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DIPLOMARBEIT

Metabolic profiling of *Vaccinium macrocarpon* using ^{1}H-NMR:
Application for quality control of cranberry juice

angestrebter akademischer Grad

Magistra der Pharmazie (Mag.pharm.)

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Wien, im März 2009

Acknowledgements

I wish to thank Univ. Prof. Mag. Dr. Dr. h.c. Brigitte Kopp for offering me the possibility to perform researches for my thesis at the University of Leiden, her fast correction and support.

Further, I would like thank Prof. Dr. Rob Verpoorte for welcoming me cordially for an internship at the Department of Pharmacognosy at Leiden University, and the interesting research topic.

I owe special thanks to Hye Kyong Kim for her scientific supervision, support and correction and Young Hae Choi for his dedicated support with NMR.

Moreover, I really enjoyed the time in the laboratory with my colleagues at the multinational Pharmacognosy Department and would like to thank them all for the great time and their support in all possible ways.

I would also like to express my deep gratefulness to my family and friends, who have been a great support during my whole studies.

1. Abstract

In folklore and natural medicine, American cranberry is believed to be active in the prevention and treatment of urinary tract infections (UTIs). Clinical studies show a correlation of cranberry juice consumption and decreased occurrence of UTIs. Becoming important for the maintenance of human health, the quality of cranberry juice has to be monitored and assured. In the present work, a profile of the phenolic secondary metabolites of a *Vaccinium macrocarpon* extract, which seem to be the bioactive compounds, was established, using NMR spectrometry. Subsequently, cranberry juice samples of four different brands were analyzed by NMR in combination with multivariate data analysis. The analyzed juices showed differences depending on the manufacturer mostly by the concentrations of benzoic acid, benzoic acid glucoside, quercetin and catechin present in the juice.

In der Naturheilkunde wird *Vaccinium macrocarpon* (Großfrüchtige Moosbeere, „Cranberry“, Kultur-Preiselbeere) als Mittel zur Prophylaxe und Behandlung von Harnwegsentzündungen verwendet. Klinische Studien belegen eine positive Korrelation zwischen regelmäßigem (täglichen) Konsum von Cranberrysaft und geringerer Häufigkeit von Harnwegsentzündungen. Wird der Cranberrysaft aufgrund seiner Wirkung als gesundheitsförderndes Nahrungsergänzungsmittel konsumiert, sollte eine Qualitätskontrolle der bioaktiven Inhaltsstoffe, der phenolischen Sekundärmetaboliten, erfolgen. Im Rahmen dieser Arbeit wurde mithilfe von NMR-Spektroskopie ein Profil der phenolischen Sekundärmetaboliten in einem *Vaccinium-macrocarpon*-Extrakt erstellt. Anschließend wurden Cranberrysäfte von vier Herstellern mittels NMR-Spektroskopie untersucht und die Daten einer Multivariaten Datenanalyse unterworfen. Die untersuchten Cranberrysäfte zeigten unterschiedliche Konzentrationen von Benzoesäure, Benzoesäure-Glucosid, Quercetin und Catechin.

2. Introduction and Objectives

American cranberry (*Vaccinium macrocarpon* Ait.) is a low-growing perennial shrub, native to North America. It´s fruits, "cranberries", can be consumed as fresh fruits or in different preparations, such as juice cocktails, jams, sauces or relish. Folklore ascribes multiple health-promoting properties to cranberry, most famously, a preventive effect against urinary tract infections (UTI). In the recent years, several studies were conducted to investigate this effect (Bailey *et al*., 2007; McMurdo *et al*., 2005; Kontiokari *et al*., 2001). Cranberry extract has been shown to inhibit the adherence of uropathogenic *Escherichia coli* to urinary tract epithelial cells *in vitro* (Zafriri *et al*., 1989). Unique A-type linked proanthocyanidins (condensed tannins) and fructose were determined as the bioactive compounds (Foo *et al*., 2000). A recent review (Jepson and Craig, 2007) concludes, that there is positive clinical evidence for the UTI-preventive effect provided by regular cranberry juice consumption, although it seems that only in certain subpopulations. Urinary tract infections represent a major problem and were shown to be more prevalent among women than men, throughout all age groups. It has been estimated that around 25% of women have had at least one UTI in their lifetime and will suffer several (Henig and Leahy, 2000). With the increasing numbers of resistant bacteria to the antibiotic agents in use, the focus is increasingly drawn to the prevention of urinary tract infections and the role of cranberry therein. According to current knowledge, daily dosages of 240 – 300 ml of cranberry juice cocktail are recommended for the prevention of UTIs (Howell, 2007). In this way, cranberry juice cocktail is becoming a medical product and plays a role in the contribution to and preservation of human health, therefore its quality has to be controlled, unified and guaranteed. A metabolomics approach to establish a quality control of cranberry juice cocktail, offers a good method to monitor the quality and quantity of (secondary) metabolites, which are directly linked to the health-beneficial effects exerted. The use of NMR provides a macroscopic observation of a broad range of compounds (metabolites) present in cranberry juice cocktail, which allows to detect poor quality or adulteration in respect to different criteria (quality/quantity of proanthocyanidins, addition of preservatives or sugar etc.).

The specific objective of the present work consists of:

1. profile of secondary metabolites in *Vaccinium macrocarpon* extract using NMR
2. analysis of cranberry juices for the development of a quality control technique

To achieve the specific aims, an extract of *Vaccinium macrocarpon* was analyzed by one- and two-dimensional nuclear magnetic resonace spectrometry. Subsequently, samples of four different manufacturers of cranberry juice were analyzed using NMR, followed by multivariate data analysis.

The next chapter gives an introduction in the field of "metabolomics", followed by chapter 4, in which a closer look to the taxonomy and morphology of *V. macrocarpon* is taken and previous studies about its health-beneficial properties are discussed. Chapter 5 deals with urinary tract infections, risk factors, treatment and prevention strategies. Chapter 6 and 7 give a short introduction to the principle of nuclear magnetic resonance spectrometry and the basic theory of principal component analysis, respectively. In chapter 8, materials and the physico-chemical methods used in the present work are summed up. Finally, results are summarized and discussed in chapter 10.

3. Metabolomics

3.1 General term definition

The term metabolomics can be defined as the aim of a qualitative and quantitative measurement of all low molecular weight metabolites in an organism at a specified time under specific environmental conditions (Colquhoun, 2007; Eisenreich and Bacher, 2007). The ultimate goal is to provide a broad and essentially non-targeted snapshot representing the greatly diverse composition of the plants metabolites (Hall, 2006). No absolute numbers are known, but it is estimated that an organism contains about the same quantity of compounds as it has genes. For plants the number would be around 30 000 compounds (Verpoorte *et al*., 2008). These compounds are present in a wide range of quantities (hormones vs. sugars) and show totally different chemical properties (lipids vs. sugars). By this complexity, plants can be defined at every level of genotype, phenotype, tissue and cell. This approach provides a deeper insight into the metabolic composition of plant tissues and the obtained data can be exploited effectively for both their diagnostic and predictive value (Hall, 2006).

Generally, the metabolome (entity of small molecules in an organism) consists of two types of compounds - primary and secondary metabolites. Primary metabolites are responsible for the basic functions of living cells, such as respiration or amino acid biosynthesis. Basically, all organisms share the same type of primary metabolites, though per class of organisms large differences may result from different biosynthetic pathways (e.g. mammalians are not capable of amino acid biosynthesis). In contrast to this, secondary metabolites are species specific and are involved in the interaction of cells with their surrounding environment (Verpoorte *et al*., 2007). The plant needs to compensate temporal and seasonal challenges, which is facilitated by metabolic changes. Therefore, plants have developed a complex arsenal of secondary metabolites to deal with abiotic (light, UV, water) and biotic (herbivores, parasitism and pathogen attack) stress factors (Hall, 2006). The primary metabolites are therefore important for the growth and agricultural yields, whereas the secondary metabolites are responsible for flavor, color and the resistance of plants against various stress factors (Verpoorte *et al*., 2007). It is also the secondary metabolites in our food that are linked to health-beneficial properties, provided by plants, such as are proanthocyanidins in cranberry or alkaloids in various plant species.

Different approaches are in use for metabolite analysis. On the one hand, “metabolic fingerprinting” is a term defining a high throughput qualitative screening of the metabolic

composition of the analyzed sample, e.g. a plant tissue exract, with the primary aim of sample comparison and discrimination analysis. Generally, no attempt is initially made to identify the detected metabolites. Sample preparation, separation and detection should be as rapid and simple as possible. On the other hand, "metabolic profiling" represents the identification and quantification of the detected metabolites. For practical reasons this is only feasible for a limited number of compounds, which can be selected on the basis of analysis or molecular relationships in molecular pathways/networks.

It is essential to consider that all metabolomic measurements result in a so-called "metabolic snapshot" which is specific to the time of sampling. The obtained information provide a valuable metabolic insight, but also puts emphasis to proper care of the cultivation and sampling of the analyzed material. Samples originating from different sources, collected at different times, etc. can be compared, but conclusions should be drawn in context of the initial sample differences (Hall, 2006). Metabolomics studies can be divided into three phases. In the first, as many signals as possible are assigned to compounds, followed by the second phase, in which a large number of samples is measured, e.g. young and old leaves, different times of the day, and different stages of development of the plant, to determine the biological variation for the system studied. In the third phase, the effect of different experimental conditions on the model system is studied, e.g. diseased or transgenic plants compared with the normal wild type (Verpoorte *et al*., 2007).

Ultimately, metabolic analysis aims towards providing an efficient and comprehensive predictive tool for analyzing the performance of the complex metabolic network. Metabolic analysis seems to represent the key to predictive tools, which allow the detection of multiple metabolites and fluxes. The identification of components in a biological system is an essential first step towards explaining their biological function (Ratcliffe and Shachar-Hill, 2005).

3.2 Analytical methods in use for metabolomics

The main requirements for analytical methods employed for metabolomics purposes include a broad coverage of detected metabolites, qualitative and quantitative results, high reproducibility and signal robustness, as well as preferably short time for measurement (Choi, 2008). Five major approaches are currently employed for metabolomic analyses: HPLC- or TLC-UV, GC-MS, MS^n and NMR-spectroscopy (Verpoorte *et al*., 2008). Mass

spectrometry (MS) and nuclear magnetic resonance spectrometry (NMR) are considered to be the primary, because most universal approaches among the available analytical tools (Verpoorte *et al*. 2007). The technologies applied in metabolomics show characteristic advantages and drawbacks considering the diversity of metabolites detected, sensitivity, or resolution (Verpoorte *et al*., 2008). The selection of the most suitable technique results in a compromise between speed, selectivity, and sensitivity. Usually, a first step of chromatographic separation is carried out before further analysis of the sample by GC or LC-MS (Mattoli *et al*., 2006).

Currently, none of the techniques applied for metabolomic measurements is able to provide complete qualitative and quantitative information of all the metabolites that are present in a tissue extract. The available methods either provide a metabolite "profile", in which metabolites of a selected group are identified and quantified, or a metabolite "fingerprint", in which metabolites are detected and subjected to pattern recognition analysis (Ratcliffe and Shachar-Hill, 2005).

In the present work, the metabolomics data were acquired by NMR measurements, therefore the focus is mainly drawn to this method. The basic workflow, as well as the main advantages and challenges are discussed in chapter 6.

3.3 Applications

The increased information on the biochemical composition of plant tissues can be exploited in a wide range of both scientific and applied fields (Hall, 2006). One of the most established applications of plant metabolomics is the analysis of dynamic metabolite changes during stress (Guy *et al*., 2008). The most common use for plant metabolomics is comparative analysis. By application of multivariate data analysis, plants of different genotypes or plants subjected to different treatments, can be distinguished (Ratcliffe and Shachar-Hill, 2005). Metabolomics information provides a deeper insight into the complex and highly interactive nature of the plant metabolic network. It also contributes to a deeper understanding of the nature of plant phenotypes in relation to development, physiology and environment (Ward *et al*., 2007).

The quality of crop plants is a direct function of their metabolite content. The quality of plant tissues also determines their commercial value in terms of factors, such as flavor, fragrance, shelf life, physical attributes, etc. Each of these attributes can be defined by a metabolic profile of the concerned material at a specific time. Metabolomic analysis can be

applied as an efficient tool to monitor crop quality or to identify potential biochemical markers, which enable the detection of product contamination and adulteration (Hall, 2006). Metabolomics contributes to gaining a deeper knowledge and have better control of the biochemical basis of our food. This knowledge can be exploited to design modified programmes, which are aimed at better quality, optimised food processing and ultimately, improved (micro)nutrient bioavailability and bioefficacy. The understanding of pathways, which are responsible for the biosynthesis of nutritionally relevant metabolites is essential to gain control of the absence/level of presence of these compounds in our food . The identification of molecular markers can contribute future development of high quality food products (Hall *et al*., 2008).

One of main aims of plant metabolic analyses is the development of a detailed view of the control hierarchy in the metabolic network. This knowledge would allow rational design of strategies to genetically engineer metabolism for the targeted overproduction of desirable end-products. However, these efforts have until now mosty failed. This might be explained by the fact, that the control of a metabolic pathway is generally shared among all enzymes of this pathway. Hence, engineering of a single enzyme is not likely to result in significant flux change. The goal is to generate a more complete view of the whole metabolic network, including a quantification of the control exerted by each enzyme, instead of dealing with individual pathways. Subsequently, the gene-regulatory network will be superimposed on the metabolic network model to account for the the consequences of altered environmental or developmental circumstances on the structure and balance of the metabolic network (Sweetlove *et al*., 2008).

Another application of metabolomics is the determination of the extent and effectiveness of a modification introduced by genetic engineering. Genetically modified (GM) products are subjected to metabolic analysis to assess so-called “unintended effects”. In the highly interactive metabolic network, which is present in plant tissues, even minor changes have the potential to result in significant changes in nutritionally relevant metabolite content. On the one hand, metabolomics finds application in the investigation of the impact of environmental conditions (spatial/seasonal etc.) on food quality. On the other hand, it is also used for the development of tools and markers for quality monitoring. Nutrigenomics is a science trying to link nutrition and the genome. Metabolomics represents an integral component and is predicted to play an important role by correlating bioactive food metabolites and disease prevention. The aim would be a switch from single biomarkers to increasingly comprehensive (metabolomic) profiles. Metabolomics can be

applied to study the complex interaction between food production, processing, consumption and long-term health status and to provide both plant and human biomarkers. Metabolomics is currently finding application in exploring important biochemical pathways in plants. This information linked with knowledge of the genetics, can be exploited for the design of breeding programmes, which target to nutrition-related issues more closely (Hall *et al*., 2008).

Metabolic NMR fingerprinting has been used for many years to authenticate foodstuffs, especially in the beverage industry (Brescia *et al*., 2002; Le Gall *et al*., 2001; Charlton *et al*., 2002; Duarte *et al*., 2002; Vogels *et al*., 1996). The quality control of phytomedicinal preparations is becoming increasingly important. In recent years, nutraceuticals and "bioprotective" or "functional" foods have attracted much attention. Increasing numbers of food products reach the market bearing health-related claims often focused on e.g. anti-oxidant activity. The metabolomics approach is predicted to play an important role in this regard for both the plant and human sides (Hall *et al*., 2008).

3.4 Limitations

Currently, no single analytical technique is able to generate sufficient visualization of the metabolome, and multiple methodologies are therefore employed to gain a comprehensive view (Mattoli *et al*., 2006). All currently applied extraction and detection methods, have characteristic and unavoidable intrinsic bias towards certain metabolite groups, irrespective of their high level of sophistication (Hall, 2006). The major limitation of metabolomic analysis is the inability to completey profile the metabolome. Difficulties faced in this context are directly linked to the chemical complexity of the metabolic composition and the dynamic range limitations of most instrumental approaches (Mattoli *et al*., 2006). Hence, for comprehensive coverage multiparallel, complementary extraction/detection technology combinations using hyphenated systems can be employed to build up a metabolic picture towards an ever-increasing degree of completeness (Hall, 2006).

Major limitations also occur by the currently limited ability to chemically identify many of the detected compounds and the lack of reference compounds for these metabolites, which results in a major obstacle for their definite quantification. The quantification of discriminative components is essential when exploiting metabolic datasets beyond profiling and particularly when investigating the metabolic flux (Hall, 2006). Another

limitation is that the methodologies in use for metabolomic measurements require the analysis of plant tissue extracts. Ideally, metabolomics analyses would be performed at the cellular or subcellular level without extraction. This is of particular importantance, when analysing the metabolic flux. Currently, the total content of a metabolite is measured, which does not consider a potential physical separation within the cell (Hall, 2006; Sweetlove *et al*., 2008; Ratcliffe and Shachar-Hill, 2005).

4. *Vaccinium macrocarpon* Ait.

4.1 Scientific classification

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Ericales
Family:	Ericaceae
Genus:	*Vaccinium* L.
Species:	*Vaccinium macrocarpon* Aiton
Common names (engl.):	American cranberry, large cranberry, cranberry

4.2 Botanical description

Fig. 1. *Vaccinium macrocarpon*

Vaccinium macrocarpon is a low-growing, trailing, woody shrub with a perennial habit. The plant produces stolons (horizontal stems), which can be up to 2 m long. Short vertical branches or uprights, 5 to 20 cm long, grow from buds on the stolons and can be either vegetative or fruiting. Each fruiting upright contains up to seven flowers. The flowers, which are pink or pale rose colored, are tube-shaped in terminal clusters, and deeply 4-parted. The lobes are lanceolate, reflexed and the anthers exserted, awnless. The flowers bloom in the time from June – August and are pollinated primarily via domestic honey bees. The flowers are followed by a globous dark red edible fruit in the size of 12 – 20 mm

containing many seeds. The leaves are leathery, evergreen and the shape is elliptic to oblong (7 – 17 mm). During the growing season, the leaves are dark green and glossy, and turn reddish-brown during the dormant season. The habitus of *Vaccinium oxycoccus* (European cranberry) is very similar. The larger fruits ("cranberries") of *Vaccinium macrocarpon* (American cranberry), however, represent the main differentiating morphologic factor between these two species, also indicated by the common name "large cranberry".

4.3 Habitat and distribution

Vaccinium macrocarpon (American cranberry) is, as the name suggests, a native American heath. Its native range extends in temperate climate zones from the East Coast to the Central U.S. and from Southern Canada to the north to the Appalachians. The plants thrive on the special combination of soils and hydrology found in wetlands. American cranberry is cultivated for commercial purposes in sandy "bogs", marshes or rain-soaked salt meadows in Massachusetts, New Jersey, and the Canadian provinces of Quebec and British columbia. Commercial harvests occur in September and October (Raz *et al*. 2004; Foo *et al*. 2000).

Fig. 2. Distribution of *V. macrocarpon* in North America

4.4 Medical effects

4.4.1 History

According to Native American legends and reports from European explorers, cranberries were extensively used by the Amerindians in their diet, medicine, and for commercial purposes. The fresh berries were ground into a damp meal containing meat or fish, which was subsequently sun dried to make pemmican, which is a dried meat to sustain travellers and hunters. Later, cranberries were discovered by American sailors as a remedy to prevent scurvy. Native Americans were the first to use cranberries for their medicinal properties. The fruits were used in poultices for treating wounds, blood poisoning (Henig and Leahy, 2000) and various other complaints, including stomach ailments, liver problems and fever (Raz *et al*., 2004). The plant leaves were used for urinary disorders, diarrhea, and diabetes (Henig and Leahy, 2000).

In recent years, cranberry has gained strong public attention for its putative role in maintaining urinary tract health by prevention of infections. Particularly in the form of cranberry juice, it has been widely used for several decades for this purpose (Jepson and Craig, 2007).

Cranberries can be consumed as fresh fruits, or processed into concentrate, sauce products, and juice drinks. The most common preparation is cranberry juice cocktail (CJC), comprising a mixture of cranberry juice, sweetener, water, and added vitamin C (ascorbic acid). Also, dried cranberry powder formulated in capsules or tablets is available (Raz *et al*., 2004).

4.4.2 Literature on biological activity of V. macrocarpon

Throughout its range, *Vaccinium macrocarpon* is believed to provide with various health beneficial properties. Many studies were conducted to find scientific evidence for its putative effects.

Phenolic cranberry compounds were shown to possess significant oxygen radical absorbing capacity (Zheng and Wang 2003; Määttä-Riihinen *et al*. 2005). It is suggested that potentially cancer-inducing oxidative damage might be balanced by dietary antioxidants. Therefore, berry extracts containing high amounts of flavonoids were tested for their ability to inhibit the growth of selected human tumor cell lines *in vitro*. With increasing concentration of cranberry extract, increasing inhibition of cell proliferation in

all of the cell lines tested could be observed, with different degrees of potency between cell lines (Seeram *et al*. 2006; Neto, 2007).

