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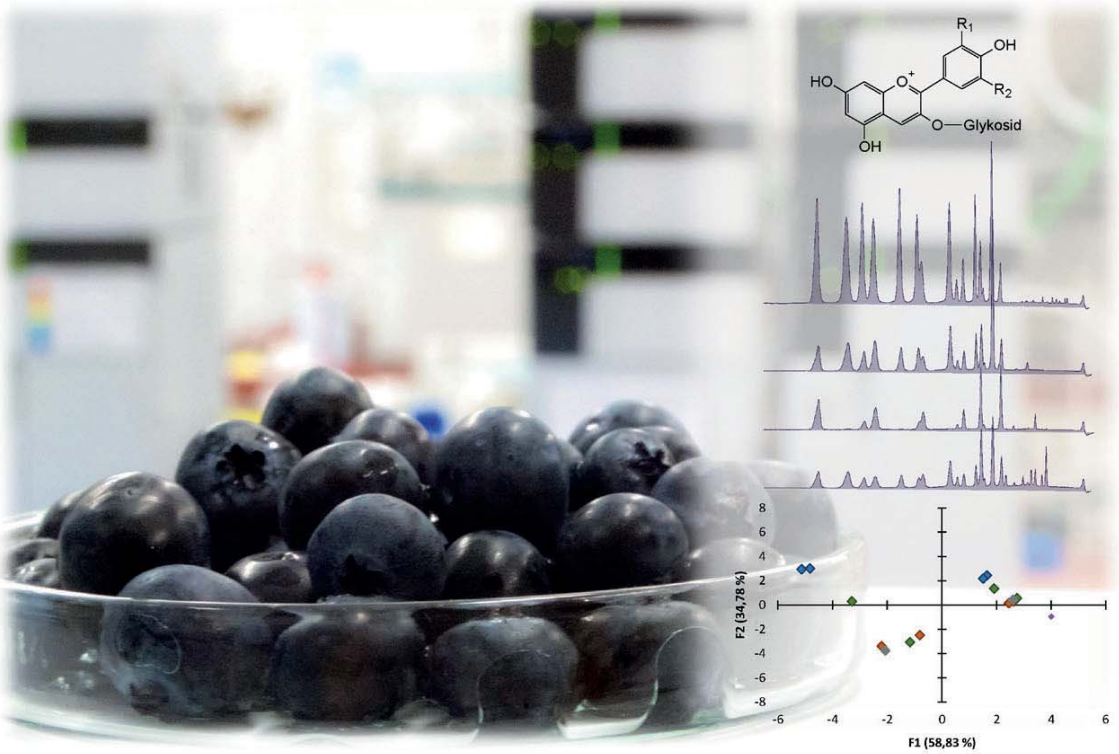
Secondary plant metabolites for authentication of *Vaccinium* L. species



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Secondary plant metabolites for authentication of *Vaccinium* L. species

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Cookery, or the art of preparing good and wholesome food, and of preserving all sorts of alimentary substances in a state fit for human sustenance, or rendering that agreeable to the taste which is essential to the support of life, and of pleasing the palate without injury to the system, is, strictly speaking, a branch of chemistry; but, important as it is both to our enjoyments and our health, it is also one of the latest cultivated branches of the science.

Friedrich Carl Accum, 1769-1838

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Preliminary remarks

List of abbreviations

ANOVA	analysis of variance
ara	arabinoside
ASE	accelerated solvent extraction
cy	cyanidin
Da	unified atomic mass unit dalton
DAD	diode array detector
DNS	dinitrosalicylic acid
dp	delphinidin
DW	dry weight
eV	electron volt
F1	first factor of a principal component analysis
F2	second factor of a principal component analysis
FW	fresh weight
g	g-force
gal	galactoside
GC	gas chromatography
glu	glucoside
HACCP	hazard analysis and critical control points
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
LC	liquid chromatography

LDA	linear discriminant analysis
lyo	lyophilized
m/z	mass-to-charge ratio
MEP	2-C-methyl-D-erythritol-4-phosphate
MS	mass spectrometry
mv	malvidin
MVA	mevalonic acid
n	number of samples
nkat	nanokatal
PBXXL	enzyme preparation pectinex BE XXL
PCA	principal component analysis
pn	peonidin
pt	petunidin
R10L	enzyme preparation Rohapect 10 L
RCL	enzyme preparation Rohament CL
rpm	revolutions per minute
RPTE100	enzyme preparation Rohapect PTE 100
RSK	Richtwerte und Schwankungsbreiten bestimmter Kennzahlen
Rt	retention time
UAE	ultrasound assisted extraction
UHPLC	ultra high-performance liquid chromatography
UV	ultraviolet
v/v	volume fraction of two liquids
v/v/v	volume fraction of three liquids

VHC	enzyme preparation Vegazym HC
VinoUFC	enzyme preparation Vinozym Ultra FCE

List of publications

Heffels, P., Bührle, F., Schieber, A., & Weber, F. Influence of common and excessive enzymatic treatment on juice yield and anthocyanin content and profile during bilberry (*Vaccinium myrtillus* L.) juice production. *European Food Research and Technology* **2017**, *243*, 59–68.

Heffels, P., Müller, L., Schieber, A., & Weber, F. Profiling of iridoid glycosides in *Vaccinium* species by UHPLC-MS. *Food Research International* **2016**, doi: 10.1016/j.foodres.2016.11.018.

Weber, F., Heffels, P., & Schieber, A. Polyphenolprofile von „Superfruits“ zur Authentizitätsbestimmung. *Flüssiges Obst* **2016**, 03, 100–104.

Heffels, P., Weber, F., & Schieber, A. Influence of accelerated solvent extraction and ultrasound-assisted extraction on the anthocyanin profile of different *Vaccinium* species in the context of statistical models for authentication. *Journal of Agricultural and Food Chemistry* **2015**, *63*, 7532–7538.

Dinkova, R., Heffels, P., Shikov, V., Weber, F., Schieber, A., & Mihalev, K. Effect of enzyme-assisted extraction on the chilled storage stability of bilberry (*Vaccinium myrtillus* L.) anthocyanins in skin extracts and freshly pressed juices. *Food Research International* **2014**, *65*, 35–41.

Conferences

Weber, F., Heffels, P., Bührle, F., Kaumans, D., & Schieber, A. Effects of thermal and enzymatic treatment on polyphenol profiles during bilberry juice production. 250th ACS National Meeting & Exposition in Boston, MA, United States, August 16-20, 2015, *Abstracts of Papers* **2015**, pp AGFD-10. [Oral presentation by Fabian Weber].

Heffels, P.; Hofem, S.; Boner, M.; Schieber, A.; Weber, F. Stabilisotopen-Verhältnisse und Polyphenolprofile als Marker zur Authentizitätskontrolle von *Vaccinium*-Spezies. 43. Deutscher Lebensmittelchemikertag in Gießen, Germany, September 22-24, 2014, *Lebensmittelchemie* **2015**, 69, 5–6. [Poster]

Heffels, P.; Weber, F.; Schieber, A. Einfluss der beschleunigten Lösungsmittelextraktion und der Ultraschallextraktion auf die Polyphenolprofile von *Vaccinium*-Spezies. Regionalverband Nordrhein-Westfalen - Tagung in Bonn, Germany, March 19, 2014, *Lebensmittelchemie* **2014**, 68, 53–57. [Oral presentation]

Heffels, P.; Weber, F.; Schieber, A. Effects of accelerated solvent extraction and ultrasonication on the anthocyanin profile of bilberry extracts. 7th INSAH Congress on Polyphenol Applications, Bonn, Germany, June 6-7, 2013, *Abstracts of Papers* **2013**, p 64. [Poster]

Heffels, P.; Weber, F.; Schieber, A. Einfluss der beschleunigten Lösungsmittelextraktion und der Ultraschallextraktion auf das Anthocyanprofil von *Vaccinium*-Spezies. 42. Deutscher Lebensmittelchemikertag in Braunschweig, September 16-18, 2013, *Lebensmittelchemie* **2014**, 68, 28-29. [Poster]

Declaration of contribution as co-author

The contribution of the co-authors to the papers presented in Chapter 2, Chapter 3, and Chapter 4 are as follows:

Prof. Dr. Andreas Schieber proofread all manuscripts and contributed to the publication as the supervisor of this thesis.

