlPO_4$ or $FePO_4$. Field studies have shown that *P. sativum* is poorer at accessing residual P than is *L. albus*, and is relatively responsive to applications of soluble P. A possible explanation for the lack of ability to access $AlPO_4$ or $FePO_4$ is that carboxylate release by these species does not occur in a way that is conducive to P uptake. That is, carboxylate release may not be structured in time and space as it is for cluster root-bearing species such as *L. albus*.

Carboxylate release by *C. arietinum* is not conducive to obtaining P from sparingly soluble forms. *Lupinus angustifolius* released fewer carboxylates into its rhizosphere than *L. albus* and *L. cosentinii*, yet was comparatively effective in making use of P from the sparingly soluble forms. Species accessed different forms of sparingly soluble P, but no species was superior in accessing all forms. *Lupinus albus* and *L. cosentinii* presumably rely more heavily on carboxylate release and cluster-root production to obtain nutrients, rather than on a larger root system.[26]

4.3. Phytoremediation

4.3.1. Biological remediation of contaminated soil

Various physiochemical and biological methods have been developed to remediate or cleanup contaminated soils. For successful decontamination it is very important to use a cleanup strategy whose selection is based on the contaminant or mixture of contaminants in question, soil type, and other environmental factors. Remediation approaches differ in soils contaminated with inorganic contaminants from those contaminated with organics. Inorganic compounds are immutable and cannot be degraded. There are very few remediation alternatives for soils contaminated with inorganic when compared to organic contaminants. Unfortunately, completely effective and economically affordable remediation methods have not yet been developed. Soil remediation can be performed either in place (*in situ*), or by excavating and removing contaminated material (*ex situ*). Special attention has been given to biological *in situ* cleanup processes because of lower cost and the larger areas that could be remediated simultaneously. Phytoremediation and bioremediation are biological processes that are emerging as potentially effective remediation techniques.

4.3.1.1. Phytoremediation

Phytoremediation comes from the Greek word *phyto* or plant and Latin *remedium,* which mean to correct and remove evil. It involves the use of plants to remove and contain hazardous environmental pollutants. Plants may participate in decontamination directly by the uptake of pollutants and their immobilization, degradation or transformation within the plant, or indirectly by supporting rhizosphere microorganisms through the exudation of substrates and nutrients and the physical presence of the roots.

Polluted sites often contain complex mixtures of organic and inorganic compounds, commonly released from the same anthropogenic sources. Plant remediation mechanisms, especially those dependent on uptake from the soil

solution, may differ when pollutants are present as mixtures rather then separately in the environment.

The advantages of phytoremediation over other cleanup strategies are numerous: it is cheaper then other conventional technologies, reduces erosion by wind and water, reduces exposure risk to the community, leaves usable topsoil intact, doesn't require an extra source of energy because it is solar driven, and is aesthetically pleasing and accepted by the public. The main disadvantage of this method is the longevity of the clean-up process. Other potential problems include contamination of the food chain and the volatilization of some compounds.

Phytoremediation strategies may be either to stabilize or sequester the contaminants into the soil matrix or to remove the contaminant by uptake or degradation. Phytostabilization and phytoextraction are the main mechanisms for decontamination of soils polluted with inorganic compounds. In phytoextraction plants extract metals from the soil and translocate them to the aboveground plant parts that can be harvested and incinerated. The final product is an ash rich in one or more metals that can either be smelted for metal recovery or disposed at designated areas such as landfills. If perennial plants that translocate metals to the shoots are used, the above ground biomass can be removed, allowed to regrow, and be harvested again. With perennials, repeated sowing would be avoided, further decreasing the cost of the phytoremediation process. Mature plants and/or regrowth, after being excised at ground level, may contain more metal then the juvenile tissues.

Phytoextraction varies between different plant species, with two major categories of plants suitable for phytoremediation. One group consists of hyperaccumulator plants such as *Thlaspi caerulescens*, that can store high concentrations of metals per g of tissue of above ground parts but have overall small biomass. The other group contains plants that can store lower metal concentrations per tissue weight, but have much larger biomass. One such plant is *Brassica juncea* (Indian mustard), which produces a large amount of biomass. There was no correlation between the biomass of roots and/or shoots and degradation outcome.

4.3.2. Uptake of metals and polycyclic aromatic hydrocarbons

Many soils are contaminated with heavy metals or polycyclic aromatic hydrocarbons (PAHs), which can have negative impact on ecosystems and human health.

Heavy metals are inorganic elements normally found in the environment. Since the beginning of the industrial revolution anthropogenic activity has increased their mobilization and contamination. Some metals are essential nutrients for living organisms that can become toxic when present in excess, while others are toxic even in small amounts. They can be transferred into agricultural crops and animals and biomagnified along the food chain before consumption, and thus may pose serious threat to human health. All plants require elemental ions including metals for their primary and secondary metabolism. The selectivity of elemental uptake by plants is limited, so they may also take up nonessential or even toxic metals. Upon the entrance into the plant, metals can accumulate in the root free space where they are either trapped by the negatively charged carboxylic groups on the cell walls or are transported across cell membranes into the cytoplasm. From this symplastic space, metals can be translocated to the other parts of the plant including shoots where they usually form complexes with organic acids such as citrate and malate. The uptake and fate of individual metals within the plant may be altered by the presence of mixtures composed of either multiple metals or metals and organic compounds as a result of competitive or synergistic interaction between them. This interaction may be limited just to the root surface or may affect accumulation and translocation of metals within the tissues.

4.3.3. Phytoremediation of soils contaminated with metals and polycyclic aromatic hydrocarbons

Phytoremediation is an emerging clean-up technology in which plants remove contaminants from the environment. Plants may have multiple

beneficial effects as they can simultaneously affect the dissipation of pollutants in the soil and improve overall soil quality. The use of plants is also much cheaper and aesthetically pleasing then other remediation techniques available on the market. Because of these positive qualities, interest in phytoremediation as a clean up strategy is constantly growing. Under controlled conditions, plants have proved effective at remediation and removal of individual contaminants.

Unfortunately, in the natural environment contaminated sites may contain mixtures of pollutants rather than individual contaminants. These mixtures are usually composed of inorganic elements and/or various organic compounds. As phytoremediation depends on a living system, it is much more sensitive to harsh environmental conditions, phytotoxicity, and is more dependent on the contaminant's properties. The presence of multiple pollutants with different chemical, physical and structural properties may further add complexity to the already fragile situation and alter plants efficiency for removal and/or detoxification. Study Bukvic, A.[1] revealed a strong relationship between metal exposure levels, metal depletion, and plant metal uptake. More subtle was the effect of other metals and PAHs, on the uptake of individual metals. In general, the presence of other metals decreased uptake, while PAHs eliminated this inhibition. In this study, phytotoxicity was observed in almost all single metal and metal mixture treatments when compared with the controls. Although all metals in soil solution were available for the plant uptake, less than 1% of the total metal added to the soil was taken up into the plant tissue. There was no significant difference between the uptake from soil spiked with multiple metals or metal mixtures with PAHs. Mineralization was notably different only between soils previously spiked with PAHs with or without metals and soils only spiked with metals.

One potential mechanism of phytoremediation is called phytoextraction, where plants take up contaminants from the soil solution and translocate them to aboveground plant biomass that can be harvested and incinerated.