Being a rich source of natural anthocyanin antioxidants, cranberries are reported to have positive influence on age-induced oxidative stress, inflammatory responses, and various degenerative diseases. Berry anthocyanins were also reported to improve neuronal and cognitive brain functions, ocular health, as well as protect genomic DNA integrity (Zafra-Stone *et al*., 2007).

Cranberry consumption is reported to provide cardioprotective benefits, including improvements in plasma lipids and vascular function, as well as reduction of the systemic and vascular inflammatory response (Ruel and Couillard, 2007; Comalada *et al*. 2005).

A study shows that long-term fruit juice consumption might provide protection against Alzheimer´s disease. However, the protective effects may be enhanced by consumption of a combination of juices rich in phenolics, one of which being cranberry juice (Dai *et al*. 2007).

A study has found that a cranberry fraction inhibits colonization of oral *streptococci* to the tooth surface and might in this way slow the development of dental plaque, which is a major cause of peridontal and gum diseases (Yamanaka *et al*. 2004). It was also shown that high molecular weight compounds of cranberry possess an inhibitory effect on influenza virus adhesion and infectivity (Weiss *et al*. 2005).

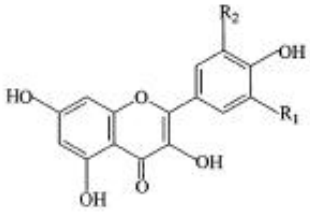
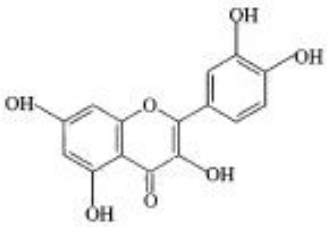
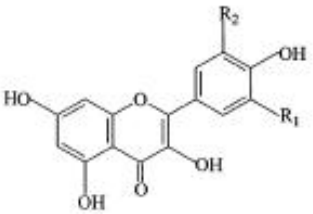
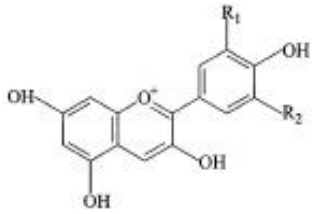
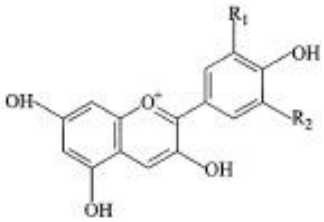
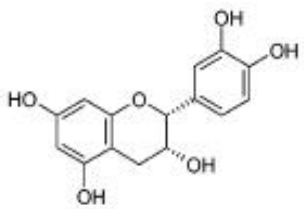
There is evidence for cranberry products indicating their positive effect in reducing urinary tract infections (Jepson and Craig, 2007). Being an important aspect of this work, the role of cranberry in the maintenance of urinary tract health is discussed in more detail in chapter 5.7.2.

4.5 Chemical composition of Vaccinium macrocarpon

Fresh cranberry fruits (“cranberries”) contain >80% water and 10% carbohydrates (glucose and fructose). Other constituents are flavonoids, triterpenoids, organic acids and ascorbic acid. The major organic acids are citric, malic, and benzoic acids, as well as quinic and glucuronic acids. The high amount of organic acids makes the single-strength juice very acidic (pH < 2.5) and unpalatable (Raz *et al*., 2004). Cranberries are rich sources of dietary flavonoids including anthocyanins and proanthocyanidins (Jepson and Craig, 2007). A total amount of 400 mg of flavonoids and phenolics per liter of a freshly

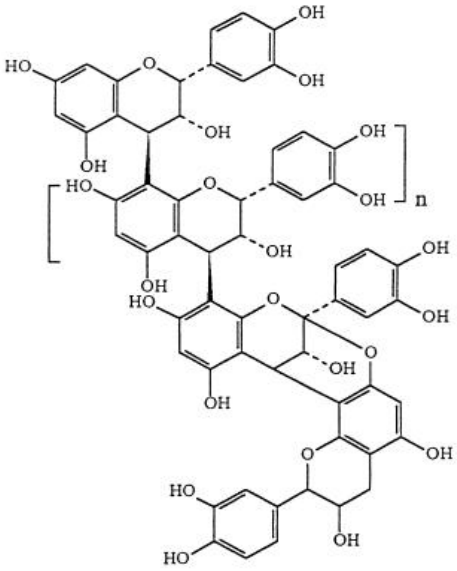
squeezed cranberry juice was reported, distributed as about 44% of phenolic acids and 56% of flavonoids (Chen *et al*., 2001).

There are eight major classes of flavonoids: flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, chalcones and dihydrochalcones. They result from variations in the structural arrangement and positions of the functional groups. Possible glycosilation at multiple sites with a various saccharides and their further acylation produce a group containing more than 5000 chemically distinguishable compounds (Lin and Harnly, 2007). The unique flavonoids of cranberry, including anthocyanins, proanthocyanins and flavonol glycosides are responsible for the attractive bright appearance and distinctive flavor of the cranberry fruit. They are also believed to contribute to the health promoting effects of cranberry products. Foo *et al*. (2000) elucidated the structures of the cranberry proanthocyanidins (condensed tannins), which inhibit the adherence of uropathogenic *Escherichia coli in vitro*. Rare A-type low molecular weight procyanidins were found in cranberry at higher concentrations than the more common B-type procyanidins (Määttä-Riihinen *et al*., 2005). Fruit phenolics, including flavonoid and non-flavonoid compounds, are also of interest in food science, as they have impact on the color quality, adstringency and shelf stability of fresh and processed fruits (Kalt *et al*., 2008). A summary of flavonoids and their glycosides present in *V. macrocarpon* is given in table 1 (Mullen *et al*. 2007; Lin and Harnly 2007; Vvedenskaya *et al*. 2004). The vast majority of flavonoids and phenolic acids in cranberry juice, up to 85%, occur as esters or bound phenolic compounds (Chen *et al*., 2001).

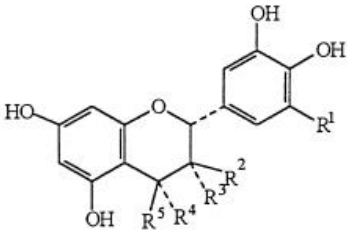
Myricetin-3-β-galactoside Myricetin-3-α-xylopyranoside Myricetin-3-α-arabinofuranoside Dimethoxymyricetin-hexoside Methoxymyricetin-pentoside	 R1,2 = OH
Quercetin-3-β-galactoside Quercetin-3-β-glucoside Quercetin-3-β-xylopyranoside Quercetin-3-α-arabinofuranoside Quercetin-3-α-rhamnopyranoside Quercetin-3-O-(6''-p-coumaroyl)-β- galactoside Methoxyquercetin-pentoside 3'-methoxyquercetin-3-α-xylopyranoside	
Kaempferol-3-glucoside Methoxykaempferol derivatives	 R1,2 = H
Cyanidin-3-arabinoside Cyanidin-3-galactoside Cyanidin-3-glucoside	 R1,2 = OH
Peonidin-3-galactoside Peonidin-3-glucoside Peonidin-3-arabinoside	 R1 = OCH3 R2 = H
Epicatechin	

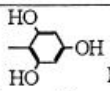
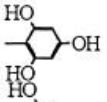
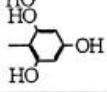
Tab. 1. Reported flavonoids and their glyocosides in *V. macrocarpon*

Proanthocyanidin oligomers (n = 1 or 2)

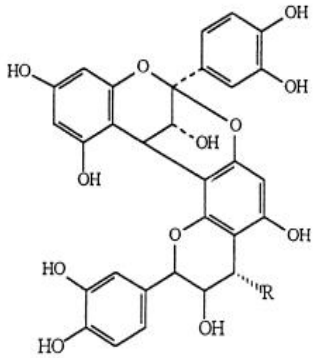


Catechin-(4α-2)-phloroglucinol (3)
Epicatechin-(4β-2)-phloroglucinol (4)
Epigallocatechin-(4β-2)-phloroglucinol (5)



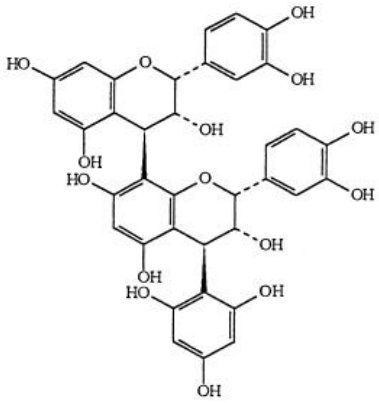
	R^1	R^2	R^3	R^4	R^5
3	H	OH	H		H
4	H	H	OH	H	
5	OH	H	OH	H	

Epicatechin-(4β-8, 2β-O-7)-epicatechin



R = H

Epicatechin-(4β-8)-epicatechin-(4β-2)-phloroglucinol



Tab. 2. Structures of bioactive proanthocyanidins in *V. macrocarpon* (Foo *et al.*, 2000)

5. Urinary tract infection

5.1 Urinary tract infection

Under normal conditions, urine in the bladder is sterile, and the term urinary-tract infection (UTI) refers to the presence of microorganisms in the urinary tract, including the bladder, prostate, collecting system, and kidneys (Henig and Leahy, 2001). Most uncomplicated UTIs in women seem to result from the interaction of infecting agents with uroepithelial cells in the bladder (Hooton, 2001). The vast majority of UTIs is caused by bacteria and *Escherichia coli* (*E.coli*) is the most common uropathogen, detected in 70 – 95% of the cases. Other uropathogenic strains are *Staphylococcus saprophyticus* (isolated in 5 – 20% of cases), *Proteus mirabilis*, *Klebsiella* species or *Enterococci* (Franco, 2005). This is the case for community-acquired infections. Most uropathogens originate from the rectal flora and follow an ascending way through the urethra into the bladder with an interim phase of periurethral and distal urethral colonization (Hooton, 2001). In contrast to this, hospital-acquired infections or infections complicated by underlying host factors tend to be polymicrobic and multi-drug-resistant (Franco, 2005). Throughout all age groups, UTIs are more prevalent among women than among men. It has been estimated that approximately 25% of women have had at minimum one UTI in their lifetime and will suffer several (Henig and Leahy, 2000).

5.2 Mechanism of infection

As mentioned in chapter 5.1, healthy urine is sterile and bacterial entry with subsequent proliferation in the urinary tract is required for disease to occur. Bacteria located within the urinary tract will not cause disease unless they proliferate. The phase of proliferation seems to occur through bacterial attachment to the urinary tract mucosal surface cells, followed by bacterial multiplication and infection of the host tissue (Henig and Leahy, 2000). Therefore, bacterial adherence to uroepithelial cells appears to be the crucial step in the pathogenesis of UTIs, especially cystitis (bladder infection) (Howell, 2007; Henig and Leahy, 2000). Specific bacterial surface-structures - fimbriae or pili - facilitate bacterial binding to host-receptors, located on the cell surface (Raz *et al*. 2004). An adhesion molecule - adhesin - is displayed at the distal end of each such pilus (Franco, 2005).

The two most important uropathogenic *E. coli* adhesins are P fimbriae (papenconded adhesins) and type 1 pili. P fimbriae bind to host cells by means of their tip adhesins, encoded by PapG, which attach to cell-surface glycosphingolipids of the globoseries family. *In vitro* studies have demonstrated that the attachment of P fimbriated strains to the tissue leads to cytokine release and ceramide signalling (Stapleton, 2003). Type 1 fimbriae are found nearly universally among uropathogenic and nonpathogenic, e.g. fecal commensal, *E. coli* isolates. They enable adhesion to uroepithelial cells followed by invasion of the bladder. Type 1 fimbriae display the FimH adhesin on their surface, which binds mannose (mannose-sensitive fimbriae). In the bladder, mannosylated residues can be found across the luminal uroepithelial surface (Franco, 2005).

Once attached, the uropathogenic bacteria will divide rapidly and form small clusters, building up intracellular bacterial communities (IBCs). Due to their intracellular location, they are not killed by antibiotic agents. After going through some developmental stages, it comes to the formation of bacterial communities, which possess biofilm-like properties. Biofilms represent collections of microorganisms, attached to a surface or to each other, which display community behaviour and provide protection from environmental changes or insult. The formation of biofilms leads to resistance of bacteria to the host defence system by protecting the bacteria inside the matrix, and to antibiotic treatment, as the drugs are not able to penetrate the biofilm matrix. Single bacteria can detach from the intracellular biofilm and rebind to another part of the epithelium to form a new generation of IBCs or a reservoir. During the reservoir phase, host and bacteria are quiescent. Despite antibiotic treatment, the uropathogen can persist in this state for the duration of months following the intial infection (Franco, 2005).

5.3 Recurrent urinary tract infection (RUTI)

The term recurrent urinary tract infection (RUTI) refers to a symptomatic UTI that follows the state of a previous infection, generally, but not necessarily, after treatment (Hooton, 2001). The diagnosis RUTI is made at the occurrence of three or more symptomatic episodes over a 12-month period (Franco, 2005). Most recurrences occur during the first three months after the intial infection (Hooton, 2001). Recurrent UTIs are common and can affect also young, healthy women with anatomically and physiologically normal urinary tracts.

Occasionally, recurrences occur due to a persistent focus of infection (relapse). The initially infecting strain may persist in the fecal flora after elimination from the urinary tract, re-colonize the bladder and lead to recurrent UTI. The vaste majority of RUTI however, is believed to result from re-infection, which indicates that it is caused by a different strain than the original infection (Hooton, 2001).

It has been shown that women with recurrent UTI are more susceptible to vaginal colonization with uropathogens in comparison to women without a history of recurrent UTI. The prevalence of vaginal colonization with *Escherichia coli*, *Enterococcus faecalis*, *Proteus mirabilis*, and *Klebsiella* was significantly higher in women suffering from recurrent infections. It has been demonstrated, that women with recurrent UTI were subject to colonization with gram-negative bacilli, that was heavier and lasted longer, compared with controls (Hooton, 2001).

The development of recurrent UTI in healthy women mostly starts with vaginal colonization by enteric flora, especially with uropathogens, such as *Escherichia coli*. Uropathogenic *E. coli* (UPEC) subsequently passes an interim phase of periurethral and distal urethral colonization. This colonization in combination with the presence of an anatomically given shorter urethra facilitate bladder colonization and infection. Uropathogenic *E.coli* strains are able to produce an acidic polysaccharide capsule to protect themselves from phagocytosis by human leucocytes and to inhibit complement activation (Franco, 2005).

5.4 Diagnosis of UTI

The "gold standard" bacteriological criteria for diagnosis of UTI include microbiological confirmation by urine culture from a midstream specimen of urine (MSU). For young, symptomatic women the diagnostic criterion should be 100 CFU/mL (Franco, 2005), which is often associated with pyuria (white cells in the urine) (Jepson and Craig, 2007). Classic symptoms of cystitis (bladder infection) include dysuria (pain on passing urine), frequent and urgent need to urinate, cloudy urine and suprapubic discomfort. Upper tract involvement leads to loin pain, haemuria (blood in the urine) and fever. In the elderly, these symptoms may result in a general feeling of being unwell and confusion (Franco, 2005; Henig and Leahy, 2000).

Cystitis only very rarely progresses to pyelonephritis, although the bladder is usually also affected during a kidney infection. Host and bacterial factors play a role in kidney

involvement: pregnancy increases the risk of movement of bacteria into the kidney, and bacteria with special characteristics cause pyelonephritis (Foxman and Brown, 2003).

5.5 Risk factors

Generally, all humans are susceptible to UTI. However, certain circumstances increase the exposure to potential uropathogens and others increase the susceptibility to the development of UTI symptoms following bacterial colonization. Important markers of host susceptibility to (recurrent) UTI include age, gender, pregnancy, diabetes and prostatic enlargement (Foxman and Brown, 2003). Throughout all age groups, women have a significantly higher incidence of UTI than men. This fact can be partly explained by differences in anatomy: women have moister periurethral spaces and shorter distances between the anus and urethral opening and between the urethral opening and the bladder. Most UTIs result from an ascending way of infection (Foxman and Brown, 2003). These factors increase exposure to potential uropathogens and increase their ability to colonize the urinary tract. Table 3 sums up the UTI risk factors for different pupulation segments.

Risk factors for urinary tract infection (UTI)	
young premenopausal women	sexual intercourse contraception (diaphragm, spermicide) pregnancy anatomy (distance urethra – anus) non-secretor status genetics antimicrobial use history of recurrent UTI
elderly postmenopausal women	urogenital atrophy (postmenopausal estrogen deficiency) diabetes mellitus 2 high post-void residual incontinence presence of cystocoele urogenital surgery non-secretor status history of recurrent UTI
hospitalized patients	indwelling catheter ureteric stent nephrostomy tube obstruction (e.g. urolithiasis) malignancy renal cyst neurogenic bladder urethral diverticulum

Tab. 3. Summary of risk factors in different population segments

Young premenopausal women

In the population of young premenopausal women, sexual intercourse, diaphragm-spermicide use, and a history of recurrent UTI have been demonstrated to be strong and independent risk factors. Suffering the first infection at an early age and having a mother with a history of UTIs were associated with a two – four-fold increased risk of UTI (Hooton, 2001). Bladder trauma, associated with sexual activity, and potentially increased by (spermicide-coated) condom use, offers uropathogens the opportunity to enter and interact with the bladder walls. Contraception strategies such as diaphragms, (spermicide-coated) condoms, and spermicides are all correlated with an increased risk of UTI. (Miller and Krieger, 2002) Spermicides, e.g. Nonoxynol 9, increase the risk of UTI by altering the vaginal ecosystem in favor of uropathogens through harming the physiological *lactobacilli*, while having little or no direct effect on uropathogenic *E.coli* (Franco, 2005). In this way, spermicides reduce the vaginal *lactobacilli* population, facilitating colonization of vagina with *E. coli* (Miller and Krieger, 2002).

UTIs often occur during pregnancy. The majority of UTIs during the time of pregnancy pass asymptomatically, but they bear the risk of leading to a low-birth-weight infant or preterm birth.. Pyelonephritis can also cause severe maternal and fetal morbidity and mortality (Henig and Leahy, 2000). Pregnancy and anatomic abnormalities increase the ability of uropathogens to ascend to the bladder and kidney. Data indicate that anatomic differences may have an influence on predisposing young women to recurrent UTI, especially when other exogenous risk factors for UTI are absent (Hooton, 2001).

It is believed that an increased susceptibility to UTI can also result from genetic determination. Women with recurrent UTI were shown to be three – four times more likely non-secretors of ABO histo-blood group antigens. Uropathogenic *E.coli* (UPEC) adheres better to uroepithelial cells from women who are non-secretors. The biochemical explanation for the increased adherence of *E. coli* to these cells seems to be the presence of unique globoseries glycolipid receptors (sialosyl galactosylgloboside and disialosyl galactosylgloboside), which are exclusively found on epithelial cells of non-secretors. These receptors can bind uropathogenic *E. coli* more effectively than other globoseries glycosphingolipids present in the vaginal epithelial cells (Franco, 2005; Stapleton, 2003).

Another factor with genetic variability and possible influence on the development of UTI is the interleukin-8-receptor CXCR1. IL-8 is an inflammatory cytokine which causes neutrophil migration across infected uroepithelial cells. Studies have shown that knockout mice without CXCR1 were not able to clear bacteria from the kidney, ultimately leading to

bacteremia. Analysis of the CXCR1 expression in neutrophils of children with a history of recurrent pyelonephritis has detected a defective version of the IL-8 receptor, which might explain their increased susceptibility to recurrent pyelonephritis (Hooton, 2001).

Voiding and drinking habits do not seem to play a role in the development of UTIs (Miller and Krieger, 2002).

Elderly postmenopausal women

After menopause, the reduced estrogenic levels seem to contribute to the more frequent occurence of UTI in healthy postmenopausal women. It has been shown that topically applied intravaginal estrogen lead to normalization of the vaginal flora and significantly decreased the incidence of recurrent UTI (Hooton, 2001). Diabetes, mostly affecting the elderly, is a risk factor for asymptomatic bacteriuria. Among postmenopausal women, diabetes leads to a twofold increase of acute UTI. Patients suffering from diabetes may also have a weaker host response, hence, their uroepithelial cells may be more susceptible to bacterial binding (Foxman and Brown, 2003).