Dr. Fabian Weber advised on experimental work and supported interpretation and publication of the results. He proofread all manuscripts and was as corresponding author responsible for all formal aspects of the publications.

Franziska Bührle conducted the enzymatic juice processing and contributed to the subsequent UHPLC analysis.

Laura Müller conducted the sample preparation of bilberries for the analysis of iridoids and contributed to the subsequent UHPLC-MS analysis.

Chapter 1

General introduction

1 History of food fraud

The longing for economic success has tempted food sellers to maximize their profit margins ever since. Unfortunately, there have always been people trying to gain their profits using dishonorable strategies. Thus, food fraud is a widespread phenomenon since the beginning of food commerce. For example, in Ancient Rome, fresh food was mixed with spoilt food, expensive products were blended with inferior goods, and coloring or flavoring additives were introduced to mask impurities (Schieber, 2008). The first food frauds entailed the first regulating processes as well. In Ancient Rome and Athens, laws considering the adulteration of wine with flavors and colors were enacted (Sumar & Ismail, 1995). In medieval times, France and Germany passed food control statutes, King John in England made a decree regarding penalties for the adulteration of bread, and extensive laws were enacted by Henry III regarding the adulteration of human food. For centuries, food adulteration was common, but suitable analytical methods were missing to disclose such practice. In consequence, consumers were largely unaware of the extent of food fraud they were exposed to. In the 19th century, the German chemist Frederick Carl Accum was one of the first who elucidated the practice with his most famous book *A Treatise on Adulterations of Food and Culinary Poisons* published in 1820 (Shears, 2010). He availed the principle of "naming and shaming" by naming individual merchants, their offence and the resulting penalty. Apart from this, he described methods for detecting adulteration and can therefore be considered as one of the pioneers of food authentication. Dr. John Postgate and Arthur Hill Hassall have expedited the awareness that systems for the detection and monitoring of food adulteration were necessary. After a comprehensive investigation of foods in the British food trade, the first Food Adulteration Act was passed in 1860 and revised in 1872. The demand of food inspectors came up and in 1874 the Society of Public Analysts was founded with Hassall as its first president. Since then, the

growing knowledge about the composition of food due to advances in analytical chemistry has evolved a competition between adulterators and analysts.

2 Adulterated food today

Today, the manipulation of foodstuff can be categorized into food quality, food safety, food fraud, and food defense. Food quality and food safety reflect unintended adulterations, whereas food fraud and food defense imply intentional actions. Apart from this, a further categorization can be made considering economical aspects or the health risks of manipulation. Risks belonging to the fields of food quality and food fraud generally underlie financial aspects such as the motivation of gaining higher economic profits. Thus, a food quality risk would be the accidental bruising of a fruit which occurs due to mishandling caused, for example, by an inappropriate cost reduction in the production process. On the other hand, the addition of melamine to milk in order to pretend a high protein content with the motivation to increase the margin is considered a food fraud. In contrast to these economically motivated actions, harmful food alterations are considered in food safety and food defense. Food safety covers, for example, the unintended contamination of raw vegetables with pathogenic microorganisms due to poor protection and control during the production process. A risk of food defense is reflected by the intentional contamination of food with toxic substances with a revenge intent against a store or manager by injuring consumers (Spink & Moyer, 2011).

In order to protect consumers from all these types of food manipulation, various actions of prevention and controlling are enforced. In Germany, for example, in the first place, food business operators are responsible for the safety of the foodstuff they distribute. Via self-monitoring and quality management systems they have to ensure the compliance of established laws. This includes, among others, the application of a HACCP concept to ensure safe processing and production of foodstuff as well as the traceability of every product on the basis of labeling and documentation. This embodies the first step of food control which minimizes the occurrence of unintended food contaminations and makes a quick identification of the source of occurring problems possible. The official food control conducts the second step by a supervision of the control accomplished by the economy. Through randomized and risk-oriented controls of foodstuff and food production sites, it is intended to protect the consumers' health and information. All levels from growers and breeders via processors to vendors are covered by about one million factory inspections and approximately 400,000 analyzed food samples in Germany per year. In case of an offense of established food law, the authorities are

enforced to apply various sanctions from cautioning via penalty charge to criminal process. If foods are hazardous to health, they are not allowed to be placed on the market or have to be removed thereof.

According to an EU report, the current "top ten" products at risk of food fraud are olive oil, fish, organic foods, milk, grains, honey and maple syrup, coffee and tea, spices, wine and fruit juices (de Lange, 2013). Olive oil, for example, has superior organoleptic properties and health benefits as compared to other oils. Blending with cheaper seed oils, refined olive oil or mildly deodorized olive oils in order to draw the high profit of extra virgin olive oil are considered the main adulteration problems (Wójcicki et al., 2015). Fish underlies wrong labeling in terms of species, geographical origin or production (European Commission, 2015; Upton, 2015). Similar labeling offenses occur for organic foods if they contain conventional food products (Capuano et al., 2013). In terms of milk, valuable compounds such as milk fat are often extracted or the milk is diluted, which is often masked by the addition of various adulterants (Handford et al., 2016). Honey is controlled in regard to the compliance of sugar content and labeling the correct botanical source and geographic origin (European Commission, 2015). Coffee can show an inappropriate quality of the beans considering species, geographical origin and defective beans. Apart from this, other substances (coffee husks and stems, maize, barley, chicory, wheat middlings, brown sugar, soybean, rye, triticale, and açaí) have been added to coffee blends in order to increase profit margins (Toci et al., 2016). Tea is also diluted via the addition of foreign plant materials or inorganic matter (Dhiman & Singh, 2003), whereas spices even show harmful additives such as sudan red or other red dyes (Moore et al., 2012). Wine and fruit juices underlie the dilution with water and the addition of cheap ingredients to mask adulteration. In addition, high prized fruits are sometimes blended with cheaper species (Widmer et al., 1992; Zhang et al., 2009; Nuncio Jauregui et al., 2014). In conclusion, the main food product adulterations occur because of the dilution with inferior additives and false labeling in terms of species, geographical origin, and way of production. In order to avoid an unfair competition and ensure consumer protection, there has to be the possibility to detect these adulterations. This problem is addressed by the analytical discipline of food authentication.

3 Food authentication

One of the most important processes of food control and quality control is the detection of fraudulent practice. Food authentication fulfills this demand as its purpose is the verification of the compliance of foodstuff with the specifications of the products. Reviewed features are,

among others, the origin (species, geographical or genetic), the production method (conventional, organic, traditional procedures, free range) or the processing technologies applied (irradiation, freezing, microwave heating, enzymatic treatment) (Danezis et al., 2016). In the context of globalized food markets, which lead to an increasing product variability and availability, consumers demand reliable knowledge about the origin and composition of the food products they eat and drink. Producers are also interested in authentic commodities and food preparations used for high-value products as they have to fulfill national legislation, international standards and guidelines. In the course of expanding food markets, the scientific interest in the development of food authentication increases, too. The number of published studies on food authentication increased exponentially in the past 10 years from 409 articles between 2006 and 2008 to 907 for the period from 2012 to 2014 (Danezis et al., 2016). Its great importance is also reflected by numerous research projects funded by the German Federal Ministry for Economic Affairs and Energy and representatives of industries. The present dissertation has also arisen from such a project dealing with fruits and fruit juices, which are part of the "top ten" counterfeit products as mentioned above. It was the IGF research project 16645 N laying the foundation of the present work. Its assignment was the examination of proper authenticity markers and quality attributes for various fruits. For this purpose, more than 200 authentic fruit samples and fruit juice samples from all over the world were acquired in order to establish a valid database. On that account, the polyphenol profiles of the *Vaccinium* L. species cranberry, bilberry, and lingonberry as well as of pomegranate (*Punica granatum* L.) were investigated, which enabled a species authentication. Moreover, the geographical origin was authenticated by the analysis of stable isotopes. The ongoing topicality of food authentication is also reflected by the initiation of further projects. In 2016, a comprehensive project called "FoodAuthent" funded by the German Federal Ministry of Food and Agriculture was launched. A collaboration of the German Federal Ministry of Risk Assessment, IT specialists, and further analytical institutes aims at the development of a system for the acquisition, analysis, and utilization of food authenticity data. The modern approach of fingerprinting analysis shall deliver a database of characteristic fingerprints for various foodstuffs, which will be accessible via a cloud computing system (BfR, 2016).