This study was conducted to investigate the influence of mixtures on aspect of phytoextraction. Although phytoextraction is a promising remediation

technique, it is still not completely clear how its efficiency changes in the presence of both inorganic and organic contaminants. The presence of organic contaminants did not affect uptake of metals into the plant tissue, although it had an influence on the metal quantities recovered by total metal extraction.[1]

5. EXPERIMENTAL PART

5.1. Chemicals

- distilled water prepared with machine QPAK Alpha Q or MILLI Q® Gradient A 10, MILLIPORE, Massachusetts, USA
- oxalic acid, Sigma Aldrich, France, M_r = 126.10
- L-(+)-tartaric acid, Sigma Aldrich, France, M_r = 150.09
- formic acid, Sigma Aldrich, France, M_r = 46.03
- DL-malic acid, Sigma Aldrich, France, M_r = 134.09
- trisodium DL-*iso*-citrate, Sigma Aldrich, France, M_r = 258.07
- diammonium lactate, Sigma Aldrich, France, M_r = 184.15
- sodium acetate, Sigma Aldrich, France, M_r = 136.1
- citric acid, Sigma Aldrich, France, M_r = 192.13
- succinic acid, Sigma Aldrich, France, M_r = 118.09
- fumaric acid, Sigma Aldrich, France, M_r = 116.10
- *cis*-aconitic acid, Sigma Aldrich, France, M_r = 174.11
- *trans*-aconitic acid, Sigma Aldrich, France, M_r = 174.11
- sodium sulphate decahydrate, Normapur, France, M_r = 322.19
- methanesulfonic acid, FLUKA Chemika, Switzerland, M_r = 96.10
- orthophosphoric acid, Fisher chemicals, Pennsylvania, USA, M_r = 98.00
- perchloric acid (70%), Fisher chemicals, Pennsylvania, USA, M_r = 100.46
- methanol, Fisher Scientific HPLC Grade, Pennsylvania, USA, M_r = 32.04
- hydrogen peroxide 30%, MERCK, France, M_r = 34.03

5.2. Equipment

Columns:

- Acclaim Organic Acid, 150 x 4.0 mm (particle size 5 µm), Dionex, California, USA
- ProntoSIL, 250 x 3 mm (particle size 5 µm), Bischoff, Germany

- PLRP-S, 250 x 4.6 mm (particle size 5 µm), Polymer Laboratories, Massachusetts, USA

HPLC system No 1:

UV Detector:	L – 4000, Merck, France
Pump:	L – 6000, Merck, France
Autosampler:	AS – 2000 A, Merck, France
Computer:	x86 Family, 96 MB RAM, MS Win 2000, SP 2
PC programme:	HPLC System Manager, Version 4.1, Merck HITACHI Model D - 7000, France

HPLC system No 2:

Diode Array Detector:	L – 7455, La Chrom Merck HITACHI, France
Pump:	L – 7100, La Chrom Merck HITACHI, France
Autosampler:	L – 7200, La Chrom Merck HITACHI, France
Interface:	D – 7000, La Chrom Merck HITACHI, France
Computer:	x86 Family, 128 MB RAM, MS Win NT
PC programme:	HPLC System Manager, Version 4.1, Merck HITACHI Model D - 7000, France

Analytical balance:

PRECISA 262 SMA, France

PRECISA QB 4200 C

Digital pH-meter:

GLP 22, Crison, Spain

5.3. Preparation of solutions

5.3.1. Preparation of solutions for HPLC

Mobile phase for the column Acclaim OA 4 x 150 mm, Dionex

16.11 g of Na_2SO_4 was dissolved in 500 ml distilled water in a volumetric flask to prepare 500 ml of 100 mM Na_2SO_4. This solution was acidified with methanesulfonic acid to pH 2.65.

Mobile phase for the column ProntoSIL 250 x 3 mm, Bischoff

1.70 g of H_3PO_4 was dissolved in 500 ml distilled water in a volumetric flask to prepare 500 ml of 50 mM H_3PO_4.

Mobile phase for the column PLRP-S 250 x 4.6 mm, Polymer Laboratories

1.29 ml of perchloric acid was diluted with distilled water in a volumetric flask to prepare 500 ml of 30 mM perchloric acid.

5.3.2. Preparation of standard solutions

Standard solutions of 12 organic acids were prepared. 0.01 g of oxalic, tartaric, formic, malic, *iso*-citric, lactic, acetic, citric, succinic, fumaric, *cis*-aconitic and *trans*-aconitic acid was weighted on analytical balance, completed in 10 ml with distilled water in a volumetric flask, dissolved and stored in 50 ml reclosable containers.

Mixture of organic acids for HPLC analysis was prepared as it is mentioned in corresponding literature.[27] 15 µl of oxalic acid stock solution, 120 µl of tartaric acid stock solution, 180 µl of formic acid stock solution, 120 µl of malic acid stock solution, 120 µl of *iso*-citric acid stock solution, 180 µl of lactic acid stock solution, 120 µl of acetic acid stock solution, 120 µl of citric acid stock solution, 120 µl of succinic acid stock solution, 7 µl of fumaric acid stock solution and 7 µl total for *cis*- and *trans*- aconitic stock solutions was measured with automatic pipette and mixed.

Solutions of individual acids were prepared to obtain individual chromatograms of the acids. The same volumes of acids as shown in the preceding paragraph were taken for preparation of the mixture of these acids. Only first ten acids were measured.

5.4. Preparation of samples

Places for germination of seeds were prepared at first. Five plastic wheels with diameter 4 cm were covered with polyamide squares (10 x 10 cm) from bellow and it was fixed with rubber bands. Two squares of polystyrene were wrapped in paper to waterlog and placed on a plate.

Seeds – Ray-Grass Anglais Fourrager Tetraploide, CAILLARD, France - were humidified and so sterilized for 30 minutes with 6 % hydrogen peroxide, made from 20 ml of 30% H_2O_2 and 80 ml of distilled water. Then the seeds were well washed with distilled water and 1 g was placed to every wheel. The wheels were placed on the wrapped polystyrene squares that were floating in distilled water that the plate was filled with. It was covered with carton altogether to prevent getting light to the seeds. Next 4 days it was necessary only to add distilled water. Then distilled water was commuted for nutrient solution (Table 1) for next 3 days.

Table 1: Composition of nutrient solution according to Coïc, Y.[28]

Composition	**Concentration**	
	g.l^{-1}	**mmol.l^{-1}**
$Ca(NO_3).\ 4H_2O$	0.4	2.29
KNO_3	0.4	3.96
K_2HPO_4	6.45×10^{-3}	0.037
NaCl	0.02	0.34
KH_2PO_4	0.1	0.74
$MgSO_4$	0.2	1.66
NH_4NO_3	0.2	2.50
$MnSO_4.\ 1H_2O$	1.69×10^{-3}	0.01
$CuSO_4.\ 5H_2O$	2.4×10^{-4}	0.96×10^{-3}
$ZnSO_4.\ 7H_2O$	3.3×10^{-4}	1.15×10^{-3}
H_3BO_3	1.86×10^{-3}	0.03

After 7 days of germination the germinated seeds were taken out from every wheel individually. The root of each small plant from one wheel was cut and placed in 2 ml vessel with 1 ml of distilled water. The roots were taken out from the solution after two hours, cut and dried for 3 hours at 80°C. Then it was weighed. The solutions were used for HPLC as samples 1 - 5.

5.5. Separation conditions

HPLC system No 1 with column Acclaim OA 4 x 150 mm, Dionex:

Temperature:	ambient
Mobile phase:	100 mM Na_2SO_4, pH 2.65 (adjusted with methanesulfonic acid)
Flow rate:	0.6 ml/min
Injection volume:	40 µl
Detection:	UV, 210 nm
Time of analysis:	25 min. for mixture, 12 min. for individual acids, nutrition solution, anions, 20 min. for samples of exudates

Measurements were done under isocratic conditions.

HPLC system No 2 with column ProntoSIL 250 x 3 mm, Bischoff:

Temperature:	ambient
Mobile phase:	50 mM H_3PO_4
Flow rate:	0.7 ml/min
Injection volume:	10 µl
Detection:	diode-array detector
Time of analysis:	30, 35 or 40 min.

6. RESULTS AND DISCUSSION

6.1. HPLC system No 1 with column Acclaim OA 4 x 150 mm, Dionex

6.1.1. HPLC analyses of standards

Chromatogram obtained from our measurement (Fig. 2) was compared with a model chromatogram for the column 4 x 250 mm (Fig. 3) because of unavailability of the precise one for the column 4 x 150 mm.