Hospitalized patients

The likelihood of bacteriuria increases with the patient´s advancing age and is highest among elderly institutionalized women. The presence and prolonged use of an urogenital medical device, such as catheter or urethral stent enhances the risk and severity of UTI significantly (Henig and Leahy, 2000). Obstruction of urinary flow, e.g. due to prostate enlargement in men, and residual urine in the bladder, e.g. due to spinal cord injury, decreased bladder tone or neuropathy, increase the risk of bacteriuria. Incrased residual bladder volume enables the maintenance of bacterial colonization, which provides the uropathogen additional opportunities to interact with the host epithelial cells (Foxman and Brown, 2003). Complicated UTIs are more likely to origin from infections with muli-resistant pathogens. Factors causing urinary stasis, such as urolithiasis, malignacy, renal cyst and neurogenic bladder increase the UTI risk. The main UTI risk factors in hospitalized patients include an indwelling catheter, ureteric stent, or nephrostomy tube. Also, some diseases or medical conditions, e.g. diabetes mellitus, renal failure, renal transplantation, and immunosuppression, can cause a higher susceptibility to uropathogens by bypassing normal host defences (Franco, 2005).

5.6 Management/Treatment

Before medication is prescribed, reversible risk factors should be considered, and as far as possible, corrected. Women using spermicides or diaphragm, might consider alternative strategies of contraception. For hospitalized patients with catheters, catheter management should be reviewed. In the elderly, perineal hygiene, hydration, and bladder or bowel incontinence are important factors to be managed. Postmenopausal women suffering from recurrent UTI might consider estrogen therapy (Franco, 2005)

The standard treatment for an acute urinary tract infection is antibiotic therapy (Howell, 2007; Reid, 1999; Franco, 2005). Trimethoprim-sulfamethoxazole (TMP-SMX) in a three-day regimen is considered the treatment of first choice for acute and uncomplicated bladder infections. Three-day course regimens are generally prefered, due to their better compliance, lower cost, and lower frequency of adverse reactions compared to the seven – ten day administration (Hooton, 2001). Nitrofurantoin is a safe and effective drug for the treatment of uncomplicated UTIs, but it should be administered for at least seven days. The use of fluoroquinolones is a reasonable alternative for women who are known or suspected of having resistant organisms or who are allergic to more conventional treatments. Fluoroquinolones are also prescribed in areas with high resistance to trimethoprim-sulfamethoxazole (>15 – 20%). Risk factors for the development of a TMP-SMX resistance include diabetes, recent hospitalization, recent antibiotic use and recent TMP-SMX use (Hooton, 2001).

5.7 Prevention

Prophylactic treatment is relevant for women who experience two or more symptomatic UTIs over a 6-month period, or three or more over a 12-month period (Hooton, 2001). Each infection causes two – three days of symptomatic illness and chronic UTI can lead to long-lasting pain and discomfort. An acute UTI causes the loss of several working days and requires about one week for recovery. Hence, patients are eager to prevent a recurrence and a motive for prevention exists. Current key strategies in UTI prevention are: antibiotic prophylaxis, cranberry products, *lactobacillus*-based probiotic preparations, and vaccine development (Stapleton, 2003).

5.7.1 Antibiotic prophylaxis

In the situation where the infection cycle has no known cause, the use of antibiotics represents the method used most to manage recurrent UTIs. This approach represents treatment of the patient with antibiotics in amounts that prevent bacterial multiplication and infection of the bladder (Reid, 1999).

a) long-term prophylaxis

Following antimicrobials can be used in long-term prophylaxis: trimethoprim/ sulfamethoxazole (TMP-SMX) (40/200 mg), trimethoprim (TMP), nitrofurantoin (50 mg) or norfloxacin (200 mg). In the long-term preventive treatment, antibiotics are administered for six – twelve months. In some cases this period can be extended to two – five years. The medication can be given on alternate nights or 3 nights a week (Franco, 2005; Reid, 1999). This approach effectively prevents recurrent infections in 95% of patients during antibiotic intake. However, it has been shown, that 50% of the patients will suffer an infection within only three months after prophylaxis has been finished (Franco, 2005). The effect of prophylactic long-term antibiotic intake therefore seems to be only short-lasting.

b) post-coital therapy

Post-intercourse prophylaxis is an option for women, who have defined sexual intercourse as their main UTI risk factor. Post-coital therapy consists of the intake of a low dose of antibiotics immediately after voiding post-intercourse. Studies compared this approach with daily antibiotics intake for UTI prevention. Post-coital prophylaxis was found to be more effective than placebo and as effective as daily antibiotics intake (Franco, 2005).

c) patient initiated therapy

Patient-initiated therapy is an ideal UTI prevention method for women who are not suitable or not willing to take long-term daily prophylaxis. Women can initiate therapy at the onset of their known symptoms, starting antibiotics intake empirically and continuing the intake for three days (Franco, 2005).

Side effects of antimicrobial therapy:

Normal vaginal flora comprises *lactobacilli* and *staphylococci* which provide effective protection from infection caused by uropathogens. Some antimicrobials agents have adverse effects on the vaginal ecology. Perturbation of the normal flora leads to reduced adherence of *lactobacilli* to vaginal epithelial cells *in vivo*, enabling persistent colonization by *Escherichia coli*. It has been demonstrated that vaginal fluid of women suffering from recurrent UTI binds *E.coli* more effectively. β-lactam antibiotics cause significant changes in the physiological vaginal flora followed by increased genital uropathogen colonization. Studies have demonstrated that TMP and nitrofurantoin have less negative effects on the vaginal flora compared to amoxycillin (Franco, 2005).

The contribution of continual low-dose antibiotic regimens for UTI prevention to increasing bacterial resistance, represents a major problem. Additionally to the patient´s wish to prevent UTI, the increasing number of *E.coli* isolates resistant to antimicrobial agents has stimulated interest in novel, nonantibiotic approaches for the prevention of infections. Antibiotics intake causes increased presence of drug-resistant microorganisms in the rectum, perineum, and urethra. Depending on the antibiotic agent in use, many patients suffer from yeast vaginitis following antibiotics treatment, as a result of disruption of the physiological flora. Trends in health care and society show a movement toward the preferred use of natural remedies instead of chemotherapeutic regimens (Reid, 1999).

5.7.2 Role of cranberry in prevention of (R)UTI

Mode of action: anti-adherence activity

Cranberry (*Vaccinium macrocapron* Ait.) ingestion, particularly in the form of cranberry juice, has long been associated with prevention of urinary tract infections (Howell, 2007; Jepson and Craig, 2007; Reid, 1999). The beneficial mechanism was historically thought to be due to the fruit acids causing a bacteriostatic effect in the urine. However, it has been shown that cranberry juice, in contrast to earlier thinking, apparently does not significantly alter the urinary pH (Lowe and Fagelman, 2001; Hooton, 2001; Stapleton, 2003). Ingestion of large amounts of cranberry juice would be required to only slightly reduce urinary pH and increase the concentration hippuric acid. Therefore, the UTI preventive effect of cranberry has to be ascribed to a different mechanism of action (Raz *et al*., 2004).

In vitro and *ex vivo* studies demonstrate anti-adherence effect of cranberry products to uropathogenic bacteria (Bailey *et al*., 2007). Specific proanthocyanidins (PACs), condensed tannins with A-type linkages, were isolated from cranberry, which exert antiadhesion activity against antibiotic susceptible and resistant strains of uropathogenic P-fimbriated *E. coli* (Howell, 2007). As described in chapter 5.2, infection is initiated by bacterial adherence to epithelial cells, followed by bacterial multiplication and colonization of the urinary tract. Therefore, the adherence of uropathogens to uroepithelial cells can be seen as the crucial step in the pathogenesis of UTIs. Virulence factors in respect to the pathogenesis of UTI represent adhesins, which are displayed on the bacterial surface and facilitate the adherence to carbohydrate receptors on the surface of uroepithelial cells. The vast majority of uropathogenic *E. coli* strains expresses type 1 fimbriae that bind mannose-like receptors. P-fimbriated (mannose- resistant) *E. coli* adhere to oligosaccharide sequences [α-Gal(1 → 4)β-Gal] of receptors located on the uroepithelial surface and are therefore correlated with cystits and pyelonephritis (Howell, 2007).

Zafriri *et al*. (1989) identified two bioactive compounds in cranberry that exert inhibitory effect on *E.coli* adhesins. The monosaccharide fructose inhibits adhesion of mannose-sensitive fimbrial type 1 adhesins to uroepithelial cells, which has only been demonstrated *in vitro*. The other, proanthocyanidins (PACs), inhibits mannose-resistant (P-fimbriated) adhesins of uropathogenic *E. coli* strains. Bioavailability studies suggest that these compounds are absorbed after intake.

Although all fruit juices contain fructose, only juices from *Vaccinium* berries (cranberries and blueberries) contain proanthocyanidins, the mannose-resistant adhesin inhibitors (Henig and Leahy, 2000; Raz *et al*., 2004).

PACs are tannins, polyphenolic compounds, which are a part of the plant defence system responding to environmental stress and microbial infection (secondary metabolites). The adstringency of PACs serves the young berries as protection against animal and insect predators and is responsible for the unique adstringent taste of cranberry juice.

A special characteristic of PACs is their ability to bind proteins, suggesting one possible mechanism of antiadherent action. PACs, acting as receptor analogues can competitively inhibit adhesion of *E. coli* to uroepithelial surface by binding their fimbrial tips (Howell, 2007). The group of PACs isolated from cranberry comprises generally epicatechin units with at minimum one A-type linkage. The molecular structures of the bioactive A-linked PACs were determinded by Foo *et al.* (2000) and found to be active in the antiadhesion

assays (Howell, 2007). Studies have demonstrated that proanthocyanidins containing A-type linkage were more effective regarding their antiadherent activity compared to PACs containing only B-type linkages. Therefore it can be concluded, that A-linkage in cranberry proanthocyanidins, which can occur at the terminal unit or between the extension units of the PAC molecule, appears to represent an essential structural characteristic for bacterial antiadhesion activity. Heterogeneous structures of cranberry PACs may arise during fruit ripening or resul from post harvest processing. (Howell, 2007).

Representing a different mode of action, cranberry compounds may also lead to changes of P-fimbriated uropathogenic *E. coli* in various ways, resulting in decreased adhesion capabilities. Cranberry PACs may for example influence adhesion by altering cell surface properties of the bacteria resulting in a shift of the zeta potential distribution (electrical potential across the interface of solids and liquids) in the positive direction. pH-neutralized cranberry juice was also shown to cause conformational changes in surface macromolecules of P-fimbriated *E. coli*, leading to decreased fimbrial length and density. Cranberry PACs may also induce a change in the bacterial shape from rods to spheres. Data also suggest that cranberry compounds may reduce fimbrial expression at the genetic level (Howell, 2007; Bailey *et al*., 2007). Bacteria incubated in cranberry-containing media demonstrated a 95% reduction in their fimbrial expression and showed extreme cellular elongation (Henig and Leahy, 2000).

Cranberry juice cocktail

The most common form of cranberry consumption is cranberry juice cocktail (CJC), a sweetened beverage comprising approximately 25% single-strength cranberry juice, sweetener, water and vitamine C (ascorbic acid). The single-strength juice is highly acidic, pH ~2.5, and quite adstringent. These properties make the fruit juice quite unpalatable at full strength.

Side effects and compliance

Generally, the safety of cranberries and derived products is considered to be excellent. Some patients though, may experience a mild laxative effect, when larger amounts are ingested. In high-risk patients, however, the regular ingestion of large amounts of cranberry products over a long period may increase the risk of urinary stones, due to the increased urinary excretion of oxalate and slight urinary acidification (Raz *et al*., 2004). Other reported side effects include reflux, mild nausea and frequency of bowel movements.

In some of the trials the number of withdrawals was very high (20 – 47%), indicating that cranberry juice cocktail does not represent an acceptable therapy when taken over a long duration (Jepson and Craig, 2007). Also the unique taste, main withdrawal reason for children, and caloric load of cranberry juice cocktail decrease the acceptance of many patients. Capsules of cranberry concentrate could represent an alternative. Ultimately, the cost of consuming larger amounts of cranberry juice may represent a limiting factor in general acceptance. Cranberry products are currently not covered by health insurance (Jepson and Craig, 2007; Raz *et al*., 2004).

Clinical studies/Evidence

In recent years, cranberry consumption has been more and more recommended as a safe alternative for UTI prevention. There seems to be positive evidence that cranberry juice consumption can decrease the number of symptomatic UTIs.

In vitro studies detected a strong antiadherence activity when uropathogenic bacteria were preincubated with cranberry juice (Henig and Leahy, 2000). Preincubation of uropathogenic *E.coli* strains in fresh cranberry juice for only 30 seconds was shown to result in inhibitory activity. After 24 and 48 hours, the number of *E. coli* colonies was reduced to approximately 50% (Lowe and Fagelman, 2001). *In vivo* studies have demonstrated that urine from mice and humans, collected after drinking cranberry juice containing PAC with A-type linkages, exerted significant inhibitory effects on bacterial adherence compared to control urine (Howell, 2007; Henig and Leahy, 2000; Stapleton, 2003).

Additionally to antiadherence effects, *in vitro* studies have reported decreased fimbrial production of *E. coli* grown on agar medium containing cranberry juice. This was in correlation with fimbrial functional assays. Cranberry juice in liquid growth medium has also shown antimicrobial activity against a broad spectrum of uropathogenic bacteria (Stapleton, 2003).

Cranberry juice was added to a culture epithelial cells with adherent *E. coli* to study its antiadherent effect, when bacteria were already attached to receptors on the cell surface. An occasional and variable release response was observed. The variable efficacy of cranberry juice in releasing already adhered uropathogens limits its potential as a treatment strategy for an acute infection (Lowe and Fagelman, 2001). In contrast to UTI prevention, where cranberry is believed to be effective at least in certain population segments, there is currently no evidence that cranberry can treat UTI once an infection is present.

The evidence for cranberry and derived products indicates that it can be effective in reducing recurrent UTIs in certain subpopulations. The strongest evidence available is for sexually active women with a history of recurrent UTI (Raz *et al*., 2004). The evidence is more inconclusive regarding its effectiveness in elderly men and women, although one trial demonstrated a decreased incidence of bacteriuria and pyuria in this high-risk population after regular consumption of 300 ml of cranberry juice cocktail (Lowe and Fagelman, 2001). There is currently no evidence that cranberry juice consumption for the purpose of preventing recurrent UTIs is effective in patients with neuropathic bladder (Jepson and Craig, 2007). Also, cranberry consumption did not alter the frequency of UTIs or bacteriuria in the population segment of patients with intermittent catheterization. The lack of benefit in this population may be explained by a low volume of cranberry concentrate ingested or the patients´ inherent dysfunction, which might have overridden any clinical effect of cranberries in this case (Lowe and Fagelman, 2001; Raz *et al*., 2004).

A cranberry preparation with a standardized phenolic content of 30% (25% minimum proanthocyanidins) was reported to be successful in the prevention of UTIs in women who were subject to recurrent infections. The standardized cranberry preparation was administered twice daily (one capsule) for twelve weeks, each capsule contained 200 mg standardized cranberry extract. The preparation used was much higher in phenolic content than both dried cranberries with 0.5% and dried juices with 3% to 7%. None of the women developed a UTI in the course of the study, neither on the basis of symptoms nor laboratory results, which was in contrast to their UTI history of an infection every two months. Women who continued the intake of the phenolic-rich cranberry product remained free from infections during the following two years. These results suggest a possible correlation between the amount of phenolic compounds ingested and the UTI preventive effect. Results of this preliminary study indicate that women with a history of recurrent UTIs can prevent these recurrent infections by daily intake of a cranberry extract. However, further studies are required, including a control group. (Bailey *et al*., 2007).

Summarizing, there seems to be evidence that cranberry and derived products are effective agents for the prevention of UTIs, particularly in women suffering from recurrent infections and the elderly, but not in patients with abnormal bladder function requiring catheterization, regardless their age. However, none of the trials justified the dosage of the administered cranberry product. Additionally, the chemical composition varies between different cranberry products. There is no standardization, and the bioequivalence between

cranberry juice and other formulations, such as capsules/tablets is not clear (Jepson and Craig, 2007). Future trials should be conducted with cranberry products comprising known, characterized, and quantified ingredients. A standard analytical method for cranberry products combining accurate PAC quantification with antiadhesion bioactivity analysis would enable manufacturers to formulate efficacious cranberry products (Howell, 2007).

Currently, there is no evidence regarding the amount and concentration as well as duration of cranberry intake to be most effective. It has been postulated that future trials may need to cover a longer time period, so that the natural course of the illness is taken into account. Current knowledge suggests a glass of cranberry juice twice a day (daily dosage: 240 – 300 ml) (Jepson and Craig, 2007, Howell, 2007).

It is believed that proanthocyanidins with A-linkage are the main bioactive ingredients in cranberry products, but only little is known about their metabolism (Stapleton, 2003). No data are available, which compare cranberry products with established strategies, such as antibiotics for UTI prevention. Theoretically, the use of cranberry products instead of antibiotics to prevent UTIs might lead to a decreased number of antibiotics-resistant organisms, but there is currently no evidence supporting this theory (Jepson and Craig, 2007).

5.7.3 Probiotics

defences against UTI: the normal vaginal flora

The physiological vaginal flora of estrogenized, premenopausal, nonpregnant women, represents a complex microbial ecosystem comprising 10^8 to 10^9 cfu/ml of vaginal fluid, containing over 50 bacterial species. 90% of these bacteria are *lactobacilli* with *L. crispatus* being the most common species found in the vaginal flora of healthy premenopausal women. This benign vaginal flora prevents colonization by virulent, uropathogenic microorganisms. (Stapleton, 2003)

Lactobacilli protect the vagina from colonization by uropathogens through various modes of action, including steric hindrance or blocking potential sites of attachment, competition for nutrients, production of hydrogen peroxide and maintenance of a low pH (Hooton, 2001; Miller and Krieger, 2002). The reduction or complete loss of *lactobacilli*, particularly the H_2O_2-producing species *L. crispatus* or *L. jenseii*, e.g. following antibiotic treatment or use of spermicides, is correlated with an increased risk of colonization with

uropathogenic *E. coli* strains. Conditions causing perturbations of the normal vaginal ecology are associated with increased risk of UTI (Stapleton, 2003).

Probiotics can be defined as biotherapeutic agents comprising living microorganisms, which are administered to promote the host´s health by treating or preventing infections owing to strains of pathogens (Reid, 1999). *Lactobacillus* probiotic therapy supports the restoration of the normal vaginal flora, which might be disturbed by antimicrobial agents, a common problem following antibiotic therapy for UTI. The number of *lactobacilli* required for the maintenance of vaginal flora capable to resist uropathogens has not been defined (Reid, 1999). However, many *lactobacilli* present in dairy products do not seem suitable for UTI prevention in humans (Miller and Krieger, 2002).

5.7.4 Estrogen

In healthy postmenopausal women, the reduced levels of estrogenic hormones occuring after menopause seem to contribute to an increased incidence of recurrent UTI. Topically applied intravaginal estrogen has been shown to normalize the vaginal flora and significantly reduce the occurrence of recurrent UTI (Miller and Krieger, 2002; Hooton, 2001). Therefore, estrogen seems to play a role in the maintenance of the normal vaginal flora and to reduce the risk of UTI. However, increased estrogen amounts may enhance the risk of UTI by facilitating adherence of uropathogens to uroepithelial cells. Local estrogen therapy is considered to lack the adverse effects of systemic estrogen intake (Franco, 2005).

5.7.5 Others

In addition to long-term administered antibiotics, methamine hippurate 1 g (twice daily) and povidone-iodine urogenital washes (twice daily) have been employed for long-term UTI prevention, but patient compliance and efficacy have not been satisfactory enough to make these the treatments of choice (Reid, 1999). For patients with renal insufficiency, cephalexin 125 mg can be prescribed (Franco, 2005).

Deliberate bacterial colonization of the bladder has been reported to significantly decrease the incidence of recurrent UTIs among patients with spinal cord injury. In this approach, a relatively benign isolate of *E. coli* is used to colonize the patient´s bladder in order to competitively inhibit the formation of colonies by more pathogenic strains. This concept of deliberate colonization finds appeal in a patient population where urinary tract

colonization with some organism, generally an uropathogen, is inevitable (Stapleton, 2003).

