Plant Root Exudates:

Variation between Species and Reaction to Water Deficit

Dissertation

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List of Abbreviations

Conc.	Concentration
CV	Cultivar
Da	Dalton
DAD	Diode array detector
DPV	Differential pulse voltammetry
ESI	Electrospray interface
FW	Fresh weight
GC	Gas chromatography
HPLC	High performance liquid chromatography
MS	Mass spectrum
MS agar	Muraghige-Skoog medium
MDS	Multidimensional scaling
MW	Molecular weight
PM	Primary metabolite
RP	Reversed phase
SM	Secondary metabolite
TIC	Total ion concentration
UPLC	Ultrahigh performance liquid chromatography
TOF-MS	Time-of-flight mass spectrometry
UV	Ultraviolett spectrum
v/v	Volume per volume
WD	Water deficit

Content

1. Root Exudates: Definition, Collection, Expectable Analytes, and Research Questions

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Abstract: Root exudates comprise primary and secondary plant metabolites that are exuded by roots. They oligomerize with microbial metabolites to form mucilage. Contact with soil particles then induces the formation of a slimy polymer called mucigel that covers roots. Different methods exist to recover root exudates from either soil-grown plants or hydroponic cultures. This low-molecular-weight fraction of analytes can be subjected to GC and LC analyses. The results provide a basis for asking specific questions. Their nature depends on the focus of the research, either phenotyping characterization or metabolic diversity exploration.

1.1 Introduction

Root exudates comprise a wide range of low- and high-molecular-weight organic compounds that are present in the intercellular space of root tip tissues and root hairs. They may leak either from root cells or be transported via the phloem from other tissues (Rovira *et al.*, 1983; Bertin *et al.*, 2003). The low-molecular-weight metabolites comprise primary and secondary metabolites that can vary depending on the plant species, age and the sum of all biological, physical and chemical stresses (Uren, 2007). Uren (2007) offers two recommendations for research on root exudates that were adopted also as guidelines for the research within this thesis:

(1) "Root exudates released into the soil surrounding the root have been implicated in many mechanisms for altering the level of soluble ions and molecules within the rhizosphere. However, very few have been critically evaluated" (Jones *et al.*, 1996);

(2) "Root exudation cannot be simply explained by a single mechanism but is moreover a combination of complex multidirectional fluxes operating simultaneously. While we currently possess a basic understanding of root exudation, its overall importance in plant nutrition and response to microbial pathogens and root-feeding predators remains largely

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unknown. Future research should therefore be directed at quantifying the significance of root exudates in realistic plant-soil systems" (Farrar & Jones, 2003).

Fast and reliable phenotyping represents an ultimate prerequisite for assessing and quantifying genetic versus environmental effects in plant physiological research. This also applies to root exudates. To achieve this, Kuijken *et al.* (2015) recommend to use a sterile hydroponic cultivation system. Notwithstanding of the advantages of this approach—collecting the root exudate reduces itself to analysing the nutrient solution itself; the absence of microbial degraders, amongst others—this thesis does not follow the recommendations of these authors. Instead, the preferred method is soaking tap-water-rinsed roots of soil-grown plants in distilled water for several hours. For clarification of this decision, the ongoing text will review of the general terms that are used in root exudate research, the collection methods, and the low-molecular-weight analytes that are detectable in root exudates by gas chromatography (GC–MS) and liquid chromatography (LC–DAD, UPLC–ESI/TOF MS).

1.2 Rhizosphere and rhizodeposition

The term rhizosphere was first coined by the German scientist Lorenz Hiltner (1904). It denotes that volume of soil that is affected by plant root metabolic activities (Bertin *et al.*, 2003). The rhizosphere (Figure 1.1) represents a complex environment that results from multiple physical and chemical interactions of plant roots on one hand and fungi, bacteria, numerous members of the soil fauna as well as humic and clay soil particles on the other hand (Singer & Munns, 2006; Pierret *et al.*, 2007).

The term rhizodeposition specifies the exudation of low- and high-molecular-weight metabolites from plants via their roots during their lifetime, altogether up to 17 % of the photosynthetically fixed carbon (Nguyen, 2003). The reported amount, however, can vary depending on the author; maximum values range around 30 %.

Direct specific effects on soil microbial communities are difficult to assess, however, because the originally exuded chemical structures are modified usually by diverse biotically and even abiotically monitored chemical reactions (Dennis *et al.*, 2010). Root-exuded metabolites have to be more or less water-soluble and usually include sugars, amino- and organic acids from the central metabolism; furthermore, variable amounts of secondary metabolites can be present (Uren, 2007; van Dam & Bouwmeester, 2016). Secondary plant metabolites, or specialized metabolites are assumed to either contribute to efficacy in nutrient uptake by

mobilization or direct coordination complex formation of micronutrients (Cesco *et al.*, 2010; Mimmo *et al.*, 2014) or affect microbial community structure in the rhizosphere (Scheffknecht *et al.*, 2006; Hartmann *et al.*, 2009; Philippot *et al.*, 2013). Root density, species identity, plant age and environmental stress can affect the quality and quantity of root exudation (Neumann & Römheld, 2007).

1.3 Root mucilage and mucigel

Root mucilage represents a high-molecular-weight gelatinous layer that forms on the root surface (Figure 1.1). It incorporates plant root exudates as well as other low-molecular-weight compounds of either microbial or abiotic oxidative origin. In terms of chemistry, its structure is often reported as polysaccharide, but more detailed studies have revealed the presence of amino acids, sugar and sugar acid units in the polymer, most of which are of plant origin (Moody *et al.*, 1988; McNear, 2013; Vranova *et al.*, 2013). By contrast, seed mucilage is thought to be made up predominately by pectic polysaccharides (Willats *et al.*, 2001).

Rovira *et al.* (1983) categorized mucilages into four different classes depending on their source:

(1) Root cap Golgi vesicles

(2) Hydrolyzation of polysaccharide-rich primary cell walls of sloughed root cap cells

(3) Epidermal cells (including root hairs)

(4) Bacterial degradation of primary cell walls of old, dead epidermal cells

Strictly speaking, mucilages represent polymers that are formed by precursor molecules of plant and microbial origin exclusively. This applies to studies in hydroponic cultures. But if plant roots come into contact with non-sterile soil, the term mucigel is recommended (Jenny & Grossenbacher, 1963). Together with soil organic matter, plant mucilage forms a colloid that is characterized by distinctive morphological, physical and chemical properties. The chemical composition of mucilage is known to differ between plants (Moody *et al.*, 1988). Depending on the soil type, the same plant species probably will form a specific mucigel depending on the soil type.

The general view is that the mucigel layer on the root surface acts (1) as a lubricant in the soil environment supporting root elongation and radial expansion (Morel *et al.*, 1991; Hawes *et al.*, 2002) and (2) as nutritional niche for specific communities of bacteria and fungi (Philippot

et al., 2013). For the first benefit, the presence of border cells that are associated with the root apex seems is regarded to be essential though there exist considerable differences between plant species in terms of their numbers; Brassicaceae, including Arabidopsis, even lack those (Hawes *et al.*, 2002). The mechanical impedance of the soil particles enhances the secretory activity of border cells (Iijima & Kono, 1992). Root exudation, in turn, can reduce the mechanical strength of soil in proximity of the root tip (Whiteley, 1989). Additionally, the forming mucigel was suggested to increase the water-holding capacity of the rhizosphere soil and might protect the root against desiccation (Young, 1995). Other authors, by contrast, suggested only an indirect role for the mucigel (McCully & Boyer, 1997). More recent studies with neutron radiography confirmed that the rhizosphere soil of lupines at least contains more water than the surrounding bulk soil (Carminati *et al.*, 2010).

Phosphatidylcholines of predominantly saturated fatty acids represent important lipid root mucigel components that alter the interactions of soil solids with water and most certainly affect microbial activities in the rhizosphere; furthermore, they can mobilize phosphate efficiently and generally allow plants to draw water from smaller pores than they could access otherwise (Read *et al.*, 2003).

1.4 Commonly applied root exudate collection methods

No perfect method for the collection of roots exudates exists. The debate ranges from sterile hydroponic cultivation systems for root exudate phenotyping purposes (Kuijken *et al.*, 2015). to non-sterile modifications of rhizobox setups (Oburger *et al.*, 2013). The most widely used hydroponic system consists of plants that are germinated in small plastic vials (PCR or culture) or pipette tips filled with nutrient agar (< 200 µL), of which the bottom was cut after solidification of the agar. The vials are then put into pipette boxes with nutrient solution. Nutrient compositions follow recommendations for the Murashige-Skoog (MS) medium (Murashige & Skoog, 1962). After germination and development of roots (usually 3 weeks) the plantlets are transferred each into 50 mL glass bottles filled with nutrient solution and perforated screw caps to accommodate the pipette tip or vial. For several weeks the nutrient solutions are exchanged weekly and combined for further analysis (Kuijken *et al.*, 2015; Mönchgesang *et al.*, 2016). Figure 1.2 illustrates a setup of hydroponic root exudate collection that is used by Kujiken and co-workers (2015). Roots are in a sterile and shoots in a non-sterile environment.

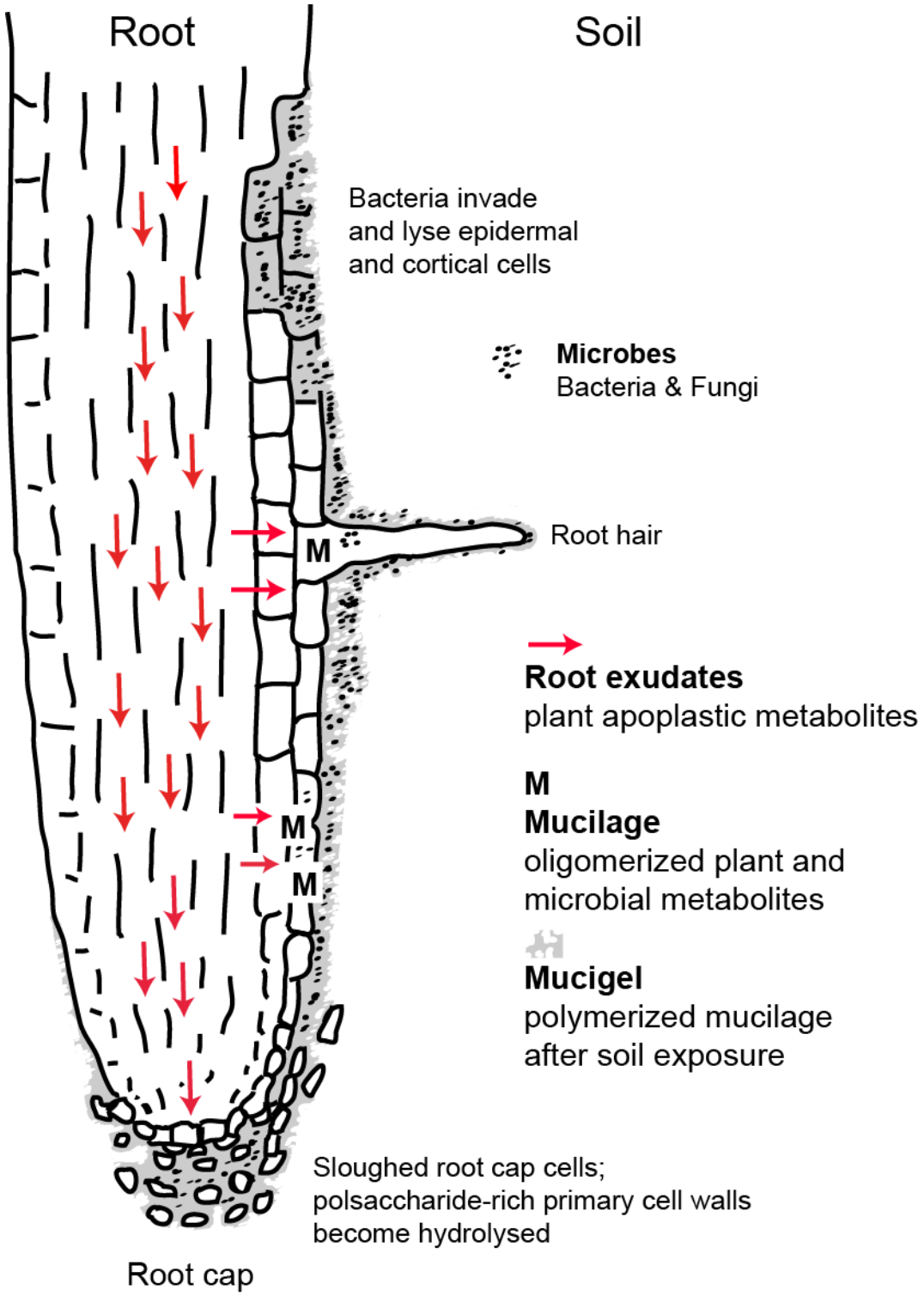

Figure 1.1. Origin of organic materials in the rhizosphere

Rhizobox setups represent the other extreme (Wenzel *et al.*, 2001) with specific modifications to collect root exudates (Oburger *et al.*, 2013) as illustrated by Figure 3. Micro-suction cups allow determining concentration gradients by in situ sampling in the rhizosphere soil compartment and in a soil-free compartment (Figure 1.3).

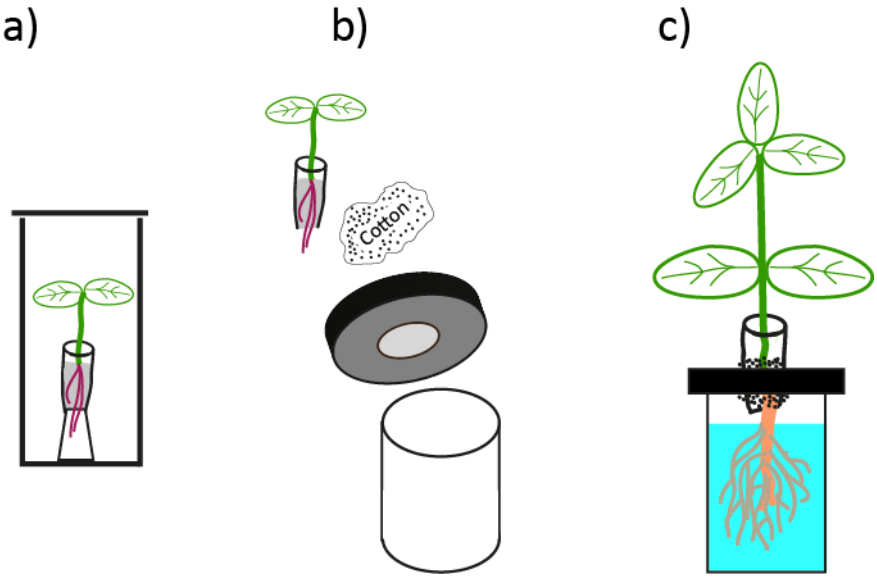

Figure 1.2. Sterile hydroponic root exudate collection setup: (a) germination in sterile culture tubes with cut-off bottom containing MS medium; (b) seedling transfer into a sterile container; (c) root exudate collection of developed plant.

Classically, root exudates are sampled from soil-grown plants. The soil is carefully removed and the roots are rinsed with tap water, the roots are transferred into a beaker or flask with distilled water, in which the plants remain for time periods of up to 24 h, and after which it is filtered to remove soil particles (Steinkellner *et al.*, 2008). Figure 1.4 illustrates the procedure that also was performed throughout this thesis.

1.5 Common analytes in root exudates and possible functions

This exemplary survey will exclusively focus on primary and secondary plant metabolites that are detectable with GC and LC methods. Microbial metabolites, by contrast, are produced in much lower quantities and only in rare cases detections were successful, e.g. for 2,4-diacetylphloroglucinol from rhizosphere-colonizing *Pseudomonas* ssp (Bonsall *et al.*, 1997).

The most prevalent analytes are primary metabolites, sugars, mainly monosaccharides, sugar alcohols, organic- and amino acids (Vranova *et al.*, 2013). Figure 1.5 presents selected structures, all of which have been detected also in the analyses of the present thesis. The methylated derivative of citramalic acid deserves special mentioning because it was detected in the rhizosphere of *Beta vulgaris* in an investigations carried out in the same lab as this thesis (Khorassani *et al.*, 2011). One detail question of the thesis was to explore to what extent this rather unusual organic acid, which is not involved in the citric acid cycle, can be detected in the root exudates of other plant species. Lactic acid, by contrast, is a well-known bacterial

fermentation product, an occasional metabolite of some yeasts and mould fungi, and a glycolysis product in oxygen-deficient muscle tissues (Lang & Gänzle, 2011).

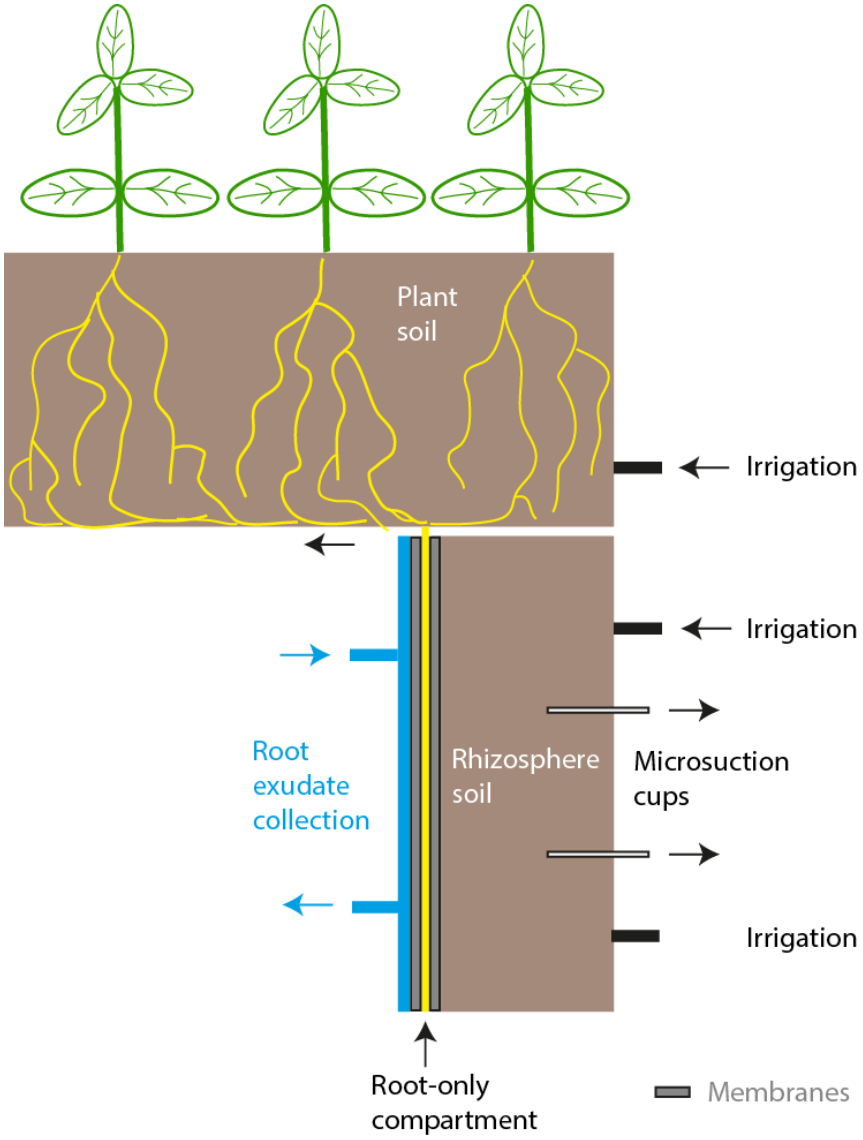

Figure 1.3. A rhizobox setup allows simultaneous root exudate and soil water collection (with the help of microsuction cups) from the identical plant accession. This, however, requires the incorporation artificial membranes.

In much lower quantities, secondary plant metabolites have been detected in root exudates. Figure 1.6 presents selected examples for often-mentioned compounds. The exudation of scopoletin and other coumarins contribute to improved Fe(III) uptake of Arabidopsis roots (Schmid *et al.*, 2014; Ziegler *et al.*, 2016). Kaempferol represents just one example of flavonoids that are exuded by members of the plant family Fabaceae and assist in attracting root nodule forming symbiotic bacteria (Steele *et al.*, 1999). Within the same family a specific structural type of flavonoids occurs, isoflavones, which contribute to the same activity; Pisatin is a characteristic isoflavone of pea (Makarova *et al.*, 2016). Phenolic acids, such as p-

hydroxybenzoic acid, have been especially focused on in studies of rice root exudates (Seal *et al.*, 2004) and probably occur in the root exudates of many other grass species as well. Cinnamic acid represents another probably even more widespread phenolic acid, or more specifically a phenylpropanoid, that can occur in root exudates and was even detected in those of Arabidopsis (Strehmel *et al.*, 2014). The hitherto mentioned plant secondary metabolites all classify as phenols but non-phenolic metabolites can occur also in root exudates. A very well investigated group of non-proteinaceous amino acids represent mugineic acid and its derivatives that, similarly as the exuded coumarins for Arabidopsis, facilitate iron uptake by barley roots by formation of Fe(III) coordination complexes (Tsednee *et al.*, 2012). A derivative of an aromatic amino acid, tryptophan, is also reported from the root exudates of several grass species (Friebe *et al.*, 1995). Strigolactones, such as strigol, were initially identified as germination stimulants of the parasitic weed *Striga hermonthica* and believed to represent sesquiterpene structures; later studies, however reveals that they induce the colonization of plant roots by beneficial arbuscular-mycorrhizal fungi and actually are apocarotenoids (Akiyama *et al.*, 2005). *Sorghum* root hairs excrete sorgolenone is a resorcinol derivative, a phenolic fatty acid derivative despite its quinone moiety, which is assumed to be responsible for soil sickness that is caused by this grass species through allelopathic activity (Dayan *et al.*, 2010). The sulphur-containing a-terthienyl actually represents another fatty acid derivative, a thiophene polyacetylene, which raised attention due to its nematicidal activity, from which its producer, various *Tagetes* ssp. (marigold) benefits (Weidenhamer *et al.*, 2009).

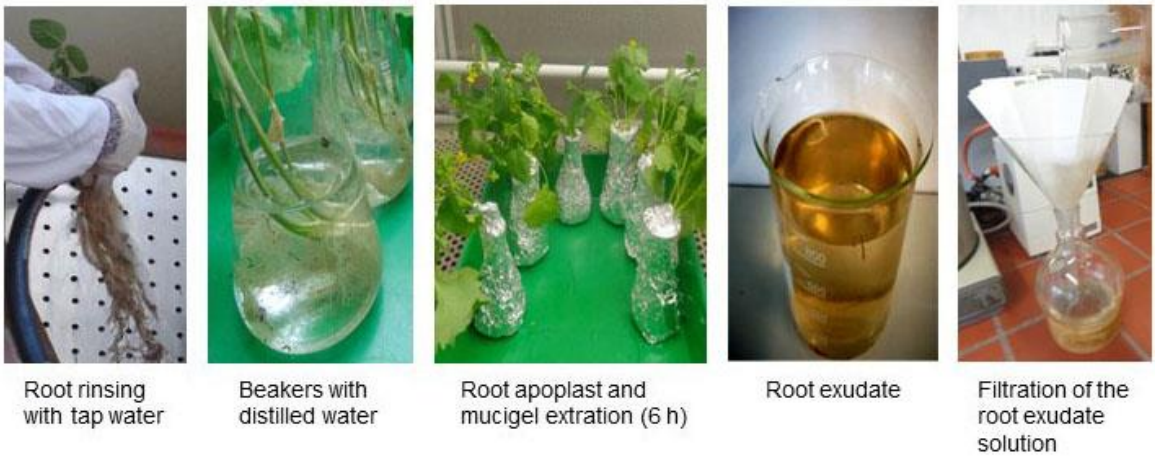

Figure 1.4. Direct root exudate recovery procedure from soil-grown plant

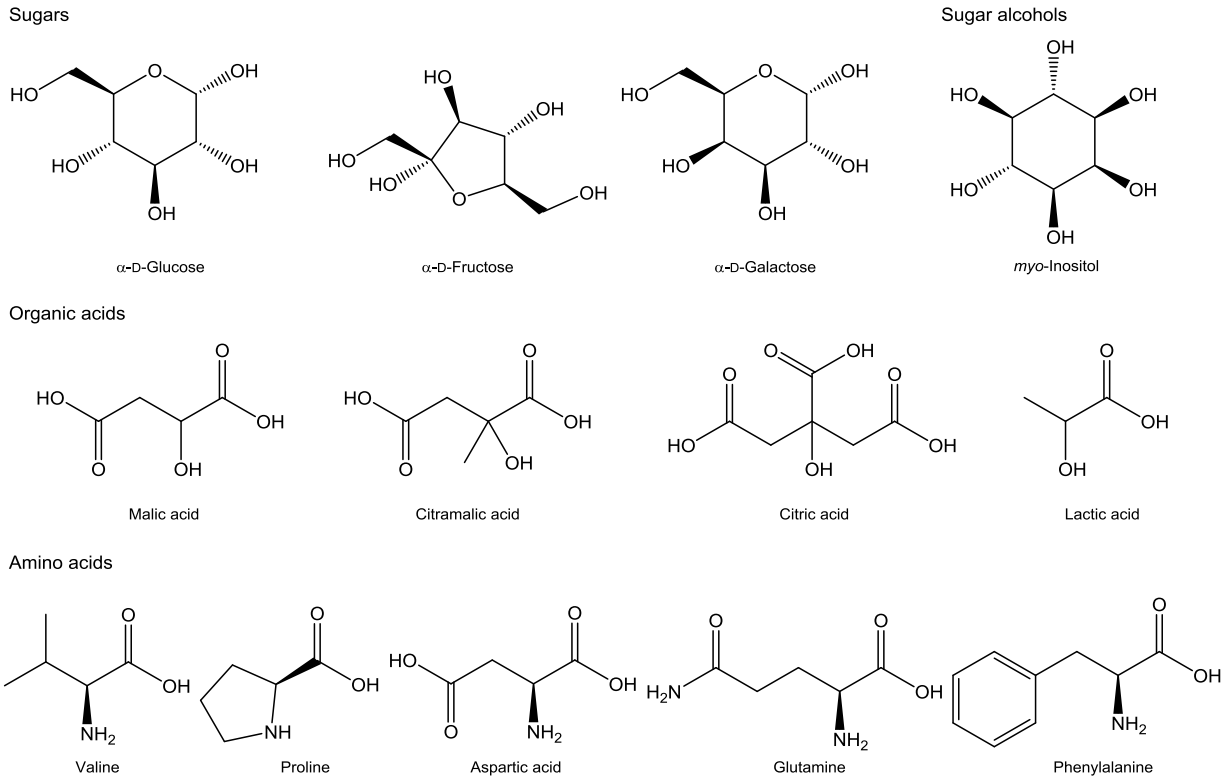

Figure 1.5. Primary, or as recently more often called, central metabolites from plant root exudates (by the majority, lactic acid is most probably of microbial origin).

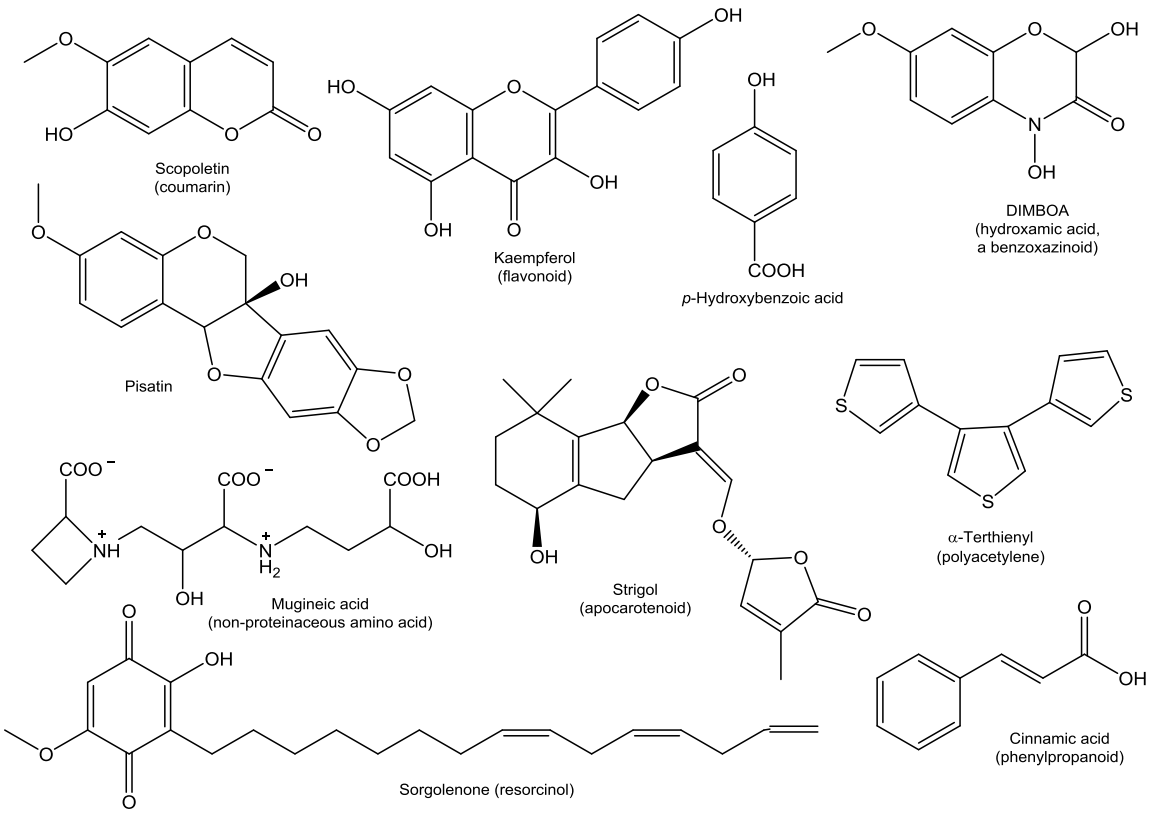

Figure 1.6. Selected secondary, or specialized, plant metabolites from root exudates (for more information see text)

The potential benefits of root exudation fall into two categories: (1) chemical defence and information and (2) nutrient mobilization. The first category comprise several types of biotic interactions (van Dam & Bouwmeester, 2016): effects on insect herbivores and their natural enemies, phytophagous nematodes, plant–plant communication, and plant–plant communication. In terms how these interactions actually function in complex soil environments, many issues are unclear so that most conclusions have to remain rather speculative.

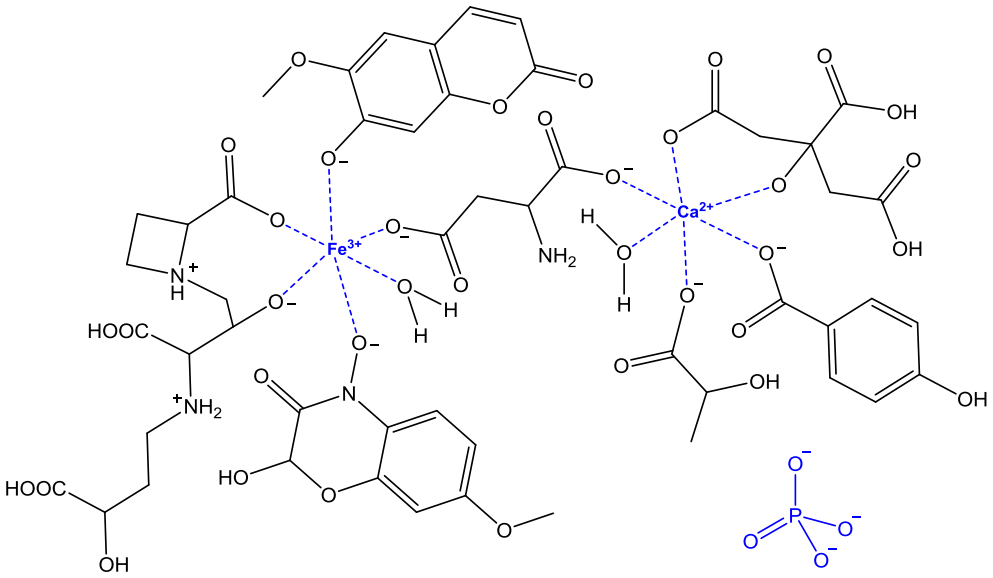

Figure 1.7**.** Putative coordination complex scenario with primary plant metabolites from Figure 1.5 and secondary plant metabolites from Figure 1.6 as ligand examples. This chemistry facilitates either mobilization of nutrient anions or uptake of cations with low water solubility.

By contrast, more evidence that is substantial exists that root exudate components, both primary and secondary metabolites, can contribute to mobilization and uptake of phosphorus and various mineral nutrients. Iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) are important enzyme cofactors that rely on coordination complex formation with low-molecular-weight organic ligands as take-up mechanism (Williams & Fraústo da Silva, 2006; Crichton, 2008; Marschner, 2012; Williams & Rickaby, 2012). Coordination complex formation of organic acids of Ca^{2+} ions, especially citric acid, has been pointed out as one important mobilization mechanism of the essential plant nutrient phosphorus (Gerke, 2015). Figure 1.7 presents a putative scenario in which plant primary metabolites from Figure 1.5 and plant secondary metabolites from Figure 6 act as ligands in coordination complexes with Fe^{3+} and Ca^{2+} ac central atoms. The formation of the latter coordination complex may produce free phosphate ions for uptake by plant roots. In reality, most probably, such coordination

complexes not only contain ligands of plant metabolite origin but also bacterial and fungal metabolites as well as low-molecular-weight oxidation products from humic acids.

1.6 What we see and what we can't see in root exudate analysis

The term "root exudates" designates plant metabolites that have passively leached out or actively been secreted from root tissues. Already in the apoplast of root tissues, mucilage forms and on the root surface, which comes into contact with soil organic matter, clay minerals and various prokaryotic and eukaryotic soil organisms, a specific mucigel forms (Rovira *et al.*, 1983). Both mucilage and mucigel are predominately made up of polymers that are elusive to standard GC and HPLC analyses techniques. Consequently, the major portion of detectable analytes predominantly have to be unmodified plant metabolites. Oligomerization products of both primary and secondary metabolites have especially been found in studies in which HPLC– or UPLC–TOF/MS was used as analysis method (Strehmel *et al.*, 2014; Mönchgesang *et al.*, 2016). By contrast, GC, which is routinely used for metabolic profiling of primary metabolites,—an extensive library of electron impact MS spectra of nearly all known plant primary metabolites is available in the public domain (Kopka *et al.*, 2005)—is limited in detecting higher-molecular-weight molecules because their limited volatility often prevents analysis. Furthermore, another fact can possibly contribute to reduced detectability of reaction products outside of the cell. In contrast to their original site of biosynthesis, in which the chemical reactions are catalysed by a tightly coordinated enzymatic machinery, possible chemical modifications in the apoplast are less controlled and occur more in the fashion of oxidative decomposition of organic material in the soil that results in the formation of fulvic and humic acids (Stevenson, 1994). If we assume that we have an analyte with a molecular weight of approx. 300, ≈ 50 ng correspond to hundred trillion (10^{14}) molecules (Meinwald, 2003). If the majority of analytes range in amounts below this threshold, detection by chromatographic methods becomes difficult to impossible and only direct infusion in a Fourier transform ion cyclotron resonance mass spectrometer (FTICR/MS) can still provide information to some extent. One disadvantage of chemical ionization is that, besides of the analyte itself, it usually contains also various adducts of the analyte and each analyte can be the parent ion of further fragments. This varies from analyte to analyte. If chromatography is possible, the spectra are clearer in terms of which fragments belong the same analyte because they contain less complex fragment patterns. Two papers illustrate this problem: the first one reviews the analysis of marine dissolved organic matter that might reflect a similar chemical

reaction system as it might exist in the apoplast (Dittmar & Paeng, 2009), the second provides an extensive analysis of tea fermentation products with classic chromatorgraphic and high resolution MS methods (Kuhnert *et al.*, 2010). In addition, many plant metabolites can serve as ligands in coordination complexes with metals as central atoms, which creates a huge structural diversity that is nearly impossible to analyse (Fan *et al.*, 1997), so far, coordination complexes have detected by LC–ESI–TOF/MS in simplified hydroponically recovered root exudates (Tsednee *et al.*, 2012).

One often mentioned drawback of root exudate analysis is the fact that it requires rather huge amounts of water in comparison to the low amounts of analytes. This disadvantage, however, might represent an advantage because not only analytes but also their potential microbial degraders become highly diluted during the procedure. Both methods, collection from hydroponic cultures or from soil-grown plants predominantly yield complex mixtures of primary and secondary plant metabolites when analysed with chromatographic methods (Tawaraya *et al.*, 2013; Vranova *et al.*, 2013; Strehmel *et al.*, 2014; Mönchgesang *et al.*, 2016). The previous paragraph attempts to explain why we do not see much more. There exists, however, a fundamental difference between hydroponic cultures and soil.-grown plants, which may not be evident at first glance:

(1) The root apoplast of the soil-grown plants is extracted only for a short-time period whereas hydroponically grown plants are confronted with continuous apoplast extraction for a considerable portion of their life period;

(2) The potential stress that may be caused by the constant extraction of the apoplast could be mitigated by the provision of ionic nutrient solutions and artificial coordination complexes of weakly water-soluble iron, e.g. Fe.

1.7 Research questions

Six plant species were chosen as model plants on basis of their status as crop plant and tolerance of the conditions in the available climate chamber: Arabidopsis, Rapeseed, Phaseolus, Pisum, Tobacco and Maize. They were grown in identical light, humidity and nutrient supply regimes to allow addressing of the following questions under controlled conditions:

(1) Do root exudates differ between plant species in terms of primary and secondary metabolite profiles?

(2) Do root exudates differ generally from root tissue extracts?

(3) Is collection by soaking of cleaned soil-grown roots in distilled water an efficient method to obtain specific root exudate metabolites?

(4) Does water deficit as abiotic stress affect root exudation?

(5) Do root-exuded metabolites correlate with nutrient concentrations in leaves?

1.8 References

Ahuja, I, Kissen, R, Bones, AM. 2012. Phytoalexins in defense against pathogens. *Trends in Plant Science* **17**: 73–90.

Akiyama, K, Matsuzaki, K, Hayashi, H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**: 824–827.

Albalasmeh, AA, Ghezzehei, TA. 2014. Interplay between soil drying and root exudation in rhizosheath development. *Plant and Soil* **374**: 739–751.

Atkinson, J, Morand, P, Arnason, JT, Niemeyer, HM, Bravo, HR. 1991. Analogs of the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA): decomposition to benzoxazolinones and reaction with β-mercaptoethanol. *Journal of Organic Chemistry* **56**: 1788–1800.

Badri, DV, Vivanco, JM. 2009. Regulation and function of root exudates. *Plant, Cell and Environment* **32**: 666–681.

Bar-Even, A, Flamholz, A, Noor, E, Milo, R. 2012. Rethinking glycolysis: on the biochemical logic of metabolic pathways. *Nature Chemical Biology* **8**: 509–517.

Baumert, A, Milkowski, C, Schmidt, J, Nimtz, M, Wray, V, Strack, D. 2005. Formation of a complex pattern of sinapate esters in *Brassica napus* seeds, catalyzed by enzymes of a serine carboxypeptidase-like acyltransferase family? *Phytochemistry* **66**: 1334–1345.

Bertin, C, Yang, XH, Weston, LA. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil* **256**: 67–83.

Betina, V. 1995. Differentiation and secondary metabolism in some prokaryotes and fungi. *Folia Microbiologica* **40**: 51–67.

Blair, AC, Nissen, SJ, Brunk, GR, Hufbauer, RA. 2006. A lack of evidence for an ecological role of the putative allelochemical (+/-)-catechin in spotted knapweed invasion success. *Journal of Chemical Ecology* **32**: 2327–2331.

Bonsall, RF, Weller, DM, Thomashow, LS. 1997. Quantification of 2,4-diacetylphloroglucinol produced by fluorescent *Pseudomonas* spp. in vitro and in the rhizosphere of wheat. *Applied and Environmental Microbiology* **63**: 951–955.

Brett, CMA, Brett, AMO. 1993. Electrochemistry. Principles, Methods, and Applications. Oxford, New York: Oxford University Press.

Broeckling, CD, Broz, AK, Bergelson, J, Manter, DK, Vivanco, JM. 2008. Root exudates regulate soil fungal community composition and diversity. *Applied and Environmental Microbiology* **74**: 738–744.

Carlson, RE, Dolphin, DH. 1981. Chromatographic analysis of isoflavonoid accumulation in stressed *Pisum sativum*. *Phytochemistry* **20**: 2281–2284.

Carminati, A, Moradi, AB, Vetterlein, D, Vontobel, P, Lehmann, E, Weller, U, Vogel, H-J, Oswald, SE. 2010. Dynamics of soil water content in the rhizosphere. *Plant and Soil* **332**: 163–176.

Cesco, S, Neumann, G, Tomasi, N, Pinton, R, Weisskopf, L. 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant and Soil* **329**: 1–25.

Clarke, KR. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Ecology* **18**: 117–143.

Clericuzio, M, Tabasso, S, Garbarino, JA, Piovano, M, Cardile, V, Russo, A, Vidari, G. 2007. Non-phenolic dicinnamamides from *Pholiota spumosa*. Isolation, synthesis and antitumour activity. *European Journal of Organic Chemistry* **2007**: 5551–5559.

Cooper, JE. 2007. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *Journal of Applied Microbiology* **103**: 1355–1365.

Crichton, CR. 2008. Biological Inorganic Chemistry — An Introduction. Amsterdam: Elsevier.

Dagne, E, Bekele, A, Waterman, PG. 1989. The flavonoids of *Millettia ferruginea* subsp. *ferruginea* and subsp. *darassana* in Ethiopia. *Phytochemistry* **28**: 1897–1900.

Dakora, FD, Phillips, DA. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* **245**: 35–47.

Davies, KJA. 2000. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life* **50**: 279–289.

Dayan, FE, Rimando, AM, Pan, Z, Baerson, SR, Gimsing, AL, Duke, SO. 2010. Sorgoleone. *Phytochemistry* **71**: 1032–1039.

de Vasconcelos Silva, Maria Goretti, de Abreu Matos, Francisco José, Lacerda Machado, MI, Craveiro, AA. 2003. Essential oils of Ocimum basilicum L., O. basilicum. var. minimum L. and O. basilicum. var. purpurascens Benth. grown in north-eastern Brazil. *Flavour and Fragrance Journal* **18**: 13–14.

Dennis, PG, Miller, AJ, Hirsch, PR. 2010. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiology Ecology* **72**: 313–327.

Dittmar, T, Paeng, J. 2009. A heat-induced molecular signature in marine dissolved organic matter. *Nature Geoscience* **2**: 175–179.

Escudero, N, Marhuenda-Egea, FC, Ibanco-Cañete, R, Zavala-Gonzalez, EA, Lopez-Llorca, LV. 2014. A metabolomic approach to study the rhizodeposition in the tritrophic interaction. Tomato, Pochonia chlamydosporia and Meloidogyne javanica. *Metabolomics* **10**: 788–804.

Fan, TW, Lane, AN, Pedler, J, Crowley, D, Higashi, RM. 1997. Comprehensive analysis of organic ligands in whole root exudates using nuclear magnetic resonance and gas chromatography-mass spectrometry. *Analytical Biochemistry* **251**: 57–68.

Farrar, JF, Jones, DL. 2003. The control of carbon acquisition by the growth of roots. In: Kroon, H de, Visser, EJW, eds. Root Ecology. Berlin: Springer, 91–124.

Fiehn, O, Kopka, J, Trethewey, RN, Willmitzer, L. 2000. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. *Anal. Chem.* **72**: 3573–3580.

Foyer, CH, Noctor, G. 2009. Redox regulation in photosynthetic organisms. signaling, acclimation, and practical implications. *Antioxidants & Redox Signaling* **11**: 861–905.

Friebe, A, Schulz, M, Kück, P, Schnabl, H. 1995. Phytotoxins from shoot extracts and root exudates of *Agropyron repens seedlings*. *Phytochemistry* **38**: 1157–1159.

Fukai, T, Qing-Hua, W, Nomura, T. 1991. Six prenylated phenols from *Glycyrrhiza uralensis*. *Phytochemistry* **30**: 1245–1250.

Gerke, J. 2015. The acquisition of phosphate by higher plants. Effect of carboxylate release by the roots. A critical review. *Journal of Plant Nutrition and Soil Science* **178**: 351–364.

Hadacek, F, Bachmann, G. 2015. Low-molecular-weight metabolite systems chemistry. *Frontiers in Environmental Science* **3**: 12.

Hartmann, A, Schmid, M, van Tuinen, D, Berg, G. 2009. Plant-driven selection of microbes. *Plant and Soil* **321**: 235–257.

Hartmann, T. 1985. Principles of plant secondary metabolism. *Plant Systematics and Evolution* **150**: 15–34.

Hartmann, T. 2007. From waste products to ecochemicals. Fifty years research of plant secondary metabolism. *Phytochemistry* **68**: 2831–2846.

Hawes, MC, Bengough, G, Cassab, G, Ponce, G. 2002. Root caps and rhizosphere. *Journal of Plant Growth Regulation* **21**: 352–367.

Henry, A, Doucette, W, Norton, J, Bugbee, B. 2007. Changes in crested wheatgrass root exudation caused by flood, drought, and nutrient stress. *Journal of Environment Quality* **36**: 904–912.

Hiltner, L. 1904. Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngung und Brache. *Arbeitsberichte der Deutschen Landwirtschaftsgesellschaft* **98**: 59–78.

Hofmann, A, Wittenmayer, L, Arnold, G, Schieber, A, Merbach, W. 2009. Root exudation of phloridzin by apple seedlings (*Malus* x *domestica* Borkh.) with symptoms of apple replant disease. *Journal of Applied Botany and Food Quality-Angewandte Botanik* **82**: 193–198.

Hulme, AC. 1954. The isolation of L-citramalic acid from the peel of the apple fruit. *Biochimica et Biophysica Acta* **14**: 36–43.

Huo, C-H, Wang, B, Lin, W-H, Zhao, Y-Y. 2005. Benzoxazinones from Acanthus ilicifolius. *Biochemical Systematics and Ecology* **33**: 643–645.

Iijima, M, Kono, Y. 1992. Development of Golgi apparatus in the root cap cells of maize (*Zea mays* L.) as affected by compacted soil. *Annals of Botany* **70**: 207–212.

Irigoyen, JJ, Einerich, DW, Sánchez-Díaz, M. 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologia Plantarum* **84**: 55–60.

Jacob, C, Jamier, V, Ba, LA. 2011. Redox active secondary metabolites. *Omics* **15**: 149–155.

Jenny, H, Grossenbacher, K. 1963. Root-soil boundary zones. As seen in the electron microscope. *Soil Science Society of America Journal* **27**: 273.

Jones, DL, Darah, PR, Kochian, LV. 1996. Critical evaluation of organic acid mediated iron dissolution in the rhizosphere and its potential role in root iron uptake. *Plant and Soil* **180**: 57–66.

Jones, DL, Nguyen, C, Finlay, RD. 2009. Carbon flow in the rhizosphere. Carbon trading at the soil–root interface. *Plant and Soil* **321**: 5–33.

Jung, C, Maeder, V, Funk, F, Frey, B, Sticher, H, Frossard, E. 2003. Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification. *Plant and Soil* **252**: 301–312.

Jung, Y-J, Park, J-H, Cho, J-G, Seo, K-H, Lee, D-S, Kim, Y-C, Kang, H-C, Song, M-C, Baek, N-I. 2015. Lignan and flavonoids from the stems of *Zea mays* and their anti-inflammatory and neuroprotective activities. *Archives of Pharmacal Research* **38**: 178–185.

Kerem, Z, Regev-Shoshani, G, Flaishman, MA, Sivan, L. 2003. Resveratrol and two monomethylated stilbenes from Israeli *Rumex bucephalophorus* and their antioxidant potential. *Journal of Natural Products* **66**: 1270–1272.

Khorassani, R, Hettwer, U, Ratzinger, A, Steingrobe, B, Karlovsky, P, Claassen, N. 2011. Citramalic acid and salicylic acid in sugar beet root exudates solubilize soil phosphorus. *BMC Plant Biology* **11**: 121.

Kopka, J, Schauer, N, Krueger, S, Birkemeyer, C, Usadel, B, Bergmüller, E, Dörmann, P, Weckwerth, W, Gibon, Y, Stitt, M, Willmitzer, L, Fernie, AR, Steinhauser, D. 2005. GMD@CSB.DB. the Golm Metabolome Database. *Bioinformatics* **21**: 1635–1638.

Kuhn, BM, Geisler, M, Bigler, L, Ringli, C. 2011. Flavonols accumulate asymmetrically and affect auxin transport in Arabidopsis. *Plant Physiology* **156**: 585–595.

Kuhnert, N, Drynan, JW, Obuchowicz, J, Clifford, MN, Witt, M. 2010. Mass spectrometric characterization of black tea thearubigins leading to an oxidative cascade hypothesis for thearubigin formation. *Rapid communications in mass spectrometry : RCM* **24**: 3387–3404.

Kuijken, RCP, Snel, JFH, Heddes, MM, Bouwmeester, HJ, Marcelis, LFM. 2015. The importance of a sterile rhizosphere when phenotyping for root exudation. *Plant and Soil* **387**: 131–142.

Kuo, P-C, Yang, M-L, Hwang, T-L, Lai, Y-Y, Li, Y-C, Thang, TD, Wu, T-S. 2013. Anti-inflammatory diterpenoids from *Croton tonkinensis*. *Journal of Natural Products* **76**: 230–236.

Lang, C, Gänzle, M. 2011. Lactic acid. https://roempp.thieme.de/roempp4.0/do/data/RD-13-02408.

Lanoue, A, Burlat, V, Henkes, GJ, Koch, I, Schurr, U, Roese, Ursula S. R. 2010. De novo biosynthesis of defense root exudates in response to Fusarium attack in barley. *New Phytologist* **185**: 577–588.

Legseir, B, Cherif, A, Richard, B, Pusset, J, Labarre, S, Massiot, G, Le Men-Olivier, L. 1986. Alkaloids of *Alstonia lenormandii*, a structural revision of 10-methoxycompactinervine. *Phytochemistry* **25**: 1735–1738.

Lion, CJ, Matthews, CS, Stevens, MFG, Westwell, AD. 2005. Synthesis, antitumor evaluation, and apoptosis-inducing activity of hydroxylated (*E*)-stilbenes. *Journal of Medicinal Chemistry* **48**: 1292–1295.

Luo, Q, Sun, L, Hu, X, Zhou, R. 2014. The variation of root exudates from the hyperaccumulator *Sedum alfredii* under cadmium stress: metabonomics analysis. *PloS one* **9**: e115581.

Lv, J-L, Zhang, L-B. 2013. Flavonoids and polyacetylenes from the aerial parts of *Bidens tripartita*. *Biochemical Systematics and Ecology* **48**: 42–44.

Macias, FA, Marin, D, Oliveros-Bastidas, A, Chinchilla, D, Simonet, AM, Molinillo, Jose M. G. 2006. Isolation and synthesis of allelochemicals from gramineae: benzoxazinones and related compounds. *Journal of Agricultural and Food Chemistry* **54**: 991–1000.

Mada, RJ, Bagyaraj, DJ. 1993. Root exudation from *Leucaena leucocephala* in relation to mycorrhizal colonization. *World Journal of Microbiology & Biotechnology* **9**: 342–344.

Makarova, LE, Dudareva, LV, Petrova, IG, Vasil'eva, GG. 2016. Secretion of phenolic compounds into root exudates of pea seedlings uponi inoculation with *Rhizobium leguminosarum* bv. *viceae* or *Pseudomonas siringae* pv. *pisi*. *Applied Biochemistry and Microbiology* **52**: 205–209.

Marschner, P, ed. 2012. Marschner's Mineral Nutrition of Higher Plants. Amsterdam: Elsevier.

McCully, ME, Boyer, JS. 1997. The expansion of maize root-cap mucilage during hydration. 3. Changes in water potential and water content. *Physiologia Plantarum* **99**: 169–177.

McDowell, N, Pockman, WT, Allen, CD, Breshears, DD, Cobb, N, Kolb, T, Plaut, J, Sperry, J, West, A, Williams, DG, Yepez, EA. 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologist* **178**: 719–739.

McNear, DH. 2013. The rhizosphere - Roots, soil and everything between. *Nature Education Knowledge* **4**: 1.

Meinwald, J. 2003. Understanding the chemistry of chemical communication. Are we there yet? *Proceedings of the National Academy of Sciences of the United States of America* **100**: 14514–14516.

Mimmo, T, Del Buono, D, Terzano, R, Tomasi, N, Vigani, G, Crecchio, C, Pinton, R, Zocchi, G, Cesco, S. 2014. Rhizospheric organic compounds in the soil–microorganism–plant system. their role in iron availability. *European Journal of Soil Science*: n/a–n/a.

Mithöfer, A, Boland, W. 2012. Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology* **63**: 431–450.

Mönchgesang, S, Strehmel, N, Schmidt, S, Westphal, L, Taruttis, F, Müller, E, Herklotz, S, Neumann, S, Scheel, D. 2016. Natural variation of root exudates in *Arabidopsis thaliana*-linking metabolomic and genomic data. *Scientific Reports* **6**: 29033.

Moody, SF, Clarke, AE, Bacic, A. 1988. Structural analysis of secreted slime from wheat and cowpea roots. *Phytochemistry* **27**: 2857–2861.

Morel, JL, Habib, L, Plantureux, S, Guckert, A. 1991. Influence of maize root mucilage on soil aggregate stability. *Plant and Soil* **136**: 111–119.

Morris, C, Grossl, PR, Call, CA. 2009. Elemental allelopathy. Processes, progress, and pitfalls. *Plant Ecology* **202**: 1–11.

Murashige, T, Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–497.

Nakabayashi, R, Saito, K. 2015. Integrated metabolomics for abiotic stress responses in plants. *Current Opinion in Plant Biology* **24**: 10–16.

Nascimento, IAd, Braz-Filho, R, Carvalho, MGd, Mathias, L, Fonseca, FdA. 2012. Flavonoides e outros compostos isolados de *Mimosa artemisiana* Heringer e Paula. *Química Nova* **35**: 2159–2164.

Neal, AL, Ahmad, S, Gordon-Weeks, R, Ton, J. 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PloS one* **7**: e35498.

Neumann, G, Römheld, V. 2007. The release of root exudates as affected by the plant physiological status. In: Pinton, R, Varanini, Z, Nannipieri, P, eds. The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Boca Raton, London, New York: CRC Press, 41–93.

Nguyen, C. 2003. Rhizodeposition of organic C by plants. Mechanisms and controls. *Agronomie* **23**: 375–396.

Niemeyer, HM. 2009. Hydroxamic acids derived from 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one. Key defense chemicals of cereals. *Journal of Agricultural and Food Chemistry* **57**: 1677–1696.

Niessen, WMA. 2006. Liquid Chromatography-Mass Spectrometry. Boca Raton, FL: CRC Press.

Oburger, E, Dell'mour, M, Hann, S, Wieshammer, G, Puschenreiter, M, Wenzel, WW. 2013. Evaluation of a novel tool for sampling root exudates from soil-grown plants compared to conventional techniques. *Environmental and Experimental Botany* **87**: 235–247.

Pedras, MSC, Adio, AM, Suchy, M, Okinyo, DPO, Zheng, Q-A, Jha, M, Sarwar, MG. 2006. Detection, characterization and identification of crucifer phytoalexins using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. *Journal of Chromatography A* **1133**: 172–183.

Penaloza, E, Corcuera, L, Martinez, J. 2002. Spatial and temporal variation in citrate and malate exudation and tissue concentration as affected by P stress in roots of white lupin. *Plant and Soil* **241**: 209–221.

Petho, M. 1992. Occurrence and physiological role of benzoxazinones and their derivatives. III. Possible role of 7-methoxybenzoxazinone in the iron uptake of maize. *Acta Agronomica Hungarica* **41**: 57–64.

Philippot, L, Raaijmakers, JM, Lemanceau, P, van der Putten, W. H. 2013. Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology* **11**: 789–799.

Pichersky, E, Noel, JP, Dudareva, N. 2006. Biosynthesis of Plant Volatiles: Nature's Diversity and Ingenuity. *Science* **311**: 808–811.

Pierret, A, Doussan, C, Capowiez, Y, Bastardie, F, Pagès, L. 2007. Root functional architecture. A framework for modeling the interplay between roots and soil. *Vadose Zone Journal* **6**: 269.

Read, DB, Bengough, AG, Gregory, PJ, Crawford, JW, Robinson, D, Scrimgeour, CM, Im Young, Zhang, K, Zhang, X. 2003. Plant roots release phospholipid surfactants that modify the physical and chemical properties of soil. *New Phytologist* **157**: 315–326.

Riviere, C, Pawlus, AD, Merillon, J-M. 2012. Natural stilbenoids: distribution in the plant kingdom and chemotaxonomic interest in Vitaceae. *Natural Products Reports* **29**: 1317–1333.

Rovira, AD, Bowen, GD, Foster, RC. 1983. The significance of rhizosphere microflora and mycorrhizas in plant nutriation. In: Läuchli, A, Bieleski, RL, eds. Inorganic Plant Nutrition. Berlin, Heidelberg: Springer Berlin Heidelberg, 61–93.

Salinger, MJ. 2005. Climate Variability and Change: Past, Present and Future – An Overview. *Climatic Change* **70**: 9–29.

Sánchez-Moreiras, AM, La Peña, TC de, **Reigosa, MJ. 2008.** The natural compound benzoxazolin-2(3*H*)-one selectively retards cell cycle in lettuce root meristems. *Phytochemistry* **69**: 2172–2179.

Scheffknecht, S, Mammerler, R, Steinkellner, S, Vierheilig, H. 2006. Root exudates of mycorrhizal tomato plants exhibit a different effect on microconidia germination of Fusarium oxysporum f. sp lycopersici than root exudates from non-mycorrhizal tomato plants. *Mycorrhiza* **16**: 365–370.

Schmid, NB, Giehl, Ricardo F H, Döll, S, Mock, H-P, Strehmel, N, Scheel, D, Kong, X, Hider, RC, Wirén, N von. **2014.** Feruloyl-CoA 6'-hydroxylase1-dependent coumarins mediate iron acquisition from alkaline substrates in Arabidopsis. *Plant Physiology* **164**: 160–172.

Schmidt, H, Günther, C, Weber, M, Spörlein, C, Loscher, S, Böttcher, C, Schobert, R, Clemens, S. 2014. Metabolome analysis of *Arabidopsis thaliana* roots identifies a key metabolic pathway for iron acquisition. *PloS one* **9**: e102444.

Schmidt, W, Tittel, J, Schikora, A. 2000. Role of hormones in the induction of iron deficiency responses in Arabidopsis roots. *Plant Physiology* **122**: 1109–1118.

Seal, AN, Pratley, JE, Haig, T, An, M. 2004. Identification and quantitation of compounds in a series of allelopathic and non-allelopathic rice root exudates. *Journal of Chemical Ecology* **30**: 1647–1662.

Shindy, WW, Smith, OE. 1975. Identification of plant hormones from cotton ovules. *Plant Physiology* **55**: 550–554.

Singer, MJ, Munns, DN. 2006. Soils. An Introduction. Upper Saddle River, N.J.: Pearson Prentice Hall.

Steele, HL, Werner, D, Cooper, JE. 1999. Flavonoids in seed and root exudates of *Lotus pedunculatus* and their biotransformation by *Mesorhizobium loti*. *Physiologia Plantarum* **107**: 251–258.

Steinkellner, S, Mammerler, R, Vierheilig, H. 2008. Effects of membrane filtering of tomato root exudates on conidial germination of *Fusarium oxysporum* f. sp. *lycopersici*. *Journal of Phytopathology* **156**: 489–492.

Stephan, UW, Scholz, G. 1993. Nicotianamine - mediator of transport of iron and heavy-metals in the phloem. *Physiologia Plantarum* **88**: 522–529.

Stevenson, FJ. 1994. Humus Chemistry. Genesis, Composition, Reaction. New York: Wiley.

Strehmel, N, Böttcher, C, Schmidt, S, Scheel, D. 2014. Profiling of secondary metabolites in root exudates of *Arabidopsis thaliana*. *Phytochemistry* **108**: 35–46.

Styles, E, Ceska, O. 1975. Genetic control of 3-hydroxy- and 3-deoxy- flavonoids in *Zea mays*. *Phytochemistry* **14**: 413–415.

Tan, J, Bednarek, P, Liu, J, Schneider, B, Svatoš, A, Hahlbrock, K. 2004. Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected *Arabidopsis thaliana* roots and leaves. *Phytochemistry* **65**: 691–699.

Tawaraya, K, Horie, R, Saito, A, Shinano, T, Wagatsuma, T, Saito, K, Oikawa, A. 2013. Metabolite profiling of shoot extracts, root extracts, and root exudates of rice plant under phosphorus deficiency. *Journal of Plant Nutrition* **36**: 1138–1159.

Tawaraya, K, Horie, R, Saito, S, Wagatsuma, T, Saito, K, Oikawa, A. 2014a. Metabolite profiling of root exudates of common bean under phosphorus deficiency. *Metabolites* **4**: 599–611.

Tawaraya, K, Horie, R, Shinano, T, Wagatsuma, T, Saito, K, Oikawa, A. 2014b. Metabolite profiling of soybean root exudates under phosphorus deficiency. *Soil Science and Plant Nutrition* **60**: 679–694.

Tsednee, M, Mak, YW, Chen, YR, Yeh, KC. 2012. A sensitive LC-ESI-Q-TOF-MS method reveals novel phytosiderophores and phytosiderophore-iron complexes in barley. *New Phytologist* **195**: 951–961.

Turtola, S, Manninen, A-M, Holopainen, JK, Levula, T, Raitio, H, Kainulainen, P. 2002. Secondary metabolite concentrations and terpene emissions of scots pine xylem after long-term forest fertilization. *Journal of Environment Quality* **31**: 1694.

Uren, NC. 2007. Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. In: Pinton, R, Varanini, Z, Nannipieri, P, eds. The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Boca Raton, London, New York: CRC Press, 1–23.

Vamerali, T, Saccomani, M, Bona, S, Mosca, G, Guarise, M, Ganis, A. 2003. A comparison of root characteristics in relation to nutrient and water stress in two maize hybrids. *Plant and Soil* **255**: 157–167.

van Dam, NM, Bouwmeester, HJ. 2016. Metabolomics in the rhizosphere. Tapping into belowground chemical communication. *Trends in Plant Science* **21**: 256–265.

Vanderlinde, EM, Hynes, MF, Yost, CK. 2014. Homoserine catabolism by *Rhizobium leguminosarum* bv. *viciae* 3841 requires a plasmid-borne gene cluster that also affects competitiveness for nodulation. *Environmental Microbiology* **16**: 205–217.

Vranova, V, Rejsek, K, Skene, KR, Janous, D, Formanek, P. 2013. Methods of collection of plant root exudates in relation to plant metabolism and purpose. A review. *Journal of Plant Nutrition and Soil Science* **176**: 175–199.

Wang, P, Bi, S, Wang, S, Ding, Q. 2006. Variation of wheat root exudates under aluminum stress. *Journal of Agricultural and Food Chemistry* **54**: 10040–10046.

Warren, CR. 2015. Wheat roots efflux a diverse array of organic N compounds and are highly proficient at their recapture. *Plant and Soil* **397**: 147–162.

Weidenhamer, JD, Boes, PD, Wilcox, DS. 2009. Solid-phase root zone extraction (SPRE). a new methodology for measurement of allelochemical dynamics in soil. *Plant and Soil* **322**: 177–186.

Weng, J-K, Philippe, RN, Noel, JP. 2012. The rise of chemodiversity in plants. *Science (New York, N.Y.)* **336**: 1667–1670.

Wenzel, WW, Wieshammer, G, Fitz, WJ, Puschenreiter, M. 2001. Novel rhizobox design to assess rhizosphere characteristics at high spatial resolution. *Plant and Soil* **237**: 37–45.

Whiteley, GM. 1989. The deformation of soil by penetrometers and root tips of *Pisum sativum*. *Plant and Soil* **117**: 201–205.

Willats, WG, McCartney, L, Knox, JP. 2001. In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of Arabidopsis thaliana. *Planta* **213**: 37–44.

Williams, RJP, Fraústo da Silva, JJR. 2006. The Chemistry of Evolution. The Development of our Ecosystem. Amsterdam, Boston: Elsevier.

Williams, RJP, Rickaby, REM. 2012. Evolution's Destiny. Co-evolving Chemistry of the Environment and Life. Cambridge, UK: Royal Society of Chemistry.

Wu, J-H, Tung, Y-T, Chien, S-C, Wang, S-Y, Kuo, Y-H, Shyur, L-F, Chang, S-T. 2008. Effect of phytocompounds from the heartwood of *Acacia confusa* on inflammatory mediator production. *Journal of Agricultural and Food Chemistry* **56**: 1567–1573.

Yamada, K, Higashinakasu, K, Shigemori, H, Hasegawa, K. 2005. Isolation and identification of an allelochemical exuded from germinating Pea (*Pisum sativum*) seeds. *Heterocycles* **65**: 267.

Yang, J, Zhang, J, Wang, Z, Zhu, Q, Liu, L. 2002. Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta* **215**: 645–652.

Young, IM. 1995. Variation in moisture contents between bulk soil and the rhizosheath of wheat (*Triticum aestivum* L. cv. Wembley). *New Phytologist* **130**: 135–139.

Yu, H, Shen, G, Gao, X. 2013. Determination of tobacco root exudates by GC-MS. *Zhongguo Yancao Xuebao* **19**: 64–72.

Zhang, J, Zhang, D, Mao, Z, Li, Z, Zhao, M. 2009. Isolation and identification of root exudates in different kinds of apple rootstock seedlings. *Hebei Nongye Daxue Xuebao* **32**: 29–32.

Zhang, Q, Pettolino, FA, Dhugga, KS, Rafalski, JA, Tingey, S, Taylor, J, Shirley, NJ, Hayes, K, Beatty, M, Abrams, SR, Zaharia, LI, Burton, RA, Bacic, A, Fincher, GB. 2011. Cell wall modifications in maize pulvini in response to gravitational stress. *Plant Physiology* **156**: 2155–2171.

Zheng, X, Chen, S, Li, Q, Lin, R, Lin, W. 2014. Determination of phenolic acids in root exudates of allelopathic rice by solid phase extraction-ion chromatography with conductivity detection. *Analytical Letters* **47**: 2156–2164.

Zhou, X, Zhang, Y, Niklas, KJ. 2014. Sensitivity of growth and biomass allocation patterns to increasing nitrogen: a comparison between ephemerals and annuals in the Gurbantunggut Desert, north-western China. *Annals of Botany* **113**: 501–511.

Ziegler, J, Schmidt, S, Chutia, R, Muller, J, Bottcher, C, Strehmel, N, Scheel, D, Abel, S. 2016. Non-targeted profiling of semi-polar metabolites in Arabidopsis root exudates uncovers a role for coumarin secretion and lignification during the local response to phosphate limitation. *Journal of Experimental Botany* **67**: 1421–1432.

2. Material and Methods

Pervin Akter[1]

2.1 Chemicals

All chemicals and solvents were of analytical grade and used without further purification. HPLC-grade ethyl acetate and cyclohexane were provided by Carl Roth (Karlsruhe, Germany). Methanol (LC-grade) was acquired from Th. Geyer (Renningen, Germany). Phosphoric acid was provided by Carl Roth (Karlsruhe, Germany). All other chemicals, of which the source is not specifically stated, were obtained from Sigma Aldrich (Taufenkirchen, Germany). Double distilled water was prepared in-house (GFL, Burgwedel, Germany).

2.2 Plant Material and Culture

Seeds of the six selected plant species (Table 2.1) were surface sterilized by soaking in 70 % ethanol for 30 sec, washed thoroughly three times with autoclaved water, and prepared for sowing. The temperature was set to 22 °C during day (14 h) and 15 °C during night (10 h). Minimal humidity was 65 %. Illumination was provided by standard growth chamber fluorescent lamps with a photosynthetically active photon flux density of 210–250 µmol m^{-2} sec^{-1} (Figure 1). Potting soil was prepared by mixing of 2 parts of sieved (3 mm) commercial soil (Einheitserde T25, Hawita GmbH, Vechta, Germany) with 1 part of sand (v/v).

Seeds (3–10) were sown in previously prepared plastic pots filled with potting soil and placed in the plant growth chamber. Later, only the most vigorous individual was left, the others removed. The culture regime was based on recommendations from gardeners and colleagues who had maintained plant cultures in the same climate chamber. Pots were watered every 2–3 days with and allowed to grow for 14 days. From day 14 onwards, fertilizer was added to the water (Hakaphos® Blau, 3 g/L, Compo Expert GmbH, Münster, Germany). All six species received the identical regime. At day 47, root exudates were collected as described in the ongoing text. Fresh leaves free of damage, senescence or disease symptoms were dried at 65 °C for mineral nutrient analysis and fresh roots were frozen in liquid nitrogen for metabolite

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analysis. Each experiment was repeated in the same climate chamber under identical conditions.

Figure 1. Cultures of Arabidopsis, Rapeseed, Phaseolus, Pisum, Tobacco and Maize in the available growth chamber (22° C 14 h, 15° C 10 h, min humidity 65 %, photosynthetically active photon flux density: 210–250 µmol m^{-2} sec^{-1}).

2.3 Water deficit stress

All plants were regularly watered until day 22. The pots received no water from day 23 until day 36. After day 36 water was provided until the harvest at day 47. This regime was decided upon after discussion with gardeners, colleagues and literature study (Yang *et al.*, 2002) and in consideration of the available facilities.

2.4 Plant biomass

Fresh weights were determined after root exudate collection as the weight of the shoot/root material after removal and drying with paper cloth (roots), dry weights after drying at 60 °C until constant weight.

2.5 Root exudate collection

Individual plants were removed from the pots and the root washed thoroughly with tap water to remove adhered soil. The final rinse was performed with distilled water. The complete root system was immersed then into a conical flask wrapped into aluminium foil and filled with aqua dest. Root exudate collection was performed for 6 hours in the growth chamber under daylight conditions. Table 2.2 informs about the specific setup for every plant species. After collection, the root exudate solutions were pooled for each species and watering regime and filtered to remove still present soil and root particles (Whatman qualitative filter paper, grade 1, GE Healthcare Life Sciences, Freiburg, Germany). The thus treated root exudate solutions were kept at –20 °C until further work-up.

Every root exudate collection solution was concentrated to 100 ml using a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) and extracted twice with 100 mL ethyl acetate and polar lower layer water-soluble fraction. The combined ethyl acetate and the water fraction were concentrated to 20 mL for storage at –20 °C until analysis. One mL (water) was dried on a speed vac (RVC 2-25, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) to calculate the weight of the total extract.

2.6 Root extraction

Frozen roots (1 g) were ground and extracted with 2 mL MeOH for 24 h at room temperature. The extract was filtered (Whatman qualitative filter paper, grade 1, GE Healthcare Life Sciences, Freiburg, Germany) and speed-vac dried. The work-up and sample preparation procedure was identical to that of the root exudate.

2.7 Analysis of primary and secondary metabolites

2.7.1 Water fraction (primary plant metabolites)

Two mg of dried sample were dissolved in 100 µL pyridine to which methoxyamine hydrochloride (30 mg mL^{-1}) was added. The solution was kept at room temperature for 18–24 hours. Then 50 µL MSTFA was added and the sample well vortexed. GC–MS analysis was performed on an Agilent Network 5973 quadrupole mass detector linked to an Agilent 6890 GC (Agilent, Waldbronn, Germany). The column was an HP5-MS (30 m x 0.25 mm x 0.25 µm) obtained from the same company. Helium was used a carrier gas (1 mL min^{-1}). The injector

Table 2.1

Plant species	Cultivar name (cv.)	Family	Source
Arabidopsis thaliana L. (**A**)	Ecotype Columbia	Brassicaceae	General Phytopathology & Crop Protection*
Brassica napus L. (**R**)	Miniraps (Rapid cycling OSR)	Brassicaceae	Plant Biology, Univ. of Winsconsin, USA
Nicotiana tabacum L. (**T**)	Xanthi	Solanaceae	General Phytopathology & Crop Protection*
Zea mays L. (**M**)	Rolandino	Poaceae	KWS Saat SE, Einbeck, Germany
Pisum sativum L. (**Pi**)	Topaz	Fabaceae	General Phytopathology & Crop Protection*
Phaseolus vulgaris L. (**Ph**)	Hangdown	Fabaceae	Dürr Samen, Reutlingen, Germany

*Georg-August Universität Göttingen

Table 2.2: Root exudate collection design for each individual species

Species	Individuals/ experiment	Pot size	Total water volume (mL)	Water volume/ plant (mL)
Arabidopsis thaliana L.	40	5 x 5 x 5	560	14
Brassica napus L.	15	11 x 11 x 15	800	53
Nicotiana tabacum L.	8	12 x 12 x 11	2000	250
Zea mays L.	8	12 x 12 x 11	2400	300
Pisum sativum L.	15	11 x 11 x 15	1500	100
Phaseolus vulgaris L.	15	11 x 11 x 15	1500	100

was used in the splitless mode and thermostatted at 230 °C. The temperature gradient was as follows: 0–2 min: 50 °C, 3-58 min: 50–330 °C, 59–60: 330 °C. Transfer line temperature was 330 °C, ion source temperature 230 °C, electron energy 70 eV for EI ionization. Spectra were recorded in the range of 70–600 Da. TIC chromatograms were baseline detected, peak identified (after deconvolution with AMDIS 2.65), and peak-integrated with OPENChrom community edition 1.1.0 (Lablicate GmbH, Hamburg, Germany). Databases for tentative

analyte identification included NIST Mass Spectral Library 2.0 f, build Jun 25 2008, and the Golm metabolome database (GMD) (Kopka *et al.*, 2005). Retention indices were calculated on basis of alkane standards (dodedecane, pentadecane, and nonadecane).

2.7.2 Ethyl acetate fraction (secondary plant metabolites)

Samples were dried and re-dissolved in 100 µL MeOH: acetic acid (99: 1, v/v) to yield a final concentration of 10 mg/mL. (0.25 ml/6x31 mm, Macherey-Nagel GmbH & Co. KG, Germany) and transferred into glass autosampler vials with low-volume inlets ((Macherey Nagel GmbH and Co. KG, Düren, Germany).

Samples were first analysed by a HPLC-DAD system consisting of a Jasco A2-2059-SF Plus autosampler, Jasco PU-2085 Plus pump, Jasco LG 2080-04S gradient former, DG2080-54 degasser (Jasco Labor-und Datentechnik GmbH, Groß-Umstadt, Germany). The analyses were performed with a RP-18 column, C18 Varian Polaris (150 mm × 2 mm, 5 µm; Varian, Walnut Creek, CA, USA) thermostatted at 40 °C. Ten µL of each samples were injected. The mobile phases consisted of 0.5% aqueous H_3PO_3 (v/v, solvent A) and MeOH (solvent B). The constant flow rate was 0.2 mL min^{-1}. The gradient was as follows: 0–1 min: 5 % B, 1–101 min: 5–100 % B, 116 min 100 % B. A photodiode array UV/VIS detector (Varian Prostar, Varian, Walnut Creek, CA, USA) was used to record spectra from 220–590 nm. Galaxy Chromatography Workstation 1.9.3.2 software (Varian, Walnut Creek, CA, USA) was used for data peak analysis and integration. Peaks eluting between 5–107 min were incorporated into the peak list. Peak quantitation was performed on maximum absorbance chromatograms in which the maximal absorption wavelength defined the peak intensity of each detected analyte.

Additionally samples were analysed also by UPLC–TOF/MS (LCT Premier, Waters, Millford, MA, USA) after drying, re-dissolving in MeOH: water (4:6, v/v) and defatting with 250 µL cyclohexane. The system also contained a photodiode array detector. The column was a C18 ACQUITY HSS T3 (100 x 1 mm, 1.8 mm) with a constant flow rate of 0.2 mL min^{-1}. The mobile phase consisted of water: formic acid (100:0.1, v/v solvent A) and acetonitrile: formic acid (100:0.1, v/v, solvent B). A binary gradient was used: 0–3 min: 1 % B, 3–8 min: 1–100 % B. The eluent was introduced by an ESI interface both in positive and negative mode with the following parameters: capillary voltage, 2500 V (neg.) / 2700 V (pos.); cone voltage: 30 V; nitrogen flow as desolvation gas: 800 L h^{-1}; nitrogen flow as con gas: 30 L h^{-1}; desolvation temperature: 350 °C; source temperature: 80 °C. The MS analyser was operated in full scan

mode over 10 min in a mass range *m/z* = 85–1200 with a scan speed of 5000 Da sec^{-1} and 3 scans averaged. Data acquisition is carried out by the software MassLynx (V4.1, Waters, Milford, MA, USA) in centroid data format. All analysis were calibrated by applying leucine-enkephaline ($[M+H^+]^+$ 556.2771 or $[M–H^+]^-$ 554.2615) as lock spray reference compound.

Identification of selected secondary metabolites was performed on basis of a comparison of UV and MS spectra. For comparison of UV spectra, an in-house spectra library (ACD-ROM/Spectrus DB 2015, 2.5, ACD-ROM Labs, Toronto, Canada) was used. For MS spectra, KNApSACK v1.200.03 (NAIST Comparative Genomics Laboratory, Nara, Japan) and CAS SciFinder™ (American Chemical Society; Washington DC, USA) were used.