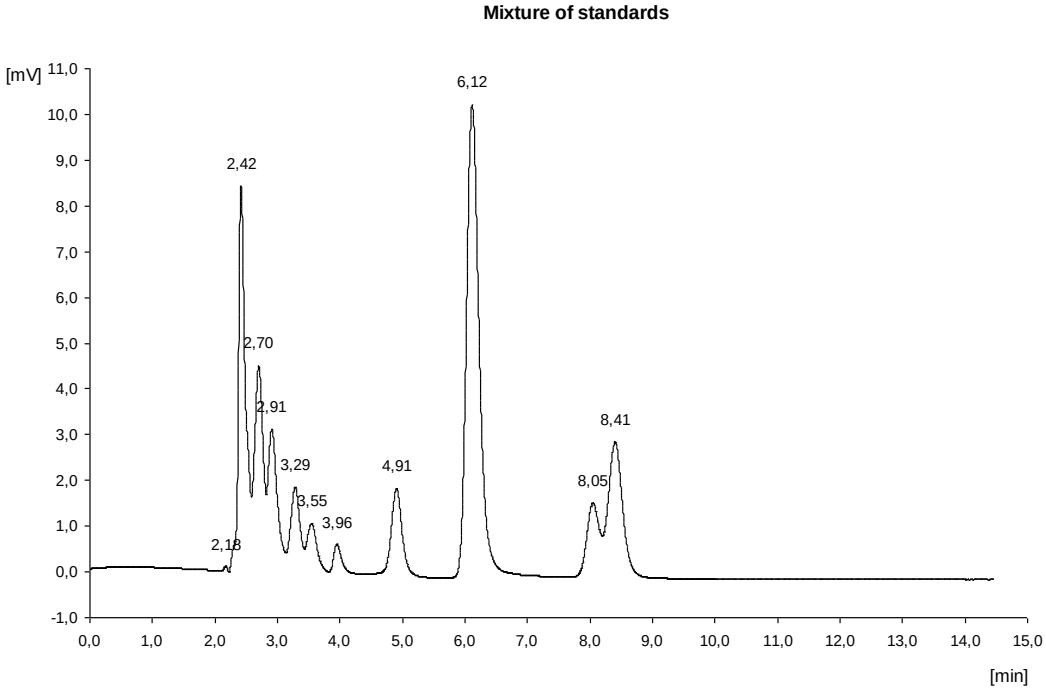

Fig. 2: Chromatogram obtained from our measurement of the mixture of standards

(The numbers above peaks represent times of retention of these acids:

2.18 - oxalic acid; 2.42 - tartaric acid; 2.70 - formic acid; 2.91 - malic acid; 3.29 - *iso*-citric acid; 3.55 - lactic acid; 3.96 - acetic acid; 4.91 - citric acid; 6.12 – no-separated peaks of succinic acid and fumaric acid; 8.05 - *cis*-aconitic acid; 8.41 - *trans*-aconitic acid)

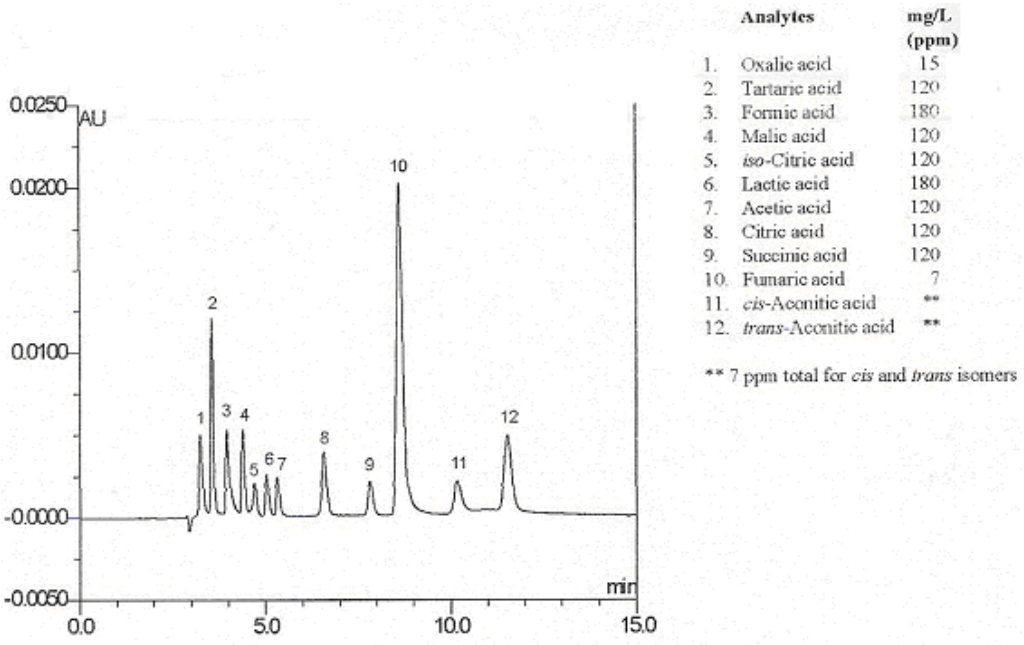

Fig. 3: Model chromatogram for highly hydrophilic organic acids[27]

Then chromatograms of some individual standard acids were made because the peaks in mixture of standards were not all separated. Results of these analyses and comparison of retention times of all analyses of standards are in Table 2.

Table 2: Comparison of retention times (T_r) of all analyses of standards

Standard acids	The mixture of acids (T_r [min])	The individual injections of acids T_r [min])
oxalic acid	2.18	2.40
tartaric acid	2.42	2.66
formic acid	2.70	2.93
malic acid	2.91	3.32
***iso*-citric acid**	3.29	3.60
lactic acid	3.55	3.96
acetic acid	3.96	3.87
citric acid	4.91	4.14
succinic acid	6.12	5.69
fumaric acid	6.12	4.80
***cis*-aconitic acid**	8.05	
***trans*-aconitic acid**	8.41	

6.1.2. HPLC analysis of root exudates

Five samples (Sample 1 – 5) of root exudates were prepared. Every sample was measured three times, each time the sample was differently diluted, according to height of peaks in previous measurement. Resultant chromatograms and tables of acids that were recognized in these chromatograms are bellow. (Fig. 4 – Fig. 8 and Table 3 – Table 7) Published figures are chromatograms of concentrated samples. Retention times of tartaric acid were taken from chromatograms of diluted samples.

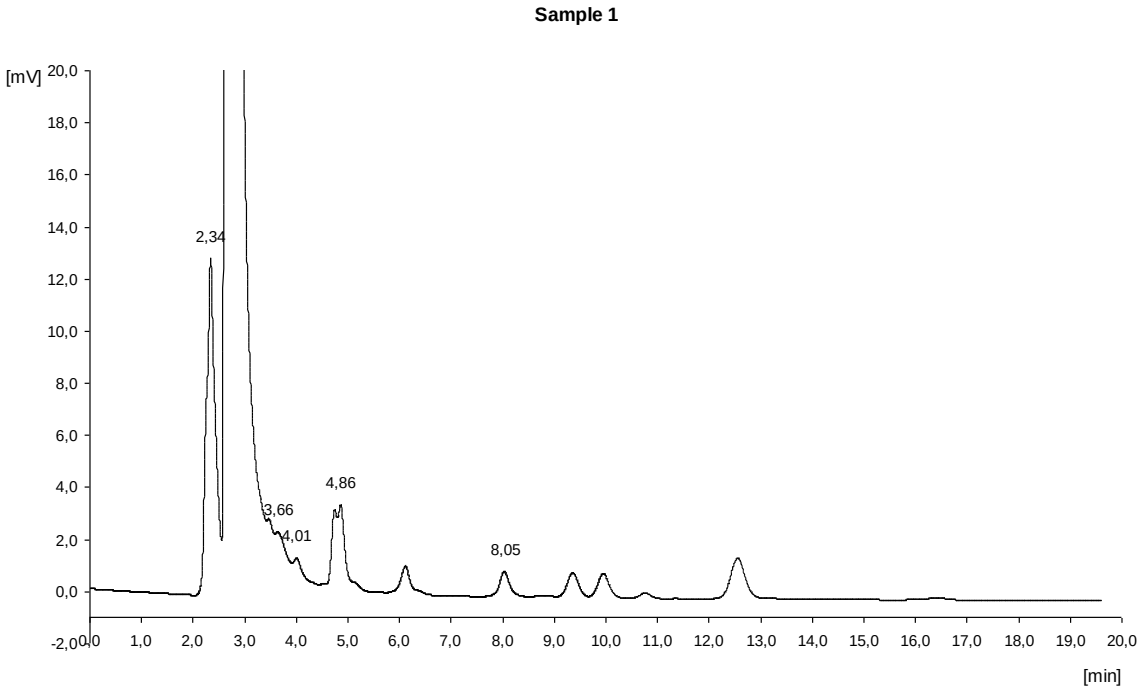

Fig. 4: Chromatogram of sample 1

Table 3: Acids that were recognized in the chromatogram of sample 1

T_r [min]	**The found acids**
2.34	Oxalic
2.72	Tartaric
3.66	*iso*-citric
4.01	Lactic
4.86	Fumaric
8.05	*cis*-aconitic