4 Modern techniques detecting food fraud

There are multiple ways of testing food authenticity reflected by numerous analytical techniques and principally differing approaches. The classical authenticity assessment of food

usually follows the principle of a targeted analysis. This “bottom-up” approach implies that specific marker compounds are analyzed, which were previously defined. Subsequently, the results are evaluated on the basis of a univariate data analysis, which verifies the presence or absence of marker compounds and the compliance or violation of established limits. Combined with a great effort to validate the analytical method, the targeted analysis provides a reliable authenticity assessment. The drawback is the fact that only those adulterations can be detected which are covered by the defined target compounds. Any fraudulent practice affecting so far unconsidered compounds would remain undisclosed.

On that account, the non-targeted approach found its way into the field of food control. It derives from the principle of “metabolomics”, which describes the scientific study of small molecules, the metabolites, of a biological system. Based on a comprehensive chemical analysis, it is the aim to detect as many substances as possible (Roessner et al., 2011). After gaining popularity in the fields of pharmacy and toxicology, this “top-down” approach has also gained importance in food and feed science in recent years. The great amount of data obtained thereby has to be evaluated on the basis of a multivariate data analysis in order to receive comprehensible results. Often applied multivariate models are hierarchical cluster analysis (HCA), principal component analysis (PCA), linear discriminant analysis (LDA), and partial least squares (PLS) (Riedl et al., 2015).

The application of non-targeted analysis in food science can be categorized into the two groups of food profiling and food fingerprinting, similar to the distinction made in metabolomics (Koek et al., 2011). Food profiling reflects a hypothesis-driven approach concentrating on a limited number of compounds or a compound class. After a non-targeted analysis, compounds have to be identified and can be quantified in order to build a compound database to perform profile comparison during the authentication of further samples. For the description of the mentioned profile, usually multivariate data analyses have to be used. Advantages are a high sensitivity and selectivity due to compound specific sample preparations. Food fingerprinting renounces the identification and quantification of individual compounds and reflects a high throughput screening. If any sample preparation is applied, it is usually conducted in a simple way and all compounds, or as many as possible, are detected. Thereby, unexpected additives or deviations can be revealed. This design allows the investigation of multiple objectives in one analysis run. Yet, a good sample database is necessary for authentication based on multivariate modeling. A comprehensive overview of the non-targeted approaches is given by Esslinger et al. (Esslinger et al., 2014).

Analytes applied for the described approaches can derive from the whole compound setup of the plant or animal derived ingredients of the investigated food. However, there is another approach called proteomics, which focuses exclusively on the total protein content of a sample. Originally only used in biomedical research, proteomics has frequently been applied in food science in recent years. This approach can be categorized into three different areas. Firstly, qualitative proteomics identifies and characterizes the complete proteome or a subset of the proteins of a sample. Post-translational modifications of proteins due to a wide range of biological signals can make the proteome more complex by phosphorylation, acetylation, glycosylation or oxidation and are also covered by qualitative proteomics. Apart from this, also non-enzymatic post-translational modifications caused by food storage or food processing can affect the structure of proteins via carbonylation, thiol oxidation, aromatic hydroxylation, Maillard glycation, condensation, elimination of side chains or peptide backbone breakdown. For a proper identification of proteins, their presence in a database is necessary. The huge variety of food ingredients, which has developed due to the complexity of applied species, origins, and processing techniques, causes the risk of various proteins not being represented in the current databases. Thus, qualitative proteomics of food is a challenging approach. Secondly, quantitative proteomics is used to determine the relative amount of proteins. This quantitative profile of proteins is affected by the foodstuff composition, technological processing, and biological variability of food components, which are important aspects of food authentication. Thirdly, the functional proteomics has the aim of revealing functional interactions between proteins or between proteins and other compounds. The consequences of such interactions are interesting in order to improve food quality aspects. When applying proteomics for food authentication, either the whole protein is applied for profiling or, after protein digestion, peptides are used to verify the authenticity of food (Ortea et al., 2016). The following survey, presenting some of the most common analytical techniques used for food authentication, excludes techniques of proteomics as it would go beyond the scope of the present dissertation.

4.1 Extraction

The first step of analytical processes is usually the sample preparation including the extraction and purification of analytes. The approaches for extraction vary widely as non-instrument-based techniques (Nour et al., 2013; Zhang et al., 2012), classical techniques using Soxhlet extraction (Luque de Castro & García-Ayuso, 1998), and modern techniques like microwave-assisted extraction, supercritical fluid extraction, ultrasound-assisted extraction (UAE), and

accelerated solvent extraction (ASE) (Wang & Weller, 2006) are applied. Used for the analysis of foodstuff, all these techniques have the shared objective of liberating analytes from cellular structures. The principles of cell wall disruption and a selective solubility of analytes are used to achieve this goal. There are a lot of studies which are using these numerous extraction techniques covering the topic of detecting food fraud and food authenticity. As the principles they are based on differ greatly, the question is raised if the results of studies applying different extraction methods are comparable. This topic is covered in **chapter 2** in respect of UAE and ASE.

4.2 Analysis

The variability of commodities and compounds in food analysis entails a wide range of analytical techniques. A brief survey of the most important analytical methods applied in food authentication is given below.

4.2.1 Chromatographic techniques

The principle of chromatography is based on the adsorption and/or partition of analytes between a mobile and a stationary phase. Thus, it is a technique used for compound separation and has to be coupled with a detecting technique in order to conduct qualitative or quantitative analyses. The technique can be classified into the two groups of gas chromatography (GC) and liquid chromatography (LC). As the names imply, during GC, the mobile phase is gaseous, whereas it is liquid for LC (Schieber, 2008).

In terms of GC, analytes typically have to be easily vaporized or have to become volatile via derivatization. Thus, volatile food compounds such as food flavors (Hansen et al., 2016; Cagliero et al., 2012) and essential oils (Krupcik et al., 2015; Wong et al., 2015) are often subjected to GC analyses in the field of food authentication.

LC is an extremely versatile technique as both polar and non-polar non-volatile compounds can be analyzed. Commonly, it is referred to as high-performance liquid chromatography (HPLC). There are polar stationary phases applied with non-polar eluents (normal-phase HPLC) as well as hydrophobic stationary phases used with polar eluents (reversed-phase HPLC). Combined with an isocratic or gradient elution and a great diversity of detectors, the possible applications are numerous. The usage in the field of food authentication reaches from the analysis of amino

acids, organic acids, via triglycerides, carotenoids, tocopherols, and phytosterols to polyphenolic compounds (Schieber, 2008).

4.2.2 Spectroscopic techniques

Spectroscopic techniques applied for food authenticity analysis are UV-Vis spectroscopy, infrared (IR) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy. In terms of UV-Vis spectroscopy, both visible and ultraviolet light is applied to excite non-bonding electrons to higher unoccupied anti-bonding molecular orbitals. The compound specific absorption spectra are used for identification and quantification. IR spectroscopy deals with the infrared region of the electromagnetic spectrum, which excites the characteristic vibrations and rotations of molecular bindings. This rapid, non-destructive and cost-efficient technique is frequently applied in authenticity studies and maturity testing (Wang & Yu, 2015). NMR spectroscopy exploits the resonance frequencies of certain atoms in a molecule. This provides detailed information about the electronic structure of a molecule as the intramolecular magnetic field influences these frequencies. Primarily being a tool for structure elucidation, it is increasingly used for food authenticity purposes (Markus et al., 2014; Ogrinc et al., 2003).

4.2.3 Stable isotope analysis

Stable isotope analysis is based on the characteristic distribution of stable isotopes of hydrogen, oxygen, carbon, nitrogen, sulfur, and others due to biochemical and physicochemical effects. This technique can deliver information about the type of plant material (C_3 vs. C_4 vs. CAM), the geographical origin due to the typical distributions of stable isotopes in water and soil as well as the conditions of agricultural production (organic vs. conventional). The two analytical techniques of isotope ratio mass spectrometry (IR-MS) (Boner & Förstel, 2004; Förstel, 2007) and site-specific natural isotope fractionation nuclear magnetic resonance spectroscopy (SNIF-NMR) (Perini et al., 2014) are powerful tools, although associated with high costs for purchasing and operating.