2.8 Differential pulse voltammetry (DPV)

The potentiostat was a 797 VGA Computrace (Methrom AG, Filderstadt, Germany) An electrochemical cell containing a glassy carbon working electrode (3 mm diameter), a platinum wire counter electrode (3 mm diameter, 10 cm in length) and an Ag/AgCl (saturated KCl) reference electrode were used for all measurements. Prior to each measurement, the GC electrode was rinsed with distilled water and then polished manually with an aqueous slurry of aluminium oxide powder (0.3 µm, diameter) on a damp smooth polishing cloth for 2 min. The conditions were as follows: start potential –200 mV, end potential 1200 mV. Voltage step: 6 mV, voltage step time 0.4 sec, sweep rate 0.015, pulse amplitude 25 mV, pulse time 0.05 sec. Fifteen mL of 1 M acetate buffer (0.67 mL acetic acid and 0.059 g sodium acetate in 120 mL water, pH = 3.6) were transferred into the electrochemical cell and 5 mL of the original conc. root exudate solution added. Analysis was performed after 5 min degassing with argon. All DPV analyses were performed by Dr. Gert Bachmann, Department of Ecogenomics and Systems Biology, Division of Molecular Systems Biology, University of Vienna, Austria.

2.9 Leaf nutrient analyses

Leaves were dried for 2 days at 60 °C and stored in paper bags at room temperature until analyses. One hundred mg tissue was transferred to 2 mL Eppendorf tubes and ground with a mixer mill for 1–3 min (MM 200, Retsch, Haan, Germany). The metal beads were cleaned thoroughly with 0.1 % HCl followed by sterile water before use. Ground samples were weighed (1–5 mg tissue), placed in small Pyrex tubes and digested with 1 mL of 7 N nitric acid (Roth, Karlsruhe, Germany) for 6 h, at 120 °C in a block heater. Elemental analyses for P, S, K, B, Na,

Ca, Mg, Zn, Mn, Mo, Cu and Fe were performed with a Vista-PRO simultaneous inductively coupled plasma-optical emission spectrometer (ICP-OES, Varian, Palo Alto, CA, USA). A plant tissue sample (SMR 1515 apple leaf) with certified elemental concentrations was used for calibration. The analyses were performed by Kirsten Fladung, Institute of Applied Plant Nutrition, Georg-August-Universität Göttingen. Micro- and macronutrient concentrations are reported in mg kg^{-1} (ppm).

2.10 Statistical analysis

2.10.1 Metabolite similarity

All root exudate and extract samples consisted of metabolite lists based on unique combinations of retention times and UV or MS spectra. Peak areas were calculated by the respective software (see analysis-specific sections). Undetected metabolites were assigned with peak area values below the detection level but > 0. The samples were standardised by dividing by the sum of the total chromatogram's peak area. The thus obtained variables were analysed by SIMPER (similarity percentages) of Bray-Curtis resemblance matrices and visualized by MDS (non-metric multidimensional scaling) plots. In these plots, distance resembles similarity. Every sample category is represented by two repeats. All repeats were pooled to obtain sufficiently concentrated samples for some analyses. Consequently, no repeats exist for each repeat but each repeat somehow represents a mean, for *n* see Table 2.2.

Variable vectors were generated by exploring for multiple correlations > 0.1. This low value was chosen because one variable rarely contributes to all explored cases and the sum of contributions of many variables determine similarity and dissimilarity of many cases. Group differences were analysed by the ANOSIM procedure. All analyses were performed with Primer 6.1.13 (Primer-E Ltd, UK). Procedures in this statistical software package were developed originally to analyse marine community structures (Clarke, 1993). Our view is that metabolites in root exudates also represent communities that resemble those of living organisms. Consequently, the same statistical procedures are applicable.

2.10.2 Leaf nutrient patterns and metabolite–nutrient correlations

Boxplots were created with Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

For comparison of resemblance matrices, the procedure RELATE (Primer 6.1.13, Primer-E Ltd, UK) was used. It represents a non-parametric version of a Mantel test that utilizes Spearman rank correlations.

2.11 References

Clarke, KR. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Ecology* **18**: 117–143.

Kopka, J, Schauer, N, Krueger, S, Birkemeyer, C, Usadel, B, Bergmüller, E, Dörmann, P, Weckwerth, W, Gibon, Y, Stitt, M, Willmitzer, L, Fernie, AR, Steinhauser, D. 2005. GMD@CSB.DB. the Golm Metabolome Database. *Bioinformatics* **21**: 1635–1638.

Yang, J, Zhang, J, Wang, Z, Zhu, Q, Liu, L. 2002. Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta* **215**: 645–652.

3. Primary and Secondary Plant Metabolites in Root Exudates and Tissues: Comparison Within and Between Species

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Abstract: Six crop plants, Arabidopsis (*A. thaliana*), Rapeseed (*Brassica napus*), Phaseolus (*Phaseolus vulgaris*), Pisum (*Pisum sativum*), Tobacco (*Nicotiana tabaccum*) and Maize (*Zea mays*) were grown for 36 days in pots with soil. Root exudates were collected, partitioned into an ethyl acetate and water fraction, the former of which was analysed by GC–MS and the latter by HPLC–DAD and UPLC–TOF/MS. Corresponding root tissue was extracted by methanol and subjected to the same workup and analysis procedures.

The exuded amounts of primary (PM) and secondary plant metabolites (PM) were more comparable than those present in the roots. SMs were more specific than PMs and were root- or root exudate-specific in some instances. In the latter case, dissimilarity between roots and root exudates was caused by quantitative differences by the majority.

Similarity and dissimilarity of the root exudates and the root extracts (two pooled repeats each) were analysed by nonparametric multivariate statistics that included more than 180 PMs and more than 130 SMs. The majority of PMs was identified on basis of alkane retention indices and database MS spectra comparison. For 18 SMs, all of which had been pointed out a substantial contributors to root exudate and root extract similarity and dissimilarity, tentative structures are proposed on basis of UV and MS spectra.

3.1 Introduction

Low- and high-molecular-weight organic compounds that occur in root tip and root hair tissues are described by the collective term root exudates. Recent reviews agree in that they are comprised of a mixture of primary and secondary plant metabolites (Uren, 2007; Badri & Vivanco, 2009; van Dam & Bouwmeester, 2016). Studies are, however, scarce due to the difficult procedures to collect root exudates for chemical analyses. No common agreement exists over how to collect them efficiently. Some authors argue that plant roots should come into contact with soil (Farrar & Jones, 2003; Uren, 2007), others favour hydroponic or agar

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Author contributions: concept: PA, FH; text: PA; figures: PA, FH; GC–MS: FH, UPLC–MS: KF; DPV: GB data analysis: FH, PA

cultures based on MS medium (Kuijken *et al.*, 2015). The latter approach is especially favoured in phenotype characterization of Arabidopsis.

A further problem is that, compared to tissue-accumulated plant metabolites, root exudate metabolites occur in much lower amounts, a problem that affects the analysis of primary metabolites (PM) less than that of secondary metabolites (SM). To address this complication, the collected root exudates that were obtained from a plant species at a given time point were pooled for further analyses, especially in respect to SMs. The culture of every plant species was repeated once.

The present study utilized soil-grown plants to study root exudate composition of six plant species. All were grown in the same climate chamber under identical light and moisture conditions, in the same soil and with the identical watering and fertilizing regime. The choice of the plant species was governed by their status as crop plant and by their successful development under the provided condition, all of which was extensively explored and tested in preliminary experiments. As a result, Arabidopsis, Rapeseed, both members of Brassicaceae, Phaseolus and Pisum, two Fabaceae, Tobacco (Solanaceae) and Maize (Poaceae) were choses to be included into the survey. The available climate chamber space allowed the growing of six plant species including one complete repeat of each. The focus of the study was more on the variation between species than on the phenotypic characterization of a specific one.

The collected root exudates were partitioned into an ethyl acetate-soluble and a water-soluble fraction. The first one contained, by majority, SMs and was analysed by HPLC–DAD and UPLC–TOF/MS. A combination of UV and MS spectra was aimed at providing substantial clues to propose tentative structures. The water fraction was analysed by GC–MS. Comprehensive, online available databases, e.g. GMD (Kopka *et al.*, 2005), allow a tentative identification of the majority of PMs. These methodologies are used in the majority of studies (van Dam & Bouwmeester, 2016).

As a rule, methodologies with high sensitivity tend to discriminate certain groups of analytes, whereas less discriminant methodologies lack sensitivity. Nuclear magnetic resonance spectroscopy (proton of C13 carbon) represents the latter category that has also been applied in one root exudate study (Escudero *et al.*, 2014). Generally, spectra of complex mixtures suffer from substantial signal overlap that affects structure elucidation of specific analytes,

many of which cause the appearance of more than one signal. This also applies to mass spectrometry. In an attempt to include an additional method providing information that is based on different principles than those to which standard chromatography–spectroscopy linked methodologies adhere, an electrochemical method was included. Differential pulse voltammetry represents an electrochemical technique that explores electro-oxidation and –reduction of the analytes and is especially suited for organic molecule that show a tendency to polymerize on the electrode surface (Brett & Brett, 1993). A specific mixture of analytes yields a specific combination of peaks in a voltammogram that, if changed, allows concluding that the quality and quantity of the analytes has changed too.

This available experimental setup allowed posing several questions:

(1) Do different plant species exude comparable amounts of root exudates and do the exuded amounts reflect those present in root tissues?

(2) Do root exudates contain specific primary or secondary metabolites?

(3) How common is the exotic citramalic acid, a methylated derivative of citric acid, that was detected as a component of sugar beet root exudates (Khorassani *et al.*, 2011)?

(4) Does a soil culture-based root-exudate-collection approach provide acceptable results?

3.2 Material and Methods

See Chapter 2.

3.3 Results

3.3.1 Root exudate versus root extract yields

To facilitate some comparison between the exuded and the root-accumulated metabolites, the obtained yields were recalculated to represent one g fresh weight, in case of root exudates (unfrozen) and roots (frozen). Figure 3.1 presents a bar graph of the amounts of PMs (water-soluble) and SMs (ethyl acetate-soluble) fractions in root exudates compared to whole root extracts. A comparison of the mean amounts of each extract category illustrates the situation: 0.4 mg and 0.01 mg for exudate PMs and SMs, 9.1 and 0.8 mg for root PMs and SMs. Roots accumulated roughly 20-times higher amounts of primary metabolites and 80-times more secondary metabolites compared to what could be found in the corresponding root exudates.

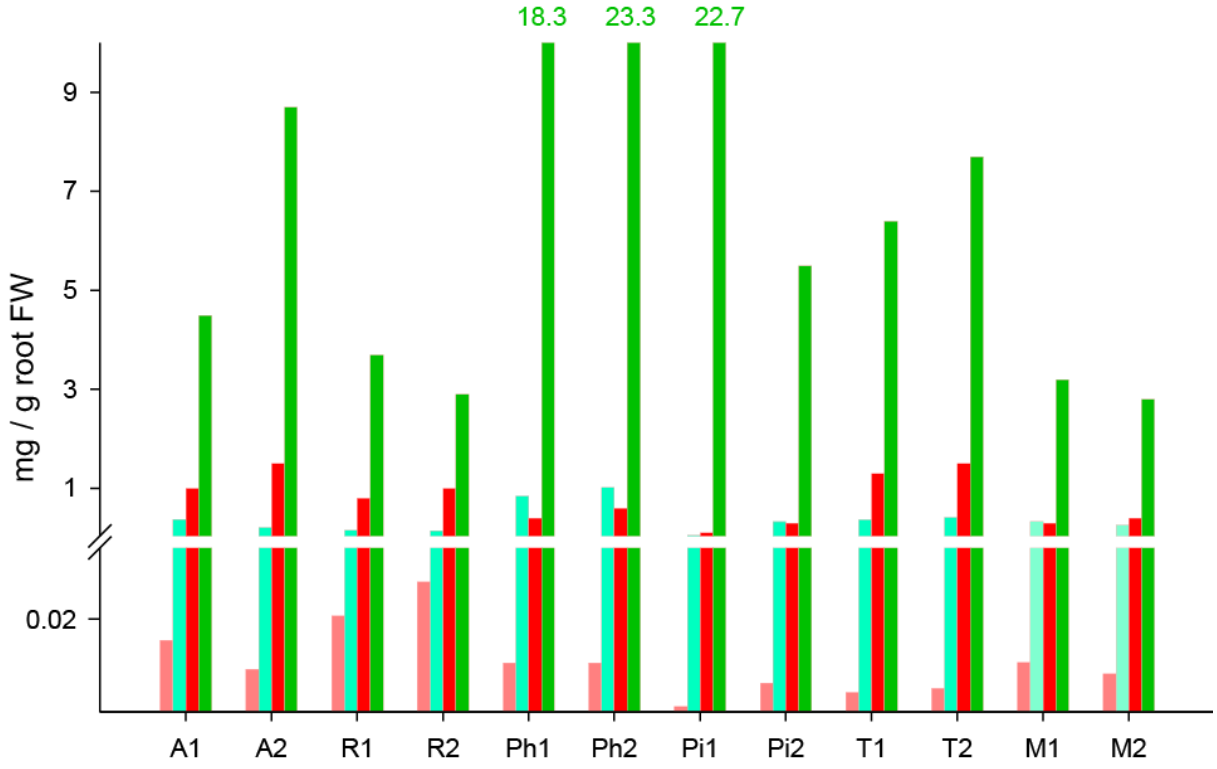


Figure 3.1. PMs and SMs in root exudates extracts (mg/g root fresh weight): root exudate PMs, root exudate SMs, total root PMs, total root SMs; A, Arabidopsis; R, Rapeseed; Ph, Phaseolus; Pi, Pisum; T, Tobacco; M, Maize.

The two repeats yielded more or less comparable result, only Pisum deviated. In the first experiment, root exudate SM amounts were clearly lower than in the second. Conversely, in the second experiment, root primary metabolite amounts were less. Rapeseed root exudates yielded the highest amounts of PMs and SMs. Concerning root yields, by contrast, the highest amount of PMs was found in Phaseolus and the highest amount of SMs in Tobacco, closely followed by Arabidopsis and Rapeseed. Concerning the lowest amounts, no clear candidate emerged within the PMs of root exudates; Pisum and Tobacco were joint winners for the display of the lowest amount of SMs. Rapeseed and Maize showed the lowest amount of PMs, Pisum and Maize that of secondary metabolites in root extracts. A Spearman rank correlation analysis between primary and secondary metabolites amounts in root exudates pointed to the fact that ratios between exuded and accumulated PMs correlate better than those of exuded and accumulated SMs. ρ-Values were 0.41 for PMs and–0.04 for SMs.

3.3.2 Primary metabolites (PM) in roots and root exudates

PM profiling—a few secondary metabolites were detected too (less than 1 %)—of the root exudates and the corresponding root extracts was performed with GC–MS (see Chapter 2).

The tentative identification of the analytes is based on comparison of retention indices and mass spectra that are available in the Golm metabolome database (Kopka *et al.*, 2005). Appendix 2 (see CD-ROM) provides a summary of all metabolites that were identified in this thesis together with their retention times and MS spectra. Figure 3.2 summarizes the results that were obtained by similarity analysis of the GC–MS profile data. Brassicaceae (Figures 3.2a and 3.2b) and Fabaceae (Figures 3.2c and 3.2d) were represented by two species each within the investigated plant species, Solanaceae and Poaceae with one each (Figures 3.2e and 3.2f).

3.3.2.1 Arabidopsis

The root PM profiles of Arabidopsis and Rapeseed were comprised of more than 100 metabolites, many of which were present as minor amounts only. The PMs that contributed most to the similarity of the two repeats (average 55 %) included the sugars fructose, mannose and galactose, the sugar alcohol pinitol, the amino acids alanine, threonine, valine and serine, the glutamic acid oxidation product GABA, as well as malic acid, succinic acid and glycerol. The number of the detected PMs in the root exudates was roughly 20 % lower. The PMs contributing most to similarity of the two repeats (average 58 %) comprised the sugars glucose, fructose, arabinose, mannose, ribose, and galactose, the sugar alcohol myo-inositol, the non-proteinogenic amino acid pyroglutamic acid, lactic acid, citric acid and glycerol. The dissimilarity between root and root exudates (average 58 %) was caused as follows (with decreasing contribution): glucose, mannose, malic acid, arabinose, phosphoric acid, citric acid, myo-inositol, pinitol, pyroglutamic acid, lactic acid, amongst others (Figure 3.2a).

3.3.2.2 Rapeseed

Rapeseed root similarity (66 %) was again caused by amino acids, in particular by threonine, asparagine, serine, aspartic acid, and pyroglutamic acid, the glutamic acid oxidation product GABA, and glycerol. The root exudates (average similarity 29 %), by contrast, were characterised by the sugars glucose, galactose and arabinose, the sugar alcohol *myo*-inositol, the amino acid leucine, ethanolamine, a derivative of the amino acid serine, uracil and glycerol. The dissimilarity (average 64 %) between root and root exudates was caused as follows (with decreasing contribution): GABA, phosphoric acid, *myo*-inositol, benzoic acid, glycerol, lactic acid, pyroglutamic acid, xylose, glucose and tryptophan, besides of many less pronounced differences of other metabolites (Figure 3.2b).

3.3.2.3 Phaseolus

In contrast to the Brassicaceae, the PM profiles of two Fabaceae Phaseolus and Pisum roots revealed less metabolites, around 80 in Phaseolus and only around 60 in Pisum, but root exudate PM numbers were more or less similar to the roots. Accordingly, less metabolites contributed to the similarity and dissimilarity of root and root exudate PM profiles. The similarity of the Phaseolus root PMs (average 73 %) was caused by the amino acids asparagine and alanine, the non-proteinogenic amino acid pyroglutamic acid, and the glutamic acid oxidation product GABA. Phaseolus root exudate similarity (average 71 %) was supported by the amino acids aspartic acid and alanine, the sugar glucose, the sugar alcohol *myo*-inositol, and phosphoric acid. The dissimilarity (average 68 %) between root and root exudates was caused as follows (with decreasing contribution): aspartic acid, asparagine, and phosphoric acid, besides of many less pronounced differences of other metabolites (Figure 3.2c).

3.3.2.4 Pisum

The Pisum root PM profiles (average similarity 84 %) were characterised by the amino acids asparagine and homoserine, the sugar glucose and phosphoric acid. The root exudate PM profiles showed a low similarity (average 28 %). One of the pooled samples E1 showed a very low number of detectable PMs, only 36, compared to 67 from the pooled sample from the other repeat. The sugars glucose, galactose and ribose, the sugar alcohol *myo*-inositol, glycerol and 2,4-dihydroxybutanoic acid contributed to similarity. The dissimilarity (average 75 %) between root and root exudates was caused as follows (with decreasing contribution): *myo*-inositol, asparagine, phosphoric acid, homoserine, xylulose, 2,4-dihydroxybutanoic acid, glycerol and aspartic acid, besides of many less pronounced differences of other metabolites (Figure 3.2d).

3.3.2.5 Tobacco

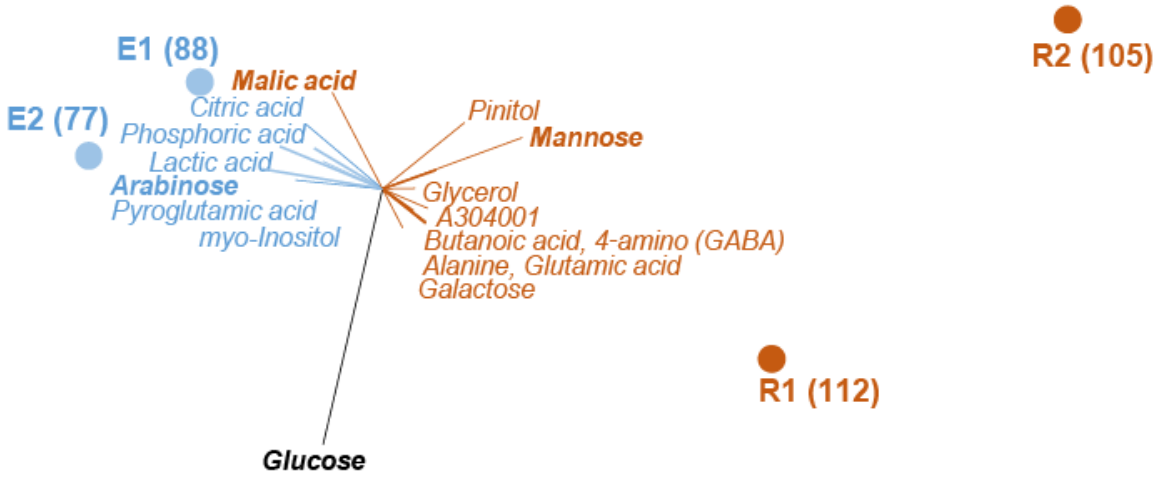
In Tobacco root PM profiles the amino acids proline, the sugars glucose and fructose, the sugar alcohol *myo*-inositol, phosphoric acid and glycerol contributed to similarity (average 72 %).

Figure 3.2. MDS plot of Bray-Curtis similarity of root exudate (**E1, E2**) and root (**R1, R2**) primary metabolite (PM) profiles that were obtained by GC–MS analyses of the water phase of the crude exudate collection/extract; **(a)** Arabidopsis, **(b)** Rapeseed, **(c)** Phaseolus, **(d)** Pisum, **(e)** Tobacco, and **(f)** Maize. PM contributions to root exudate similarity and root extract similarity are indicated as respective vectors. Black vectors indicate metabolites that contribute more to the variation within root exudates and root extracts.

(a) Arabidopsis

Root exudate similarity: 58 %, root similarity: 55 %
Dissimilarity: 58 %

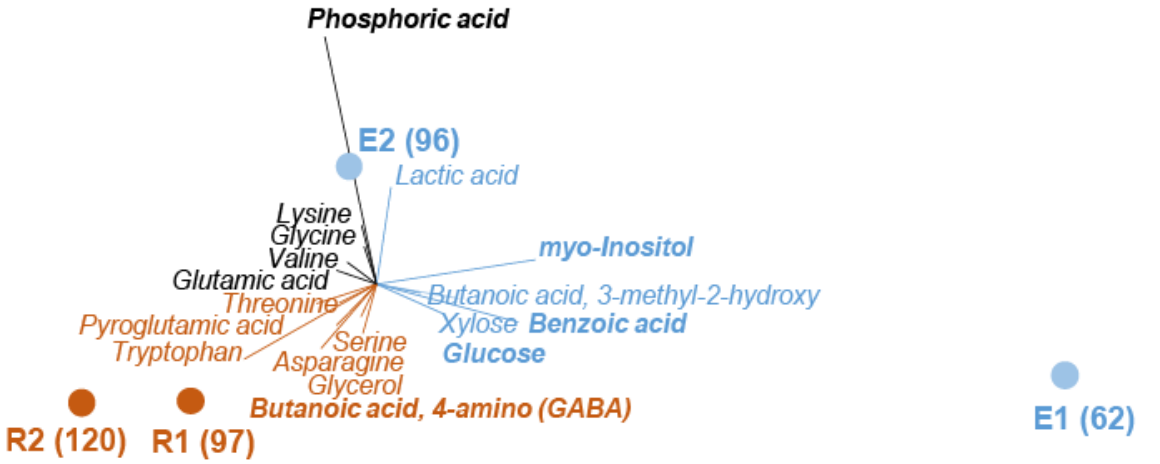
2D Stress: 0



(b) Rapeseed

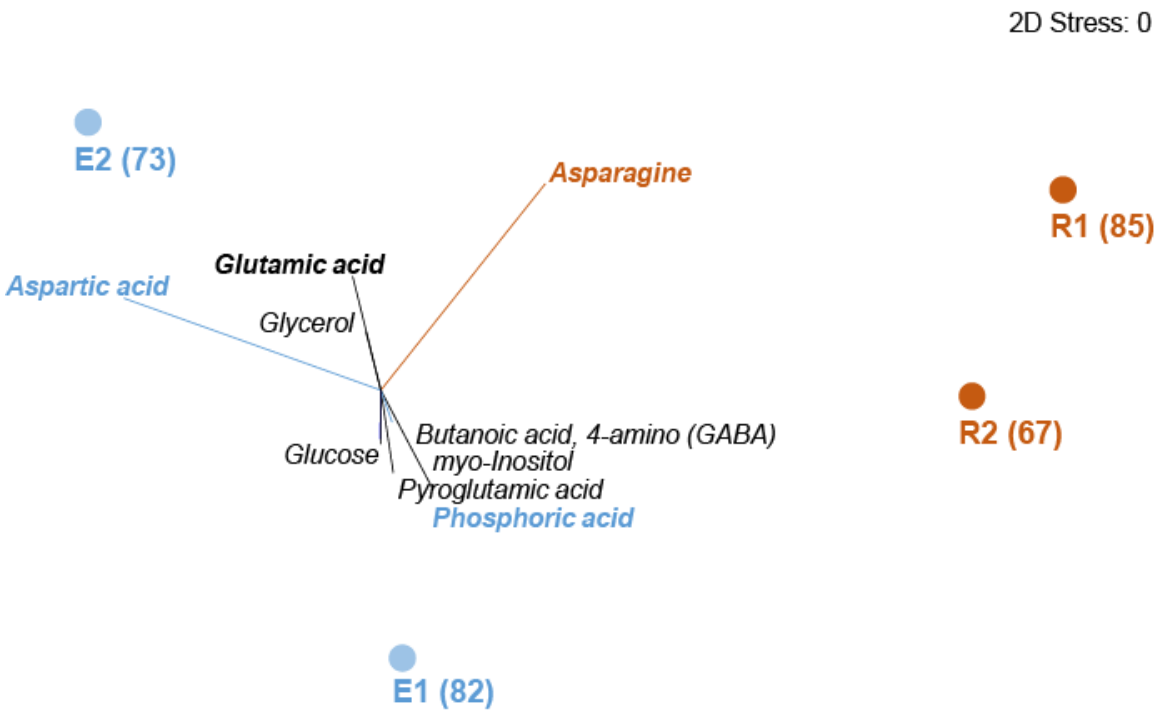
Root exudate similarity: 29 %, root similarity: 66 %
Dissimilarity: 64 %

2D Stress: 0



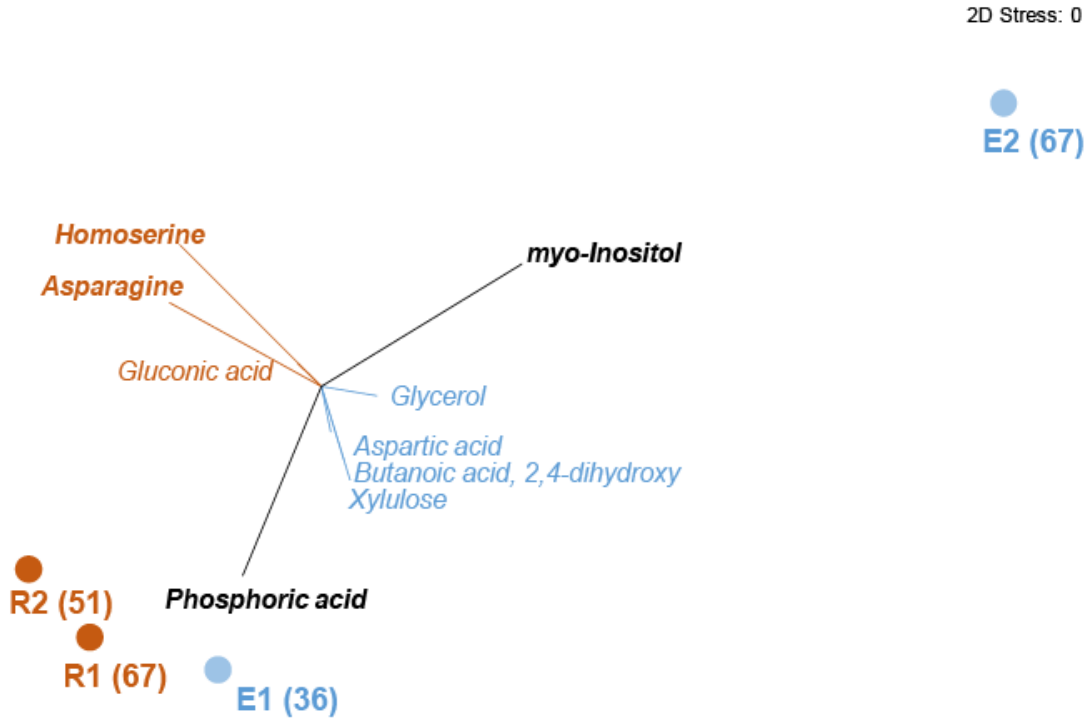
(c) Phaseolus

Root exudate similarity: 71 %; root similarity: 73 %
Dissimilarity: 68 %



(d) Pisum

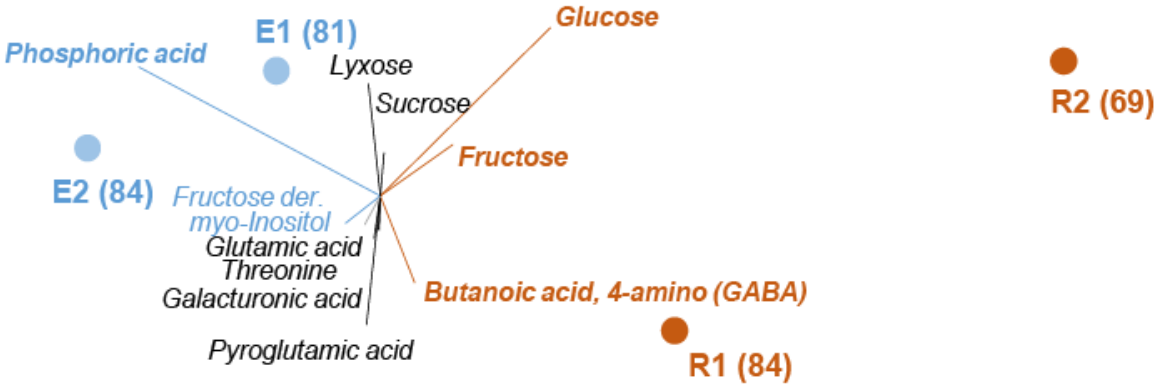
Root exudate similarity: 58 %, root similarity: 84 %
Dissimilarity: 75 %



(e) Tobacco

Root exudate similarity: 82 %, root similarity: 88 %
Dissimilarity: 63 %

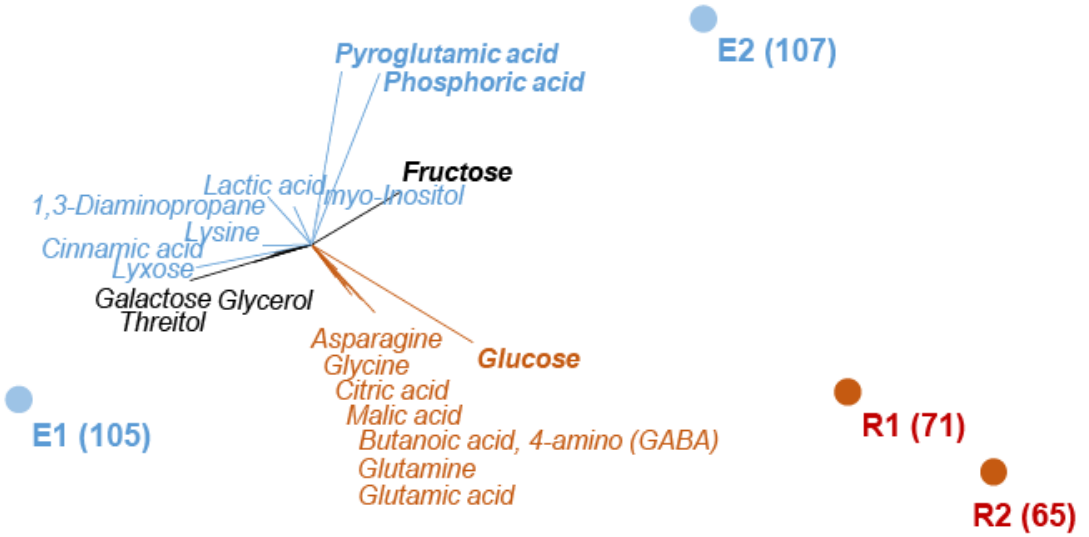
2D Stress: 0



(f) Maize

Root exudate similarity: 28 %, root similarity: 70 %
Dissimilarity: 66 %

2D Stress: 0



The numbers of detected PMs in roots and root exudates were rather similar, 84 and 69 in roots, and 81 and 84 in root exudates. Root exudate PM profiles showed an average similarity of 82 %, to which phosphoric acid, the sugars fructose, glucose and lyxose, and succinic acid contributed most. The dissimilarity (average 63 %) between root and root exudates was caused as follows (with decreasing contribution): phosphoric acid, glucose, the glutamic acid oxidation product GABA, fructose, lyxose and proline, besides of many other metabolites (Figure 3.2e).

3.3.2.6 Maize

Maize root PM profile similarity (average 70 %) was determined by the sugars glucose and fructose, gluconic acid, the amino acids alanine and glutamic acid, the glutamic acid oxidation product GABA, malic acid, citric acid, and phosphoric acid. Root exudate PM profiles showed only low similarity (average similarity 28 %) despite similar numbers of detected metabolites, 105 and 107; which however was much higher than in roots (65 and 71). 1,3-Diaminopropane, the amino acids, alanine and serine, lactic acid, succinic acid and glycerol contributed to root

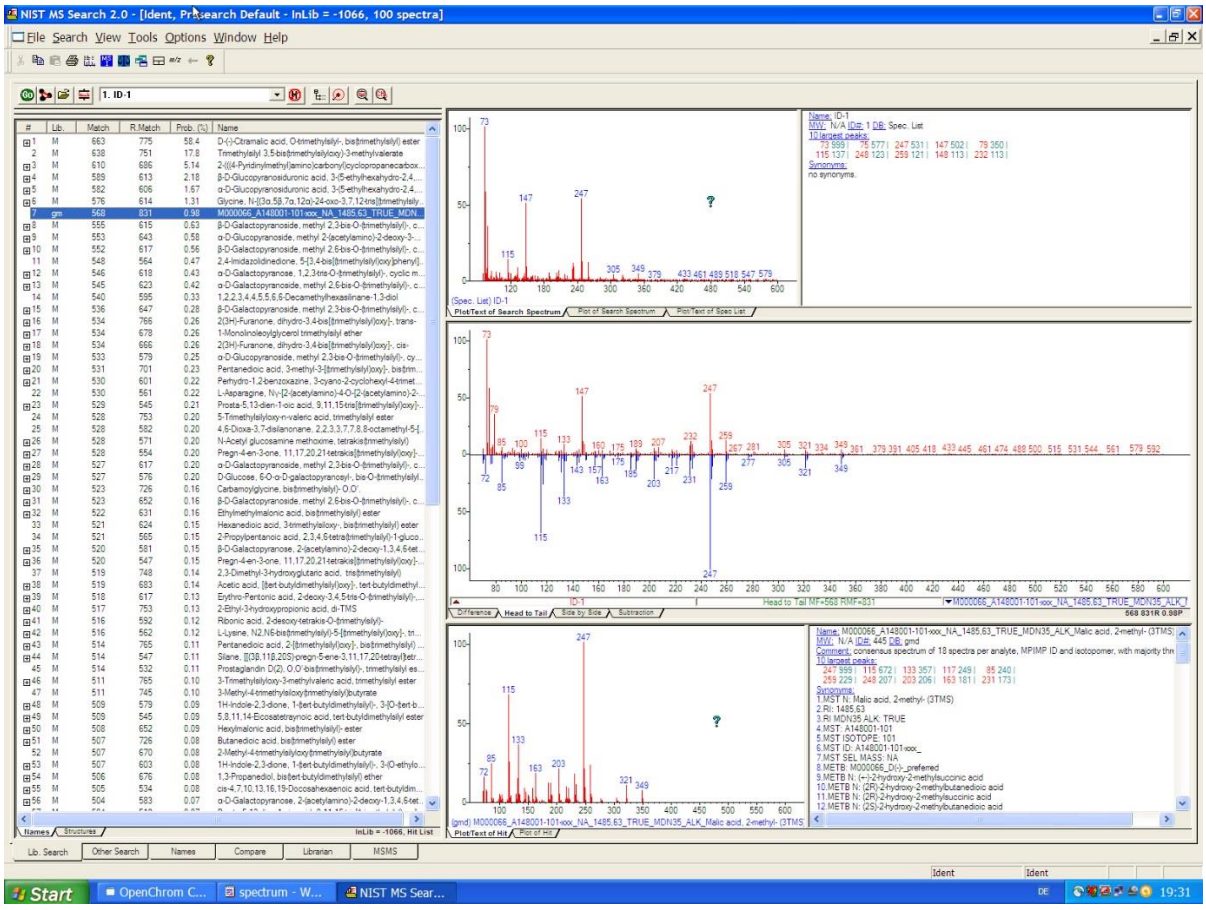


Figure 3.3. Tentative identification of citramalic acid on basis of its EI-MS spectrum

PM similarity. The dissimilarity (average 66 %) between root and root exudates was caused as follows (with decreasing contribution): glucose, fructose, phosphoric acid, pyroglutamic acid, threitol, glycerol, malic acid 1,3.diaminopropane, and citric acid, besides of many less pronounced differences of other metabolites (Figure 3.2f).

3.3.2.7 Citramalic acid

This organic acid was detected in the root exudates and root extracts of Arabidopsis but not in Rapeseed (Figure 3.3). Further, it showed in the root exudates of Phaseolus, Pisum and Maize but not in Tobacco. It was not detectable in the root extracts.

3.3.3 Secondary metabolites (SM) in roots and root exudates

SM profiling of the root exudates and the corresponding extracts was performed by HPLC–DAD (max. absorbance). UPLC–TOF/MS analyses were available only for the root exudate samples and used to obtain additional information for structure elucidation. For analytes, that SIMPER analysis identified as prominent contributor to similarity and dissimilarity, tentative structures are presented that were obtained on basis of a comparison of UV and MS data with the literature if possible. Appendix 3 (see CD-ROM) provides a summary of all SMS with their corresponding UV spectra that were detected in all analyses within the present thesis. Figure 3.4 summarizes the results that were obtained by a non-parametric multivariate analysis of the HPLC–DAD profiles.

3.3.3.1 Arabidopsis

Arabidopsis root exudate SM profiles revealed 29 and 28 detectable SMs in the two repeats, only a few more than in the roots (26 in both repeats) (Figure 3.4a). Analytes that were detected at 17.85, 33.68, 63.03 and 25.12 min and contributed to root exudate SM similarity (average 69 %), the first three represented exclusive root exudate SMs; analytes that were detected at 47.20, 44.90, 46.40 and 47.80 min to that of roots (average 78 %) as exclusive root SMs. The dissimilarity between root exudate and root SM profiles (average 55 %) was caused by analytes detected at 17.85, 38.45 and 42.77 min (in decreasing order), the latter two of which occurred in both organs.

The 17.85 min analyte was identified tentatively as a methoxylated 3,4,2',3',4'-pentahydroxy-trans-chalcone ($[M+H^+]^+$: 303.14, calc. 303.09; $[M+CH_3CN+H^+]^+$: 344.16, calc. 344.11; M^-: $[M-H^+]^-$: 301.12, calc. 301.08; $[M+HCOO^-]^-$: 347.12, calc. 347.08). The methoxylated chalcone

has not been described in the literature yet, 3,4,2',3',4'-pentahydroxy-trans-chalcone reportedly occurs in the heartwood of *Acacia confusa* (Wu *et al.*, 2008) and the aerial parts of *Bidens tripartita* (Lv & Zhang, 2013)

A further characteristic SM of the Arabidopsis root exudate was an analyte that eluted at 45.70 min. The UV spectrum suggested an indole structure and MS^+ data ($[M+H^+]^+$: 207.16, calc. 207.11; $[M+NH_4^+]^+$: 224.19, calc. 224.14). The proposed structure is fully methoxylated indole derivative (Figure 3.4a). The corresponding indole structure with one hydroxyl group not methylated was identified as component of hydroponically obtained root exudates from Arabidopsis (Strehmel *et al.*, 2014).

The third characteristic root exudate SM eluted at 63.03 min, the UV spectrum pointing to an aromatic compound. In the positive mode, a $[M+H^+]^+$ fragment of 404.19 was detected. The proposed non-phenolic unsaturated dicinnamoyl spermidine (Figure 3.4a) has a calc. mass of 404.23. If the unsaturated bond in the non-phenolic part is saturated, the structure reflects that of maytenine, a basidiomycete metabolite (Clericuzio *et al.*, 2007). Interestingly, two structurally similar spermidine conjugates are described, not with cinnamic but sinapic acid from Rapeseed seeds (Baumert *et al.*, 2005.) and *p*-coumaric acid from hydroponic Arabidopsis root exudates (Strehmel *et al.*, 2014), both of which add some further support for the proposed structure.

The fourth root exudate-specific SM eluted at 33.68 min. The UV spectrum suggested an aromatic compound but indoles with a conjugated formyl group can also show spectra with UV maxima above 300 nm (Pedras *et al.*, 2006). The M^+ spectrum showed a prominent fragment at 176.0717 and the M^- spectrum at 174.0563. This concurs with C_9H_5NOS (calc. $[M+H^+]^+$: 176.02; calc. $[M-H^+]^-$: 174.00). This fragment is the only stable MS fragment of the indole caulilexin A, an indole with two adjacent sulphur atoms, which stabilizes in both ionisation modes. The UV spectrum was in agreement with published data (Pedras *et al.*, 2006).

The fifth root exudate specific SM eluted at 25.12 min and showed a UV spectrum that was highly similar to that of a dioxomethylene cinnamide structure from the Rapeseed root exudates that will be discussed in more detail in the next paragraph. The M^+ spectrum showed a major fragment at 210.06. This agreed with a cinnamide structure with two hydroxyl and one methoxy group (calc. $[M+H^+]^+$: 210.07. A SciFinder search offers a commercial source but

no references for this structure, but the all-methoxy derivative was identified in extracts of leaves and stem bark of *Alstonia lenormandii*, an Apocynaceae (Legseir *et al.*, 1986).

Only the latter of these five root exudate-specific SMs was also detected in root extracts, but in minor relative amounts. Just as root exudates, the Arabidopsis roots were accumulated specific SMs (44.90, 46.40, 47.20 and 47.80 min).

3.3.3.2 Rapeseed

Rapeseed root exudate SM profiles were comprised of 26 detectable metabolites in each of the two repeats, which exceeded those of the roots (21 and 20) (Figure 3.4b). Analytes that were detected at 38.27, 40.30, 42.77 and 33.68 min contributed to root exudate SM similarity (average 85 %), amongst others, other analytes that were detected at 76.64 and 95.76 min to that of roots (average similarity 90 %). SM dissimilarity between root and root exudates (average 69 %) was supported by analytes that eluted at 40.30, 38.27 and 76.64 min (in decreasing order).

A prominent root exudate SM eluted at 38.27 min. The UV spectrum was identical with that of cinnamic acid in the library (Figure 3.4b). In the negative MS mode, the corresponding fragment showed: $[M–H^+]^-$: 147.04, calc. 147.04. Cinnamic acid was also present in the Arabidopsis root exudates, also exclusively but in less prominent amounts.

A second prominent root exudate SM that eluted at 40.30 min showed a similar UV spectrum as cinnamic acid (Figure 3.4b). Its MS in the positive mode agreed with a dioxomethylene cinnamide structure ($[M+H^+]^+$: 192.07, calc. 192.07; $[M+CH_3CN+H^+]^+$: 233.10, calc. 233.09) (Figure 3.4b). Even the di-hydroxylated derivative is not known as naturally occurring metabolite. This SM also occurred in Arabidopsis root exudates and extracts, but did not contribute substantially to their similarity or dissimilarity.

A third root exudate-specific SM eluted at 42.77 min; its UV spectrum suggesting an indole structure. The MS in the positive mode ($[M+H^+]^+$: 192.07, calc. 192.07) lent support for a 1-hydroxy-3-formyl-4-methoxyindole structure (Figure 3.4b). A very similar MS^+ has brassicanal A ($[M+H^+]^+$: 192.05), but the UV maximum shows a prominent additional maximum at 325 nm (Pedras *et al.*, 2006). Similarly as the dioxomethylene cinnamide, this SM was also present in Arabidopsis roots and root exudates.

Rapeseed root similarity was determined by numerous, mostly lipophilic organ-specific SMs, the majority of which was not detectable in the root exudates (Figure 3.4b).

3.3.3.3 Phaseolus

Phaseolus root exudate profiles were comprised of 28 detectable SMs in both repeats and considerably lower numbers in roots, 12 and 13 respectively (Figure 3.4c). SMs that were detected at 38.27 min, again cinnamic acid, 34.64 min, 60.35, 63.68 and 55.97 min contributed to the SM similarity of root exudates (average 97 %); different, more unipolar SMs to root SM similarity (average (84 %), which included peaks eluting at 79.60 and 104.61 min, amongst others (Figure 3.4c). The dissimilarity between root exudate and root SM profiles (average 78 %) was mostly caused by the root SM eluting at 79.60 min and the root exudates SM eluting at 38.27 min (cinnamic acid), 34.64 min, 60.35 and 55.97 min, besides of many less pronounced differences of other SMs.

The second most prominent root exudate SM in Phaseolus eluted at 34.64 min. Its UV spectrum suggested an aromatic compound. On basis of its MS data, a potential structure could be 4-hydroxy-1,4-benzoxazinone (HBOA). This is supported by the corresponding MS fragments: MS^+: $[M+H^+]^+$: 166.06, calc. 166.05, and $[M+CH_3CN+H^+]^+$: 207.08, calc. 207.08; MS^-: $[M–H^+]^-$: 164.04, calc. 164.04, and $[M+HCOO^-]^-$: 210.04, calc. 210.04. The UV spectrum (Figure 3.4c) agrees with the literature (Atkinson *et al.*, 1991).

Further characteristic SMs included two stilbene derivatives. They eluted at 60.35 and 63.68 min. Both showed resveratrol-type UV spectra with prominent maxima above 300 nm (Figure 3.4c). The first one could be 4-*O*-methylresveratrol ($[M+H^+]^+$: 243.06, calc. 2043.10; $[M–H^+]^-$: 241.05, calc. 241.09) (Kerem *et al.*, 2003), the second lacks one of the hydroxyl groups ($[M+H^+]^+$: 227.07, calc. 227.11; $[M+CH_3CN+H^+]^+$: 268.09, calc. 268.13; $[M–H^+]^-$: 225.05, calc. 225.09). The second is only known as synthetic analogue to naturally occurring stilbenes (Lion *et al.*, 2005). The position of the oxygen functions on the ring system could be different though the high correlation of the UV spectrum with that of resveratrol suggests a similar substitution pattern.

3.3.3.4 Pisum

Exudate profiles of Pisum roots were comprised of 15 and 17 SMs and root profiles of 19 in both repeats (Figure 3.4d). SMs that were detected at 38.27 min, again cinnamic acid, 51.01, 51.49, and 34.64 min (4-hydroxy-1,4-benzoxazinone) contributed most to SM similarity of root

exudates (average 70 %), besides a number of other root exudate specific SMs with minor contributions. Other, more unipolar SMs detected at 79.60, 76.64, and 79.04 min contributed to root similarity (average 89 %), amongst others (Figure 3.4d). The dissimilarity between root exudate and root SM profiles (average 48 %) was caused by the root SM that eluted at 79.60 min, and root exudate SMs eluting at 51.01, 51.49, and 34.64 min (4-hydroxy-1,4-benzoxazinone), besides of many less pronounced differences of other SMs.

The UV spectrum of the peak eluting at 51.01 min suggested an isoflavone structure. UPLC–ESI-TOF/MS analysis identified two related structures, but only in the positive mode. The first one was anhydropisatin ($[M+H^+]^+$ 297.08, calc. 297.08) (Dagne *et al.*, 1989), the second probably is its hydroxylated derivative ($[M+H^+]^+$ 313.24, calc. 313.07) (Figure 3.4d).

3.3.3.5 Tobacco

Exudate profiles of Tobacco roots were comprised of 18 and 17 detectable SMs in both repeats, the root profiles of 19 and 21 SMs (Figure 3.4e). SMs that were detected at 38.27 (cinnamic acid), 40.91, 24.91, and 77.60 min, besides of many other root-exudate-specific SMs, contributed to root exudate similarity (average 74 %), other, more unipolar SMs, e.g. eluting at 76.64, 90.45, 72.61, and 89.12 min, to root SM similarity (average 76 %). The dissimilarity between root exudate and root (average 74 %) was caused by the root SMs eluting at 76.64, 103.17, and 89.12 min and the root exudate SM cinnamic acid (38.27 min), besides of many less pronounced differences of other SMs (Figure 3.4e). The hitherto identified HBOA (34.64 min) was also present in tobacco root exudates though with less contribution to the differences between root and root exudates as in the investigated Fabaceae species.

The UPLC–TOF/MS analyses of the root exudates showed two prominent peaks in the TIC trace that could not be correlated to the major UV trace peaks with prominent UV spectra. Conversely, a notable peak with an unspecific UV spectrum could not be correlated with mass fragments at the corresponding retention time albeit the used stationary phases were of the

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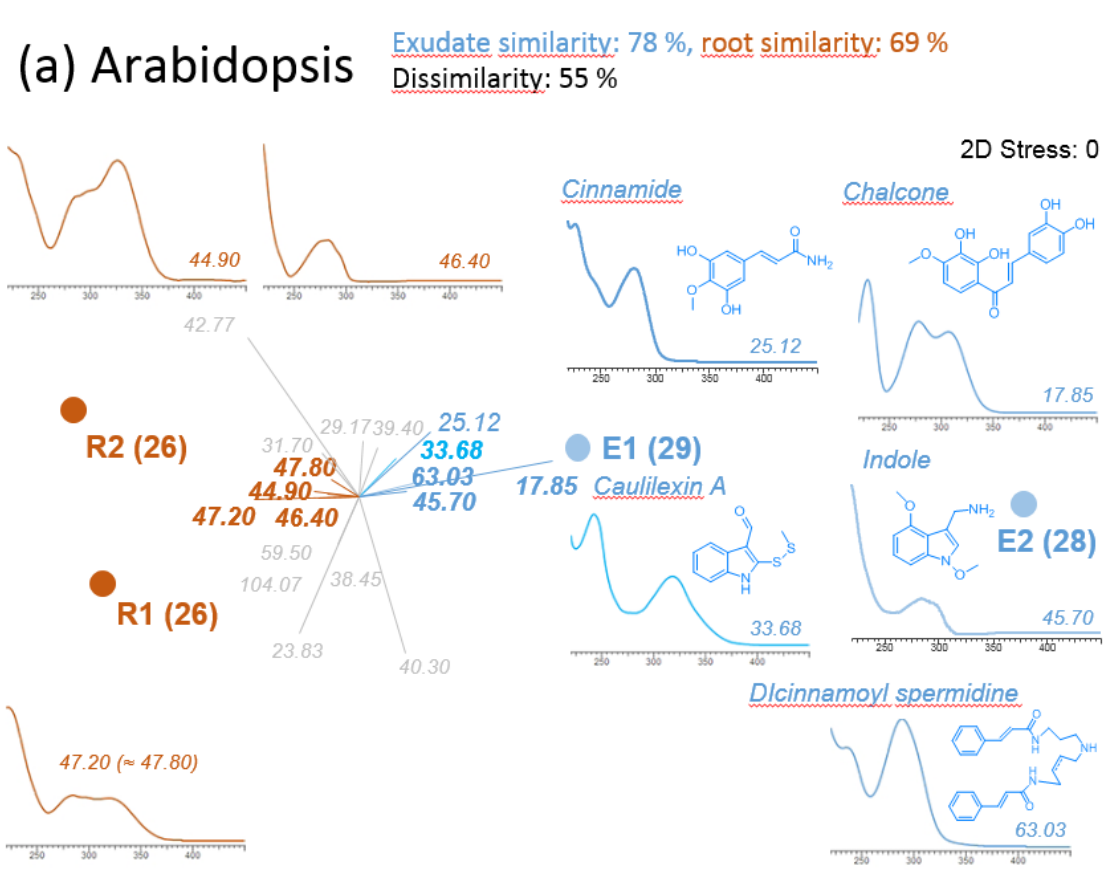
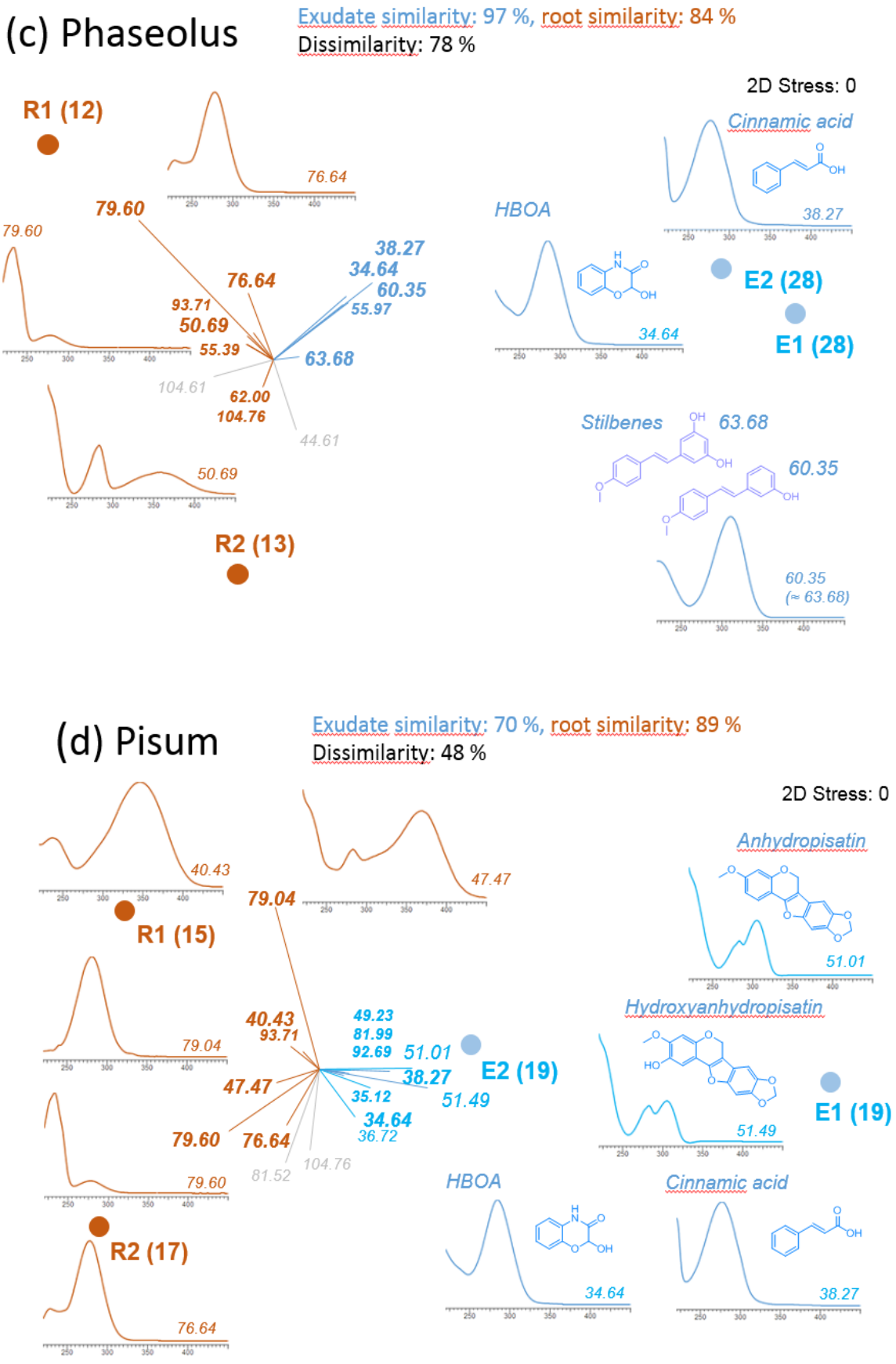
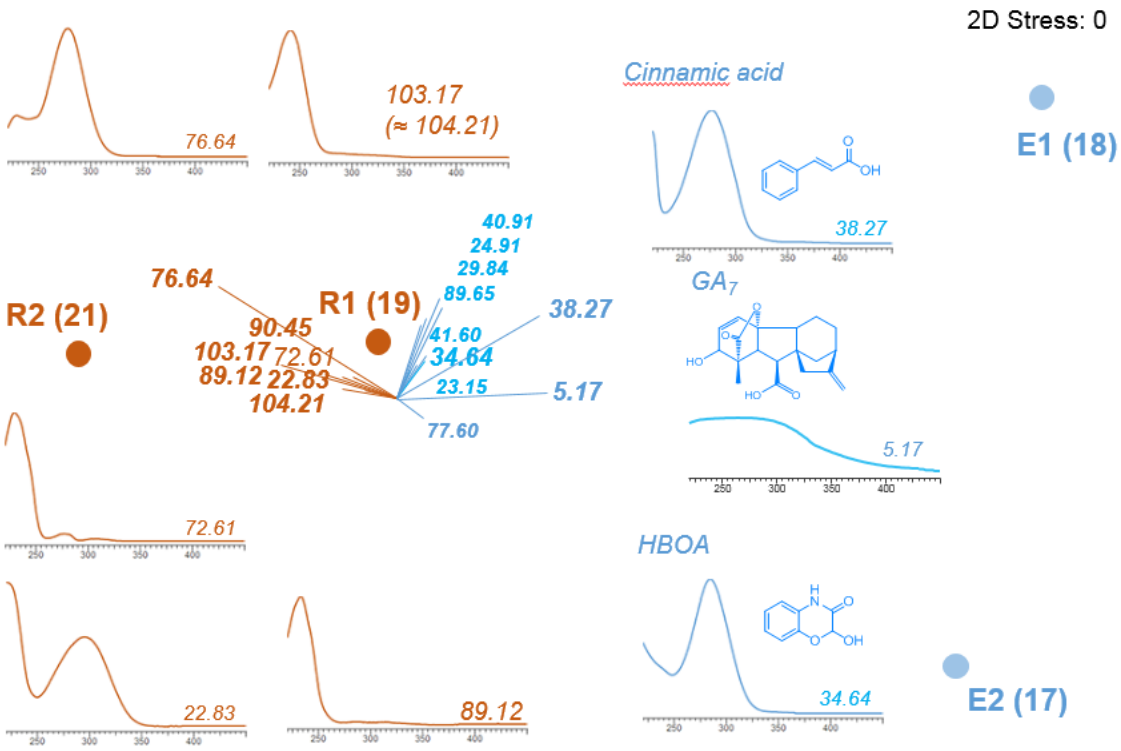
Figure 3.4. MDS plot of Bray-Curtis similarity of root exudate (**E1, E2**) and root (**R1, R2**) secondary metabolite (SM) profiles that were obtained by HPLC–DAD analyses of the ethyl acetate phase of the crude root exudate collection/extract; **(a)** Arabidopsis, **(b)** Rapeseed, **(c)** Phaseolus, **(d)** Pisum, **(e)** Tobacco, and **(f)** Maize. Contributions to root exudate similarity and root extract similarity are indicated as respective vectors. Grey vectors indicate metabolites that contribute more to the variation within than between groups. Bold analyte retention times indicate exclusive occurrence. Font size reflects contribution.


(a) Arabidopsis
Exudate similarity: 78 %, root similarity: 69 %
Dissimilarity: 55 %
2D Stress: 0
44.90
46.40
42.77
R2 (26)
R1 (26)
29.17
39.40
25.12
31.70
33.68
47.80
63.03
44.90
45.70
47.20
46.40
59.50
104.07
38.45
23.83
40.30
E1 (29)
17.85
Caulilexin A
Cinnamide
25.12
Chalcone
17.85
Indole
E2 (28)
45.70
33.68
DIcinnamoyl spermidine
63.03
47.20 (≈ 47.80)
(b) Rapeseed
Exudate similarity: 90 %, root similarity: 85 %
Dissimilarity: 69 %
2D Stress: 0
95.76
(≈ 94.75, 96.99, 104.21)
Cinnamic acid
38.27
104.21
73.32
95.76
96.99
91.81
94.75
76.16
31.70
76.64
38.45
R1 (20)
R2 (21)
38.27
40.30
42.77
33.68
Dioxomethylene
cinnamide
E1 (26)
E2 (26)
40.30
Caulilexin A
33.68
Indole
42.77
76.64


(c) Phaseolus
Exudate similarity: 97 %, root similarity: 84 %
Dissimilarity: 78 %
2D Stress: 0
R1 (12)
76.64
79.60
79.60
38.27
34.64
60.35
55.97
76.64
93.71
50.69
55.39
63.68
104.61
62.00
104.76
44.61
50.69
R2 (13)
HBOA
Cinnamic acid
38.27
34.64
E2 (28)
E1 (28)
Stilbenes
63.68
60.35
60.35
(≈ 63.68)
(d) Pisum
Exudate similarity: 70 %, root similarity: 89 %
Dissimilarity: 48 %
2D Stress: 0
40.43
47.47
79.04
R1 (15)
Anhydropisatin
51.01
79.04
40.43
93.71
49.23
81.99
92.69
51.01
38.27
51.49
35.12
34.64
36.72
47.47
79.60
76.64
104.76
81.52
79.60
R2 (17)
76.64
E2 (19)
Hydroxyanhydropisatin
51.49
E1 (19)
HBOA
Cinnamic acid
34.64
38.27

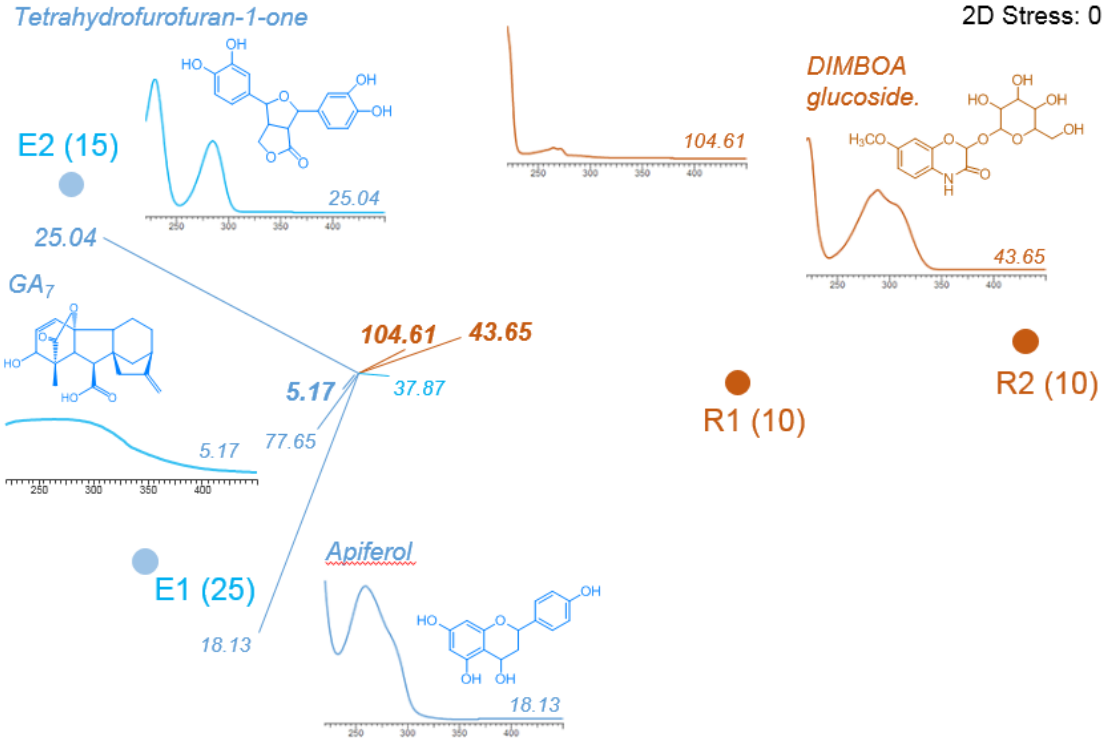
(e) Tobacco

Exudate similarity: 74 %, root similarity: 76 %
Dissimilarity: 74 %



(f) Maize

Exudate similarity: 63 %, root similarity: 87 %
Dissimilarity: 66 %



same type. The larger of the peaks eluting at 5.1 min in the TOF/MS TIC trace of the two could be a gibberellin derivative GA_7 ($[M+NH_4^+]^+$: 348.27, calc. 348.18; $[M–H^+]^–$: 329.23, calc. 329.14). The second peak at 4.9 min showed mass fragments that were lower by 2 Daltons ($[M+NH_4^+]^+$: 346.25, calc. 346.17; $[M–H^+]^–$: 327.22, calc. 329.14). The calculated masses are identical; either if the unsaturation is located on a C–C or C–O bond. A CAS SciFinder™ similarity structural search revealed only a saturated form of GA_7. The correlation with the prominent peak at 5.17 min., a retention time that is far too low, is corroborated by the fact, that the same phenomenon with the identical analytes could be observed in the Maize root exudates (see ongoing text). The difference in the retention times between the HPLC–DAD and UPLC–TOF/MS analyses could be explained by the contrasting application of phosphoric and formic acid in the eluent, which could cause different effects on the dissociation behaviour of GA_7. The gibberellins were detected in the root exudates but in the root extracts.

3.3.3.6 Maize

Exudate profiles of Maize roots were comprised of 26 and 16 SMs in both repeats. Despite of the considerable differences in numbers between the two root exudate repeats, both root extract repeats showed 10 SMs (Figure 3.4f). SMs that eluted at 25.04 min, 18.13 and 100.63 min contributed to root exudate SM similarity (average 63 %), the same SMs, eluting at 25.04 and 18.13 min, and a further SM eluting at 43.65 min to root SM similarity (average 87 %), amongst others. The dissimilarity between root exudate and root SMs (average 66 %) was caused by the SMs eluting at 25.04 min and 18.13, both of which occurred in much higher proportions in root exudates than in roots.

The UV spectrum of the SM eluting at 25.04 min resembled a tetrahydrofurofuran lignan (Figure 3.4f). Despite displaying a prominent peak in the UV trace, this SM was nearly absent in the MS TIC trace, both in the positive and negative mode. The fragment with the highest intensity was 234.0536 in the negative mode. This would correspond to a $C_{12}H_{10}O_5^{2–}$ fragment (calc. 234.0539) of a tetrahydro-4,6-bis(4-hydroxy-3-methoxyphenyl)-1H,3H-furo[3,4c]furan-1-one that was characterized recently from Maize stems (Jung *et al.*, 2015). A weak fragment barely distinguishable of the noise with a mass of 343.2117 provides some support, though a weak one, for a structure of all-hydroxyl derivative of the described furane-1-one lignan ($[M–H^+]^–$ calc. 343.0823). Unfortunately, no UV data have been provided for the Maize furan-1-one lignan.

A tentative structure for the SM eluting at 18.13 min is the flavanol apiferol (Figure 3.4f), sometimes also called apiforol, ($[M+H^+]^+$: 275.14, calc. 275.09; $[M–H^+]^–$: 273.13, calc. 273.08; $[M+ HCOO^–]^–$: 319.1311, calc. 319.0823). This structure is reported to occur in maize cobs as phlobaphene precursor (Styles & Ceska, 1975). Similarly as Tobacco, GA_7 was present at 5.17 min, again only in the root exudates. Cinnamic acid (38.27 min) and HBOA (34.64 min) were detectable in the root exudates but did not contribute to similarity as substantially than in other investigated plant species.

3.3.4 Total comparison of root exudates with roots

Two ANOSIM analyses were performed to explore if any general differences between root exudate and the root-extractable metabolites exist, one for PMs (Figure 3.5a), one for SMs (Figure 3.5b).

The PM profiles differentiated root exudates and roots significantly at the 0.1% level when SMs from all six investigated plant species were considered. About one quarter off all detected PMs (186, see Appendix 2 on the CD-ROM) contributed to the ordination that is based on Bray-Curtis similarity. Phosphoric acid, albeit being more a nutrient than a PM, was the one with the highest contributors (Figure 3.5a). Among the "true" PMs, the sugars glucose and fructose, the sugar alcohol *myo*-inositol, various proteinogenic amino acids, such as asparagine, aspartic acid, and alanine, non-proteinogenic amino acids, such as pyroglutamic acid and homoserine, and the glutamic acid oxidation product GABA, and the organic acids malic and lactic acid were most involved in structuring the grouping. Homoserine is well-known to occur in Pisum root exudates (Vanderlinde *et al.*, 2014). Root PMs profiles showed more similarity between species than root exudate PMs.

Likewise, SM profiles caused root exudates and root extracts ton differ significantly at a level of 0.1% when all six investigated plant species were included into the analysis (Figure 3.5b). Here the proportion of metabolites that supported the grouping was even higher, roughly by 10 %, which also was reflected in the better separation of the two groups (lower MDS stress level). The similarity within groups and between repeats was lower than in PMs, the dissimilarity between the two groups higher. Specific SMs contributed to the similarity of root

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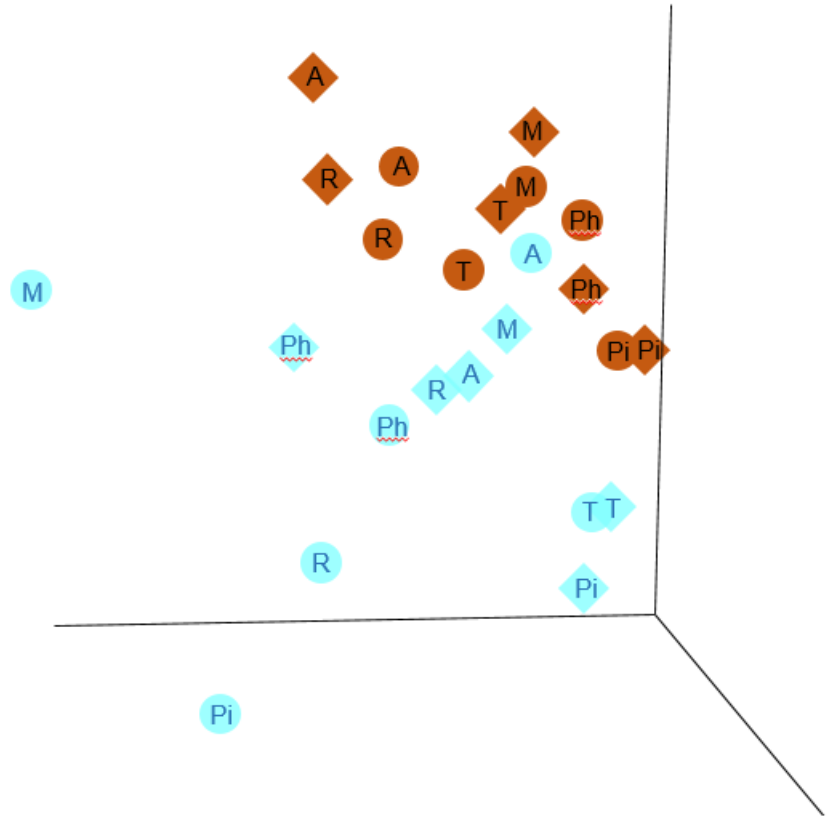
Figure 3.5. MDS plot of Bray-Curtis similarity of **(a)** primary metabolites (PM) and **(b)** secondary metabolites (SM) in root exudates and root extracts. Contributions to similarity and dissimilarity were determined by SIMPER, group difference by ANOSIM analysis; A, Arabidopsis; R, Rapeseed; Ph, Phaseolus; Pi, Pisum; T, Tobacco; M, Maize; circle symbol, first repeat, diamond symbol, second repeat.

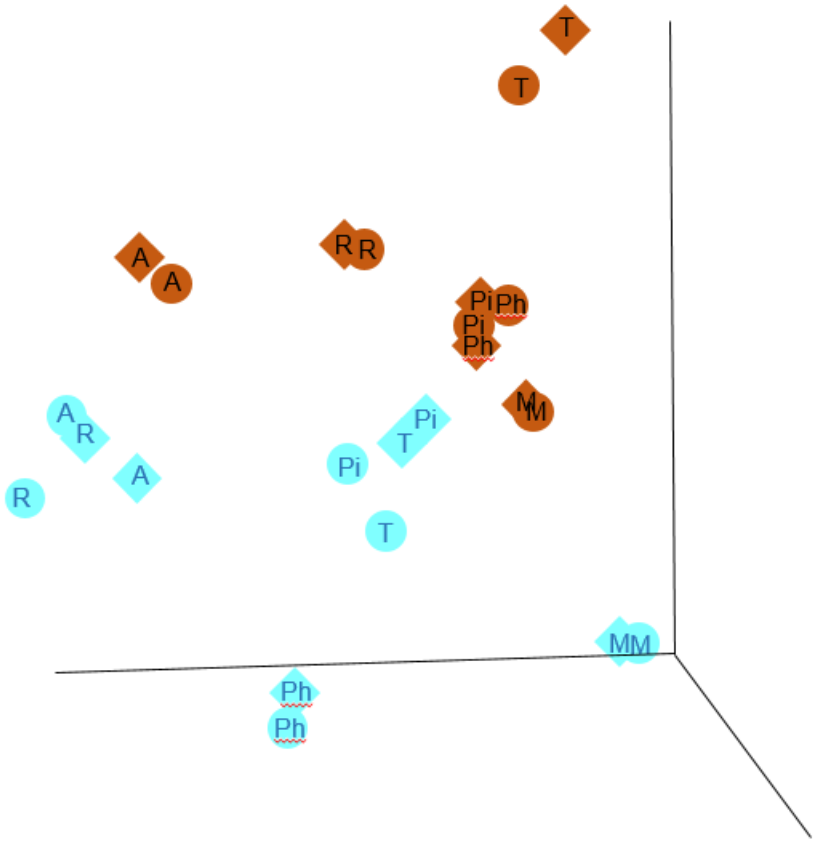
a)

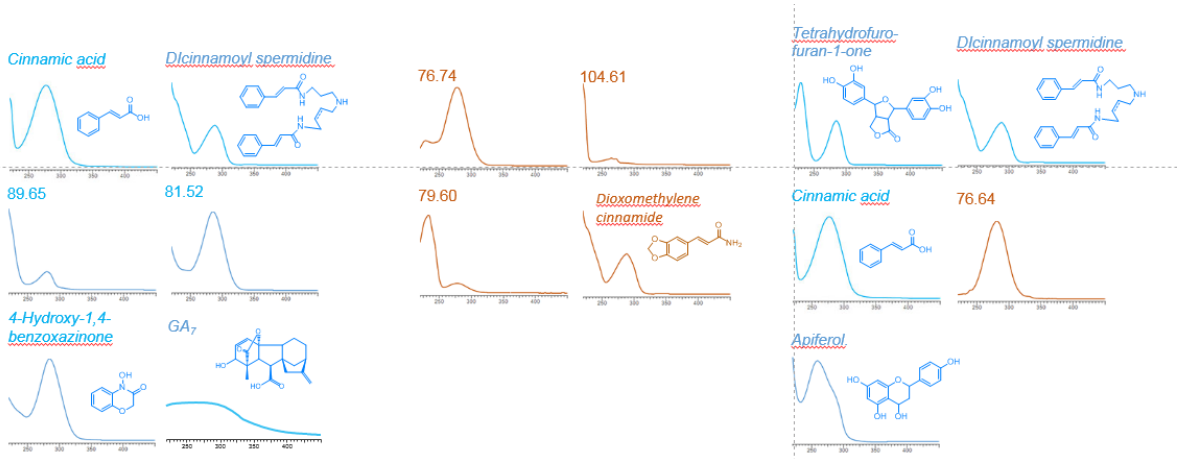
Primary Metabolites (PMs)
3D Stress: 0.09
Significance level: 0.1 %
Global R: 0.232



Similarity (% contrib.)		Dissimilarity (% contrib.)
Phosphoric acid (21)	**Phosphoric acid (11)**	Phosphoric acid (11)
Glucose (11)	**Glucose (10)**	Asparagine (8)
Fructose (4)	**Fructose (9)**	Aspartic acid (7)
Asparagine (0.5)	**Asparagine (9)**	Glucose (7)
GABA (-)	**GABA (7)**	*myo*-Inositol (6)
Glycerol (4)	Glycerol (5)	Fructose (5)
Alanine (2)	Alanine (4)	GABA (3)
***myo*-Inositol (15)**	*myo*-Inositol (4)	Pyroglutamic acid (2)
Pyroglutamic acid (3)	Pyroglutamic acid ((3)	Glycerol (2)
Serine (0.5)	Serine (3)	Malic acid (2)
Lactic acid (6)	Lactic acid (2)	Homoserine (2)
Aspartic acid (3)	Aspartic acid (3)	Lactic acid (2)
Galactose (2)	Galactose (1)	Alanine (2)
Average: 42 b%	Average 69 %	Average 69 %

b) **Secondary Metabolites (SMs)**
3D Stress: 0.07
Significance level: 0.1 %
Global R: 0.327





Similarity (% contrib.):

Cinnamic acid (14)
Dicinnamoyl spermidine (7)
89.65 (5)
81.52 (4)
4-Hydroxy-1,4-benzoxazinone (3)
GA_7 (0.69, but much higher in TIC!)

Average 31 %

76.74 (5)
104.61 (4)
79.60 (3)
Dioxomethylene cinnamide (2)

Average 42 %

Dissimilarity (% contrib.):

Tetrahydrofurofuran-1-one (7)
Dicinnamoyl spermidine (7)
Cinnamic acid (5)
79.04 (4)
Apiferol (3)

Average 74 %

exudate and root profiles. Cinnamic acid contributed most to root exudate SM similarity. In fact, it was the only SM that was present in every analysed root exudate sample but in none of the root extracts.