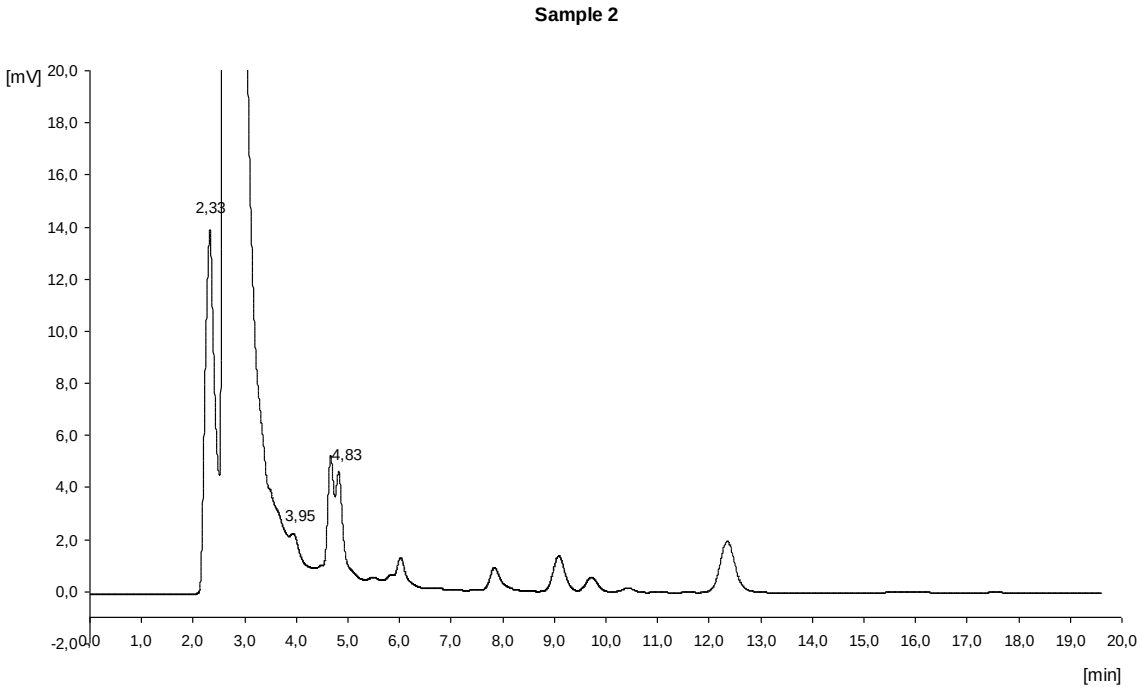

Fig. 5: Chromatogram of sample 2

Table 4: Acids that were recognized in the chromatogram of sample 2

T_r [min]	**The found acids**
2.33	oxalic
2.72	tartaric
3.95	lactic
4.83	fumaric

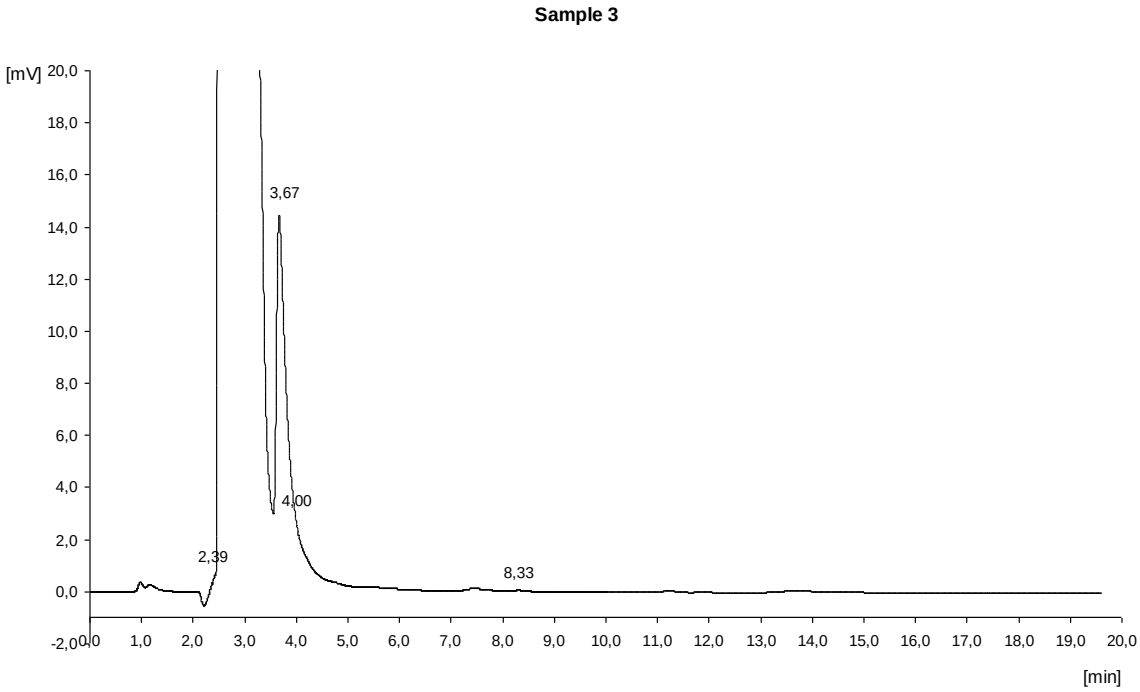

Fig. 6: Chromatogram of sample 3

Table 5: Acids that were recognized in the chromatogram of sample 3

T_r [min]	**The found acids**
2.39	oxalic
2.70	tartaric
4.00	lactic
3.67	*iso*-citric
8.33	*trans*-aconitic

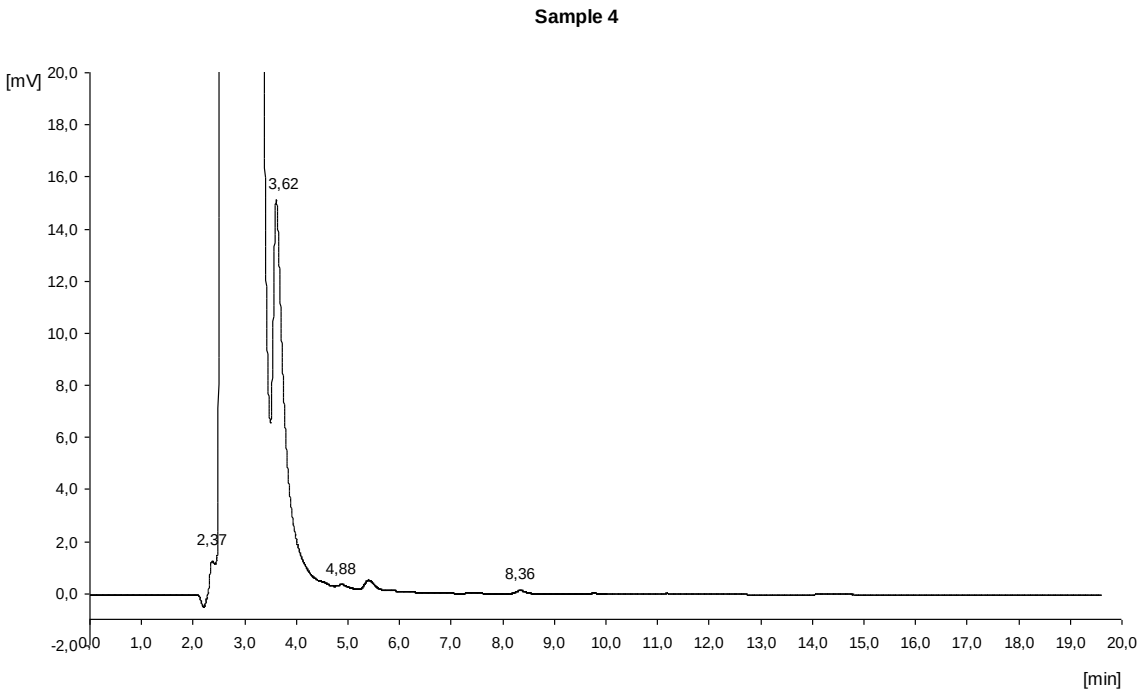

Fig. 7: Chromatogram of sample 4

Note: Published figure is chromatogram of concentrated samples. Retention times of tartaric acid and malic acid were taken from chromatogram of diluted sample.