4.2.4 Enzyme-linked immunosorbent assay (ELISA)

Enzymes can be utilized in various respects during food authentication, for example, to release specific compounds during sample preparation or as indicators of the efficiency of heat treatment. Utilized as an auxiliary reaction for quantification, they are applied in enzyme-linked immunosorbent assays (ELISA). Bound to an antibody, which binds to an antigen representing the analyte, the enzyme reaction indicates the analytes (Asensio et al., 2003).

4.2.5 Polymerase chain reaction (PCR)

The most important DNA-based method in food authentication is the polymerase chain reaction (PCR). Briefly, after the denaturation of the template DNA (95 °C), primers hybridize with the two formed single strands (50-65 °C). Finally, the elongation of the primers is conducted with a thermostable polymerase (72 °C). The manifold repetition of this cycle allows a million-fold amplification of the template DNA and enables the identification of the source material via gel electrophoresis (Kumar et al., 2014; Uncu et al., 2015).

4.3 Multivariate data analysis

The data acquired in modern food authenticity assessments are often multi-dimensional as the sample set is usually characterized by a complex marker compound profile and not only by one or two single compounds. Thus, a univariate or bivariate evaluation is not possible and the application of multivariate data analysis is necessary to identify patterns in large sets of analyzed variables. Factor analysis is one method of multivariate data analysis. It takes advantage of the phenomenon that with a growing number of variables, the chance of overlapping variables increases. From a statistical point of view, these overlaps represent correlations. High correlations between variables allow a mathematical reduction of variables without the loss of any important information of the dataset (Backhaus, 2008). In terms of authenticity, it is usually necessary to compare samples and references in regard to their authenticity marker setup representing a large set of variables. The most frequently applied techniques of multivariant data analysis using factor analysis are principal component analysis (PCA) and linear discriminant analysis (LDA), whose main differences will be briefly introduced in the following.

4.3.1 Principal component analysis

PCA calculates principal components for a dataset with the hypothesis that the complete variance of the dataset is expressed by the sum of all principal components. For a dataset described by n variables, a total sum of n principal components can be calculated. Thereby, the i^{th} principal component spans the direction of the i^{th} most variation of the dataset. This means in turn, the n^{th} principal component represents the least variation and the least importance for the description of the dataset, whereas the first principal component is most important for the description of the original dataset. Focusing on the first two or three principal components allows an interpretation of the dataset without the loss of important information as they usually

express the majority of the total variation. This reduction of factors enables the plotting of a multi-dimensional dataset as a two or three-dimensional graph and it reveals correlations between samples, between variables, and between these two. Thus, the aim of a principal component analysis is a comprehensive reproduction of a dataset with a minimum number of factors (Backhaus, 2008).

4.3.2 Linear discriminant analysis

The principle of LDA is similar to the one of PCA as both reduce the number of variables of a multi-dimensional dataset applying factor analysis. However, there are certain differences regarding the selection of the principal components. During LDA the dataset is categorized into previously defined groups prior to analysis. In terms of authentication analysis, this could be categorization according to the origin or species of samples. The questions LDA aims to answer are whether groups differ significantly and which variables are suitable for a distinction between groups. The criteria for the selection of new principal components are to separate the means of each group as far as possible while simultaneously minimizing the scatter of each group. This distinguishes LDA from PCA as the latter fulfills the criteria to maximize the variation for each principal component.

5 Berry fruits

According to the botanical terminology, berry fruits are indehiscent fruits deriving from a single ovary and show a fleshy pericarp. They contain one or often multiple seeds but no stone. Well-known berries are grapes, currants, bilberries or cranberries but also tomatoes, bananas or cucumbers belong to this type of fruits. In contrast, strawberries, blackberries or raspberries which are also commonly referred to as berries do not fulfill the botanical criteria of a berry. In fact, the appearance as a small, round, juicy, and fleshy fruit leads to the non-scientific but trivial classification because most fruits which are berries in the botanical meaning meet these characteristics as well. Fruits discussed in the present dissertation derive from plants of the genus *Vaccinium* L. and are berry fruits in terms of both the trivial and botanical definition. They are small, fleshy, red fruits which are consumed fresh, dried, processed into juices, jams or food additives. Berry fruits and berry fruit juices show a growing economic importance, which can be explained by the consumers' increased preference for healthy food (Gagliardi, 2015). The high amount of secondary plant metabolites, which are associated with various potential health benefits, let berry fruits suit the demand of a health-conscious nutrition. This

renders berry fruits to be high-value commodities for various high-priced foods, beverages and nutraceutical products. The predominant secondary plant metabolites are phenolic compounds and, in particular, polyphenols. The latter include flavonoids (e.g.: anthocyanins, flavonols, and flavanols), condensed tannins (proanthocyanidins), hydrolyzable tannins (ellagitannins and gallotannins), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), stilbenoids, and lignans (Nile & Park, 2014). Causing the typically red to purple color of most berries, anthocyanins belong to the predominant compounds in berries and will be introduced in more detail below.

5.1 Authentication of berry fruits and juices

Berry fruits and berry fruit juices are susceptible to adulterations as they yield high profits due to their high amount of value adding compounds and an increasing demand. Blending with cheaper fruits or dilution with other inferior ingredients appear to be easy ways of increasing profit margins. As a consequence, there is a special interest in the detection of fraudulent practice, which has been covered by various previous studies. Frequently, some of the above mentioned phenolic compounds like anthocyanins, flavonols, and proanthocyanidins are used for authentication purposes. A brief review of published research covering the topic of berry fruit authenticity is given in the following.

Research on Finnish bilberries (*V. myrtillus* L.) and bog bilberries (*V. uliginosum* L.) revealed a habitat dependence of the complex anthocyanin profiles, which are composed of the anthocyanidins delphinidin, cyanidin, peonidin, petunidin, and malvidin, each attached to one of the sugar moieties arabinose, galactose, and glucose. On a south-north axis of about 1000 km, southern berries showed the lowest total anthocyanin content and contained the highest cyanidin glycoside proportions, whereas northern berries were characterized by high delphinidin glycoside proportions (Lätti et al., 2008; Lätti et al., 2010). A comparison of the anthocyanin fingerprint of bilberries form Turkey and Finland showed consistency in respect of aglycones, which points to a species identification based on the aglycone pattern. On the other hand, the grouped sugar moieties of anthocyanins revealed a logistic regression model classifying 96.7 % of the investigated samples correctly into their geographical region of origin. Thus, the authentication of origin based thereon seems possible (Primetta et al., 2013). The influence of solar radiation on flavonoid synthesis in bilberry leaves was investigated as well. Increased amounts of various flavonoids in plants exposed to direct sunlight indicate a protective role of flavonoids against high solar radiation (Jaakola et al., 2004). In another study, a screening of

phenolic compounds in eighteen berries was conducted by HPLC-UV/Vis. In particular, hydroxycinnamic acids, flavonol glycosides, and anthocyanins were analyzed in berries from six families. The distribution of conjugated forms of phenolic compounds from species of the same family showed distinctive similarities. However, for anthocyanins the chromatographic profiles of conjugates and compositions of aglycones showed differences (Määttä-Riihinen et al., 2004).

A further development of the International Federation of Fruit-Juice Producers (IFU) Method No. 71 was presented, which is used to determine anthocyanin profiles by HPLC with UV detection. The method was modified by adding fluorescence detection in order to simplify the complex pattern of peaks obtained when using UV/Vis detection. It was stressed that a complete polyphenol database is of great importance for nutritionists and the food industry as health claims as well as authenticity and quality of fruit juices have to be evaluated (Obón et al., 2011; Díaz-García et al., 2013). Apart from this, profiles of anthocyanins and anthocyanidins of berry fruits, juices, fruit extracts, and food products were obtained using thin layer chromatography (TLC) and HPLC. The fingerprints were considered to establish the authenticity of fruit juices in conformity with the labeling (Filip et al., 2012).