Another SM that was present in all root exudates except those of the Brassicaceae, was 4-hydroxy-1,4-benzoxazinone. The contribution of GA_7 and its unsaturated derivative is most probably higher than indicated by the UV trace due to its unspecific and weaker absorption. Similarly, as root extracts, root exudates profiles share a number of more unpolar metabolites

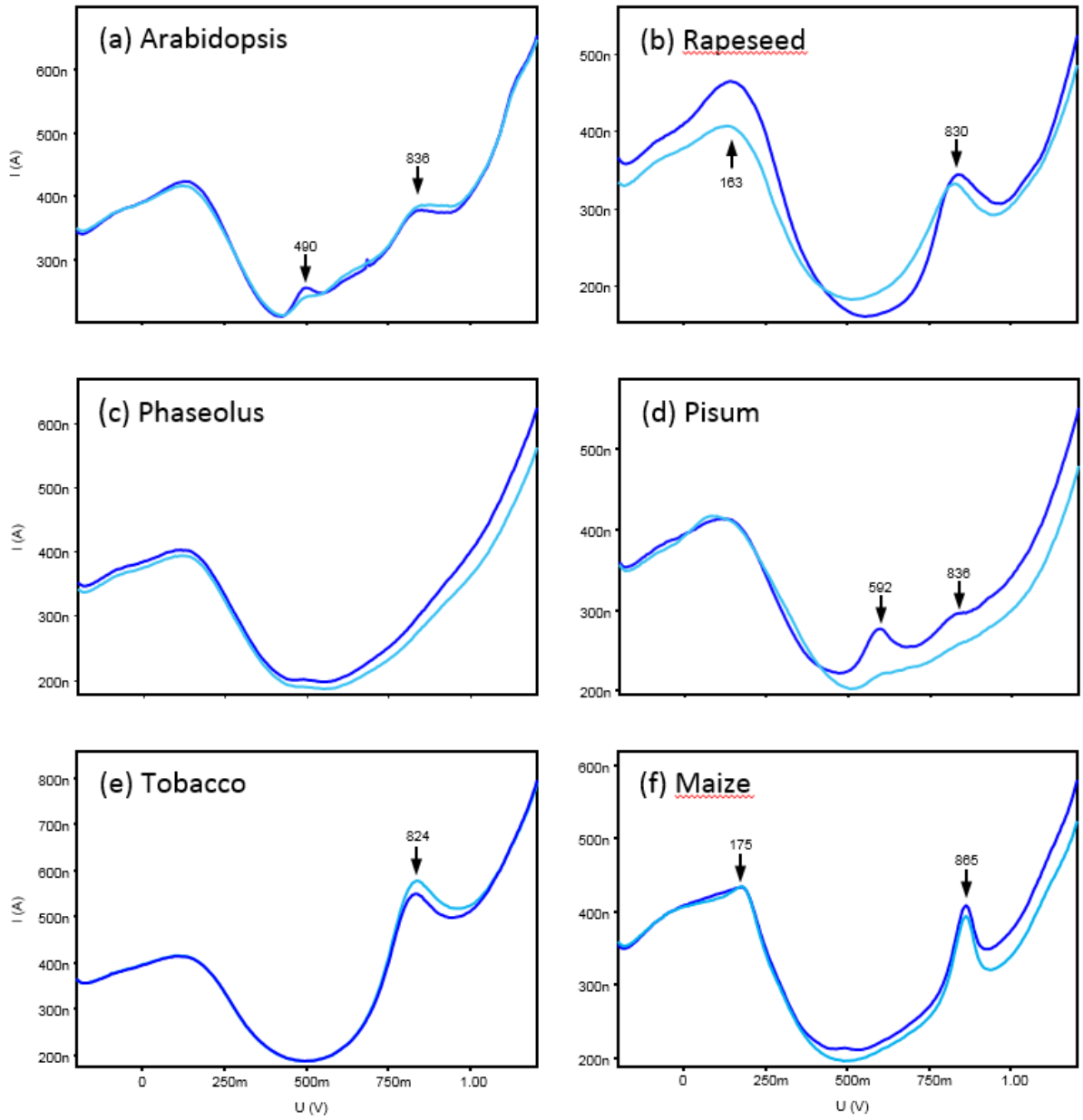


Figure 3.6. Differential pulse voltammograms of crude root exudates, first repeat, second repeat; working electrode, glassy carbon; counter electrode, platinum wire; reference electrode, Ag/AgCl; 1 M acetate buffer (pH = 3.6).

that, however, differ between the two groups. Prominent characteristic SMs in the profiles of the single species contribute to dissimilarity. In contrast to the PMs, only a handful of SMs are shared between species. Affiliation to a specific plant family reflects itself in ordination proximity in cases when two genera of the same plant family were included into the investigation.

3.3.5 Differential pulse voltammetry (DPV) of crude root exudates

Figure 7 compares differential pulse voltammograms (DPV) of the two repeats from the crude (unfractionated) root exudates of the six plant species that were included into this study. By the majority, the repeats compared to each other. Only Phaseolus showed no distinctive peaks; Arabidopsis at 490 and 836 mV (Figure 3.6a), Rapeseed at 163 and 830 mV (Figure 3.6b), Pisum at 592 and 836 mV (Figure 3.6d), Tobacco at 824 mV (Figure 3.6e), and Maize at 175 and 865 mV (Figure 3.6f). The analysis is destructive and requires certain sample amounts. Only the water phases could be analysed separately in addition. Without exception, however, the characteristic peaks from the crude root exudates vanished (data not shown).

3.4 Discussion

The six investigated plant species yielded similar amounts of root exudates, around 1 mg/g FW PMs, and about 0.01–0.02 mg/g FW SMs. The ration between SMs and PMS is 1–2 to 100. The situation in roots is completely different. Roots of Rapeseed and Maize contained just three-times as much PMs as SMs (Figure 3.1). The culture regime that was used for this study was especially aimed at achieving optimal conditions to compare six different plant species in a soil environment because this physiological scenario reflects natural conditions much more than hydroponic cultures do (Farrar & Jones, 2003). A recent review about carbon flow in the rhizosphere—root exudation represents one major component of this process—states that the concepts of tackling with this phenomenon are believed to be clear but the mechanistic insights are still lacking in many aspects (Jones *et al.*, 2009). A major aim of this study was to contribute to a better understanding of soluble PMs and SMs pattern formation in root exudation by comparing six plant species that were subjected to more or less comparable growth conditions. To our knowledge, no study with a comparable scope was performed so far. Interestingly, in terms of amounts, more variation was found in the root extractable PMs and SMs than in those from the root exudates. This suggests that the root–soil concentration gradient and the permeability of the plasma membrane could affect exudation dynamics more

than the spatial location of the metabolites in the root tissue. Chemical and physical parameters might apply more than accommodation facilities for metabolite accumulation that is determined both genetically and epigenetically.

PMs, or central metabolites, as they are called more often recently, represent the major portion of plant metabolites in root exudates (Jones *et al.*, 2009). This was also the case in this study. PM profiles differed between species. In some cases such as Rapeseed and Maize, root exudate PM profiles showed much lower similarity than root extract PM profiles. From the data that have been obtained in this study, it is difficult to decide if this is an analytical artefact or if the polymerization dynamics of the PMs into mucilage affected the results. Mucilage is not a polygalacturonic acid polymer, as some of the literature suggests and even though the chemical properties may be similar (Albalasmeh & Ghezzehei, 2014), but its soluble analysable fraction consist besides various uronic acids of sugars and amino acids (Moody *et al.*, 1988). In this study, the most common analyte was phosphoric acid in the root exudates and the roots, which was to be expected as the plants were provided with ample fertilizer. Besides, the sugars glucose and fructose were prominent components but, due to their wide and variable occurrence, did not contribute to the specificity of the PM patterns of each investigated species (Figures 3.2a–f, Figure 3.5a). If the obtained data could point out to a more root exudate-specific PM, the sugar alcohol *myo*-inositol would rank as the most promising candidate. Conversely, the amino acids asparagine, serine and GABA earned similar significance for the root extracts. Uronic acids were detected only in minor amounts in all species. The differences in the PM patterns were substantial enough, however, to divide root exudate PMs and root extract PMs into two significantly different groups though considerable overlap characterized many metabolites (R = 0.232, Figure 3.5a).

A special PM is citramalic acid, which represents a methylated citric acid derivative that was first isolated from apple peels (Hulme, 1954). Further, it occurs in root exudates of sugar beet (Khorassani *et al.*, 2011), and, more recently, in Green Bean (Phaseolus) and Soybean (*Glycine* max)root exudates (Tawaraya *et al.*, 2014a; Tawaraya *et al.*, 2014b). In this study, both investigated Fabaceae, Phaseolus and Pisum, also showed citramalic acid in their root exudates. It was further detected in Arabidopsis, both in root exudates and root extracts, and Maize, for both of which it is known to occur as metabolite in aerial parts (Fiehn *et al.*, 2000; Zhang *et al.*, 2011).

In contrast to PMs, secondary plant metabolites, or as they are called in more recent studies, specialized metabolites, represented only minor components of root exudates. The main attempts to propose structures on basis of UV and MS data was focussed on those peaks that contributed to their similarity and dissimilarity in the root exudate–root extract comparison that was calculated on basis of the two repeats by SIMPER analysis. Though average similarity was a little bit lower than for PMs, the ANOSIM analyses yielded revealed that root exudates and root extracts form also two groups when compared on basis of their SMs. (Figure 3.5b). This can be more expected in case of SMs as they are considered to be more unique and less widespread in terms of occurrence within plants than the PMs (Hartmann, 2007). During the first survey of the HPLC analyses results, a prominent peak at the unpolar end of the chromatographic run at 100.63 min (see Appendix 3) caught attention because it was present in every root exudate analysis. It was decided to exclude this analyte from the various statistical analyses because of doubts about its nature of original root exudate components. Still, several rather unpolar analytes in the root exudates and even more so in the root extracts contributed to their respective similarity (Figure 4.5b). Apart from the lipophilic analytes, one hydrophilic metabolite was present in all root exudate samples that was identified tentatively as cinnamic acid (Figures 3.4 and 3.5b). Cinnamic acid was also detected by GC–MS, but only in some root exudate samples. Cinnamic acid is known from many root exudate studies. Furthermore, it was detected by applying various methodologies. Those included GC–MS in soybean (*Glycine max*) (Tawaraya *et al.*, 2014b),) ion chromatography with conductivity detection in rice *Oryza sativa* (Zheng *et al.*, 2014), GC–MS in tobacco (Yu *et al.*, 2013), HPLC–DAD in apple rootstock seedlings (Zhang *et al.*, 2009) and HPLC–DAD as well as HPLC–Q-TRAP/MS in barley by (Lanoue *et al.*, 2010).

The second most widespread SM was HBOA that was detected in all root exudates apart from Arabidopsis and Rapeseed (Figures 5 and 6). HBOA is known as benzoxazinone precursor in many cereals (Niemeyer, 2009) but was detected also as metabolite of dicotyl plants (Huo *et al.*, 2005). Its occurrence in all of the non-Brassicaceae species suggests that it might constitute a rather widespread root exudate SM.

Another SM in Arabidopsis root exudates is a cinnamide derivative with two hydroxyl and one methoxy group (Figure 5a). This is not the first proposal for the occurrence of cinnamides in root exudates, a hydroxylated derivative was reported as constituent of wheat root exudates (Warren, 2015). The cinnamide was also present in the root extract. Further, a chalcone

derivative with four hydroxyl and one methoxy group was detected. Another chalcone, phloridzin, with four hydroxyl groups, is to occur in root exudates of apple seedlings (Hofmann *et al.*, 2009). The chalcone structure that was also present in Rapeseed root exudates. This finding is rather unexpected for Brassicaceae, but not for root exudates in general.

The at first glance most exotic root exudate SM was a potential cinnamoyl spermidine structure (Figure 3.4a). In hydroponically obtained Arabidopsis root exudates, however, a similar structure is to occur, but instead of cinnamic acid with *p*-coumaric acid a partial structure (Strehmel *et al.*, 2014). Additionally, two indole derivatives can be proposed as further characteristic root exudate SMs of Arabidopsis. One of them showed substantial agreement with caulilexin A, an indole with two adjacent sulphur atoms. The second indole derivative could possess a second amino group and two methoxy groups, 1-methoxy-3-formyl-4-methoxyindole. The hydroxylated derivative—in this study it was detected in the Rapeseed root exudates—was detected as a component of hydroponically obtained Arabidopsis root exudates (Strehmel *et al.*, 2014). The former sulphur indole belongs to a group of phytoalexins that has been intensively studied in Rapeseed and Cauliflower (Pedras *et al.*, 2006). Summing up, a first survey of major SMs in Arabidopsis root exudates revealed some conformance but also some substantial differences to the reported SMs in the hydroponically obtained root exudate of Arabidopsis (Strehmel *et al.*, 2014). Further studies have to explore if the different collection method and or sample preparation are the reason.

The root exudate SM profiles of Rapeseed showed much similarity to Arabidopsis. This was not so pronounced in case of the root extracts (Figure 3.4b). Both species were characterized by different sets of specific SMs. At the present time point, the UPLC–TOF/MS data were not available yet but a thorough analysis of the root extract SMs that compares to that of the root exudate SMs is projected. The similarity of Arabidopsis and Rapeseed root exudate SMs was caused by the co-occurrence of several SMs. One of the most prominent was the in all investigated root exudates present cinnamic acid that contributed also substantially to Rapeseed root exudate similarity (Figure 3.4b). Indoles were also present, caulilexin A this time both in roots and in root exudates. 1-Hydroxy-3-formyl-4-methoxyindole was the monomethoxylated precursor (42.77 min) of the dimethoxylated 1-methoxy-3-formyl-4-methoxyindole in Arabidopsis (45.70 min). Furthermore, a different cinnamide derivative, this time with a dioxomethylene moiety, was present in larger amounts. In contrast to the Arabidopsis-specific cinnamide, this derivative was present in both species. The chalcone from

the Arabidopsis root exudates was also present in Rapeseed, however only in minor concentrations. Apart from these metabolites, a coumarin (29.17 min) was detected in Arabidopsis and flavonoids (39.40 min, 43.96 min) in both Arabidopsis and Rapeseeds, but only in minor amounts. The detected SMs in the Arabidopsis and Rapeseed root exudates reflected the classification of the two families to the same family, Brassicaceae, much more the extractable root SMs.

Phaseolus and Pisum presented the second pair of species that belong to the same plant family, in this case Fabaceae. In contrast to Brassicaceae, the majority of SMs differed between the two species, in root exudates and in root extracts. Both had only cinnamic acid and HBOA in common, but a similar situation applies to Tobacco and Maize too. The root exudates of Phaseolus contain stilbenes (Figure 3.4c). This is not unexpected as Fabaceae, together with Dipterocarpaceae, Gnetaceae and Vitaceae belong to plant families that are well-known to produce this type of SMs (Riviere *et al.*, 2012). Pisum, by contrast, produces isoflavones in the root exudates, anhydropisatin and its hydroxylated derivative (Figure 3.4d), but not pisatin that is mentioned in many other reports (Carlson & Dolphin, 1981; Yamada *et al.*, 2005; Makarova *et al.*, 2016). Isoflavones represent well-known SMs within Fabaceae that were even shown to stimulate *Rhizobium* sp. to colonize their roots and form nodules (Makarova *et al.*, 2016).

Tobacco root exudate and root extract SMs differed as to be expected. Their dissimilarity was caused by numerous specific SMs, most of them in lower amounts. HBOA was present among the root exudate SMs. One of the more prominent SMs was cinnamic acid but there was a further, very polar metabolite, the spectral information of which pointed to a gibberellic acid derivative, GA_7 (Figure 3.4e). According to literature, this is not unexpected (Mada & Bagyaraj, 1993; Tawaraya *et al.*, 2014a).

Maize root exudate SMs also differed from those in their root extract. The number of detectable SMs in root exudates was the lowest of all investigated plant species (Figure 3.4f). The UV spectra identified two prominent aromatic compounds that were also present in the root extracts but in lower amounts. The root extracts contained a SM with characteristic UV spectrum of a benzoxazinone and its probable identity is DIMBOA glucoside. The only identified benzoxazinone in root exudates was HBOA however. This is quite notable because DIMBOA and other benzoxazines are regarded as root exudate components of maize and other grasses (Macias *et al.*, 2006; Niemeyer, 2009; Neal *et al.*, 2012). One of the two aromatic

SMs could be apiferol, a flavanol, that resembles catechin, another flavanol with a more prominent record in root exudate research (Blair *et al.*, 2006). The second aromatic SM probably could have a tetrahydrofurofuran-1-one structure. A similar lignan structure was described from stems at least (Jung *et al.*, 2015).

No chromatographical methodology exists that allows a complete detection of all metabolites in a sample. For this reason, an electrochemical technique, differential pulse voltammetry was employed to provide alternative information about the chemical characteristics of plant root exudates from the six investigated species (Figure 4.6). Impressively, the method suggested a high proportion of repeatability for a single investigated species in terms of oxidizable analytes. The presented voltammograms demonstrate the applicability of this electrochemical technique for studying root exudates. So far, differential pulse voltammetry has also been used to study the coordination complex formation of copper with isoflavones that are present in the root exudates of *Lupinus albus* (lupine) (Jung *et al.*, 2003). It is quite possible that the peaks in the voltammogram are not caused by the analytes that we have identified by GC and HPLC, but by coordination complexes of these compounds in which the serve as ligands for mineral nutrient central atoms. Unfortunately, a coordination complex does not survive the chromatographical procedures. In case of GC, the considerably higher molecular weight prevents the required volatilization; in case of LC, the usual low-pH milieu results in its decomposition.

3.5 Conclusion

Four questions were posed in context with this study:

1) The exuded amounts of primary (PM) and secondary plant metabolites (SM) compared more than those extractable from the roots did.

2) SMs were more specific for an investigated plant species than PMs were. Some of them only occurred in roots and some of them only in root exudates. PM dissimilarity between roots and root exudates was caused more by quantitative than qualitative differences.

3) Citramalic acid can occur in the root exudates of many plant species, but it was also detected in Arabidopsis roots.

4) The applied collection procedure of root exudates provided good yields. SMs, however, require the pooling of the samples from different plant individual. The so far obtained analyses results points to a potential selection pressure to small SM molecules—for instance, no glycosides were detected. This contrasts a published study with excellent research on the chemical composition of hydroponically obtained root exudates from Arabidopsis in which many dimers and some oligomers are reported. In soil, mucilage formation might incorporate these analytes into non-analysable polymer structures, a process that might be considerably slowed by the higher dilution in the nutrient solution in a hydroponic regime.

3.6 References

Albalasmeh, AA, Ghezzehei, TA. 2014. Interplay between soil drying and root exudation in rhizosheath development. *Plant and Soil* **374**: 739–751.

Atkinson, J, Morand, P, Arnason, JT, Niemeyer, HM, Bravo, HR. 1991. Analogs of the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA): decomposition to benzoxazolinones and reaction with β-mercaptoethanol. *Journal of Organic Chemistry* **56**: 1788–1800.

Badri, DV, Vivanco, JM. 2009. Regulation and function of root exudates. *Plant, Cell and Environment* **32**: 666–681.

Baumert, A, Milkowski, C, Schmidt, J, Nimtz, M, Wray, V, Strack, D. 2005. Formation of a complex pattern of sinapate esters in *Brassica napus* seeds, catalyzed by enzymes of a serine carboxypeptidase-like acyltransferase family? *Phytochemistry* **66**: 1334–1345.

Blair, AC, Nissen, SJ, Brunk, GR, Hufbauer, RA. 2006. A lack of evidence for an ecological role of the putative allelochemical (+/-)-catechin in spotted knapweed invasion success. *Journal of Chemical Ecology* **32**: 2327–2331.

Brett, CMA, Brett, AMO. 1993. Electrochemistry. Principles, Methods, and Applications. Oxford, New York: Oxford University Press.

Carlson, RE, Dolphin, DH. 1981. Chromatographic analysis of isoflavonoid accumulation in stressed *Pisum sativum*. *Phytochemistry* **20**: 2281–2284.

Clericuzio, M, Tabasso, S, Garbarino, JA, Piovano, M, Cardile, V, Russo, A, Vidari, G. 2007. Non-phenolic dicinnamamides from *Pholiota spumosa*. Isolation, synthesis and antitumour activity. *European Journal of Organic Chemistry* **2007**: 5551–5559.

Dagne, E, Bekele, A, Waterman, PG. 1989. The flavonoids of *Millettia ferruginea* subsp. *ferruginea* and subsp. *darassana* in Ethiopia. *Phytochemistry* **28**: 1897–1900.

Escudero, N, Marhuenda-Egea, FC, Ibanco-Cañete, R, Zavala-Gonzalez, EA, Lopez-Llorca, LV. 2014. A metabolomic approach to study the rhizodeposition in the tritrophic interaction. Tomato, Pochonia chlamydosporia and Meloidogyne javanica. *Metabolomics* **10**: 788–804.

Farrar, JF, Jones, DL. 2003. The control of carbon acquisition by the growth of roots. In: Kroon, H de, Visser, EJW, eds. Root Ecology. Berlin: Springer, 91–124.

Fiehn, O, Kopka, J, Trethewey, RN, Willmitzer, L. 2000. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. *Anal. Chem.* **72**: 3573–3580.

Hartmann, T. 2007. From waste products to ecochemicals. Fifty years research of plant secondary metabolism. *Phytochemistry* **68**: 2831–2846.

Hofmann, A, Wittenmayer, L, Arnold, G, Schieber, A, Merbach, W. 2009. Root exudation of phloridzin by apple seedlings (*Malus* x *domestica* Borkh.) with symptoms of apple replant disease. *Journal of Applied Botany and Food Quality-Angewandte Botanik* **82**: 193–198.

Hulme, AC. 1954. The isolation of L-citramalic acid from the peel of the apple fruit. *Biochimica et Biophysica Acta* **14**: 36–43.

Huo, C-H, Wang, B, Lin, W-H, Zhao, Y-Y. 2005. Benzoxazinones from Acanthus ilicifolius. *Biochemical Systematics and Ecology* **33**: 643–645.

Jones, DL, Nguyen, C, Finlay, RD. 2009. Carbon flow in the rhizosphere. Carbon trading at the soil–root interface. *Plant and Soil* **321**: 5–33.

Jung, C, Maeder, V, Funk, F, Frey, B, Sticher, H, Frossard, E. 2003. Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification. *Plant and Soil* **252**: 301–312.

Jung, Y-J, Park, J-H, Cho, J-G, Seo, K-H, Lee, D-S, Kim, Y-C, Kang, H-C, Song, M-C, Baek, N-I. 2015. Lignan and flavonoids from the stems of *Zea mays* and their anti-inflammatory and neuroprotective activities. *Archives of Pharmacal Research* **38**: 178–185.

Kerem, Z, Regev-Shoshani, G, Flaishman, MA, Sivan, L. 2003. Resveratrol and two monomethylated stilbenes from Israeli *Rumex bucephalophorus* and their antioxidant potential. *Journal of Natural Products* **66**: 1270–1272.

Khorassani, R, Hettwer, U, Ratzinger, A, Steingrobe, B, Karlovsky, P, Claassen, N. 2011. Citramalic acid and salicylic acid in sugar beet root exudates solubilize soil phosphorus. *BMC Plant Biology* **11**: 121.

K**opka, J, Schauer, N, Krueger, S, Birkemeyer, C, Usadel, B, Bergmüller, E, Dörmann, P, Weckwerth, W, Gibon, Y, Stitt, M, Willmitzer, L, Fernie, AR, Steinhauser, D. 2005.** GMD@CSB.DB. the Golm Metabolome Database. *Bioinformatics* **21**: 1635–1638.

Kuijken, RCP, Snel, JFH, Heddes, MM, Bouwmeester, HJ, Marcelis, LFM. 2015. The importance of a sterile rhizosphere when phenotyping for root exudation. *Plant and Soil* **387**: 131–142.

Lanoue, A, Burlat, V, Henkes, GJ, Koch, I, Schurr, U, Roese, Ursula S. R. 2010. De novo biosynthesis of defense root exudates in response to Fusarium attack in barley. *New Phytologist* **185**: 577–588.

Legseir, B, Cherif, A, Richard, B, Pusset, J, Labarre, S, Massiot, G, Le Men-Olivier, L. 1986. Alkaloids of *Alstonia lenormandii*, a structural revision of 10-methoxycompactinervine. *Phytochemistry* **25**: 1735–1738.

Lion, CJ, Matthews, CS, Stevens, MFG, Westwell, AD. 2005. Synthesis, antitumor evaluation, and apoptosis-inducing activity of hydroxylated (*E*)-stilbenes. *Journal of Medicinal Chemistry* **48**: 1292–1295.

Lv, J-L, Zhang, L-B. 2013. Flavonoids and polyacetylenes from the aerial parts of *Bidens tripartita*. *Biochemical Systematics and Ecology* **48**: 42–44.

Macias, FA, Marin, D, Oliveros-Bastidas, A, Chinchilla, D, Simonet, AM, Molinillo, Jose M. G. 2006. Isolation and synthesis of allelochemicals from gramineae: benzoxazinones and related compounds. *Journal of Agricultural and Food Chemistry* **54**: 991–1000.

Mada, RJ, Bagyaraj, DJ. 1993. Root exudation from *Leucaena leucocephala* in relation to mycorrhizal colonization. *World Journal of Microbiology & Biotechnology* **9**: 342–344.

Makarova, LE, Dudareva, LV, Petrova, IG, Vasil'eva, GG. 2016. Secretion of phenolic compounds into root exudates of pea seedlings uponi inoculation with *Rhizobium leguminosarum* bv. *viceae* or *Pseudomonas siringae* pv. *pisi*. *Applied Biochemistry and Microbiology* **52**: 205–209.

Moody, SF, Clarke, AE, Bacic, A. 1988. Structural analysis of secreted slime from wheat and cowpea roots. *Phytochemistry* **27**: 2857–2861.

Neal, AL, Ahmad, S, Gordon-Weeks, R, Ton, J. 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PloS one* **7**: e35498.

Niemeyer, HM. 2009. Hydroxamic acids derived from 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one. Key defense chemicals of cereals. *Journal of Agricultural and Food Chemistry* **57**: 1677–1696.

Pedras, MSC, Adio, AM, Suchy, M, Okinyo, DPO, Zheng, Q-A, Jha, M, Sarwar, MG. 2006. Detection, characterization and identification of crucifer phytoalexins using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. *Journal of Chromatography A* **1133**: 172–183.

Riviere, C, Pawlus, AD, Merillon, J-M. 2012. Natural stilbenoids: distribution in the plant kingdom and chemotaxonomic interest in Vitaceae. *Natural Products Reports* **29**: 1317–1333.

Strehmel, N, Böttcher, C, Schmidt, S, Scheel, D. 2014. Profiling of secondary metabolites in root exudates of *Arabidopsis thaliana*. *Phytochemistry* **108**: 35–46.

Styles, E, Ceska, O. 1975. Genetic control of 3-hydroxy- and 3-deoxy- flavonoids in *Zea mays*. *Phytochemistry* **14**: 413–415.

Tawaraya, K, Horie, R, Saito, S, Wagatsuma, T, Saito, K, Oikawa, A. 2014a. Metabolite profiling of root exudates of common bean under phosphorus deficiency. *Metabolites* **4**: 599–611.

Tawaraya, K, Horie, R, Shinano, T, Wagatsuma, T, Saito, K, Oikawa, A. 2014b. Metabolite profiling of soybean root exudates under phosphorus deficiency. *Soil Science and Plant Nutrition* **60**: 679–694.

Uren, NC. 2007. Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. In: Pinton, R, Varanini, Z, Nannipieri, P, eds. The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Boca Raton, London, New York: CRC Press, 1–23.

van Dam, NM, Bouwmeester, HJ. 2016. Metabolomics in the rhizosphere. Tapping into belowground chemical communication. *Trends in Plant Science* **21**: 256–265.

Vanderlinde, EM, Hynes, MF, Yost, CK. 2014. Homoserine catabolism by *Rhizobium leguminosarum* bv. *viciae* 3841 requires a plasmid-borne gene cluster that also affects competitiveness for nodulation. *Environmental Microbiology* **16**: 205–217.

Warren, CR. 2015. Wheat roots efflux a diverse array of organic N compounds and are highly proficient at their recapture. *Plant and Soil* **397**: 147–162.

Wu, J-H, Tung, Y-T, Chien, S-C, Wang, S-Y, Kuo, Y-H, Shyur, L-F, Chang, S-T. 2008. Effect of phytocompounds from the heartwood of *Acacia confusa* on inflammatory mediator production. *Journal of Agricultural and Food Chemistry* **56**: 1567–1573.

Yamada, K, Higashinakasu, K, Shigemori, H, Hasegawa, K. 2005. Isolation and identification of an allelochemical exuded from germinating Pea (*Pisum sativum*) seeds. *Heterocycles* **65**: 267.

Yu, H, Shen, G, Gao, X. 2013. Determination of tobacco root exudates by GC-MS. *Zhongguo Yancao Xuebao* **19**: 64–72.

Zhang, J, Zhang, D, Mao, Z, Li, Z, Zhao, M. 2009. Isolation and identification of root exudates in different kinds of apple rootstock seedlings. *Hebei Nongye Daxue Xuebao* **32**: 29–32.

Zhang, Q, Pettolino, FA, Dhugga, KS, Rafalski, JA, Tingey, S, Taylor, J, Shirley, NJ, Hayes, K, Beatty, M, Abrams, SR, Zaharia, LI, Burton, RA, Bacic, A, Fincher, GB. 2011. Cell wall modifications in maize pulvini in response to gravitational stress. *Plant Physiology* 156: 2155–2171.

Zheng, X, Chen, S, Li, Q, Lin, R, Lin, W. 2014. Determination of phenolic acids in root exudates of allelopathic rice by solid phase extraction-ion chromatography with conductivity detection. *Analytical Letters* **47**: 2156–2164.

4. Plant Metabolites in Root Exudates: Effect of Water Deficit Compared in Six Plant Species

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Abstract: Six crop plants, Arabidopsis (*A. thaliana*), Rapeseed (*Brassica napus*), Phaseolus (*Phaseolus vulgaris*), Pisum (*Pisum sativum*), Tobacco (*Nicotiana tabaccum*) and Maize (*Zea mays*) were exposed to water deficit (WD) for 14 days after 22-day growth. Root exudates of the soil-grown plants were collected 9 days after re-watering. The control treatment was watered regularly.

WD treatment reduced the biomass production until the time point of root exudate collection and increased the shoot: root ratio. Phaseolus and Maize, by contrast, showed a more opportunistic behaviour by increasing root development in attempts to counter the stress in this way resulting in a decreased shoot: root ratio. A more conservative strategy was chosen by the other four species.

WD treatment increased levels of stress-associated primary metabolites (PM), such as glucose, fructose, proline and GABA. Changes in secondary metabolite (SM) profiles included the new appearance of dihydrophenanthrenes in Pisum and the higher frequency of occurrence of aldehyde structures. Total amounts of PM and SM fractions increased after WD treatment. PM changes were more general and allowed differentiation of the two treatment groups. By contrast, SM changes were highly species-specific.

The obtained results clearly demonstrated that a stress event such as water deficit reflects itself in the amounts and in the chemical composition of root exudates.

4.1 Introduction

Climate warming is expected to increase extreme weather events such as flooding caused by heavy rainfall and drought (Salinger, 2005). This will especially affect forests and rain-fed crop productions systems. The physiological mechanisms of plants are still poorly to understood to make predictions or develop counteractive measures (McDowell *et al.*, 2008). In an attempt to address this, the present study was aimed at exploring how a water deficit (WD) period affects plant root exudation in terms of amounts and profiles of plant primary and secondary

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metabolites (PM and SM). Due to the difficult collection procedure, studies on plant metabolites in root exudates are much more scarce than on plant tissues (van Dam & Bouwmeester, 2016).

Existing studies that explored the effect of abiotic factors on root exudation rather focussed on nutrient uptake, especially that of phosphorus (Penaloza *et al.*, 2002; Tawaraya *et al.*, 2014b), or on heavy metal stress (Wang *et al.*, 2006; Luo *et al.*, 2014), but not on water deficit. Looking at more recent reviews of plant root exudates, one might get the impression that participation in biotic interactions, rather trendily termed "chemical communication in the rhizosphere", such as effects on insect herbivory and their natural enemies, phytophagous nematodes, plant–plant communication, and plant–microbe interactions, represent more important issues (Badri & Vivanco, 2009; van Dam & Bouwmeester, 2016).

One climate chamber was available for this study and thus it was decided to affect water deficit by not watering 22 days-old plants for two consecutive weeks. After this water deficit period, the plants were again regularly watered for 9 days until root exudate collection. The 9-day recovering period was regarded as necessary to make certain that (1) water supply conditions were comparable at the analysis time point and (2) any metabolic reprogramming that occurred in plant tissues shows in the root exudate profiles. Control plants were watered regularly for the whole period.

Six plant species were included into the investigation on basis of two requirements: (1) the candidate plant should be of economic importance, and (2) it should sufficiently resilient to the stress treatment. The final choice included Arabidopsis and Rapeseed, two Brassicaceae. Phaseolus (Green Bean) and Pisum (Pea) represented Fabaceae. Further, Tobacco (Solanaceae) and Maize were included, the latter to cover the agriculturally important grasses. These six plant species represented a compromise between available climate chamber space and manageable sample numbers. Preliminary experiments were carried out to make certain that the chosen plants developed properly within the experiment duration and tolerated the projected water deficit treatment.

Chapter 3 presents a comparative analysis of the root exudates from the chosen six plant species. The pooled crude root exudates were partitioned into a water and ethyl acetate fraction, the former of which was analysed by GC–MS and the latter of which by HPLC–DAD and UPLC–TOF/MS. All of which represent methodologies that are used routinely in root

exudate analysis (van Dam & Bouwmeester, 2016). Additionally, an electrochemical method, differential pulse voltammetry (Brett & Brett, 1993), was used as alternative method for detecting changes in the chemical composition of root exudates.

Several questions were central to this study:

(1) How does water deficit (WD) affect biomass production?
(2) How does WD affect amounts of PMs and SMs in root exudates?
(3) Are qualitative changes in root exudate PMs and SMs more general or species-specific after WD treatment?

4.2 Material and Methods

See Chapter 2.

4.3 Results

4.3.1 Water deficit (WD) effect on plant biomass

Appendix 1 (see CD-ROM) illustrates how plants looked alike after 14 days of water deficit (WD) and 9 days later when watering supply was applied regularly again. WD stress reduced shoot and root biomass whenever applied (Figure 4.1a). Most striking was the only rather low retardation of Phaseolus root growth (three quarters of the untreated plants). The two Brassicaceae, Arabidopsis and Rapeseed, had their root and shoot biomass reduced roughly to one half. The two Fabaceae, Phaseolus and Pisum, by contrast, showed striking differences: shoot biomass was more decreased in Phaseolus than in Pisum; the root biomass showed the opposite picture. Tobacco had its shoot and root biomass reduced to one-quarter; maize the shoot biomass to one-quarter and the root to one-half compared to the untreated plants.

A comparison of shoot to root ratios of untreated (C) versus stressed (WD) plants provides even more differentiated insights (Figure 4.1b). Compared to root biomass, shoot biomass of

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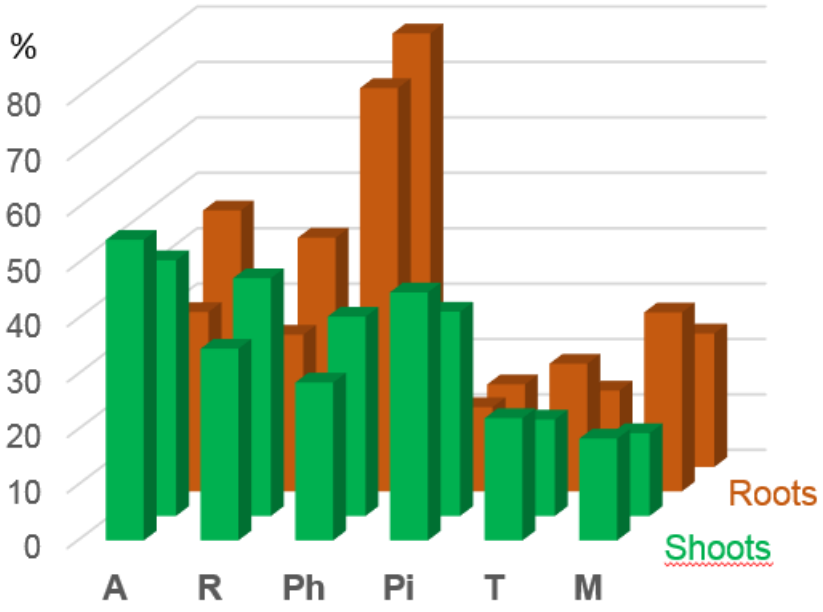
Figure 4.1. Effect of water deficit (WD) treatment (14 days) on plant biomass production and metabolite biosynthesis; A, Arabidopsis, R, Rapeseed, Ph, Phaseolus; Pi, Pisum; T, Tobacco; M, Maize: **(a)** Biomass of WD treated plants in % of control (both repeats); **(b)** shoot : root ratio of control and WD treated plants (both repeats); **(c)** Primary metabolites (PM) and secondary metabolites (SM) in the root exudates of WD treated and control plants (C); total amount of water (PM) and ethyl acetate (SM) fraction (mg/g FW); numbers indicate replicate.

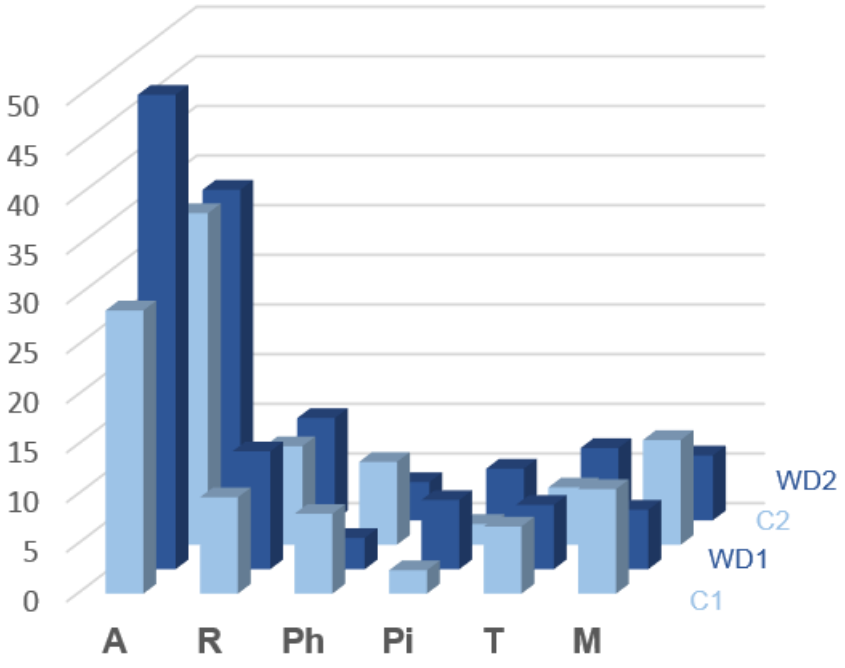
(a)

WD1, WD2 (both repeats):
% dry wt of control (C1, C2)

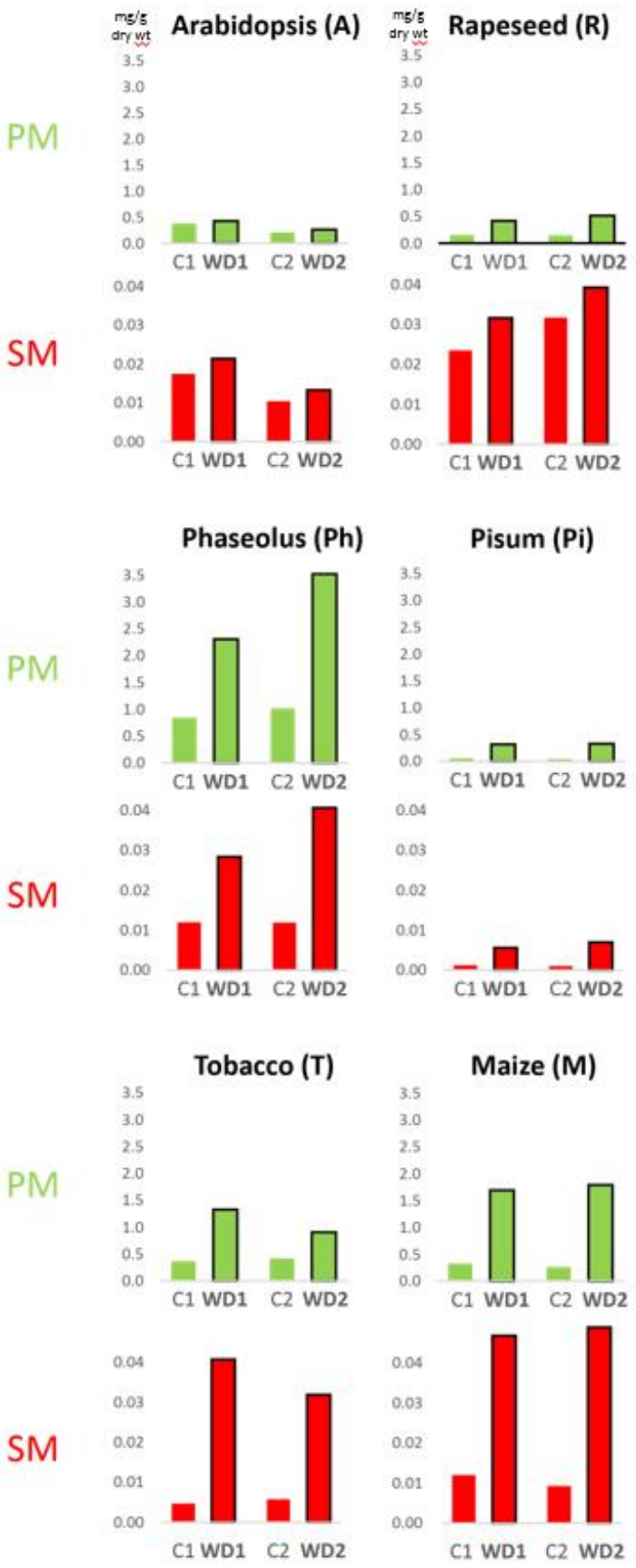


(b)

Shoot : root ratio of control and treatment plants (both repeats)



(c)


mg/g dry wt
Arabidopsis (A)
Rapeseed (R)
Phaseolus (Ph)
Pisum (Pi)
Tobacco (T)
Maize (M)
PM
SM
C1 WD1 C2 WD2

Arabidopsis is much higher than that of the other investigated plant species. In all cases, where flower development was under way in control, the stress application caused delayed development (Appendix 1). In the majority of cases, the shoot: root ratio was higher in the stressed plants, only in Phaseolus and Maize it decreased.

4.3.2 Water deficit (WD) effect on primary and secondary plant metabolite (PM and SM) amounts

Figure 4.1c facilitates a rough comparison by providing the recalculated mg/g root amounts from the pooled root exudate samples and the mean root fresh weight. A general trend is visible in both repeats: both PMs and SMs amounts increase in the root exudates of the stressed plants, albeit by variable amounts. The identical scales of the bar graphs allows a relative comparison. The Brassicaceae Arabidopsis and Rapeseed resemble each other. Both are comprised of comparatively low PM amounts but rather high SM amounts, both of which moderately increase following water deprivation. Likewise to the biomass development, the two Fabaceae Phaseolus and Pisum differed considerably in the general amounts of PMs and SMs in the root exudate. In both species, however, the amounts increased roughly three-times after exposure to WD. Tobacco and maize, albeit unrelated, compared more to each other than to any of the other investigated species. In terms SM increase after WD treatment, both species showed the largest percentage.

4.3.3 Water deficit (WD) effect on primary plant metabolite (PM) profiles

Primary metabolite profiling—a few secondary metabolites were detected too—of the root exudates was performed with GC–MS after chemical derivatisation. The tentative identification of the analytes is based on comparison of retention indices and mass spectra that are available in the Golm metabolome database (Kopka *et al.*, 2005). A summary of all metabolites that were identified in this thesis can be found in Appendix 2 (see CD-ROM-ROM). Figure 4.2 provides a summary of the results that were obtained by the non-parametric multivariate analysis of the metabolite profiles.

4.3.3.1 Arabidopsis

The WD treatment caused several PMs in Arabidopsis to increase, among of which the sugars fructose and glucose, and the amino acid proline were most prominent. These metabolites also contributed most to the similarity of the WD plants (average 56 %). The similarity of the

control plants (average 31 %) was much lower and determined by the sugars fructose, mannose, ribose and arabinose. Further metabolites included the amino acids aspartic acid and pyroglutamic acid, phosphoric acid, lactic acid and the sugar alcohol *myo*-inositol. The dissimilarity between the two treatment groups was 64 % (average) and mainly caused by the sugars glucose, talose, fructose, mannose, arabinose, the sugar alcohols pinitol and *myo*-inositol, and the amino acid proline, besides many other PMs with more minor contributions. The Arabidopsis root exudates showed 88 and 77 PMs that changed to 77 and 74 by WD treatment. Figure 4.2a provides a summary of the Arabidopsis results.

4.3.3.2 Rapeseed

The second Brassicaceae, Rapeseed, compared to Arabidopsis by accumulating the amino acid proline after water deficit treatment, which, together with the sugar galactose contributed specifically to WD PM similarity (average 34 %). Again, control similarity was lower (29 %) and specifically caused by accumulation of the sugar alcohol *myo*-inositol. The average dissimilarity was similar to that of Arabidopsis (67 %). The major contributors to dissimilarity in PM patterns were the sugar alcohol *myo*-inositol, phosphoric acid, the amino acids proline, and pyroglutamic acid, isocitric acid, and benzoic acid, amongst many other PMs with more minor contributions. The lower similarity between root exudate PMs could be affected by the high difference between the detected PM numbers in the control root exudates, 62 and 96. Similarly as in Arabidopsis, the detected PM numbers in the water deficit treatment were more close, 90 and 88 respectively. Figure 4.2b provides a summary of the Rapeseed results.

4.3.3.3 Phaseolus

The first of the two investigated Fabaceae species, Phaseolus, differed from Arabidopsis and Rapeseed, the two Brassicaceae. The WD treatment repeats showed low similarity (average 23 %), to which increased amounts of the sugar alcohol *myo*-inositol and the sugar glucose contributed most. By contrast, the controls showed much higher similarity (average 71 %) that was caused the amino acid aspartic acid, alanine, and pyroglutamic acid, phosphoric acid, glucose and *myo*-inositol. The major contributors to the dissimilarity (average 60 %) of

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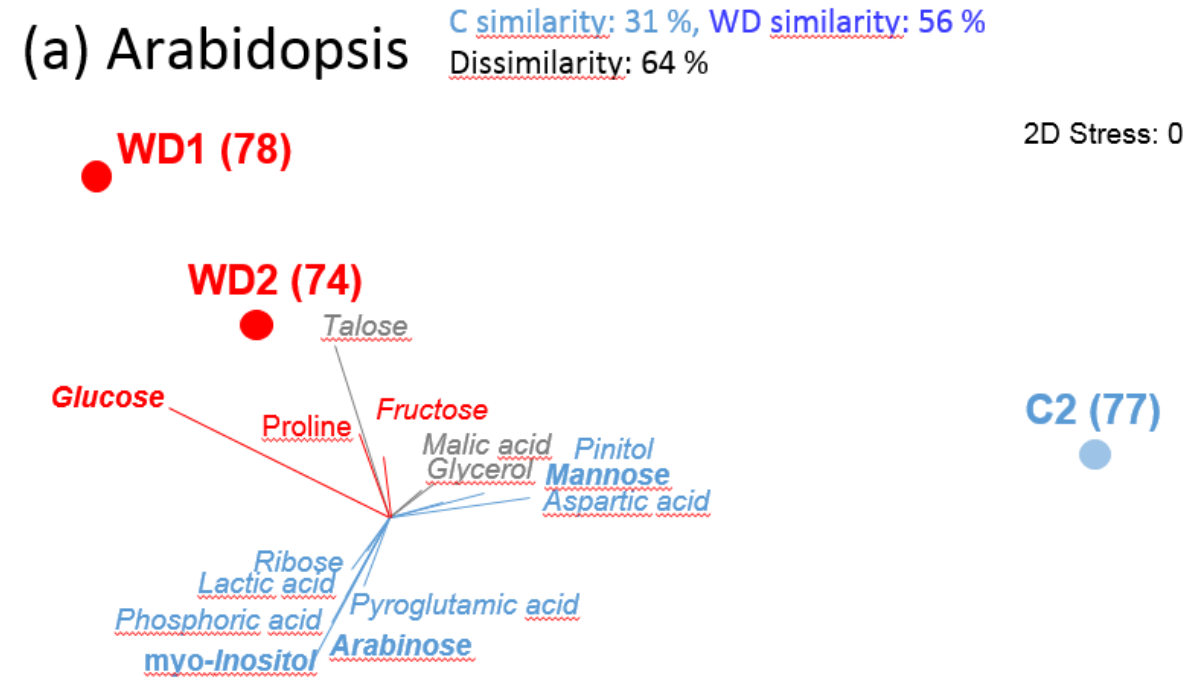
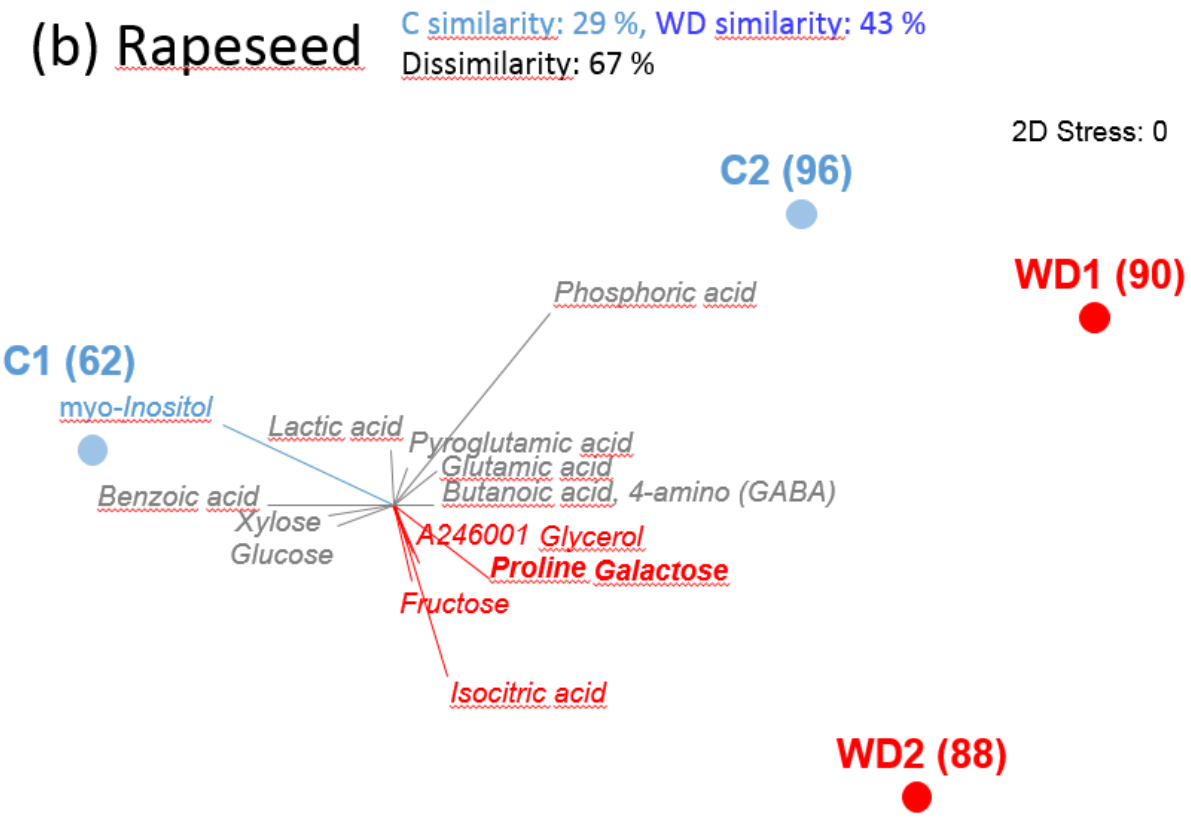
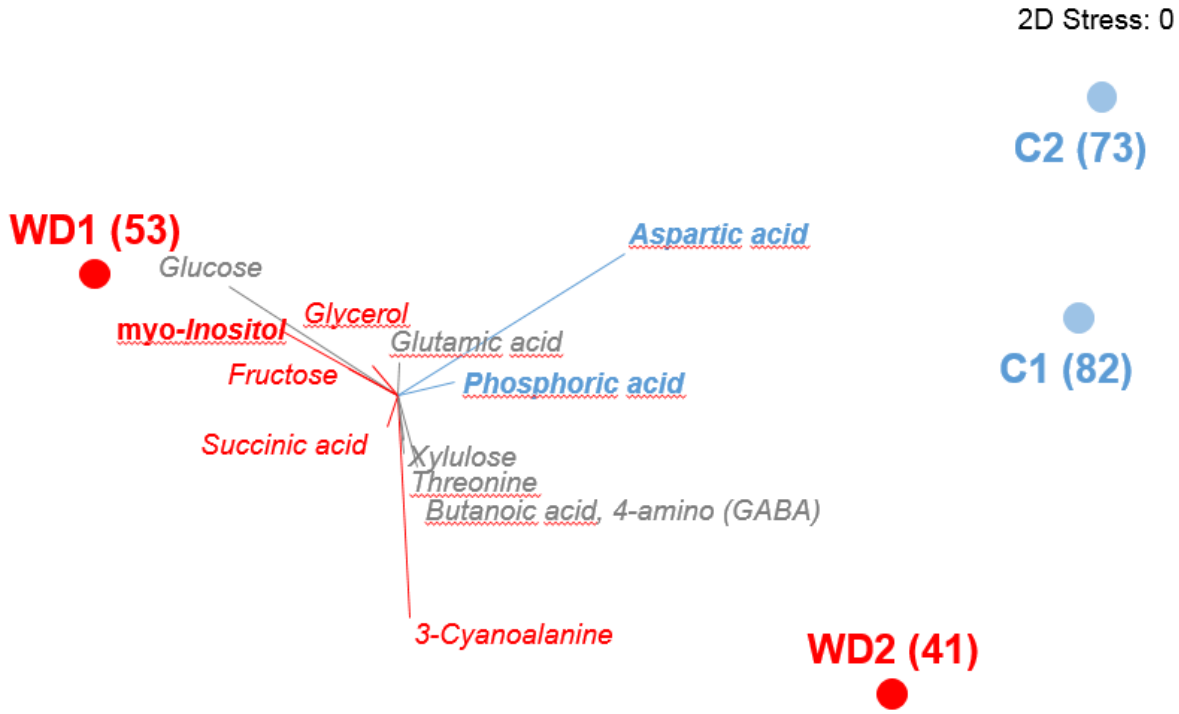
Figure 4.2. MDS plot of Bray-Curtis similarity of primary metabolites (PM) in root exudates of control (**C1, C2**) and water deficit-treated plants (**R1, R2**) that were obtained by GC–MS analyses of the water phase of the crude exudate collection/extract; **(a)** Arabidopsis, **(b)** Rapeseed, **(c)** Phaseolus, **(d)** Pisum, **(e)** Tobacco, and **(f)** Maize. PM contributions to treatment similarity are indicated as vectors in the respective colour of the treatment group. Grey vectors indicate metabolites that contribute more to the variation within than between treatment groups.


(a) Arabidopsis
C similarity: 31 %, WD similarity: 56 %
Dissimilarity: 64 %
2D Stress: 0
WD1 (78)
WD2 (74)
C2 (77)
Talose
Glucose
Proline
Fructose
Malic acid
Pinitol
Glycerol
Mannose
Aspartic acid
Ribose
Lactic acid
Pyroglutamic acid
Phosphoric acid
Arabinose
myo-Inositol


(b) Rapeseed
C similarity: 29 %, WD similarity: 43 %
Dissimilarity: 67 %
2D Stress: 0
C2 (96)
WD1 (90)
C1 (62)
WD2 (88)
Phosphoric acid
myo-Inositol
Lactic acid
Pyroglutamic acid
Glutamic acid
Benzoic acid
Butanoic acid, 4-amino (GABA)
Xylose
Glucose
A246001 Glycerol
Proline Galactose
Fructose
Isocitric acid

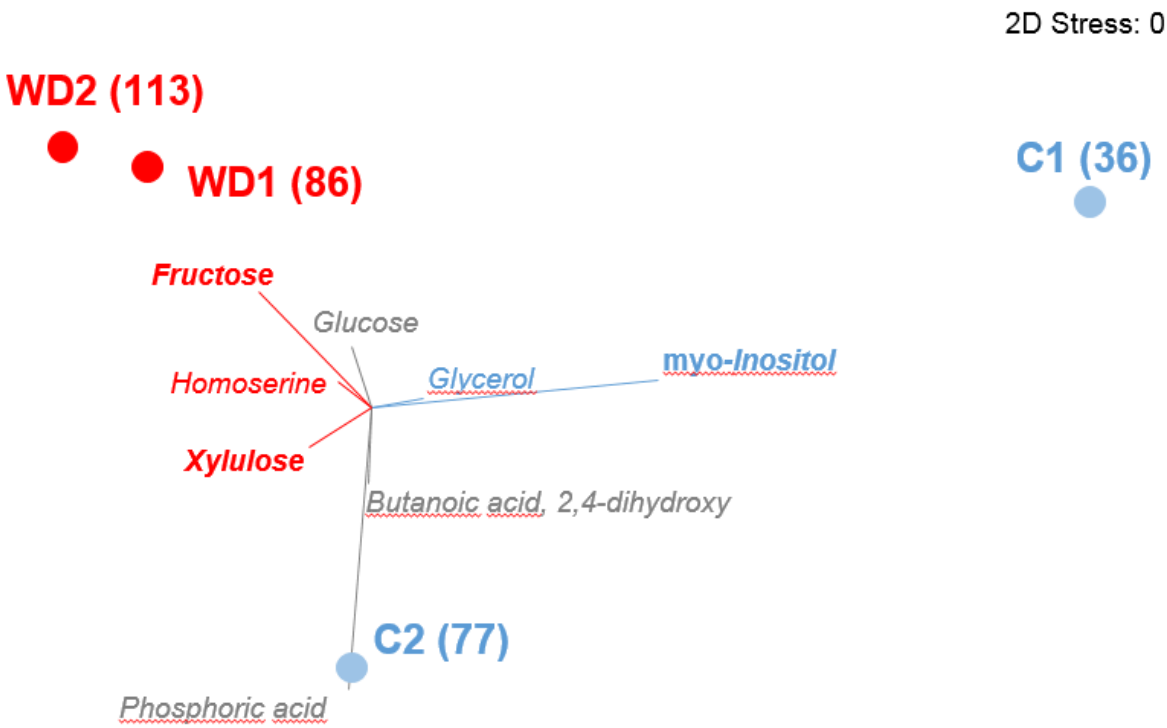
(c) Phaseolus

C similarity: 71 %, WD similarity: 23 %
Dissimilarity: 60 %



(d) Pisum

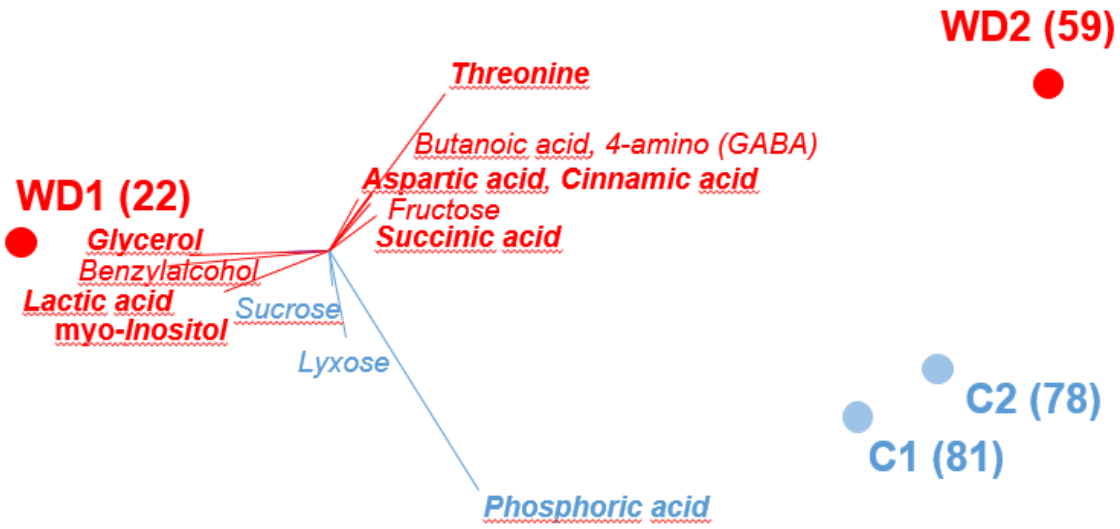
C similarity: 28 %, WD similarity: 79 %
Dissimilarity: 72 %



(e) Tobacco

C similarity: 82 %, WD similarity: 24 %
Dissimilarity: 65 %

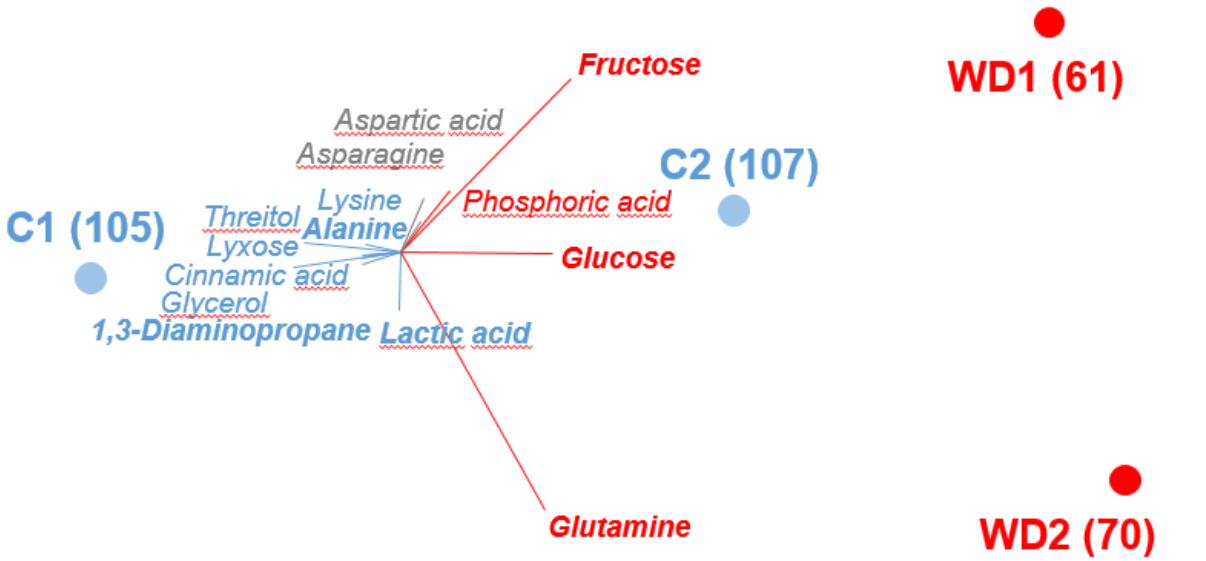
2D Stress: 0



(f) Maize

C similarity: 28 %, WD similarity: 52 %
Dissimilarity: 64 %

2D Stress: 0



the two treatment groups included aspartic acid, glucose, *myo-inositol*, 3-cyanoalanine, phosphoric acid, fructose, and pyroglutamic acid, besides many other PMs with more minor contributions. Phaseolus root exudates showed 82 and 73 PMs that were reduces to 53 and 41 by the WD treatment. Figure 4.2c provides a summary of the Phaseolus results.

4.3.3.4 Pisum

The second species of the two Fabaceae, Pisum, resembled the two Brassicaceae species in terms of treatment group similarity. The WD (Ziegler *et al.*, 2016)repeats showed a comparatively high similarity (average 79 %) to which the sugars fructose and xylose contributed most, to a lesser extent the amino acid homoserine. Similarly, as in Phaseolus, however, control repeat similarity (average 28 % was determined by the sugar alcohol *myo*-inositol and phosphoric acid. The dissimilarity between the two treatment groups was 72 % (average), to which *myo*-inositol, phosphoric acid, fructose, xylulose, glucose, 2,4-dihydroxy-butanoic acid, homoserine and glycerol contributed, besides many other PMs with more minor contributions. Pisum root exudates showed 36 and 77 PMs in the two repeats that increased to 86 and 113 by the WD treatment. Figure 4.2d summarizes the results of Pisum.

4.3.3.5 Tobacco

Tobacco as representative of Solanaceae showed a low PM similarity in the WD treatment (average 24 %). The first repeat was characterized by increased amounts of the sugar alcohol *myo*-inositol, lactic acid and glycerol, the second repeat, by contrast, by accumulation the amino acids aspartic acid and threonine succinic acid, and cinnamic acid. The two control repeats were more similar (average 82 %), to which, besides phosphoric acid, *myo*-inositol, fructose, glucose, glycerol, lactic acid and the amino acid aspartic acid contributed most. The dissimilarity of the two treatment groups (average 65 %) was caused by phosphoric acid, lactic acid, threonine, glycerol, lyxose, *myo*-inositol, GABA (γ-aminobutyric acid) and aspartic acid, amongst other PMS with more minor contributions. Tobacco root exudate showed 81 and 78 PMs that were reduced 22 and 59 by the WD treatment. Figure 4.2e provides a summary of the Tobacco results.

4.3.3.6 Maize

Maize, the only grass species that was included in the experiments, showed an average similarity of the WD treatment repeats of 52 %, to which the sugars glucose, fructose and galactose, the amino acids pyroglutamic acid and glutamine, and phosphoric acid contributed

most. The control treatments showed low similarity (average 28 %), to which 1,3-diamino-propane, lactic acid, alanine, glycerol, succinic, lysine, serine, and leucine contributed most. The dissimilarity of the two treatment groups was 64 % (average) and was caused by glutamine, glucose, fructose, phosphoric acid, glycerol and pyroglutamic acid, amongst many other PMs with lower contributions. Maize root exudates showed 105 and 107 PMS in the two repeats that were reduced to 61 and 70 by WD treatment. Figure 3f provides a summary of the Maize results.

4.3.4 Water deficit (WD) effect on secondary plant metabolite (PM) profiles

SM profiling of the root exudates was performed by LC–DAD (max. absorbance). UPLC–TOF/MS analyses were available only for the root exudate samples and used to obtain additional information for structure elucidation. For analytes, that SIMPER analysis identified as prominent contributor to similarity and dissimilarity, tentative structures are presented that were obtained on basis of a comparison of UV and MS data with the literature if possible. A summary of all analytes with their corresponding UV spectra that were obtained within the present thesis is provided by Appendix 3. Figure 4.3 summarizes the results that were obtained by a non-parametric multivariate analysis of control (C) and water deficit-treated (WD) root exudate profiles.

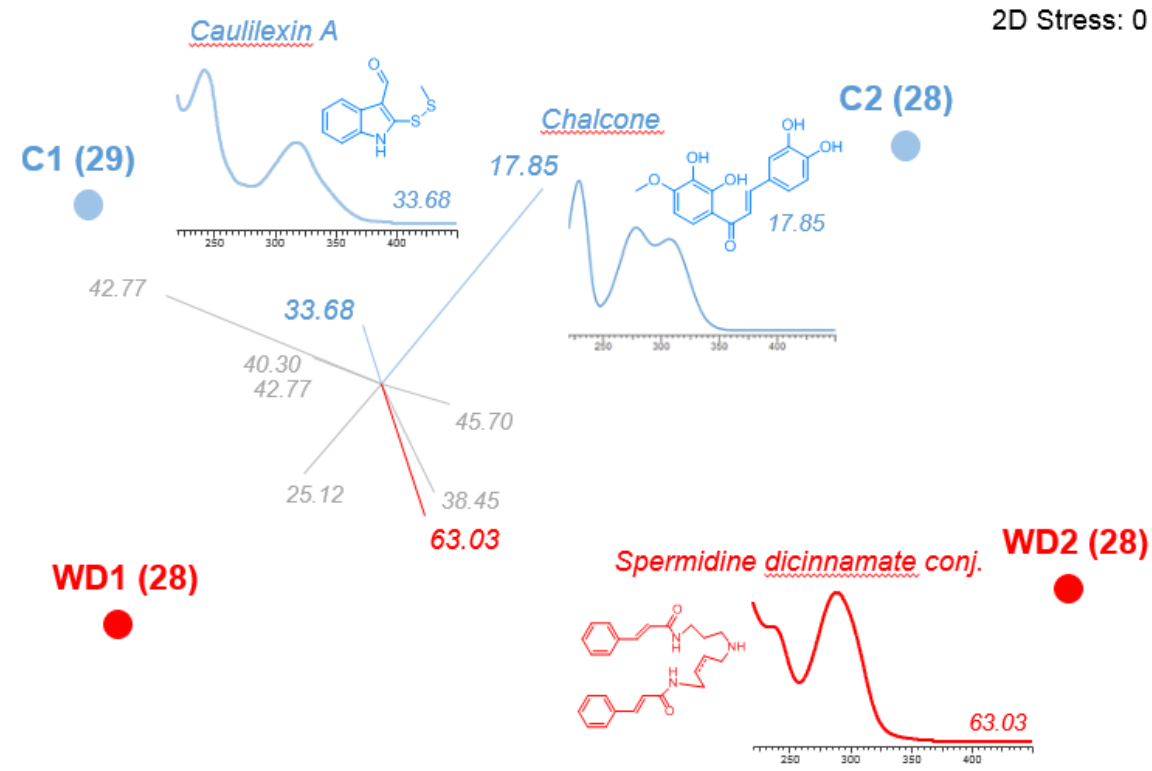
4.3.4.1 Arabidopsis

WD treatment did not change the SM numbers in Arabidopsis root exudates, 29 and 28 in the control treatment, 28 in both WD repeats. Average similarity was comparable between both treatments, 69 % for the control, 62 % for the WD treatment (Figure 4.3a). Average dissimilarity was low, 33 %. Many more SMs contributed more to dissimilarity of repeats than to similarity of treatments. The indole Caulilexin A and the chalcone derivative decreased after WD treatment, whereas the spermidine dicinnamate conjugate increased (for structure assignment see 3.3.3.1).

Figure 4.3. MDS plot of Bray-Curtis similarity of secondary metabolites (PM) in root exudates of control (**C1, C2**) and water deficit-treated plants (**R1, R2**) that were obtained by LC–DAD and UPLC–MS analyses of the ethyl acetate phase of the crude exudate collection/extract; **(a)** Arabidopsis, **(b)** Rapeseed, **(c)** Phaseolus, **(d)** Pisum, **(e)** Tobacco, and **(f)** Maize. PM contributions to treatment similarity are indicated as vectors in the respective colour of the treatment group. Grey vectors indicate metabolites that contribute more to the variation within than between treatment groups.