Table 6: Acids that were recognized in the chromatogram of sample 4

T_r (min)	**The found acids**
2.37	oxalic
2.68	tartaric
3.30	malic
3.62	*iso*-citric
4.88	fumaric
8.36	*trans*-aconitic

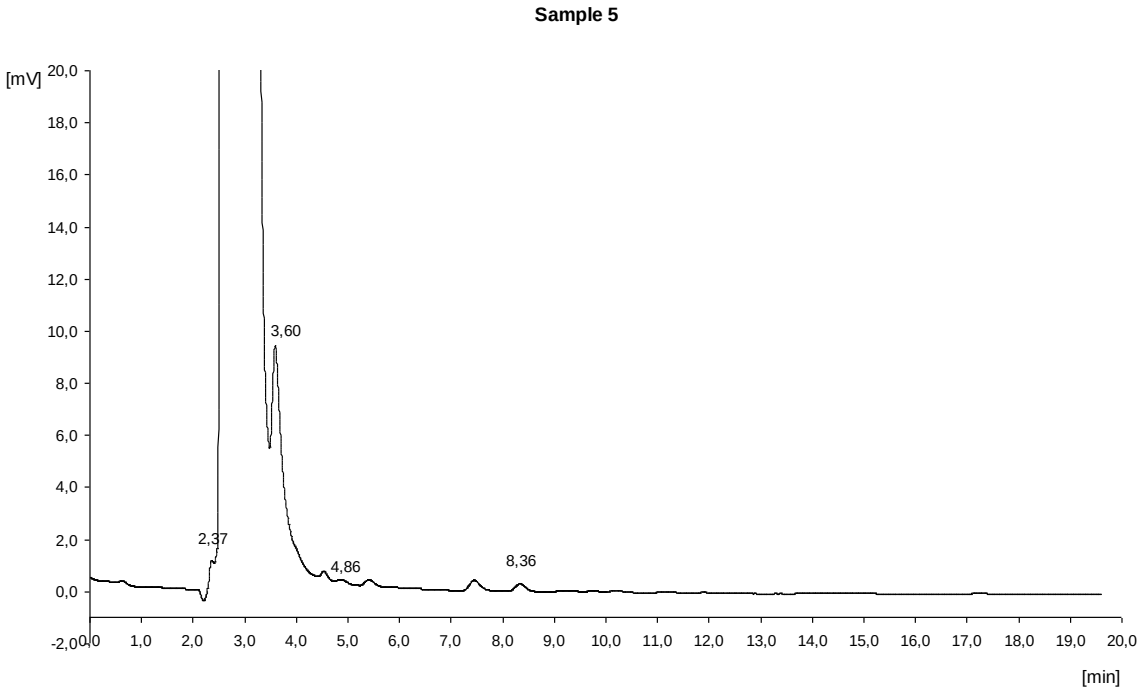

Fig. 8: Chromatogram of sample 5

Table 7: Acids that were recognized in the chromatogram of sample 5

T_r (min)	**The found acids**
2.37	oxalic
2.68	tartaric
3.60	*iso*-citric
4.86	fumaric
8.36	*trans*-aconitic

These chromatograms shown on figures 4 – 8 were compared to one another and with regard to literature it was proposed that oxalic and tartaric acid and maybe malic and acetic acid are present in the exudates. This supposition was proved by HPLC with diode-array detector (see Chapter 6.2).

After that, nutrition solution and then inorganic ions (nitrate, phosphate, and sulphate) were injected to find out, if the peaks of the inorganic anions do not coincide with the peaks of the organic acids. (Fig. 9)

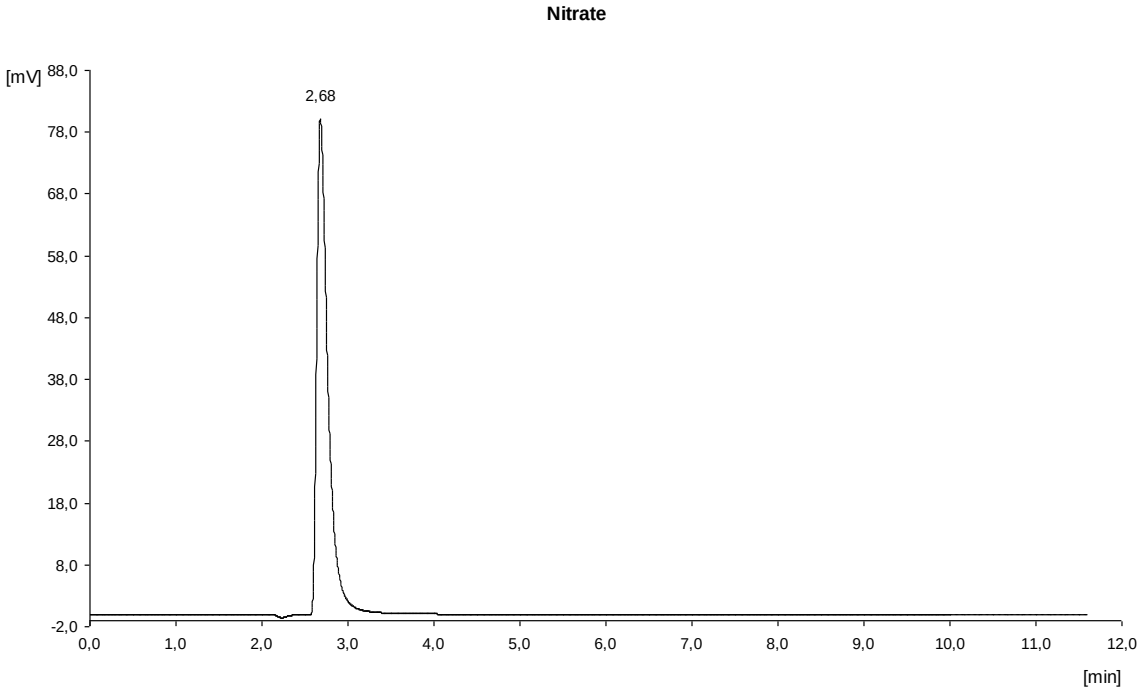

Fig. 9: Chromatogram of nitrate

After these experiments possible use of another two columns was examined. These columns were ProntoSIL 250 x 3 mm, Bischoff and PLRP-S 250 x 4.6 mm, Polymer Laboratories. Analyses of samples 1 and 4 were done with these columns and on basis of these measurement ProntoSIL column was used for analyses on HPLC system with diode-array detector.

6.2. HPLC system No 2 with column ProntoSIL 250 x 3 mm, Bischoff

6.2.1. Analyses without and with gradient elution

During work with HPLC system No 2, there was possibility to use also gradient elution. The mixture of acids (standards) (Fig. 10, Table 8), individually the standard acids supposed to be in the exudates (Table 9) and

samples 1 and 4 were measured again because of similar chromatograms of samples 1 and 2, resp. chromatograms of samples 3, 4 and 5.

The mixture was analysed with ProntoSIL and Dionex column too. Because of better resolution confirmed, we continued to work with ProtoSIL column.