Mono- or oligosaccharides containing carbohydrates cleaved from native glycosphingolipids were reported to have inhibitory effects on bacterial colonization on UTI mouse models. These compounds do not cause bacterial resistance to antibiotics and can be synthesized in nonhydrolyzable forms (Stapleton, 2003).

Ultimately, two peptides, FALL-39 and cecropin P1 were reported to be active against uropathogens but not against *lactobacillus* strains (Reid, 1999).

6. Nuclear magnetic resonance (NMR) spectrometry

6.1 Basic theory of NMR*

NMR technology is based on the fact that in a magnetic field certain nuclei can absorb energy in form of electromagnetic radiation in the radio frequency (rf) region at frequencies dependent on the characteristics of the sample. An NMR spectrum represents a plot of absorption peaks versus peak intensities.

All nuclei carry a charge, which in some nuclei spins on the nuclear axis, generating a magnetic dipole along this axis. Its spin number *I* describes the angular momentum of the spinning charge and can have values of 0, 1/2, 1, 3/2, etc. $I = 0$ indicates no spin, therefore no magnetic properties. Hence, these nuclei, e.g. ^{12}C, ^{16}O cannot be observed in NMR experiments. Relevant nuclei for NMR spectrometry are ^{1}H, ^{3}H, ^{13}C, ^{15}N and ^{31}P. They are characterized by a spin number of $I = ½$. The most important nuclei for NMR experiments are ^{1}H and ^{13}C. The spin states of nuclei are quantified by the magnetic quantum number m.

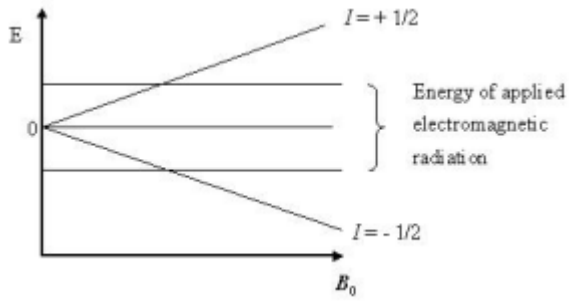


Fig. 3. transition of nuclei to higher energy levels
(adapted from Silverstein *et al*., 2005)

Two energy levels can be taken by $I = 1/2$ nuclei in a constant external magnetic field $\boldsymbol{B_0}$: m = +1/2 representing orientation against the magnetic field and higher energy level, or m = -1/2, with the nucleus orientated in the direction of $\boldsymbol{B_0}$ in the lower energy level, respectively. The energy levels in a magnetic field are splitted according to the type of nucleus (^{1}H, ^{13}C, ...) and $\boldsymbol{B_0}$ with the difference between the two energy levels being directly proportional to the magnetic field applied. ^{1}H has a higher frequency than ^{13}C at

* summary from Silverstein *et al*., Spectrometric identification of organic compounds (pp 127 – 175)

the same field strength. Hence, the nuclei will be observed at different, well separated frequencies and only one nucleus can be detected at one time. After the two energy levels for the nucleus have been established, energy in form of rf radiation (v_1) is introduced to disturb the system causing a transition between the two energy levels. The introduced radiofrequency energy is given in megahertz (MHz) and the frequency of 100 MHz at a magnetic field strength of 2.35 tesla (T) is required to change the orientation of the ^{1}H nucleus. At this ratio, the system is in resonance. The proton absorbes energy, which causes its rise to the higher energy level. A spectrum can be obtained by observing the nuclei returning to equlibrium state.

Generally, there are two approaches of introducing energy to the system. Either the radiofrequency energy is introduced by continuous-wave (CW) scanning of the frequency range or by pulsing the whole range of frequencies with one single burst of radiofrequency energy (pulsed FT NMR) to excite all nuclei simulaneously. Nowadays, the initial CW spectrometers are mostly replaced by the more powerful FT instruments.

The pulsed spectrometer introduces radiofrequency energy in form of a pulse with a sufficiently large frequency to the sample, which is placed in a constant magnetic field. This high power pulse simultaneously excites all sensitive nuclei in the measured sample. During the return of the excited protons to equilibrium state they radiate the absorbed energy, which is collected by a detector resulting in a free induction decay (FID), a signal containing all emitted frequencies over time. The FID is a function of time and can be converted into a conventional NMR spectrum, which is a function of frequency, using a computer algorithm (Fourier transform, FT). A basic NMR experiment consists of three phases: a relaxation delay (tr), which allows the spins to reach equilibrium state is followed by a short rf pulse (tp), lifting the nuclei to the higher energy state. During the subsequent acquisition (tacq) time, the FID is recorded. Since relaxation times for protons are usually on the order of a few seconds or fractions of a second, rapid repetitive pulsing with signal accumulation is possible.

The resulting NMR spectrum is depicted as a series of peaks whose areas are proportional to the number of protons they represent. Therefore, NMR can be used as a quantitative technique, when the peak areas are measured by an electronic integrator. Additionally, a good baseline and resolution are required for accurate integration.

Instruments which are in general wide use are operating at 300 – 600 MHz. Spectrometers above 100 MHz contain helium-cooled superconducting magnets

(solenoids) and use the pulsed FT mode. Basic requirements for NMR experiments include a generally high field, good field stability and homogeneity and a computer interface.

The exact radiation frequency absorbed by a nucleus ("chemical shift") depends on the observed nuclear isotope (^{1}H, ^{13}C, etc.) and variations in its local magnetic field. A nucleus in a molecule is shielded by its electron cloud, the density of which depends on its chemical environment, such as binding partners, bond lengths, etc. The symbol σ represents the "shielding constant", which gives information on the degree of shielding by the electron cloud. This depends on the density of the circulating electrons, which is well described by the inductive effect exerted by groups attached to the carbon atom. Variations of shielding effects within a molecule give rise to different chemical shifts in the recorded spectrum. The discrimination of individual absorptions defines the term of high-resolution NMR spectrometry.

The positions of chemical shifts are measured in frequency units from a reference peak. The Hz and δ scales depict increased applied frequency on the left and decreased applied frequency on the right side from the TMS signal (internal standard, 0 ppm). The chemical shifts are given in dimensionless units labeled δ or ppm. They are not dependent on the frequency of the employed instrument.

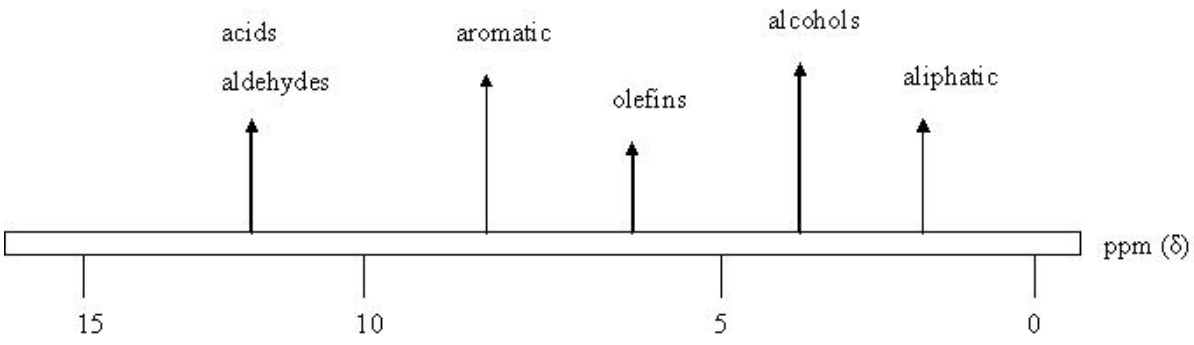


Fig. 4. Functional groups absorb energy at characteristic frequencies (Schripsema, 2008)

The form of the signal is strongly influenced by spin-spin coupling. Scalar coupling is mediated through chemical bonds and usually not important beyond three bonds. The chemical shifts of two coupling protons can be quite widely separated resulting from differences in their chemical environment. However, the spin of each proton is slightly affected by the other proton, so that each absorption peak will appear as a doublet. The frequency difference (Hz) between the two peaks of a doublet is proportional to the strength of the coupling and is represented by the coupling constant *J*. Coupling constants are independent of the applied magnetic field between protons and rarely exceed 20 Hz.

If the chemical shift difference betwenn protons ($\Delta\nu$) is much larger than the coupling constant ($\Delta\nu/J > 8$), two doublets can be observed. As $\Delta\nu/J$ decreases, the doublets will approach each other, resulting in a "roofing" effect: the inner two peaks increase in intensity, while the outer two peaks decrease, until they result in one single line.

One neighboring proton induces the formation of a doublet, while two equally coupled neighboring protons will give rise to a triplet. The multiplicity is n + 1 (n = number of neighboring, equally coupled protons), which is visualized in the Pascal´s triangle. More *J* coupling induces more complex signals.

The sensitivity of NMR measurements is described in the signal to noise ratio (*S / N*), which is influenced by the gyromagnetic ratio (γ), magnetic field ($\boldsymbol{B_0}$), acquisition time (t) and the number of nuclei (n). Noise in NMR measurements is mainly caused by thermal noise in the probe and first-stage receiver electronics. The development of cryogenically cooled probes led to significant improvements of NMR sensitivity, resulting in decreased sample amount requirements. These probes possess cryogenically cooled rf coils (~20°K) and show *S/N* improvement of ~4 x standard probes.

The sensitivity for ^{1}H nuclei is way higher in comparison to ^{13}C NMR, as almost all H in nature represent ^{1}H, in contrast to the low natural abundance of ^{13}C (~1%). Hence, only as little as ~1% of the carbons can be observed in a ^{13}C NMR experiment and higher amounts of the sample are required.

A routine ^{1}H NMR sample measured on a 300 MHz spectrometer requires approximately 10 mg of the compound in about 0.5 ml solvent in a 5-mm o.d. NMR glass tube. Also, microprobes with 1.0 mm, 2.5 mm, or 3 mm o.d. tube are available to reach higher sensitivity.

An ideal NMR solvent should comprise no protons, be inert, low boiling and inexpensive. Deuterated solvents are used in modern spectrometers, as these need a deuterium signal in order to lock or stabilize the magnetic field. A deuterium "channel" is responsible for a constant monitoring and adjustment ("locking") of the magnetic field to the frequency of the deuterated solvent. The deuterated signal is also used to "shim" the field. Modern instruments have about 20 – 30 small electromagnets, called shims, which are used to assure that the homogeneity of the magnetic field is precise at the center of the sample. These shims are computer controlled and can be adjusted in an automated manner.

Summarized information obtained from a one dimensional ^{1}H-NMR spectrum:

- position of the signals ..... chemical shift
- form of the signals ..... coupling constants
- intensity of the signals ..... number of nuclei

6.2 Two-dimensional NMR

The chemical shift range of a ^{1}H NMR spectrum is quite limited and hardly extends beyond 10 ppm. Hence, the ^{1}H spectra of multinuclear experiments, e.g. mixtures, crude plant extracts etc. are inevitably crowded as there is a large number of contributing compounds, most of them giving multiple signals (Colquhoun, 2007; Eisenreich and Bacher, 2007). Compared with ^{1}H, the chemical shift range for ^{13}C is about 20 times larger. Two-dimensional NMR-experiments allow a spreading of the overlapping signals by introducing additional frequency/chemical shift domains. In a standard one-dimensional NMR experiment a single broadband rf pulse is introduced, followed by free induction decay, whereas a minimum of two consecutive broadband pulses is required for the acquisition of two-dimensional spectra (Eisenreich and Bacher, 2007).

Homonuclear correlation experiments produce spectra with identical frequency axes corresponding to the observed nuclei, in most cases ^{1}H. The acquired ^{1}H - ^{1}H COSY (Correlation spectrometry) spectra unravel information about ^{1}H - ^{1}H coupling within a molecule. This information is useful in the elucidation of carbohydrates, organic and amino acids. Heteronuclear correlation experiments generate spectra with two different frequency axes, one corresponding to ^{1}H and the other to a different $I = 1/2$ nucleus, most commonly ^{13}C (Ratcliffe and Shachar-Hill, 2005).

Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC) spectra contain information about ^{1}H - ^{13}C coupling (correlation) between directly attached nuclei, or in long range relation (3 bonds), respectively. HSQC and HMBC represent indirect detection techniques, which provide information on the relationship between signals from two different nuclei and enable in this way the elucidation of e.g. glycosides (Ratcliffe and Shachar-Hill, 2005). The acquisition of two-dimensional advanced NMR spectra allows rapid mapping of spin networks yielding information on homotropic ^{1}H - ^{1}H and heterotropic ^{1}H - ^{13}C relations. However, the homotropic ^{13}C - ^{13}C relations will remain unknown, caused by the low abundance of natural ^{13}C (Eisenreich and Bacher, 2007).

The increased information obtained from 2D NMR techniques carries the disadvantage of an increased acquisition time. This time penalty makes the 2D-experiments unsuitable for general routine high-throughput approaches. However, they provide important information for targeted and detailed investigations, e.g. metabolic profiling. (Ratcliffe and Shachar-Hill, 2005).

The only 2D NMR method that can be employed as a metabolomic fingerprinting technique is ^{1}H *J*-Resolved (*J*-Res) NMR. *J*-Resolved spectra unravel information on the ^{1}H - ^{1}H coupling constant and the splitting pattern, while having a comparatively short acquisition time, relative to other 2D NMR techniques. (Ward *et al*., 2007).

6.3 NMR and metabolomics

^{1}H NMR represents a powerful technique for the detection and definition of (new) metabolites, since any compound in the sample containing protons will give rise to specific signals. Therefore, it is widely used as a rapid metabolite fingerprinting and profiling technique to detect a broad range of metabolites in a generally non targeted way (Mattoli *et al*., 2006). Nuclear magnetic resonance spectrometry is a non-discriminative analytical method, therefore the spectra of plant tissue extracts provide information on the compounds present, covering compounds of all chemical classes (Colquhoun, 2007). In this way NMR spectrometry represents a useful tool for gaining an overall macroscopic view on the plant metabolome (Verpoorte *et al*., 2007). Compared to NMR spectrometry, where every single atom in a molecule is observed, other spectroscopic methods provide information of partial structural elements, and can therefore only be used for partial structural elucidation or the identification of already known metabolites (Verpoorte *et al*., 2008).

NMR is currently the method of choice for medical metabolomics (metabonomics). Its application for plant metabolomic analysis, however, has been more limited due to its lower sensitivity compared to MS-based techniques (Hall, 2006). The relatively poor sensitivity of NMR is the main drawback of this method, so that only metabolites present in a µM to mM range in the NMR tube can be detected (Colquhoun, 2007). Therefore NMR spectrometry represents a favored analytical tool for major metabolites, while many other compounds will typically be missed due to their occurrence in plant extracts below the NMR detection limit (Hall, 2006). However, this disadvantage is compensated by combination of the NMR-metabolomic data (fingerprinting) with a more targeted approach

for compounds of interest (profiling). In this way, a broad information on the metabolome can be obtained.

NMR techniques show numerous advantages which can be considered complementary rather than competitive to other methods. Extracts can be measured directly by NMR spectrometry without a prior derivatization step. (Eisenreich and Bacher, 2007). NMR represents a non-destructive and non-discriminative analytical method unlike e.g. mass spectrometry, which depends on derivatization or the ability of compounds to ionize.(Ward *et al*., 2007) The possibility of unbiased quantitation represents a major advantage of NMR-acquired data, especially for metabolomics analyses (Verpoorte *et al*., 2008; Eisenreich and Bacher, 2007).

The application of ^{1}H and ^{13}C NMR spectrometry for metabolic profiling of plant tissue extracts is well established. Its scope can be extended by introduction of an in-line liquid chromatography step prior to the NMR analysis and a parallel MS analysis. Liquid chromatography solves problems caused by overlapping signals and provides the possibility of concentrating minor metabolites by solid-phase extraction (SPE). Mass spectrometry gives additional complementary structural information. Although taking the NMR method further away from the metabolomic ideal of a rapid high-throughput single-pass analytical tool, this approach provides a strategy for obtaining high-quality analytical information on pre-selected metabolite groups of interest (Ratcliffe and Shachar-Hill, 2005).

An NMR-spectrum represents a physical characteristic of the measured compound, and is therefore highly reproducible. This fact provides an important advantage, as it enables data mining from previous experiments, when following the same extraction procedures with the same NMR-solvents (Verpoorte *et al*., 2008). High reproducibility is also required for experiments studying the effect of external conditions on the plant metabolome, to define the biological variation. (Verpoorte *et al*., 2007).

High resolution NMR spectrometry has become employed for the elucidation of biosynthetic pathways and metabolite flux with quantitative analyses of various isotopologues (Eisenreich and Bacher, 2007).

7. Multivariate Data Analysis

7.1 General term definition

In contrast to univariate statistical tests (e.g. t-test, analysis of variation = ANOVA), which are carried out on one variable (column) at a time, with multivariate methods all variables are considered simultaneously. To a data set with more than three variables multivariate data analysis (MVDA) is applied. The complexity of plant extracts results in highly complex spectral data, making a direct evaluation of the analytical significance challenging. Therefore, multivariate analyses are employed to ensure good analytical rigorousness and detect clusters of similarity or difference among the analyzed samples (Mattoli *et al*., 2006). In this way, conclusions can be drawn about the classification of individual plant samples (Ward *et al*., 2007). With multivariate analytical methods, datasets can be correllated, relative differences determined and visualized. In addition, the data can be interrogated to determine the metabolites causing the separation (Stewart *et al*., 2007).

The application of MVDA forms raw data to a statistic data set, which then allows further analysis. As such, MVDA does not represent a statistics method, as the treatment of the data does not aim towards prediction, but rather vizualization. The application of MVDA to a data set leads to significant data reduction, which is an essential requirement for comparison and classification of metabolic data (Ratcliffe and Shachar-Hill, 2005).

7.2 Spectra processing

Before multivariate analytical methods are applied to the spectral traces, the recorded spectra have to undergo preprocessing steps to re-shape the data. The processing consists of phasing, (manual/automatic) baseline correction, and calibration to a stable signal, solvent or internal standard [e.g. 3-(Trimethylsilyl)-propionic acid-d_4 sodium salt, TSP], respectively. A computer software, e.g. xwin-nmr, TOPSPIN, is employed to perform these steps.

7.3 Alignment (binning or bucketing)

The number of variables is reduced by the so-called bucketing procedure. The NMR spectrum is divided into bins (buckets) of a certain width, most commonly 0.04 ppm, summing (integrating) the intensities within each bin. The resulting bins are used as variables for further multivariate data analysis. This pretreatment step causes a certain loss of resolution, but results in a decrease of sample-to-sample variability and compensates pH-shifts.

7.4 Scaling

Prior to MVDA, data are standardized, in order to give the variables equal weight in the data analysis. Principal component analysis (PCA), as described in chapter 7.5, represents a maximum variance projection method. Hence, a variable with large variance will have more influence on the model than a low-variance variable.

In order to balance the increased impact of variables with higher numerical ranges (bigger variance) on the model, the data are ”scaled”. By modifying the variance variables, it is possible to attribute different importance to them. This gives the possibility to down-weight irrelevant or noisy variables.

Normal spectroscopy data are mean-centered (Ctr), not scaled. For mean-centering, the average value of each variable is calculated and subtracted from the data. A disadvantage of this method is that minor signals might be ignored. The most common scaling technique, however, is the unit variance (UV) method. The mean centered variables (average subtracted) are divided by the standard deviation with the result that each scaled variable then has equal (unit) variance. This method enhances minor signals, and might cause exaggerated signal noise. Pareto scaling (Par) is in general the preferred option for metabolomics NMR-data. The centered variables are divided by SQRT(SD). This technique represents a compromise between the mean-centered method and unit variance (UV) scaling, enhancing minor signals (compounds) without significantly inflating baseline noise.

7.5 Principal component analysis (PCA)*

Principal component analysis (PCA) represents the basic method of multivariate data analysis. It is an unsupervised multivariate projection method enabling to extract and visualize the variation in the analyzed data matrix. The term "unsupervised" indicates, that the experimental data alone are analyzed, without hypothesis, therefore providing unbiased information. The multivariate data set is represented in form of a low-dimensional plane, consisting of two to five dimensions (principal components), providing an overview of the acquired data. In this overview, groups of observations, trends, and outliers can be observed. Additionally, also relationships between observations and variables, and among the variables themselves are unraveled.

PCA is a mathematical procedure, which serves to transform a variety of possibly correlated variables into a smaller number of uncorrelated variables - principal components. The first principal component (PC 1) accounts for as much of the variability in the data set as possible. The succeeding components account for the remaining variability. PCA therefore is a visualization method reducing the dimensionality of the data set. The resulting principal components represent descriptive dimensions indicating the maximum variation within the analyzed data set (Mattoli *et al*., 2006).