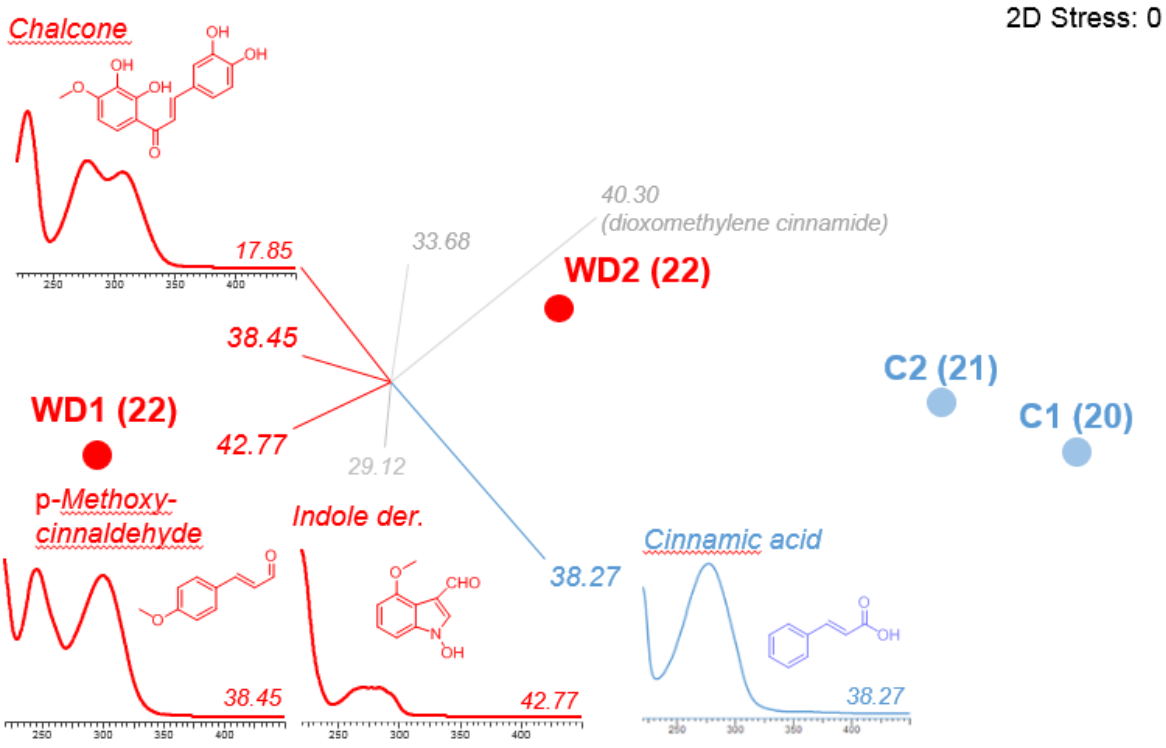
(a) Arabidopsis

Control (C) similarity: 69 %, WD similarity: 62 %
Dissimilarity: 33 %



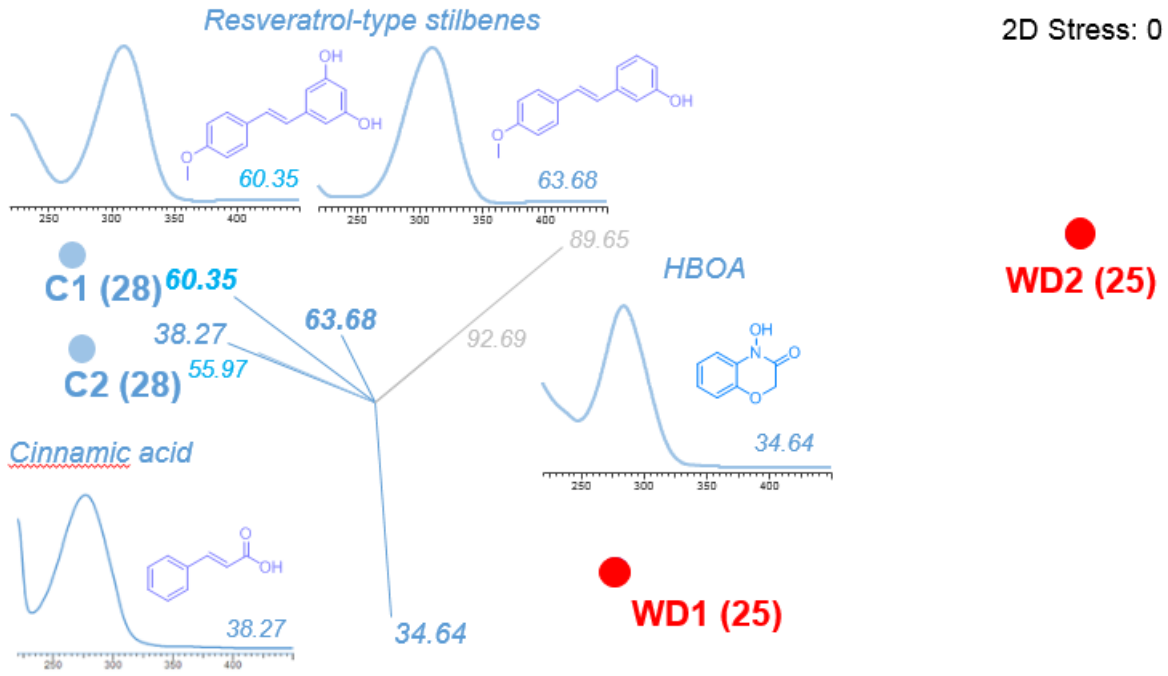
(b) Rapeseed

Control (C) similarity: 85 %, WD similarity: 75 %
Dissimilarity: 32 %



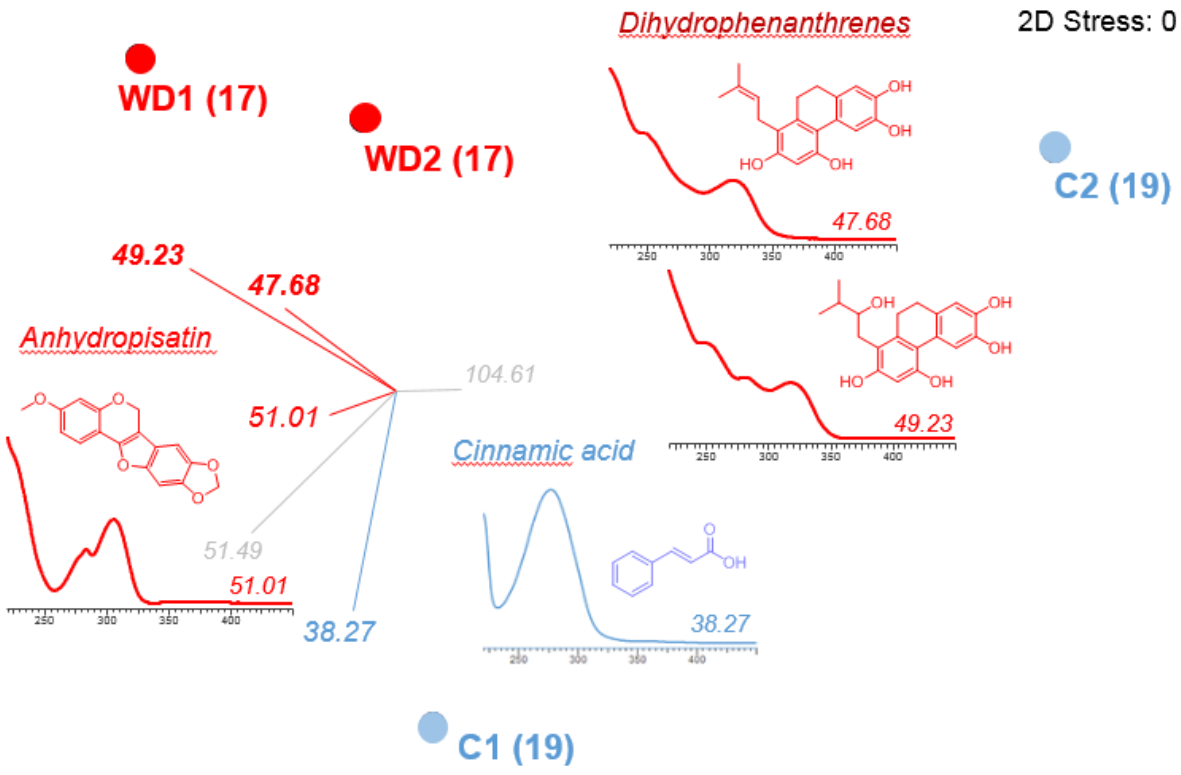
(c) Phaseolus

Control (C) similarity: 96 %, WD similarity: 67 %
Dissimilarity: 43 %



(d) Pisum

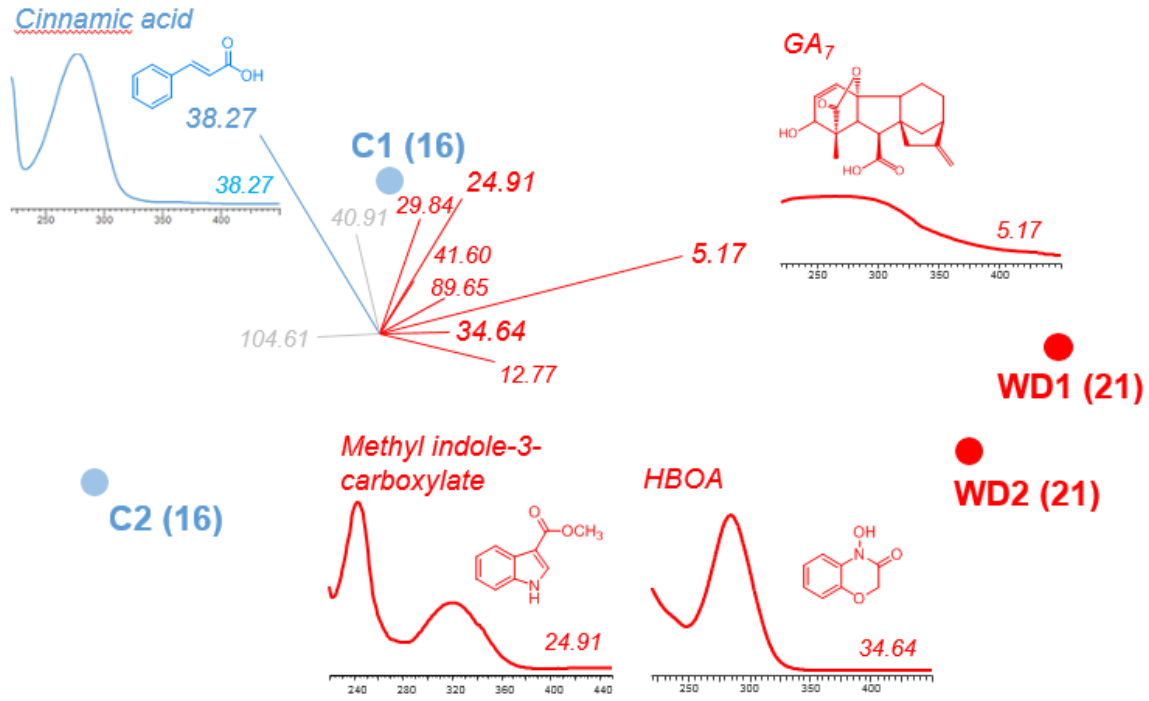
Control (C) similarity: 70 %, WD similarity: 81 %
Dissimilarity: 26 %



(e) Tobacco

Control (C) similarity: 75 %, WD similarity: 96 %
Dissimilarity: 43 %

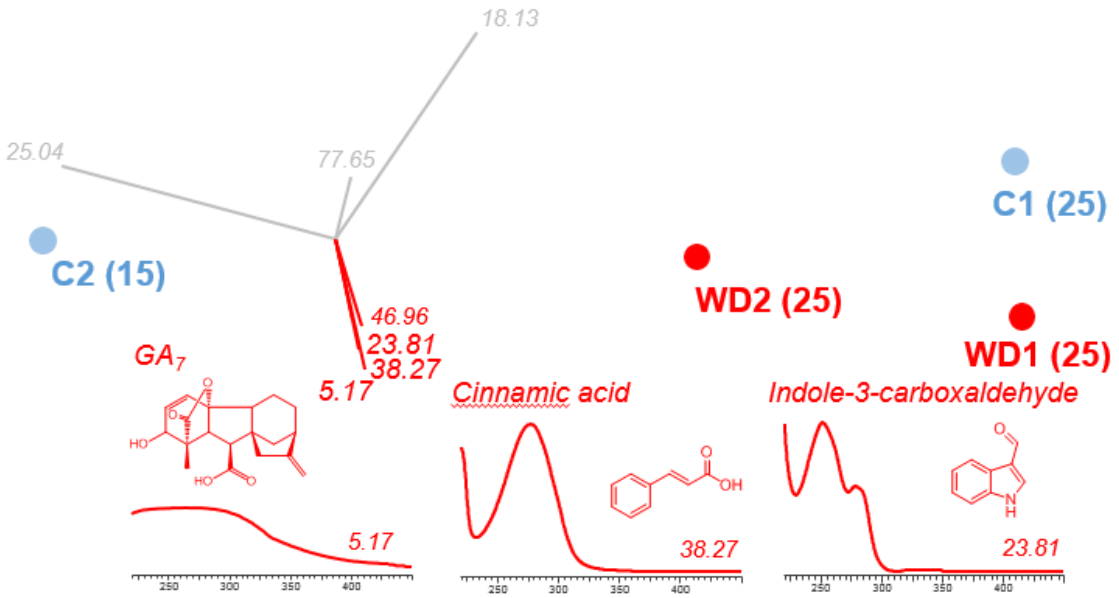
2D Stress: 0



(f) Maize

Control (C) similarity: 61 %, WD similarity: 85 %
Dissimilarity: 26 %

2D Stress: 0



Several other SMs, some of which are mentioned already in 3.3.3.1 in the root–root exudate SM profile comparison, contributed more to the dissimilarity of the repeats. Those included the indole derivatives that eluted at 42.77 and 45.70 min, and the dioxomethylene cinnamide and the one-methoxy-two hydroxyl cinnamide. The SM eluting at 38.45 min could be a *p*-methoxycinnaldehyde ($[M+H^+]^+$: 163.13, calc. 163.08; $[M+NH_4^+]^+$: 180.16, calc. 180.10; $[M+CH_3CN+H^+]^+$: 204.16, calc. 204.10). This SM has been described from many sources though not from Brassicaceae.

4.3.4.2 Rapeseed

In Rapeseed, WD treatment caused comparable effects to those of Arabidopsis. The number of detectable SMs increased only roughly by one, from 20 and 21 to 22 in both repeats (Figure 4.3b). The chalcone derivative also detected in Arabidopsis, *p*-methoxycinnaldehyde and the indole derivate eluting at 42.77 min increased from lower to higher amounts. Concomitantly, cinnamic acid decreased to lower amounts. Other metabolites that contributed more to the dissimilarity of the repeats included the indole caulilexin A and dioxomethylene cinnamide. Compared to Arabidopsis, no additional prominent metabolites were detected. The average similarity of the control was 85 %, to which dioxomethylene cinnamide and cinnamic acid contributed most. The average similarity of the WD treated repeats was 75 %, to which dioxomethylene cinnamide, the indole derivate eluting at 42.77 min, the chalcone eluting at 17.85 min, and *p*-methoxycinnaldehyde contributed most. The average dissimilarity was 43%, to which the mentioned compounds contributed substantially.

4.3.4.3 Phaseolus

WD treatment decreased SM numbers from 28 to 25 in both repeats (Figure 4.3c). Several of prominent SMs, cinnamic acid, HBOA as well two resveratrol-type stilbenes (for structure assignment see 3.3.3.3) decreased in terms of their relative amounts. Concomitantly, WD treatment caused no comparable increase of other SMs. The two resveratrol type stilbenes disappeared completely in the WD treatment (Figure 4.3c). The average similarity of the control treatment repeats was 96 %, the WD treated repeats showed only 67 %. The average dissimilarity between control and WD treatment was 41 %., to which the resveratrol-type stilbene eluting at 60.35 min, 4'-O-methylresverarol, cinnamic acid and an unipolar metabolite that eluted at 89.65 min contributed most.

4.3.4.4 Pisum

WD treatment decreased Pisum root exudate SMs from 19 to 17 in both repeats (Figure 4.3d). Cinnamic acid decreased but other metabolites increased after WD treatment. Anhydropisatin (for structure assignment see 3) was one of them. The two other SMs (Figure 4.3d) were identified as dihydrophenanthrenes, a SM class that has been identified also to occur in leaves the Fabaceae genus *Glycyrrhiza* (Fukai *et al.*, 1991). The obtained UV data (Figure 4.3d) also concur to a greater part with the literature data. One derivative (47.68 min) could be gancaoin V ($[M+H^+]^+$: 313.15, calc. 313.14; $[M+CH_3CN+H^+]^+$: 354.18, calc. 354.17; $[M-H^+]^-$: 311.1418, calc. 311.13; $[M+HCOO^-]^-$: 357.15, calc. 357.13). The second dihydrophenanthrene (49.23 min, Figure 4.3d) could be a yet undescribed hydrogenated derivative of the former one ($[M+Na^+]^+$: 348.27, calc. 348.16; $[M-H^+]^-$: 329.23, calc. 329.14; $[M+HCOO^-]^-$: 375.2384, calc. 375.1449). Average similarity of control repeats was 70 %, of WD treated repeats was 81 %, and their average dissimilarity was 64 %. To the latter, the two dihydrophenanthrenes contributed most.

4.3.4.5 Tobacco

WD treatment increased Tobacco root exudate SMs from 16 to 21 in both repeats (Figure 4.3e). Cinnamic acid decreased and many other SMs increased or even appeared. However, the latter of which only showed in minor amounts that prevented structure assignment so far and led to their absence in the MDS plot. The average dissimilarity was 43 %, average similarity of controls 75 % and of WD treatments 96 %. Cinnamic acid contributed most to control repeat similarity, several SMs that increased to the similarity of the WD-treated repeats (Figure 4.3e). The most prominent was the tentatively assigned gibberellic acid derivative GA_7 (together with its unsaturated derivative, for detailed structure assignment see 3.3.3.5), which, in reality, was even more pronounced than the UV trace suggested because of the low sensitivity of UV to analytes with predominately saturated carbon atoms. Another more saturated and yet still unidentified SM that eluted at 12.77 was prominent besides an SM that eluted at 24.91 min. The spectral data point to methyl indole-3-carboxylate ($[M+H^+]^+$: 176.06, calc. 176.07; $[M+CH_3CN+H^+]^+$: 217.10, calc. 217.10; $[M-H^+]^-$: 174.06, calc. 174.06; $[M-HCOO^-]^-$: 220.06, calc. 220.06). This compound has been identified in plant species so far, in the Fabaceae genus *Mimosa* (Nascimento *et al.*, 2012) and the Euphorbiaceae genus *Croton* (Kuo *et al.*, 2013). Further, HBOA also belongs to this group (Figure 4.3e).

4.3.4.6 Maize

The SM root exudate profiles of the control repeats differed by 10 SMs, the first showed 25 and the second 15. Both WD treatments, by contrast, yielded 25 detectable SMs. This explains the low average similarity of the control treatments (61 %) (Figure 4.3f). According to the UV trace, a tetrahydrofurofuran-1-one lignan and a chalcone (for details about structure assignment see 3.3.3.6) represented to major compounds, both of which contributed most to the similarity of the control and WD treated repeats, the latter showing a higher average similarity (85 %). The low average dissimilarity suggested contributions of more minor components. The relative concentrations of those increased in the WD treatment. Again, gibberellic acid derivative GA_7 (see Tobacco results) was affected. Further SMs included cinnamic acid, and a SM eluting at 23.81 min, the MS data of which pointed to indole-3-carboxaldehyde ($[M+H^+]^+$: 146.06, calc. 146.06; $[M+CH_3CN+H^+]^+$: 187.09, calc. 187.09; $[M–H^+]^–$: 144.05, calc. 144.05; $[M–HCOO^–]^–$: 190.05, calc. 190.05) (Figure 4.3f). This metabolite is regarded as plant hormone and classified as auxin (Shindy & Smith, 1975).

4.3.5 Total comparison of water deficit (WD) with control treatment

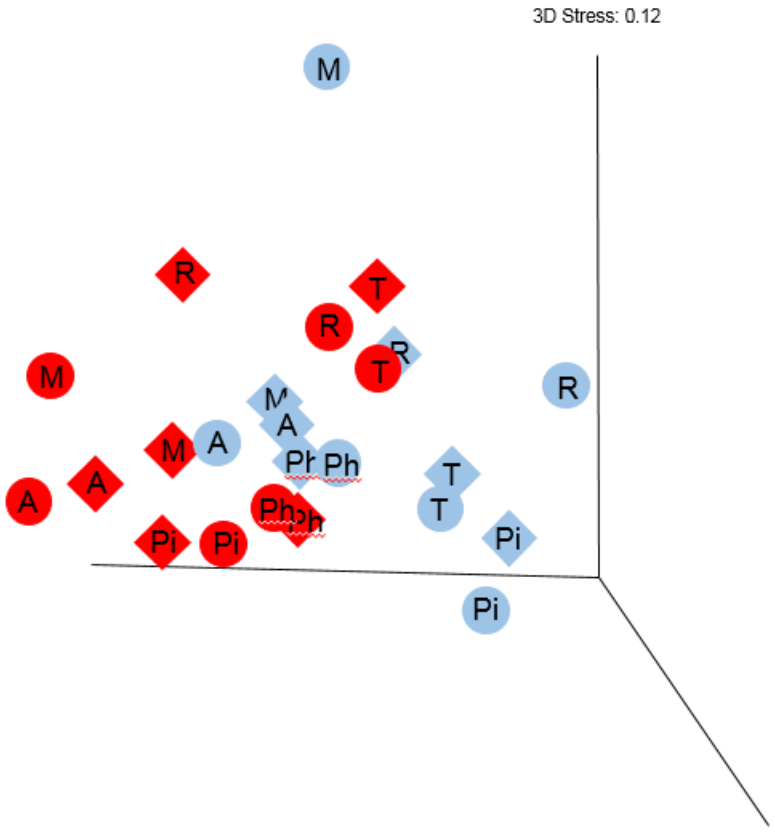
Two ANOSIM analyses were performed to explore if the control and the WD treatment caused any general differences in the root exudate profiles, one for primary metabolites (Figure 4.4a) and one for secondary metabolites (Figure 4.4b).

The PM profiles differentiated WD treatment and controls significantly when all six investigated plant species were considered. About 15 % off all PMs that were included into the analysis supported the grouping. The average similarity did not change much following WD, from 32 to 34 %. By contrast, the quality of PMs that contributed to the similarity changed. Phosphoric acid and *myo*-inositol decreased whereas glucose and fructose increased following WD treatment. Other similarly affected PMs included the amino acid proline and the glutamic acid oxidation product GABA (Figure 5a). The high stress level of the 3D plot (0.12), however, indicates a low level of dissimilarity among the samples.

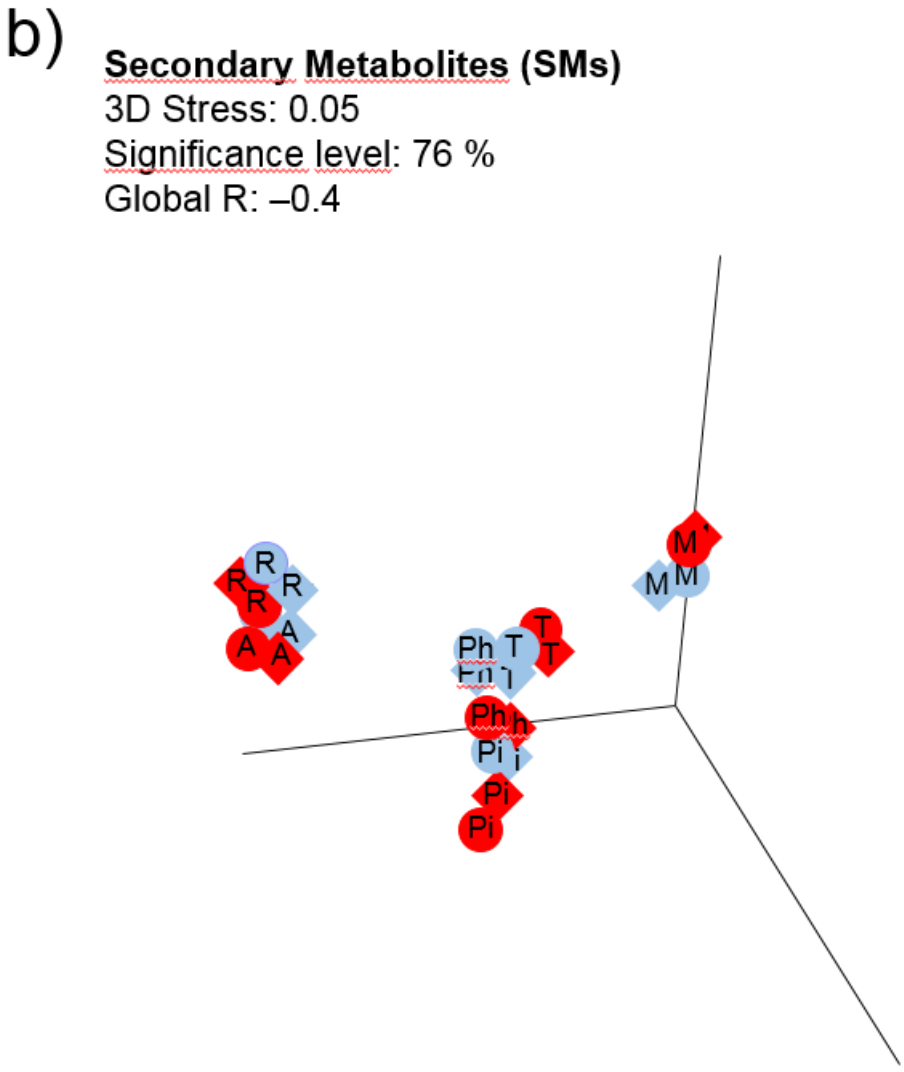
Figure 4.4. MDS plot of Bray-Curtis similarity of **(a)** primary metabolites (PM) and **(b)** secondary metabolites (SM) in control treatment and WD treatment. Contributions to similarity and dissimilarity were determined by a SIMPER, group difference by ANOSIM analysis; A, Arabidopsis; R, Rapeseed; Ph, Phaseolus; Pi, Pisum; T, Tobacco; M, Maize; circle symbol, first replicate circle, second replicate diamond.

a)

Primary Metabolites (PMs)
3D Stress: 0.12
Significance level: 1.3 %
Global R: 0.15



Similarity (% contrib.)		Dissimilarity (% contrib.)
Phosphoric acid (21)	**Glucose (22)**	Phosphoric acid (11)
***myo*-Inositol (15)**	**Fructose (19)**	Glucose (8)
Glucose (11)	Phosphoric acid (6)	Aspartic acid (7)
Lactic acid (6)	Lactic acid (5)	*myo*-Inositol (7)
Fructose (4)	*myo*-Inositol (5)	Fructose (7)
Glycerol (4)	Glycerol (5)	Glycerol (3)
Aspartic acid (3)	**Proline (4)**	Proline (3)
Pyroglutamic acid (3)	**GABA (3)**	Lactic acid (2)
Alanine (2)	Galactose (2)	Pyroglutamic acid (2)
Galactose (2)	Aspartic acid (2)	Glutamine (2)
Succininc acid (2)	Valine (2)	Talose (2)
Arabinose (1)	Arabinose (2)	Xylulose (2)
Mannose (1)	Pyroglutamioc acid (1)	GABA (2)
Average: 32 %	Average 34 %	Average 70 %



Changes in the SM profiles turned out to be more species- than treatment-specific. None of the detected SMs correlated in terms of changes of its relative amounts with the WD treatment when all six investigated species were considered as one treatment group. The higher sample dissimilarity of the SMs, compared to that of the PMs, lowered the stress level of the 3D plot to 0.05

4.3.6 Differential pulse voltammetry (DPV) of crude root exudates

Figure 4.5 illustrates the DPVs of the two control repeats in comparison to those that were obtained from the water deficit (WD)-treated repeats. In all six plant species, differences could be observed as consequence, either by the appearance of new peaks or by substantial changes

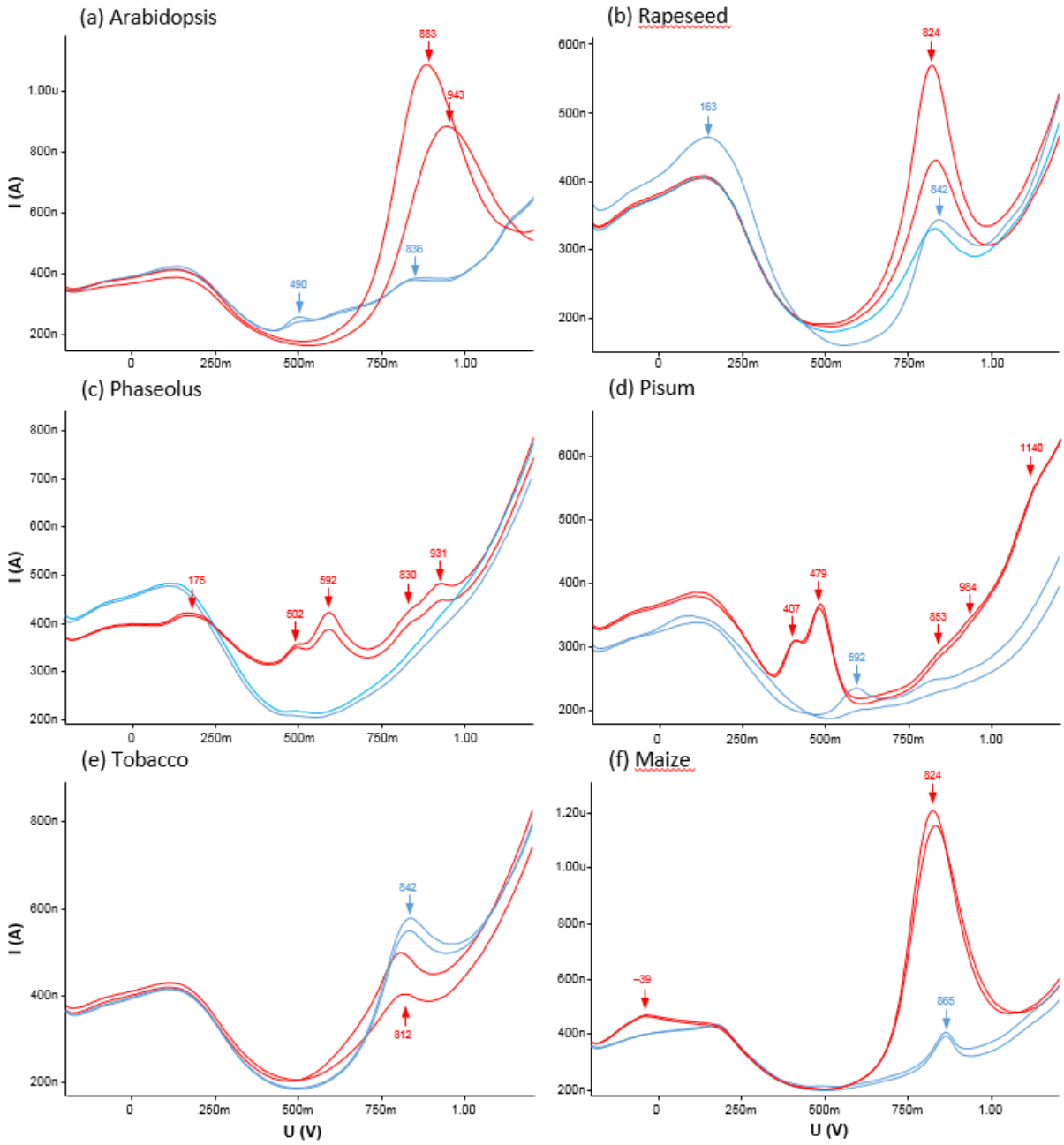


Figure 4.5. Differential pulse voltammetry of crude root exudates of control treatment (C) and water deficit (WD)-treated plants, two repeats; working electrode, glassy carbon; counter electrode; platinum wire; reference electrode, Ag/AgCl; 1 M acetate buffer (pH = 3.6).

in the intensity of already existing peaks or their disappearance. The two repeats that received identical treatment, either control or WD, resembles each other always closely. All observed effects in one repeat could be confirmed by the other.

In Arabidopsis weak peaks changed from 490 and 836 mV to strong ones at 883 and 943 mV. In Rapeseed, two weak peaks at 824 and 842 changed to two strong ones at 842 nm. In Phaseolus WD treatment caused the appearance of several new weak peaks: 175, 502, 592,

830 and 931 mV. In Pisum, the control treatments only showed a weak peak at 592 nm that was changed to several new peaks by WD treatment: 407, 479, 853, 984 and 1149 mV. In Tobacco, a relatively weak peak at 812 nm changed to a new weak peak at 842 mV. In Maize, a weak peak at 865 nm changed to a new weak peak at –39 mV and a new strong peak at 824 nm.

4.4 Discussion

WD treatment reduced the root and shoot biomass of all plants compared to the control. Phaseolus and Maize maintained a higher root biomass production than the other plant species. Shoot biomass, however, was reduced generally. WD treatment cause a decrease in shoot: root ratios (Figures 4.1a and b). This can be viewed as expression of two different strategies: Phaseolus and Maize react opportunistically to WD, the other plants try to limit the damage by reduced growth, the latter of which might help to survive more severe WD conditions (Vamerali *et al.*, 2003). The high shoot: root ratio of Arabidopsis is caused by its extreme ephemeral life style (Zhou *et al.*, 2014).

WD treatment increased the exudation of PMs and SMs, in all investigated species and in all repeats. These observations agree with a previously published study that explored root exudation of crested wheatgrass (*Agropyron cristatum*) when exposed to nutrient stress, potassium and nitrogen deficiency, drought or flooding (Henry *et al.*, 2007).

WD treatment caused changes in the PM profiles (Figure 4.2) and these changes, though quite heterogeneous at first glance, were substantial enough to secure a 1.3 % significance value to differentiate control and WD treatment groups. The R-value was rather low (0.15) pointing to a high overlap in the PM profiles. After WD treatment, the sugars glucose, fructose and the amino acids proline and GABA (γ-aminobutyric acid) increased, phosphoric acid, *myo*-inositol and the amino acids aspartic acid, alanine and pyroglutamic acid decreased in terms of contribution to treatment group similarity (Figure 4.2a). Proline and total sugars are known to increase in leafs and roots following water deficit and other abiotic forms of stress and this reflects itself in root exudates too (Irigoyen *et al.*, 1992).

SMs were also affected by the WD treatment. However, the shifts in the SM profiles were more species- and plant family-specific than general (Figure 4.3b). One phenomenon that was visible in four of the six investigated plant species was a decrease in cinnamic acid. In maize, however, cinnamic acid increased. The only plant that showed SMs that were not detectable

in the control treatment was Pisum. The dihydrophenanthrenes have so far only been identified ion the aerial parts of *Glycyrrhiza uralensis* (Fukai *et al.*, 1991) and not much is known about them. The polyhydroxylated aromatic ring, however, let expect good antioxidant activity as well as competitive ligand properties in coordination complexes with metals.

In some plants, several SMs increased in their proportions albeit less spectacular than the dihydrophenanthrenes in Pisum. *p*-Methoxycinnaldehyde is a root exudate SM of Arabidopsis and Rapeseed that was accumulated in higher amounts after WD treatment, in the latter more than in the former. Most existing reports are from odour analyses, e.g. basil (de Vasconcelos Silva *et al.*, 2003). Similarly, more indole derivatives were present the root exudates of Tobacco and Maize after WD treatment, methyl indol-3-carboxylate in Tobacco and indole-3-carboxaldehyde in Maize. Interestingly, the latter was identified as alkaline-released metabolite of Arabidopsis roots (Tan *et al.*, 2004). The same compound was also detected after fungal and bacterial infection in leaves and roots. In the present study, WD treatment triggered similar indole metabolites in Tobacco and Maize root exudates. One characteristic of biotic and abiotic stress is the enhancement of reactive oxygen species (ROS) that arise from increased but incomplete reduction of molecular oxygen which does not result in water (Foyer & Noctor, 2009). As strong oxidative agents, ROS can attack oligomers and polymers. Increased pH after alkaline treatment represents a similar chemical scenario that favours the formation of aldehyde structures (Tan *et al.*, 2004). This could explain the present finding that aldehyde structures increase in the root exudates of WD stress-treated plants.

Analysis and structure assignments of PMs and SMs are highly tentative. The detection of the single metabolites depends on specific structural properties and especially in case of mass spectrometry on specific chemical reactions. Differential pulse voltammetry (DPV) is an electrochemical method that utilizes the phenomenon that some analytes undergo chemical oxidation and reduction more easily than others do. Already in Chapter 3, the voltammograms proved a valuable addition to chromatographic analysis by proving that root exudates can be recovered with reproducible electrochemical properties that also suggest a similar chemical functionality. This functionality, however, does not even have to be necessarily supported by identical amounts of identical metabolites. Figure 4.5 demonstrates that WD treatment changes the electrochemical properties in most species except Tobacco. In the DPV of the same species, a shift of –30 mV is detectable in the peaks. In Arabidopsis the DPV reflects the differences that were observed in the SM profiles of the root exudates from the WD-treated

repeats. In addition, the DPVs of the Rapeseed repeats agree in terms that WD1 is more dissimilar to the controls by showing a more intense peak at 842 mV. Phaseolus is difficult to interpret. The chemical analysis suggests that the SM concentrations decrease after WD treatment. The DPVs somehow contradicts this conclusion by showing several new peaks, albeit of weak intensity. In Pisum, the picture is clearer as the two new peaks at 407 and 479 mV may be explained by the WD-triggered dihydrophenanthrenes. The co-occurring isoflavone are probably electrochemically less active due to a lower number of hydroxyl groups. Tobacco shows quite a contrasting picture, which could be explained by the fact that, although many SMs change, none of them is a candidate for high electrochemical activity. The gibberellic acid derivative is more an electron acceptor than a donator is. For Maize, the DPV suggests the increase of one or more highly electroactive compounds, but none with vicinal hydroxyl groups as in Pisum. Indole-3-carboxaldehyde represents a good candidate in this aspect.

When attempting to interpret the DPV results, one should not forget that the chemical reactions during voltammetry measurements are complex. The analytes can react with each other and the electrode surface, and several follow-up products may arise. The focus on SM in their interpretation is warranted. The PM fraction showed no specific peaks without exception (data not shown). The tentative identity of many structures has to be confirmed by authentic standards.

4.5 Conclusion

Water deficit (WD) treatment for 14 days reduced the biomass production and increased the shoot: root ratio. Phaseolus and Maize, by contrast, showed a more opportunistic behaviour by increasing root development in attempts to counter the stress in this way resulting in a decreased shoot: root ratio. A more conservative strategy was chosen by the other four species.

In case of PMs, WD treatment triggered elevated levels of stress-associated metabolites such as glucose, fructose, proline and GABA. Changes in SM profile included the new appearance of dihydrophenanthrenes in Pisum and the higher frequency of occurrence of aldehyde structures. Total amounts of PM and SM fractions increased after WD treatment.

PM changes were more general and allowed differentiation of the two treatment groups. By contrast, SM changes were highly species-specific.

The obtained results clearly demonstrated that a stress event such as water deficit reflects itself in the qualitative and quantitative composition of root exudates.

4.6 References

Badri, DV, Vivanco, JM. 2009. Regulation and function of root exudates. *Plant, Cell and Environment* **32**: 666–681.

Brett, CMA, Brett, AMO. 1993. Electrochemistry. Principles, Methods, and Applications. Oxford, New York: Oxford University Press.

Clarke, KR. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Ecology* **18**: 117–143.

de Vasconcelos Silva, Maria Goretti, de Abreu Matos, Francisco José, Lacerda Machado, MI, Craveiro, AA. 2003. Essential oils of Ocimum basilicum L., O. basilicum. var. minimum L. and O. basilicum. var. purpurascens Benth. grown in north-eastern Brazil. *Flavour and Fragrance Journal* **18**: 13–14.

Foyer, CH, Noctor, G. 2009. Redox regulation in photosynthetic organisms. signaling, acclimation, and practical implications. *Antioxidants & Redox Signaling* **11**: 861–905.

Fukai, T, Qing-Hua, W, Nomura, T. 1991. Six prenylated phenols from *Glycyrrhiza uralensis*. *Phytochemistry* **30**: 1245–1250.

Henry, A, Doucette, W, Norton, J, Bugbee, B. 2007. Changes in crested wheatgrass root exudation caused by flood, drought, and nutrient stress. *Journal of Environment Quality* **36**: 904–912.

Irigoyen, JJ, Einerich, DW, Sánchez-Díaz, M. 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologia Plantarum* **84**: 55–60.

Kopka, J, Schauer, N, Krueger, S, Birkemeyer, C, Usadel, B, Bergmüller, E, Dörmann, P, Weckwerth, W, Gibon, Y, Stitt, M, Willmitzer, L, Fernie, AR, Steinhauser, D. 2005. GMD@CSB.DB. the Golm Metabolome Database. *Bioinformatics* **21**: 1635–1638.

Kuo, P-C, Yang, M-L, Hwang, T-L, Lai, Y-Y, Li, Y-C, Thang, TD, Wu, T-S. 2013. Anti-inflammatory diterpenoids from *Croton tonkinensis*. *Journal of Natural Products* **76**: 230–236.

Luo, Q, Sun, L, Hu, X, Zhou, R. 2014. The variation of root exudates from the hyperaccumulator *Sedum alfredii* under cadmium stress: metabonomics analysis. *PloS one* **9**: e115581.

MCD-ROMowell, N, Pockman, WT, Allen, CD-ROM, Breshears, DD, Cobb, N, Kolb, T, Plaut, J, Sperry, J, West, A, Williams, DG, Yepez, EA. 2008. Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologist* **178**: 719–739.

Nascimento, IAd, Braz-Filho, R, Carvalho, MGd, Mathias, L, Fonseca, FdA. 2012. Flavonoides e outros compostos isolados de *Mimosa artemisiana* Heringer e Paula. *Química Nova* **35**: 2159–2164.

Penaloza, E, Corcuera, L, Martinez, J. 2002. Spatial and temporal variation in citrate and malate exudation and tissue concentration as affected by P stress in roots of white lupin. *Plant and Soil* **241**: 209–221.

Salinger, MJ. 2005. Climate Variability and Change: Past, Present and Future – An Overview. *Climatic Change* **70**: 9–29.

Shindy, WW, Smith, OE. 1975. Identification of plant hormones from cotton ovules. *Plant Physiology* **55**: 550–554.

Tan, J, Bednarek, P, Liu, J, Schneider, B, Svatoš, A, Hahlbrock, K. 2004. Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected *Arabidopsis thaliana* roots and leaves. *Phytochemistry* **65**: 691–699.

Tawaraya, K, Horie, R, Shinano, T, Wagatsuma, T, Saito, K, Oikawa, A. 2014. Metabolite profiling of soybean root exudates under phosphorus deficiency. *Soil Science and Plant Nutrition* **60**: 679–694.

Vamerali, T, Saccomani, M, Bona, S, Mosca, G, Guarise, M, Ganis, A. A comparison of root characteristics in relation to nutrient and water stress in two maize hybrids. *Plant and Soil* **255**: 157–167.

van Dam, NM, Bouwmeester, HJ. 2016. Metabolomics in the rhizosphere. Tapping into belowground chemical communication. *Trends in Plant Science* **21**: 256–265.

Wang, P, Bi, S, Wang, S, Ding, Q. 2006. Variation of wheat root exudates under aluminum stress. *Journal of Agricultural and Food Chemistry* **54**: 10040–10046.

Yang, J, Zhang, J, Wang, Z, Zhu, Q, Liu, L. 2002. Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta* **215**: 645–652.

Zhou, X, Zhang, Y, Niklas, KJ. 2014. Sensitivity of growth and biomass allocation patterns to increasing nitrogen: a comparison between ephemerals and annuals in the Gurbantunggut Desert, north-western China. *Annals of Botany* **113**: 501–511.

Ziegler, J, Schmidt, S, Chutia, R, Muller, J, Bottcher, C, Strehmel, N, Scheel, D, Abel, S. 2016. Non-targeted profiling of semi-polar metabolites in Arabidopsis root exudates uncovers a role for coumarin secretion and lignification during the local response to phosphate limitation. *Journal of Experimental Botany* **67**: 1421–1432.

5. Plant Metabolites in Root Exudates: Secondary Metabolites Affect Nutrient Uptake

Pervin Akter[1], Kirsten Fladung[2], Franz Hadacek[3]

Abstract. An previous review (Dakora & Phillips, 2002) proposed that root exudates can help in nutrient uptake, especially in nutrient-limited soils. Root exudate chemical analysis and leaf nutrient data of 24 samples from six plant species were included into this study. Arabidopsis, Rapeseed, Phaseolus, Pisum, Tobacco and Maize were cultured in an identical light, humidity and nutrient supply regime. One-half of the plants was exposed to 14 days water deficit (WD treatment). Previous analyses showed that the root exudates of these plants contained primary metabolites in variable quantities and secondary metabolites in variable quality. Spearman rank correlations were used to explore if specific metabolites correlated leaf nutrient concentrations in assumptions that coordination complex formation not only affects nutrient uptake by roots but also transport in the xylem.

Whereas primary metabolites showed no correlations with nutrient conc. in leaves, weak ones were found for secondary metabolites. A pairwise exploration identified correlation especially for more unsaturated secondary metabolites, such as chalcones, isoflavones, dihydroxy-phenanthrenes, indoles, and a dicinnamoyl spermidine conjugate. The obtained correlations provide some support for the notion that exuded plant metabolites, especially secondary, may be involved in nutrient uptake dynamics.

5.1 Introduction

Secondary plant metabolites (SMs) or, as they are recently called, specialized metabolites (Pichersky *et al.*, 2006), have a long tradition in being viewed as chemical weapons of sessile plants against a wide range of microbial and animal predators (Ahuja *et al.*, 2012; Mithöfer & Boland, 2012) albeit initial assumptions of being just waste products (Hartmann, 2007). Furthermore, SMs are not only involved in responses against biotic stress but also against

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Author contributions: concept: PA, FH; text: PA; figures: PA, FH; nutrient analysis: KF; data analysis: FH, PA

abiotic stress, such as high light, water deficit, salinity and heavy metals, amongst others (Nakabayashi & Saito, 2015). Their possible functions in root exudates are viewed similarly (Badri & Vivanco, 2009; van Dam & Bouwmeester, 2016). The analysis of plant metabolites in root exudates however is more difficult because the collectable amounts are much lower than those that were obtained from root tissues (see Chapters 3 and 4). In attempts to introduce the required amount of reproducibility for phenotype comparison, hydroponic culture setups have been recommended (Strehmel *et al.*, 2014; Kuijken *et al.*, 2015).

The functions of primary metabolites (PMs are clear-cut; this by comparison small group of plant metabolites is involved in maintaining the central metabolism that is required to sustain life. Their thermodynamic favourability, the availability of catalytic enzymes, and their physicochemical properties eliminate other biochemical alternatives (Bar-Even *et al.*, 2012). The structural diversity of SMs is much larger than that of the PMs, to which multifunctional and rapidly involving enzymes contribute. Alternative functions that have been proposed recently includes maintaining of population fitness in fluctuating and geographically dispersed environments. SM diversity itself could be allowed by a certain permissiveness—their metabolically highly specialized enzymes are more tolerant to mutations (Weng *et al.*, 2012). In root exudates, SM represent the minor portion and PM the major portion (Chapter 3).

Narrowing the perspective of potential SM functions on stress tolerance or avoidance mechanisms, however, could prevent obtaining a more comprehensive picture of the systemic functions of SMs. For example, SMs can affect the cell cycle, not only by causing just retarded growth (Sánchez-Moreiras *et al.*, 2008) but also by interfering with the auxin transport to induce shoot phenotypes (Kuhn *et al.*, 2011). These reports suggest possible roles in tissue differentiation processes as well. Similar effects have been observed in fungi, in which SM (mycotoxin) production co-occurs at the end of an enhanced growth trophophase that changes into a differentiation dominated idiophase (production of conidia) (Betina, 1995).

The functional groups of SM, mostly containing oxygen and nitrogen, point to the ability of many SMs to enter redox chemical reactions (Jacob *et al.*, 2011; Hadacek & Bachmann, 2015). Entering this type of chemistry requires the ability to donate electrons to other molecules. If these other molecules represent highly reactive radicals, SM can quench them by scavenging the radical as antioxidant. If no radicals are present, molecular oxygen (O_2) can be reduced by a one-electron transfer to superoxide anion radical ($O_2^{\bullet-}$). This Janus-headed behaviour causes the oxygen paradox (Davies, 2000).

Flavonoids, a very prominent class of plant SMs with many derivatives displaying pronounced antioxidant activities, are reported to be exuded by the roots of many plants (Cesco *et al.*, 2010). This class of SMs was identified to be involved in monitoring the early interactions between Fabaceae and the nodule-forming rhizobia (Cooper, 2007). It is generally accepted that reducing and coordination complex forming properties of flavonoids and other phenolic plant SMs can mobilize iron, copper, and other cations from mineral particles; the

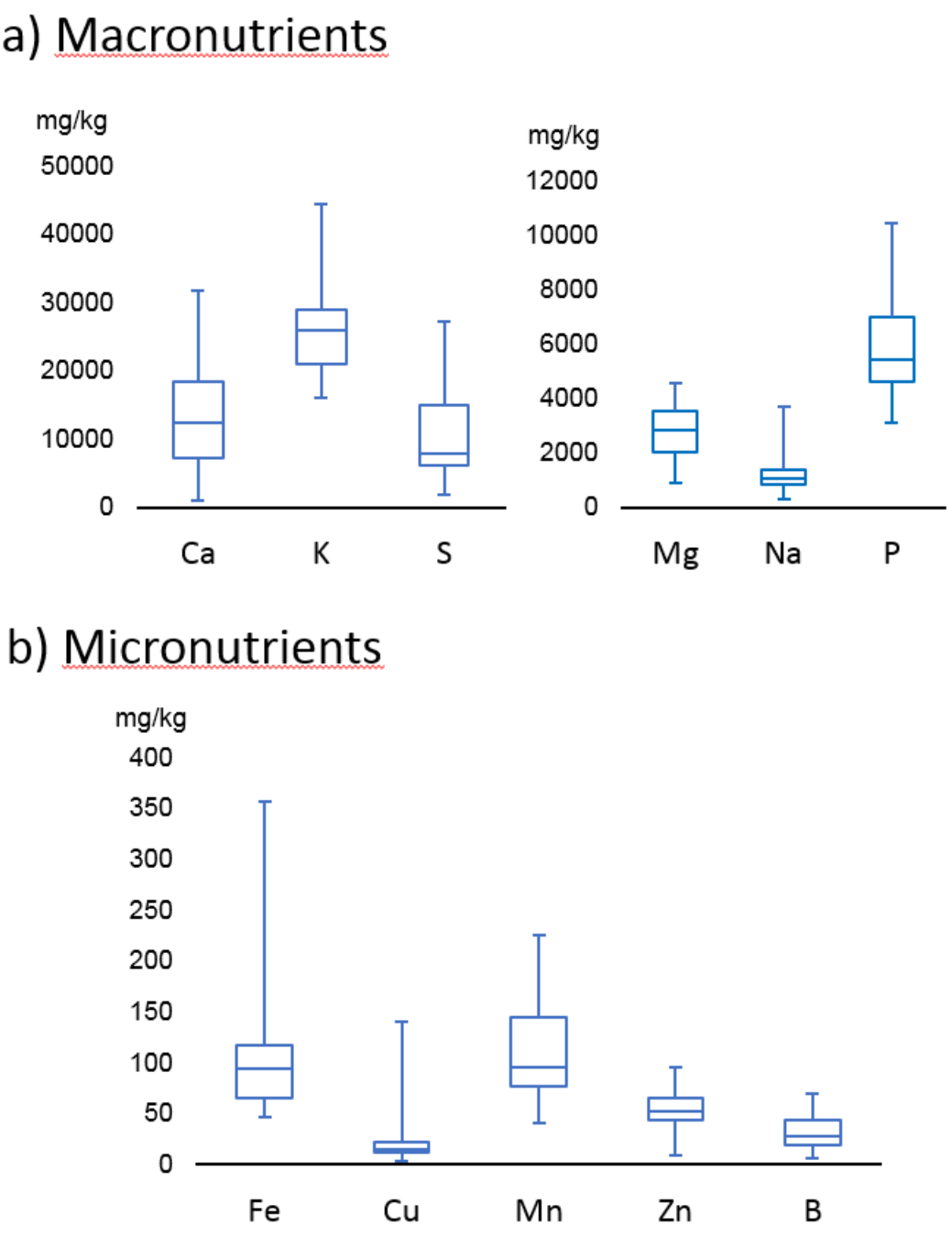


Figure 5.1. (a) Macro- and (b) Micronutrients from Arabidopsis, Rapeseed, Phaseolus, Pisum, Tobacco and Maize (Boxplots, *n* = 24).

complexation of Ca^{2+} also increases the availability of phosphorus to plants (Jung *et al.*, 2003; Neumann & Römheld, 2007; Cesco *et al.*, 2010; Hadacek & Bachmann, 2015).

The phenomenon of interference between plants by phytotoxic metabolites is coined as allelopathy, but inorganic elements can participate also in this type of interactions (Morris *et al.*, 2009). The chemical composition of root exudates could—by providing appropriate acidifiers and ligands for coordination complexes—represent a system that facilitates uptake of a wide range of nutrients, especially in those cases in which complex formation is mandatory for uptake transport due to low ion solubility. Furthermore, effects on mobilization from insoluble mineral sources, such as for phosphorus, have to be considered too. Two different uptake strategies have been suggested for iron (Neumann & Römheld, 2007):

(1) all dicotyledonous and nongraminaceous monocotyledons utilize iron reduction from Fe(III) to Fe(II), coordination complex formation with subsequent rhizosphere acidification to take up Fe(II) ions by a specific transporter;

(2) (2) grasses exudate iron-mobilizing ligands, nonproteinaceous amino acids such as mugineic acid which are also called phytosiderophores, and which can also form complexes with copper, manganese, zinc, nickel and cadmium and are reabsorbed by a specific transporter system.

All these insights somehow indicate a rather complex direct or indirect involvement of some PMs and SMs in the uptake process of nutrients via roots, which might encompass more than one mechanisms. Coordination complex formation, however, is probably central to all of them. One review specifically points out that root exudates could mediate mineral acquisition in low-nutrient environments (Dakora & Phillips, 2002). In the present thesis, root exudates from six plant species were shown to differ considerably in terms of their chemical composition from root extracts and between each other (Chapter 3). All six model plants were grown in as much as possible identical conditions concerning light, humidity and nutrient availability. Water deficit was introduced as an additional component to study root exudate dynamics (Chapter 4). The concentrations of all nutrients except nitrogen were determined in leaves in an effort to explore if the quantitative variation of PMs and SMs in root exudates

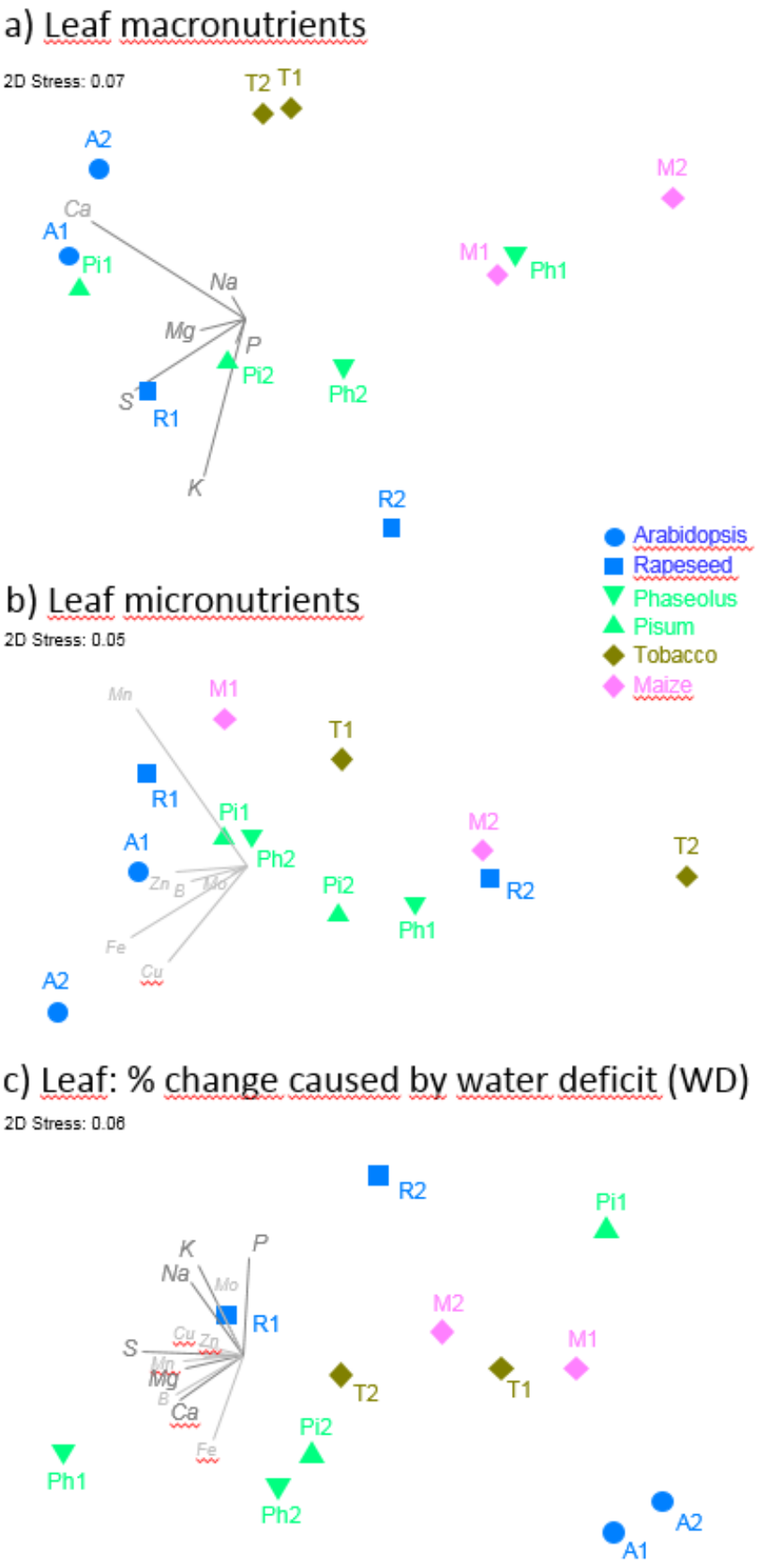


Figure 5.2. MDS plot of Bray-Curtis similarities of **(a)** macronutrients, **(b)** micronutrients, and **(c)** conc. change by water deficit (WD) treatment (% control).

complexity of the involved chemistry, the detection of weak correlations would represent an experimental hint that specific root exudate metabolites could be involved in nutrient uptake and transport.

5.2 Material and Methods

See Chapter 2.

5.3. Results

5.3.1 Macro- and Micronutrients in leaves

The considerable variation of macro- and micronutrients that was found in the control and the water deficit treatments of all six investigated plant species is illustrated by box plots in Figure 5.1. The median values (mg kg^{-1}) were as follows (in alphabetical order): calcium (12310), boron (28), copper (15), iron (94), magnesium (2858), manganese (96), molybdenum (1), phosphorus (5461), potassium (25991), sodium (1081), sulphur (7867), and zinc (52).

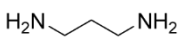
The similarity of the two repeats of the six investigated plant species is illustrated by a MDS plot based on Bray-Curtis resemblance indices by Figure 5.2, the macronutrients in Figure 5.2a, the micronutrients in Figure 5.2b. The enormous difference in concentrations was the reason to present macro- and micronutrient distributions in separate graphs. To provide some idea about the contribution of the single nutrients to the ordination, they are presented as vectors. Increasing length indicates increased weight of a nutrient to the ordination of samples, maximum correlation with the vector highest conc., minimal lowest. Figure 5.2c aims to provide a comparison of the nutrient concentration changes that were caused by the water deficit treatment.

The two repeats of a single species usually clustered close to each other, at least relative to the other species. Within the same species, some nutrients could vary up to the factor 4. However, in no case, general patterns were detectable that affected all nutrients in the same way. Generally, if two members of a family were included into the investigations, the repeats of both species showed a certain amount of resemblance in all cases. A relative comparison of the investigated species yielded the following characteristics for each of them:

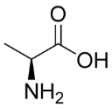
Figure 5.3. Spearman rank correlation of selected PMs and SMs with the analysed nutrients.

Primary metabolites in root exudates

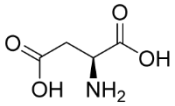
Correlation with nutrients: $\rho = 0.00$, significance of statistics: 46.9 %



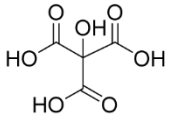
1,3-Diaminopropane
B: 0.34



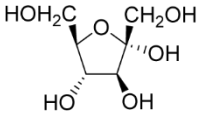
Alanine
P 0.34



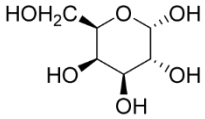
Aspartic acid
K: 0.34



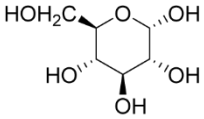
Citric acid
Cu: 0.49
Mo: 0.29
Ca: 0.15
Fe: 0.18



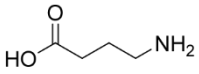
Fructose



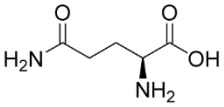
Galactose
K: 0.15



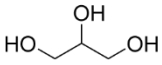
Glucose



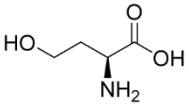
GABA
K: 0.15



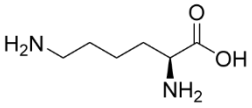
Glutamine
K: 0.19



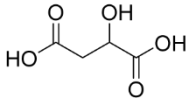
Glycerol
Cu: 0.30



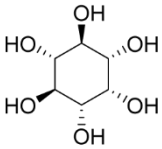
Homoserine
B: 0.36
K: 0.24



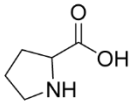
Lysine



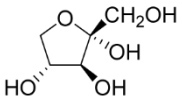
Malic acid



***myo*-Inositol**
Ca: 0.33
Na: 0.35



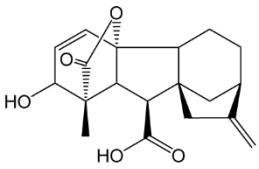
Proline



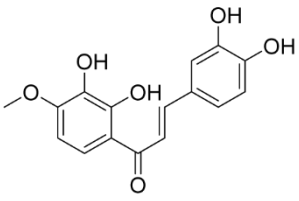
Xylose
K: 0.34

Secondary metabolites in root exudates

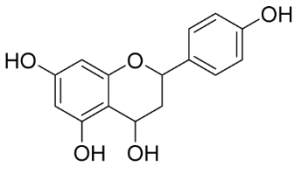
Correlation with nutrients: $\rho = 0.18$, significance of statistics: 1.0 %



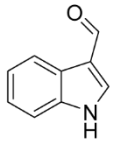
GA_7 (Diterpene)
Tobacco, Maize
Mn: 0.34
P: 0.19



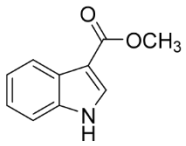
Chalcone
Arabidopsis, Rapeseed
Cu: 0.23
Fe: 0.22
Ca: 0.29
Na: 0.24
P: 0.31



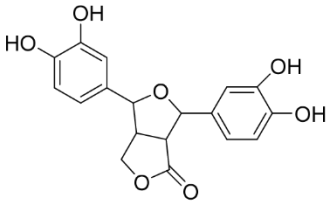
Apiferol (Flavanol)
Maize
Mo: 0.17



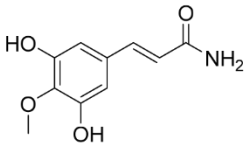
Indole-3-carboxaldehyde (Auxin)
Maize
Mo: 0.17
Zn: 0.16
Mn: 0.20
P: 0.27



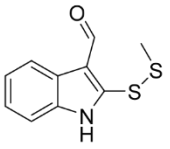
Methyl-indole-3-carboxylate (Indole)
Tobacco
Mn: 0.20
Ca: 0.39
Mg: 0.54
Na: 0.62



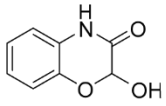
Tetrahydrofuro-furanone (Lignan)
Maize
Mo: 0.19



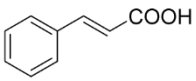
Dihydroxy-methoxy cinnamide
Arabidopsis
Cu: 0.47
Zn: 0.28
Fe: 0.46
Ca: 0.51
Mg: 0.22
Na: 0.20



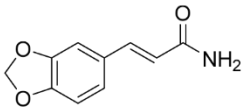
Caulilexin A (Indole)
Arabidopsis, Rapeseed
Ca: 0.19
Mg: 0.27
Na: 0.20
P: 0.30



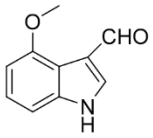
HBOA (Benzoxazinone)
Phaseolus, Pisum, Tobacco, Maize



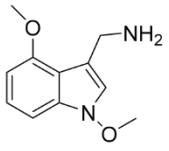
Cinnamic acid
All investigated plants
Mn: 0.28
K: 0.32



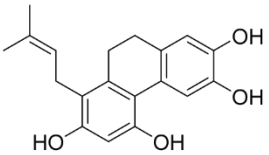
Cinnamide
Arabidopsis, Rapeseed
Ca: 0.24
Fe: 0.16
Na: 0.28



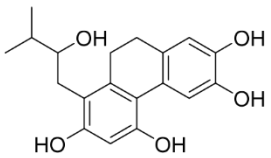
Indole
Arabidopsis, Rapeseed
Cu: 0.19
Ca: 0.25
Fe: 0.15
Na: 0.26
P: 0.27



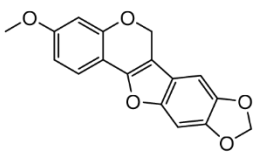
Indole
Arabidopsis
Cu: 0.46
Zn: 0.26
Ca: 0.49
Fe: 0.50
Mg: 0.26
Na: 0.18



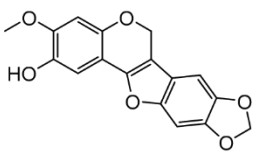
Dihydrophenanthrene
Pisum
Cu: 0.41
B: 0.55
Zn: 0.17
Ca: 0.19
Fe: 0.20
K: 0.29
Mg: 0.14
S: 0.41



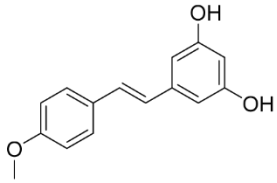
Dihydrophenanthrene
Pisum
Cu: 0.41
B: 0.55
Zn: 0.17
Ca: 0.19
Fe: 0.20
K: 0.29
Mg: 0.14
S: 0.41



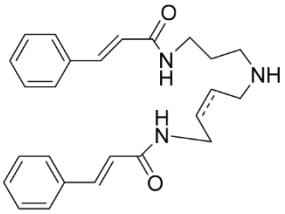
Anhydropisatin (Isoflavone)
Pisum
Cu: 0.41
B: 0.55
Zn: 0.17
Ca: 0.20
Fe: 0.21
K: 0.29
Mg: 0.14
S:0.42



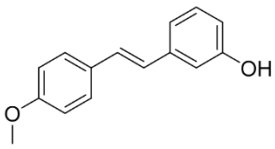
Hydroxyanhydropisatin (Isoflavone)
Pisum
Cu: 0.23
B: 0.48
Fe: 0.23
S: 0.16



4'-*O*-Methylresveratrol Stilbene
Phaseolus



Dicinnamoyl spermidine conjugate
Arabidopsis
Cu: 0.45
Zn: 0.26
Ca: 0.49
Fe: 0.48
Mg: 0.18
Na: 0.17



Stilbene
Phaseolus

Arabidopsis was especially efficient in taking up calcium and iron, one repeat also copper. Rapeseed showed variable efficiencies when the two repeats were compared directly. The first one was very efficient in manganese, potassium and sulphur uptake, the second only in potassium. Phaseolus did not excel in the uptake of any of the investigated nutrients. Pisum was very efficient in calcium; potassium, copper and iron uptake; Tobacco was only higher efficient in taking up calcium. The first repeat of Maize was very efficient in manganese uptake.

Generally, water deficit (Figure 5.3c) decreased nutrient efficacy of uptake with a few exceptions. Both Phaseolus repeats showed increased uptake of several elements except phosphorus, sodium, potassium and molybdenum. These elements, however, were found in higher concentrations in Rapeseed leaves. Arabidopsis was affected most severely by WD treatment.

5.3.2 Primary (PM) and secondary plant metabolites (SM) in root exudates and correlation of their profiles with those of leaf nutrients

Primary metabolites were analysed by GC–MS and secondary metabolites by HPLC–DAD and UPLC–TOF/MS. For this study, PMs and SMs were chosen that occurred in major amounts in the root exudates and contributed most to similarity and dissimilarity of the samples. This comprised the following PMs: 1,3-diaminopropane, 2,4-dihxydroxybutanoic acid, 3-methyl-2-hydroxybutanoic acid, GABA (γ-aminobutyric acid), alanine, arabinose, aspartic acid, benzoic acid, benzylacohol, citric acid, galactose, glucose, glycerol, glutamine, homoserine, isocitric acid, lysine, mannose, *myo*-inositol, pinitol, proline, pyroglutamic acid, ribose, succinic acid, threonine, xylose and xylulose. The following SMs were considered, including two compounds that also are classified as hormones: gibberellic acid GA_7 (hormone), a chalcone, the flavanol apiferol, indole-3-carboxaldehyde, methyl indole-3-carboxylate, a tetrahydrofurofuranone, caulilexin A, 4-hydroxy-1,4-benzoxazinone, two further indole structures, two dihydrophenanthrenes, the isoflavones anhydropisatin and hydroxyanhydropisatin, the stilbenes 4'-*O*-methylresveratrol and its dehydroxyderivate, and a dicinnamoyl spermidine. The identification of the major root exudate components is described in Chapter 3 and Chapter 4, Figure 5.3 provides several of their structures.

To explore if any correlation between root exudate metabolite profiles and nutrient uptake to the leaves exist their respective resemblance matrices were compared by a nonparametric

Mantel test using Spearman rank correlations. This procedure was performed both with the selected PM and SM group and with all PMs and SMs. The PMs showed no correlation, ρ = 0.00 (significance level of sample statistics 46.9 %) for selected major PMs and ρ = –0.14 (significance level of sample statistics 93.9 %) for total PMs (Appendix 2 on CD-ROM). By contrast, weak correlations were revealed for SMs: ρ = 0.18 (significance level of sample statistics 1.0 %) for selected major SMs and ρ = 0.23 (significance level of sample statistics 0.7 %) for total SMs (Appendix 3 on CD-ROM).

To further explore how single PMs or SMs correlate with the uptake of specific nutrients, Spearman rank correlations were determined after variable standardization. Figure 5.3 presents the detected positive correlations between the selected major PMs and SMs on one hand and leaf nutrient concentrations on the other hand.

5.4 Discussion

The median values of all nutrients were above or in the range of those concentrations that are considered sufficient for adequate growth (Marschner, 2012). This is unexpected as all investigated plants were supplied with additional fertilizer. The majority of the determined nutrient concentrations, specifically that of iron, copper, manganese were higher than the median, the highest iron conc. was 3.5-times higher, the highest copper 6-times higher, and the highest manganese 2.5-times higher than that of the median. The only further micro-nutrient that is known to benefit from coordination complex formation, zinc, showed a median that more or less divided the measurements in two halves, similarly as all other nutrients. This peculiar variation of iron, copper and manganese was a first hint that coordination complex formation with SMs in root exudates could have affected the nutrient uptake process.

A closer look at which plant species were better supplied with specific nutrients pointed to a certain efficacy of Arabidopsis in nutrient uptake, both in terms if macro- and micronutrients (Figures 3a and 3b). If this phenomenon is somehow linked to its extreme ephemeral life cycle, it merits further exploration. Conversely, water deficit affected Arabidopsis more severely than the other plant species that were included into this study (Figure 3c). One reason for these observations may be that the whole root system was not as well developed in Arabidopsis than in all the other investigated species; this was well visible in the shoot: root ratio (Chapter 4, Figure 4.1b).

The low stress values for the MDS plots that are shown by Figure 5.2 represented an important prerequisite to explore if any correlations between PMs and SMs and accumulation of specific nutrients exist. The fact that PMs, except for a few exceptions, showed no correlations (Figure 4) is not surprising. The PM system evolved for other purposes than nutrient uptake (Bar-Even *et al.*, 2012). Still, PMs represent the major portion of plant metabolites in root exudates. On the other hand, PM exudation might confer other evolutionary advantages to plants by providing more easily accessible carbon sources to root-associated microbes (Dakora & Phillips, 2002; Broeckling *et al.*, 2008). The only PM that correlated with copper, molybdenum, calcium and iron was citric acid. The preference of citric acid to form coordination complexes with these metals is well known (Dakora & Phillips, 2002; Neumann & Römheld, 2007).

In contrast to PMs, SMs showed weak correlations with some leaf-accumulated nutrients. (Figure 5.3). Basically, higher ρ values would be unrealistic, because (1) formation of coordination complexes of SM with nutrients are not specific for a specific one, and (2) weaker, less competitive ligands can compensate their in this aspect less advantageous physicochemical properties by being present in higher amounts. The correlations were calculated with two data sets, selected PMs and SMs (on basis of results from Chapter 3 and 4) and total PMs and SMs. When the total dataset was used, the correlation for PMs became worse and for SMs it remained roughly the same. One could argue that specific SMs only occur in specific species and this might have a substantial effect on the found correlations. However, the detected correlations could be negative, but they are positive. Furthermore, comparing the chemical structures with the detected correlations, it becomes evident that, by the majority, mostly unsaturated SMs with vicinal oxygen functions show chemical structures that support their potential ligand function for nutrient uptake. Examples include the chalcone, the dihydroxy-methoxy-cinnamide and indoles from Arabidopsis and Rapeseed and the dihydrophenanthrenes and isoflavones from Pisum. Another interesting metabolite in Arabidopsis root exudates is the dicinnamoyl spermidine conjugate. The spermidine moiety that is a nonproteinaceous amino acid alone turns it into a good chelator (Neumann & Römheld, 2007). A dicoumaroyl spermidine was found in hydroponically-obtained root exudates from Arabidopsis (Strehmel *et al.*, 2014). Furthermore, under iron-limiting conditions, Arabidopsis was shown to exude coumarins; coordination complexes with iron as central atom and coumarins as ligands were detected by UPLC–TOF/MS (Schmid *et al.*, 2014; Schmidt *et al.*, 2014). In this study, however, iron supply was not limited. Even though the

correlations of single nutrients with dihydrophenanthrenes and isoflavones may be high, Pisum, the exuding plant, shows lower concentrations of many nutrients than Arabidopsis. However, this is feasible as Pisum exudes much lower relative amounts of SMs than Arabidopsis (Chapter 4, Figure 4.1c). Furthermore, Phaseolus exuded the highest relative SM amounts of all investigated plants but the major components showed low correlations.

Boron, phosphorus and sulphur are taken up as anions. Phosphoric and boric acid were detected by GC–MS. Correlation of coordination complex forming PMs and SMs can arise due to mobilization effects of the corresponding cation from salts by forming a coordination complex with its cation. Iron, copper, manganese and zinc uptake can profit from coordination complex formation. Cationic nutrients that are better water-soluble, such as sodium, potassium, calcium and magnesium, can also act as central atoms in coordination complexes, but their uptake is generally assumed to be more independent of this mechanism. For the correlations, leaf nutrient concentrations were used in assumptions that SM coordination complex formation can affect transport in the xylem as for example the polyamine nicotianamine does (Stephan & Scholz, 1993). From studies in trees it is well known that SMs occur not only in the phloem but also in the xylem (Turtola *et al.*, 2002). It can be assumed that all SMs that are detectable in root exudates possess suitable dissolubility properties.

5.5 References

Ahuja, I, Kissen, R, Bones, AM. 2012. Phytoalexins in defense against pathogens. Trends in Plant Science **17**: 73–90.

Badri, DV, Vivanco, JM. 2009. Regulation and function of root exudates. *Plant, Cell and Environment* **32**: 666–681.

Bar-Even, A, Flamholz, A, Noor, E, Milo, R. 2012. Rethinking glycolysis: on the biochemical logic of metabolic pathways. *Nature Chemical Biology* **8**: 509–517.

Betina, V. 1995. Differentiation and secondary metabolism in some prokaryotes and fungi. *Folia Microbiologica* **40**: 51–67.

Broeckling, CD-ROM, Broz, AK, Bergelson, J, Manter, DK, Vivanco, JM. 2008. Root exudates regulate soil fungal community composition and diversity. *Applied and Environmental Microbiology* **74**: 738–744.

Cesco, S, Neumann, G, Tomasi, N, Pinton, R, Weisskopf, L. 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant and Soil* **329**: 1–25.

Clarke, KR. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Ecology* **18**: 117–143.

Cooper, JE. 2007. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *Journal of Applied Microbiology* **103**: 1355–1365.

Dakora, FD, Phillips, DA. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* **245**: 35–47.

Davies, KJA. 2000. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life* **50**: 279–289.

Hadacek, F, Bachmann, G. 2015. Low-molecular-weight metabolite systems chemistry. *Frontiers in Environmental Science* **3**: 12.

Hartmann, T. 2007. From waste products to ecochemicals. Fifty years research of plant secondary metabolism. *Phytochemistry* **68**: 2831–2846.

Jacob, C, Jamier, V, Ba, LA. 2011. Redox active secondary metabolites. *Omics* **15**: 149–155.

Jung, C, Maeder, V, Funk, F, Frey, B, Sticher, H, Frossard, E. 2003. Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification. *Plant and Soil* **252**: 301–312.

Kopka, J, Schauer, N, Krueger, S, Birkemeyer, C, Usadel, B, Bergmüller, E, Dörmann, P, Weckwerth, W, Gibon, Y, Stitt, M, Willmitzer, L, Fernie, AR, Steinhauser, D. 2005. GMD@CSB.DB. the Golm Metabolome Database. *Bioinformatics* **21**: 1635–1638.

Kuhn, BM, Geisler, M, Bigler, L, Ringli, C. 2011. Flavonols accumulate asymmetrically and affect auxin transport in Arabidopsis. *Plant Physiology* **156**: 585–595.

Kuijken, RCP, Snel, JFH, Heddes, MM, Bouwmeester, HJ, Marcelis, LFM. 2015. The importance of a sterile rhizosphere when phenotyping for root exudation. *Plant and Soil* **387**: 131–142.

Marschner, P, ed. 2012. Marschner's Mineral Nutrition of Higher Plants. Amsterdam: Elsevier.

Mithöfer, A, Boland, W. 2012. Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology* **63**: 431–450.

Morris, C, Grossl, PR, Call, CA. 2009. Elemental allelopathy. Processes, progress, and pitfalls. *Plant Ecology* **202**: 1–11.

Nakabayashi, R, Saito, K. 2015. Integrated metabolomics for abiotic stress responses in plants. *Current Opinion in Plant Biology* **24**: 10–16.

Neumann, G, Römheld, V. 2007. The release of root exudates as affected by the plant physiological status. In: Pinton, R, Varanini, Z, Nannipieri, P, eds. The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Boca Raton, London, New York: CRC Press, 41–93.

Pichersky, E, Noel, JP, Dudareva, N. 2006. Biosynthesis of Plant Volatiles: Nature's Diversity and Ingenuity. *Science* **311**: 808–811.

Sánchez-Moreiras, AM, La Peña, TC de, **Reigosa, MJ. 2008.** The natural compound benzoxazolin-2(3*H*)-one selectively retards cell cycle in lettuce root meristems. *Phytochemistry* **69**: 2172–2179.

Schmid, NB, Giehl, Ricardo F H, Döll, S, Mock, H-P, Strehmel, N, Scheel, D, Kong, X, Hider, RC, Wirén, N von. **2014.** Feruloyl-CoA 6'-hydroxylase1-dependent coumarins mediate iron acquisition from alkaline substrates in Arabidopsis. *Plant Physiology* **164**: 160–172.

Schmidt, H, Günther, C, Weber, M, Spörlein, C, Loscher, S, Böttcher, C, Schobert, R, Clemens, S. 2014. Metabolome analysis of *Arabidopsis thaliana* roots identifies a key metabolic pathway for iron acquisition. *PloS one* **9**: e102444.

Stephan, UW, Scholz, G. 1993. Nicotianamine - mediator of transport of iron and heavy-metals in the phloem. *Physiologia Plantarum* **88**: 522–529.

Strehmel, N, Böttcher, C, Schmidt, S, Scheel, D. 2014. Profiling of secondary metabolites in root exudates of *Arabidopsis thaliana*. *Phytochemistry* **108**: 35–46.

Turtola, S, Manninen, A-M, Holopainen, JK, Levula, T, Raitio, H, Kainulainen, P. 2002. Secondary metabolite concentrations and terpene emissions of scots pine xylem after long-term forest fertilization. *Journal of Environment Quality* **31**: 1694.

van Dam, NM, Bouwmeester, HJ. 2016. Metabolomics in the rhizosphere. Tapping into belowground chemical communication. *Trends in Plant Science* **21**: 256–265.

Weng, J-K, Philippe, RN, Noel, JP. 2012. The rise of chemodiversity in plants. *Science (New York, N.Y.)* **336**: 1667–1670.

Yang, J, Zhang, J, Wang, Z, Zhu, Q, Liu, L. 2002. Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta* **215**: 645–652.

6. General Discussion

Pervin Akter[1], Franz Hadacek[1,2]

6.1 Primary and secondary plant metabolites in root exudates of different plant species

Plant root exudates contain primary metabolites (PM) and secondary metabolites (SM). The former, by far, represent the major portion. Primary, or central metabolites, represent components of the life-sustaining biochemistry not only of plant cells, but cells of all living organisms (Bar-Even *et al.*, 2012). Substantial quality differences between plant species are thus unlikely for PMs and were not detected in root exudates and root tissues of the six investigated species. Quantitative differences, however, were substantial between species. H High fluctuations in phosphoric acid caused a low PM average similarity of only 42 % (SIMPER analysis). Other highly variable PMs included the sugars glucose and fructose, and the sugar alcohol myo-inositol. As culture conditions were not nutrient limited, organic acids, such as citric acid, which are known to be exuded in nutrient-limiting conditions (Neumann & Römheld, 2007), were only detected in Arabidopsis root exudates in notable amounts.

Secondary metabolites, which are known to be less uniform in terms of their distribution in the plant kingdom (Hartmann, 1985), reflect their status also in root exudates. The SM fractions of the investigated plants species, however, contained some compounds that were common to all of them. Besides some yet still unidentified more lipophilic metabolites, cinnamic acid was the most prominent. Literature searches in relevant databases procure many hits. HPLC–DAD is most sensitive, much more than UPLC–TOF/MS and GC–MS. SMs are much more difficult to identify.