Both gradient and isocratic elutions were employed. Results of the work without gradient differed not much from the results of the work with the gradient. (Fig. 10, Table 8) The gradient for analysis of mixture was:

10 min. 100% H_3PO_4

10 min. 0 – 100% of methanol

10 min. 100% of methanol

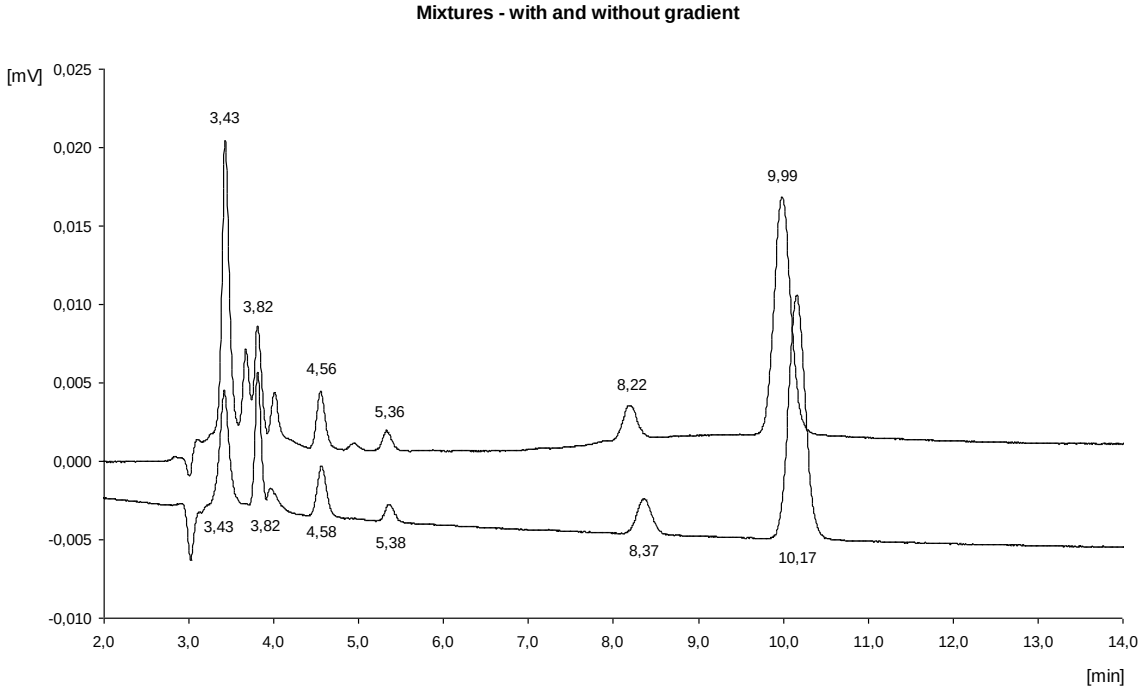

Fig. 10: The comparison of analysis of mixture without (upper line) and with (lower line) gradient

Table 8: Retention times (T_r) of acids in the mixture without and with gradient

	T_r (min)	
Acids	**Without** a gradient	**With** a gradient
oxalic	3.43	3.43
tartaric	3.82	3.82
malic	4.56	4.58
acetic	5.36	5.38
citric	8.22	8.37
fumaric	9.99	10.17

Table 9: Retention times (T_r) of individual acids without and with gradient

	T_r (min)	
The injected acids	**Without** a gradient	**With** a gradient
oxalic	3.49	
tartaric	3.87	
malic	4.73	4.71
acetic	5.45	
citric	9.14	9.17

Following figures (Fig. 11, 12) are chromatograms from work with samples 1 and 4 without gradient, table (Table 10) with acids that were recognized in the chromatograms of the samples 1 and 4, analyzed without gradient, follows.

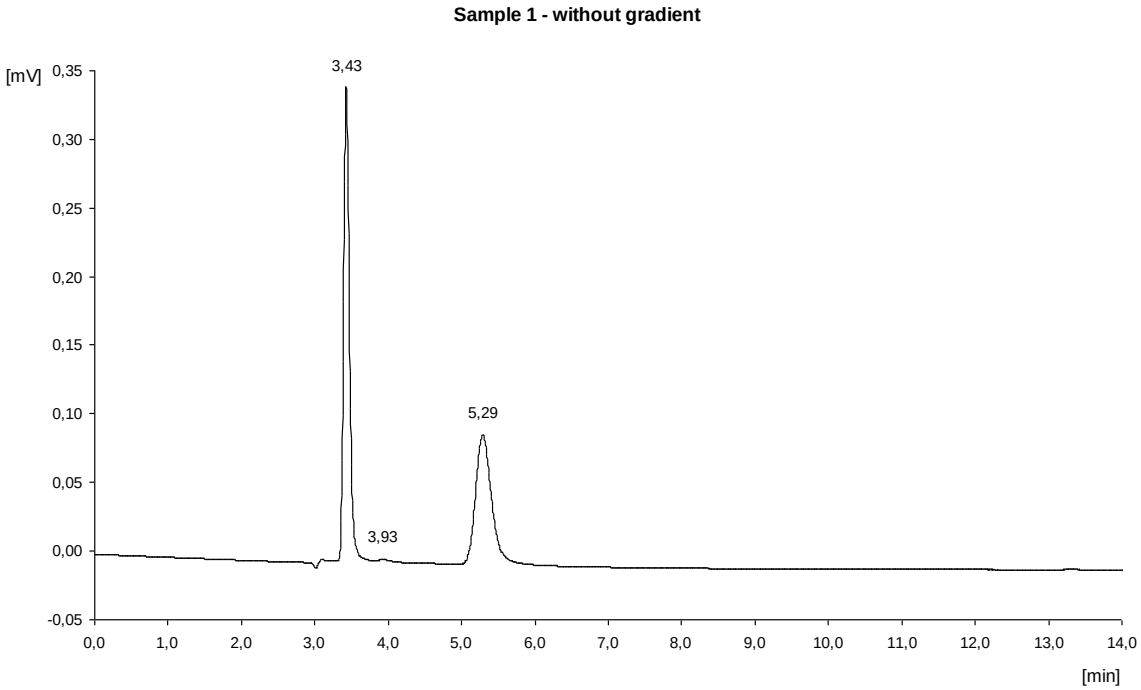

Fig. 11: Chromatogram of the sample 1 – without gradient

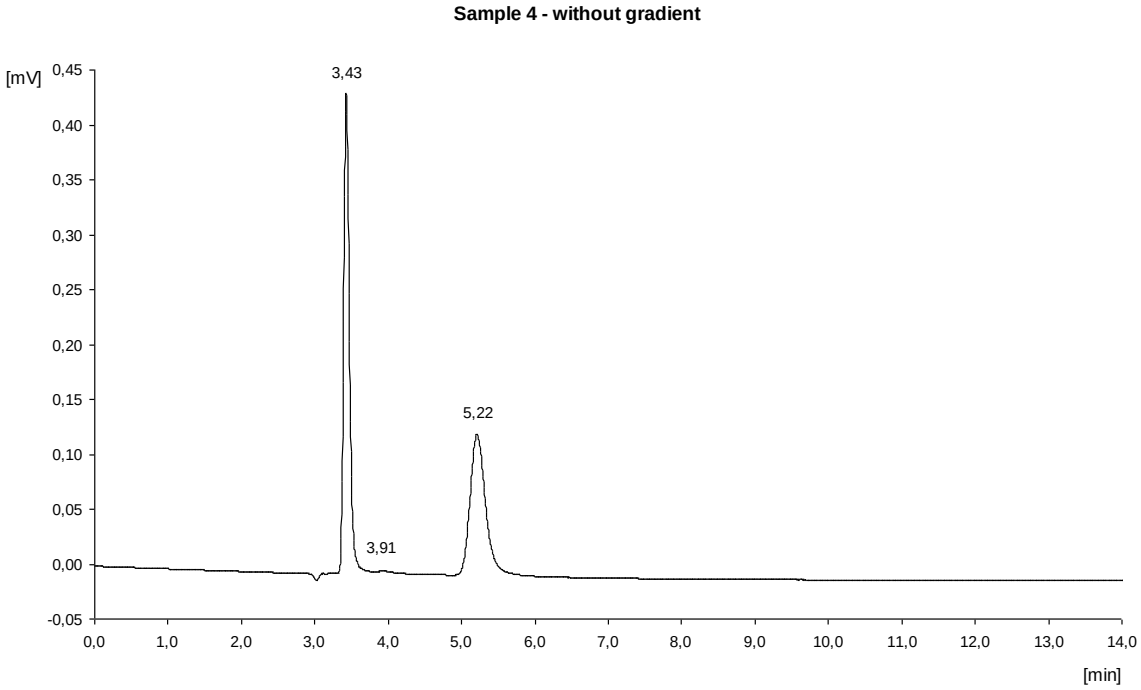

Fig. 12: Chromatogram of the sample 4 – without gradient

Table 10: Acids that were recognized in the chromatograms of the samples 1 and 4 – without gradient