Besides the assessment of potential marker compounds, various phenolic substances were also applied in order to differentiate species and prove the authenticity of commercial products. The procyanidin profile of various *Vaccinium* species obtained by UHPLC-MS allowed a discrimination between lingonberry (*Vaccinium vitis-idaea* L.), American cranberry (*Vaccinium macrocarpon* Ait.) and European cranberry (*Vaccinium oxycoccus* L.). This is of particular interest as the European cranberry lacks the procyanidin A-type trimers, which are responsible for the effectiveness of American cranberry against urinary tract infections (Jungfer et al., 2012). Additionally, anthocyanins were profiled by LC-DAD-MS in 14 bilberry extracts and 12 finished products labeled as bilberry. In comparison with a reference bilberry, 50 % of the investigated samples differed significantly, suggesting possible adulterations. It became apparent that 60 % of the extracts and 33 % of the supplements presented lower anthocyanin contents than declared. The profiles indicated admixtures of mulberry and chokeberry (Gardana et al., 2014). A similar study investigated 45 *Vaccinium* fruit dietary supplements. The anthocyanin profile assessed by HPLC-DAD revealed that 30 % of investigated samples did not contain the fruits listed as ingredients according to the anthocyanin profile, and multiple samples differed significantly in anthocyanin content according to the labeling (Lee, 2016).

This abstract of present research indicates the advance in berry fruit authentication. Various studies reported the applicability of phenolic compounds as marker substances which suit the authentication purposes by alignment of profiles with reference material. Most studies focus on the aspect of origin when testing the authenticity. Yet, considering potential side effects of processing on marker compounds is essential for the authenticity analyses of processed food. The necessity of these analyses is stressed by the mentioned studies showing high rates of counterfeit products available at retail. Apart from this, longing for a valid reference database becomes apparent as each study used its individual reference material.

5.2 Species of the genus *Vaccinium* L.

The present work discusses fruits of various species of the genus *Vaccinium* L., which is part of the tribe Vaccinieae in the Ericaceae family (Luby et al., 1991). The tribe Vaccinieae includes the Ericaceae with inferior ovaries and epigynous berries (Ballington, 2001). *Vaccinium* L. includes approximately 450 species and is morphologically diverse as it occurs mainly terrestrial as shrubs and lianas. The geographical occurrence is spread over all continents except of Australia, Antarctica, and most of Africa (Kron et al., 2002). They can be found primarily in cooler areas of the northern hemisphere, although there are tropical species on Madagascar and Hawaii (Su, 2012). Various popular berry species such as American cranberry (*V. macrocarpon* Ait.), the wild lowbush blueberry (*V. angustifolium* Ait.), cultivated highbush blueberry (*V. corymbosum* L.), bilberry (*V. myrtillus* L.), and the bog bilberry (*V. uliginosum* L.) belong to the genus *Vaccinium* L. Although they are known all over the world, their individual distributions are often narrow. *Vaccinium* L. species which naturally occur in northern Europe are *V. myrtillus* L., *V. vitis-idaea* L., *V. uliginosum* L., and *V. oxycoccos* L., whereas the occurrence of *V. angustifolium* Ait. is restricted to northern America (Smith, 2002; Ritchie, 1956).

Wild *Vaccinium* L. species have been harvested for thousands of years and have contributed to the human diet. Today three cultivated crops have developed from *Vaccinium* L. species and species hybrids, which have gained commercial relevance. These include: cultivated blueberries derived from species of the section *Cyanococcus* A. Gray, American cranberry deriving from *V. macrocarpon* Ait. in section *Oxycoccus* (Hill), and lingonberry (*V. vitis-idaea* L.) in section *Vitis-idaea* (Moench) Koch (Ballington, 2001). The development of cranberry and blueberry industries started in northern America in the beginning of the 19th century. Initially, the lowbush blueberry (*V. angustifolium* Ait.) was used commercially, but at the

beginning of the 20th century the domestication of highbush blueberry (*V. corymbosusm* L.) started (Lyrene et al., 2003). In 1990, blueberries were only grown in 10 countries while they were cultivated commercially in 27 countries in 2011 according to the Food and Agriculture Organization of the United Nations. Simultaneously, world production of blueberries and cranberries increased almost threefold during the last two decades from about 150 kt to 450 kt and from 200 kt to 550 kt, respectively (FAOSTAT). The United States of America and Canada rank among the major producers of these *Vaccinium* species. The pronounced increases reflect the economic importance of these crops. Species which are subject of the present study are closely related as they all belong to the genus *Vaccinium* L. Nevertheless, considerable biochemical differences caused by evolutionary processes lead to a chemotaxonomic differentiation into different sections as shown in **Figure 1-1.** These phytochemical differences primarily refer to the secondary plant metabolites and may also be utilized for authentication purposes. The individual species are presented in detail in the following.

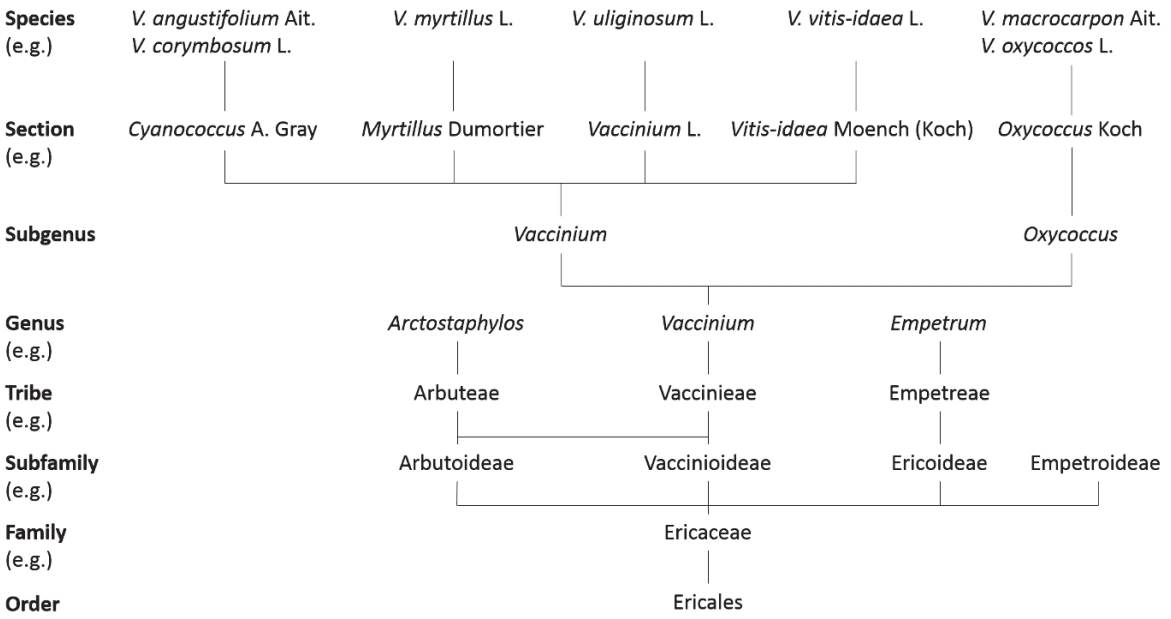


Figure 1-1: Taxonomic relations of investigated and other *Vaccinium* L. species. (USDA et al., 2015)

5.2.1 Vaccinium myrtillus L.

Vaccinium myrtillus L. is known under various names such as bilberry, European blueberry or whortleberry, to name some of them. The fruits are mainly used as coloring food additives due to their intense color ranging from dark blue to black. The appealing taste and high polyphenol content renders the berries attractive for human nutrition in the form of beverages and fruits.

They are also applied at folklore medicines (USDA et al., 2015). The distribution of bilberry ranges from Europe via the Caucasus to northern Asia and a small area in the Rocky Mountain region of the USA (Smith, 2002; Ritchie, 1956). They prefer shady places in coniferous forests as well as pine and oak woods and upland heaths (Ritchie, 1956).