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Author contributions: concept: PA, FH; text: PA; Figures: PA, FH

For PMs, public-domain databases exist that provide MS spectra of standards for comparative purposes (Kopka *et al.*, 2005). For SMs this only applies to selected cases. If liquid chromatography is linked to a mass spectrometer, no EI (electron impact) spectra are obtained, whose rich fragmentation patterns are very helpful for unambiguous identification. Instead, CI (chemical ionisation) spectra are state-of-the-art, which depend on chemical reactions that can be analyte and reactant-specific. Usually this requires to analyse samples in the positive (addition of a proton or a reactant to yield a cation) and negative ionization mode (loss of a proton or addition of a reactant to yield an anion). Although many instruments offer alternative analyses in one run, better results are obtained by separate analyses (Niessen, 2006). A further difficulty that is added to interpreting the thus obtained mass spectra is that spectra usually contain more than one peak and not all peaks belong to the same analyte. In this case, UV spectra can be very helpful und simultaneous measurement after the analytes elute from the HPLC column is possible. Unsaturated analytes usually have characteristic spectra that, with a little experience, allow identifying possible classes of SMs, for example to decide if the analyte could be a stilbene or a flavonoid. Although a high-resolution mass spectrometer was used, not in all cases the measured masses are close enough to the calculated ones, which could be by high analyte concentrations. In nearly all cases of SM identification (Chapter 3 and Chapter 4), a comparison with authentic standards or further MS/MS experiments would be desirable if not for unavailability of the former and restriction of analysis time for the latter.

Generally, representatives of more or less all reported classes of metabolites could be identified except steroids (Dakora & Phillips, 2002; Uren, 2007).

6.2 Root exudates and root tissue extracts

The profiles of PMs and SMs in root exudates and root tissues differed significantly (Chapter 3). Among PMs, the amino acids asparagine, γ-aminobutyric acid (GABA) and serine were much more prominent in root tissues than in root exudates. Phosphoric acid concentrations were also much lower in root tissues than in root exudates. This is to be expected. The average similarity for PMs in root tissues was higher than in root exudates, 69 versus 43 %.

The similarity of SMs in root exudates and root tissues was much lower. Several SMs were either specific for root exudates or root tissues, but many also occurred in both. One interesting case was the tentative DIMBOA glucoside that represented one of the few root-

specific SMs that could be identified in the root tissues. Except HBOA no benzoxazines could be found in the root exudates though literature reports point to this phenomenon (Neal *et al.*, 2012).

6.3 Efficacy of the applied extraction method

The applied classical collection method, soaking the roots of intact plants in distilled water for several hours, yielded good results in terms of detectable metabolites (Appendix 2 and Appendix 3). For secondary metabolites, however, pooling of the root exudates substantially improved result quality because otherwise more than 100 analytes could not have been detected. One problem that occurs during the analysis root exudates is that metabolites in the apoplastic space tend to form oligomers. Some impression is provided by studies of hydroponic solutions—the apoplast is extracted for a longer period, often several days—in which a high percentage of dimeric SMs can be found (Strehmel *et al.*, 2014). Not only that oligomerisation leads to higher-molecular-weight analytes, their number will be increased by this rather uncontrolled chemistry. In plant tissues, such processes are under more under enzymatic control, especially for those SMs that are accumulated specifically in more unpolar compartments. The vacuole is more aqueous and solubility of SMs is low. The low solubility of SMs in aqueous environments most probably contributes also to their low concentration in root exudates. Another fact that can hamper comparability with the literature is that both quantity and quality of exuded metabolites can be affected by various stress factors (Chapter 4). For this reason, in the water deficit treatment, root exudates were collected only after a rewetting period to make certain that water provision to the tissue was comparable.

Results from the electrochemical measurements with DPV add support the outlined scenario. The voltammograms suggest a higher reproducibility of the root exudate collection repeats than the chromatographic analyses. It could be possible, on one hand, that not all present analytes were detected by the chromatographic analyses. The voltammogram can also be affected by coordination complexes of SMs and PMs with nutrients that do not survive the chromatographic analysis. On the other hand, only specific analytes could contribute to the reproducible observed peaks. Concerning this aspect, many issues wait for future clarifications.

6.4 Water deficit

This type of stress was chosen because it is simple and more or less reproducibly to apply, especially with the available facilities (Chapter 2). The results show, that the PM and SM patterns change, the PMs more in a general way, the SMs more specifically. Not all plants are similarly affected by the water deficit. As a rule, the shoot: root ratio increases. The smaller root biomass shows increased exudation of PMs and SMs. The relative amounts vary between plant species. Arabidopsis develops much more aerial biomass than roots and thus deviates a little bit in this experiment. Phaseolus and Maize also differ by not reducing their root growth as efficiently as other plant species after exposure to water deficit. Only Pisum exuded a novel class of SMs, dihydrophenanthrenes. Though limited in scope, the obtained results recommend further exploration of root exudation dynamic in stress scenarios. The versatility of root exudates to change after exposure to stress, which is often voiced in the literature but rarely documented (Ziegler *et al.*, 2016), is corroborated by this study.

6.5 Secondary metabolites and nutrient uptake

The existing literature hints cautiously that exuded SMs can improve uptake of specific nutrients, especially micronutrients, many of which represent important cofactors for enzymes (Petho, 1992; Schmidt *et al.*, 2000; Dakora & Phillips, 2002; Schmidt *et al.*, 2014). From all stress experiments, water deficit and control, leaf nutrients were also analysed to test for possible correlations between PMs and SMs and nutrient uptake. ANOSIM analysis using Spearman rank correlation and pairwise Spearman rank correlation analysis pointed to possible effects of most the major SMs in root exudates to improved nutrient uptake. Micronutrients were more affected than macronutrients. In agreement with the proposed mechanism of coordination complex formation with micronutrient metals as central atoms and SMs as ligands, the thus identified SMs possess the required functional groups for this chemical property.

6.6 References

Bar-Even, A, Flamholz, A, Noor, E, Milo, R. 2012. Rethinking glycolysis: on the biochemical logic of metabolic pathways. Nature Chemical Biology **8**: 509–517.

Dakora, FD, Phillips, DA. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* **245**: 35–47.

Hartmann, T. 1985. Principles of plant secondary metabolism. *Plant Systematics and Evolution* **150**: 15–34.

Kopka, J, Schauer, N, Krueger, S, Birkemeyer, C, Usadel, B, Bergmüller, E, Dörmann, P, Weckwerth, W, Gibon, Y, Stitt, M, Willmitzer, L, Fernie, AR, Steinhauser, D. 2005. GMD@CSB.DB. the Golm Metabolome Database. *Bioinformatics* **21**: 1635–1638.

Neal, AL, Ahmad, S, Gordon-Weeks, R, Ton, J. 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PloS one* **7**: e35498.

Neumann, G, Römheld, V. 2007. The release of root exudates as affected by the plant physiological status. In: Pinton, R, Varanini, Z, Nannipieri, P, eds. The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Boca Raton, London, New York: CRC Press, 41–93.

Niessen, WMA. 2006. Liquid Chromatography-Mass Spectrometry. Boca Raton, FL: CRC Press.

Petho, M. 1992. Occurrence and physiological role of benzoxazinones and their derivatives. III. Possible role of 7-methoxybenzoxazinone in the iron uptake of maize. *Acta Agronomica Hungarica* **41**: 57–64.

Schmidt, H, Günther, C, Weber, M, Spörlein, C, Loscher, S, Böttcher, C, Schobert, R, Clemens, S. 2014. Metabolome analysis of *Arabidopsis thaliana* roots identifies a key metabolic pathway for iron acquisition. *PloS one* **9**: e102444.

Schmidt, W, Tittel, J, Schikora, A. 2000. Role of hormones in the induction of iron deficiency responses in Arabidopsis roots. *Plant Physiology* **122**: 1109–1118.

Strehmel, N, Böttcher, C, Schmidt, S, Scheel, D. 2014. Profiling of secondary metabolites in root exudates of *Arabidopsis thaliana*. *Phytochemistry* **108**: 35–46.

Uren, NC. 2007. Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. In: Pinton, R, Varanini, Z, Nannipieri, P, eds. The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Boca Raton, London, New York: CRC Press, 1–23.

Ziegler, J, Schmidt, S, Chutia, R, Muller, J, Bottcher, C, Strehmel, N, Scheel, D, Abel, S. 2016. Non-targeted profiling of semi-polar metabolites in Arabidopsis root exudates uncovers a role for coumarin secretion and lignification during the local response to phosphate limitation. *Journal of Experimental Botany* **67**: 1421–1432.

7. Summary

Root exudates represent complex mixtures of low-molecular-weight and high-molecular-weight compounds. The former comprise predominantly primary and secondary plant metabolites, the latter mucilage precursors and some proteins.

Different collection approaches exist. The traditionally most widely used one (also applied in this study) is soaking roots for several hours in distilled water that have been thoroughly cleaned from soil. Other collection methods comprise hydroponic cultures and rhizoboxes with microsuction devices. The former are more used by molecular biologists in attempts to characterize phenotypes reproducibly, the latter by ecologists in efforts to explore specific regions of the rhizosphere. The classical approach is one that still allows plant to be cultured in soil. This is important because mucilage formation is affected by microbial soil communities and soil physicochemical properties. Primary and secondary plant metabolites provide the majority of low-molecular precursors for mucilage development. Quality and quantity of extractable root-exuded plant metabolites is most probably affected by these parameters which are completely absent in hydroponic cultures.

Six model plants were chosen on basis of their status as crop plant and on their tolerance of the uniform culture conditions to which all model plants were subjected to in the only available climate chamber. These included the Brassicaceae Arabidopsis and Rapeseed, the Fabaceae Phaseolus and Pisum, the Solanaceae Tobacco and the grass Maize. In all experiments, the model plants received identical amounts of light, the same water supply and nutrient provision. In attempts to simulate drought stress, one-half of the plants was not deprived of water for two consecutive weeks.

One aim was to explore which and to what amounts primary as well as secondary plant metabolites do occur in the root exudates and if they differ from those present in the roots. All six plant species showed similar primary metabolite profiles that, however, varied quantitatively between the plant species. A prominent root exudate metabolite was *myo*-inositol, a sugar alcohol. Root tissues and root exudates showed different profiles with amino acids showing the most profound differences. The found primary metabolites agree with those reported in the literature.

By contrast, secondary metabolites showed characteristic profiles, in which only few compounds were common to more than one species. One metabolite that was detected in all species was cinnamic acid. Structure elucidation was focussed especially on those secondary plant metabolites that were pointed out by non-parametric multivariate statistics as substantial contributors to similarity and dissimilarity of root exudates and root tissues. Root exudates were found to contain chalcones, flavanols, isoflavones, cinnamides, a cinnamoyl spermidine, indoles, stilbenes, a hydroxamic acid benzoxazine and a gibberellic acid derivative, amongst others. Notably, no glycosides were detected among the elucidated metabolites and a considerably high proportion of aldehydes was noted. In case of Arabidopsis, an extensive analysis of hydroponically obtained root exudates exists in the literature. Many dimeric structures were reported, most of which could not be detected in the present study.

Another explored aspect was the effect of water deficit on root exudation. Primary metabolite patterns changed in a more similar way, sugars such as glucose and sucrose increased and myo-inositol proportions decreased. Amino acid pattern changes, by contrast, were more species-specific. Generally, the amounts of detectable secondary metabolites decreased as shoot: root ratios in the affected plants increased. Only Phaseolus and Maize showed higher shoot: root ratios after water deficit. This suggests a different, more opportunistic strategy to survive stress. Only Pisum exuded a new class of secondary metabolites that was absent in the regularly watered plants.

Altogether 24 different root exudate samples were available. Bases on the variability of primary and secondary root exudate metabolites correlations with nutrient supply in leaves was explored by Spearman rank correlation. Interestingly and as once suggested in a previous review, weak correlations between secondary metabolite profiles and leaf nutrients were found. Especially more unsaturated metabolites with vicinal oxygen functions correlated with the uptake of several nutrients, most of them being metal cations. The structural properties of the identified secondary metabolites allows them to act as ligands in coordination complexes in which the nutrient represents the central atom. This chemistry can add to the mobilization and uptake of nutrients by plant roots.

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9. Curriculum Vitae

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10. Appendix

The printed version provides all appendix data on the included CD-ROM

Appendix 1

Growth chamber experiments

***Arabidopsis thaliana* L.cv. Columbia**

Experiment 1

Day	Control (C)	Water deficit (WD)
36		
47		

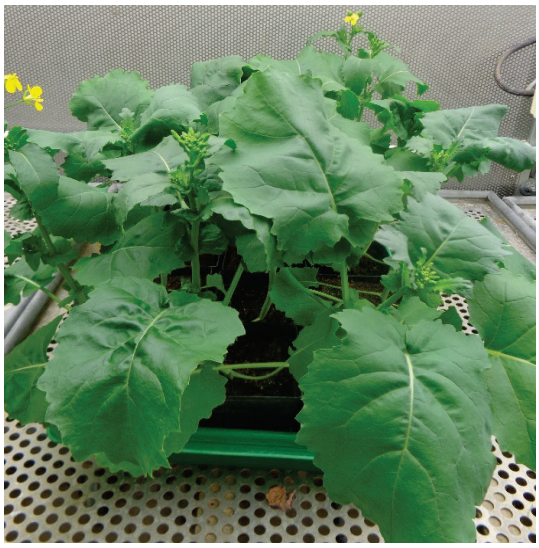
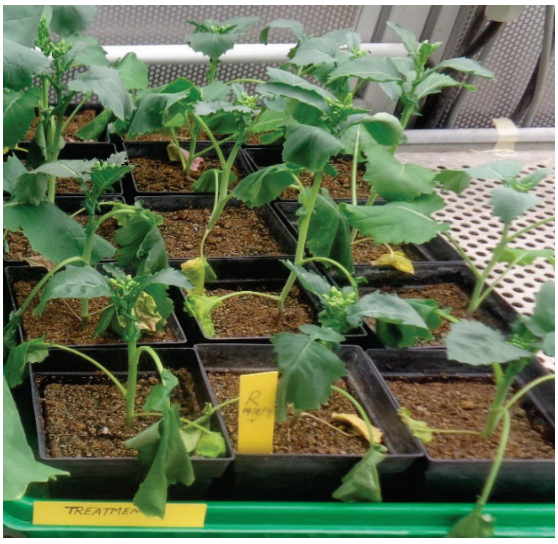
***Arabidopsis thaliana* L. cv. Columbia**

Experiment 2

Day	Control (C)	Water deficit (WD)
36		
47		

***Brassica napus* L. cv. Miniraps (Rapid Cycling OSR)**

Experiment 1

Day	Control (C)	Water deficit (WD)
36		
47		

***Brassica napus* L. cv. Miniraps (Rapid Cycling OSR)**

Experiment 2

Day	Control (C)	Water deficit (WD)
36		
47		

***Pisum sativum* L. cv. Topaz**

Experiment 1

Day	Control (C)	Water deficit (WD)
36		
47		

***Pisum sativum* L. cv. Topaz**

Experiment 2

Day	Control (C)	Water deficit (WD)
36		
47		

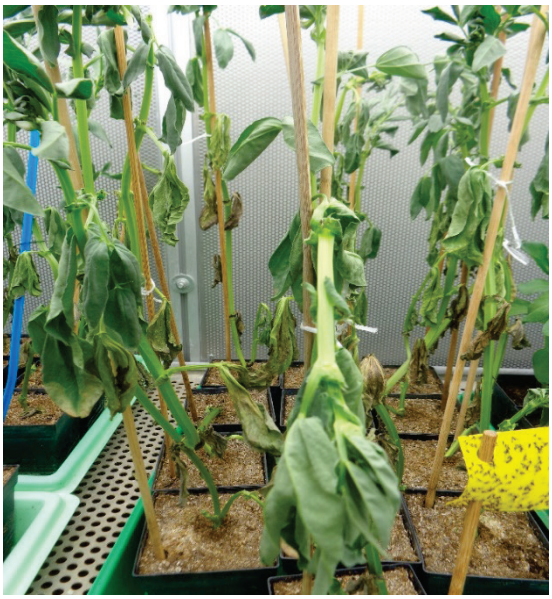
***Phaseolus vulgaris* L.cv. Hangdown**

Experiment 1

Day	Control (C)	Water deficit (WD)
36		
47		

***Phaseolus vulgaris* L.cv. Hangdown**

Experiment 2

Day	Control (C)	Water deficit (WD)
36		
47		

***Nicotiana tabacum* L. cv. Xanthi**

Experiment 1

Day	Control (C)	Water deficit (WD)
36		
47		

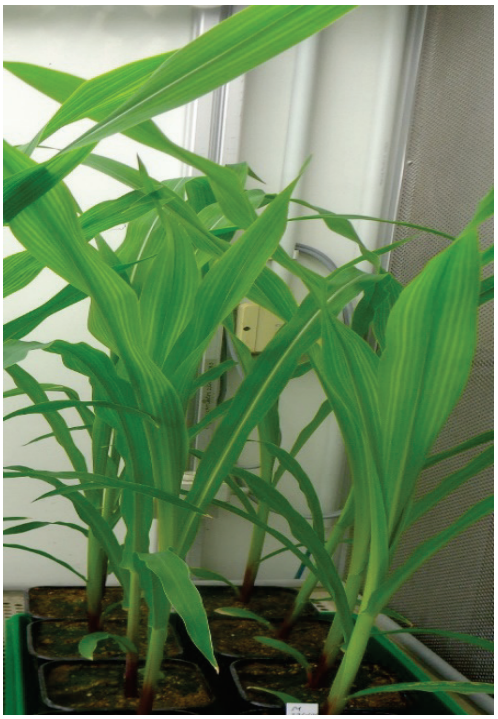
***Nicotiana tabacum* L. cv. Xanthi**

Experiment 2

Day	Control (C)	Water deficit (WD)
36		
47		

***Zea mays* L. Rolandino**

Experiment 1

Day	Control (C)	Water deficit (WD)
36		
47		

***Zea mays* L. Rolandino**

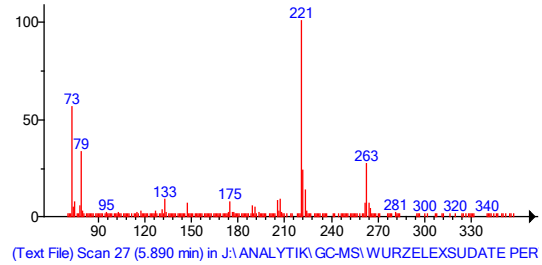
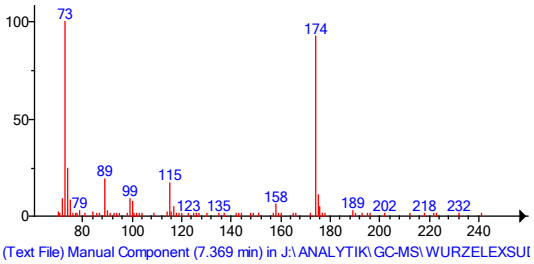
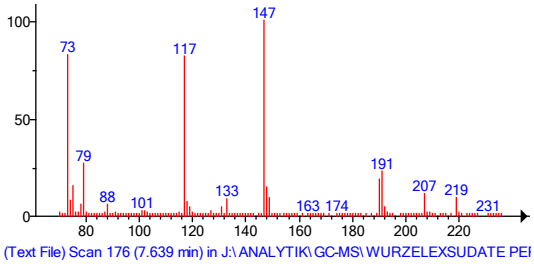
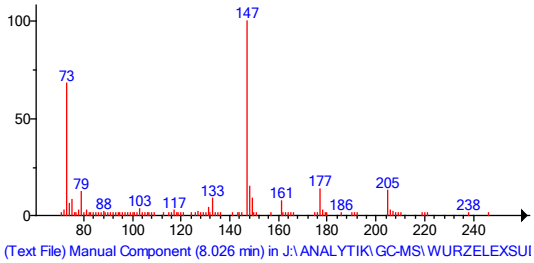
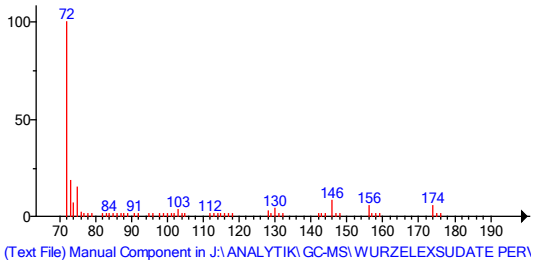
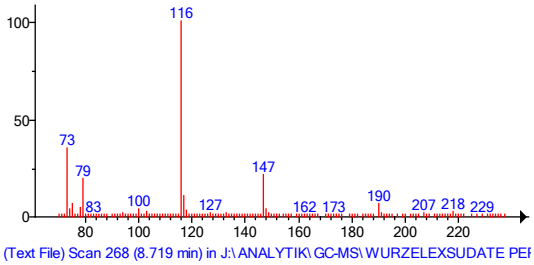
Experiment 2

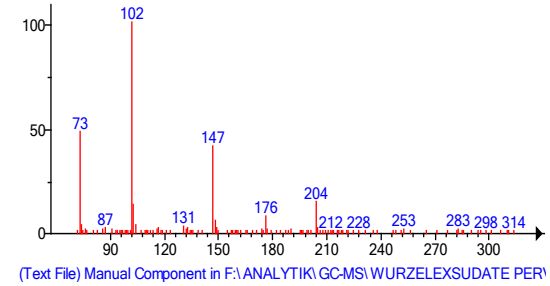
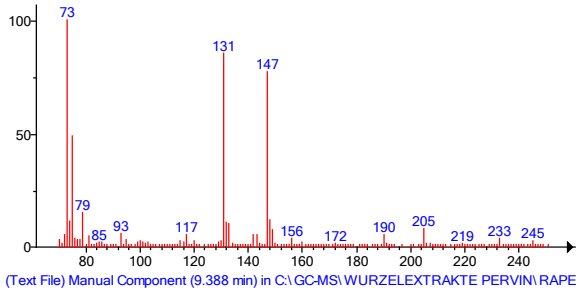
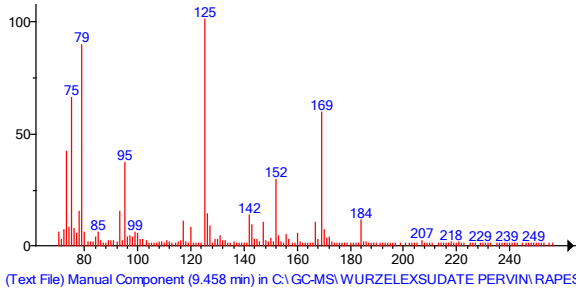
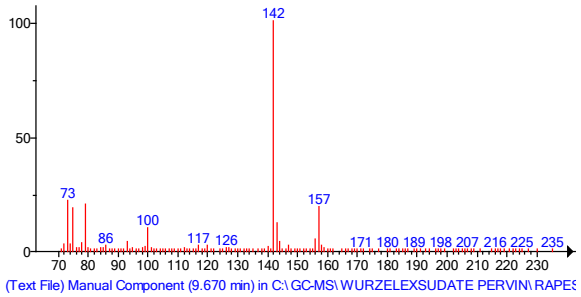
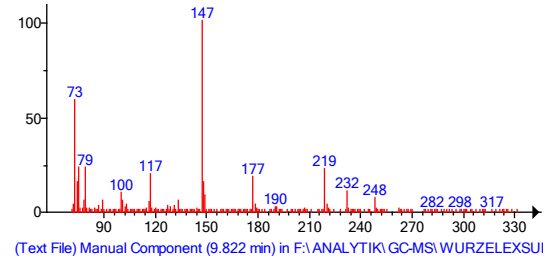
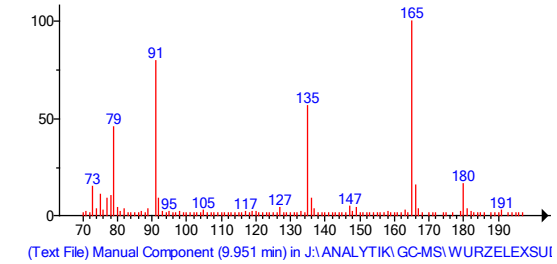
Day	Control (C)	Water deficit (WD)
36		
41		

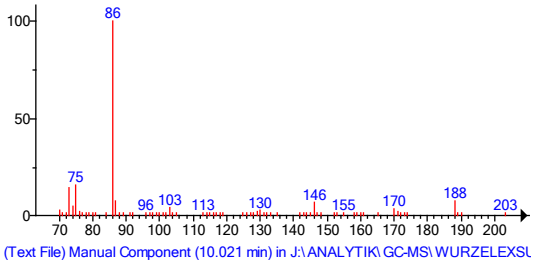
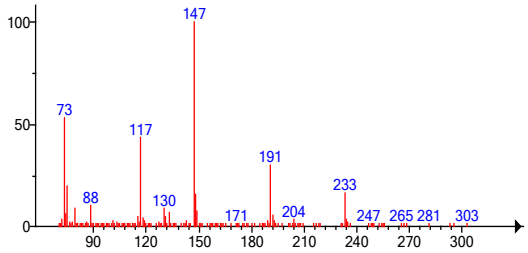
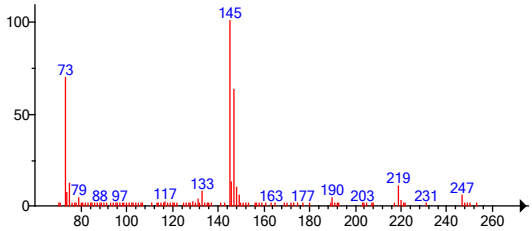
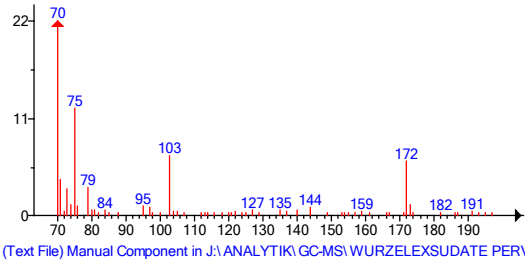
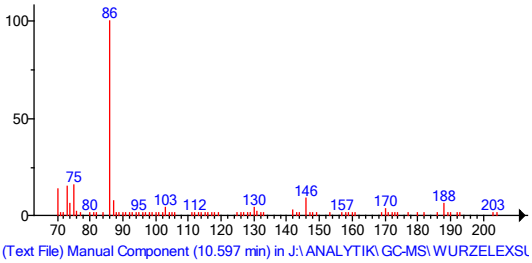
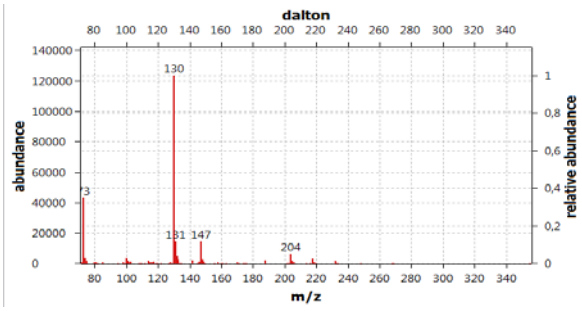
Appendix 2

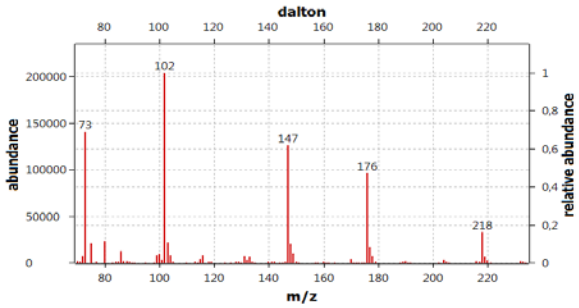
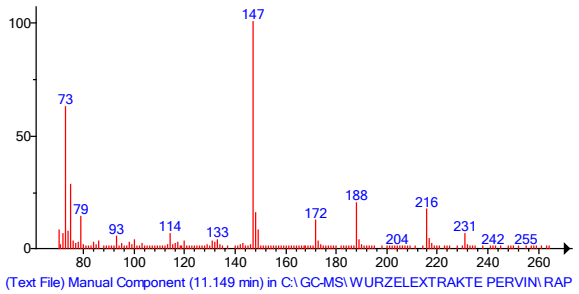
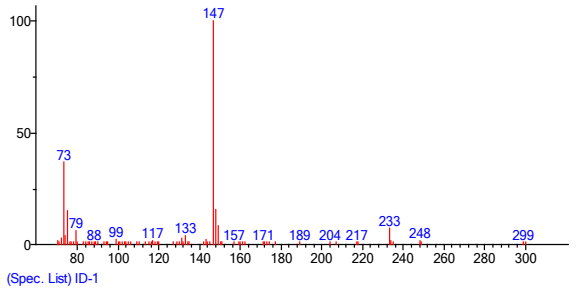
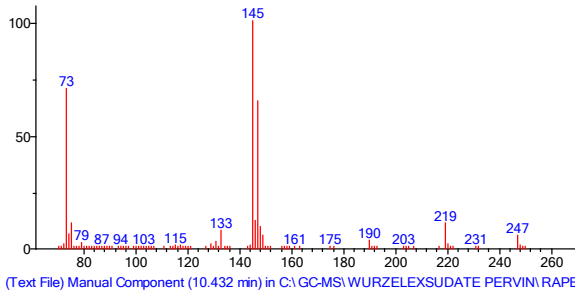
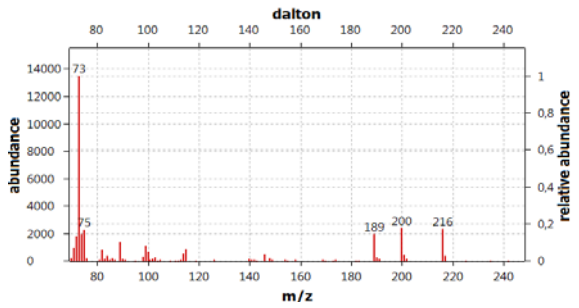
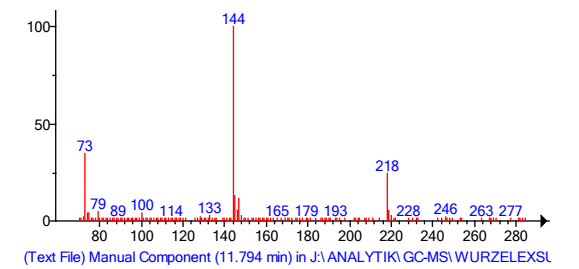
GC–MS analyses: EI/MS spectra

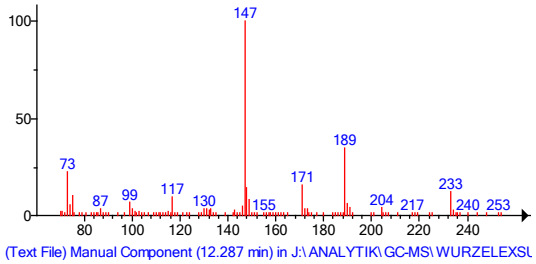
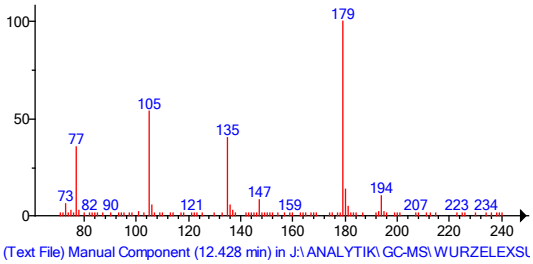
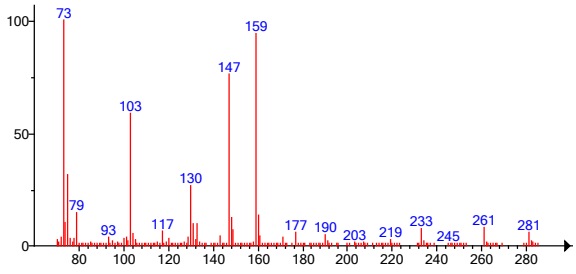
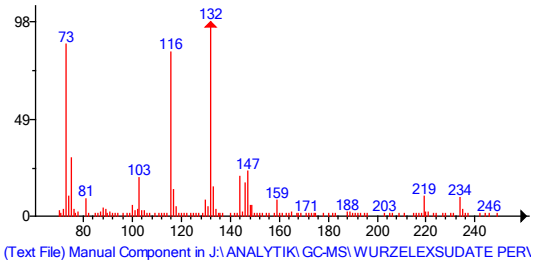
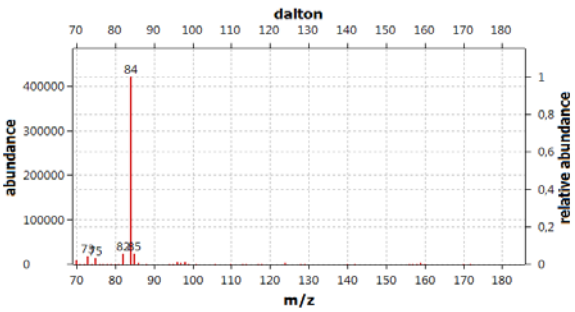
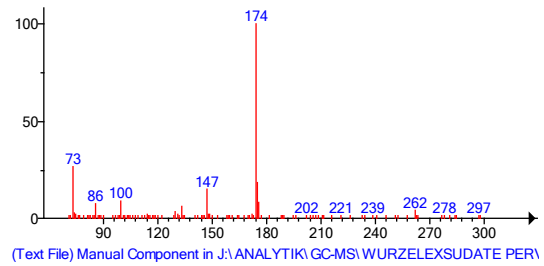
Ret.	Retention time
RI	Retention index (according to alkane standards)
RI (GMD)	Retention index (GMD)
TMS	Number of TMS (trimethylsilyl) groups after chemical derivatization
Ox	Number of oxime groups after chemical derivatization

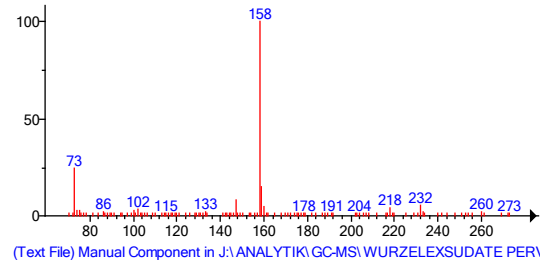
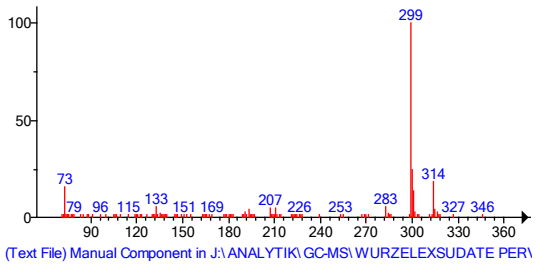
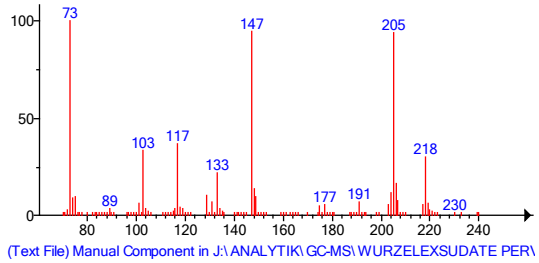
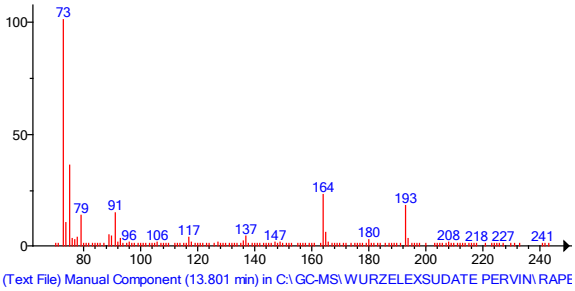
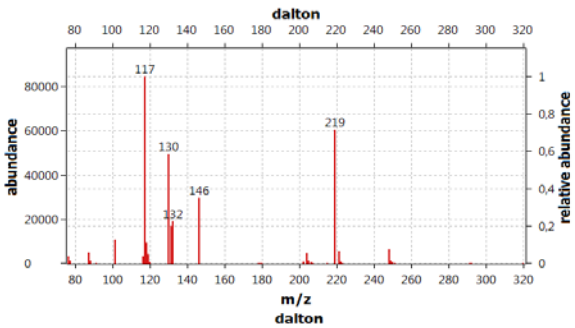
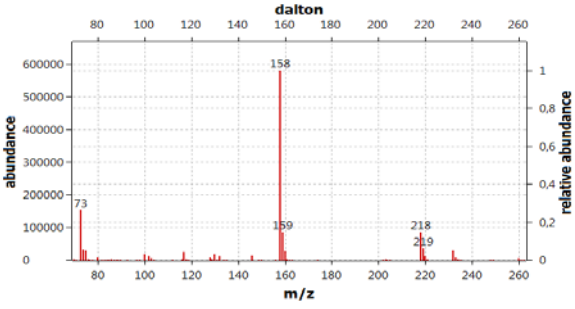
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**5.89**	**989**	*971*	Boric acid	3	
	**7.36**	**1088**	*1036*	Pyruvic acid	1	1
	**7.62**	**1096**	*1044*	Lactic acid	2	
	**8.02**	**1108**	*1062*	Acetic acid	2	
	**8.21**	**1114**	*1081*	Valine	1	
	**8.70**	**1129**	*1086*	Alanine	2	

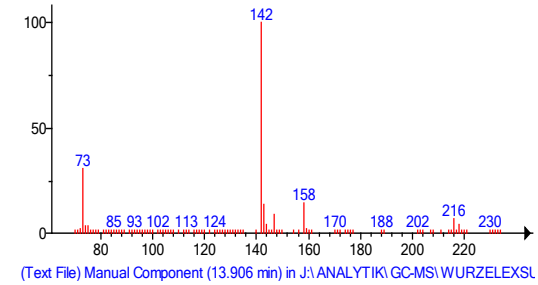
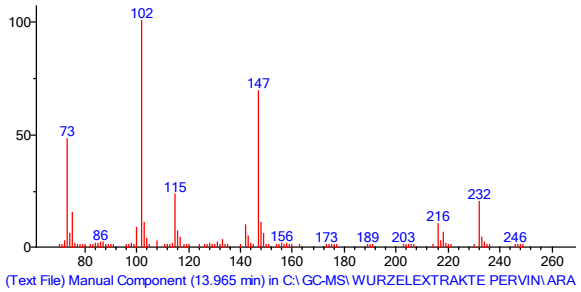
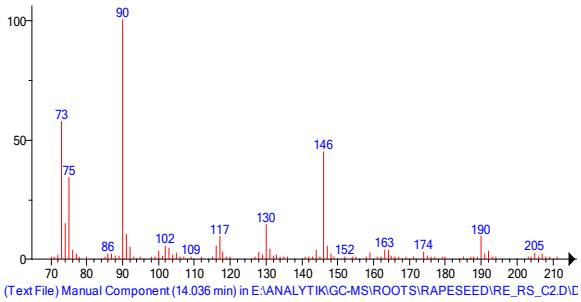
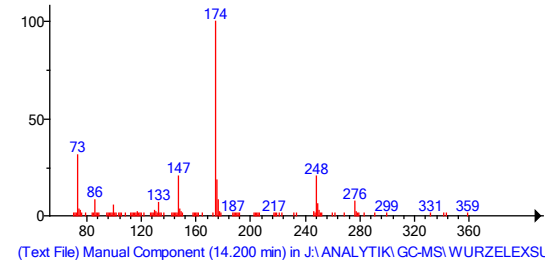
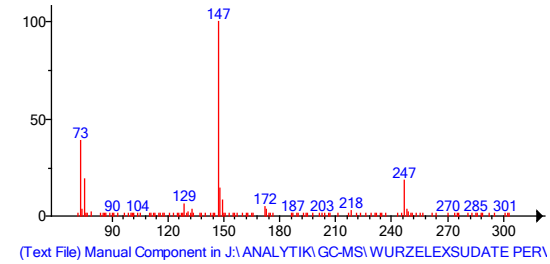
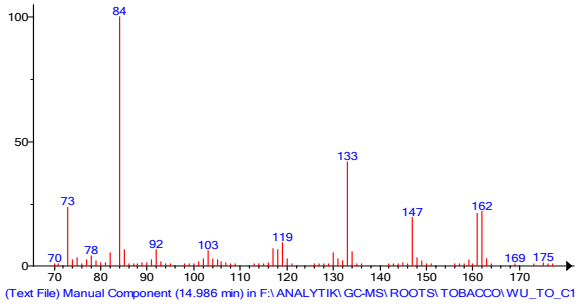
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**9.13**	**1142**	*1110*	Glycine	2	
	**9.38**	**1150**	*1111*	Butanoic acid, 2-hydroxy	2	
	**9.41**	**1151**	*1131*	Furan-2-carboxylic acid	2	
	**9.66**	**1159**	*1140*	Butyro-1,4-lactam	1	
	**9.82**	**1164**	*1131*	A115002 (GMD unknown)		
	**9.95**	**1168**	*1150*	Benzylalcohol	2	

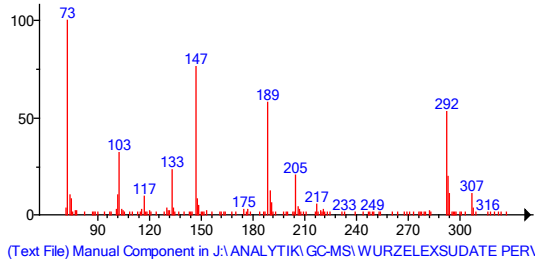
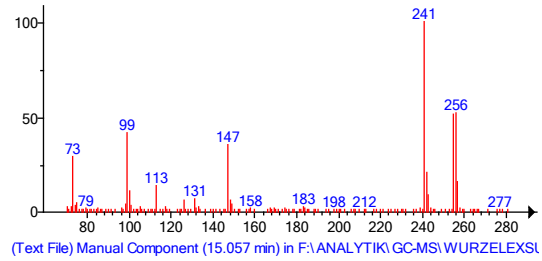
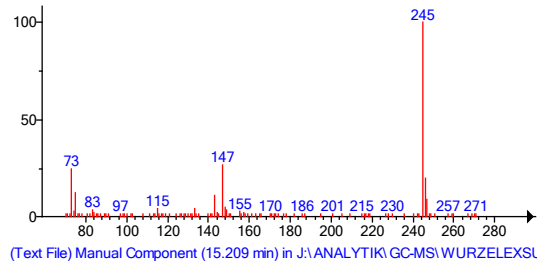
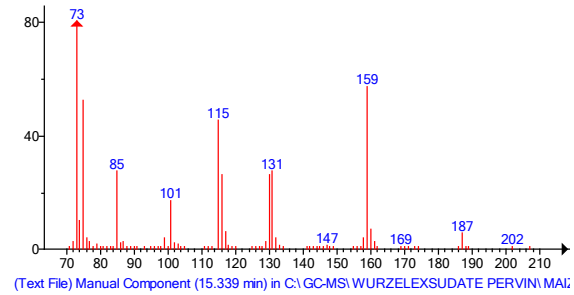
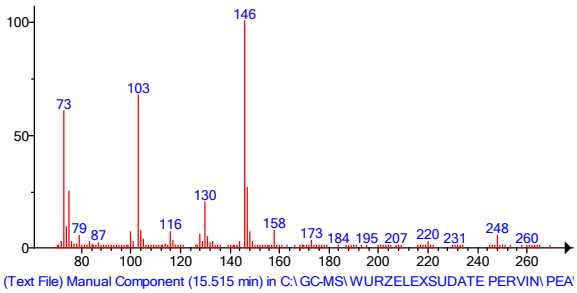
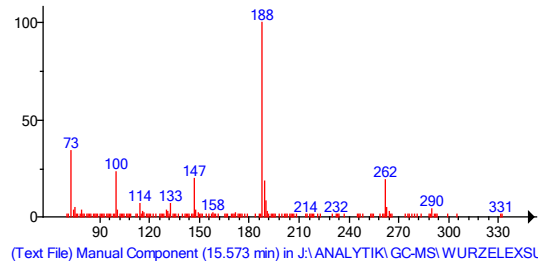
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
 (Text File) Manual Component (10.021 min) in J:\ ANALYTIK\ GC-MS\ WURZELEXSU	**10.03**	**1171**	*1151*	Leucine	1	
 (Text File) Manual Component (10.268 min) in J:\ ANALYTIK\ GC-MS\ WURZELEXSU	**10.27**	**1179**	*1160*	Butanoic acid, 3-hydroxy	2	
 (Text File) Manual Component (10.421 min) in J:\ ANALYTIK\ GC-MS\ WURZELEXSU	**10.41**	**1183**	*1178*	Butanoic acid, 3-methyl-2-hydroxy	2	
 (Text File) Manual Component in J:\ ANALYTIK\ GC-MS\ WURZELEXSUDATE PER\	**10.41**	**1183**	*1178*	Proline	1	
 (Text File) Manual Component (10.597 min) in J:\ ANALYTIK\ GC-MS\ WURZELEXSU	**10.58**	**1189**	*1174*	Isoleucine	1	
	**10.59**	**1189**	*1161*	2-Amino-butanoic acid	2	

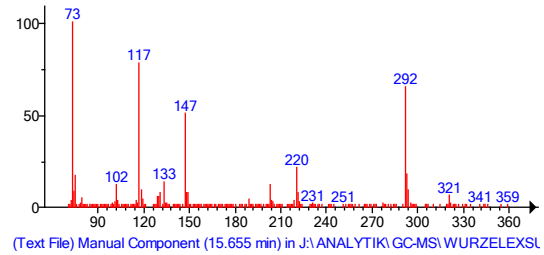
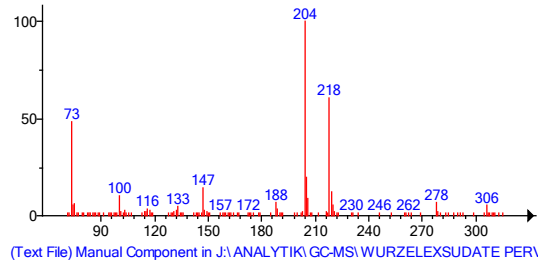
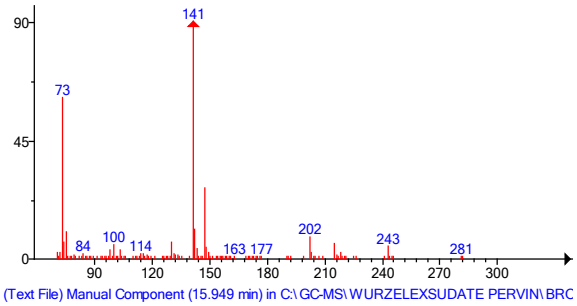
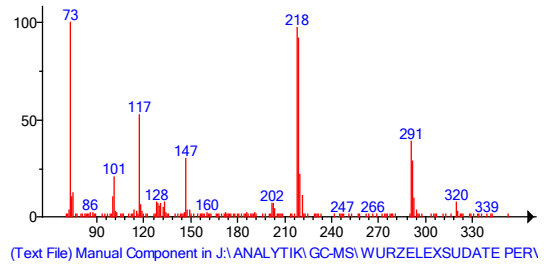
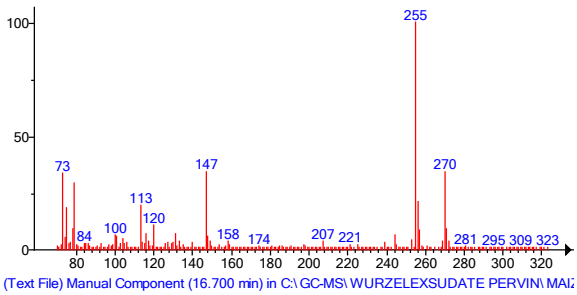
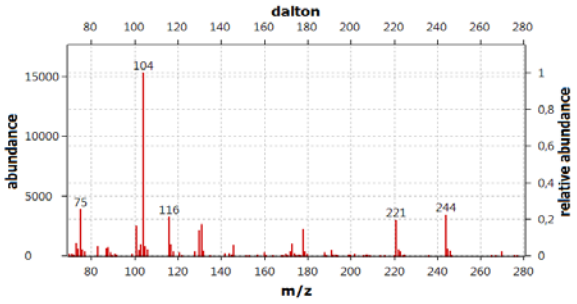
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**11.06**	**1200**	*1184*	β-Alanine	2	
	**11.17**	**1208**	*1208*	A118006 (GMD unknown)		
	**11.45**	**1217**	*1190*	Malonic acid	2	
	**11.47**	**1218**	*1050*	α-Hydroxyisovaleric acid	1	
	**11.75**	**1228**	*1168*	Isocaproic acid	1	1
	**11.79**	**1229**	*1225*	Valine	2	

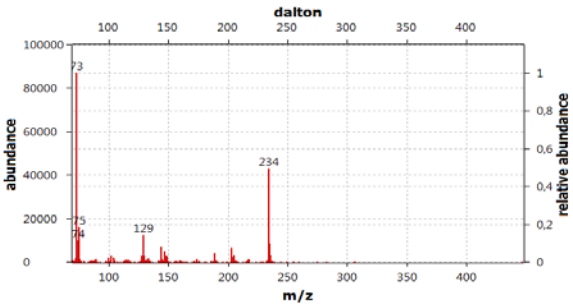
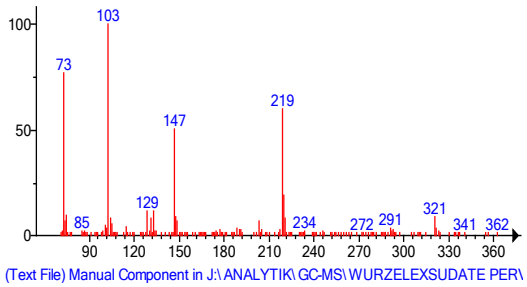
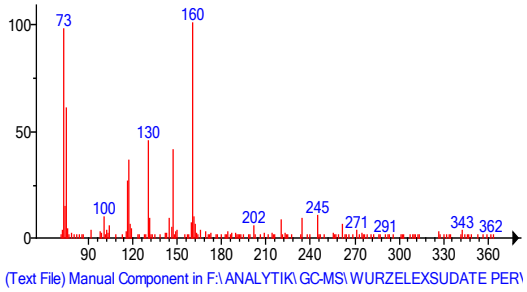
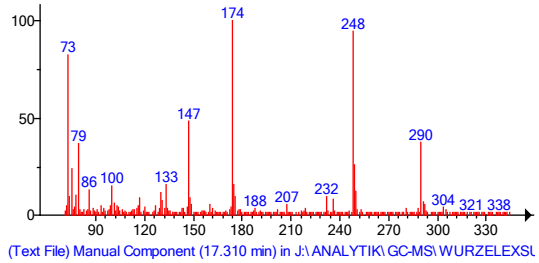
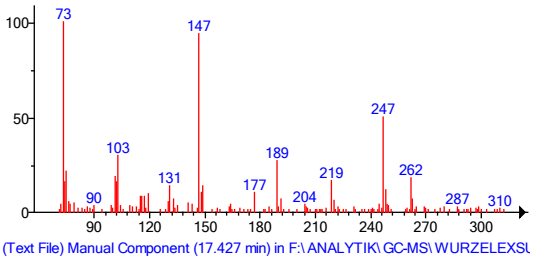
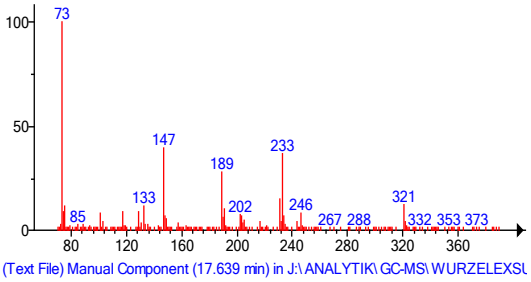
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
 (Text File) Manual Component (12.287 min) in J:\ANALYTIK\GC-MS\WURZELEXSU	**12.28**	**1245**	*1234*	Urea	2	
 (Text File) Manual Component (12.428 min) in J:\ANALYTIK\GC-MS\WURZELEXSU	**12.35**	**1248**	*1275*	Benzoic acid	1	
 (Text File) Manual Component (12.357 min) in C:\GC-MS\WURZELEXSUDATE PERVIN\MAI	**12.36**	**1248**	*1149*	Pentanoic acid, 4-methyl-2-hydroxy	2	
 (Text File) Manual Component in J:\ANALYTIK\GC-MS\WURZELEXSUDATE PER\	**12.87**	**1266**	*1252*	Serine	2	
	**12.88**	**1266**	*1364*	Pipecolic acid	2	
 (Text File) Manual Component in J:\ANALYTIK\GC-MS\WURZELEXSUDATE PER\	**13.14**	**1275**	*1259*	Ethanolamine	3	

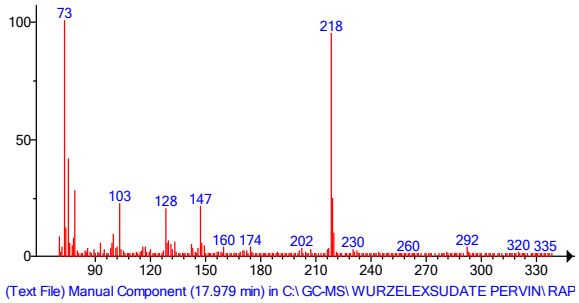
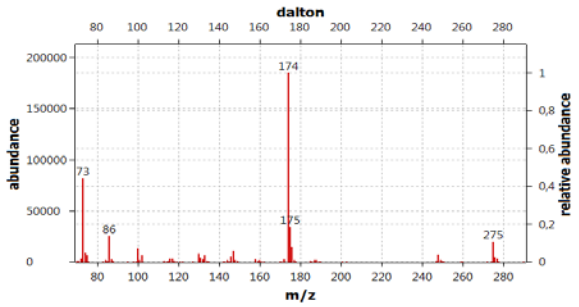
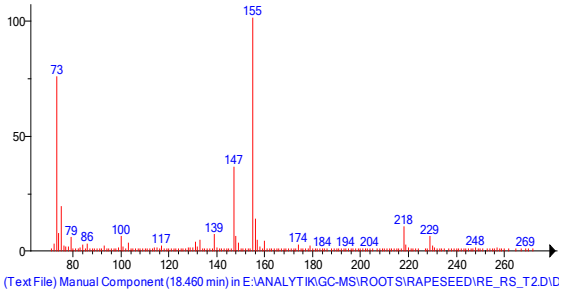
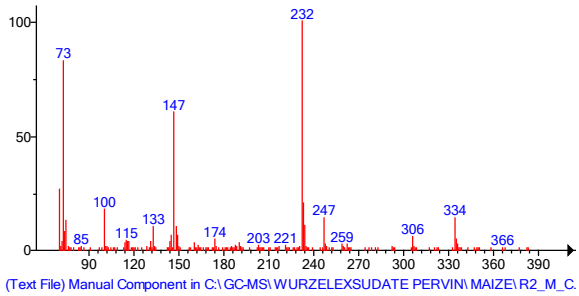
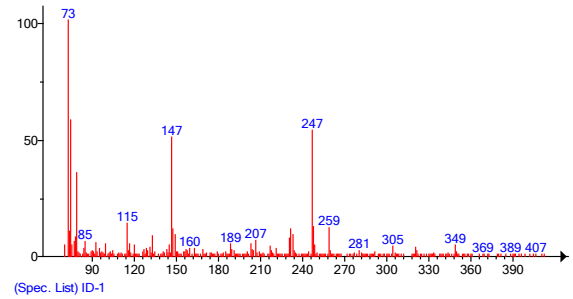
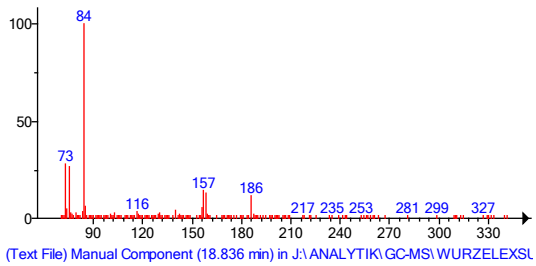
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**13.33**	**1282**	*1264*	Leucine	2	
	**13.43**	**1286**	*1273*	Phosphoric acid	3	
	**13.51**	**1288**	*1292*	Glycerol	3	
	**13.80**	**1299**	*1414*	Phenylacetic acid	1	
	**13.88**	**1302**	*1290*	Threonine	2	
	**13.90**	**1302**	*1286*	Isoleucine	2	

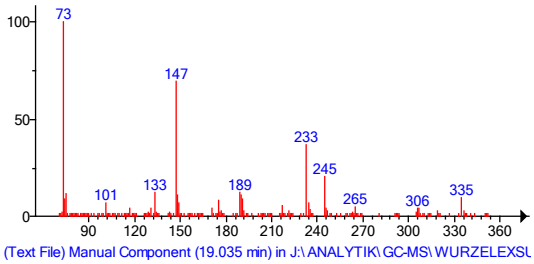
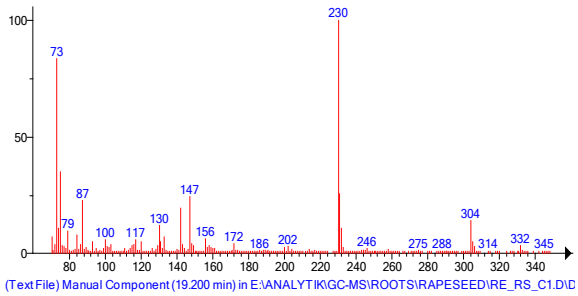
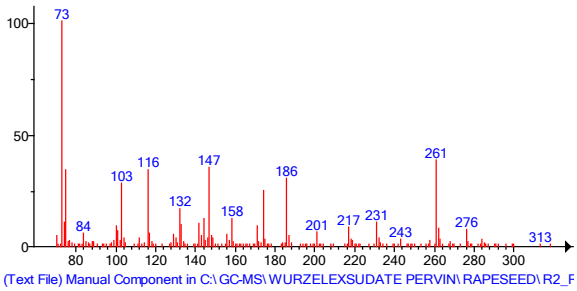
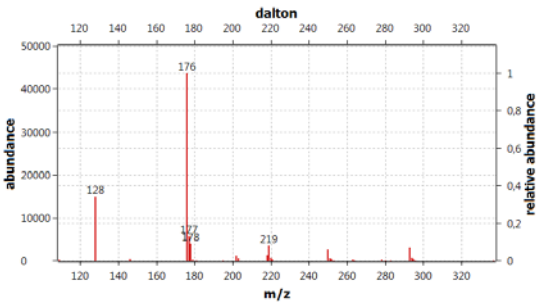
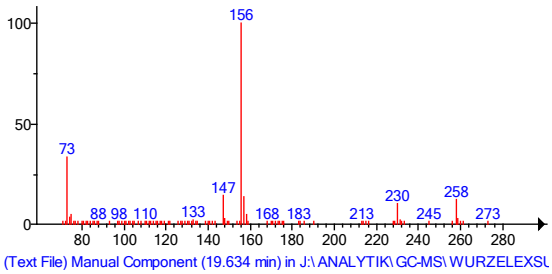
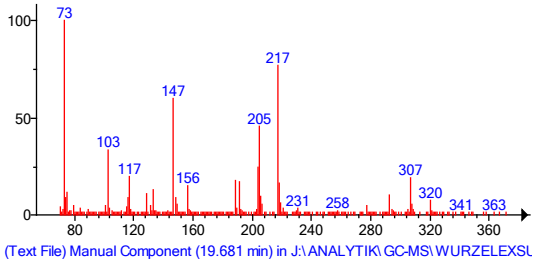
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**13.91**	**1303**	*1296*	Proline	2	
	**13.96**	**1304**	*1297*	Butanoic acid, 4-amino (GABA)	2	
	**14.01**	**1306**	*1311*	Cysteine	3	
	**14.17**	**1312**	*1302*	Glycine	3	
	**14.33**	**1318**	*1310*	Succinic acid	2	
	**14.99**	**1342**	*1369*	Nicotine		

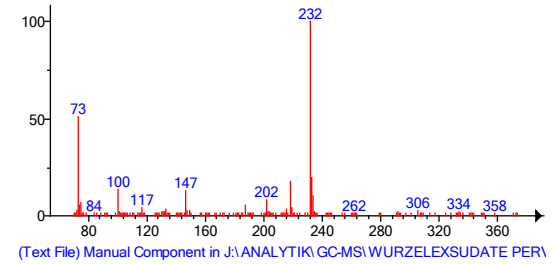
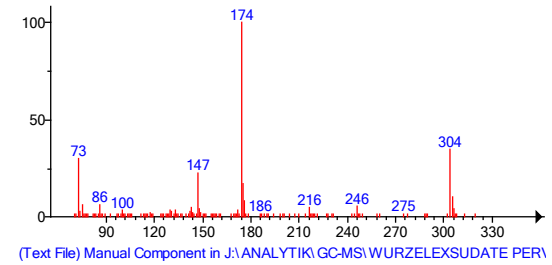
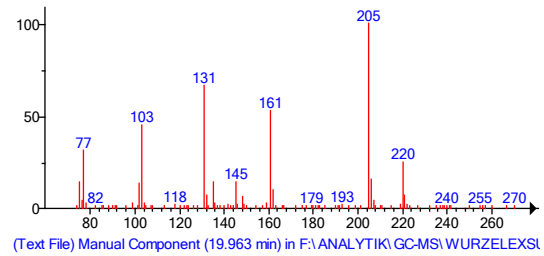
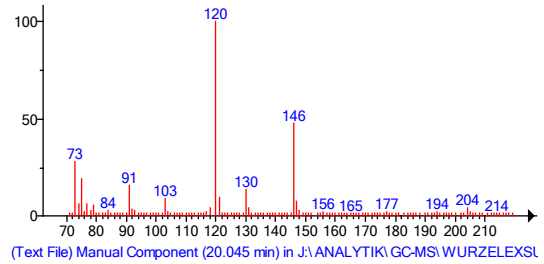
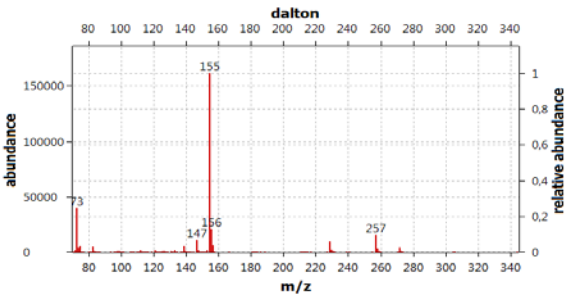
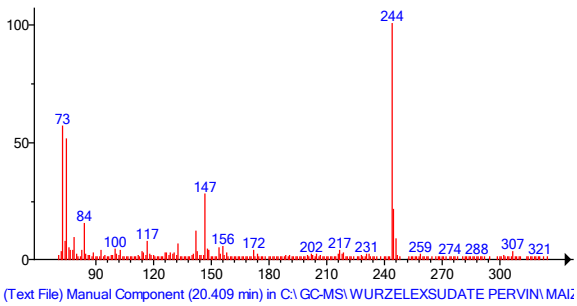
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**15.01**	**1343**	*1319*	Glyceric acid	3	
	**15.05**	**1344**	*1335*	Uracil	2	
	**15.21**	**1350**	*1346*	Fumaric acid	2	
	**15.33**	**1355**		Unknown		
	**15.51**	**1361**	*1355*	Homoserine	2	
	**15.57**	**1363**	*1360*	Alanine	3	

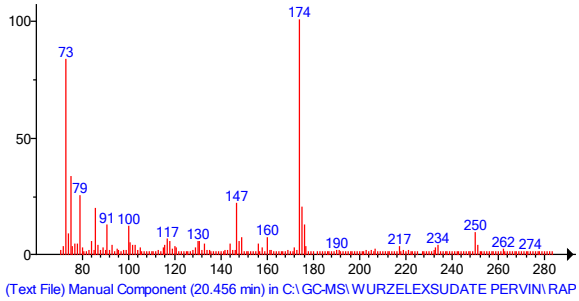
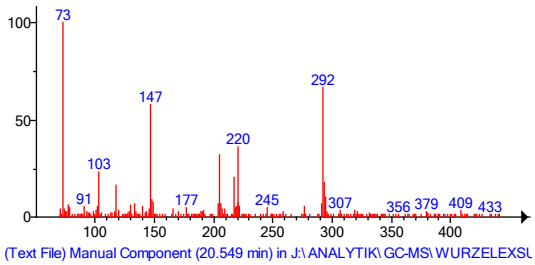
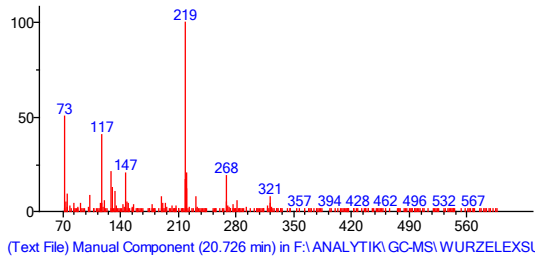
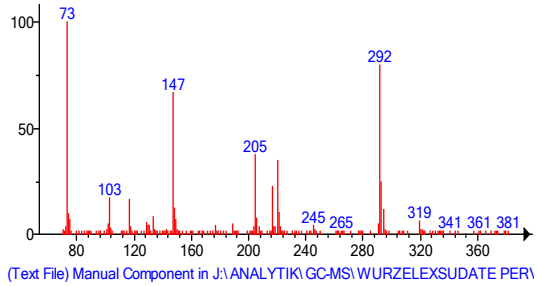
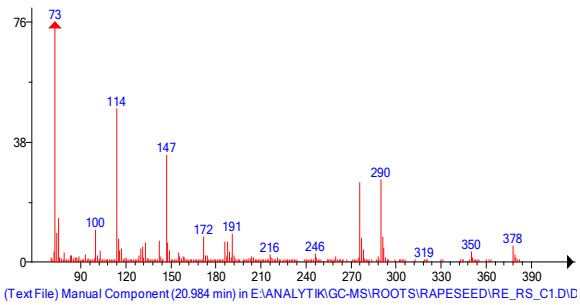
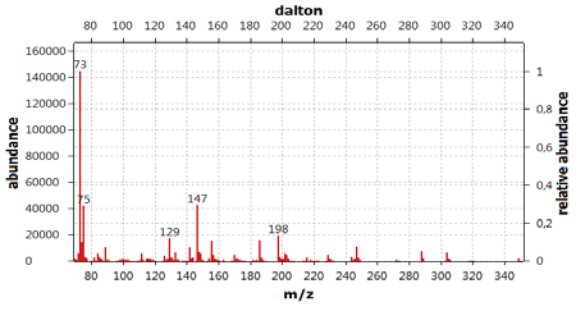
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**15.65**	**1367**	*1234*	Butanoic acid, 2,3-dihydroxy	3	
	**15.78**	**1371**	*1368*	Serine	3	
	**15.93**	**1377**	*1371*	3-Cyanoalanine	2	
	**16.49**	**1398**	*1392*	Threonine	3	
	**16.69**	**1406**	*1397*	Thymine	2	
	**16.70**	**1407**	*1416*	Methionine	1	

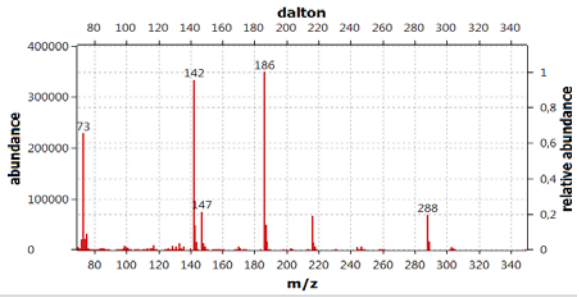
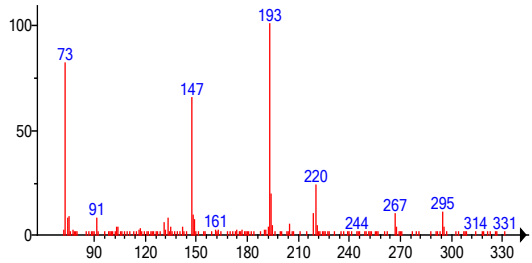
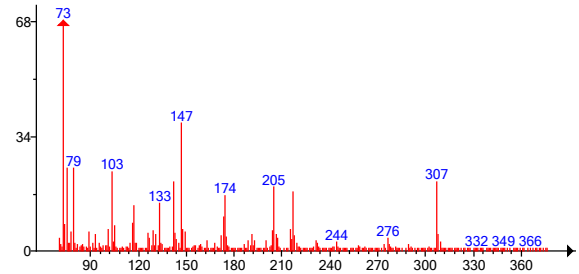
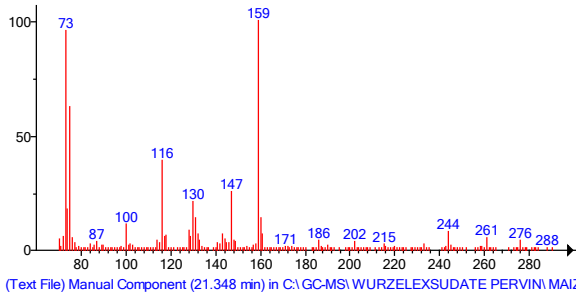
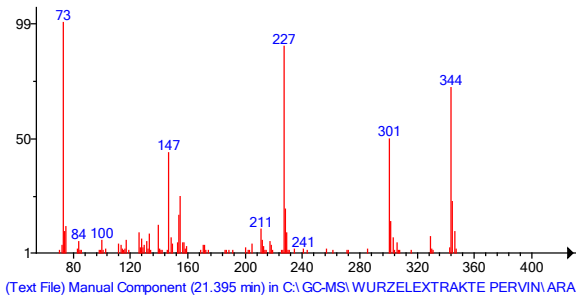
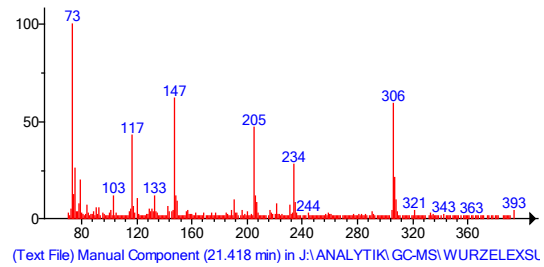
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**17.07**	**1421**	*1401*	A142003 (GMD unknown)		
	**17.11**	**1422**	*1403*	Butanoic acid, 2,4-dihydroxy	3	
	**17.18**	**1425**	*1422*	Aspartic acid	2	
	**17.31**	**1430**	*1423*	β-Alanine	3	
	**17.43**	**1435**	*1435*	Erythronic acid, 1,4-lactone	2	
	**17.62**	**1443**	*1403*	Butanoic acid, 3,4-dihydroxy	3	

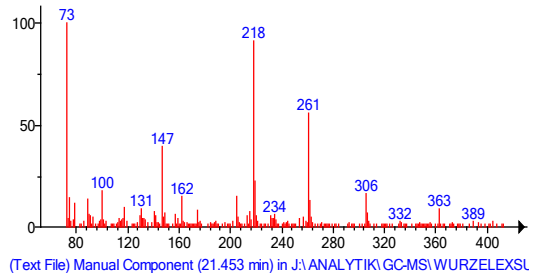
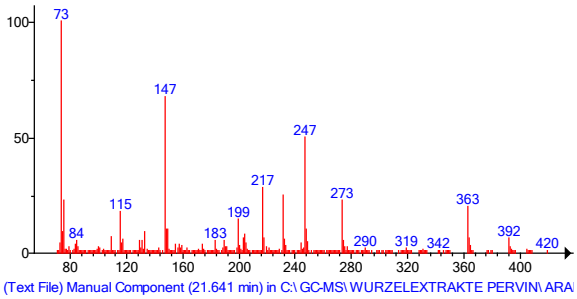
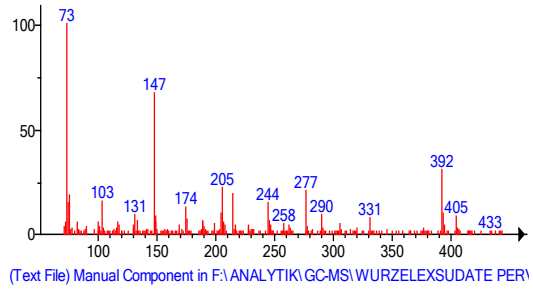
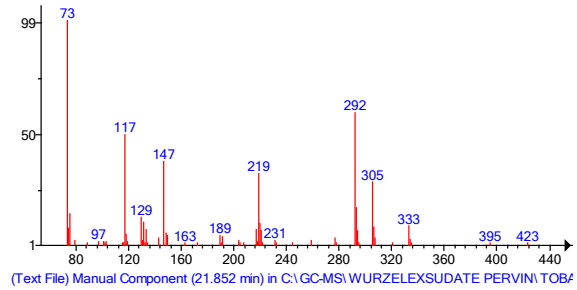
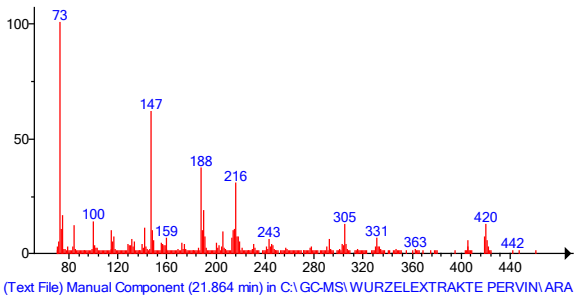
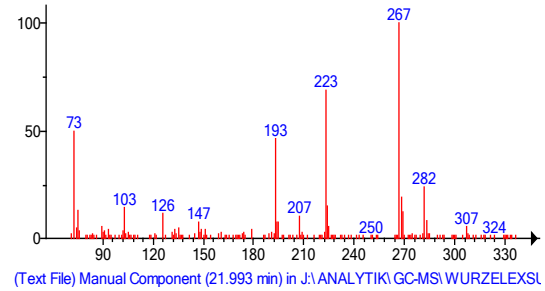
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**17.97**	**1457**	*1457*	Homoserine	3	
	**18.09**	**1461**	*1456*	Glycinamide	3	
	**18.46**	**1476**	*1469*	Glutamine (-H_2O)	2	
	**18.55**	**1480**	*1511*	Aspartic acid	3	
	**18.56**	**1480**	*1485*	Citramalic acid	3	
	**18.83**	**1492**	*1530*	Pyroglutamic acid	1	

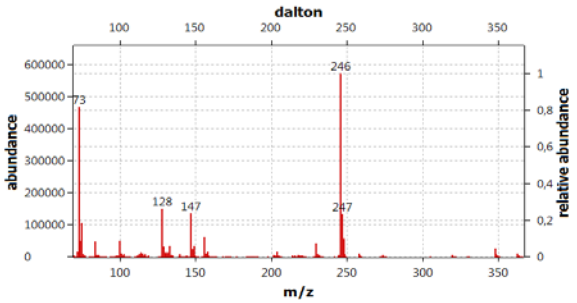
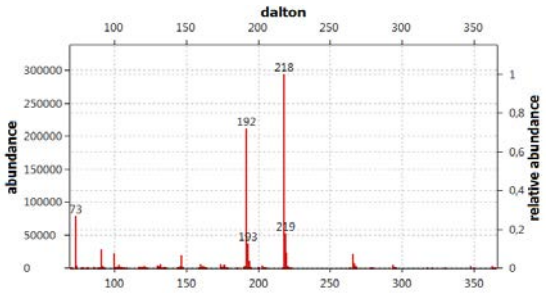
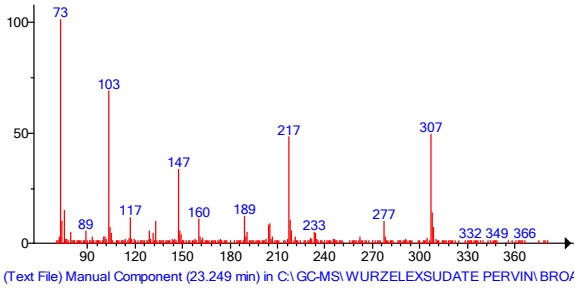
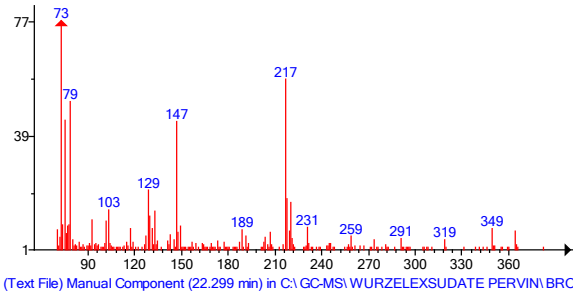
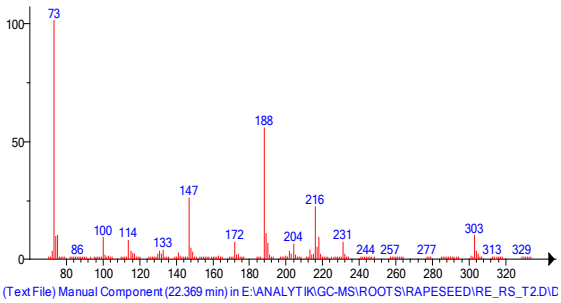
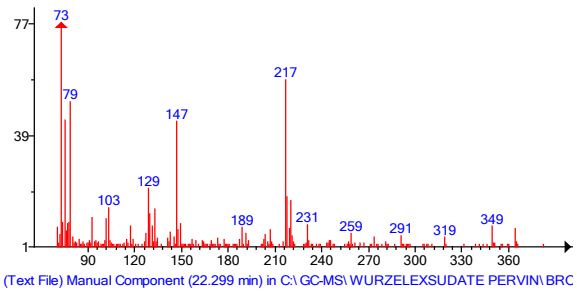
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**18.98**	**1498**	*1494*	Malic acid	3	
	**19.20**	**1507**	*1461*	Norleucine	3	
	**19.33**	**1512**	*1505*	N-Acetylserine	2	
	**19.56**	**1522**	*1515*	Methionine	2	
	**19.62**	**1524**	*1521*	Pyroglutamic acid	2	
	**19.68**	**1527**	*1501*	Threitol	4	