The data matrix consists of observations in rows, and variables in columns. The observations can be analytical samples, chemical compounds or reactions, biological individuals, etc. Variables are measured in order to characterize the properties of the observations, using e.g. chromatographic methods (HPLC, GC, TLC,...) or other techniques, including NMR. Samples can be visualized as points in a multidimensional space with each variable forming an axis. PCA determines vectors in the multidimensional space of variables, indicating the direction of greatest dispersion of the samples by fitting the data as well as possible in the least square sense (Cuny *et al*., 2008).

Before calculating the first principal component, the data are mean-centered, which results in a re-positioning of the coordinate system, such that the average point is the origin. Following mean-centering and scaling, the first principal component is calculated. PC 1 is the line that can approximate the data in the least square sense best and goes through the average point. It represents the maximum variance direction within the data. Each observation can be projected onto this line and get a co-ordinate value, named "score".

* summary from Eriksson *et al.*, Multi- and Megavariate Data Analysis (pp 43 – 69)

Usually, one principal component is not sufficient to depic the variation within a data set. Therefore, a second principal component (PC 2), represented by a line, which is orthogonal to PC 1 and passes through the average point, is calculated. Principal component 2, is oriented in a way, which represents the second largest source of variation in the data set. The most influential PCs are the first few, because they describe the greatest amount of variance. In this way, a great number of original variables is replaced by a small set of variables, principal components, without loss of information (Cuny *et al*., 2008). When dealing with plant material, the experimental design is of great importance. It is important to bear in mind that the main differences, revealed in principal component 1, can sometimes result from environmental effects (Ward *et al*., 2007).

The first two principal components together define a plane. The observations can be projected onto this plane to vizualize the structure of the analyzed data set. The co-ordinate values of the observation on this plane are named "scores" and the plotting of this projected configuration forms a score plot.

The third PC is oriented in the direction of the third largest variation in the data, is orthogonal to the first two principal components, and passes through the average point (the origin). The third principal component is perpendicular to the already existing model plane.

The descriptive ability of a PC-model may improve by calculating more principal components. However, there is no rule and several approaches to determine how many PCs are appropriate.

The loading plot unravels information about the variables, which are responsible for the separation of observations in the score plot, influential variables and also their correlation. Variables comprising similar information are clustered - they are positively correlated. The increase or decrease of the numerical value of one variable tends to influence the numerical value of the other variable in the same way. In contrast to this, negatively ("inversely") correlated variables are positioned on opposite sides of the plot origin, in diagonally opposed quadrants. The larger the distance of a variable to the plot origin, the stronger its influence on the model. The contribution factor of the principal components is calculated by the cosine of the angles, cos α. Values close to 1 (cos 0 = 1) indicate a strong influence, whereas values closer to 0 describe only little contribution (effect) of the PC. The principal component loadings give information of how the PC model plane is situated in the variable space. Loadings are used for interpretation of the scores. For the analysis a of loading plot, also column plot can be used, in which a higher signal (relative comparison) indicates bigger impact on the model.

8. Material and physico-chemical methods

8.1 *Vaccinium macrocarpon* extract

8.1.1 Samples

Five hundred gram of fresh American cranberry (Oceanspray) were purchased from the local market in Leiden (Netherlands) and stored at 4 °C.

8.1.2 Extraction

An amount of 340 g American cranberries (*Vaccinium macrocarpon*), cut in half, was extracted by maceration with 600 ml 80% methanol, under sonication for 30 min. The solid parts were separated by vacuum tank filter and the methanol fraction was evaporated until dryness. The addition of few drops of *n*-butanol was used to decrease the boiling temperature. The dried extract was reconstituted in 50 ml Millipore water. This aqueous phase was extracted three times with 50 ml ethyl acetate. The ethyl acetate fractions were collected in a flask and sodium sulfate was added to remove residual water in the organic fraction. The ethyl acetate phase was evaporated until dryness and reconstituted in ca. 5 ml methanol.

8.1.3 Separation

The methanol extract of *V. macrocarpon* was loaded onto a Sephadex LH-20 column. Degassed methanol was used as eluent and 77 fractions were collected.

8.1.4 Thin Layer Chromatography (TLC)

The collected fractions were analyzed by TLC on Silica gel plate. The mobile phase was a mixture of ethyl acetate, formic acid, acetic acid and water 100:11:11:27 (v/v). The compounds were detected under UV 254 nm and 366 nm. Fractions showing the same pattern under UV were pooled and resulted in 7 new fractions. The pooling scheme is depicted in Tab. 4.

fraction 1	1 – 23
fraction 2	24 – 31
fraction 3	32 – 39
fraction 4	40 – 44
fraction 5	45, 46
fraction 6	47, 48
fraction 7	49 - 55

Tab. 4. Pooling of Sephadex fractions

8.1.5 NMR Sample Preparation

The dried sample was reconstituted in 1 ml deuterated NMR solvent, CD3OD, sonicated to improve the solubility and centrifuged (13 000 rpm). Eight hundred µl of the supernatant were transferred in a 5 mm NMR-tube and ^{1}H-NMR-spectra were acquired at Bruker, 500MHz. For fractions 4 and 6, also following two-dimensional NMR-spectra were recorded: *J*-resolved, COSY, HMBC.

8.1.6 Spectra Processing (xwin-nmr)

Preprocessing steps of the NMR spectra were performed with xwin-nmr software (Bruker), consisting of phasing, baseline correction and calibration (on methanol signal → 3,3 ppm). All processing was done by manual.

8.1.7 High Performance Liquid Chromatography (HPLC)

Sephadex fraction 5 showed a complex ^{1}H-NMR spectrum, which suggested that it obviously contained several phenolic / flavonoid compounds, resulting in peak overlap. Preparative HPLC was chosen as a second separation step, based mainly on polarity of the compounds. For HPLC, reversed phase column (Phenomenex Luna C18, 5 □ 250 x 10 mm) was applied using a solvent system A: 0.1% formic acid in methanol, B: 0.1% formic acid in water in the ratio of 60:40, run 26 min isocratically. Automatic fraction collection (Agilent Technologies 1200 series) was used, 48 fractions (à 2 ml) were collected. Elutions were detected using UV spectrometry (Agilent Interface 35900E) at 254 nm, 220 nm, 280 nm, 360 nm. Fractions representing one peak were pooled (see Tab. 5), prepared for NMR measurement, according to protocol described in 8.1.5, and ^{1}H-NMR spectra acquired.

fraction 5A	27 – 34
fraction 5B	35 – 39
fraction 5C	40 – 43
fraction 5D	44
fraction 5E	45 - 48

Tab. 5. Pooling of HPLC fractions

8.2 Cranberry Juice

8.2.1 Samples

Four different brands of pure cranberry juices were analyzed, which were purchased at local supermarkets in Leiden, Netherlands. Two batches per brand were obtained.

	Juice (volume)	Declared content
1	Oceanspray cranberry classic juice drink (1 l)	water, cranberry juice 25%, fructose-glucose syrup, antioxidant: ascorbic acid 0.03
2	Cranberryjuice lightly sweetened (0,75 l)	cranberry juice 95%, fructose-glucose syrup 5%, pasteurized
3	Cranberryjuice pure and not sweetened (1 l)	cranberry juice 100%, concentrate of preservative
4	Natufood cranberryjuice (0.33l)	cranberry juice 100%

Tab. 6. Juices and declared content

Juice	Batch	Expiration date	Sample – ID
1	L064 C233T	12.08	CRJ 1A
	L064 C057T	12.08	CRJ 1B
	n.a.*	07.05	CRJ 1C
2	n.a.*	18.11.09	CRJ 2A
	n.a.*	25.12.09	CRJ 2B
3	n.a.*	13.1.10	CRJ 3A
	n.a.*	5.3.10	CRJ 3B
4	EL 29296	1.2.10	CRJ 4A
	DL 29559	15.2.10	CRJ 4B

Tab. 7. Cranberry juice details (* batch number not declared on juice package; batch difference ensured by purchasing these juices at different time points in different supermarkets.)

8.2.2 Sample Preparation

The goal was to find a suited extraction method to remove unwanted primary metabolites, such as sugar and organic acids, which are present in relatively high concentrations, and to concentrate flavonoids to allow their analysis.

8.2.2.1 Direct measurement

Five hundred µl of KH_2PO_4-buffer/D_2O (1:1, v/v) were added to 500 µl CRJ1A – 4B in an eppendorf tube and vortexted 1 min. After centrifugation (13 000 rpm, 5 min), 800 µl of the supernatant were transferred into a 5 mm NMR-tube and ^{1}H-NMR spectra were acquired at Bruker, 500 MHz, using water suppression mode.

8.2.2.2 Extraction with ethyl acetate

Four ml of CRJ1A – 4B were extracted with 4 ml ethyl acetate by vortexing for 1 min in a small test tube, twice. The ethyl acetate phases were collected and residual water in the organic phase was removed by sodium sulfate. After evaporation of the ethyl acetate, the dried extract was reconstituted in 1 ml MeOD and centrifuged (13 000 rpm, 5 min). Eight hundred µl of the supernatant were transferred into a 5 mm NMR-tube and ^{1}H-NMR spectra were acquired at Bruker, 500 MHz.

8.2.2.3 Extraction with ethyl acetate after neutralization

pH of the juice samples (CRJ1A – 4B) was adjusted with 1 M NaOH, using a pH-meter. A change of color from bright red to green - brown was observed as the juices approached neutral pH. With the neutralized juices, the extraction protocol using ethyl acetate, described under 8.2.2.2 was followed.

The dried extracts were reconstituted both in MeOD and MD 6 to check which NMR-solvent was most suitable. ^{1}H- and *J*-resolved spectra were recorded at Bruker, 600 MHz, cryoprobe. Triplicates of each sample were analyzed.

cranberry juice sample	pH (temperature)	after adjustment
CRJ 1A	2.72 (23.8°C)	7.08
CRJ 1B	2.70 (23.5°C)	7.01
CRJ 1C	2.67 (24.5°C)	7.09
CRJ 2A	2.42 (23.5°C)	7.03
CRJ 2B	2.43 (23.5°C)	7.13
CRJ 3A	2.44 (23.6°C)	7.08
CRJ 3B	2.42 (24.0°C)	7.02
CRJ 4A	2.61 (24.2°C)	6.93
CRJ 4B	2.60 (24.0°C)	7.07

Tab. 8. Juice pH before and after adjustment

8.2.2.4 Solid Phase Extraction

Four ml of CRJ 3A (chosen sample for preliminary extraction tests) were loaded onto a SepPak Cartridge (C18, RP, 1 cm) and eluted with:

a) water, 25%, 50%, 75%, 100% MeOH (à 4 ml)

b) water, 100% MeOH (à 4 ml)

The fractions were collected and evaporated. The dried extracts were reconstituted in 1 ml NMR-solvent, which was chosen dependent on their solubility, and transferred in an eppendorf tube.

Fraction.....	NMR solvent
25%, 50%, 75% MeOH…….	MD 6
Water…….	D_2O
100% MeOH.....	MeOD

After centrifugation (13 000 rpm, 5 min), 800 µl of the supernatant were transferred in a 5 mm NMR-tube and ^{1}H-NMR spectra were acquired at Bruker, 500MHz.

8.2.3 Preprocessing and PCA analysis

The NMR data were automatically reduced to ASCII files using AMIX (Bruker Biospin). The spectral intensities were reduced to integrated regions of the equal width of 0.04 ppm (buckets or bins) corresponding to the region of 10.0 to -0.1 ppm. The residual water and methanol signals were excluded. The files were imported into Excel files and transferred to SIMCA-P (11.0 Umetrics). Prior to PCA analysis, pareto scaling (Par) was applied to the data set, as it enhances small signals without inflating baseline noise. PCA was carried out on spectral data, which consist of the buckets representing the phenolic region of the ^{1}H-NMR-spectra (5.5 – 8.5 ppm) of all analyzed samples.

9. Results and Discussion

9.1 *Vaccinium macrocarpon* extract

The compounds present in the extract were elucidated based on the acquired one- and two dimensional NMR-spectra from the 7 Sephadex fractions (see Appendix), combined with information from literature related to phenolics / flavonoids in *V. macrocarpon*. The structures of the main phenolic / aromatic compounds are listed in Tab. 9.

Fraction	Metabolites	Chemical shifts (ppm) and coupling constants (Hz)
1	Benzoic acid glucoside*	δ 8.10 (H-2, H-6, d), δ 7.63 (H-4, t), δ 7.50 (H-3, H-5, t), 5.71 (H-1', d)
2	Citric acid Malic acid	δ 2.95 (H-β, d), δ 2.83 (H-β', d) δ 4.50 (H-α, dd), δ 2.85 (H-β, dd), δ 2.60 (H-β', dd)
3	Chlorogenic acid	δ 7.58 (H-7', d), δ 7.03 (H-2', d), δ 6.98 (H-6', d), δ 6.92 (H-5', d), δ 6.30 (H-8', d), δ 5.35 (H-3, td), δ 4.10, δ 3.70 (H-4, dd), δ 2.15 (H-2β, H-6β, m), δ 2.00 (H-2α, H-6α, m)
4	Rutin	δ 7.65 (H-2', d, J = 2.0 Hz), δ 7.58 (H-6', dd, *J* = 2.0, 8.5 Hz), δ 6.93 (H-5, d, *J* = 8.5 Hz), δ 6.42 (H-8, d, *J* = 2.0 Hz), δ 6.20 (H-6, d, *J* = 2.0 Hz), δ 5.2 (H-1'', d, *J* = 7.8 Hz), δ 0.99 (H-6''', d, *J* = 6.1 Hz)
	Myricetin-glucoside*	δ 7.57 (H-2' and H-6', s), δ 6.41 (H-8, d, *J* = 2.1 Hz), δ 6.21 (H-6, d, *J* = 2.1 Hz)
	Myricetin	δ 7.35 (H-2' and H-6', s), δ 6.40 (H-6, d, *J* = 2.0 Hz), δ 6.20 (H-8, d, *J* = 2.0 Hz)
	Quercetin-3-O-rhamnoside (Quercitrin)	δ 7.33 (H2', d, *J* = 2.0 Hz), δ 7.30 (H-6', dd, *J* = 1.9, 7.9 Hz), δ 6.90 (H-5, d, *J* = 8.2 Hz), δ 6.37 (H-8, d, *J* = 2.0 Hz), δ 6.20 (H-6, d, *J* = 2.2 Hz), δ 5.39 (H-1'', d, *J* = 2.1 Hz), δ 0.99 (H-6'', d, *J* = 6.1 Hz)
	Kaempferol	δ 8.08 (H-2' and H-6',d, *J* = 9.0 Hz), δ 6.85 (H-3' and H-5', d, *J* = 8.5), δ 6.42 (H-8,d, *J* = 2.1 Hz), δ 6.22 (H-6, d, *J* = 2.1 Hz)
5	Epicatechin	δ 6.97 (H-2', d), δ 6.80 (H-6', dd), δ 6.75 (H-5', d), δ 5.98 (H-6, d), δ 5.92 (H-8, d), δ 4.22 (H-3, m), δ 2.90 (H-4α, dd), δ 2.77 (H-4β, dd)
	Quercetin-3-O-rhamnoside (Quercitrin)	δ 7.33 (H-2', d), δ 7.30 (H-6', dd), δ 6.90 (H-5, d), δ 6.37 (H-8, d), δ 6.20 (H-6, d), δ 5.39 (H-1'', d), δ 0.99 (H-6'', d)
	Quercetin-3-O-glucoside	δ 7.83 (H-2', d), δ 7.58 (H-6', dd), δ 6.87 (H-5', d), δ 6.40 (H-8, d), δ 6.20 (H-6, d), δ 5.20 (H-1'', d)

6	Epicatechin	δ 6.97 (H-2', d,), δ 6.78 (H-6', dd), δ 6.73 (H-5', d), δ 6.00 (H-6, d), δ 5.98 (H-8, d), δ 4.35 (H-2, d) δ 4.22 (H-3, m), δ 2.85 (H-4α, dd), δ 2.77 (H-4β, dd)
	Quercetin-3-O-glucoside	δ 7.83 (H-2', d, *J* = 2.1 Hz), δ 7.58 (H-6', dd, *J* = 2.0, 8.6 Hz), δ 6.87 (H-5', d, *J* = 8.5 Hz), δ 6.40 (H-8, d, *J* = 2.1 Hz), δ 6.20 (H-6, d, *J* = 2.1 Hz), δ 5.20 (H-1'', d, *J* = 7.8 Hz)
	Quercetin-glycoside*	δ 7.52 (H-2', d, *J* = 2.1 Hz), δ 7.49 (H-6', dd, *J* = 2.1, 8.5 Hz), δ 6.89 (H-5', d, *J* = 8.5 Hz), δ 6.40 (H-8, d, *J* = 2.1 Hz), δ 6.20 (H-6, d, *J* = 2.1 Hz)
7	Gallic acid	δ 7.12 (s)
	Quercetin-glycoside*	δ 7.52 (H-2', d), δ 7.49 (H-6', dd), δ 6.89 (H-5', dd), δ 6.40 (H-8, d), δ 6.20 (H-6, d)
	Myricetin	δ 7.37 (H-2' and H-6', s), δ 6.40 (H-8, d), δ 6.20 (H-6, d)

Tab. 9. Phenolic/flavonoid compounds in *V. macrocarpon* extract
(*confirmation by 2D-NMR needed)

9.2 Cranberry Juice Analysis

9.2.1 Extraction methods

Several extraction methods of the juice samples, which are described in chapter 8.2.2., were tried to remove unwanted primary metabolites, as sugar and organic acids and to concentrate the phenolic / flavonoids to allow their analysis.

9.2.1.1 Direct measurement

The juice samples were measured without any concentration step, directly after dilution with NMR-solvent, buffer/D_2O (1:1). The acquired spectra (Fig. 5.) show peaks of high intensity in the aliphatic region (3 – 5 ppm), representing α- and β-glucose and the organic acids, citric and malic acid, respectively. Hardly any peaks could be observed in the phenolic region (5.5 – 8.5 ppm). Cranberry juice contained high concentration of sugar and organic acids in relation to the phenolic compounds. Therefore, the juice samples could not be measured directly to analyze their phenolic secondary metabolites. Extraction was required to concentrate the compounds of interest.

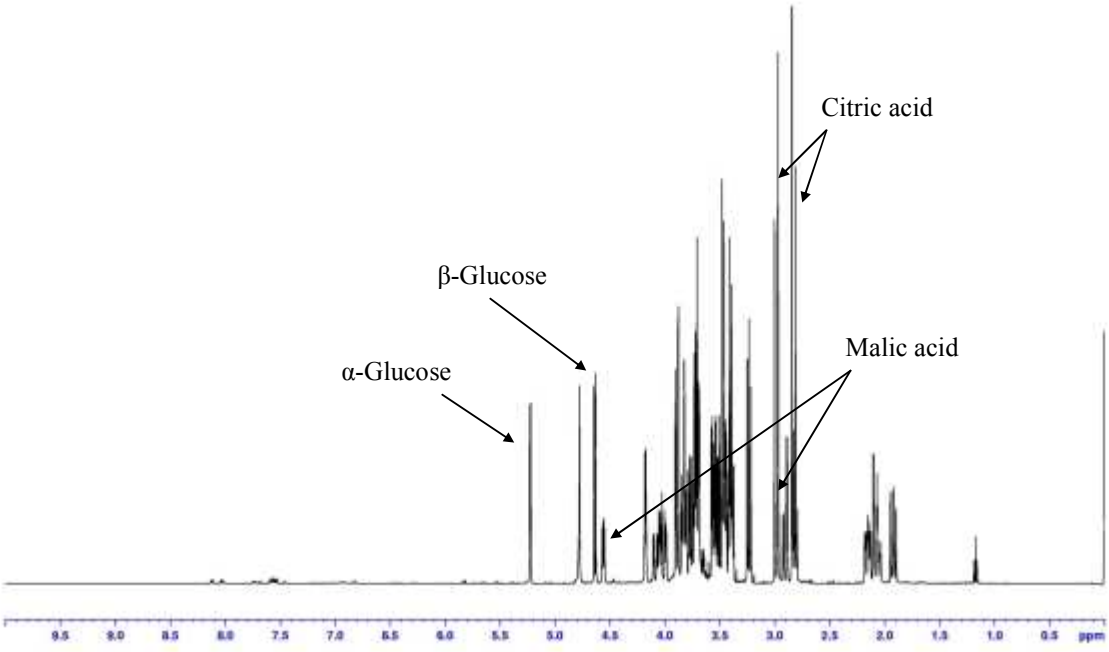


Fig. 5. ^{1}H-NMR_direct measurement

9.2.1.2 Extraction with ethyl acetate

By extraction with ethyl acetate (Fig. 6.), sugars were removed quantitatively, but still high concentrations of citric and malic acid were present in the extract. The only significant, though still minor signals in the phenolic region, could be assigned to the structure of benzoic acid. Benzoic acid is used in the beverage industry as antioxidant and preservative. The applied extraction method with ethyl acetate did not lead to satisfactory results, as it was not able to remove all primary metabolites. The high concentration of organic acids did not allow the detection of the phenolic secondary metabolites, which were present in lower concentration.