5.2.2 Vaccinium angustifolium Ait.

The fruits of wild lowbush blueberry (*V. angustifolium* Ait.) are economically most important for human nutrition. Apart from this, the genetic information is used for breeding new varieties of highbush blueberries (*V. corymbosum* L.). Specifically, disease resistance, early maturity, and over all crop quality are interesting characteristics of wild lowbush blueberry. The native distributional range is focused on the extreme north-eastern USA and maritime provinces of Canada (USDA et al., 2015).

*5.2.3 Vaccinium uligi*nosum L.

The bog bilberry (*V. uliginosum* L.) is also known as bog blueberry or western huckleberry. The fruits are used for human food and their potential for cold tolerance is considered for breeding highbush blueberry (*V. corymbosum* L.). The bog bilberry is native in waterlogged upland heaths, bogs, and in tundras of northern America, Europe, and temperate regions of Asia (USDA et al., 2015; Brochmann et al., 2004).

5.2.4 Vaccinium corymbosum L.

Today a mainly cultivated species is the highbush blueberry (*V. corymbosum* L.). It is also known as American blueberry or blueberry. The fruits are popular in human nutrition and the whole plants are used for ornamental purposes. Similar to many other *Vaccinium* species, highbush blueberries are also applied in folklore medicines. Originally only native to eastern regions of northern America, the highbush blueberry is today cultivated almost all over the world and economically belongs to one of the most important *Vaccinium* crops (USDA et al., 2015).

5.2.5 *Vaccinium macrocarpon* Ait.

The American cranberry (*V. macrocarpon* Ait.) is also known as large cranberry or cranberry. The berries are used as fresh or dried fruits or processed into beverages. The occurrence of wild and cultivated American cranberry is restricted to northern America. However, eastern Europe

is emerging as an important cranberry cultivating region (USDA et al., 2015; Smolarz & Koziński, 2014).

5.3 Plant compounds of *Vaccinium* species in authentication and chemotaxonomy

Chemotaxonomy is a tool describing the relation of species in nature, whereas authentication is a tool reviewing the composition of natural products after human handling. Although the two disciplines are looking at their subjects from different points of view, both employ the knowledge about the phytochemistry of plants and compare the compound profiles with references. While chemotaxonomy focuses on the unaltered natural profiles, authentication analyses have to take into account that natural profiles may be altered due to processing effects. The mentioned profiles may refer to primary metabolites and secondary metabolites. Primary metabolites are ubiquitous in nature and of little importance for chemotaxonomic characterization. In the field of authentication, they are included in the RSK values in Germany, but are often easy to manipulate. Secondary metabolites show a rich variety and certain compounds are of limited occurrence, which renders them very useful in chemotaxonomy and authentication studies (Singh, 2016).

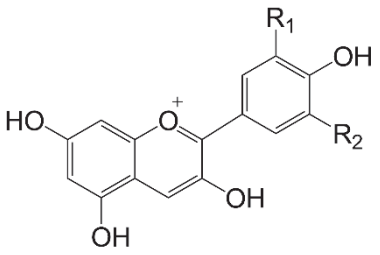
5.3.1 Polyphenols

Polyphenols are secondary metabolites widely distributed in higher plants and the subclass of anthocyanins is among others subject of the present study. Polyphenols are characterized by an aromatic phenyl ring linked to at least one hydroxyl group. Based on their structural and chemical properties, they can be categorized into five groups, which are phenolic acids, flavonoids, lignanes, stilbenes, and phenolic amides (Scalbert & Williamson, 2000; Manach et al., 2004; Tsao, 2010). *Vaccinium* species in general (Häkkinen et al., 1999a; Mikulic-Petkovsek et al., 2012) as well as bilberries and bilberry juices in particular (Buchert et al., 2005; Díaz-García et al., 2013; Laaksonen et al., 2010) show a high content of polyphenols. The most important representatives are flavonoids with the subclasses anthocyanins, flavonols, and flavanols as well as hydroxycinnamic acids of the group of phenolic acids (Gavrilova et al., 2011; Pappas & Schaich, 2009; Giovanelli & Buratti, 2009; Može et al., 2011).

Flavonoids encompass both low and high molecular weight compounds and about 10,000 molecules are known today (Cheynier et al., 2013). The general C_6-C_3-C_6 skeleton is composed of two aromatic rings (A and B) connected by a three carbon bridge, which usually forms a central heterocyclic ring C with oxygen. Various subclasses arise due to different degrees of

substitution of ring C. These are anthocyanins, flavones, isoflavones, flavanones, flavonols and flavanols. A great compound complexity within these subclasses is given by diverse substitutions of ring A and B. These include oxygenation, alkylation, glycosylation, acylation, and sulphonation (Ignat et al., 2011). Anthocyanins rank among the most important compounds in *Vaccinium* species, due to their chromophoric and value adding characteristics. Apart from this, they are frequently objects of authentication studies.

Anthocyanins are naturally occurring water-soluble dyes responsible for the intense pink, scarlet, red, mauve, violet, and blue colors in the petals, leaves, and fruits of higher plants (Jain et al., 2013). The basic structure is a 3,5,7,4'-tetrahydroxyflavylium cation (**Figure 1-2**) (Brouillard et al., 1982).



Name	**R_1**	**R_2**
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H

Figure 1-2: Chemical structures of anthocyanidins

Due to various patterns of substitution with hydroxyl and methoxy groups, a variety of different aglycones is observed in nature. The six most common aglycones in fruits are delphinidin, cyanidin, petunidin, peonidin, malvidin, and pelargonidin. Usually they are not present as a free aglycones (anthocyanidins) but bound to one or more sugar moieties.

5.3.2 Iridoids

Another group of secondary plant metabolites are iridoids. They belong to the monoterpenes and occur in the general form of a cyclopenta[c]pyran (**Figure 1-3**). The great complexity of iridoids renders them potent phytochemical marker compounds for chemotaxonomic differentiation within various plant families (Lopes et al., 2004; Jensen, 2002; Gousiadou et al., 2016).

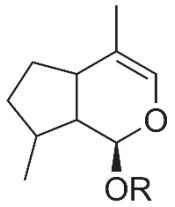


Figure 1-3: Basic structure of iridoids;
R: H or sugar moiety

However, there is only little information about the iridoid profile of *Vaccinium* species. The presence of monotropein in American and European cranberry, lingonberry, and bilberry as well as of 6,7-dihydromonotropein in cranberries and lingonberries has been reported (Jensen et al., 2002). The *p*-coumaroyl derivatives of the latter substance has also been found in American cranberry (Turner et al., 2007). Due to the limited knowledge about iridoids in *Vaccinium* L. species, they have not been considered as authenticity markers so far. The potential applicability of iridoids as marker compounds has been shown for species of the family Gentianaceae and Caprifoliaceae (Wu et al., 2016; Gao et al., 2012). The profiling of iridoid glycosides in four *Vaccinium* L. species is covered in **chapter 4** of the present dissertation.

5.4 Challenges in authenticity analysis of berry fruits and their juices

It has been shown that authentication can be conducted on the basis of secondary plant metabolites as described above. Suitable marker compounds are defined and have to be analyzed in a sample and in authentic reference materials. Qualitative and quantitative accordance of marker compounds indicates an authentic sample, whereas profile differences between an authentic reference and an unknown sample are indicative of food fraud. However, some challenges are associated with the assessment of food authenticity. One of the main difficulties is the acquisition of authentic reference material. First of all, it has to be defined what is considered to be authentic for the respective foodstuff.

In terms of the authentication of berry fruits, the selection of the correct species is crucial. Purchasing berries on the market, always entails the risk of receiving adulterated products, which excludes them from being proper reference material. In order to ensure the acquisition of authentic berries, either the berries can be obtained from trusted sources or at best their genetic identity can be verified. Having managed to obtain the correct species, further aspects have to be considered. The influence of agronomic factors has to be taken into account as they can affect the phytochemical composition of plants. It has been reported that the quantity of sugars, organic acids, and phenolic compounds of bilberries differ significantly depending on the intensity of solar radiation during growth (Mikulic-Petkovsek et al., 2015; Jaakola et al., 2004). Moreover, considerable increases in phenolic compounds were observed for various *Vaccinium* species harvested at different stages of ripeness (Prior et al., 1998). Even berries from the same plant at the same habitat and stage of ripeness may vary in their phenolic profile comparing different vintages. Each year the micro-climatic conditions may change and influence the biosynthesis of plant compounds. Thus, the phenolic compounds, which are often used as authenticity markers for berry fruits, may vary for one species depending on the habitat, maturity and vintage. As these factors are unknown for a sample which has to be authenticated, it is insufficient to acquire only a single authentic reference. In fact, in order to consider the natural variations, a comprehensive database of authentic references is necessary depicting the natural variability of marker compounds. This may be achieved either by the acquisition of multiple references or via a meta-analysis of current authenticity studies. An important consequence is the growing inaccuracy of reference data as the consideration of natural variations in the content of marker compounds leads to reference margins instead precise reference concentrations. This effect can be counteracted by expanding the group of marker compounds and applying more complex profiles for authentication.