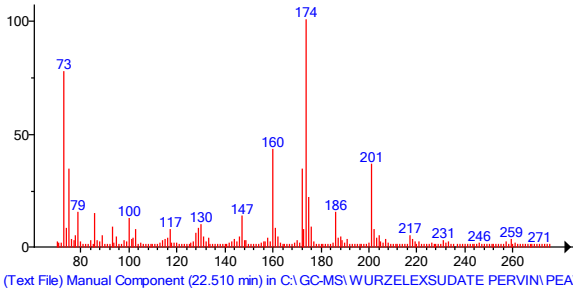
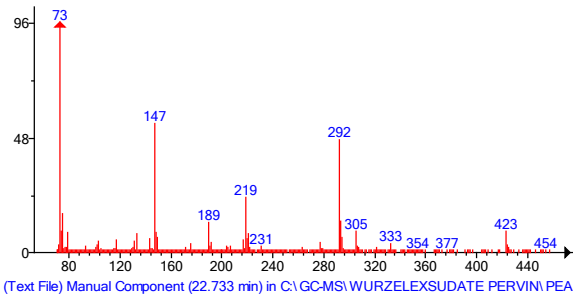
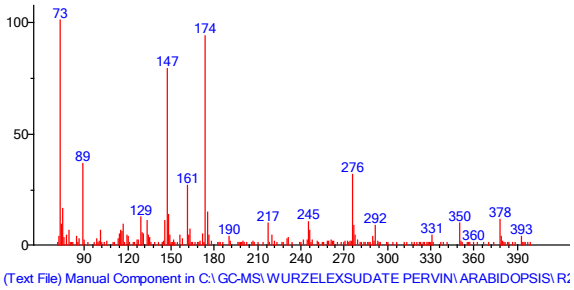
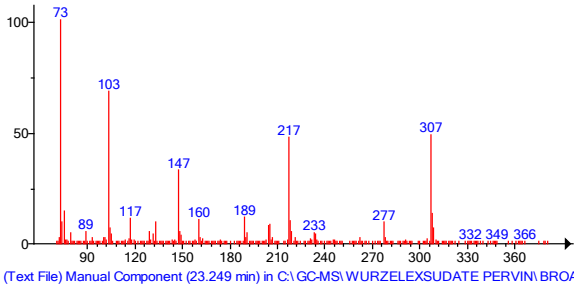
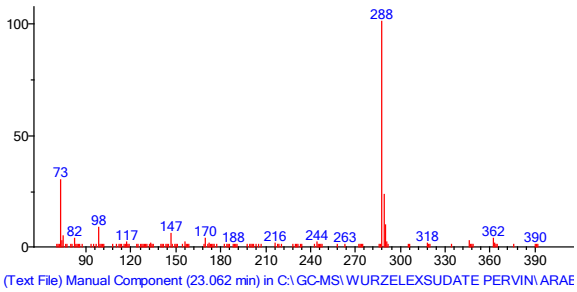
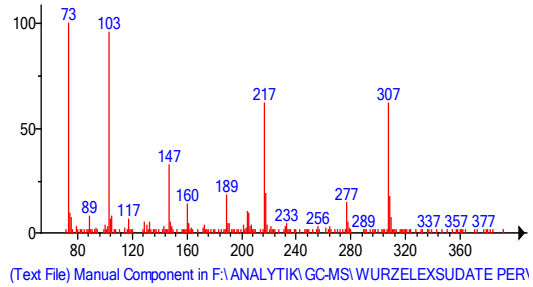
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**19.77**	**1530**	*1511*	Aspartic acid	3	
	**19.84**	**1534**	*1530*	Butanoic acid, 4-amino, (GABA)	3	
	**19.96**	**1538**	*1526*	Cinnamic acid	1	
	**20.05**	**1542**	*1565*	Phenylalanine	1	
	**20.10**	**1544**	*1529*	Glutamic acid	2	
	**20.41**	**1558**	*1663*	Maleamic acid	2	

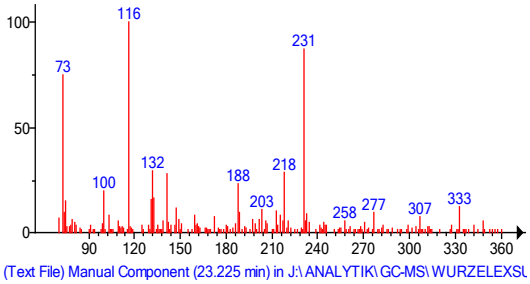
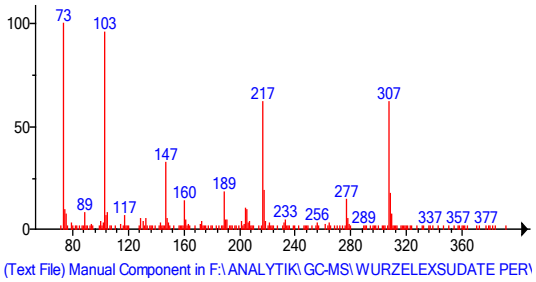
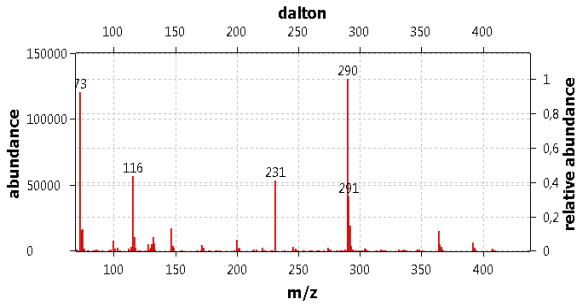
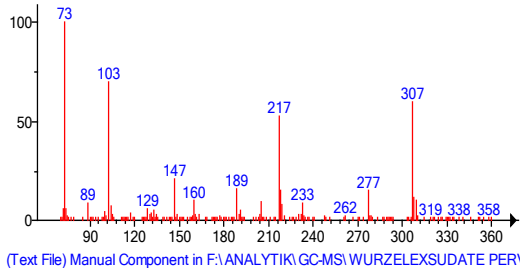
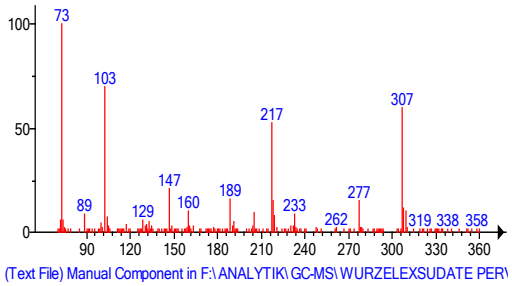
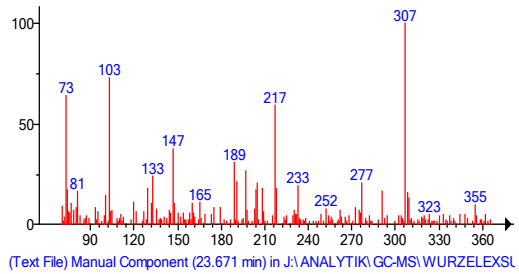
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**20.45**	**1559**	*1573*	Phenethylamine	2	
	**20.53**	**1563**	*1528*	Erythronic acid	4	
	**20.71**	**1571**	*1577*	A155004 (GMD unknown)		
	**20.95**	**1581**	*1562*	Threonic acid	4	
	**20.98**	**1582**	*1565*	Serine	4	
	**21.01**	**1584**	*1573*	2-Oxoglutaric acid	2	1

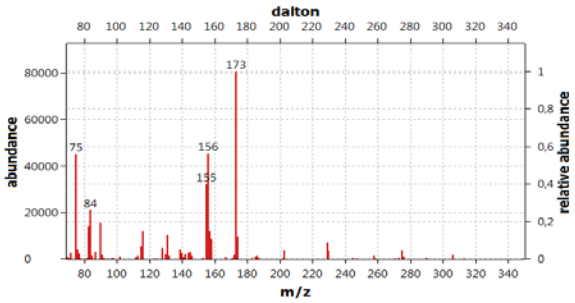
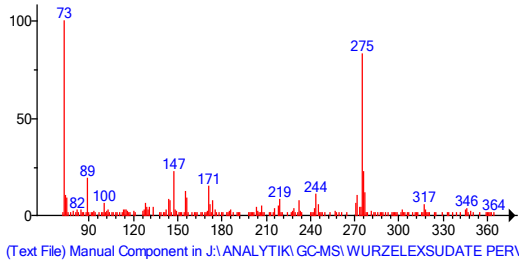
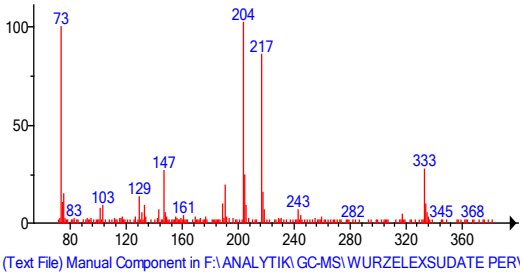
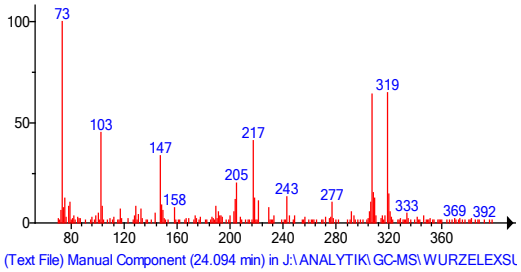
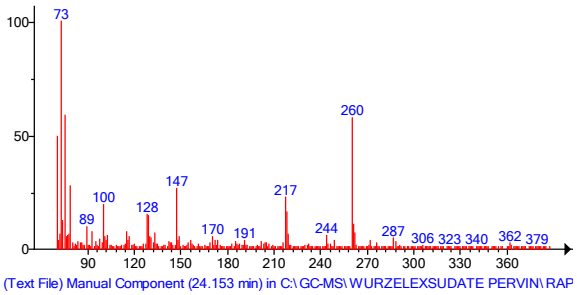
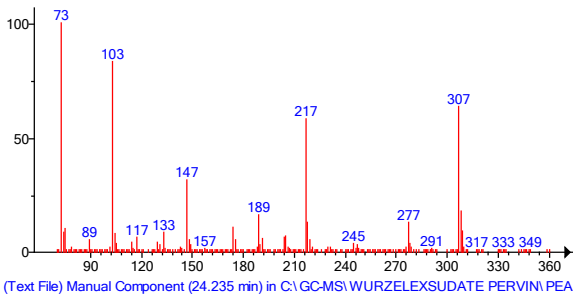
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**21.03**	**1584**	*1593*	Proline + CO_2	2	
	**21.12**	**1588**	*1585*	Lactic acid, 3-phenyl	2	
	**21.15**	**1590**	*1576*	2-Deoxyribose	3	1
	**21.34**	**1598**	*1599*	Asparagine	2	
	**21.39**	**1600**		Glutamine (-H_2O)	3	
	**21.43**	**1602**	*1664*	Xylulose	4	1

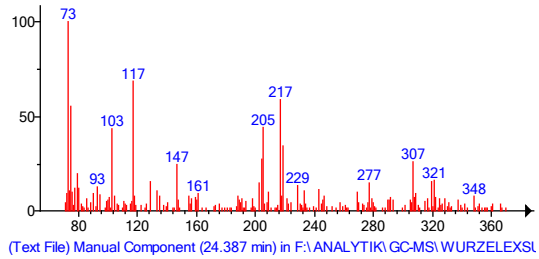
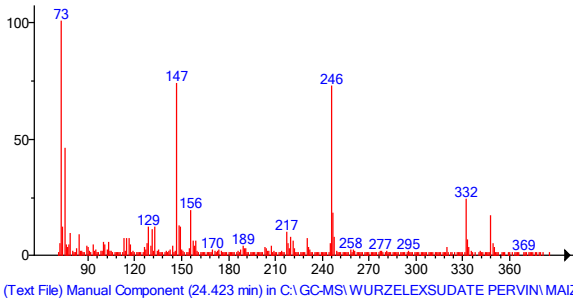
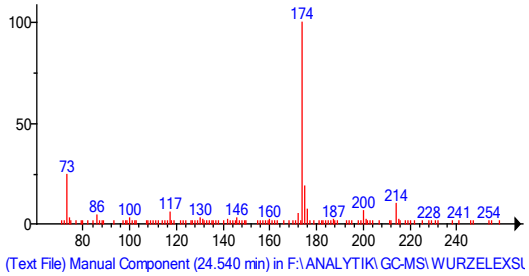
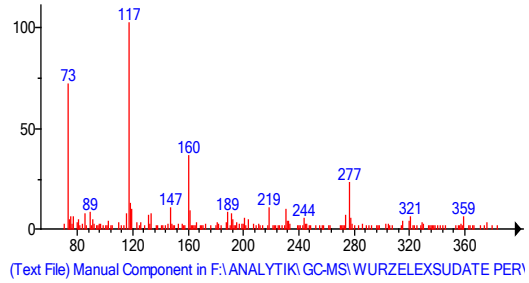
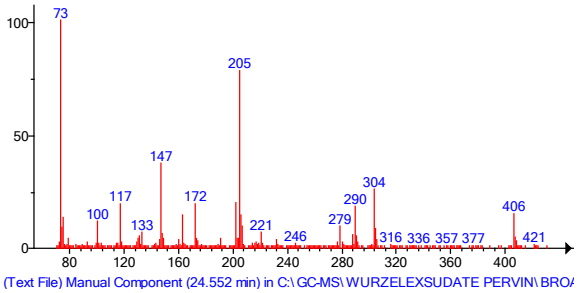
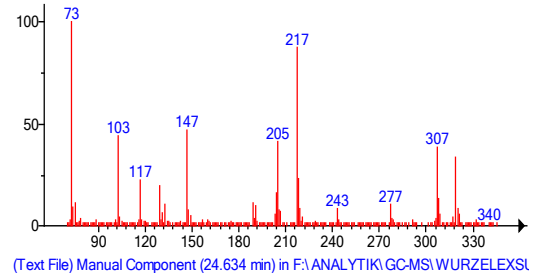
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**21.44**	**1602**	*1581*	A157012 (Asparagine derivative , GMD unknown)		
	**21.64**	**1611**	*1598*	Glutaric acid, 3-Hydroxy-3-methyl	3	
	**21.69**	**1613**	*1587*	A159013 (GMD unknown)		
	**21.84**	**1620**	*1628*	Tartaric acid	4	
	**21.85**	**1621**	*1621*	Asparagine	4	
	**21.98**	**1626**	*1640*	Benzoic acid, 4-hydroxy	2	

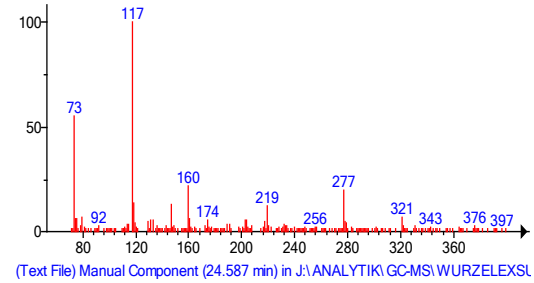
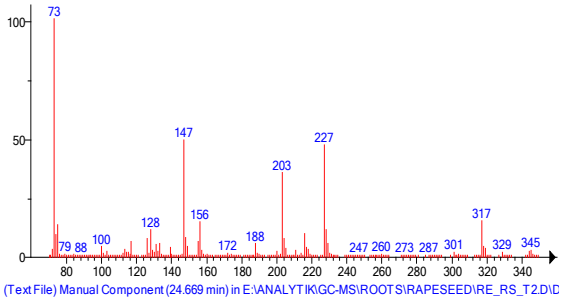
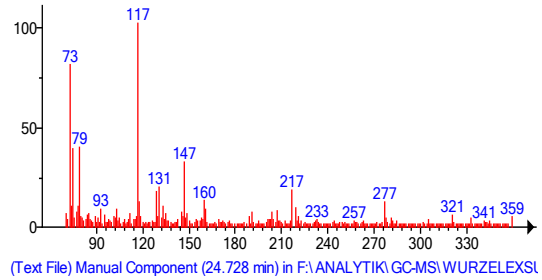
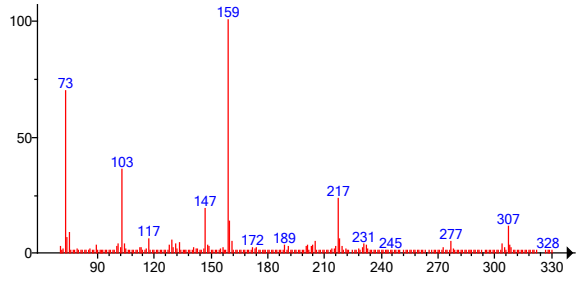
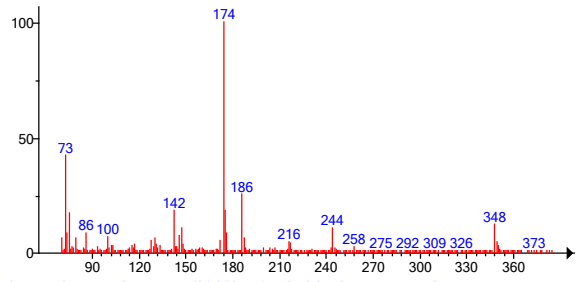
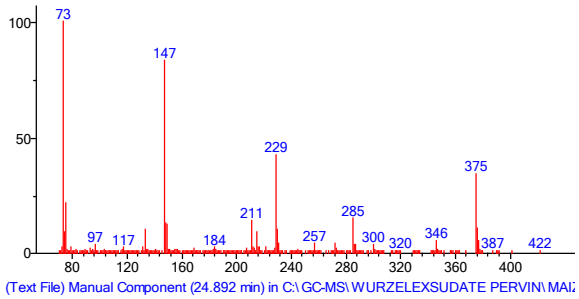
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**22.03**	**1629**	*1614*	Glutamic acid	3	
	**22.04**	**1629**	*1629*	Phenylalanine	2	
	**22.22**	**1637**	*1631*	Lyxose	4	1
	**22.29**	**1640**	*1705*	Xylonic acid	3	
	**22.37**	**1644**	*1666*	Asparagine	3	
	**22.38**	**1644**	*1705*	Xylonic acid	3	

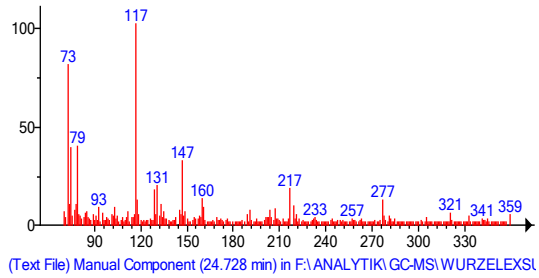
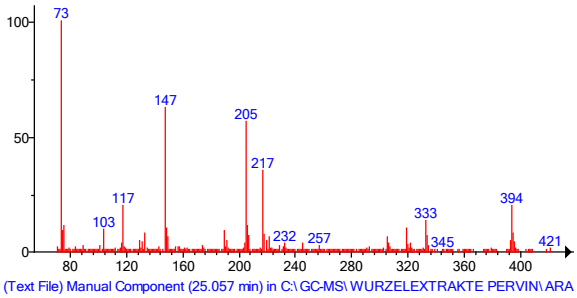
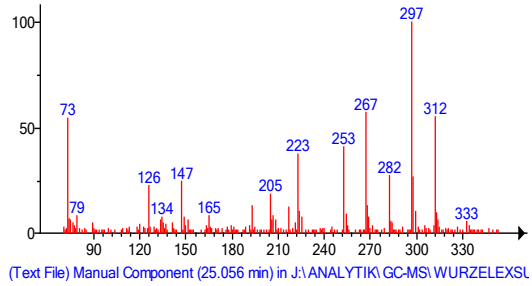
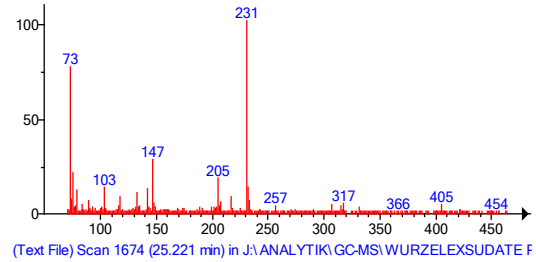
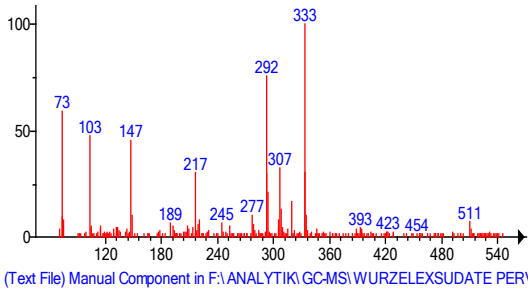
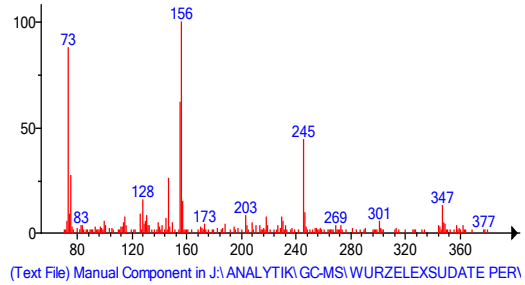
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
 (Text File) Manual Component (22.510 min) in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ PEA	**22.51**	**1650**	*1589*	1,2-Diaminopropane	4	
 (Text File) Manual Component (22.733 min) in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ PEA	**22.73**	**1660**	*1658*	Tartaric acid	4	
 (Text File) Manual Component in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ ARABIDOPSIS\ R	**22.90**	**1668**	*1590*	1,3-Diaminopropane	4	
 (Text File) Manual Component (23.249 min) in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ BRO	**22.91**	**1669**	*1692*	Lyxose	4	1
 (Text File) Manual Component (23.062 min) in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ ARAE	**23.05**	**1675**	*1659*	A167003 (GMD unknown)		
 (Text File) Manual Component in F:\ ANALYTIK\ GC-MS\ WURZELEXSUDATE PER\	**23.15**	**1680**	*1652*	Xylose	4	1

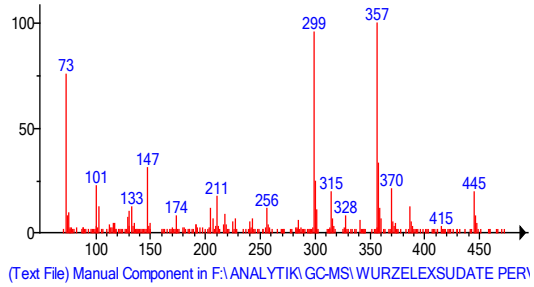
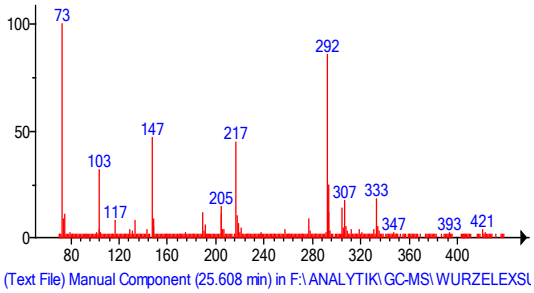
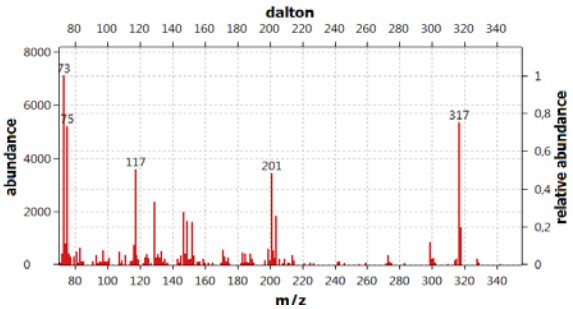
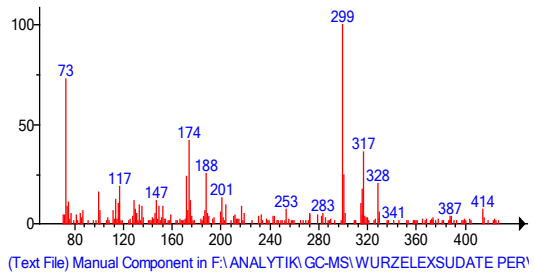
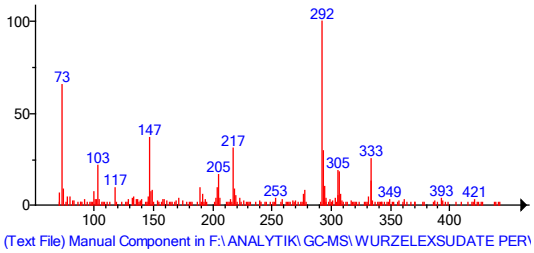
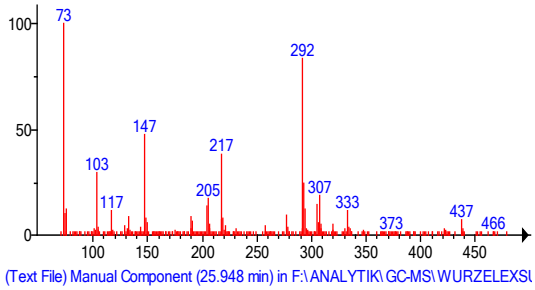
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**23.22**	**1683**	*1666*	Asparagine	3	
	**23.25**	**1684**	*1669*	Xylose	4	1
	**23.27**	**1685**	*1669*	Homoserine	4	
	**23.34**	**1688**	*1675*	Arabinose	4	1
	**23.40**	**1691**	*1675*	Arabinose	4	1
	**23.73**	**1707**	*1681*	Ribose	4	1

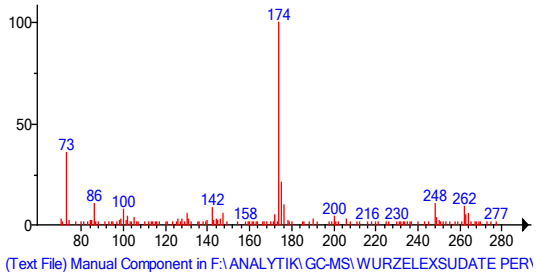
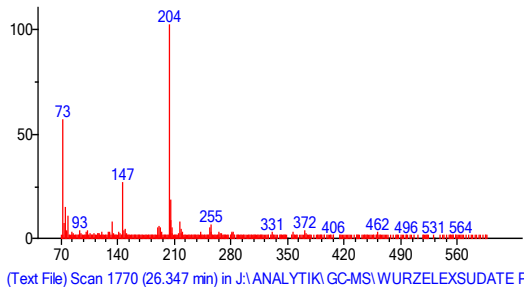
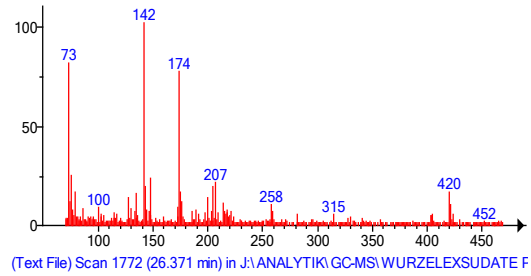
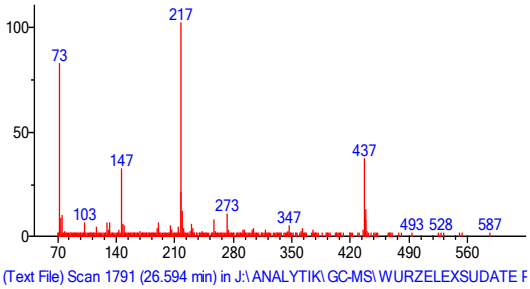
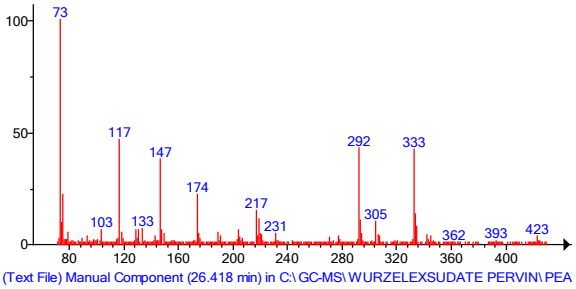
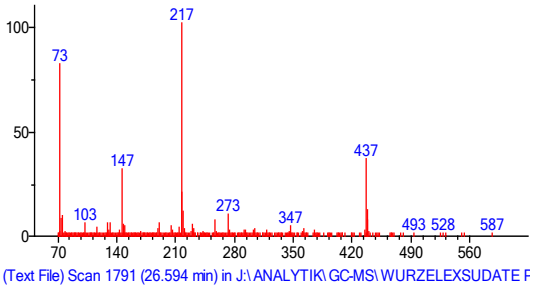
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**23.77**	**1708**	*1702*	A172005 (GMD unknown)		
	**23.89**	**1714**	*1692*	A171003 (GMD unknown)		
	**24.06**	**1722**	*1753*	Glucose, 1,6-anhydro	3	
	**24.09**	**1724**	*1718*	Xylitol	5	
	**24.18**	**1728**	*1711*	α-Aminoadipic acid	3	
	**24.23**	**1730**	*1814*	Sorbose	5	1

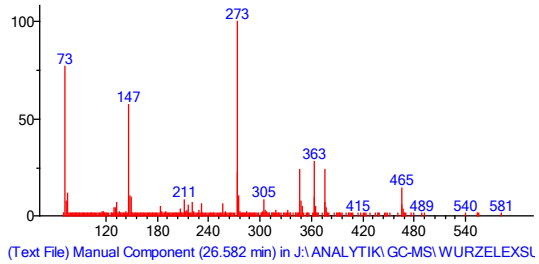
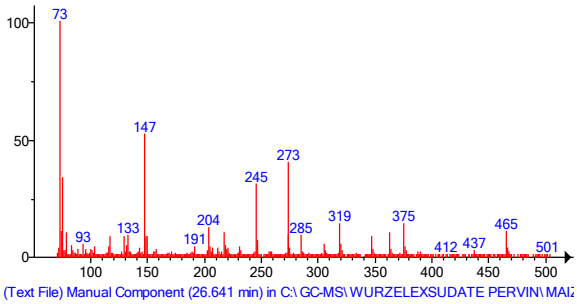
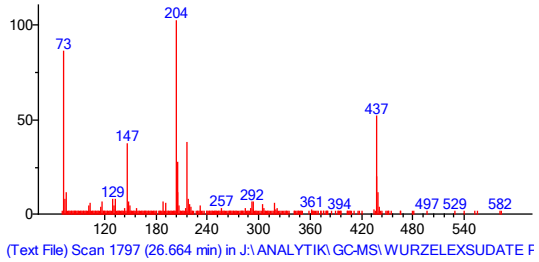
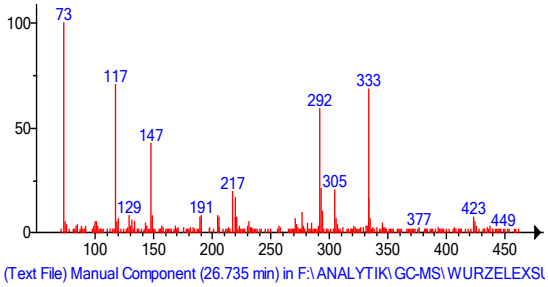
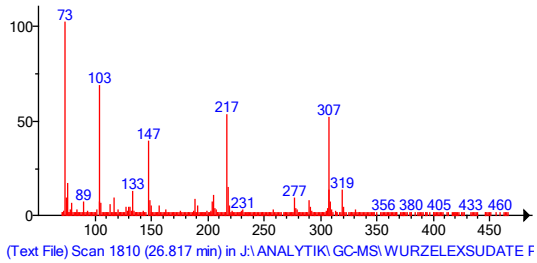
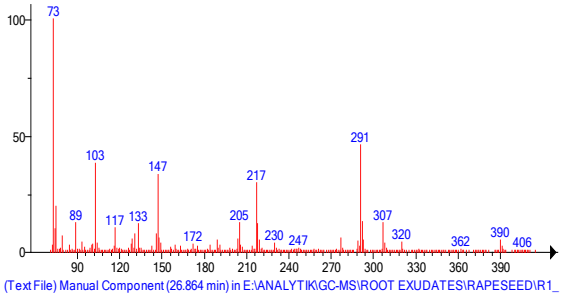
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**24.38**	**1737**	*1733*	Ribitol	5	
	**24.42**	**1739**	*1720*	Glutamine	3	
	**24.54**	**1745**	*1734*	Putrescine	4	
	**24.54**	**1745**	*1731*	Rhamnose	4	1
	**24.55**	**1745**	*1739*	Aspartic acid	4	
	**24.59**	**1747**	*1707*	Arabitol	5	

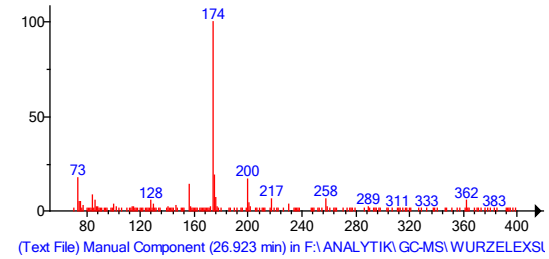
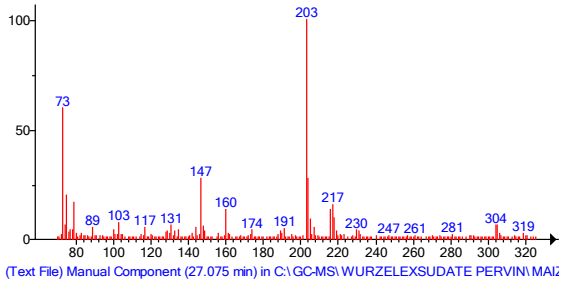
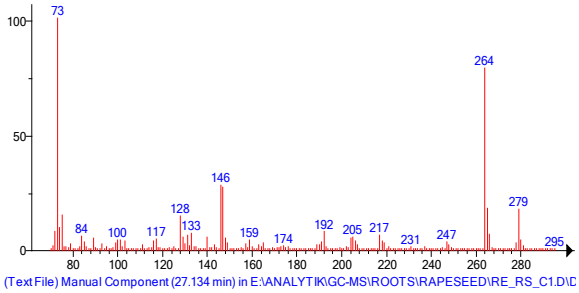
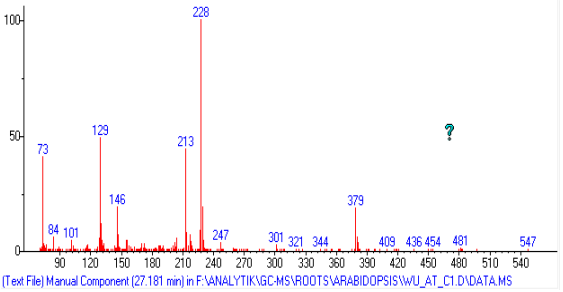
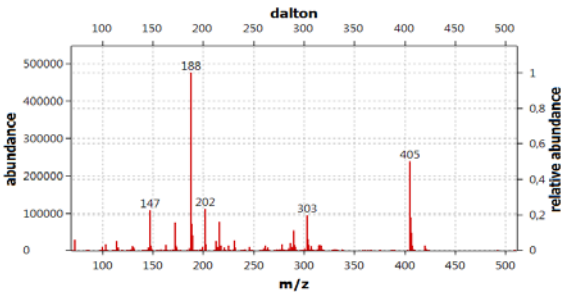
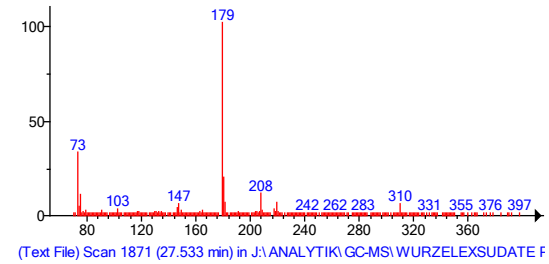
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
 (Text File) Manual Component (24.587 min) in J:\ ANALYTIK\ GC-MS\ WURZELEXSU	**24.62**	**1749**	*1733*	Rhamnose	4	1
 (Text File) Manual Component (24.669 min) in E:\ANALYTIK\GC-MS\ROOTS\RAPESEED\RE_RS_T2.D\D	**24.67**	**1751**	*1720*	Glutamine	3	
 (Text File) Manual Component (24.728 min) in F:\ ANALYTIK\ GC-MS\ WURZELEXSU	**24.72**	**1754**	*1729*	Fucose	4	1
 (Text File) Manual Component (24.763 min) in C:\ GC-MS\ WURZELEXTRAKTE PERVIN\ ARA	**24.76**	**1756**		Unknown		
 (Text File) Manual Component (24.810 min) in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ PEA	**24.81**	**1758**	*1751*	Ornithine	3	
 (Text File) Manual Component (24.892 min) in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ MAI	**24.88**	**1761**	*1804*	Aconitic acid	3	

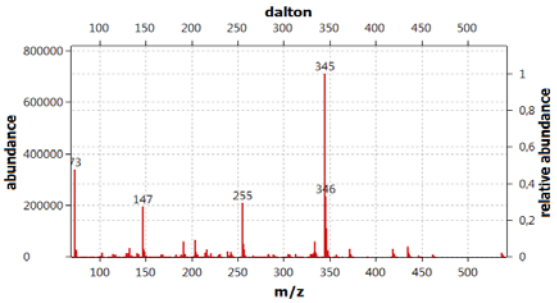
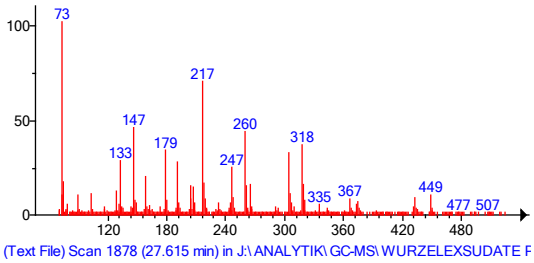
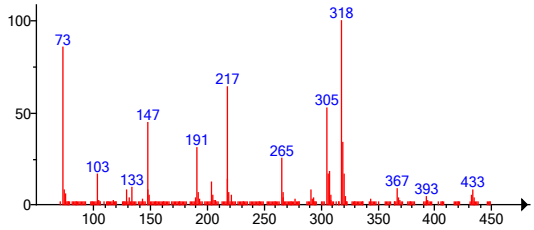
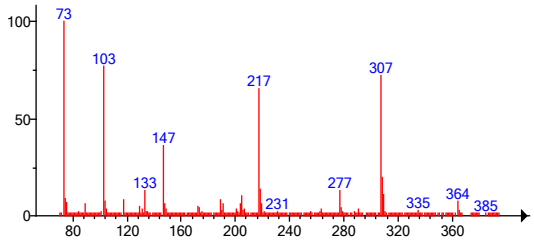
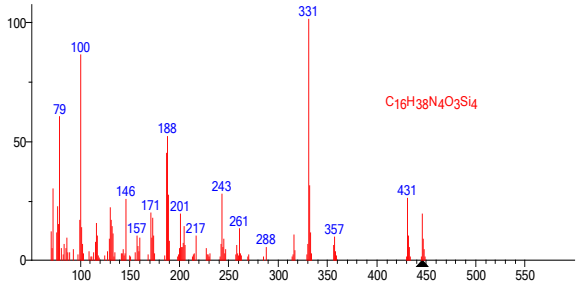
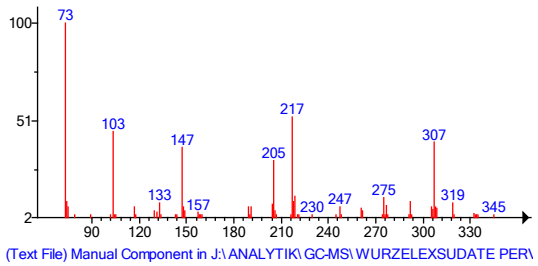
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**24.93**	**1764**	*1729*	Fucose	4	1
	**25.06**	**1770**	*1732*	A174001 (GMD unknown)		
	**25.06**	**1770**	*1776*	Vanillic acid	2	
	**25.23**	**1778**	*1741*	Glucose, 3-deoxy-2-keto	3	2
	**25.31**	**1782**	*1715*	Lyxonic acid	5	
	**25.40**	**1787**	*1782*	Glutamine	3	

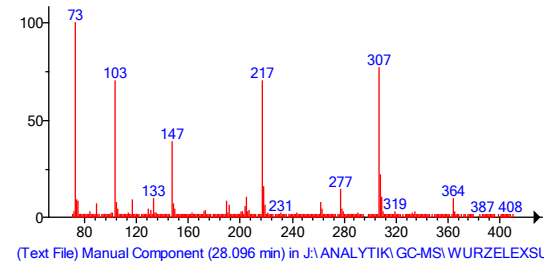
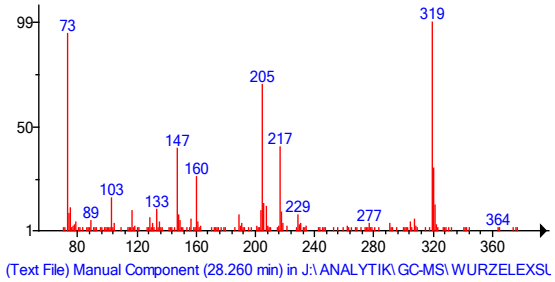
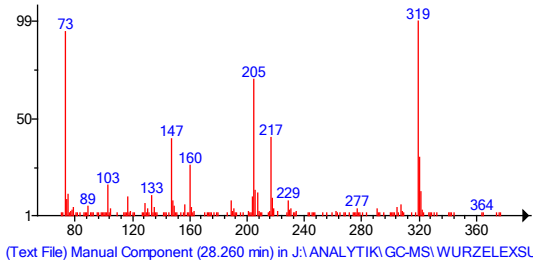
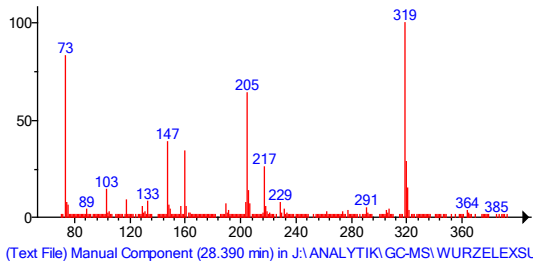
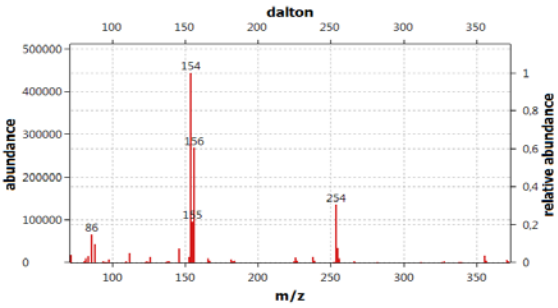
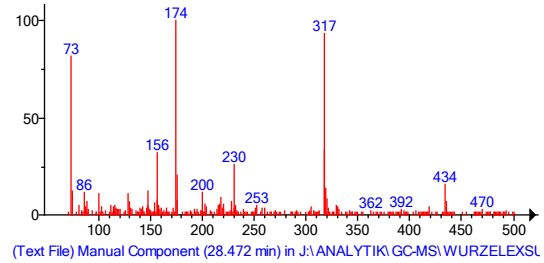
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**25.43**	**1788**	*1751*	Glycerol, 3-phosphate	4	
	**25.61**	**1797**	*1750*	Ribonic acid	5	
	**25.69**	**1801**	*1791*	Azelaic acid	2	
	**25.71**	**1802**	*1815*	Ethanolamine phosphate		
	**25.79**	**1806**	*1759*	Lyxonic acid	5	
	**25.94**	**1813**	*1765*	Arabionic acid	5	

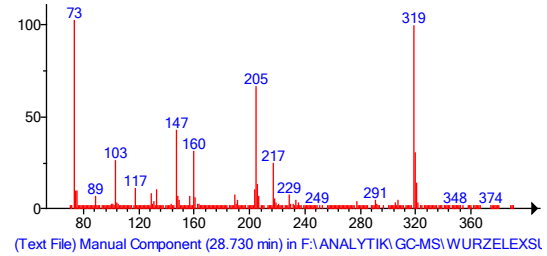
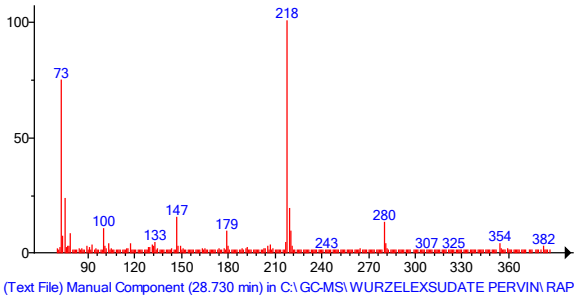
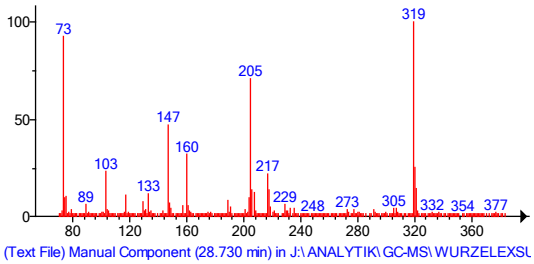
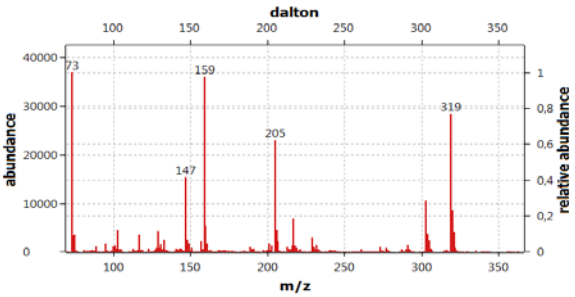
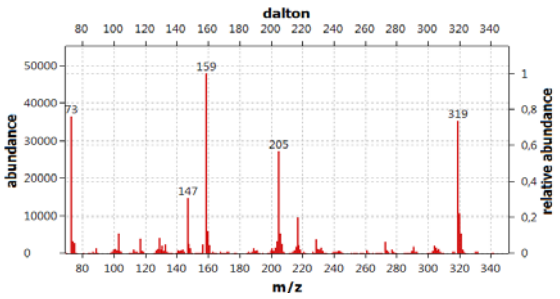
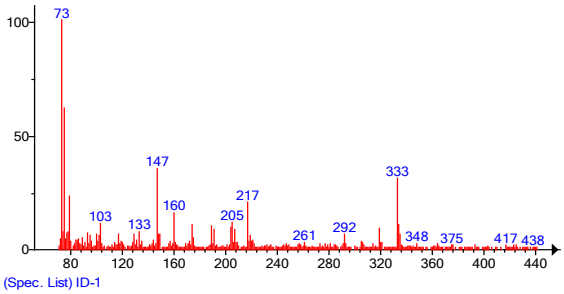
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**26.23**	**1828**	*1818*	Glycylglycine	4	
	**26.34**	**1834**	*1822*	Shikimic acid	4	
	**26.36**	**1835**	*1820*	Ornithine	4	
	**26.41**	**1837**	*1806*	Fructose der.	5	
	**26.41**	**1837**	*1807*	A181001 (GMD unknown)		
	**26.59**	**1846**	*1810*	Fructose der.	5	

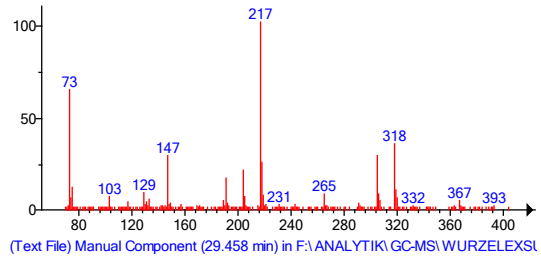
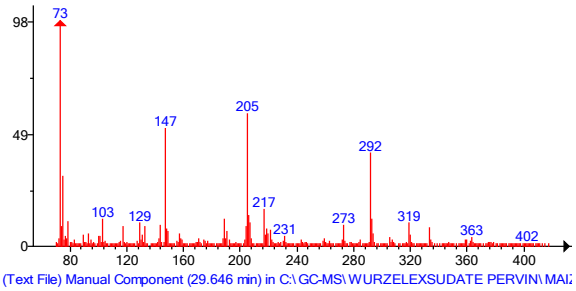
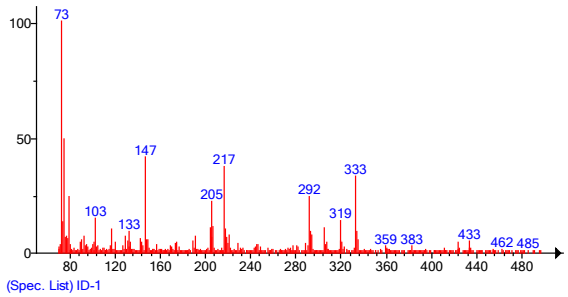
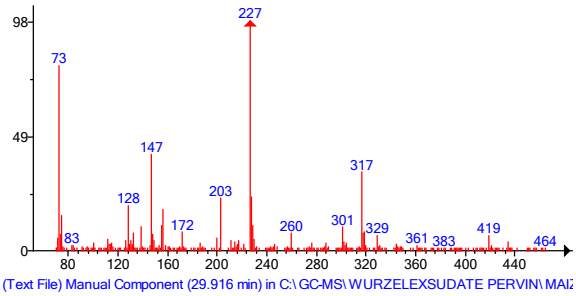
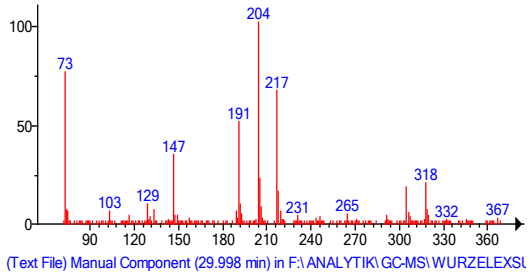
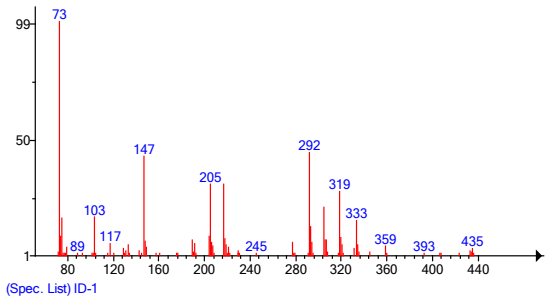
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**26.59**	**1846**	*1829*	Citric acid	4	
	**26.63**	**1848**	*1839*	Isocitric acid	4	
	**26.66**	**1850**	*1806*	Fructose der.	5	
	**26.73**	**1853**	*1944*	Saccharic acid	6	
	**26.82**	**1858**		Fructose der. (gmd)	5	
	**26.86**	**1860**	*1857*	A184032 (GMD unknown)		

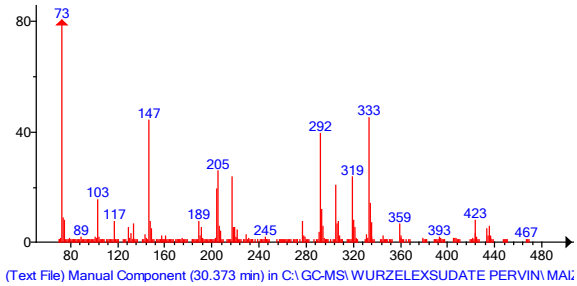
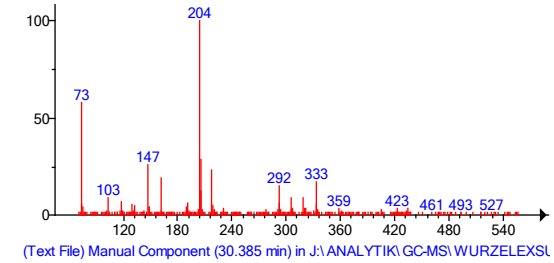
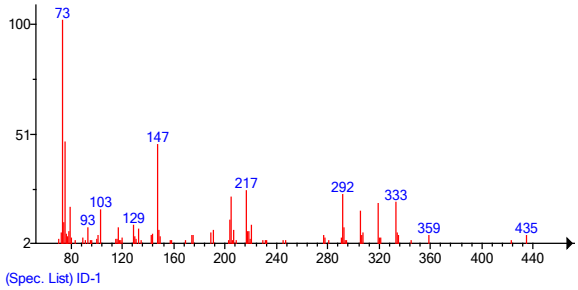
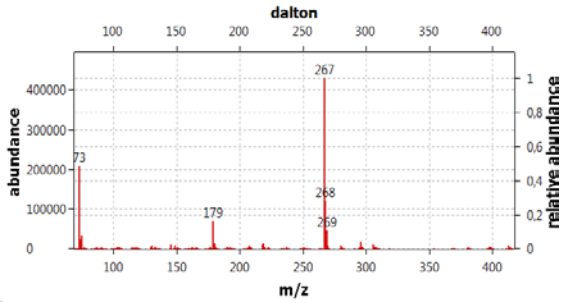
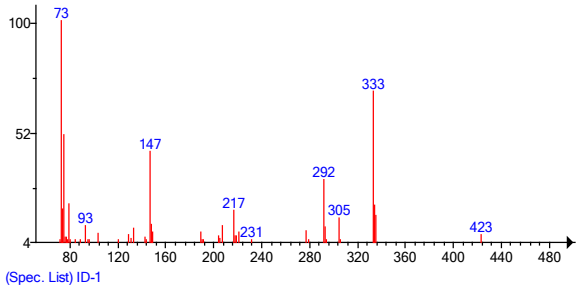
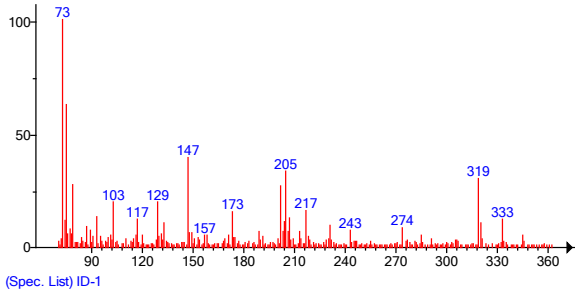
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**26.86**	**1860**	*1849*	Lysine	3	
	**27.07**	**1871**	*1845*	Deoxyglucose, 2-amino	5	1
	**27.13**	**1874**	*1874*	Adenine	2	
	**27.15**	**1875**	*1889*	Indol-3-acetonitrile	1	
	**27.36**	**1886**	*1863*	Asparagine	4	
	**27.53**	**1895**	*1845*	Tyrosine	2	

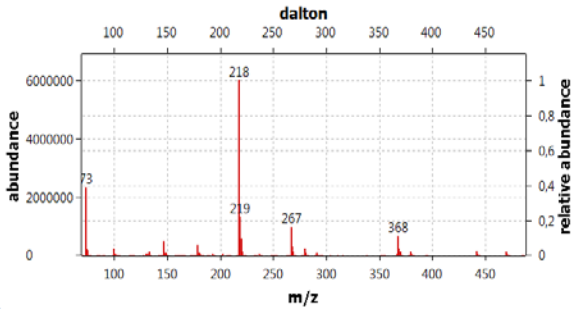
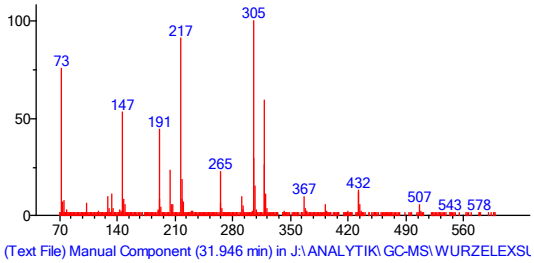
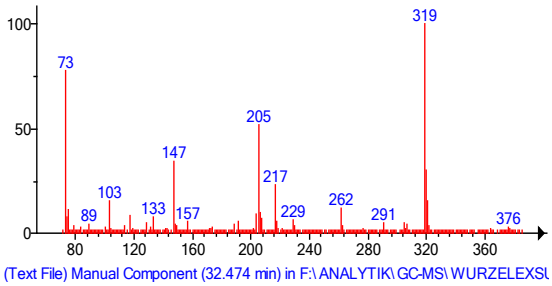
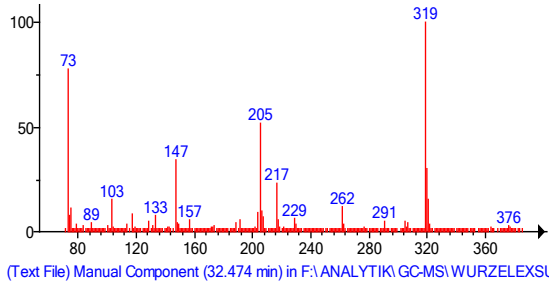
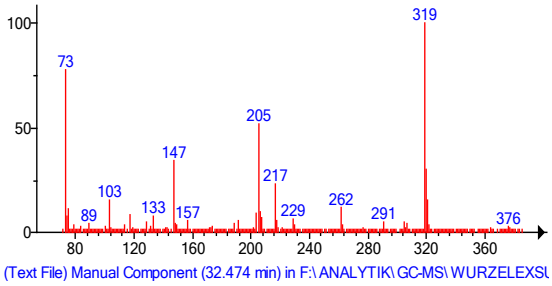
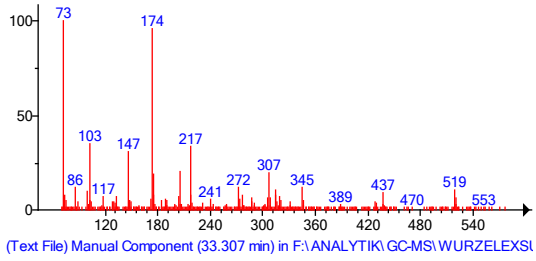
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**27.56**	**1896**	*1843*	Quinic acid	5	
 (Text File) Scan 1878 (27.615 min) in J:\ ANALYTIK\ GC-MS\ WURZELEXSUDATE F	**27.61**	**1899**	*1830*	Pinitol	5	
 (Text File) Manual Component (27.850 min) in F:\ ANALYTIK\ GC-MS\ WURZELEXSU	**27.85**	**1911**	*1951*	chiro-Inositol	5	
 (Text File) Manual Component (27.873 min) in J:\ ANALYTIK\ GC-MS\ WURZELEXSU	**27.88**	**1913**	*1874*	Fructose	5	1
 (gmd) M000092_A189007-101-xxx_NA_1876,44_PRED_VAR5_ALK_Allantoin (4TMS)	**27.89**	**1913**	*1897*	Allantoin	5	
 (Text File) Manual Component in J:\ ANALYTIK\ GC-MS\ WURZELEXSUDATE PER\	**27.99**	**1919**	*1885*	Galactose	5	1

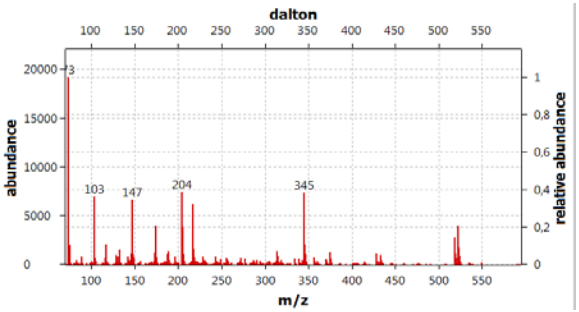
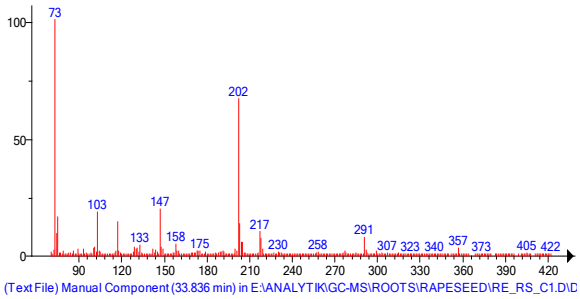
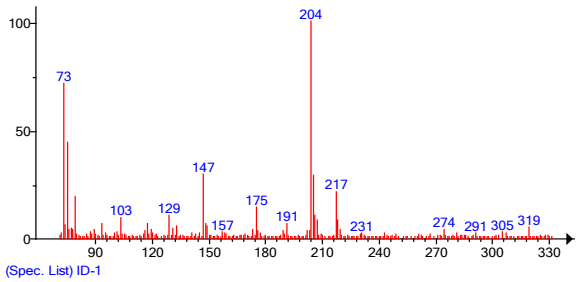
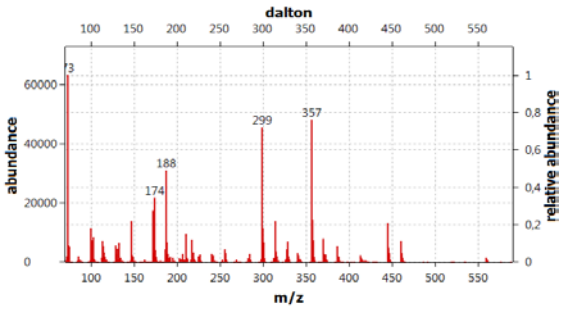
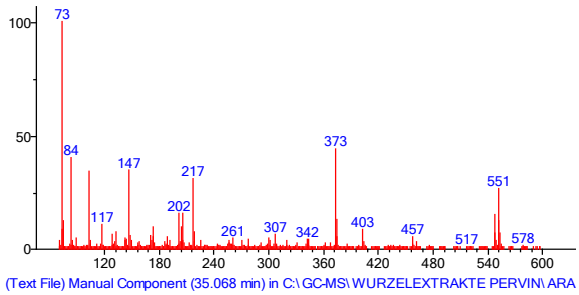
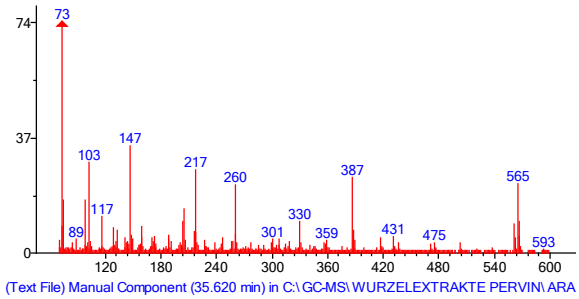
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**28.10**	**1924**	*1883*	Fructose	5	1
	**28.14**	**1927**	*1868*	Mannose	5	1
	**28.26**	**1933**	*1895*	Mannose	5	1
	**28.39**	**1940**	*1897*	Glucose	5	1
	**28.40**	**1940**	*1915*	Histidin	3	
	**28.47**	**1944**	*1913*	Lysine	4	

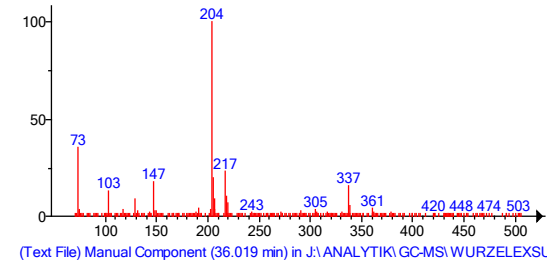
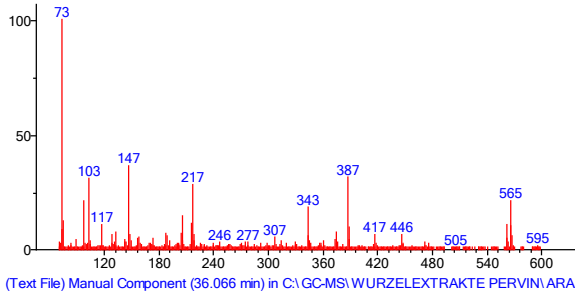
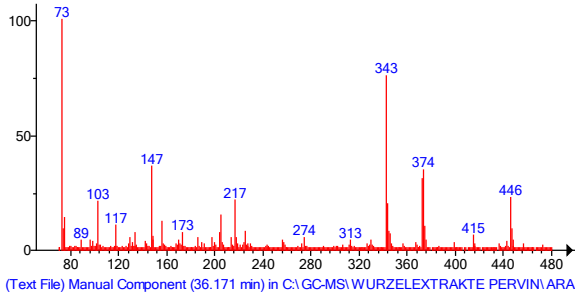
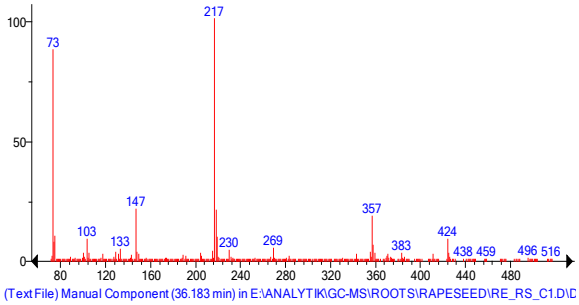
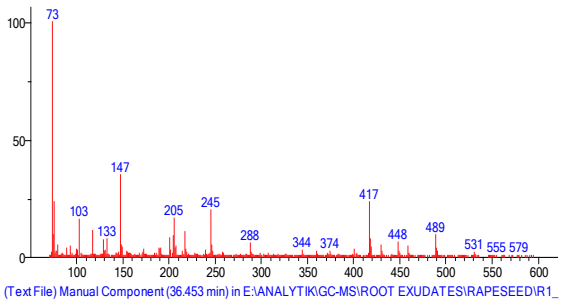
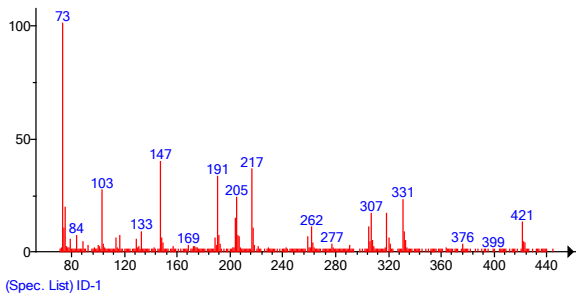
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**28.66**	**1954**	*1908*	Glucose	5	1
	**28.75**	**1959**	*1934*	Tyrosine	3	
	**28.83**	**1963**	*1912*	Galactose	5	1
	**29.08**	**1977**	*1922*	2-Deoxygalactose, 2-amino-,	5	1
	**29.22**	**1985**	*1925*	2-Deoxyglucose, 2-amino-	5	1
	**29.28**	**1988**	*1938*	Glucuronic acid	5	1

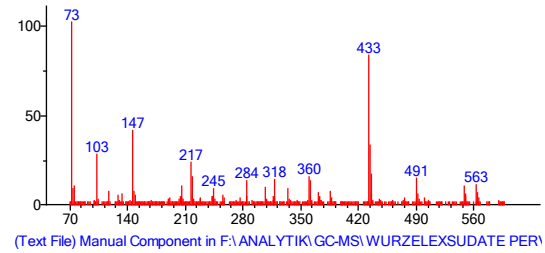
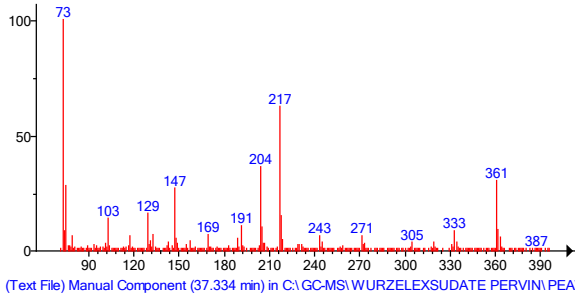
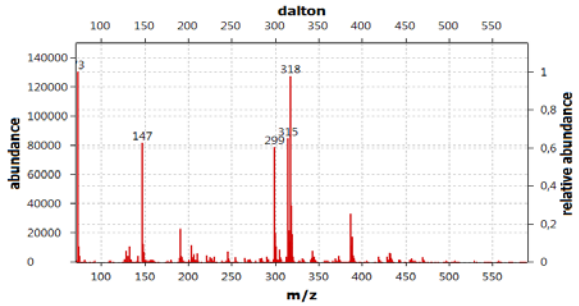
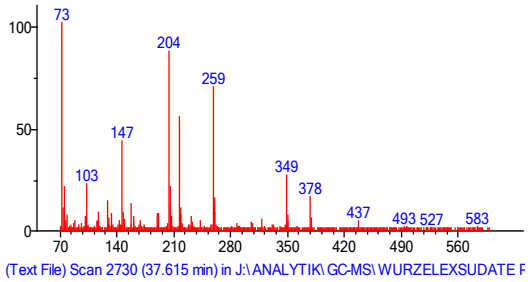
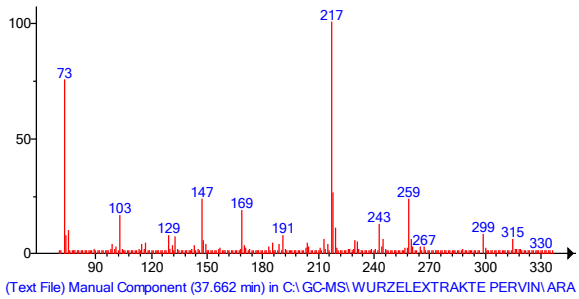
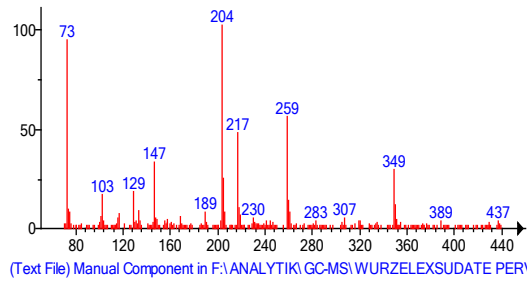
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**29.44**	**1997**	*1945*	Ononitol	6	
	**29.64**	**2008**	*1980*	Galactonic acid	6	
	**29.73**	**2013**	*1984*	Gluconic acid	6	
	**29.90**	**2022**	*1996*	Glutamine	4	
	**29.99**	**2027**	*1892*	Talose	5	
	**30.20**	**2039**	*1980*	Galactonic acid	6	

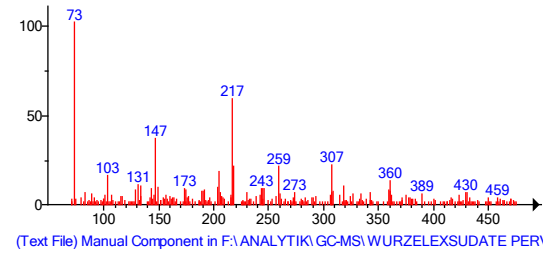
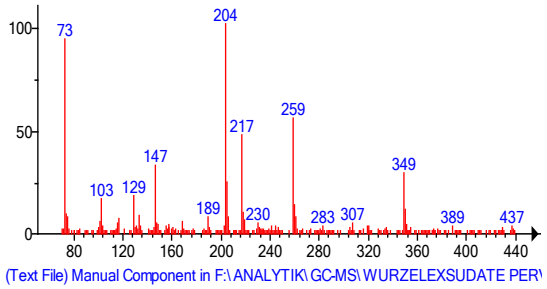
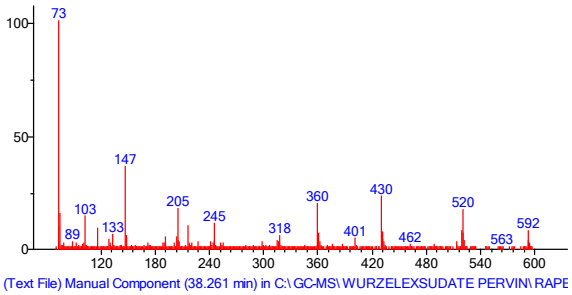
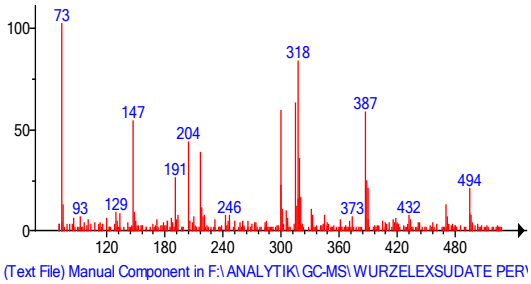
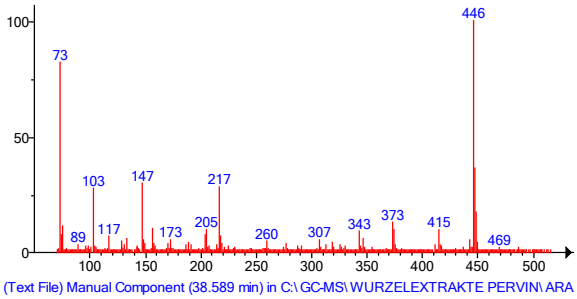
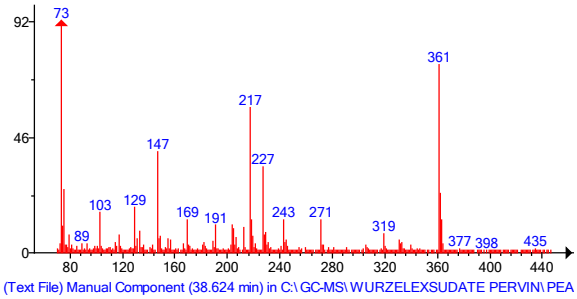
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**30.37**	**2048**	*1984*	Gluconic acid	6	
	**30.38**	**2049**	*2062*	Galacturonic acid	7	
	**30.66**	**2065**	*2250*	Glucoheptonic acid	7	
	**31.09**	**2089**	*2053*	Dihydroxyphenylalanine	3	
	**31.18**	**2095**	*2000*	Saccharic acid	6	
	**31.81**	**2131**	*2075*	*N* -Acetylglucosamine	4	1

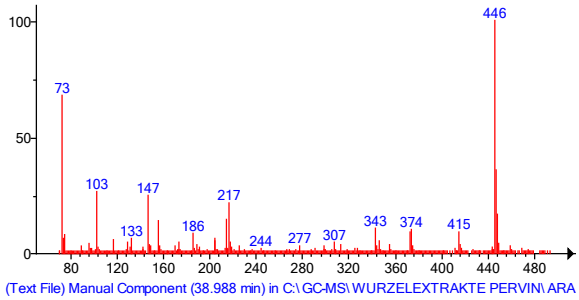
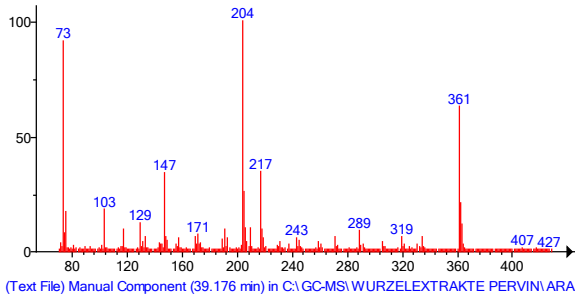
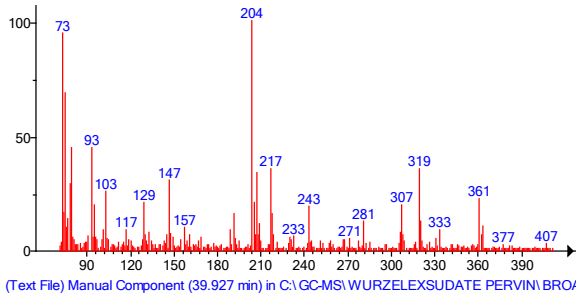
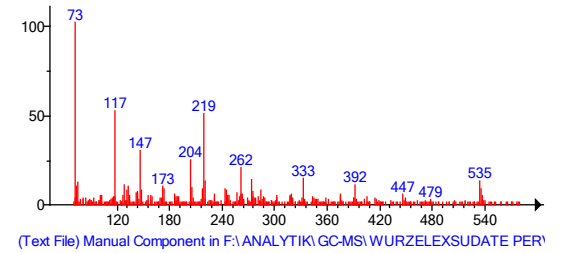
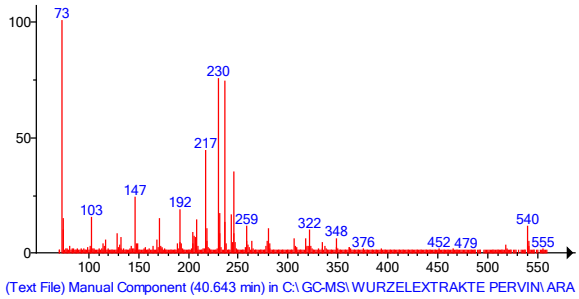
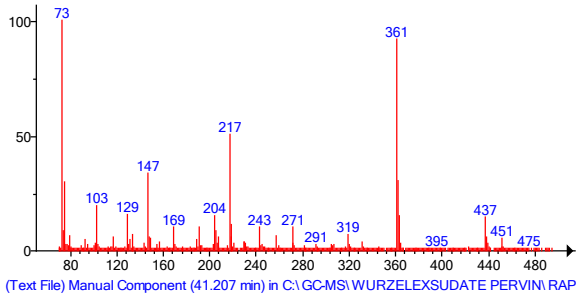
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**31.87**	**2135**	*2089*	Dihydroxyphenylalanine	4	
	**31.94**	**2139**	*2091*	*myo* -Inositol	6	
	**32.48**	**2171**	*2098*	A211001 (GMD unknown)		
	**32.73**	**2186**	*2098*	A213001 (GMD unknown)		
	**32.87**	**2194**	*2098*	A191002 (GMD unknown)		
	**33.29**	**2220**	*2155*	A216002 (GMD unknown)		