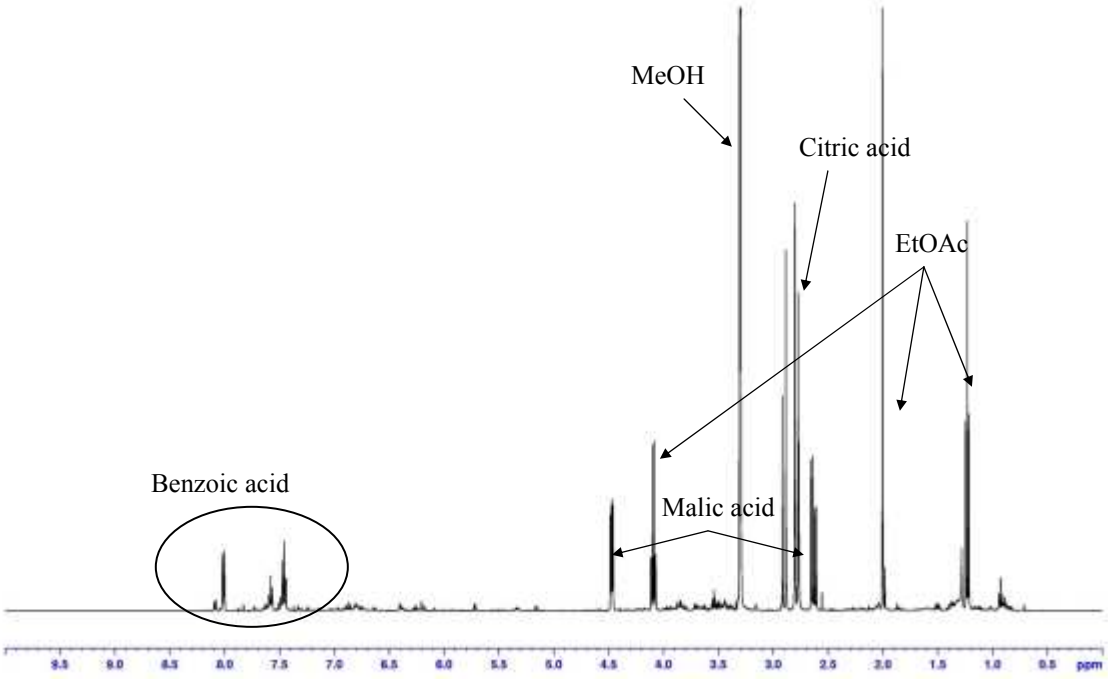


Fig. 6. ^{1}H-NMR_EtOAc-extraction

9.2.1.3 Solid Phase Extraction

Sugars were eluted in the water fraction (Fig. 7.). In the methanol fraction (Fig. 8.), no peaks in the phenolic region could be observed, the extraction-/concentration method for phenolic secondary metabolites was not successful. This might be explained by fact that interaction between solid and mobile phase is influenced by the pH of the loaded sample. The pH of the analyzed cranberry juices was between 2.4 and 2.7 due to the high content of organic acids, which could be responsible for the ineffectiveness of SEP concerning concentration of the phenolic compounds, in this case.

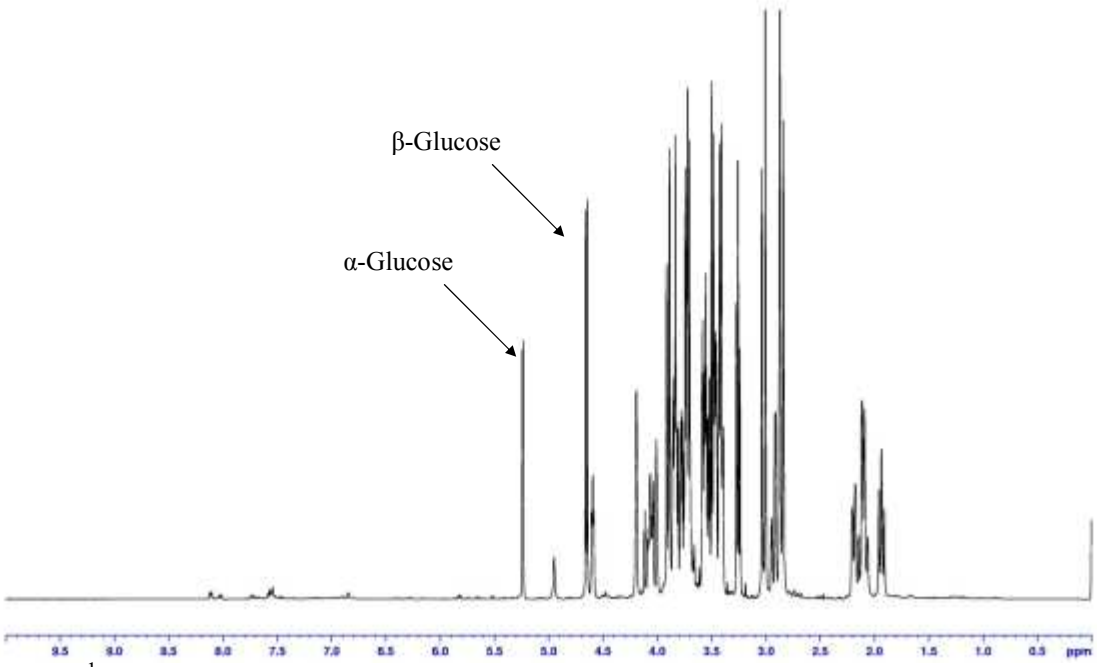


Fig. 7. ^{1}H-NMR_SPE_water

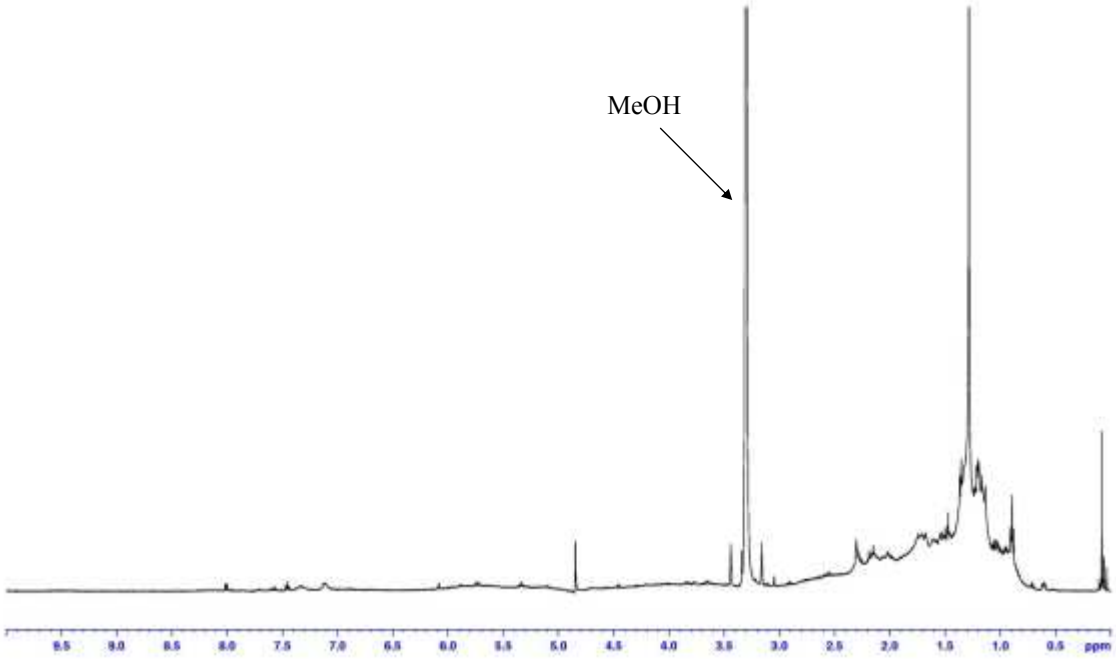


Fig. 8. ^{1}H-NMR_SPE_MeOH

9.2.1.4 Extraction with ethyl acetate after neutralization

Prior to extraction with ethyl acetate, pH was adjusted to 7 ± 0.2 using 1 M NaOH. Due to the neutral pH, organic acids were brought into their ionic form and therefore stayed in the aqueous phase. Phenolic secondary metabolites could be extracted in the ethyl acetate phase. In this way, a separation of primary and secondary metabolites was obtained, with primary metabolites (sugar, organic acids) in the aqueous, and secondary metabolites

(flavonoids) in the ethyl acetate phase. Fig. 9. and Fig. 10. show a zoom in the phenolic region of ^{1}H-spectra recorded from these extracts (5.5 – 8.5 ppm). The extraction method gave rise to a number of peaks, representing phenolic secondary metabolites. The experiment was carried out with MeOD and MD 6 as NMR-solvents, to study in which solvent the extracted compounds were better soluble and therefore provided the better spectrum. The two spectra were comparable concerning their resolution, quality and quantity of peaks. MD 6 was chosen as the NMR-solvent to be used for all samples, as it provided some advantages. It contained KH_2PO_4 buffer (pH 6), and in this way contributed to the stability of the pH, which was shown to represent an important factor. It also contained 0.1% TSP as internal standard, which was used for calibration. A database of NMR-spectra with compounds of interest contained ^{1}H-spectra recorded in MD 6, and spectra could be directly compared, as compounds measured in the same solvent are expected to show peaks at the same chemical shifts.

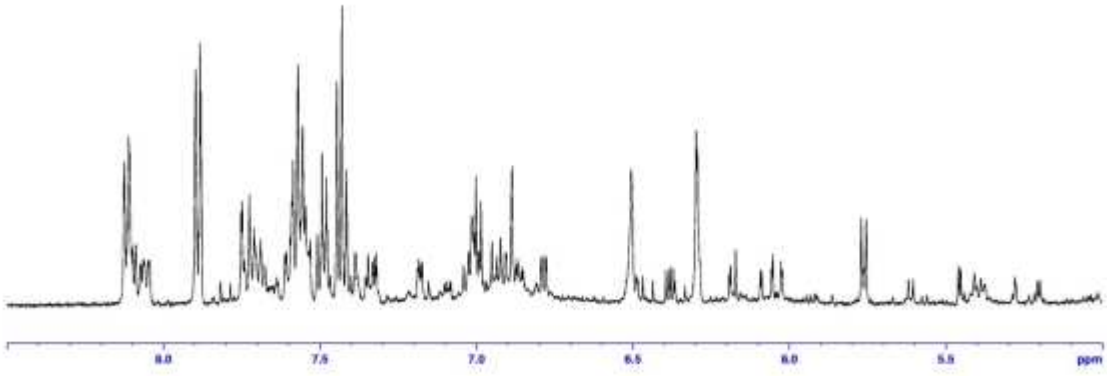


Fig. 9. ^{1}H-NMR_EtOAc_pH7_MD6

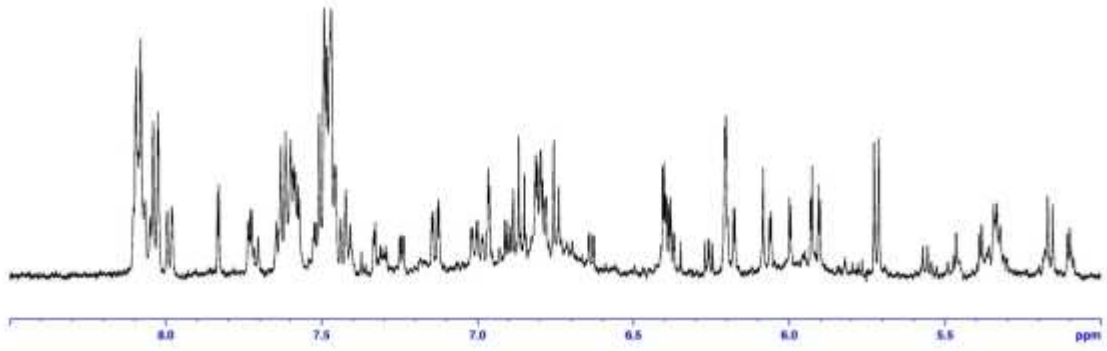


Fig. 10. ^{1}H-NMR_EtOAc_pH7_MeOD

9.2.2 Multivariate Data Analysis

Principal Component Analysis (PCA) was employed as an unsupervised multivariate data analysis method to gain an overview about the relation between the analyzed juice samples. Only the aromatic region of the measured spectra (5.5 – 8.5 ppm) was considered in the calculation, to compare the saples in respect to their phenolic / flavonoid profile. The analyzed cranberry juice samples have shown to provide significant differences to be clearly separated in the score scatter plot (Fig.11.). Three groups were obtained by employing principal component 1 (PC 1) vs. principal component 2 (PC 2) for the separation.

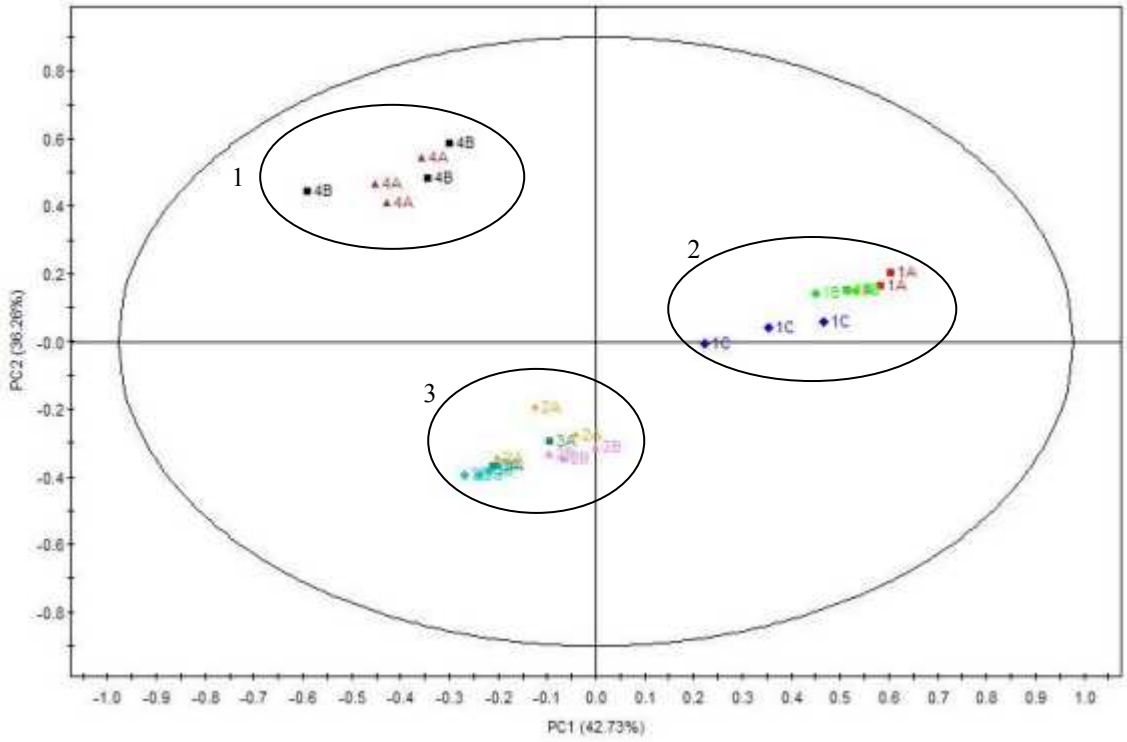


Fig. 11. Principal component analysis – score plot (PC 1 vs. PC 2)

Group	Sample	Juice
1	4A, 4B 4B	Natufood cranberryjuice Natufood cranberryjuice
2	1A, 1B, 1C	Oceanspray cranberry classic juice drink
3	2A, 2B 3A, 3B	Cranberryjuice lightly sweetened Cranberryjuice pure and not sweetened

Tab. 11. Sample ID; grouping in the score plot

In the loading plot (Fig. 12., Fig. 13.), the contribution of metabolites to the separation of the samples is revealed. The higher the column, the higher the impact on the model. Structures of compounds represented by the columns could be identified and an overview about differentiating compouds in the juice samples and concentration trends is given in Tab. 13.