T_r (min)		
Sample 01	**Sample 04**	**The found acids**
3.43	3.43	oxalic
3.93	3.91	tartaric
5.29	5.22	acetic

Following figures (Fig. 13, 14) are chromatograms from work with samples 1 and 4 with gradient, table (Table 11) with acids that were recognized in the chromatograms of the samples 1 and 4, analyzed with gradient, follows.

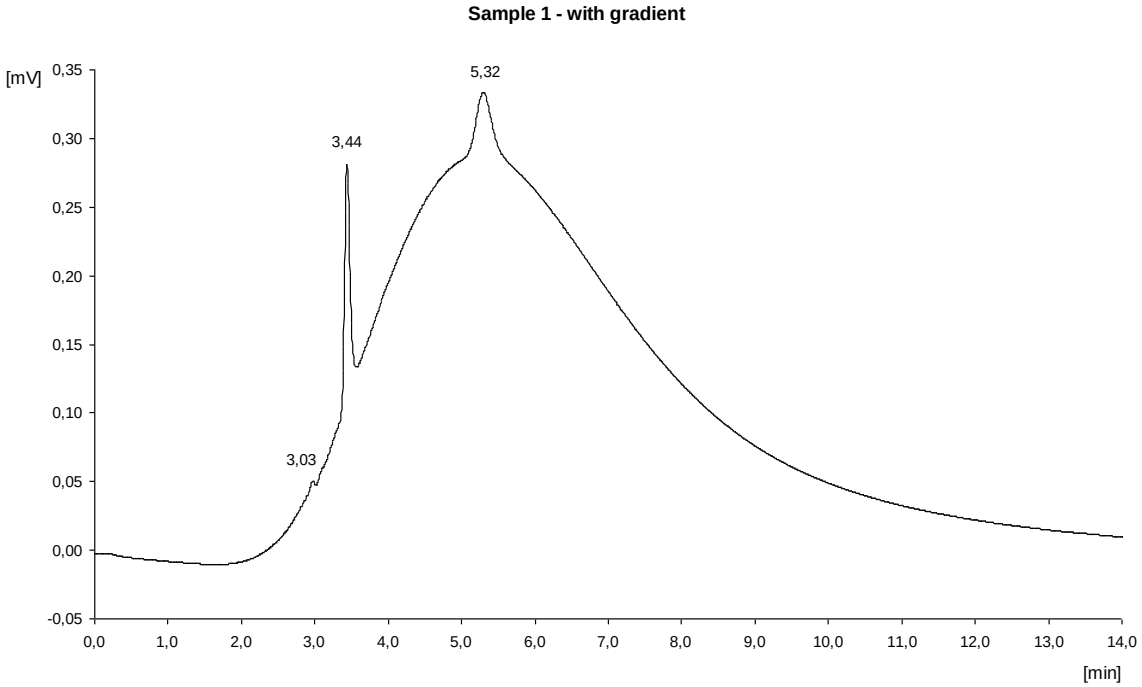

Fig. 13: Chromatogram of the sample 1 – with gradient

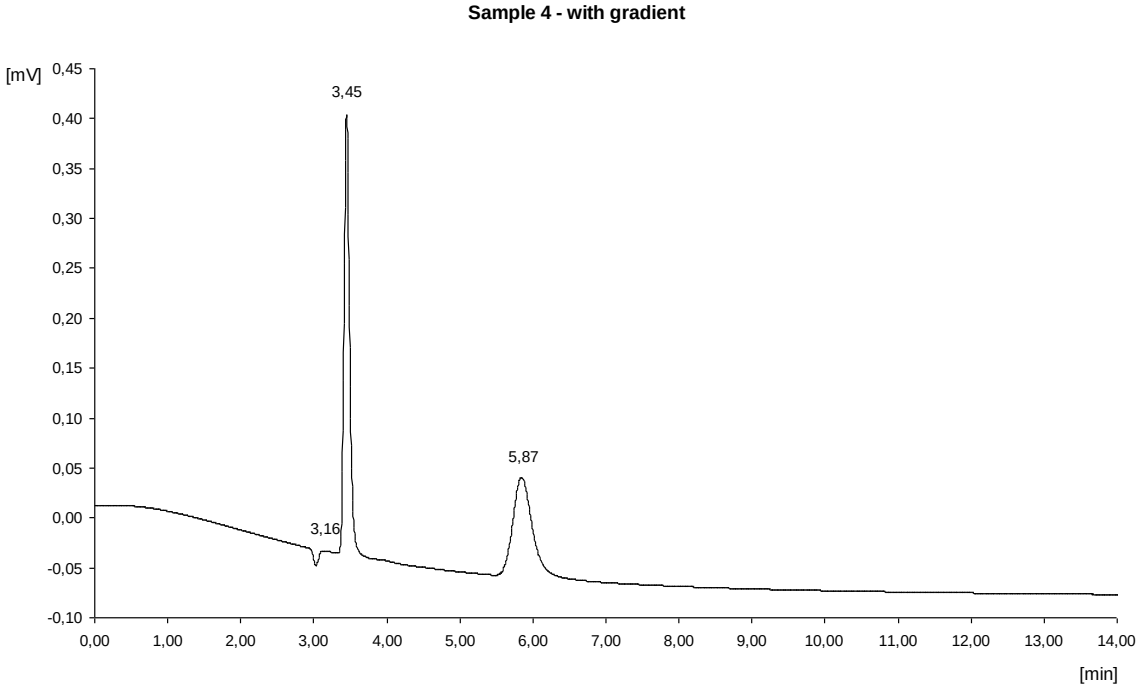

Fig. 14: Chromatogram of the sample 4 – with gradient

Table 11: Acids that were recognized in the chromatograms of the samples 1 and 4 – with gradient

T_r (min)		
Sample 01	**Sample 04**	**The found acids**
2.97	3.13	oxalic
3.44	3.45	tartaric
5.30	5.85	acetic

6.2.2. Utilization of purity spectra

There was a possibility to see also the purity spectra on the apparatus. We decided to study the spectra of the peaks obtained without gradient. But these spectra did not comply with the presumption about the content of organic acids in the exudates because the purity spectra of peaks of expected acids in samples 1 and 4 were not identical with purity spectra of peaks of the standard acids. (Fig. 15 - 16)