Moreover, the application of exclusively intact berries as reference material rises another problem of authentication. It is well known that secondary plant compounds of berry fruits are affected by various post-harvest factors concerning thermal treatment. However, these treatments are necessary as berries are seasonal goods with a short harvesting period. In order to provide these perishable products throughout the year, they have to be transformed into a more durable form. In addition, the development of novel products is necessary to meet the consumers demand for a broad selection of innovative and healthy foodstuffs (Michalska & Lysiak, 2015). One of the simplest post-harvest treatments is the production of dried fruits in order to lower the moisture and, thus, to prevent microbial spoilage. Drying of blueberries at

temperatures between 70 °C and 90 °C for example, has been shown to be associated with a degradation of anthocyanins that follows a fist-order kinetics. It has been noticeable that the thermal stability of aglycones is lower compared with glycosylated anthocyanins. In addition, the less polar aglycones are more susceptible to higher temperatures, whereas more polar aglycones are more affected by longer heating times (Reque et al., 2016). These findings show that not only a decrease in the total anthocyanin content is expectable during thermal treatment but, due to the different stabilities of individual compounds, profile changes have to be considered as well. Besides quality aspects, the latter can cause issues in terms of authenticity assessments if marker profiles are affected which are used for authentication purposes.

Besides the thermal treatment of berry fruits, enzyme assisted juice production also has to be considered during authenticity analysis. While the addition of enzyme preparations during juice processing increases the juice yield and the total anthocyanin content, a degradation of individual anthocyanins has been reported for bilberries and blackcurrants (Buchert et al., 2005; Koponen et al., 2008a). Depending on the sugar moiety of anthocyanins and the enzyme preparation applied, the quantitative profile of berry anthocyanins may be altered, which might cause problems during berry juice authentication based on the respective compounds. The complexity of enzymatic preparations necessary for efficient cell wall degradation entails the risk of multiple enzymatic side activities. This renders the prediction of profile variations based on the natural profile of fresh fruits impossible. Thus, the variations caused by common enzymes applied in the fruit juice industry have to be empirically recorded and should be described in reference databases for proper authenticity analysis. The present dissertation discusses the effects of various commercial enzyme preparations on the anthocyanins profile of *Vaccinium myrtillus* in **chapter 3**.

6 Aims of the thesis

The aim of authenticity analysis is the detection of food fraud. For this purpose, food matrices are investigated in terms of their geographical origin, species, or possible adulterations. The analyses are often based on the verification of a complex compound profile. The resulting authenticity statements are based on the comparison of these compound profiles with those obtained from authentic reference material. In consequence, interference of these profiles may evoke various problems concerning the validity of analysis and false-negative authenticity statements are a possible, worst-case outcome. This includes that besides intentional fraudulent practice also inadvertent changes of the compound profiles have to be considered. The present study addresses three challenging issues throughout the authentication process of *Vaccinium* L. fruits and juices. In particular, the analysis of raw material, adulterating effects during processing, and the profiling of a potential group of marker compounds are covered.

The analysis of raw material provides results for the assessment of authenticity and has to be carefully conducted. Especially the importance of sample preparation is often underestimated, although it is fundamental for precise and reproducible analysis. A discrimination of individual compounds due to the selectivity of the applied extraction method would alter the profile used for authentication. Thereby, an authentic product might be accidentally considered non-authentic due to the application of an inappropriate extraction method. The aspect of raw material analysis is going to be considered by the application of various extraction methods and the examination of the resulting anthocyanin profiles. The significance of possible differences will be classed by comparison with a dataset of *Vaccinium* L. species from various origins.

Considering processing, unintended changes of the compound profile are caused by post-harvest factors or processing effects. Freezing, temperature and controlled atmosphere storage as well as temperature-dependent drying are post-harvest factors leading to changes on the molecular level (Michalska & Lysiak, 2015). These changes may affect the final product quality and the assessment of its authenticity if marker compounds are involved. The most important factor influencing the compound profile of fruit products is juice processing. Enzymes applied to lower the viscosity of the mash and increase the juice yield via cell wall degradation often show glycosidase side activities. They may affect other glycosidic compounds such as a variety of secondary plant metabolites which are often essential for authentication. In the present study, the effects of enzymatic treatment on the anthocyanin profiles have been evaluated. An alteration of these marker compounds entails the risk of an incorrect authenticity assessment.

As most studies dealing with these effects apply excessive dosages, the present work includes also a commercially common dosage for comparison.

Finally, a profiling of various *Vaccinium* L. species should expand the database for chemotaxonomic and authenticity considerations. The focus is laid on the substance class of iridoids, which are so far underreported in *Vaccinium* L. species. A wider knowledge of the phytochemical profile can substantiate the discriminability of closely related species.

The specific aims of the individual studies (**chapter 2-4**) are

- to analyze the influence of accelerated solvent extraction (ASE) and ultrasound assisted-extraction (UAE) on the anthocyanin profiles of lyophilized and fresh berries of *V. myrtillus*, *V. angustifolium* and *V. macrocarpon* with two different solvent compositions (**chapter 2**).
- to interrelate the variations of anthocyanin profiles depending on the applied extraction method with the natural diversity by comparison with a dataset of 26 samples of *V. myrtillus* and *V. angustifolium* (**chapter 2**).
- to characterize the alteration of the anthocyanin profile of *V. myrtillus* caused by the application of enzymatic treatment during juice processing (**chapter 3**).
- to compare the differences in the alteration of the anthocyanin profile after the application of a commercially common dosage and an excessive dosage of enzymes during juice processing (**chapter 3**).
- to investigate the qualitative and quantitative profile of iridoids of *V. myrtillus*, *V. corymbosum*, *V. angustifolium*, and *V. uliginosum* by UHPLC-MS^n (**chapter 4**).

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Chapter 2

Influence of accelerated solvent extraction and ultrasound-assisted extraction on the anthocyanin profile of different *Vaccinium* species in context of statistical models for authentication

Anthocyanins are frequently discussed as marker compounds for fruit product authenticity. Proper analysis including sample preparation for the determination of anthocyanin concentrations is crucial for the comparability of authenticity data. The present study determined the influence of accelerated solvent extraction (ASE) and ultrasound-assisted extraction (UAE), using two different solvent compositions on the anthocyanin profile of bilberries (*Vaccinium myrtillus* L.), lowbush blueberries (*Vaccinium angustifolium* Ait.) and American cranberries (*Vaccinium macrocarpon* Ait.). Besides differences in total anthocyanin concentrations in the extracts, significant deviations ($p \leq 0.05$) in the individual anthocyanin concentration were observed, resulting in differing anthocyanin proportions. Linear discriminant analysis comparing the differences caused by the extraction method with the natural differences within a set of 26 bilberry and lowbush blueberry samples of different origins was conducted. It revealed that profile variations induced by the extraction methods are in a similar scale to profile variations due to geographic and climatic differences.

Keywords: extraction, anthocyanins, authenticity, bilberry, cranberry, *Vaccinium* spp., UHPLC, linear discriminant analysis

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1 Introduction

Today's consumers have increasing preferences for healthy food and at the same time want to satisfy their curiosity and experience new tastes. Therefore, so called 'superfuits' have enjoyed increasing popularity and hence are of growing economic importance. Besides exotic fruits like açai, mangosteen and goji berry, the group of 'superfruits' also encompasses more conventional berries like bilberry and cranberry. Their popularity arises from their appealing appearance and their high antioxidant activity, which is mainly due to polyphenols including flavonoids such as flavonols, anthocyanins, and flavanols, and non-flavonoid compounds such as phenolic acids and stilbenes (El Gharras, 2009; Motilva et al., 2013). Polyphenols have been reported to impart health benefits, e.g. protection from cardiovascular disease, chronic inflammatory diseases, various types of cancers, and possibly prevent neurodegenerative diseases (Arts & Hollman, 2005; Dai et al., 2006; Hooper et al., 2008; Pan et al., 2010; Pascual-Teresa et al., 2010; Scalbert et al., 2011).