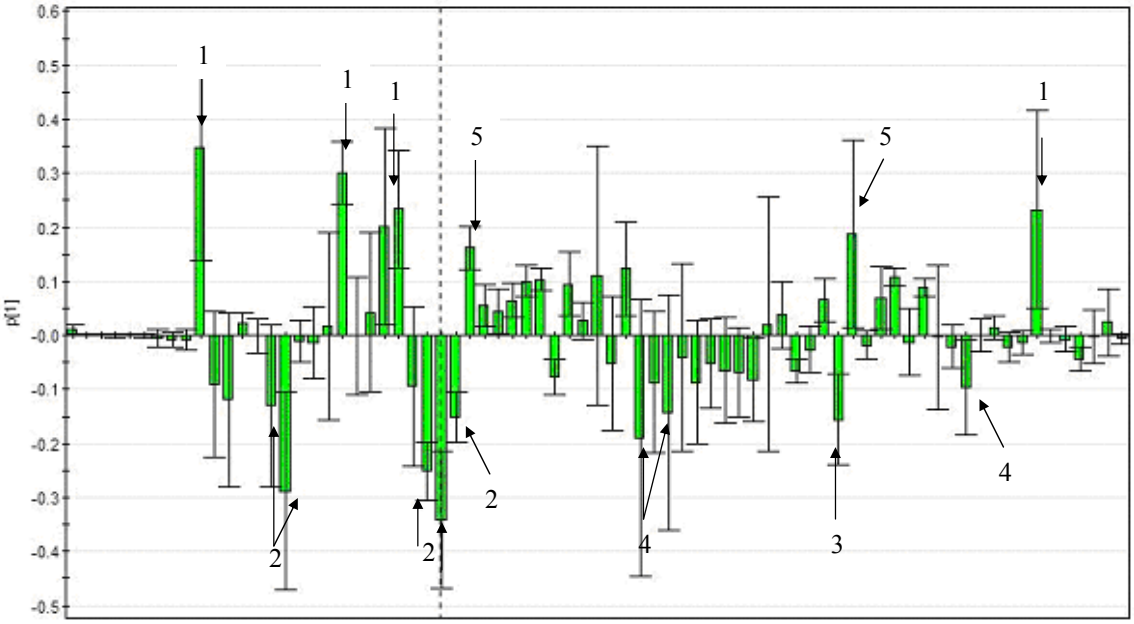


Fig. 12. Principal component analysis – loading plot for PC 1

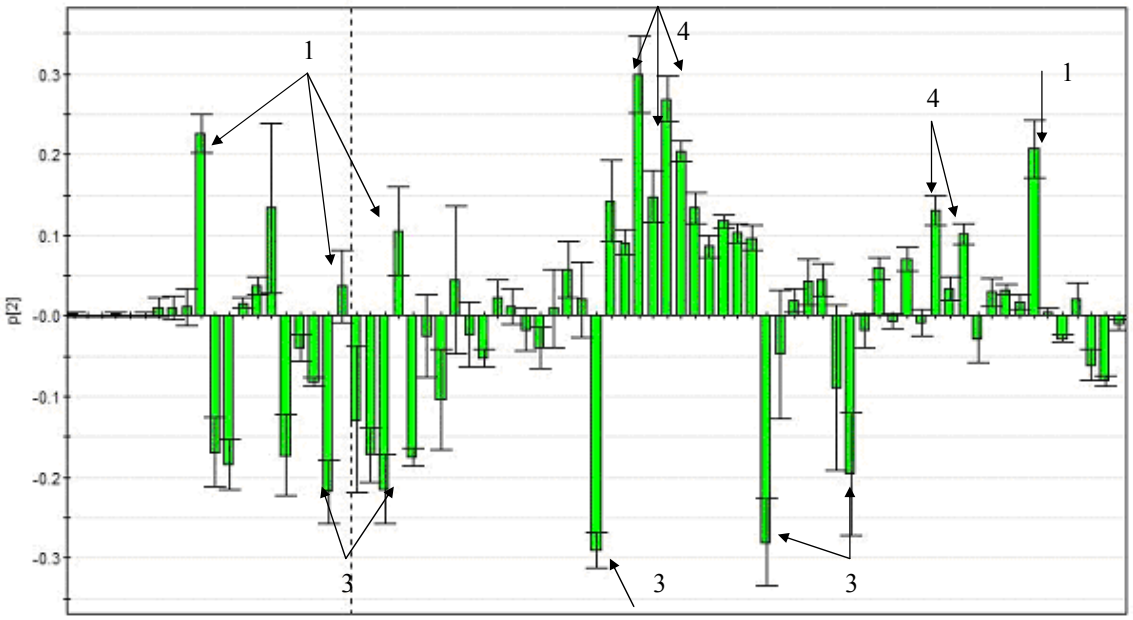


Fig. 13. Principal component analysis – loading plot for PC 2

	Metabolite	Chemical shift (ppm) and coupling constant (Hz)
1	Benzoic acid glucoside	δ 8.12 (H-2 and H-6, d, *J* = 8.0 Hz), δ 7.73 (H-4, t), δ 7.58 (H-3 and H-5, t), 5.78 (H-1', d, *J* = 8.0 Hz)
2	Benzoic acid	δ 7.90 (H-2 and H-6, d, *J* = 8.0 Hz), δ 7.49 (H-4, t), δ 7. 43 (H-3 and H-5, t)
3	Quercetin	δ 7.76 (H-2', d, *J* = 2.0 Hz), δ 7.67 (H-6', dd, *J* = 2.0, 8.5 Hz), δ 7.00 (H-5', d, *J* = 8.5 Hz), δ 6.50 (H-6, d, *J* = 2.0 Hz), δ 6.31 (H-8, d, *J* = 2.0 Hz)
4	Catechin	δ 2.54 (H-4β, dd), δ 2.84 (H-4α, dd), δ 4.10 (H-3, ddd), δ 4.69 (H-2, d), δ 5.97 (H-8, d), δ 6.04 (H-6, d), δ 6.80 (H-6', dd), δ 6.87 (H-5', d), δ 6.90 (H-2', d)
5	Myricetin	δ 7.36 (H-2' and H-6', s), δ 6.52 (H-6, d, *J* = 2.0 Hz), δ 6.30 (H-8, d, *J* = 2.0 Hz)
6	Quercitrin	δ 7.38 (H-2', d, *J* = 2.0 Hz), δ 7.35 (H-6', dd, *J* = 2.0, 8.5 Hz), δ 7.03 (H-5', d, *J* = 8.5 Hz), δ 6.50 (H-6, d, *J* = 2.0 Hz), δ 6.31 (H-8, d, *J* = 2.0 Hz), δ 5.29 (H-1''), δ 0.92 (H-6'', d, *J* = 6.2 Hz)
7	Kaempferol	δ 8.08 (H-2' and H-6', dd, *J* = 1.5, 8.0 Hz), δ 7.10 (H-3' and H-5', dd, *J* = 8.5 Hz), δ 6.48 (H-6, d, *J* = 2.0 Hz), δ 6.30 (H-8, d, *J* = 2.0 Hz)

Tab. 12 Compounds present in the analyzed cranberry juices

Sample	Characteristic
1A, 1B, 1C	Benzoic acid ↓, Myricetin ↑, Benzoic acid glucoside ↑
2A, 2B, 3A, 3B	Benzoic acid ↑ Quercetin ↑ Kaempferol ↑
4A, 4B	Benzoic acid glucoside ↑ Catechin ↑

Tab. 13. Concentration trends of differentiating compounds in the 3 groups

9.3 Discussion

9.3.1. Cranberry extract

The analysis of a cranberry extract had the pursuit of metabolite identification, using NMR. The extraction with 80% methanol and ethyl acetate allowed a concentration of the phenolic / flavonoid compounds of cranberry. There was no hydrolysis step in the extraction protocol, therefore, flavonoids could be detected in their glycosylated form.

The direct NMR-measurement of the pure cranberry extract would result in a complex and overfilled spectrum due to major peak overlap. Therefore, a Sephadex LH-20 column was chosen for the separation of the phenolic compounds, which could subsequently be observed on a TLC-plate under UV. According to the flavonoid pattern observed, the collected fractions were pooled. For the resulting 7 fractions, NMR-spectra were recorded. For Sephadex fraction 5, additional separation by HPLC followed, due to its complex ^{1}H-NMR spectrum.

The applied methods allowed the identification of the main phenolic compounds in a mixture, by measuring one- and two-dimensional NMR-spectra of the 7 Sephadex fractions obtained. The first 3 fractions contained mainly acids: benzoic acid glucoside, citric, malic and chlorogenic acid. In fractions four-seven, flavonoids were eluted and identified as rutin, quercetin-3-O-glucoside, quercitrin, myricetin, epicatechin. Moreover, other quercetin-glycosides were be detected. Further 2D-NMR measurements (HMBC) would be necessary for their exact structure elucidation.

9.3.2. Cranberry juice

Different extraction methods of the cranberry juice cocktail were performed and compared. The extraction of the neutralized juice with ethylacetate was chosen, and used for all samples, as it provided a good separation of the primary metabolites (sugar, acids) and was able to concentrate the secondary phenolic metabolites present.

^{1}H- and *J*-Resolved NMR spectra of the extracts were recorded at 600MHz, and principal component analysis was applied to the aromatic regions (5.5 – 8.5 ppm) of the obtained spectra. Three well separated clusters could be observed in the score scatter plot (Fig. 11.), originating from 4 cranberry juice samples analyzed. The best separation was obtained using principal component 1 (PC 1) vs. principal component 2 (PC 2). The formation of three groups (in contrast to four groups expected, due to four cranberry juices analyzed) might be explained by the fact, that the juice samples 2A, 2B, 3A and 3B

originated from one brand and differed only in their declared sugar content ("lighly sweetened" vs. "pure and not sweetened", see Tab. 6.). Sugars were removed by extraction with ethyl acetate and only the aromatic region of the ^{1}H-NMR spectra (5.5 – 8.5 ppm) was considered in the multivariate data analysis. Therefore, these samples formed only one cluster in the score scatter plot (Fig. 11.: group 3).

PC 1 separated group 1 from group 2 by the relatively higher concentration of benzoic acid glucoside and myricetin in the samples 1A, 1B, 1C (group 2) and the relatively higher amount of benzoic acid and catechin in the samples of group 1 (samples 4A, 4B). Principal component 2 induced a separation of group 1 and group 3. The separation criteria were the relatively higher concentration of benzoic acid glucoside and catechin in group 4 and the relatively higher amounts of benzoic acid and quercetin in group 3.

PCA was shown to be successful in differentiating the analyzed cranberry juices by in regard to their phenolic content only. Benzoic acid, benzoic acid glucoside, quercetin, and catechin were found to be the main sources of difference in the analyzed juice samples, which were revealed in the loading plot. The identification of the compounds was facilitated by the previous analysis (identification) of phenolic metabolites in a cranberry extract.

The reproducibility of the experiment was satisfactory, shown by clear clustering of the replicates. No outliers were observed. Sample 1C, an old and expired juice, was slightly, but clearly separated from the other samples from this brand (1A and 1B).

Nuclear magnetic resonance spectrometry (NMR) was shown to represent a fast and effective method for the characterization of the complex chemical composition of cranberry juice cocktail. It allowed the detection of a large number of compounds, present in a wide range of concentrations, with relatively little and fast sample preparation. Being a macroscopic method, NMR has, unlike other analytical methods, a broad scope, which allows the observation of not only the flavonoid content, but also changes/differences of other quality-contributing compounds. In this way, not only the flavonoid content could be monitored, but also adulterations, such as preservative addition could be detected. The experiments were carried out without a hydrolysis step in the protocol, therefore, flavonoid glycosides were detected, facilitated by two-dimensional NMR spectrometry. The art and degree of glycosylation significantly affect the antioxidant capacity and in this way also strongly the quality of cranberry juice and represents therefore an important factor to be well studied in the quality control of cranberry juice cocktails.

Interestingly, no proanthocyanidins (PACs) could be detected, neither in the cranberry extract, nor in any of the analyzed juice cocktails. Possible explanations might be low concentrations of the PACs compared to the relatively high detection limit of NMR spectrometry or an improvable extraction method (solvent / pH). Detection of the bioactive PACs, would be essential for the establishment of a quality control method of cranberry juice.

The applied method was successful in identification of differentiating phenolic compounds in cranberry juice cocktails, but no conclusions could be made about their impact on the quality of these juices. No definition of a “high-quality” cranberry juice cocktail, has been given yet.

10. Summary

Consumption of cranberry juice cocktails is often recommended for the prevention of urinary tract infections (UTIs), which affect mainly women in all age groups. Clinical studies show a positive correlation between ingestion of cranberry juice and a decreased incidence of UTIs. Foo *et al*. (2000) elucidated the structures of the bioactive compounds, being fructose and proanthocyanidins (condensed tannins) – phenolic secondary metabolites of American cranberry, which are responsible for the antiadhesive effect of cranberry juice against *E. coli*, the most common uropathogen. In this way becoming a health-promoting product, the quality of cranberry juice, especially regarding the active compounds (phenolic secondary metabolites) needs to be monitored.

The composition of phenolic secondary metabolites was studied in an extract of American cranberries using NMR spectrometry, with the aim to identify the main phenolic / flavonoid compounds,. The extract was subjected to a separation on a Sephadex LH-20 column, and ^{1}H-NMR spectra were recorded for the 7 resulting fractions, enabling the identification of benzoic acid glucoside, malic, citric and chlorogenic acid, rutin, quercetin-3-O-glucoside, quercitrin, myricetin, epicatechin and kaempferol.

Subsequently, cranberry juice cocktails originating from four different manufacturers were extracted and subjected to ^{1}H-NMR analysis. Principal component analysis was employed to detect differences in the phenolic / flavonoid profiles of the analyzed juice samples. The samples formed three clusters in the score scatter plot, which might be explained by the fact that two of the samples originated from one brand and differed only in the declared sugar content. Sugar was removed in the extraction procedure and only the aromatic region of the ^{1}H-NMR spectra was considered in the PCA analysis, therefore these samples form one group. The differentiating compounds between the three groups could be identified more easily on the basis of the former analysis of an American cranberry extract, as: benzoic acid glucoside, benzoic acid, quercetin and catechin.

11. Zusammenfassung

Basierend auf seiner volksmedizinischen Verwendung wird Cranberrysaft zur Prophylaxe von Harnwegsenzündungen, die vorwiegend Frauen aller Altersstufen betreffen, empfohlen. Einige klinische Studien konnten einen positiven Effekt von Cranberrysaft durch geringere Häufigkeit des Auftretens von Harnwegsinfektionen zeigen. Als wirksame Inhaltsstoffe gelten Fructose und Proanthocyanidine (kondensierte Tannine) – phenolische Sekundärmetaboliten von *Vaccinium macrocarpon* (Amerikanische Cranberry). Sie vermitteln dem Cranberrysaft antiadhäsive Eigenschaften gegen *E. coli*, dem häufigsten Erreger von Harnwegsinfektionen und unterbinden so seine Pathogenität. Cranberrysaft wird auf diese Weise zu einem gesundheitsfördernden Nahrungsergänzungsmittel, dessen Qualität, besonders hinsichtlich der bioaktiven phenolischen Sekundärmetaboliten, kontrolliert werden sollte.

Die Zusammensetzung der phenolischen Sekundärmetaboliten wurde in einem Extrakt Amerikanischer Cranberry untersucht, mit dem Ziel, die quantitativ wichtigsten phenolischen / flavonoiden Inhaltsstoffe zu identifizieren. Zur Auftrennung der Inhaltsstoffe wurde der Extrakt auf eine Sephadex LH-20 Säule aufgetragen und die daraus resultierenden sieben Sammelfraktionen wurden mittels ^{1}H-NMR Spektroskopie analysiert. Die freien Säuren Benzoesäure-Glucosid, Apfel-, Zitronen- und Chlorogensäure und die Flavonoide Rutin, Quercetin-3-O-Glucosid, Quercitrin, Myricetin, Epicatechin und Kämfperol, konnten identifiziert werden.

Anschließend wurden Cranberrysäfte, die von vier verschiedenen Herstellern stammten, extrahiert und einer ^{1}H-NMR Analyse unterzogen. Principal Component Analysis (PCA) wurde als multivariate Datenanalyse-Methode verwendet, um Unterschiede zwischen den untersuchten Proben darzustellen. Die Cranberrysaft-Proben bildeten drei Gruppen im Score Scatter Plot. Das kann dadurch erklärt werden, dass zwei der untersuchten Proben vom gleichen Hersteller stammten, und sich nur im deklarierten Zuckergehalt unterschieden. Durch die Extraktion wurde Zucker quantitativ abgetrennt und nur die aromatische Region der NMR-spektren (5.5 – 8.5 ppm) für die PCA Analyse herangezogen. Daher bildeten diese Proben eine Gruppe.

Die Strukturaufklärung der Unterscheidungsmerkmale zwischen den Cranberrysäften wurde durch die vorangegangene Analyse eines Cranberry-Extraktes erleichtert. Die untersuchten Cranberrysäfte zeigten unterschiedliche Konzentrationen von Benzoesäure-Glucosid, Benzoesärue, Quercetin und Catechin.

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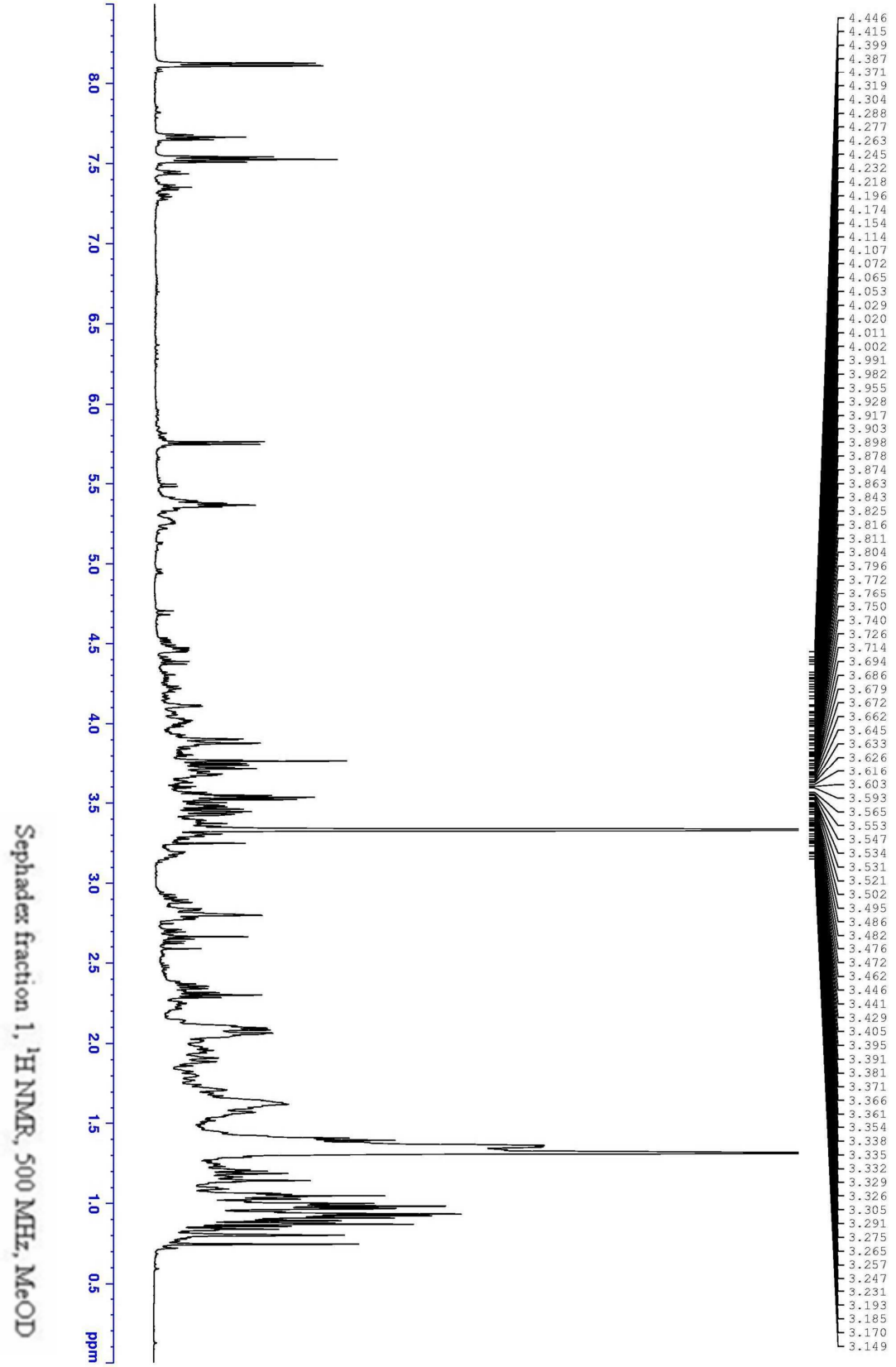
http://wisplants.uwsp.edu/scripts/bigphoto.asp?bigphoto=VACMAC_.jpg&taxon=Vaccinium%20macrocarpon%20Aiton&phog=Botanical%20Illustration&spcode=VACMAC (University of Wisconsin, herbarium)

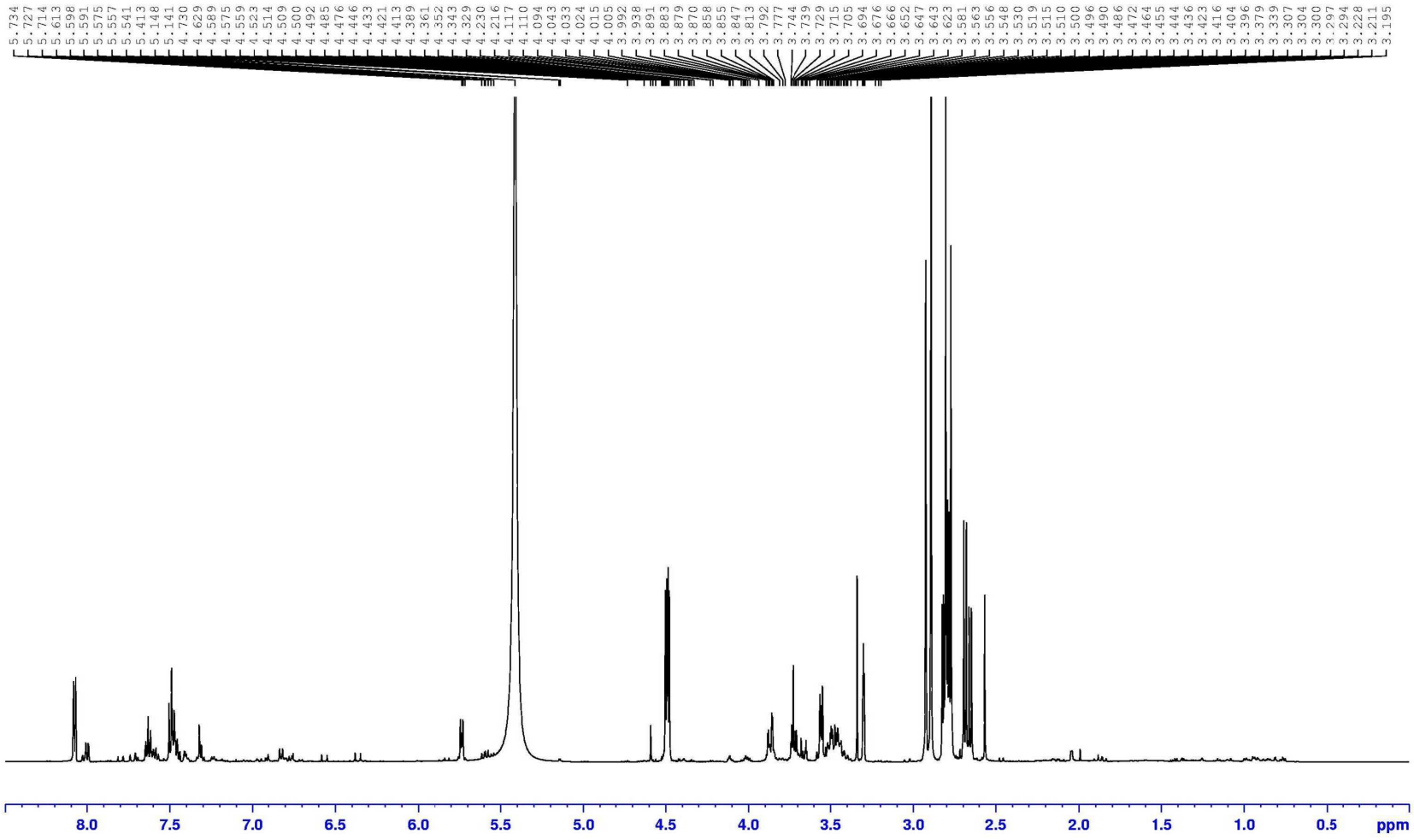
http://wisplants.uwsp.edu/scripts/maps.asp?SpCode=VACMAC