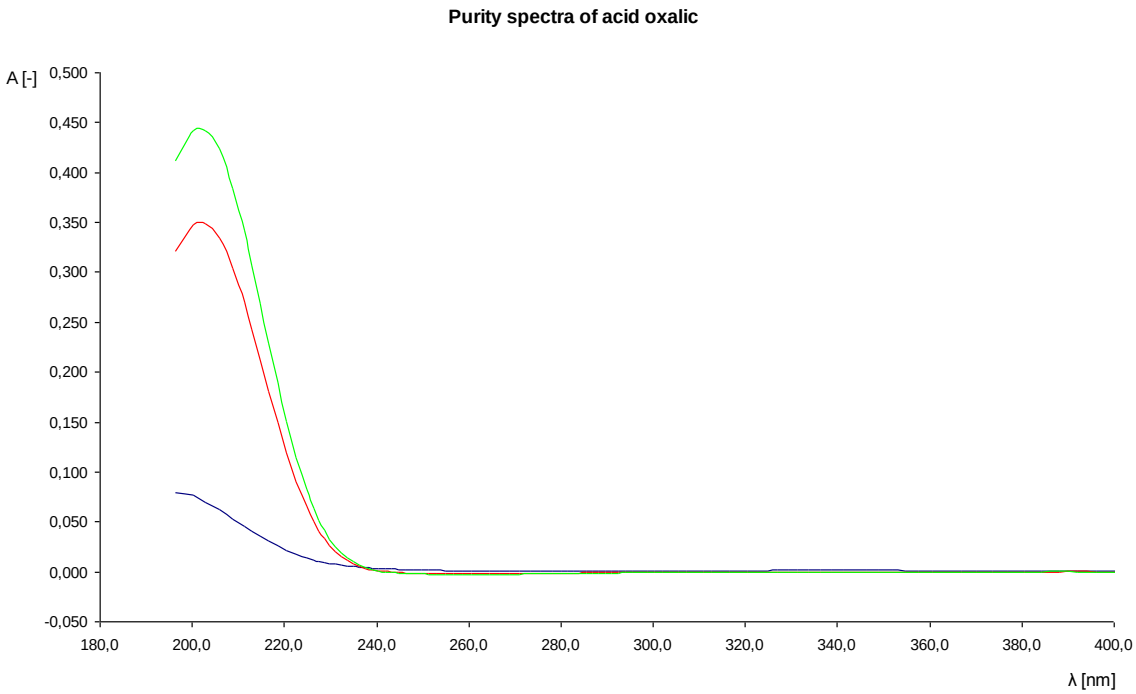

Fig. 15: Purity spectra of peaks of oxalic acid as standard (blue line) and in samples 1 (red line) and 4 (green line)

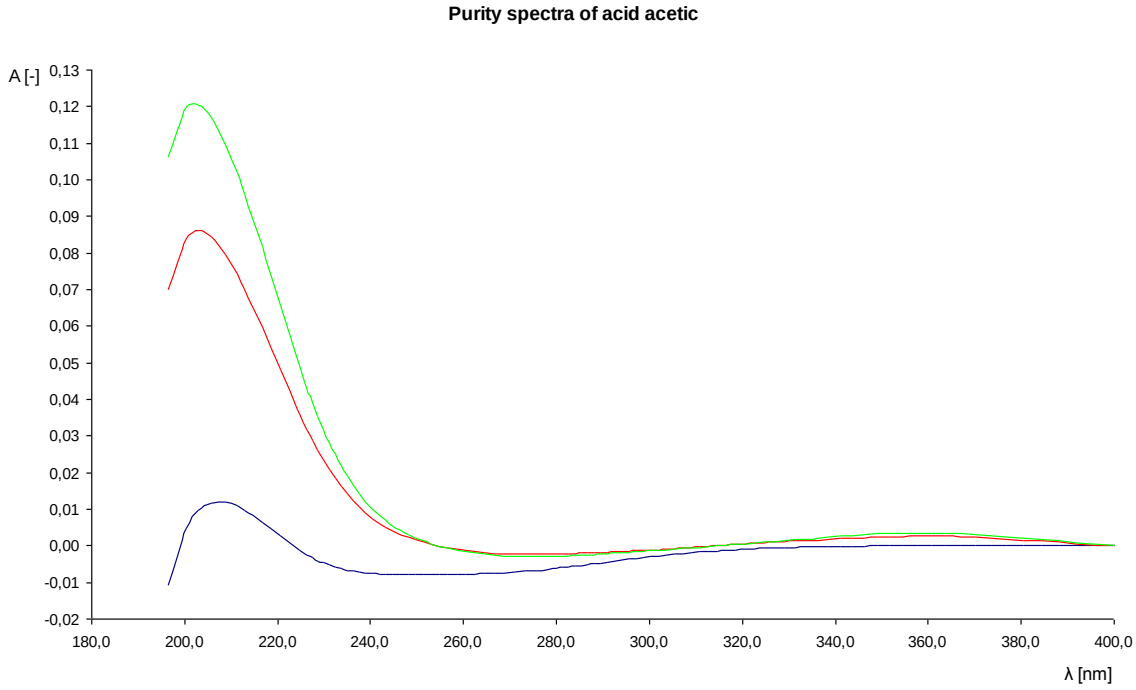

Fig. 16: Purity spectra of peaks of acetic acid as standard (blue line) and in samples 1 (red line) and 4 (green line)

Our conclusion is that we did not find any of examined organic acids in our root exudates.

7. CONCLUSIONS

Root exudates from germinated English grass seeds were analyzed by HPLC during the work on this diploma thesis. These measurements were done to test the method for the doctorate thesis called „Eco-dynamics and determination of metal elements in continuum soil – plant“. One of the objectives of this doctorate thesis is to specify methods for determination of amount of exuded organic acids and amino acids by HPLC and by ion chromatography.

Our conclusion is that we did not find any of examined organic acids in our root exudates. The reason can be insufficient rapidity of manipulation with root exudates or unsuitable storage.

8. ABSTRACT

Root exudates from germinated English grass seeds were analyzed by HPLC with UV detection (210 nm) in the first part of this thesis. Column Acclaim OA 4 x 150 mm, Dionex was used, temperature was ambient and as a mobile phase served 100 mM sodium sulphate (pH 2.65) with flow rate 0.6 ml/min. Injection volume was 40 μl, the measurement run under isocratic conditions. Standards were solutions of oxalic, tartaric, formic, malic, *iso*-citric, lactic, acetic, citric, succinic, fumaric, *cis*-aconitic and *trans*-aconitic acid and their mixture. Chromatograms from these measurements were compared to one another and with regard to literature it was gathered from, that oxalic and tartaric acid and maybe malic and acetic acid is present in the exudates. This supposition was proved by HPLC with diode-array detector.

The second part of this work consisted in analyses of samples of root exudates by means of HPLC with diode-array detection with possibility of gradient elution. Column ProntoSIL 250 x 3 mm, Bischoff was used, temperature was ambient and the mobile phase was 50 mM *ortho*-phophoric acid with flow rate 0.7 ml/min. Volume 10 μl was injected. Solutions of oxalic, tartaric, malic, acetic, citric and fumaric acids and their mixture were used as standards. Absence of any of supposed acids in samples was concluded after comparing of the chromatograms of samples and studying of purity spectra.

9. SOUHRN

V první části této práce bylo provedeno HPLC exsudátů z kořínků naklíčených semen anglického trávníku s UV detekcí při 210 nm. Při této analýze byla použita kolona Acclaim OA 4 x 150 mm, Dionex, pracovalo se za laboratorní teploty a jako mobilní fáze sloužil 100 mM síran sodný o pH 2,65 a průtoku 0,6 ml/min. Nastřikovaný objem byl 40 µl, měření proběhlo za isokratických podmínek. Standardy byly roztoky kyselin šťavelové, vinné, mravenčí, jablečné, *iso*-citrónové, mléčné, octové, citrónové, jantarové, fumarové, *cis*-akonitové a *trans*-akonitové a jejich směs. Ze srovnání chromatogramů z našich měření a s přihlédnutím k literatuře bylo usouzeno, že v exsudátech se nachází kyseliny šťavelová a vinná, příp. též jablečná a octová kyselina. Tento předpoklad byl dokazován pomocí HPLC s diode-array detektorem.

Druhá část práce spočívala v proměření vzorků exsudátů pomocí HPLC s diode-array detekcí s možností gradientové eluce. Použita byla kolona ProntoSIL 250 x 3 mm, Bischoff, pracovalo se za laboratorní teploty a jako mobilní fáze sloužila 50 mM kyselina *ortho*-fosforečná o průtoku 0,7 ml/min. Bylo nastřikováno 10 µl. Jako standardy byly použity roztoky kyselin šťavelové, vinné, jablečné, octové, citrónové a fumarové a jejich směs. Po porovnání chromatogramů vzorků a prohlédnutí spekter čistoty bylo vyhodnoceno, že žádná z předpokládaných kyselin není ve vzorcích obsažena.

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