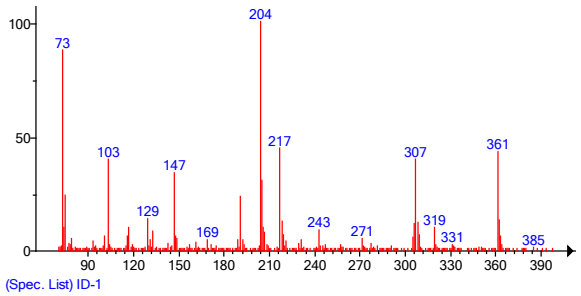
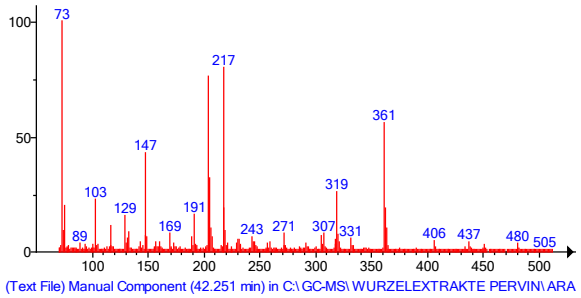
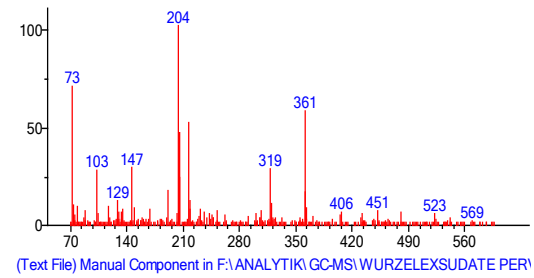
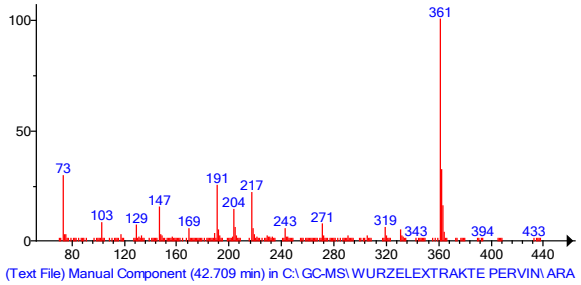
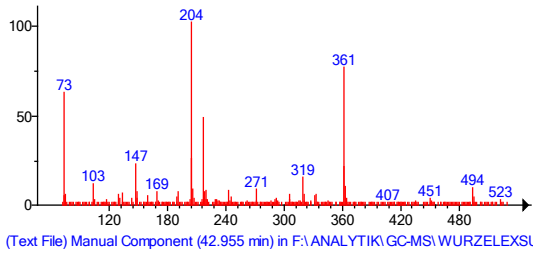
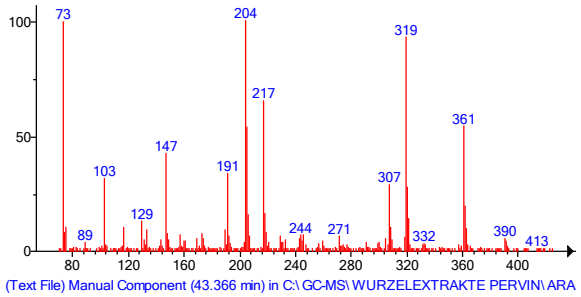
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**33.78**	**2250**	*2161*	A216003 (GMD unknown)	?	
 (Text File) Manual Component (33.836 min) in E:\ANALYTIK\GC-MS\ROOTS\RAPESEED\RE_RS_C1.D\D	**33.83**	**2253**	*2217*	Tryptophan	2	
 (Spec. List) ID-1	**33.96**	**2261**	*2161*	A217004 (GMD unknown)		
	**34.61**	**2302**		Glycerol-3-phosphate derivative	5	
 (Text File) Manual Component (35.068 min) in C:\ GC-MS\ WURZELEXTRAKTE PERVIN\ ARA	**35.07**	**2331**	*2244*	A226001 (GMD unknown)		
 (Text File) Manual Component (35.620 min) in C:\ GC-MS\ WURZELEXTRAKTE PERVIN\ ARA	**35.63**	**2367**	*2299*	A231002 (GMD unknown)		

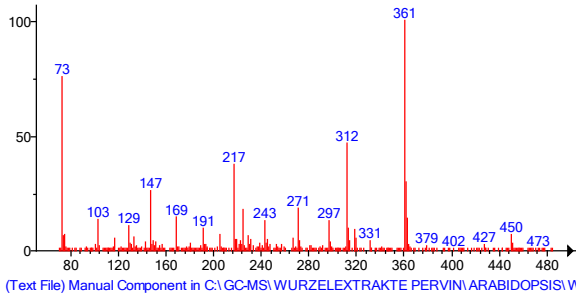
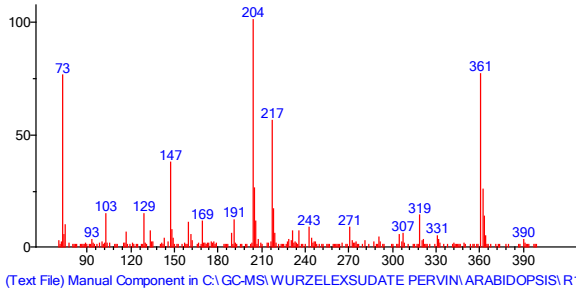
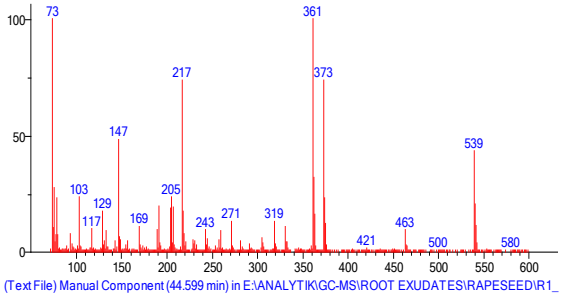
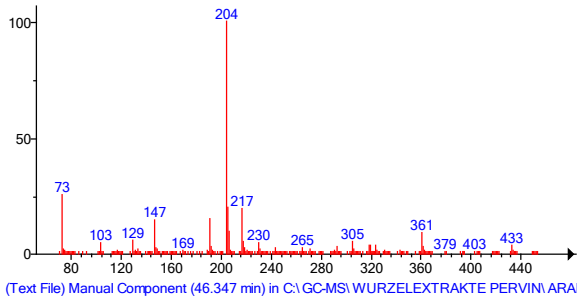
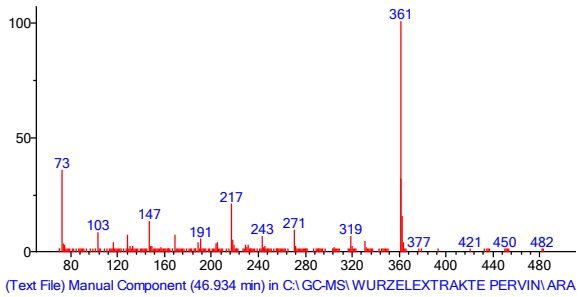
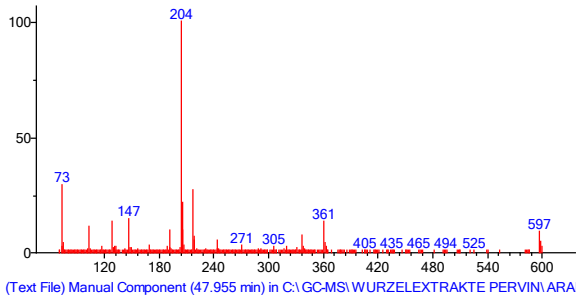
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**36.03**	**2394**	*2298*	A231002 (GMD unknown)		
	**36.07**	**2396**		Unknown		
	**36.17**	**2403**	*2503*	A252002 (GMD unknown)		
	**36.18**	**2403**	*2324*	Uridine	4	
	**36.45**	**2421**	*2336*	A234002 (GMD unknown)		
	**36.87**	**2449**	*2362*	A237002 (GMD unknown)		

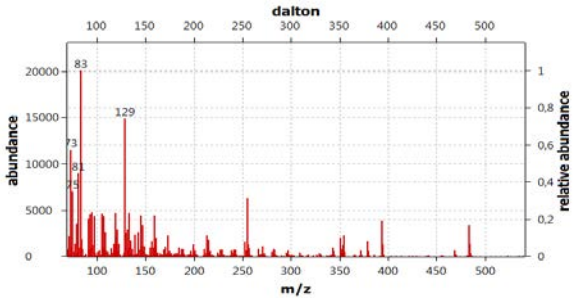
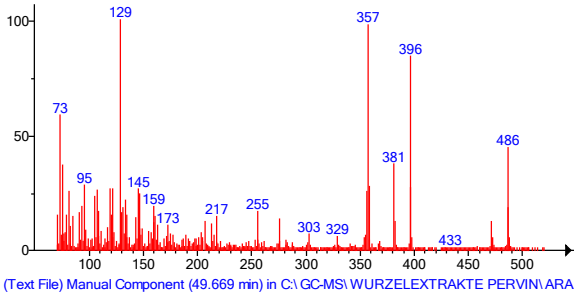
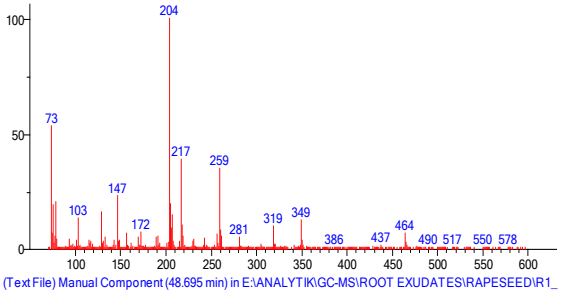
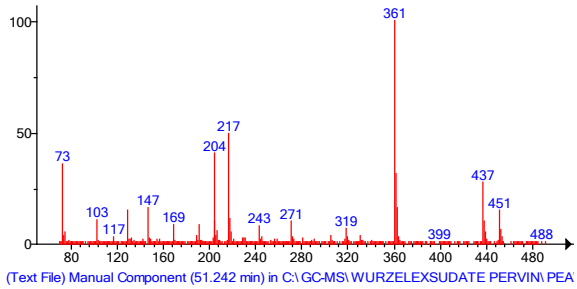
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**36.96**	**2455**	*2368*	A238003 (GMD unknown)		
	**37.33**	**2481**	*2387*	A239008 (GMD unknown)	?	
	**37.59**	**2498**	*2414*	*myo* -Inositol-1-phosphate	7	
	**37.63**	**2501**	*2404*	Xylobiose	6	1
	**37.66**	**2503**	*2468*	Uridine	3	
	**37.97**	**2525**	*2745*	Lactobionic acid	1	

EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**38.07**	**2532**	*2451*	A246001 (GMD unknown)	?	
	**38.16**	**2538**	*2798*	Lactobionic acid	1	
	**38.26**	**2545**	*2451*	A246001 (GMD unknown)		
	**38.51**	**2562**	*2485*	myo-Inositol-2-phosphate	7	
	**38.59**	**2568**	*2503*	A252002 (GMD unknown)		
	**38.63**	**2571**	*2564*	Salicylaldehyde glucoside	4	1

EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**38.99**	**2596**	*2503*	A252002 (GMD unknown)		
	**39.16**	**2609**	*2484*	A252001 (GMD unknown)		
	**39.94**	**2665**	*2484*	A252001 (GMD unknown)		
	**40.41**	**2700**	*2577*	A259001 (GMD unknown)		
	**40.65**	**2718**	*2719*	Adenosine	4	
	**41.24**	**2762**	*2654*	Sucrose	8	

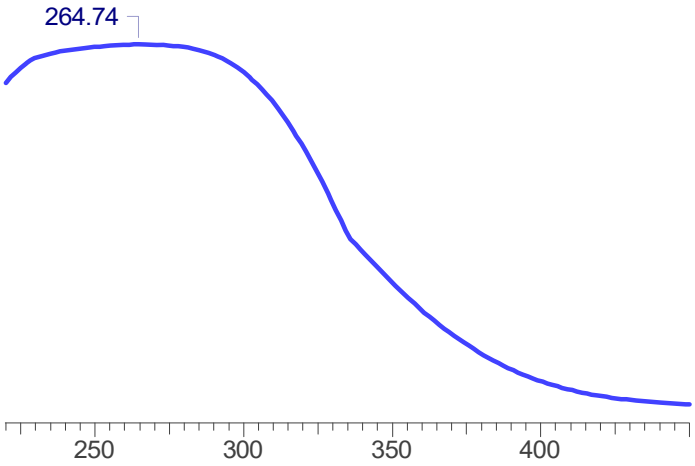
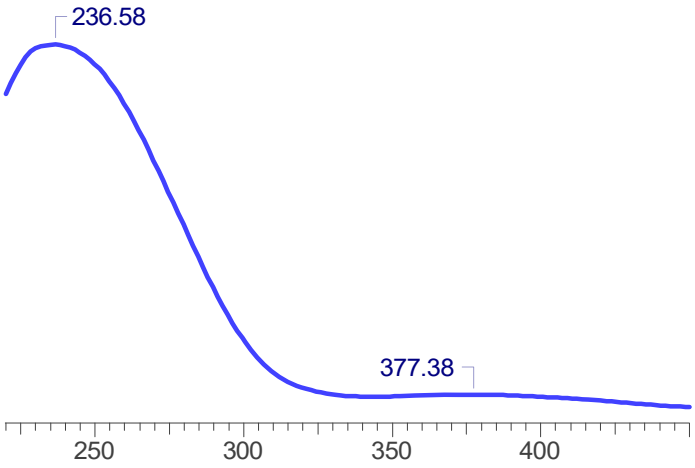
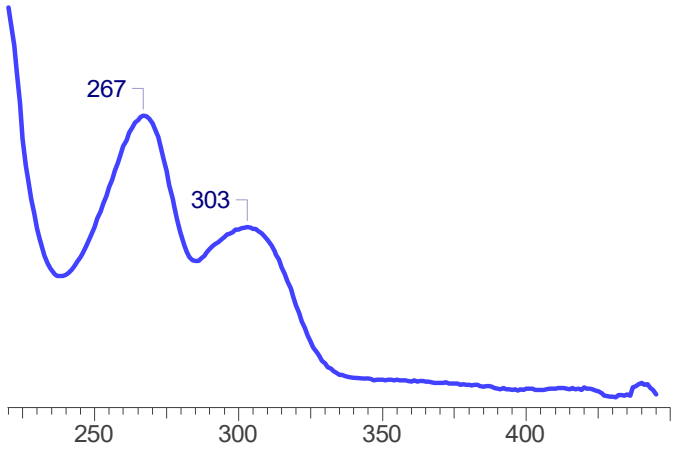
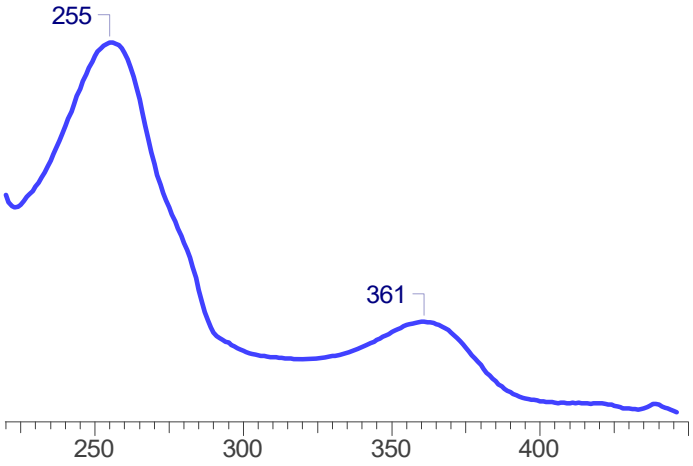
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**41.28**	**2765**	*2667*	A267006 (GMD unknown)		
	**42.27**	**2841**	*2868*	Melibiose	8	1
	**42.60**	**2867**	*2716*	Maltose	8	1
	**42.70**	**2875**	*2743*	Trehalose	8	
	**42.91**	**2892**	*2736*	A275004 (GMD unknown)		
	**43.36**	**2928**	*2751*	Sophorose	8	1

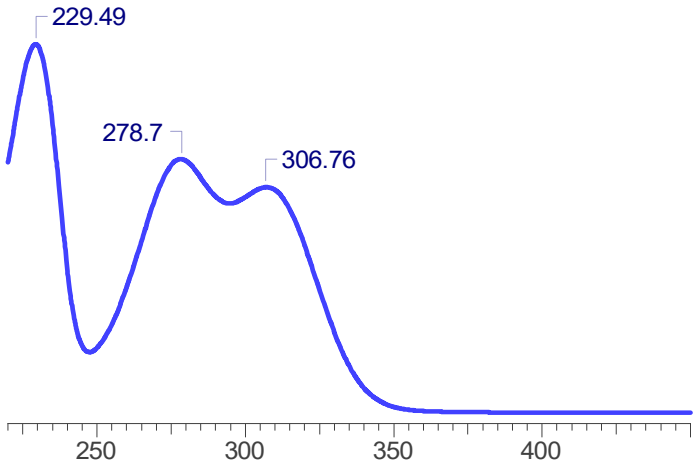
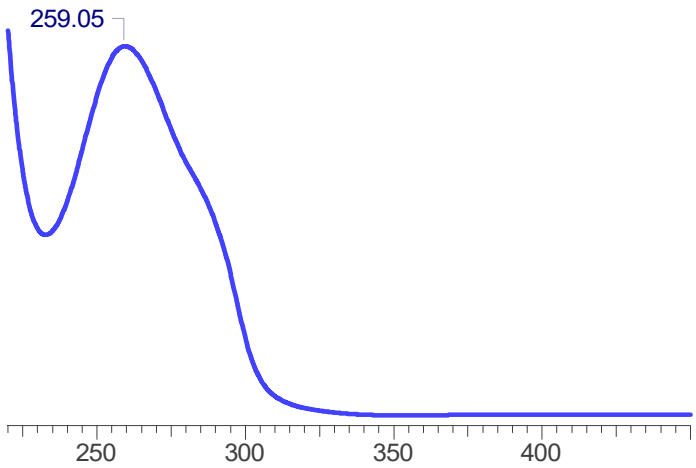
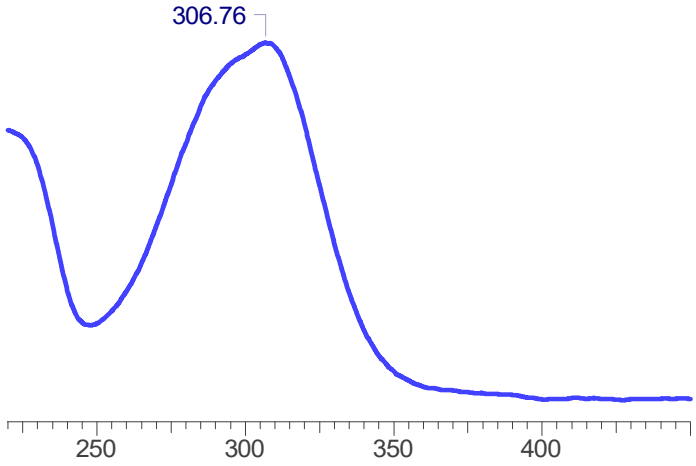
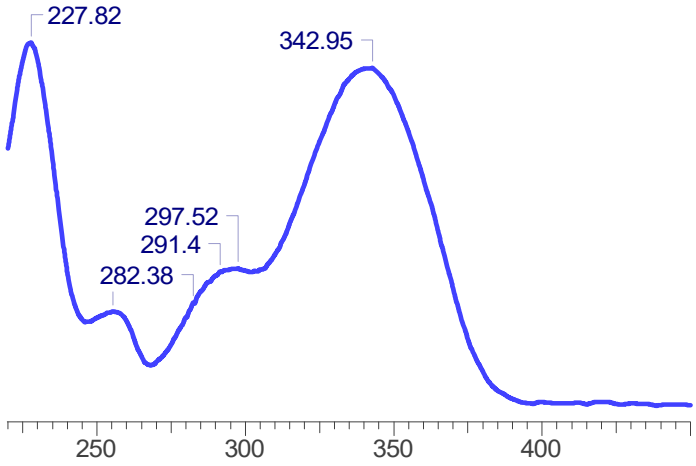
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**43.97**	**2977**	*2856*	A286005 (GMD unknown)		
	**44.57**	**3027**	*2837*	Melibiose	8	1
	**44.58**	**3028**	*2863*	A287005 (GMD unknown)		
	**46.37**	**3181**	*3003*	A301005 (GMD unknown)		
	**46.91**	**3228**	*3023*	A304001 (GMD unknown)		
	**47.96**	**3323**	*3098*	A311002 (GMD unknown)	?	

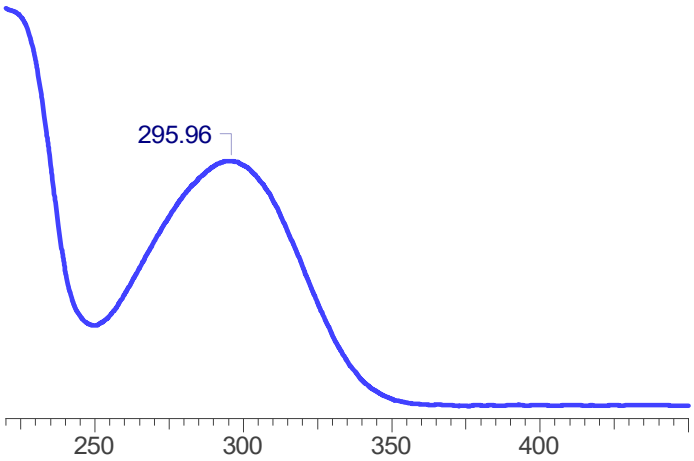
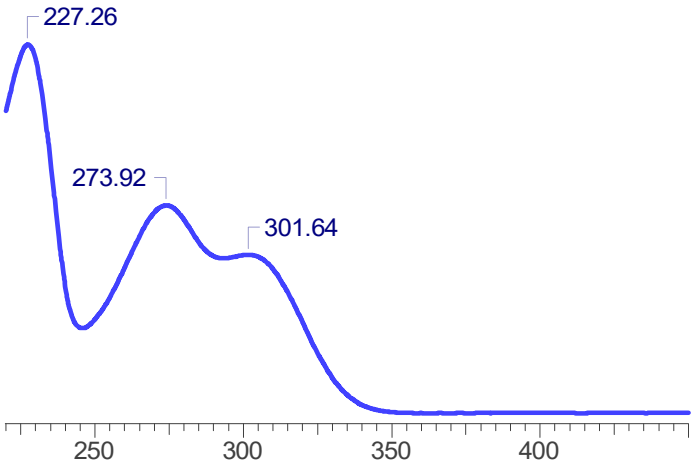
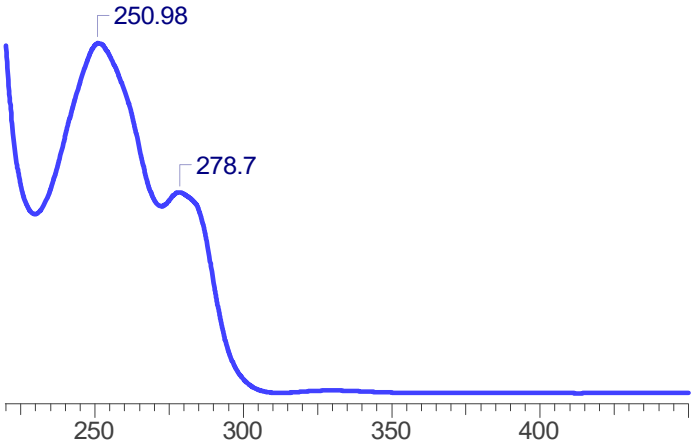
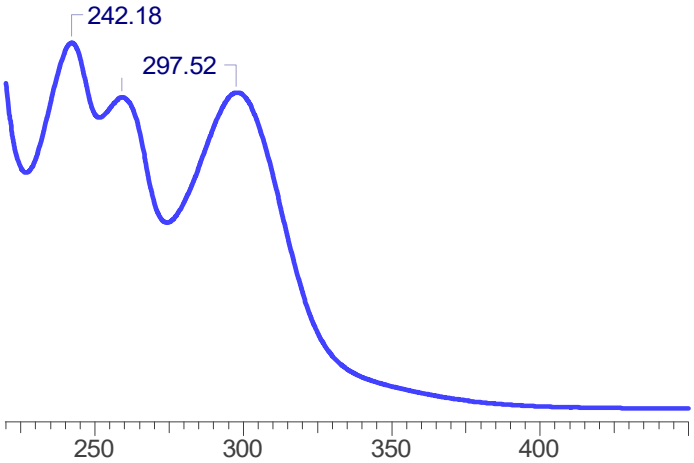
EI/MS Spectrum	Ret.	RI	RI (GMD)		TMS	Ox
	**49.01**	**3420**	*3319*	Stigmasterol	1	
 (Text File) Manual Component (49.669 min) in C:\ GC-MS\ WURZELEXTRAKTE PERVIN\ ARA	**49.86**	**3501**	*3385*	β-Sitosterol	1	
 (Text File) Manual Component (48.695 min) in E:\ANALYTIK\GC-MS\ROOT EXUDATES\RAPESEED\R1_	**48.70**	**3391**	*3562*	Maltotriose	11	1
 (Text File) Manual Component (51.242 min) in C:\ GC-MS\ WURZELEXSUDATE PERVIN\ PEA	**51.24**	**3636**	*3397*	Raffinose	11	

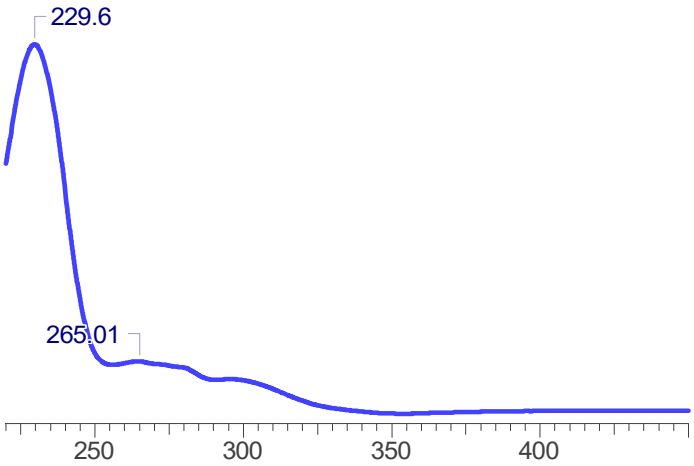
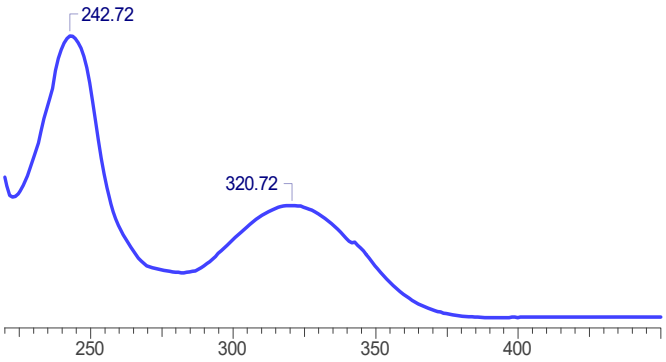
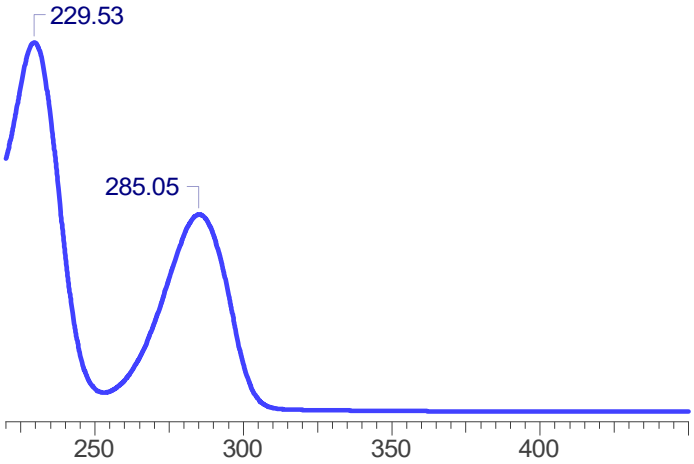
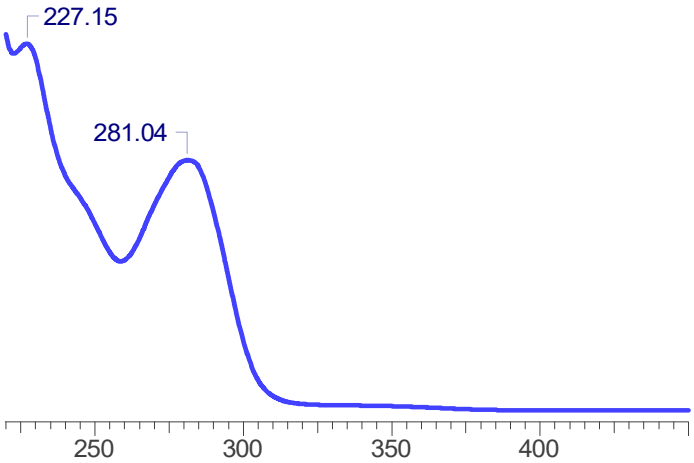
Appendix 3

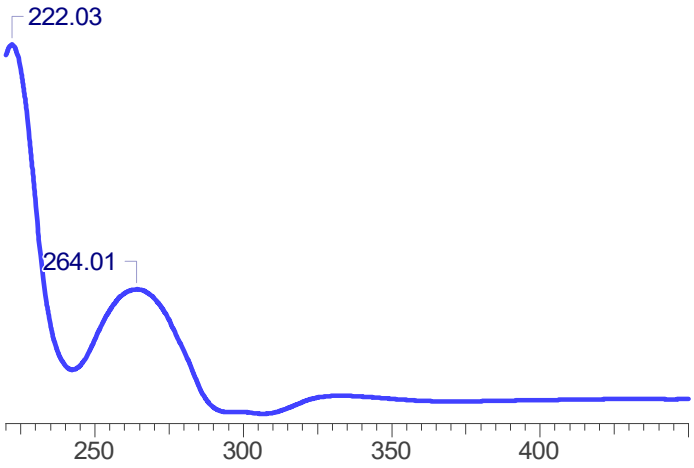
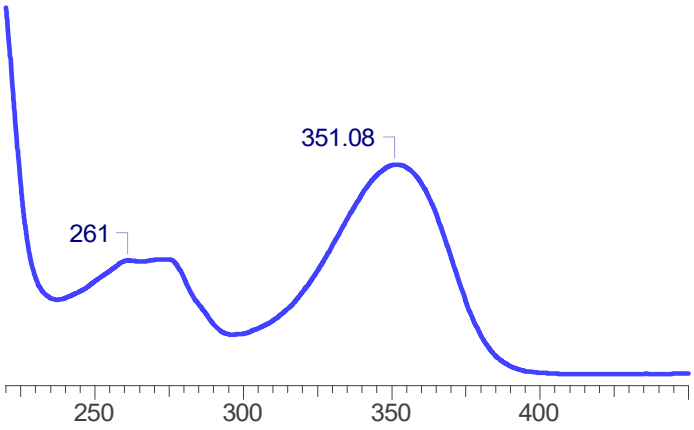
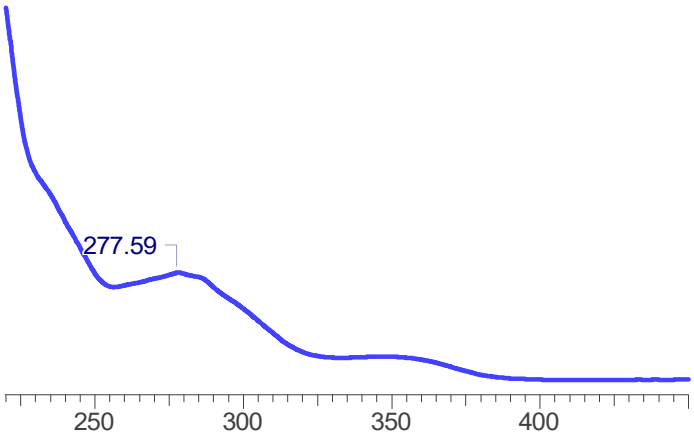
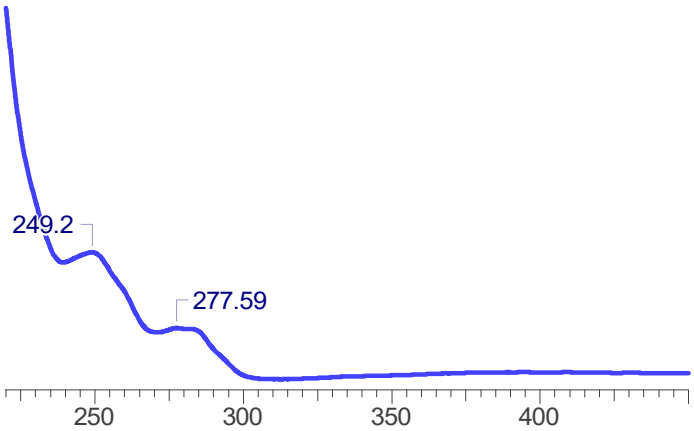
HPLC–DAD analyses: UV spectra

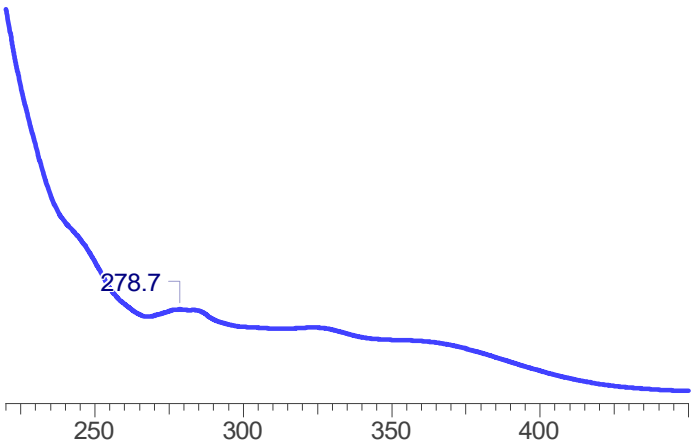
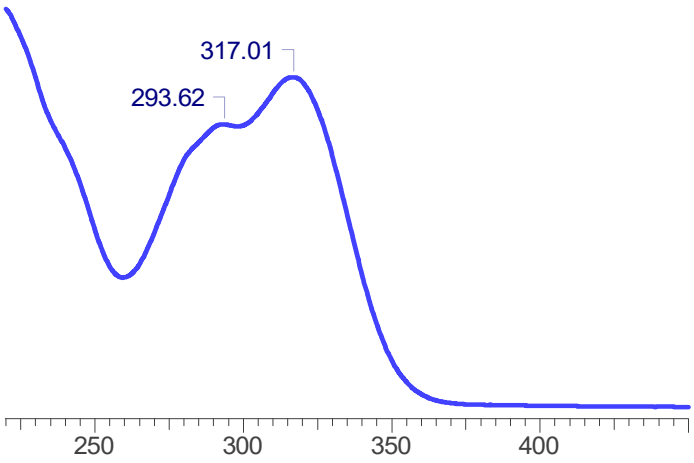
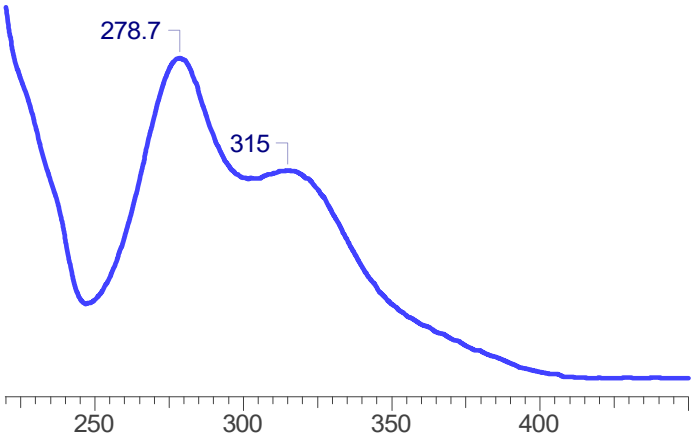
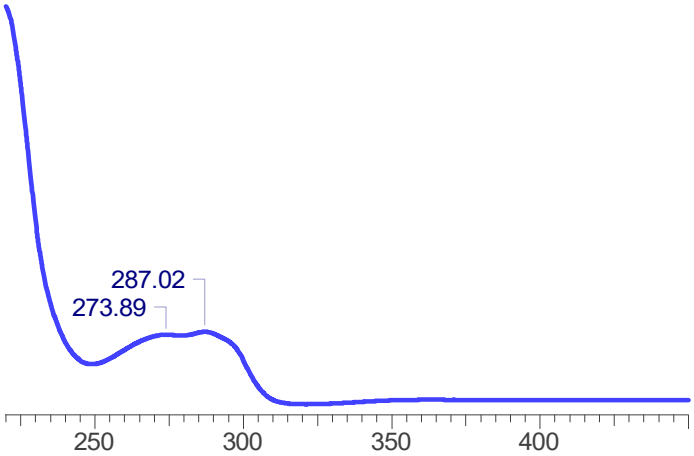
Ret. time	UV Spectrum	Identification
5.17		Gibberelline A_7
12.77		
12.83		
16.28		

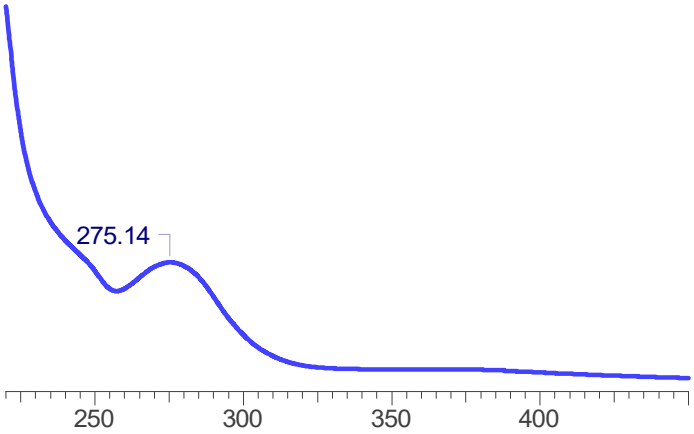
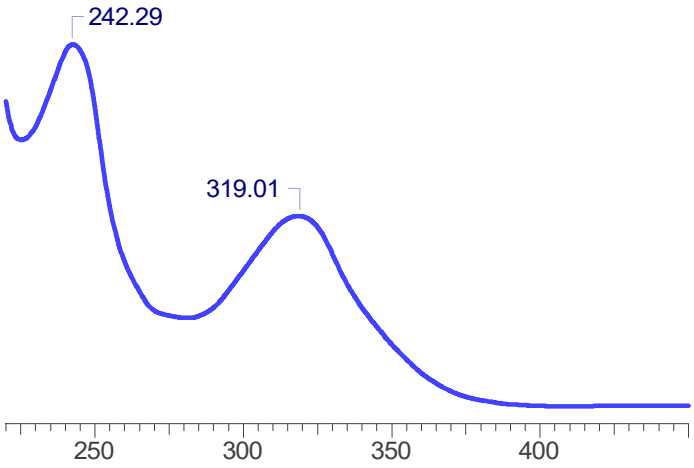
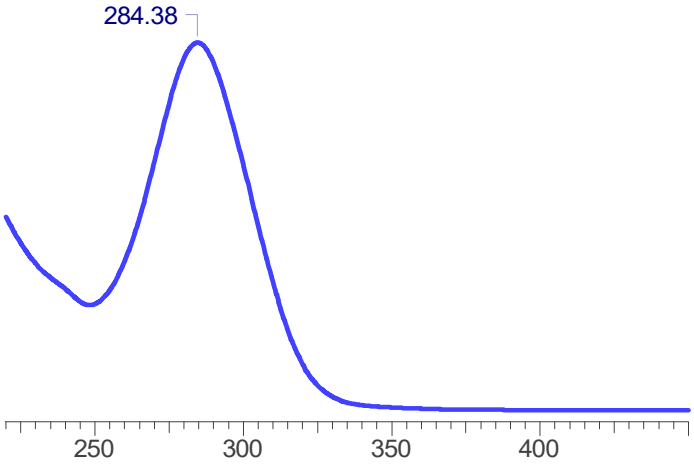
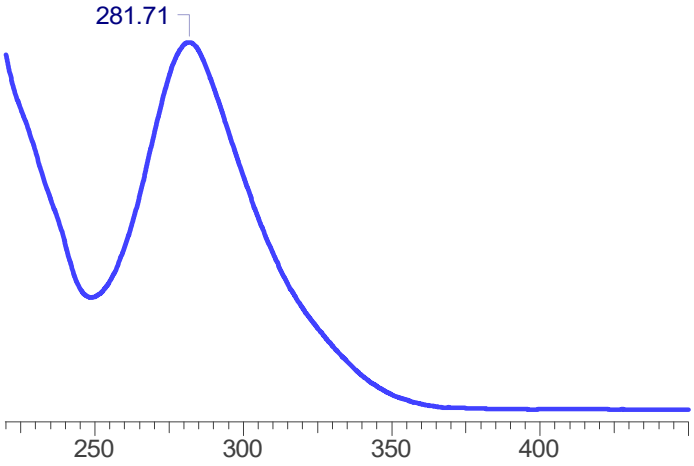
Ret. time	UV Spectrum	Identification
17.85		Chalcone, 3 x MeO, 2 x OH
18.13		Apiferiol
21.68		*para* -coumaric acid
22.24		

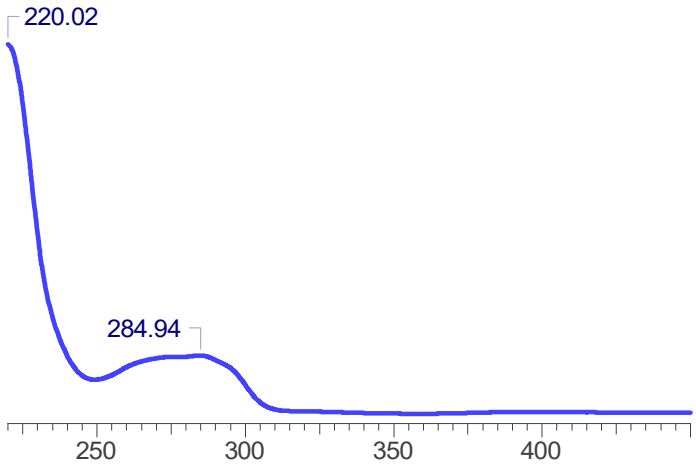
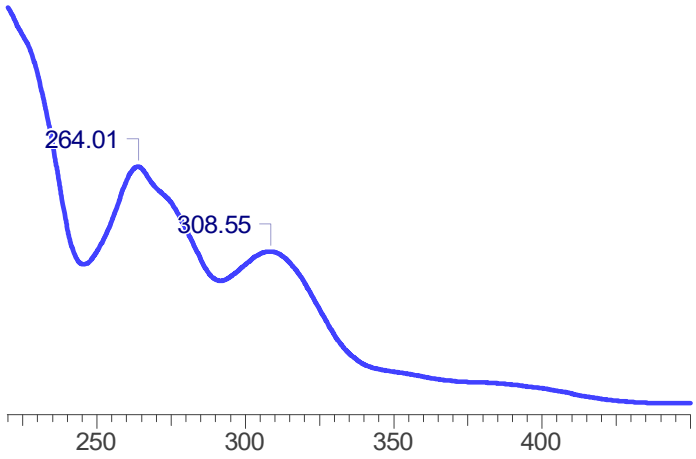
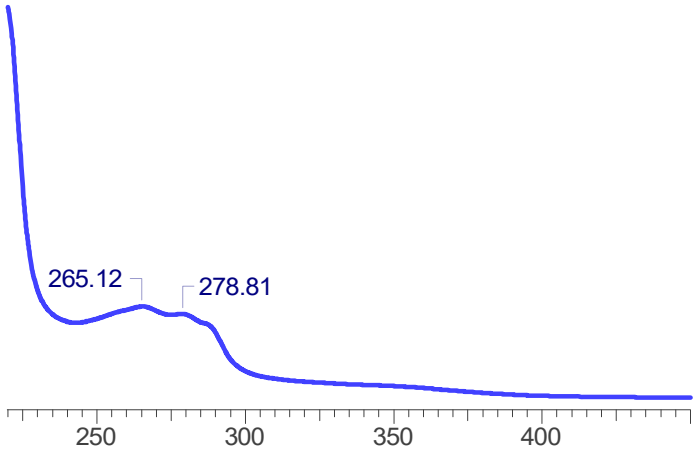
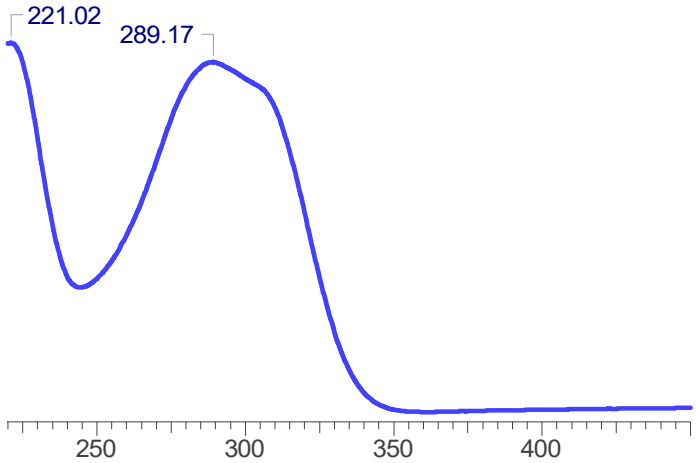
Ret. time	UV Spectrum	Identification
22.83		
23.15		
23.81		Indol-3-carboxyaldehyde
23.83		

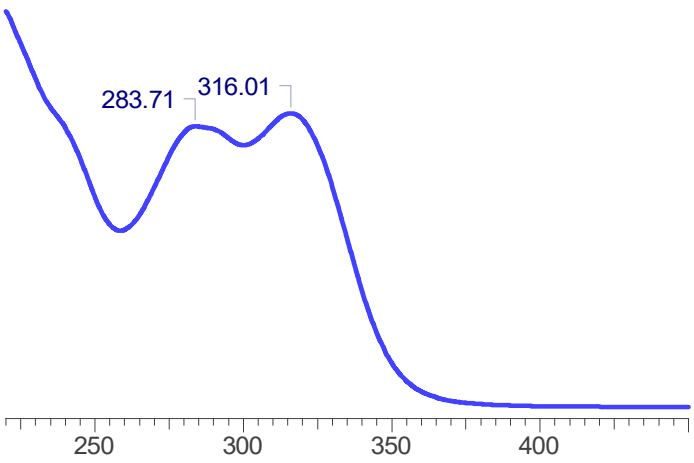
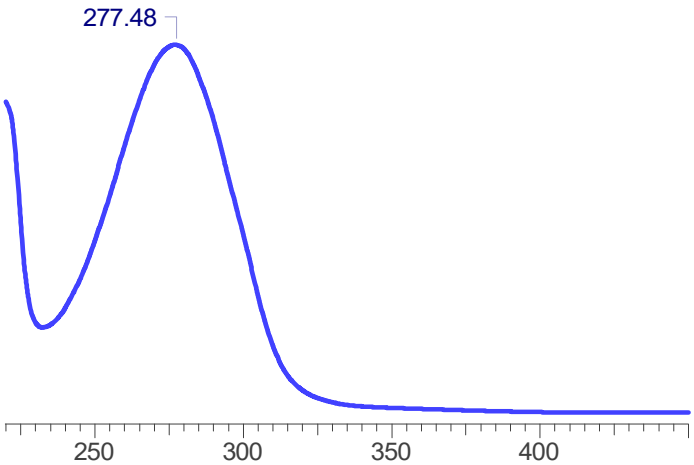
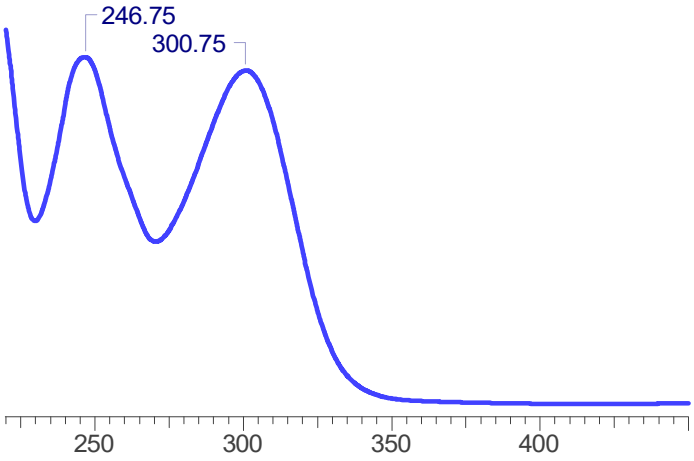
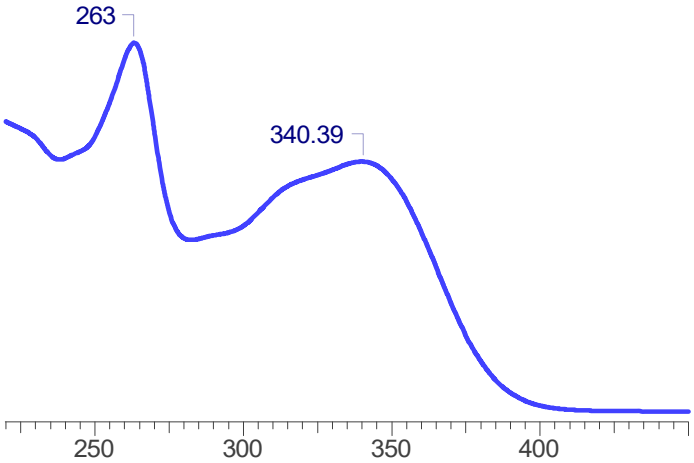
Ret. time	UV Spectrum	Identification
24.88		
24.91		3-Carbomethoxyindole
25.04		Tetrahydrofurofuran-1-one
25.12		Cinnamide der. 2 x OH, 1 x MeO

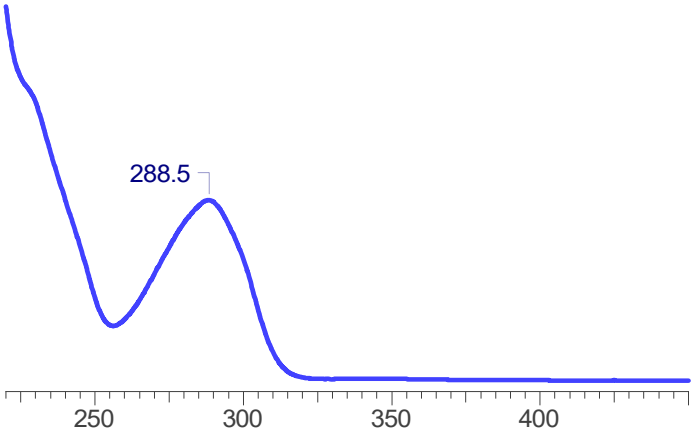
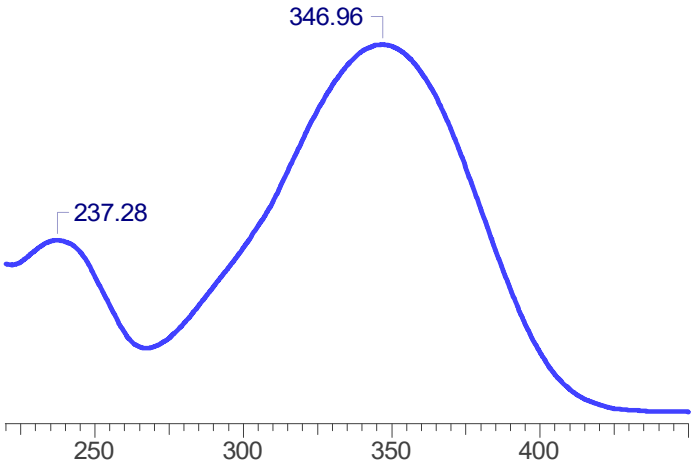
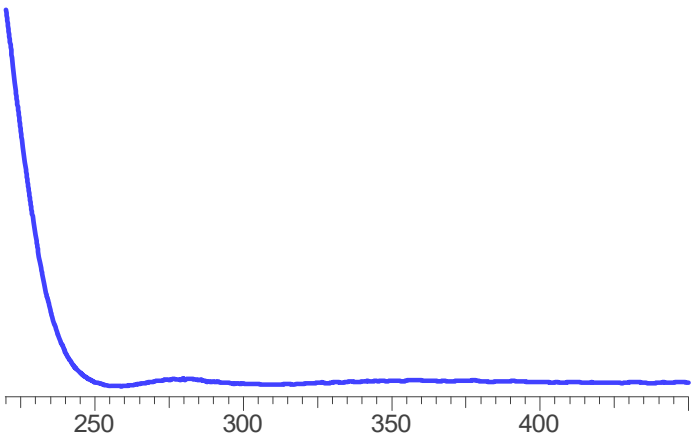
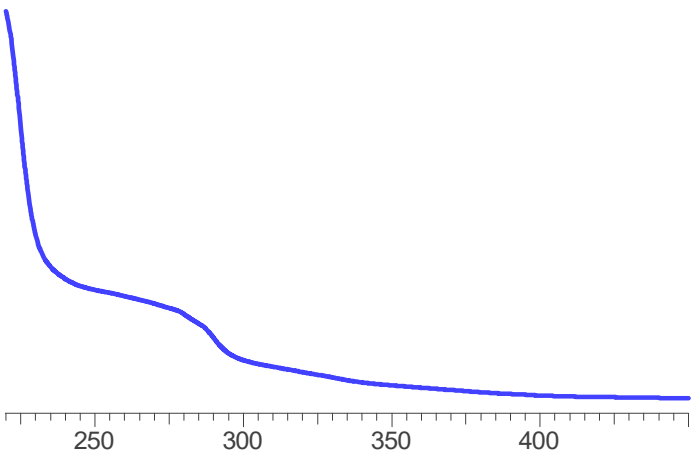
Ret. time	UV Spectrum	Identification
28.00		
29.17		
29.12		
29.84		

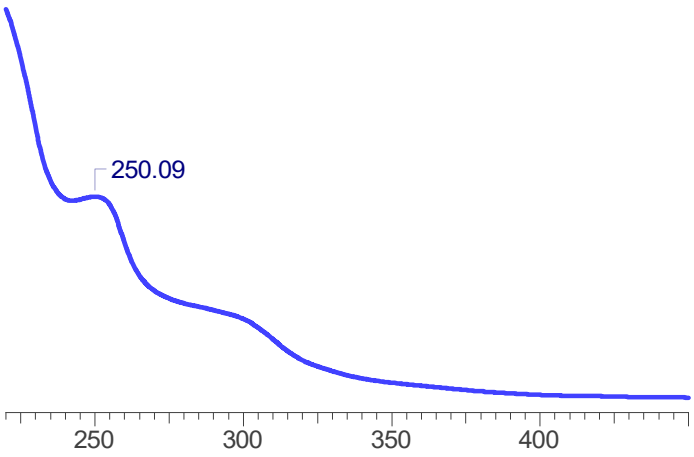
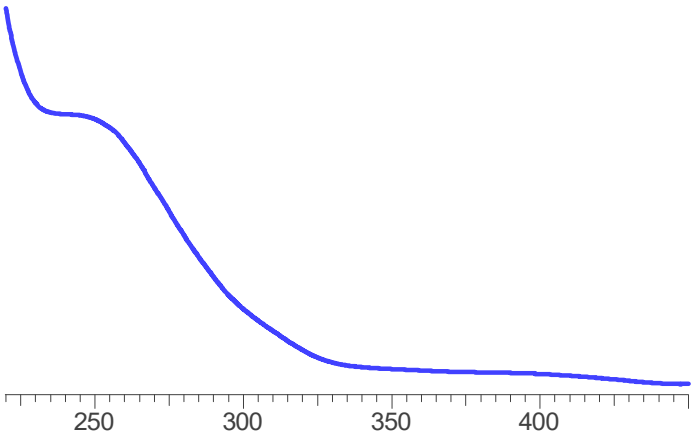
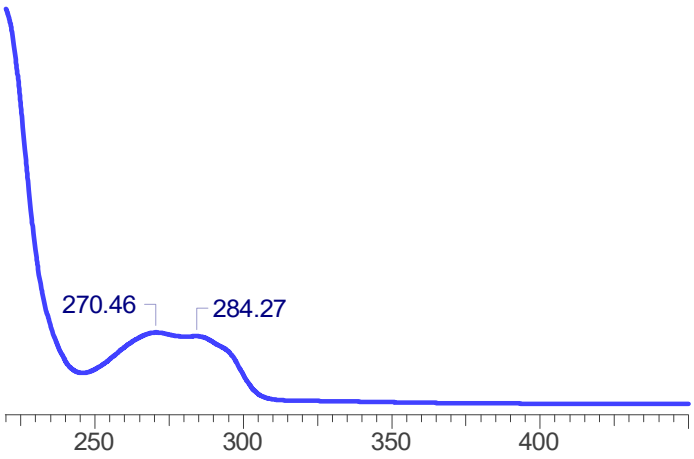
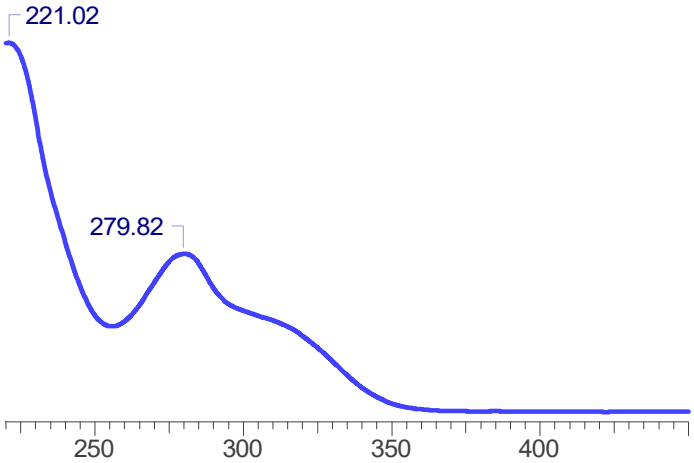
Ret. time	UV Spectrum	Identification
30.11		
30.24		
30.29		
31.70		

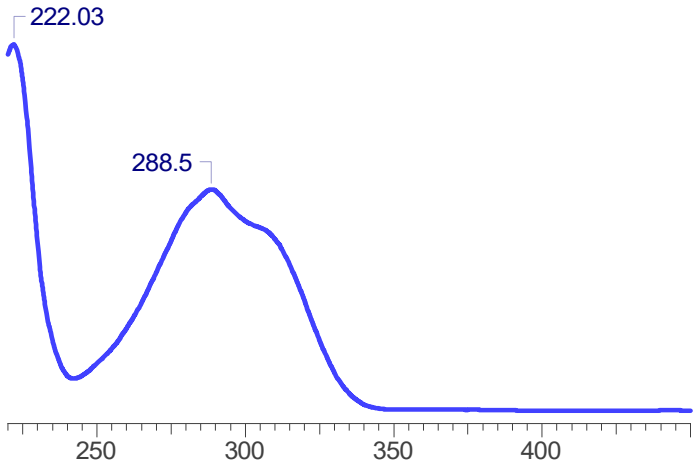
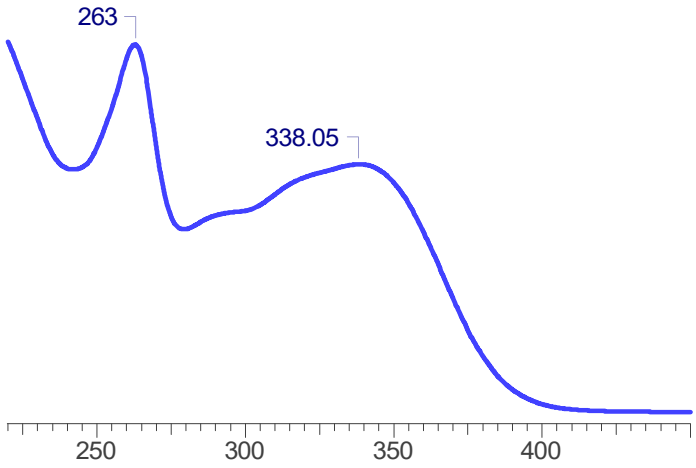
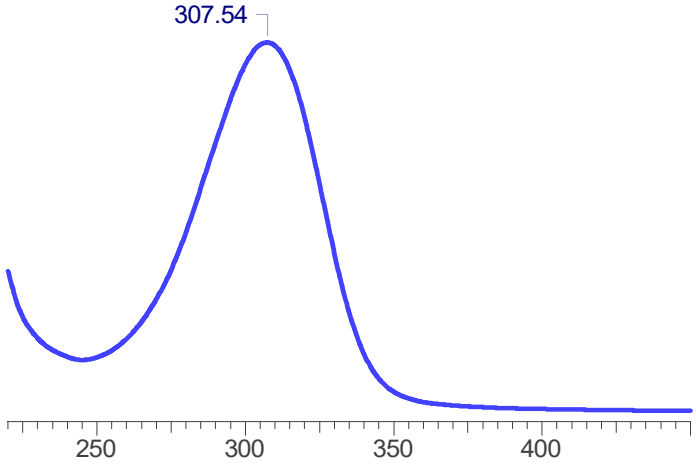
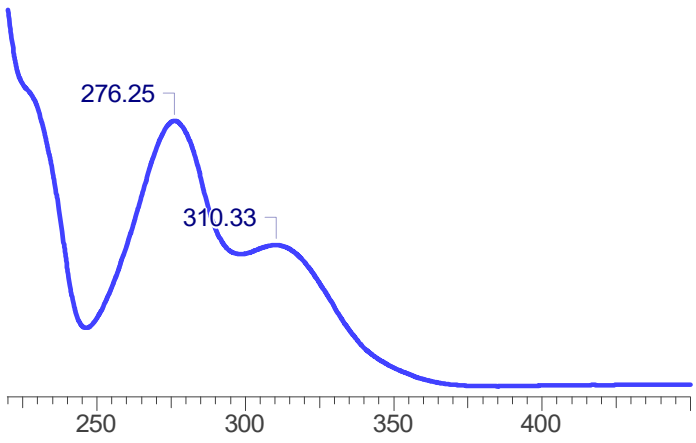
Ret. time	UV Spectrum	Identification
32.10		
33.68		Caulilexin A
34.64		4-Hydroxy- 1,4-benzoxazinone
35.12		

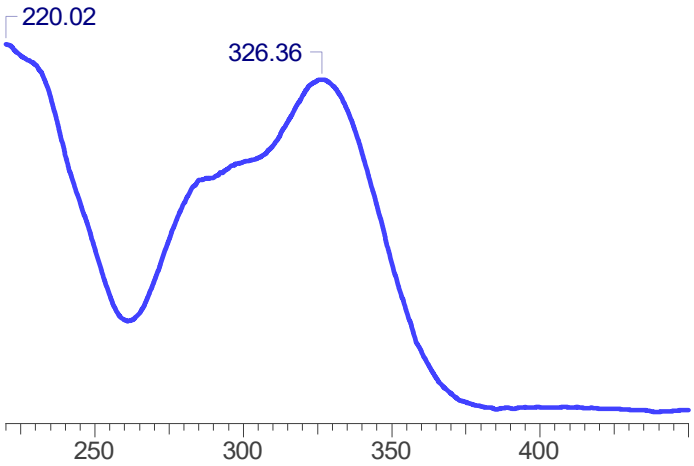
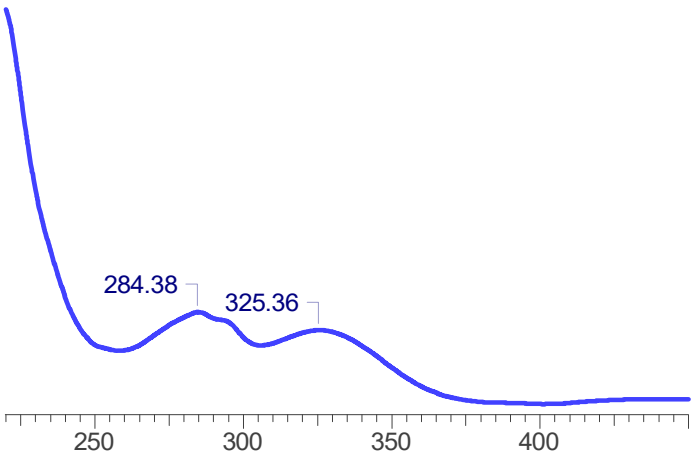
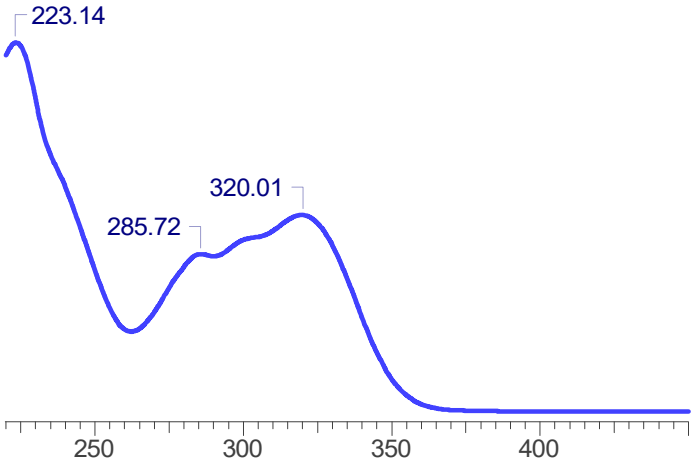
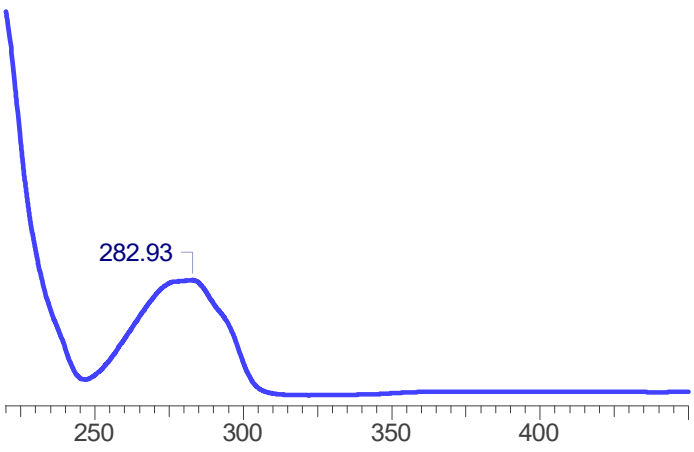
Ret. time	UV Spectrum	Identification
36.07		
36.72		
37.67		
37.87		

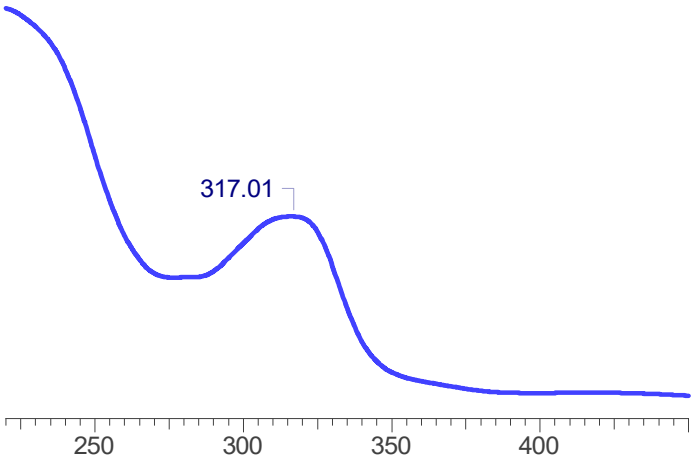
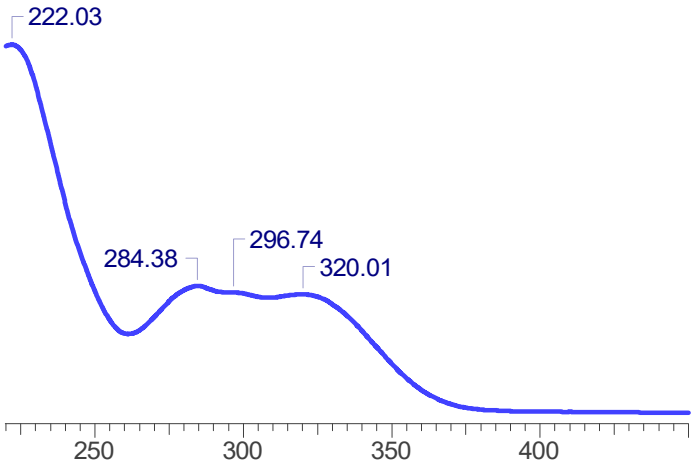
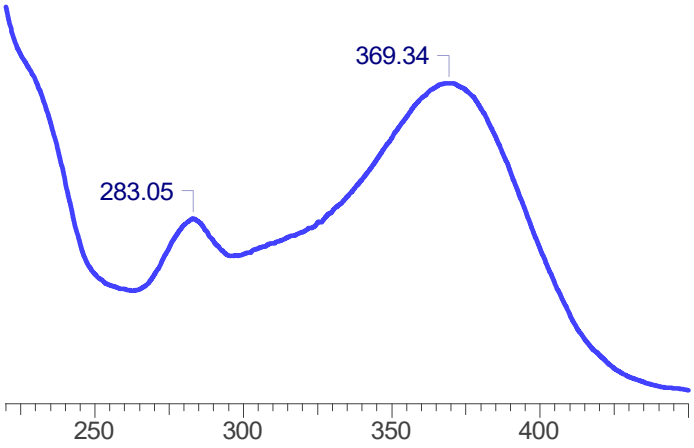
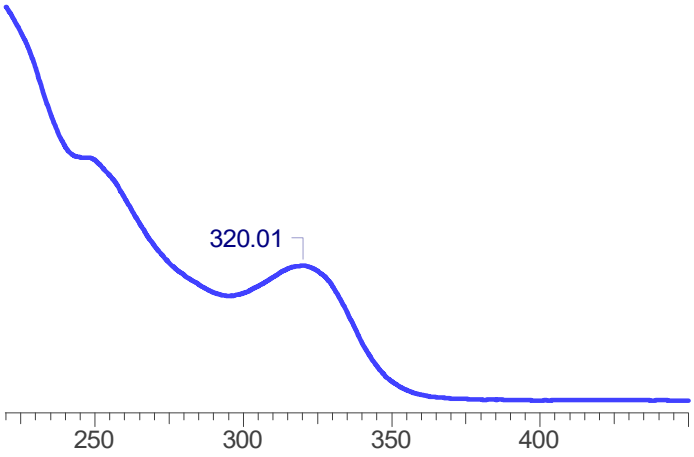
Ret. time	UV Spectrum	Identification
37.89		
38.27		Cinnamic acid
38.45		4-Methoxy cinnaldehyde
39.40		

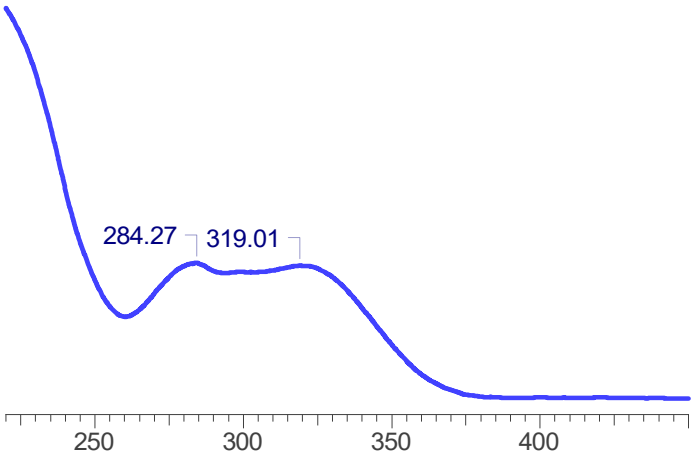
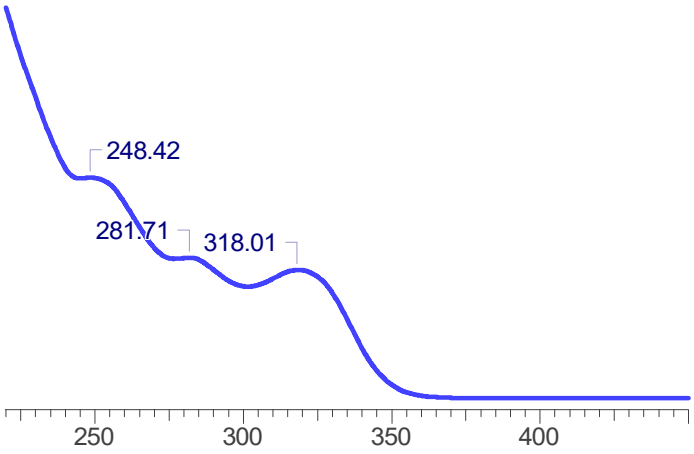
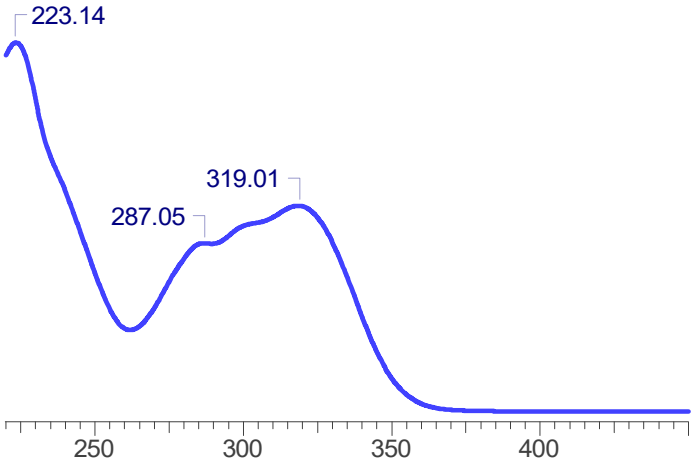
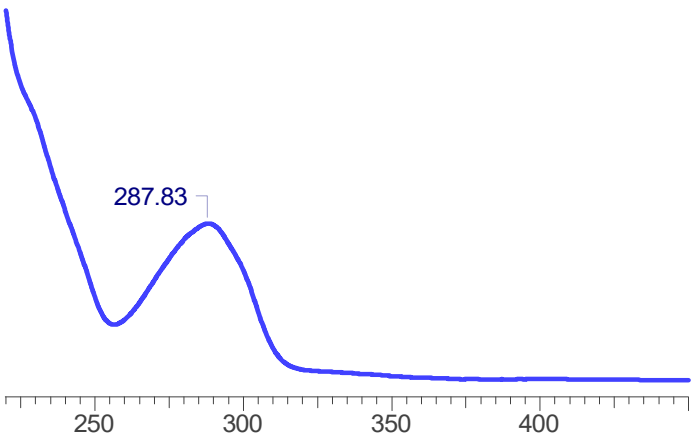
Ret. time	UV Spectrum	Identification
40.30		Dioxomethylene cinnamide
40.43		
40.88		
40.91		

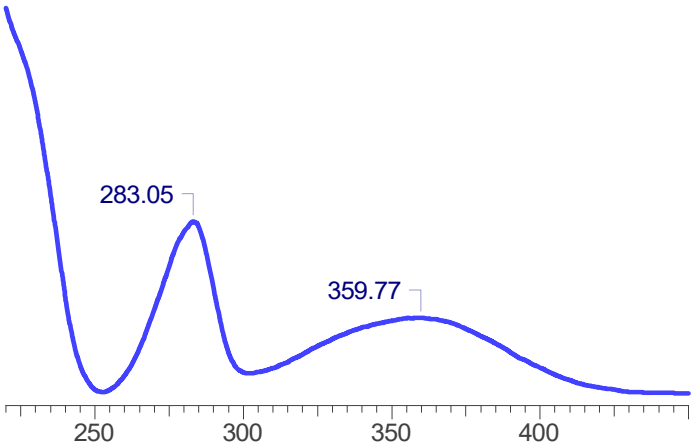
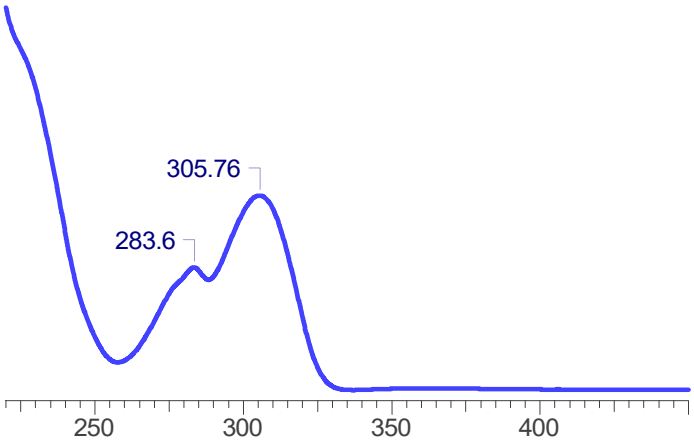
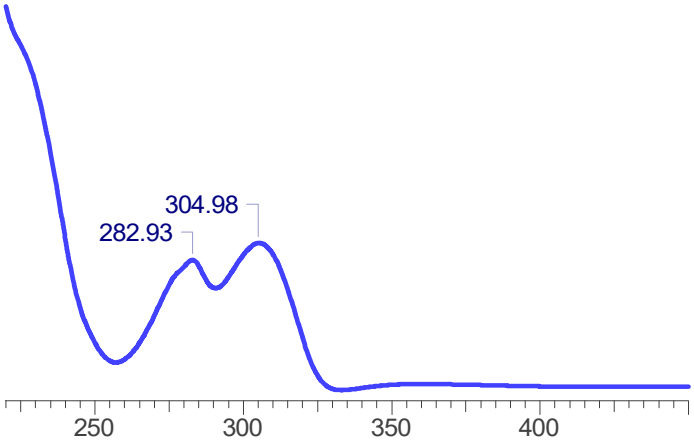
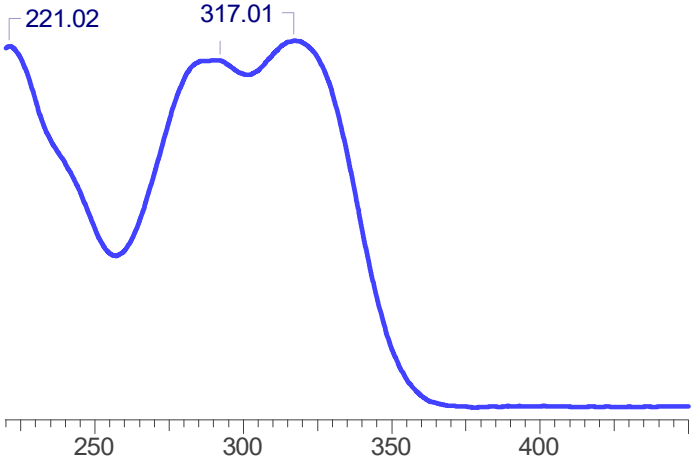
Ret. time	UV Spectrum	Identification
41.60		
41.04		
42.77		1-Hydroxy-3-formyl-4-methoxyindole
42.80		