13. Appendix

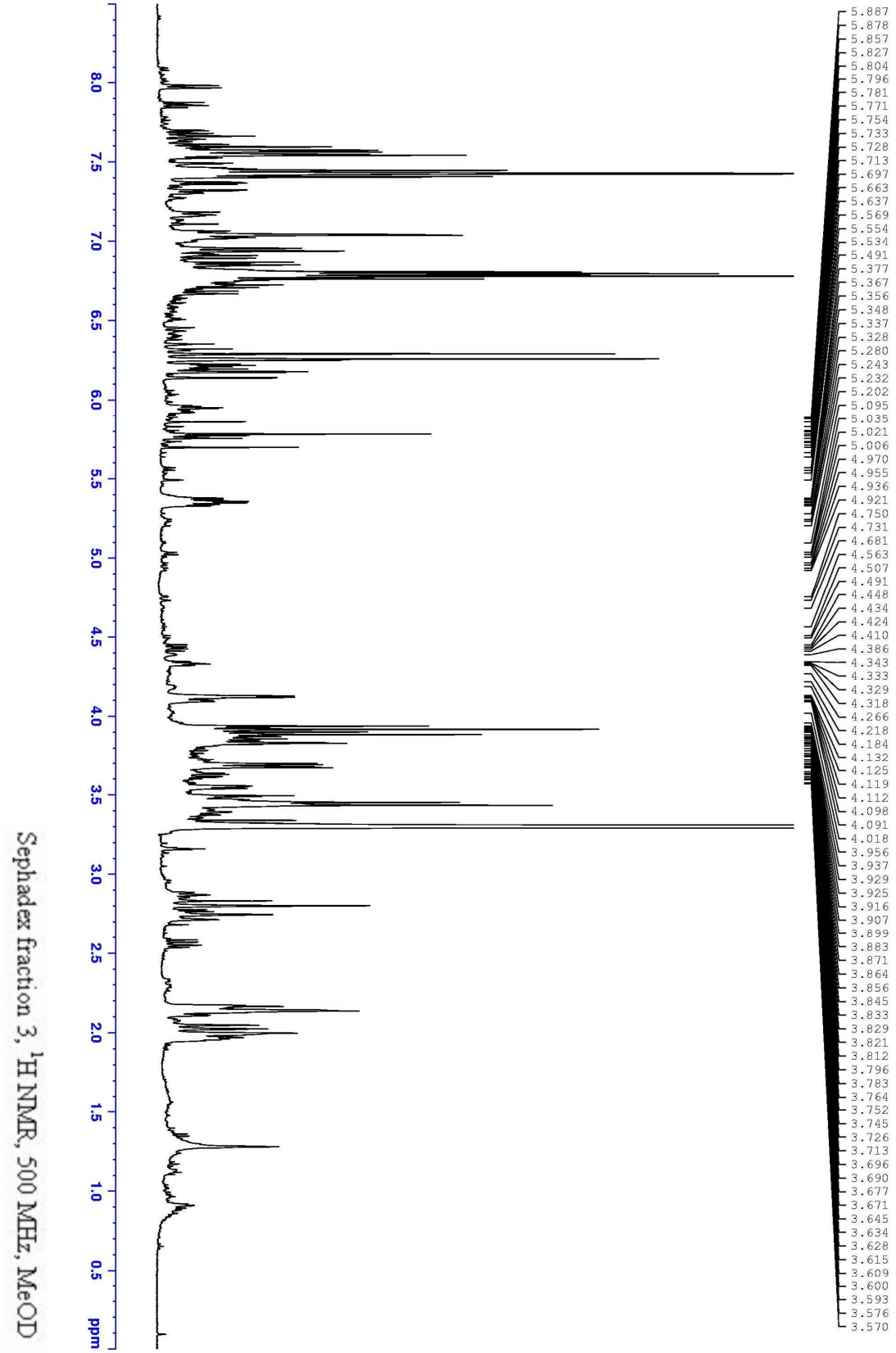
^{1}H-NMR spectra of Sephadex fractions of a *Vaccinium macrocarpon* extract (1 – 7)



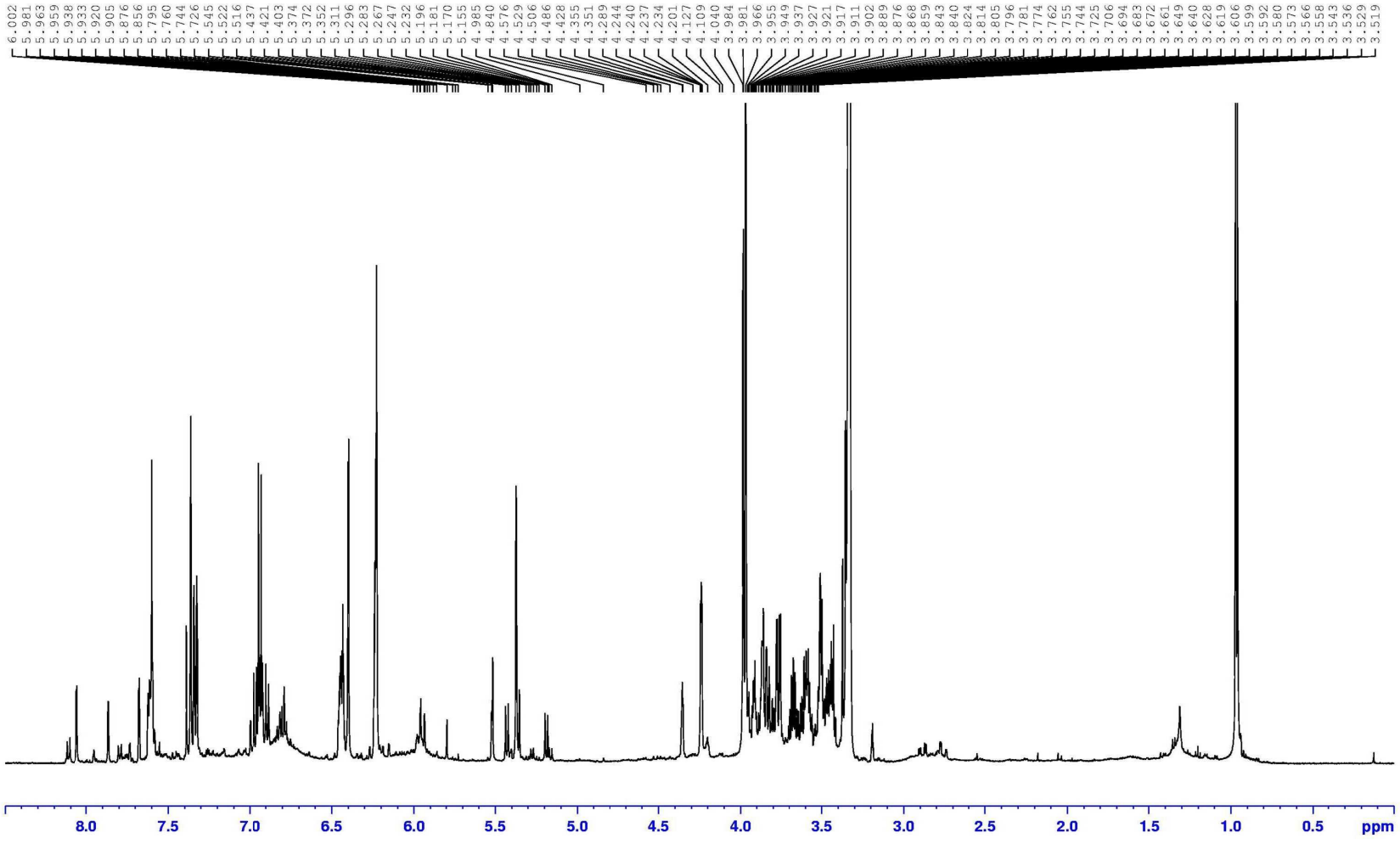
Sephadex fraction 1, ^{1}H NMR, 500 MHz, MeOD



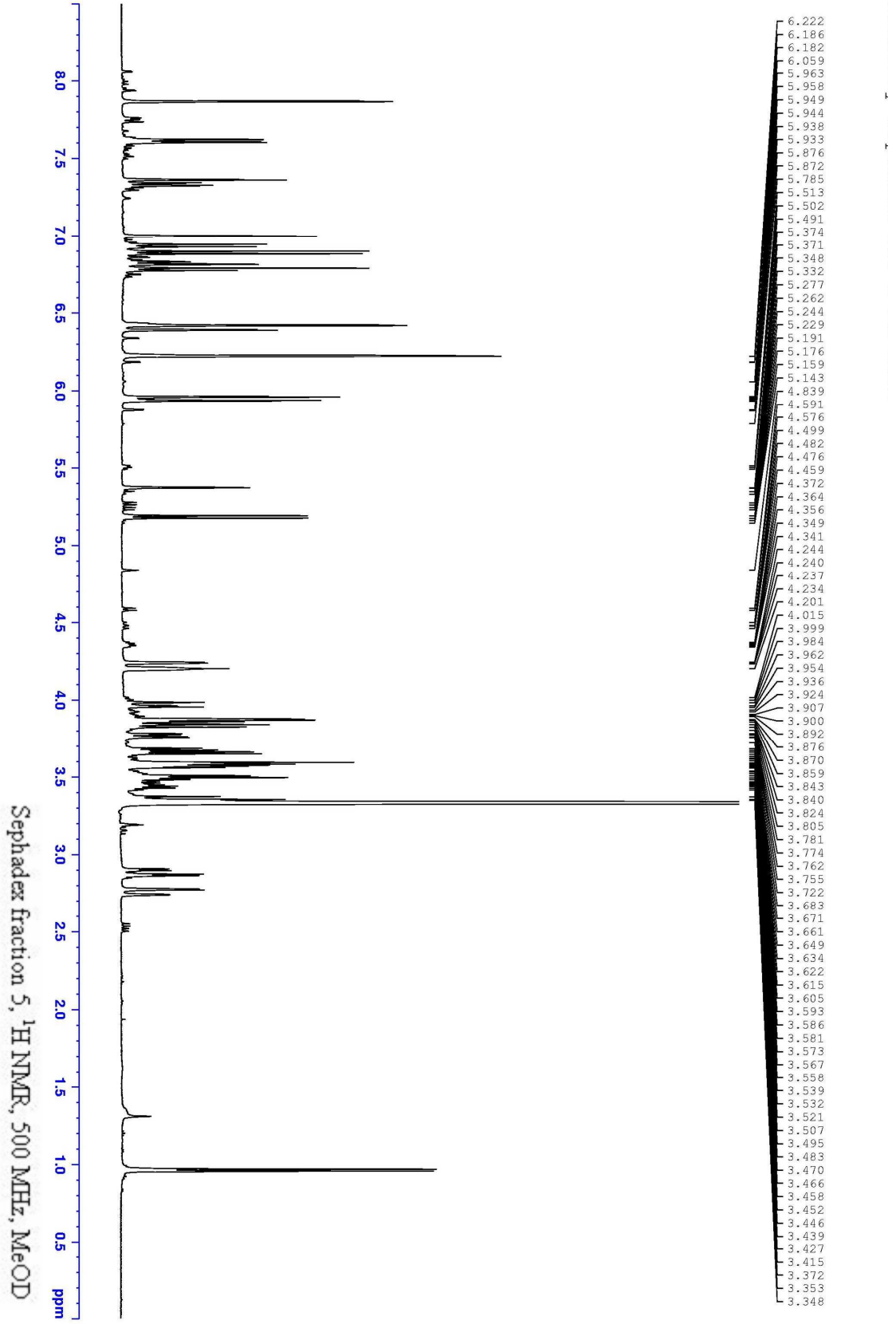
Sephadex fraction 2, ^{1}H NMR, 500 MHz, MeOD



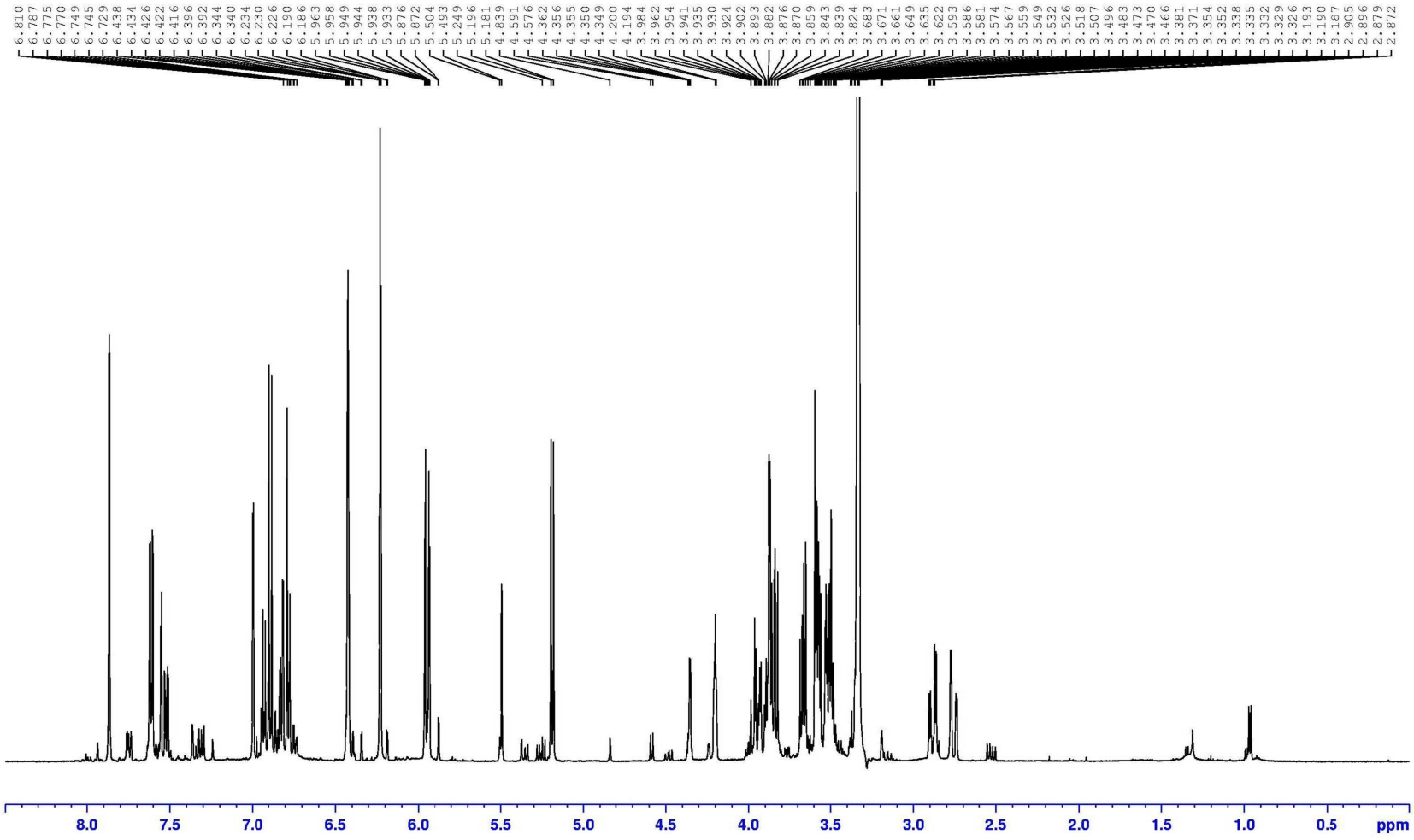
Sephadex fraction 3, ^{1}H NMR, 500 MHz, MeOD



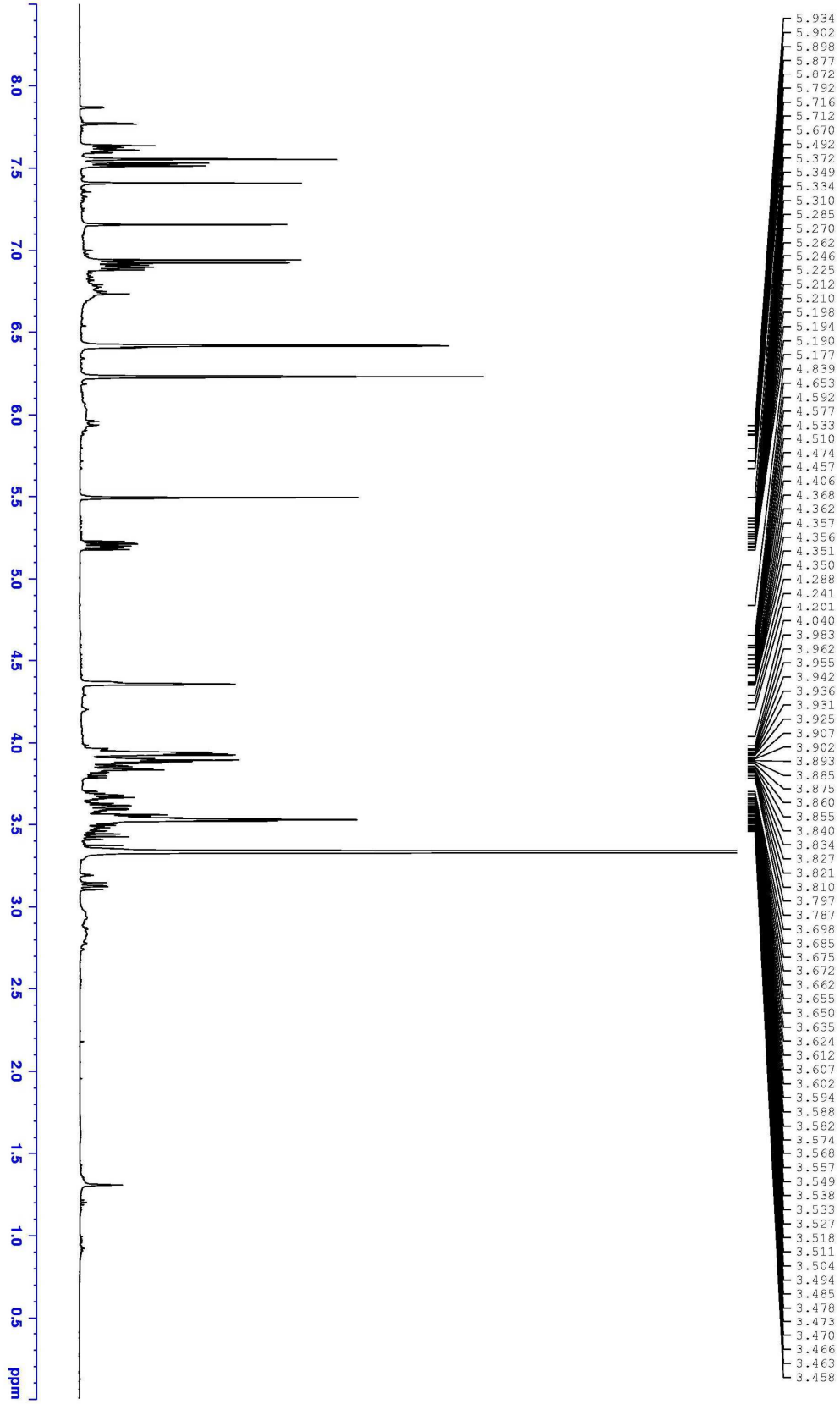
Sephadex fraction 4, ^{1}H NMR, 500 MHz, MeOD



Sephadex fraction 5, ^{1}H NMR, 500 MHz, MeOD



Sephadex fraction 6, ^{1}H NMR, 500 MHz, MeOD



Sephadex fraction 7, ^{1}H NMR, 500 MHz, MeOD

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Slowakisch	Muttersprache
Deutsch	zweite Muttersprache
Englisch	Wort und Schrift
Französisch	Grundkenntnisse
Latein	Grundkenntnisse