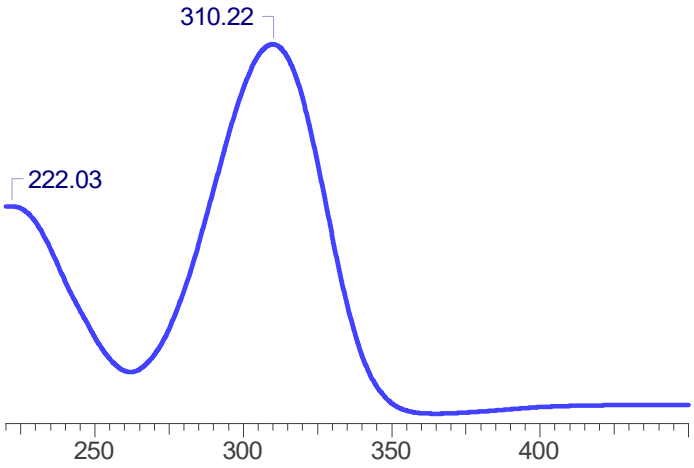
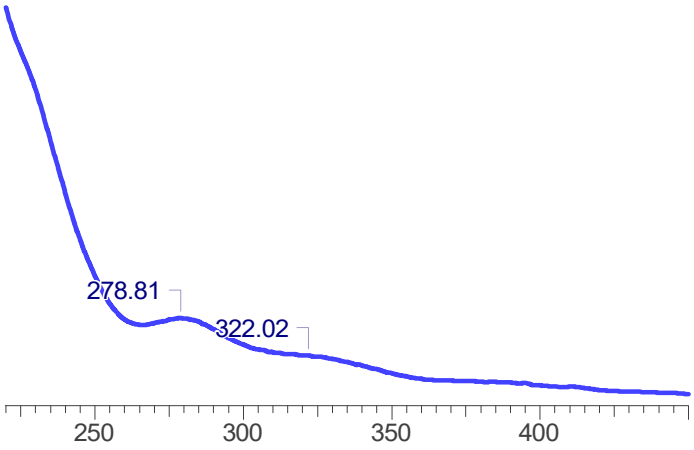
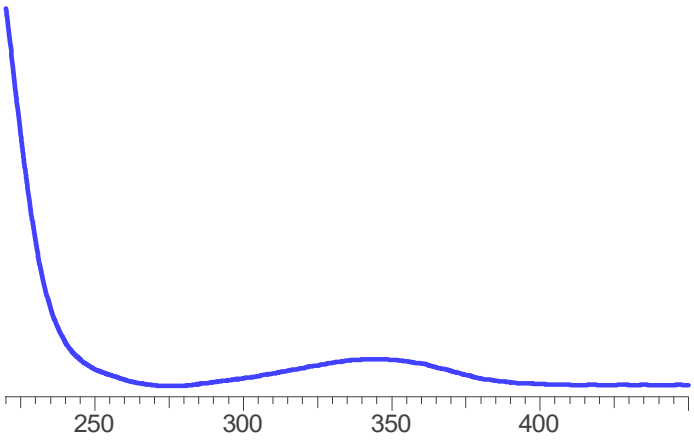
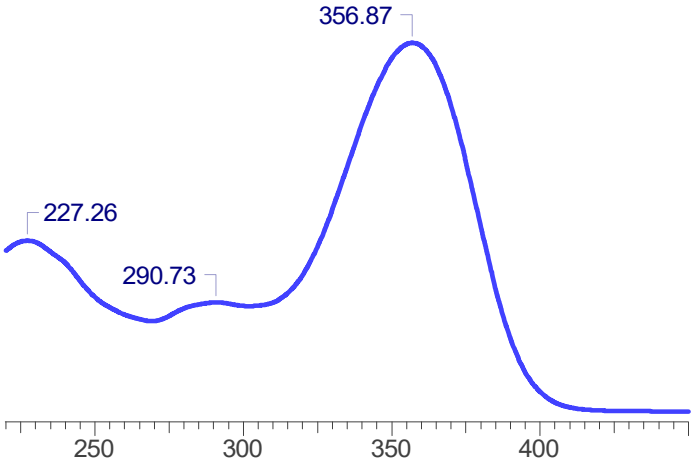
Ret. time	UV Spectrum	Identification
43.65		
43.69		
43.39		
44.61		

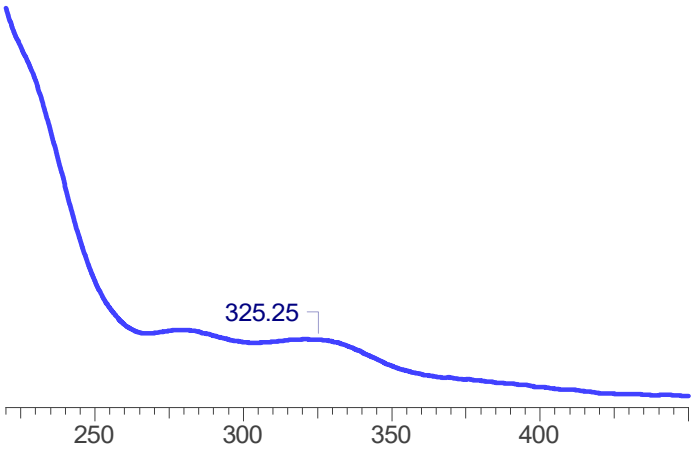
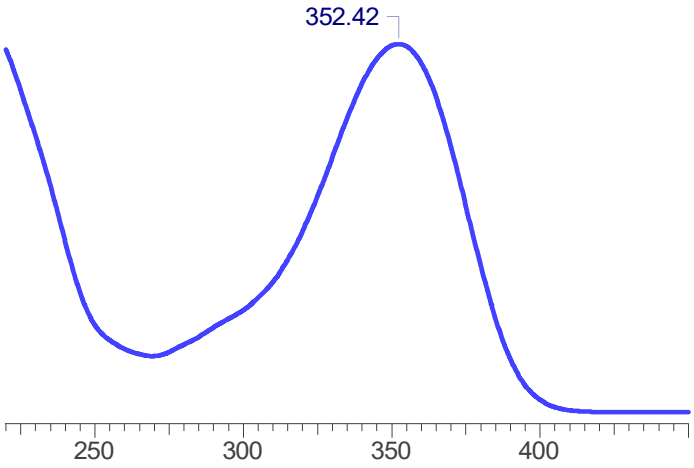
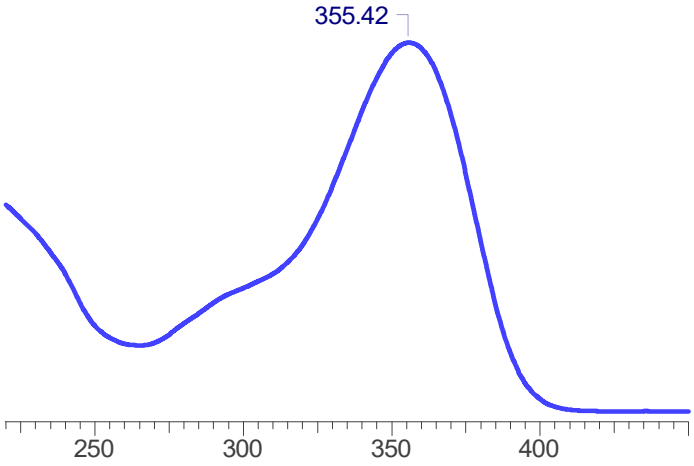
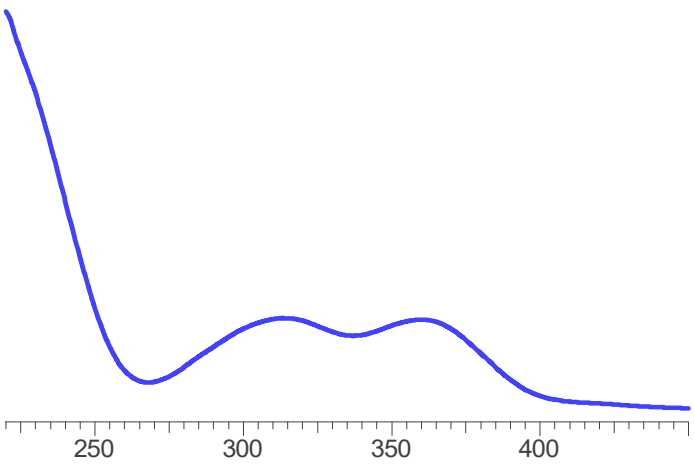
Ret. time	UV Spectrum	Identification
44.90		
45.70		Indole der.
46.35		
46.40		

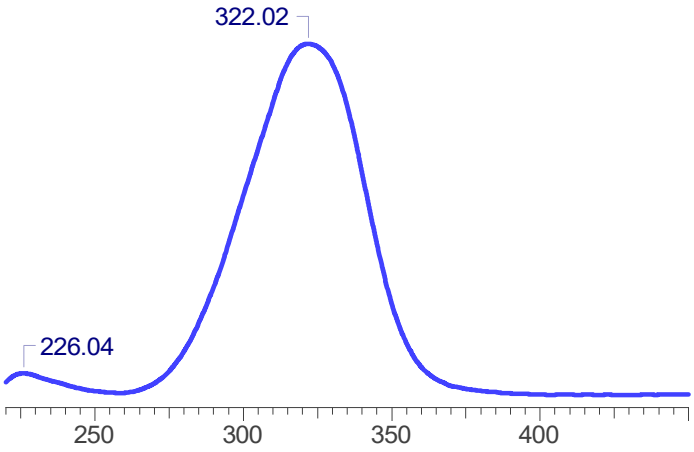
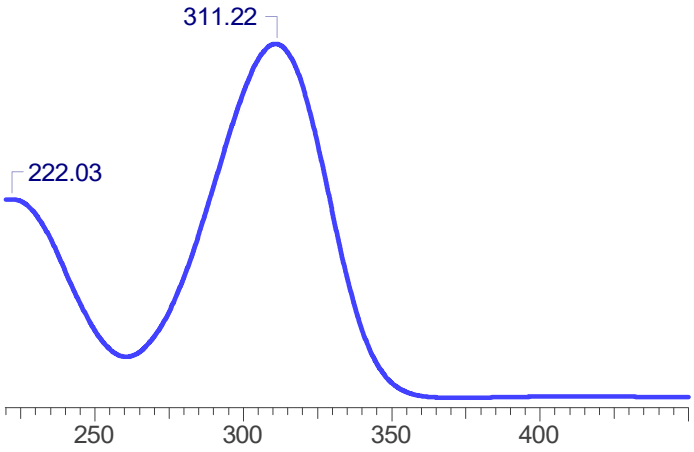
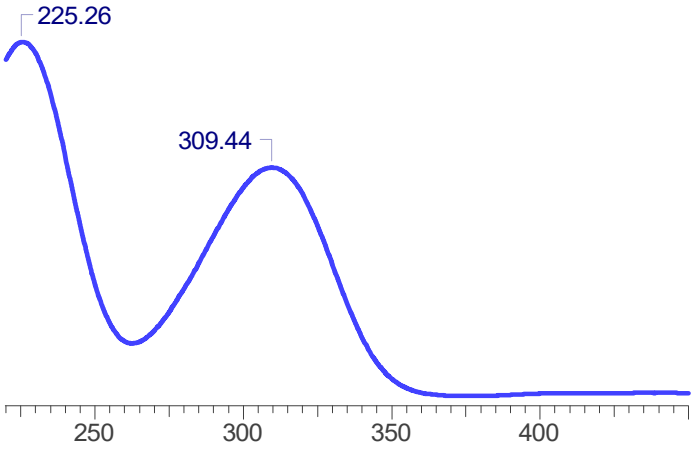
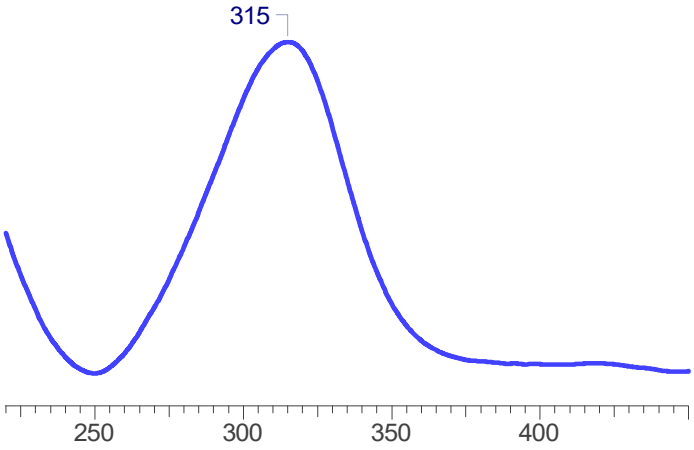
Ret. time	UV Spectrum	Identification
46.96		
47.20		
47.47		
47.68		

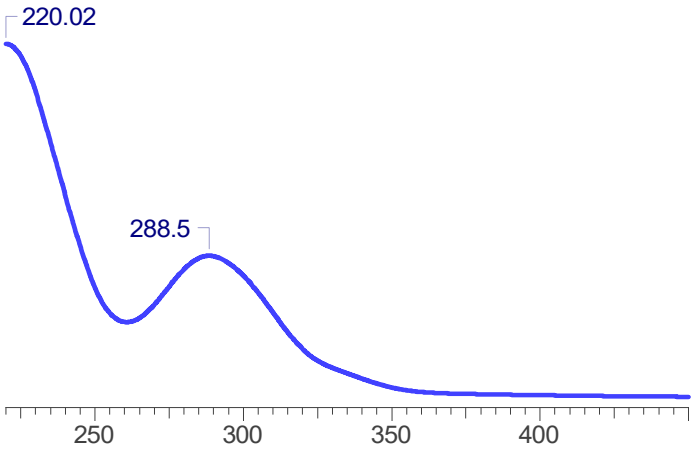
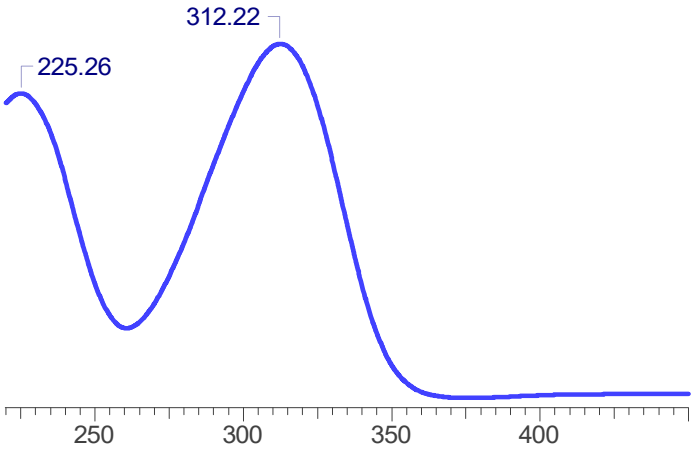
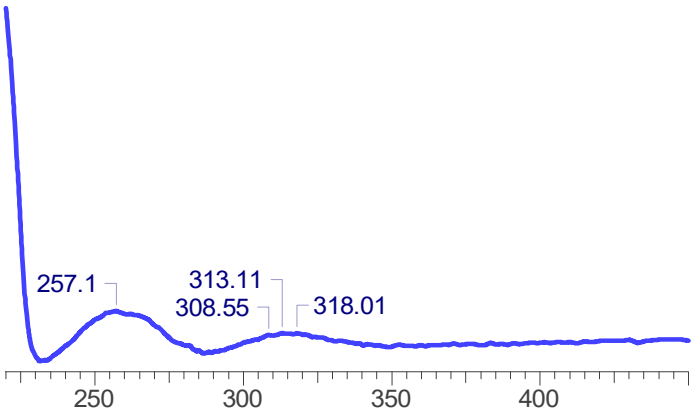
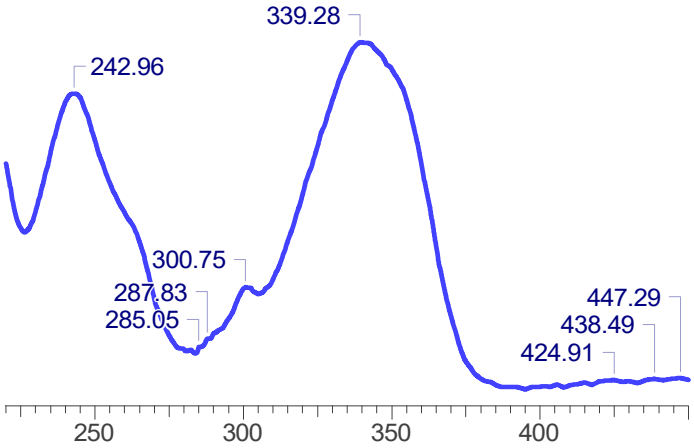
Ret. time	UV Spectrum	Identification
47.80		
49.23		Dihydrophenanthrene
49.81		
50.30		

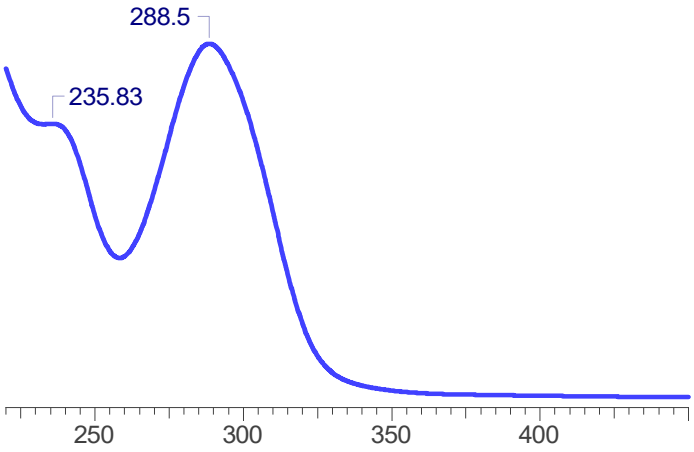
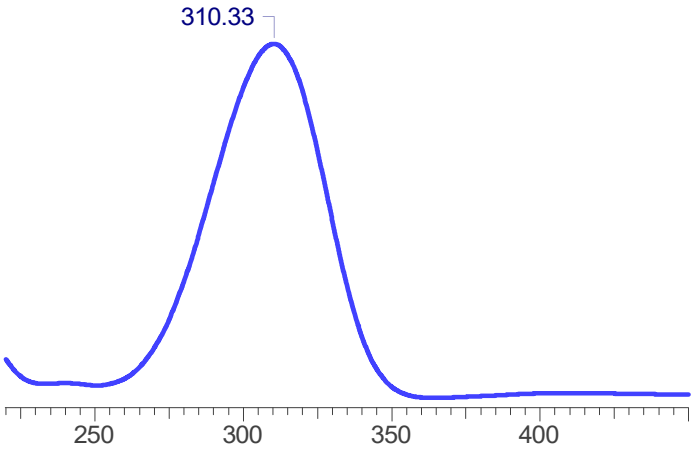
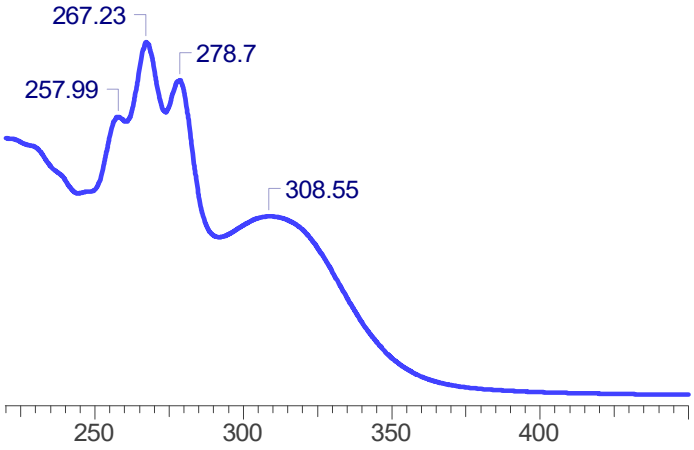
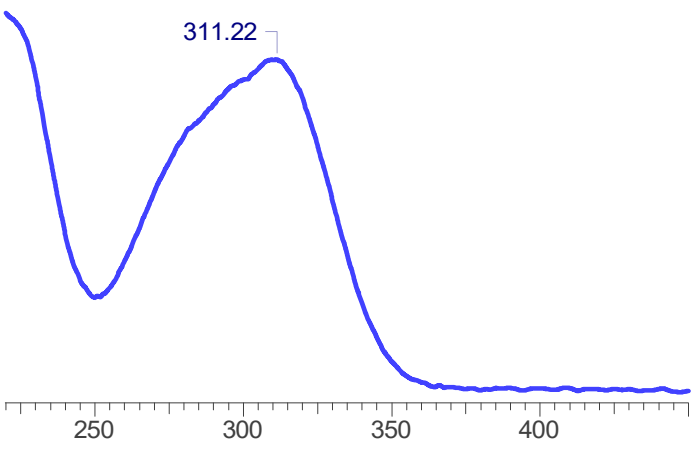
Ret. time	UV Spectrum	Identification
50.69		
51.01		Anhydropisatin
51.49		Hydroxyanhydropisatin
53.20		

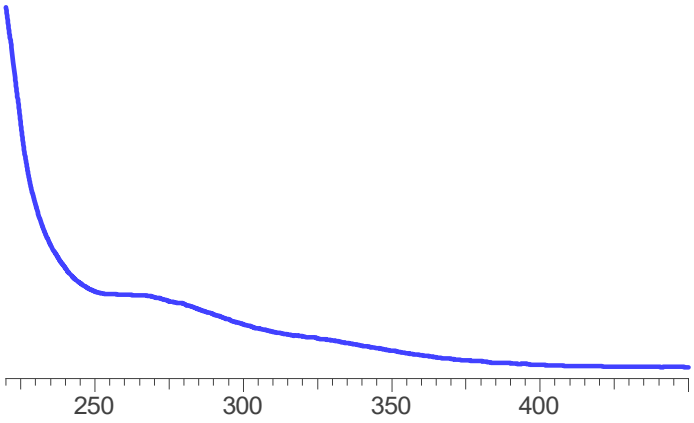
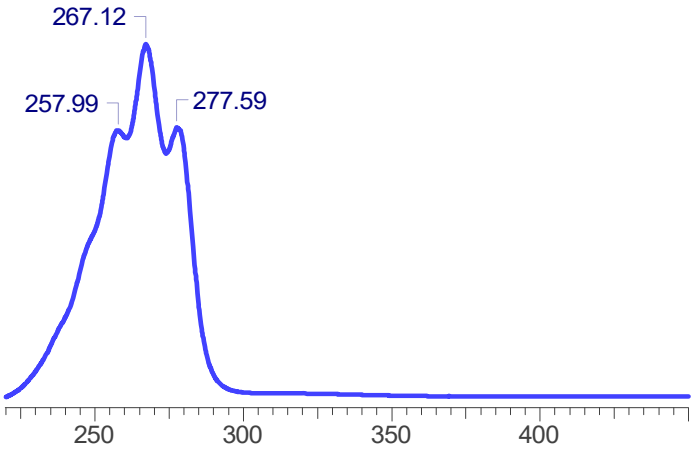
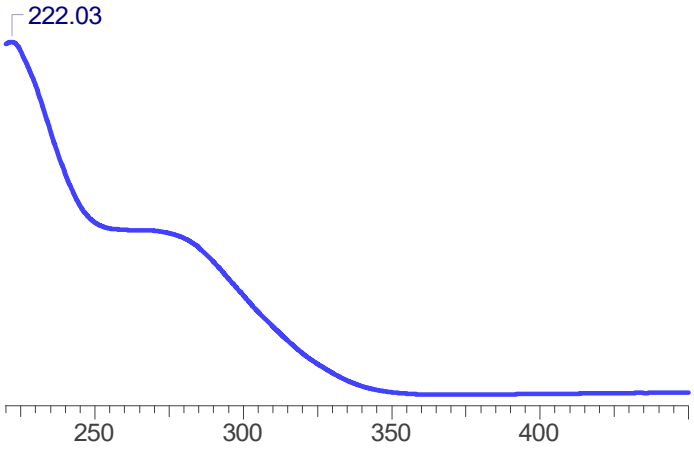
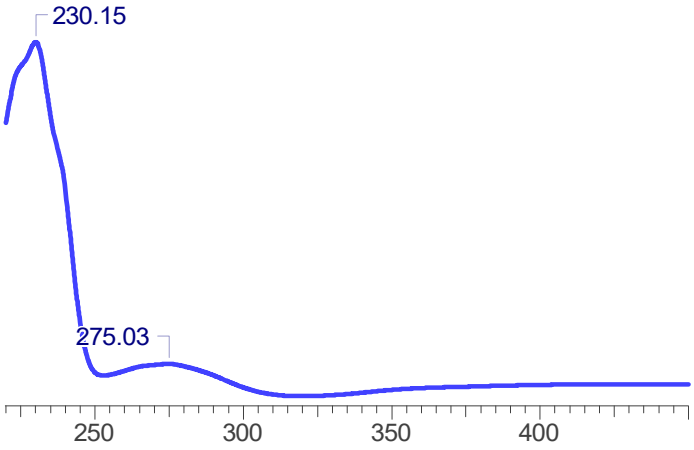
Ret. time	UV Spectrum	Identification
53.36		
55.28		
55.39		
55.97		

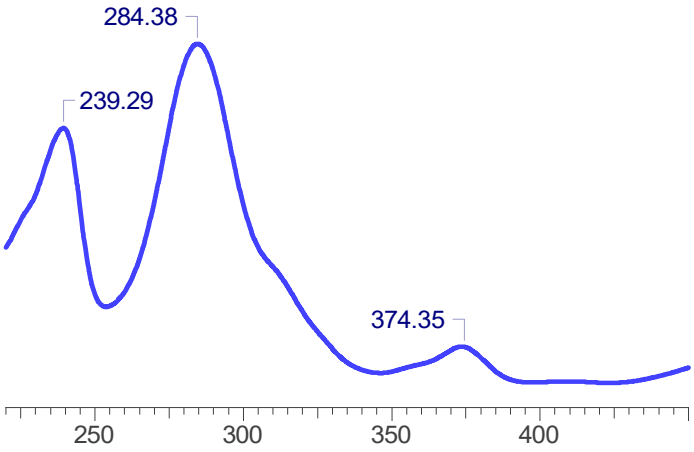
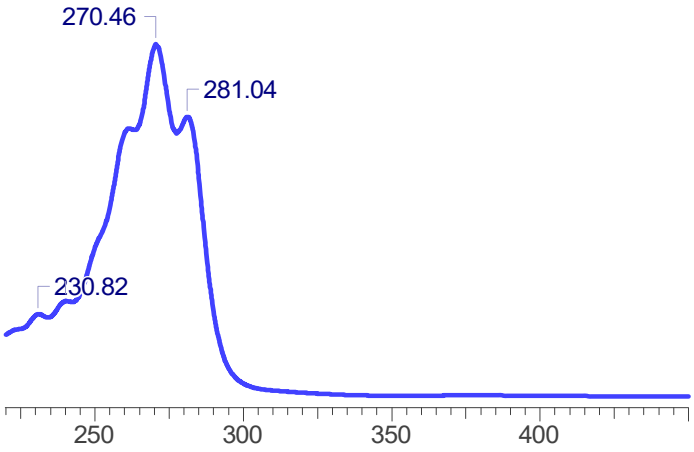
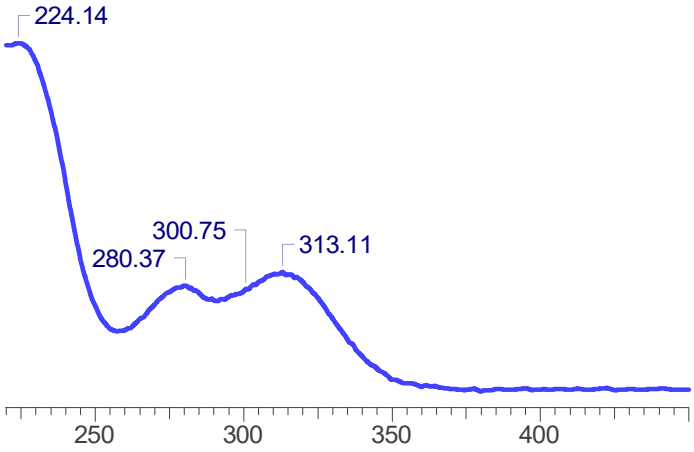
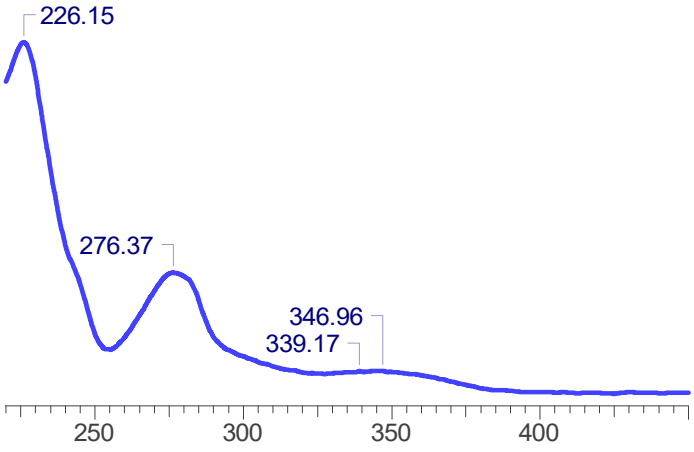
Ret. time	UV Spectrum	Identification
56.69		
56.67		
57.20		
58.32		

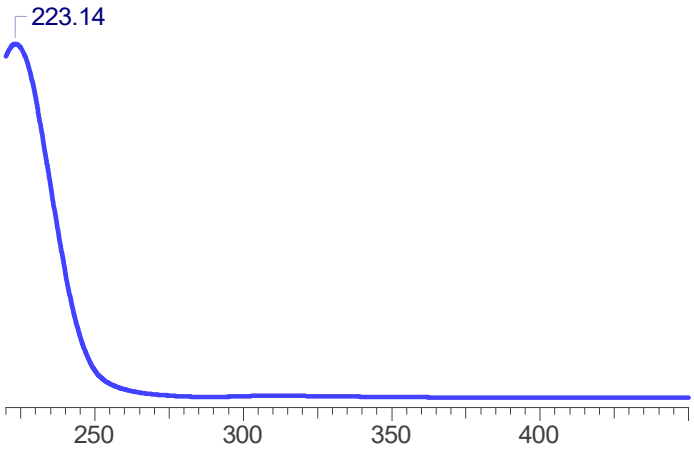
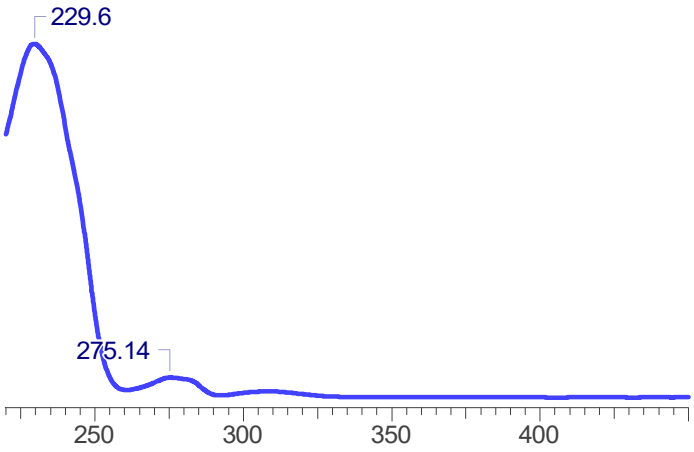
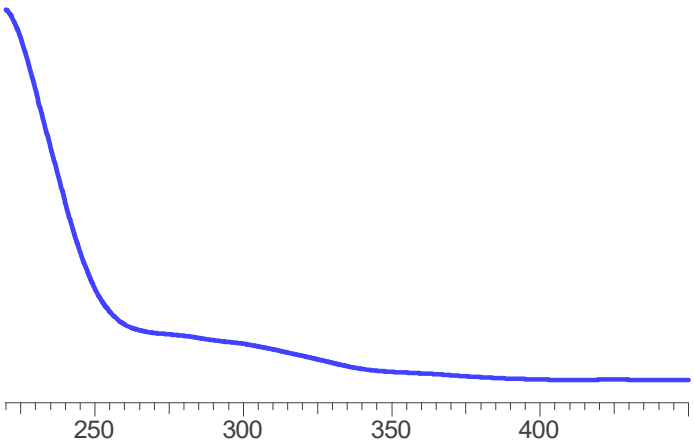
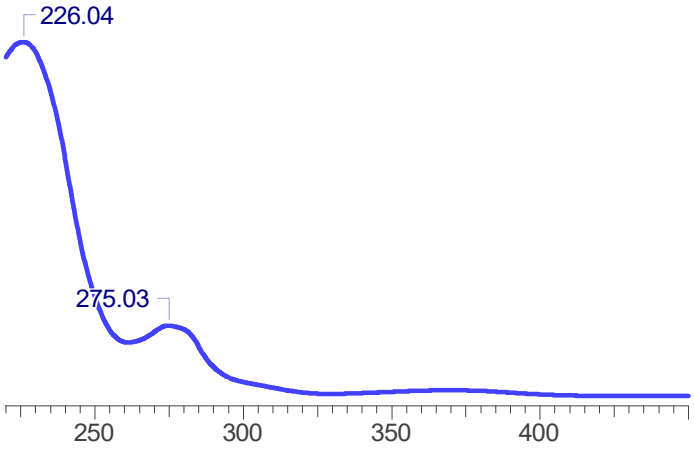
Ret. time	UV Spectrum	Identification
59.50		
60.35		4'-O-Methyl-resveratrol
61.08		
61.30		

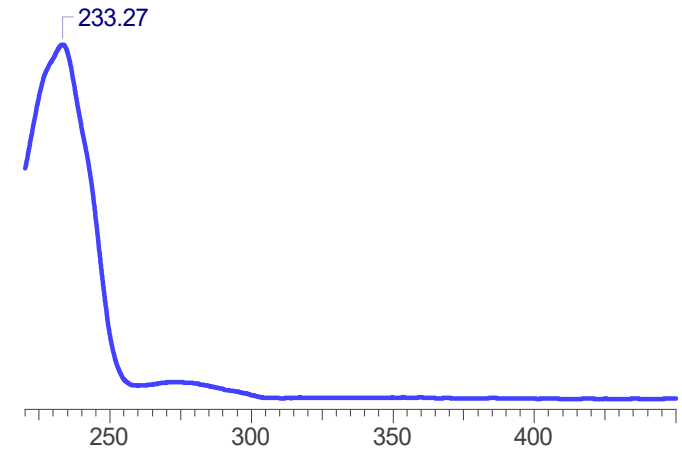
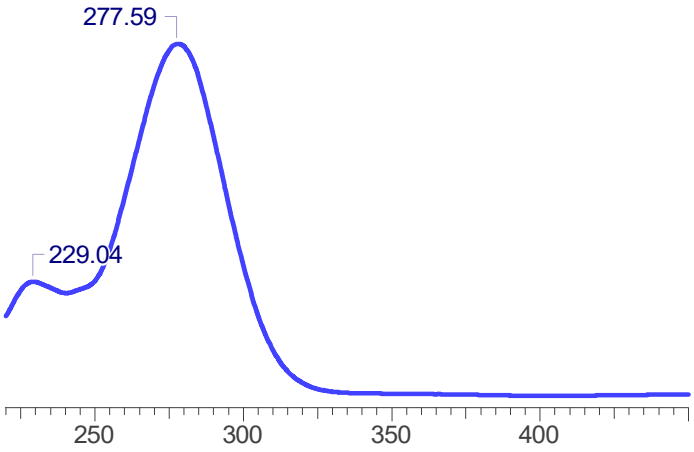
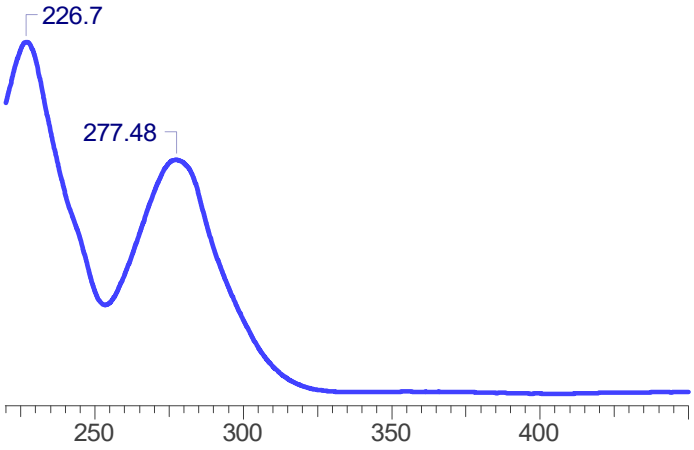
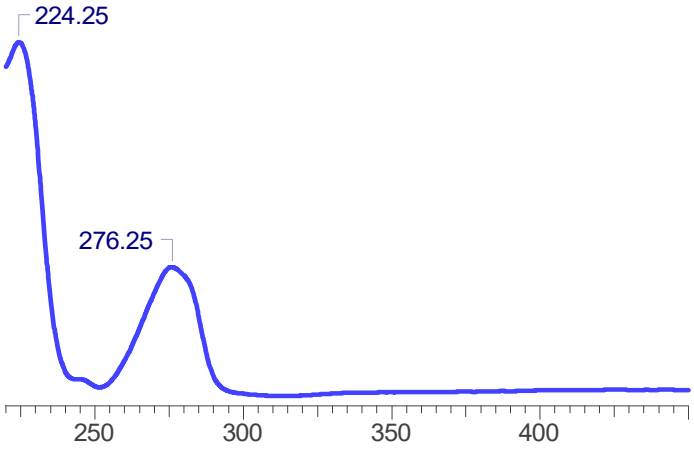
Ret. time	UV Spectrum	Identification
61.51		
61.81		
62.00		
62.88		Coumestrol

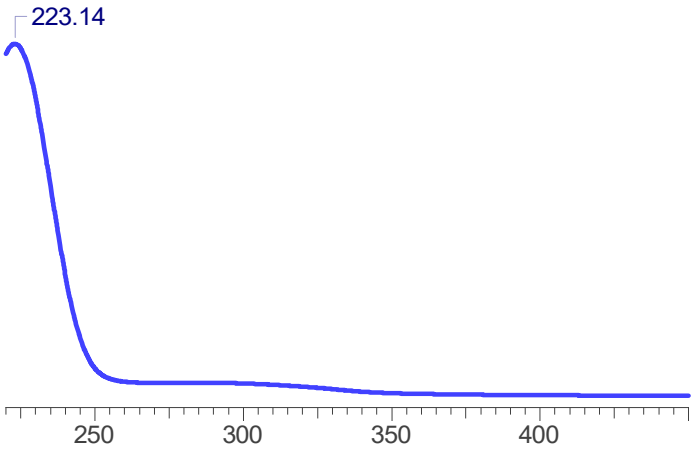
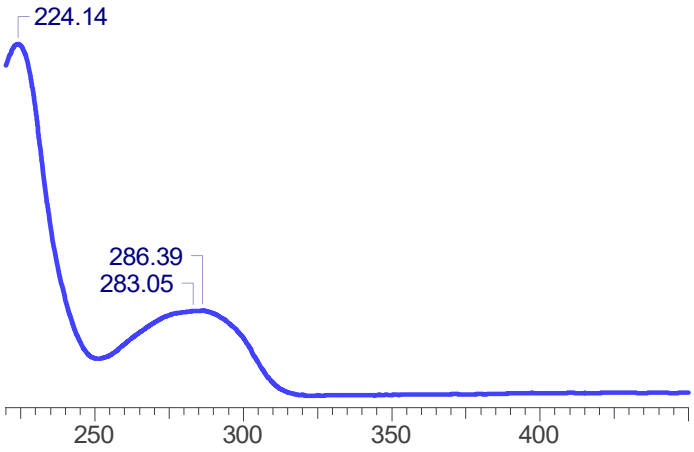
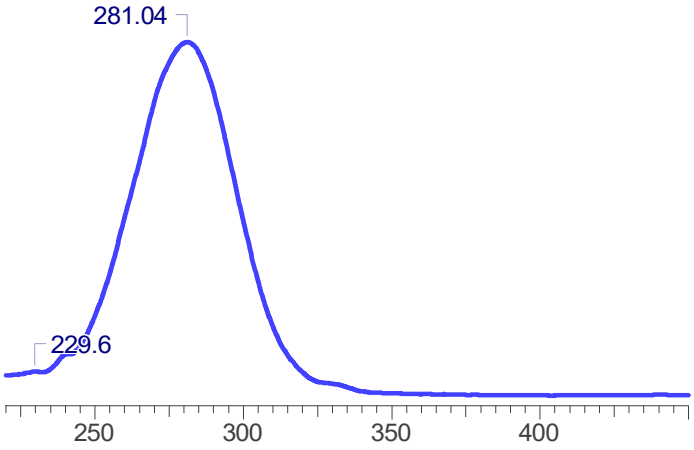
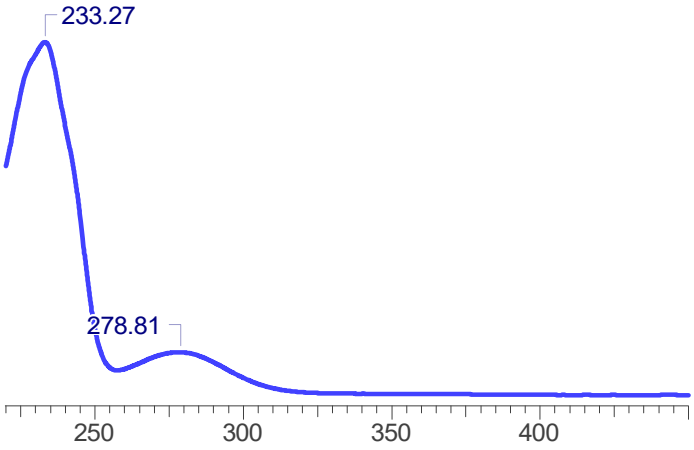
Ret. time	UV Spectrum	Identification
63.03		Sperimidine dicinnamate der.
63.68		4'-O-Methyl-resveratrol, dehydroxy
63.89		
65.28		

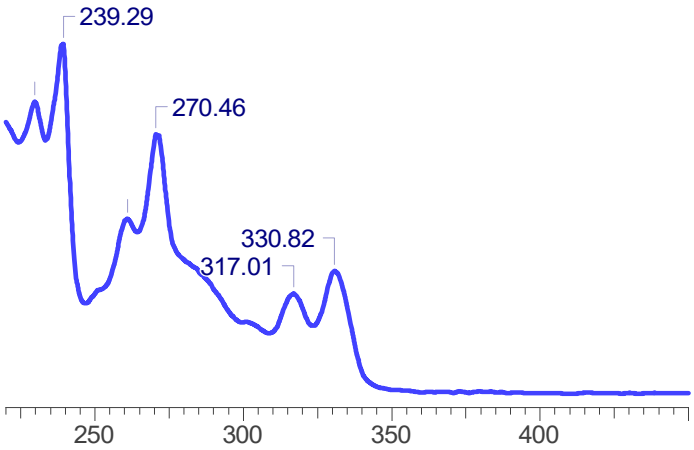
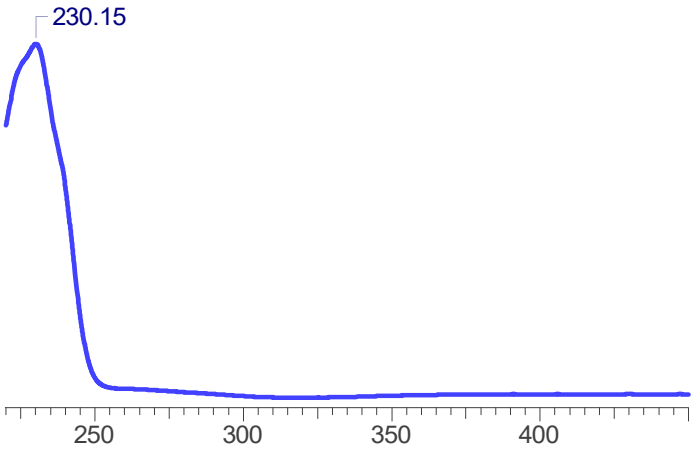
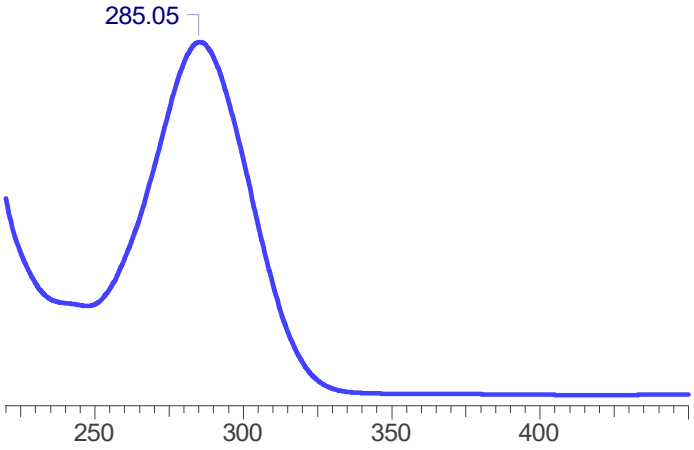
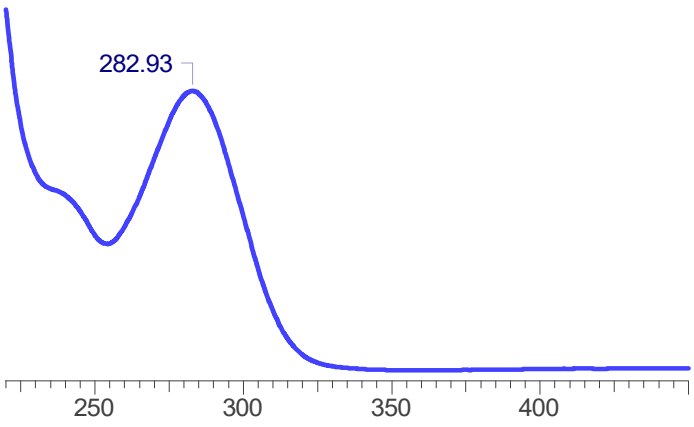
Ret. time	UV Spectrum	Identification
65.41		
65.49		
67.44		
67.71		

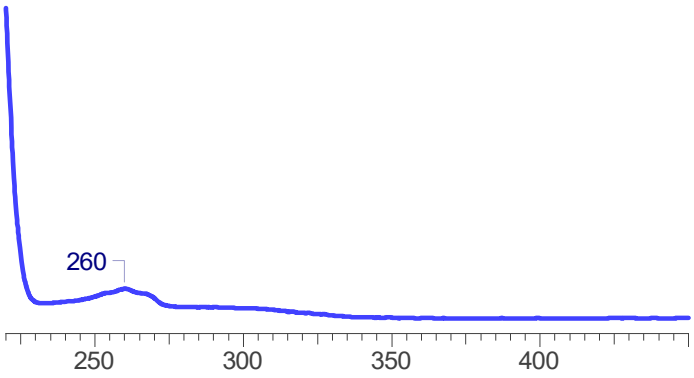
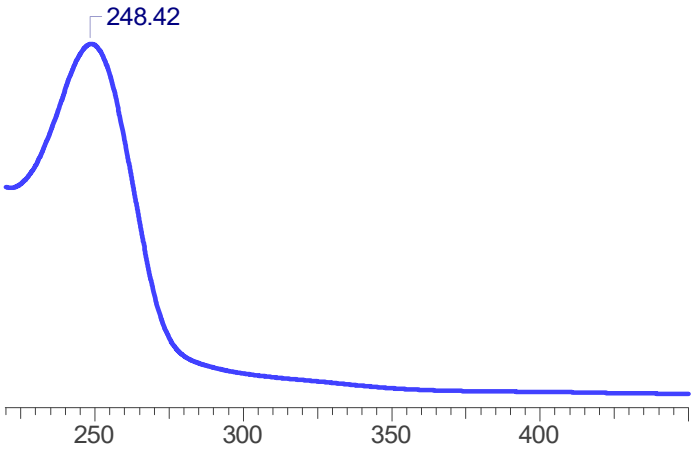
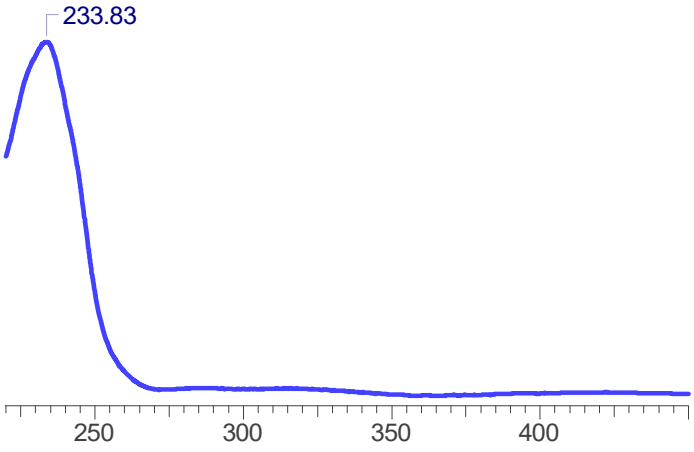
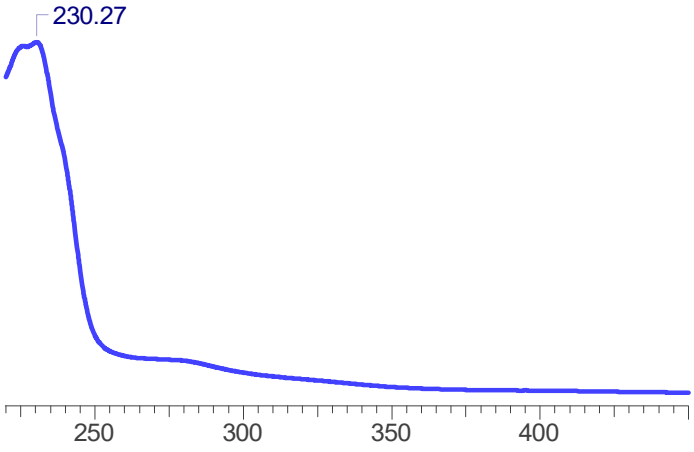
Ret. time	UV Spectrum	Identification
69.20		
69.71		
69.89		
72.83		

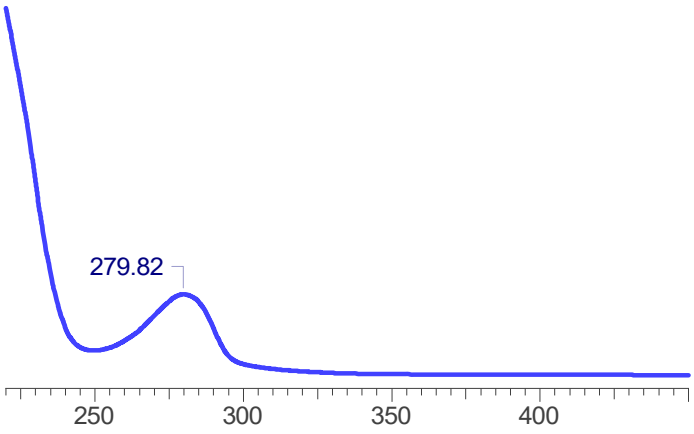
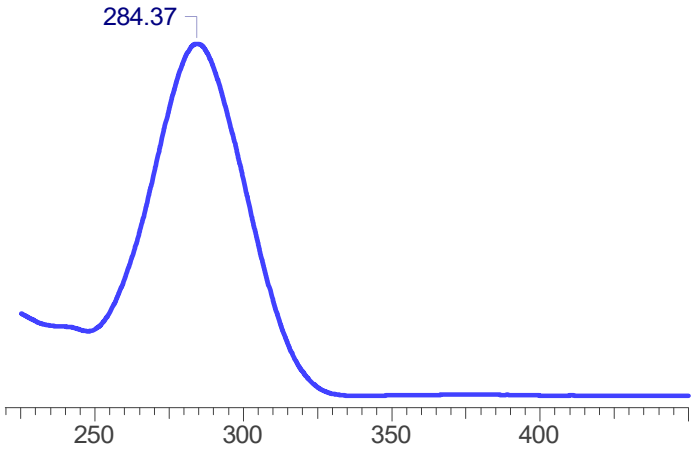
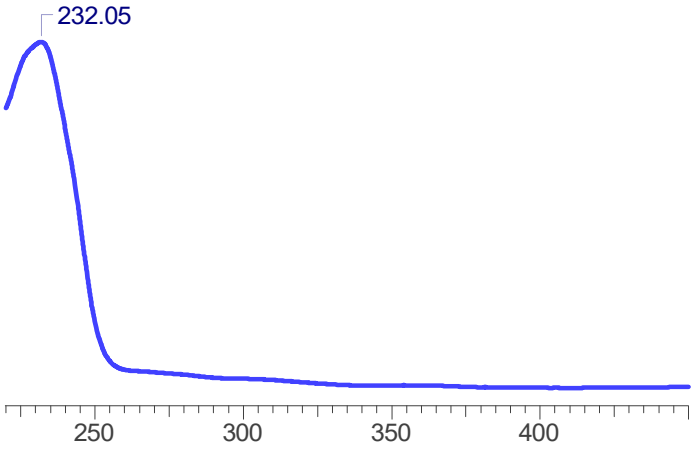
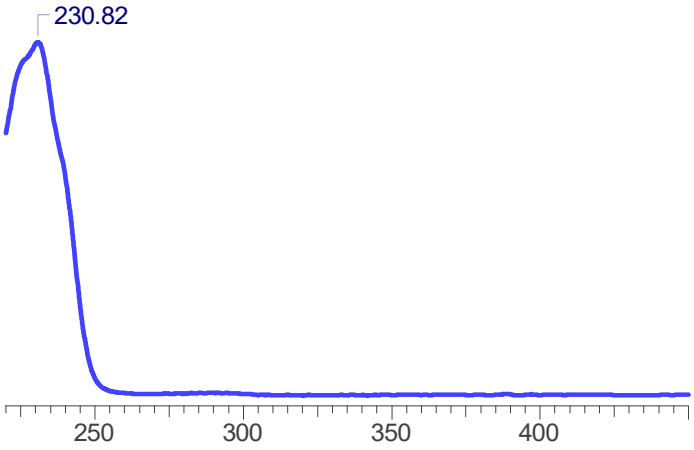
Ret. time	UV Spectrum	Identification
71.75		
72.61		
72.80		
73.32		

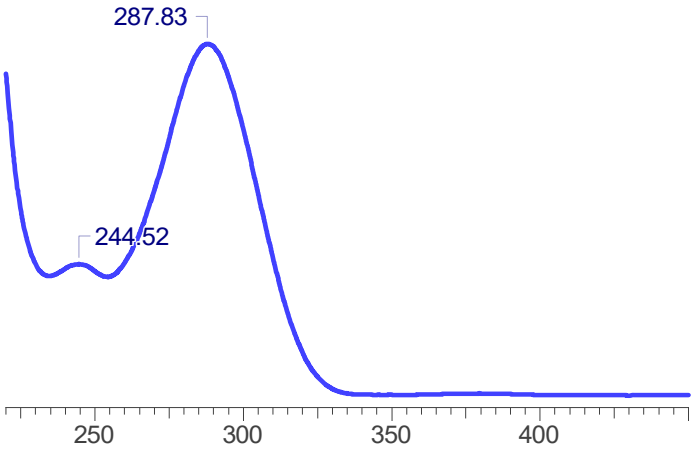
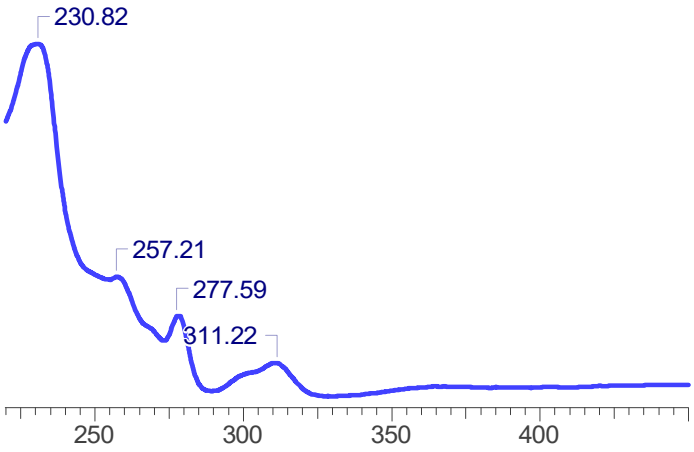
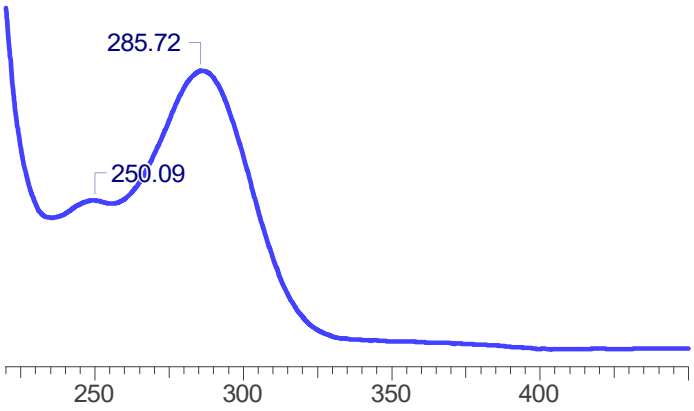
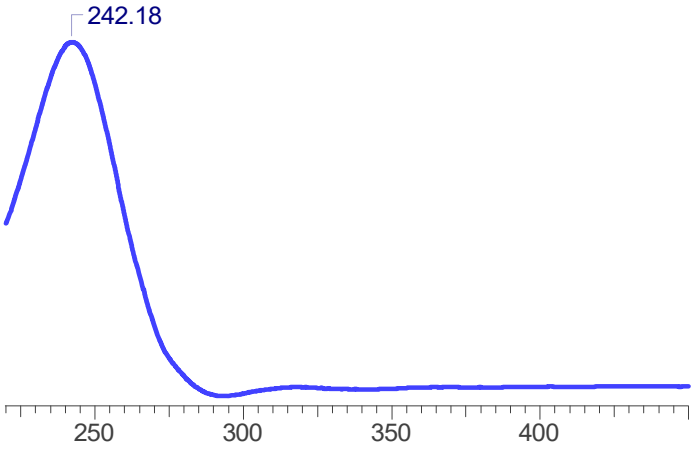
Ret. time	UV Spectrum	Identification
76.16		
76.64 77.97 79.04		
76.75		
77.60		

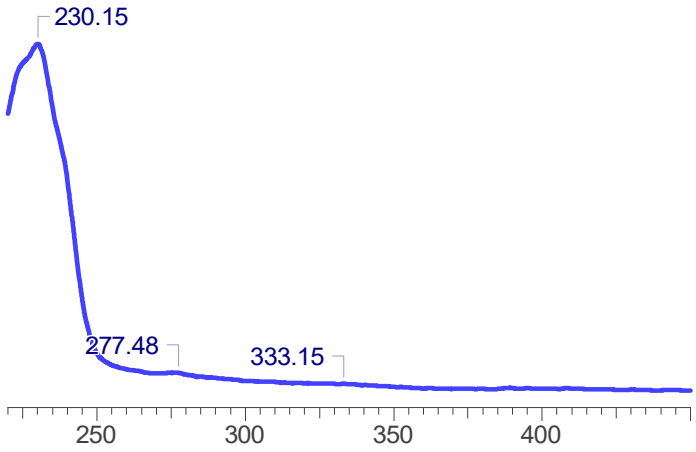
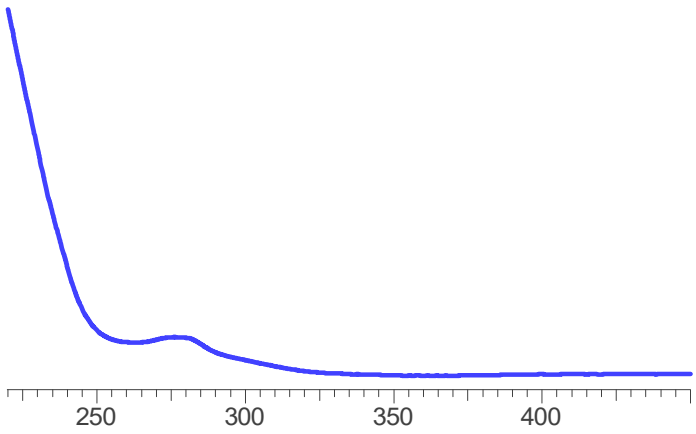
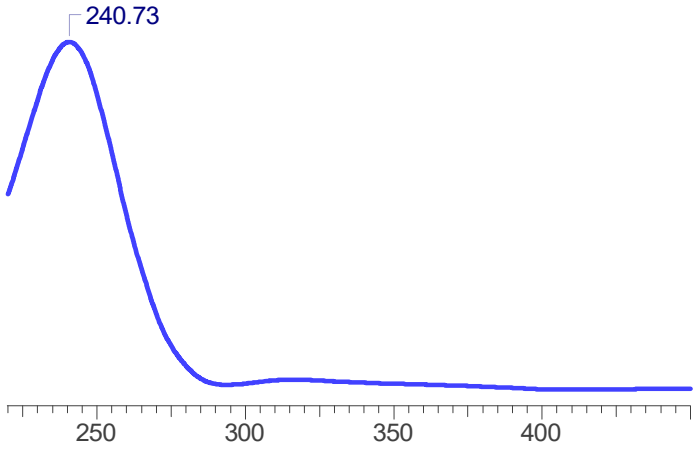
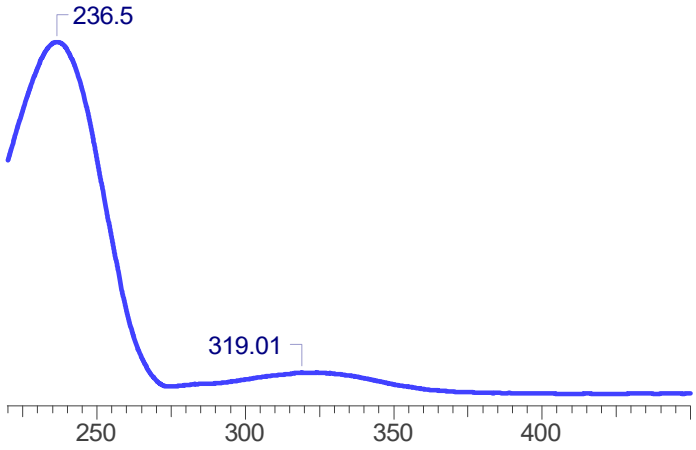
Ret. time	UV Spectrum	Identification
77.65		
77.88		
79.04		
79.60		

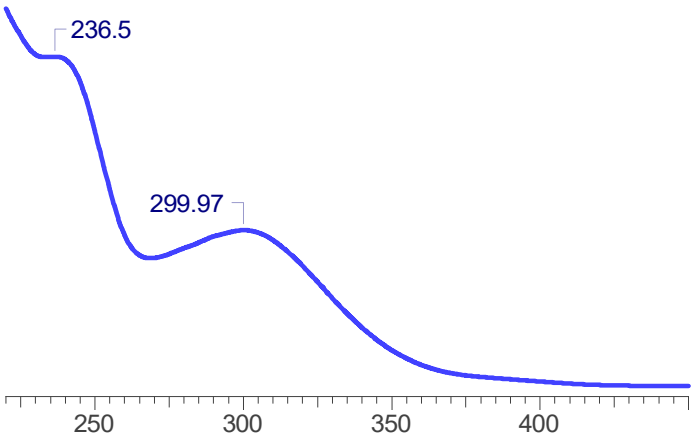
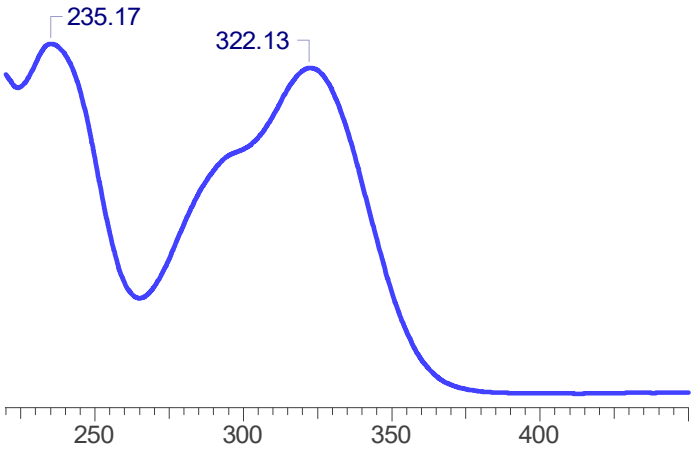
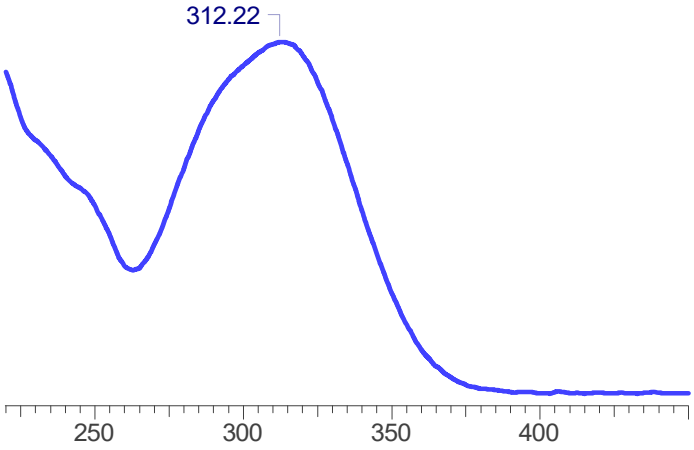
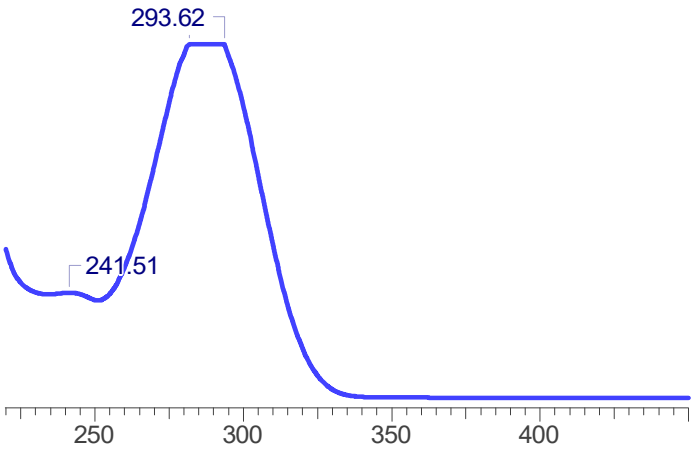
Ret. time	UV Spectrum	Identification
80.43		
81.04		
81.52		
81.99		

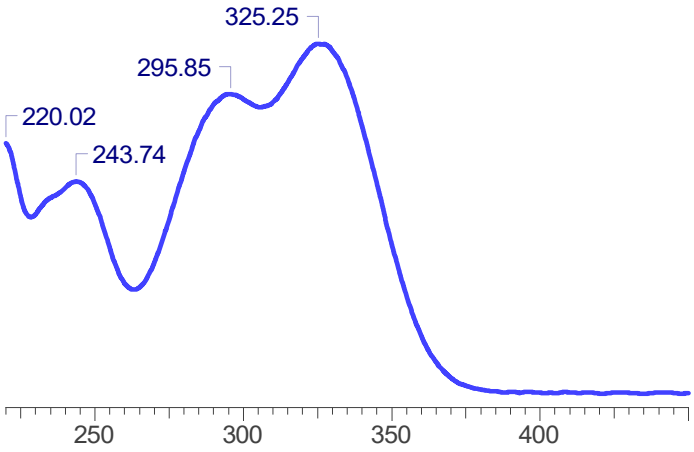
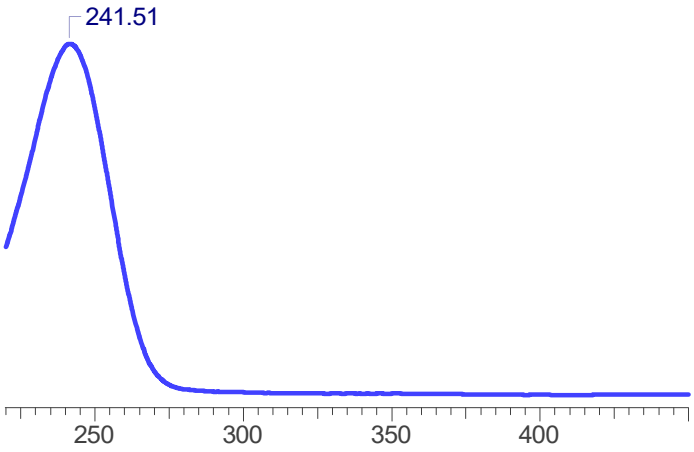
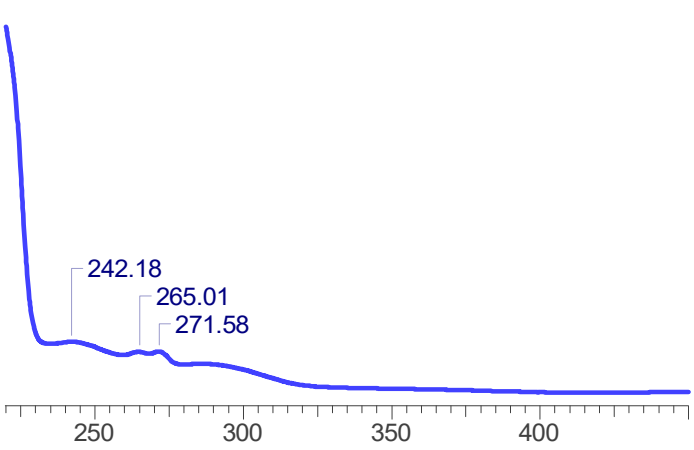
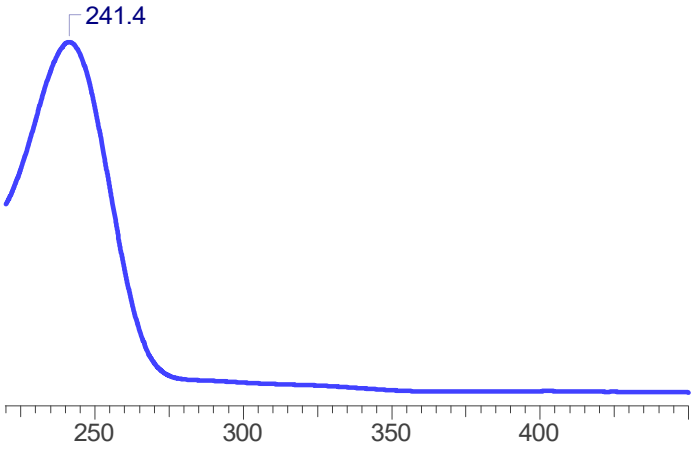
Ret. time	UV Spectrum	Identification
86.19		
88.43		
89.12		
90.21		

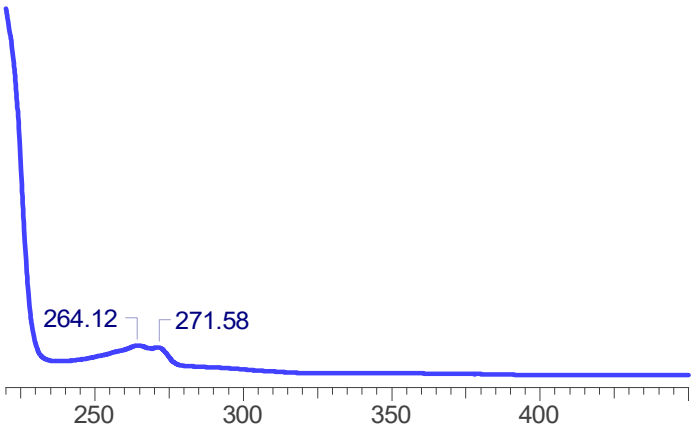
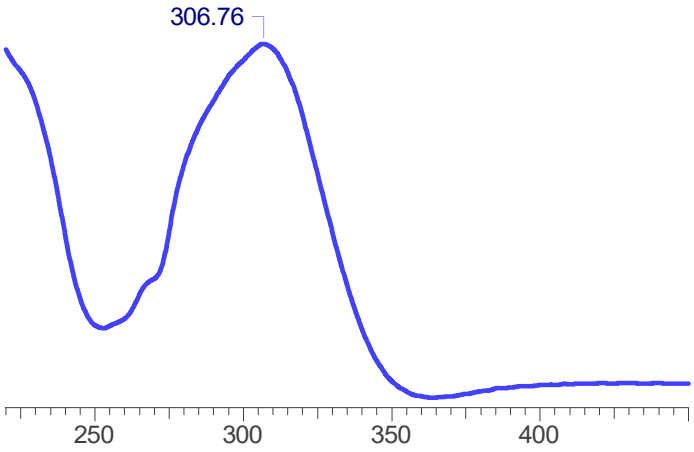
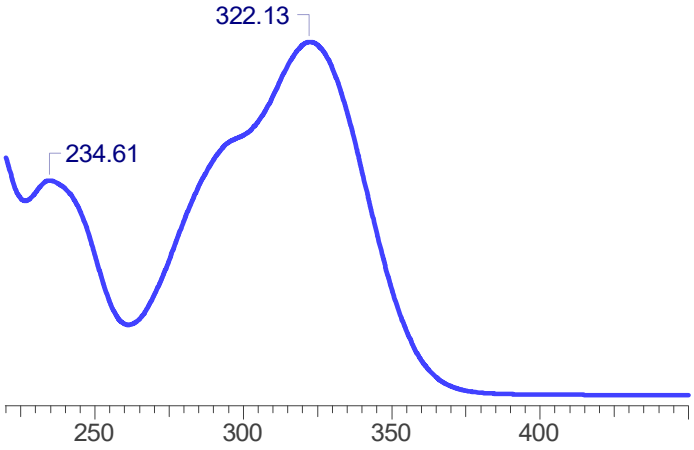
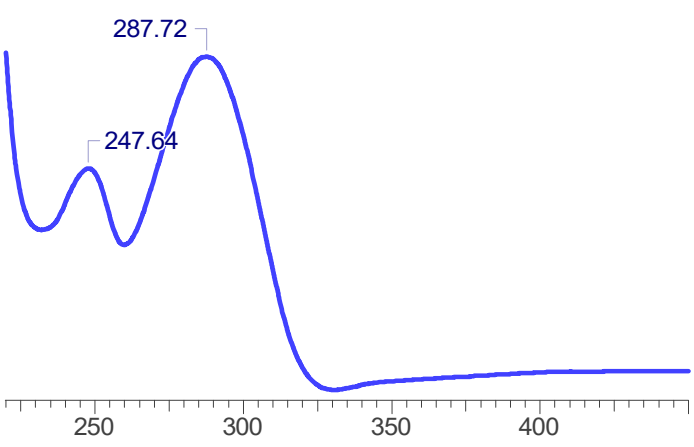
Ret. time	UV Spectrum	Identification
89.65		
90.29		
90.45		
91.81		

Ret. time	UV Spectrum	Identification
92.69		
93.71		
94.24		
94.75		

Ret. time	UV Spectrum	Identification
95.19		
95.41		
95.76		
96.99		

Ret. time	UV Spectrum	Identification
99.95		
100.11		
100.16		
100.63		

Ret. time	UV Spectrum	Identification
100.72		
103.17		
104.07		
104.21		

Ret. time	UV Spectrum	Identification
104.61		
104.76		
106.99		
108.03		

DECLARATIONS

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen, 29-09-2016

P. Akter

(Signature)

PERVIN AKTER

(Name in block capitals)

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen,.....

P. Akter

(Signature)

PERVIN AKTER

(Name in block capitals)