Besides numerous health benefits, phenolic compounds also show interesting technological properties, such as antioxidant and antimicrobial activities and coloring properties, rendering them attractive candidates for use as natural food ingredients. Anthocyanins, for example, are one of the most utilized plant-derived colorants. From an analytical perspective, the use as authenticity markers is an additional yet important application of polyphenols. Since products made from high-valued crops like bilberries and cranberries are a likely target of adulteration, the necessity of establishing reliable authenticity markers is evident. Polyphenol concentrations, profiles, and the presence or absence of some characteristic compounds may allow conclusions about potential adulteration of plant-derived foods, different varieties, origins and environmental conditions (Abad-García et al., 2012; Fügel et al., 2005; Häkkinen et al., 1999; Jungfer et al., 2012; Schieber et al., 2002; Zhang et al., 2009; Abad-García et al., 2012).

Filip et al. investigated the role of 17 anthocyanins and 5 anthocyanidins as marker compounds for the authenticity of juices from eight different fruits (Filip et al., 2012). Bilberry and lowbush blueberry fruits contain 15 major anthocyanins composed of the 5 anthocyanidin aglycones delphinidin (dp), cyanidin (cy), peonidin (pn), petunidin (pt) and malvidin (mv), found as 3-*O*-glycosides with attached galactose (gal), glucose (glu), and arabinose (ara) moieties (Cho et al., 2004; Kalt et al., 1999). Cranberry fruits show a less complex profile, which includes six major

anthocyanins composed of two anthocyanidins (cy and pn) attached to three sugar moieties (gal, glu and ara), respectively (Pappas & Schaich, 2009).

Although the anthocyanin profiles of wild bilberries from Finland and Turkey do not differ significantly, these fruits could be differentiated according to their origin by analyzing the sugar moiety ratios of anthocyanins (Primetta et al., 2013). American cranberry (*Vaccinium macrocarpon* Ait.) is mostly recognized for its health benefit to prevent urinary tract infections (Leahy et al., 2002). This effect is attributed to the high concentrations of A-type procyanidins, which are also used for authentication purposes. The differentiation of cranberry from other *Vaccinium* species can therefore be accomplished by analyzing the procyanidin profile. Especially the absence of A-type procyanidin trimers in European cranberries (*Vaccinium oxycoccus* L.) allows their differentiation from American cranberries (Jungfer et al., 2012). In addition, the anthocyanin profiles of cranberry have also been applied as authenticity markers (Obón et al., 2011).

Whereas methods for the separation and detection of anthocyanins are established and usually well reported, sample preparation including the different extraction techniques vary considerably, and frequently their description lacks detail. Besides classic approaches like Soxhlet, liquid-liquid and solid-liquid extractions, some new extraction techniques have emerged in the last decades. These include ultrasound-assisted extraction (UAE), microwave-assisted extraction and accelerated solvent extraction (ASE) (Ignat et al., 2011; Wang & Weller, 2006). The great variety of extraction methods inevitably raises the question of comparability of the corresponding results. This problem is addressed in the present study by comparing the influence of accelerated solvent extraction and ultrasound-assisted extraction on the profile of anthocyanins in bilberry, lowbush blueberry, and American cranberry extracts of lyophilized berries. In addition, fresh samples of the named fruits were investigated applying ultrasound-assisted extraction. Finally, the established method influences on the anthocyanin profiles were interrelated with the natural profile diversity by comparison with a data set of 26 bilberry and blueberry samples using a linear discriminant analysis. It should be emphasized that the scope of the study was the assessment of the anthocyanin profiles and not the optimization of anthocyanin yields.

2 Materials and methods

2.1 Chemicals

Ultrapure water was obtained from a Synergy purification system (Millipore, Molsheim, France). HPLC grade acetonitrile and acetic acid were from VWR (Mannheim, Germany). Formic acid (≥ 98 %) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol was from Th. Geyer (Renningen, Germany). Diatomaceous earth (Chromabond XTR), and regenerated cellulose filters (Chromafil RC-20/15 MS) were supplied by Macherey-Nagel (Düren, Germany). Malvidin 3-*O*-glucoside (≥ 87 %) was obtained from Phytoplan (Heidelberg, Germany).

2.2 Samples

Wild bilberries (*Vaccinium myrtillus* L., n = 21) and lowbush blueberries (*Vaccinium angustifolium* Ait., n = 5) were mainly collected manually in Germany and Poland between 2013 and 2014 or harvested in Ukraine and Canada between 2009 and 2014 and provided by Rudolf Wild GmbH & Co. KG (Eppelheim, Germany), Faethe Labor GmbH (Paderborn, Germany), and Haus Rabenhorst O. Lauffs GmbH & Co. KG (Unkel, Germany). American cranberries (*Vaccinium macrocarpon* Ait., n = 1) harvested in the USA in 2012 were obtained from Haus Rabenhorst O. Lauffs GmbH & Co. KG (Unkel, Germany). All samples were stored at -20 °C until freeze-drying or extraction.

2.3 Freeze-drying protocol

Freeze-drying was conducted using an Alpha 2-4 LSC freeze-dryer (Christ, Osterode, Germany) at 70 mbar for 3 days with a condenser temperature of -90 °C.

2.4 Extraction methods

2.4.1 Sonication

Fresh berry material (6 g) or lyophilized berry powder (1.2 g) were filled in centrifuge tubes in three replicate samples and treated with 20 mL extraction solvent consisting of methanol/water/acetic acid (A: 20/75/5, B: 80/15/5 v/v/v). The suspension was homogenized with an Ultra-Turrax and afterwards placed upright into a sonication bath (1100 W) for 10 min.

Subsequently, the samples were centrifuged at 10,947 g for 10 min. and the supernatant was decanted. The residue was extracted again under the same conditions excluding the homogenization step with 10 mL extraction solvent. Finally, the supernatants were pooled and made up to 50 mL with water. This solution was used for UHPLC analysis after membrane filtration (0.2 µm) with regenerated cellulose.

2.4.2 Accelerated Solvent Extraction

Lyophilized berry powder was extracted using an accelerated solvent extractor model ASE 350 (Thermo Scientific Inc., Braunschweig, Germany). For this purpose, 0.2 g lyophilized berry powder was mixed with diatomaceous earth and packed into a 10 mL stainless steel extraction cell. The cells were prepared with two cellulose filters on the bottom and top of the cells. The same extraction solvents as mentioned before were used and each sample was extracted in triplicate. Extraction parameters were 5 min static time, 3 cycles, 40 °C and 100 % flush volume. Finally, all extracts were adjusted to 25 mL with water and used for UHPLC analysis after membrane filtration with regenerated cellulose.

2.5 UHPLC-MS identification of anthocyanins

The identification of anthocyanins was conducted on a Waters Aquity I-Class system (Milford, MA, USA) coupled with a Thermo Scientific Inc. LTQ-XL ion trap mass spectrometer (Braunschweig, Germany). The column was a Kinetex C-18, 1.7 µm particle size (150 mm x 2.1 mm) (Phenomenex Inc., Aschaffenburg, Germany) equipped with a security guard cartridge of the same material (2.1 mm x 5 mm; 1.7 µm). At a flow rate of 0.4 mL/min the following gradient was used: 0 min, 4 % B; 4 min, 4 % B; 8 min, 4.5 % B; 14 min, 7 % B; 20 min, 15 % B; 25 min, 30 % B; 25.1 min, 100 % B; 27.9 min, 100 % B; 28 min, 4 % B; 32 min, 4 % B, where eluent A was water/formic acid (95/5, v/v) and eluent B was acetonitrile/formic acid (95/5, v/v). The experimental conditions of the MS were as follows: The capillary was set to a temperature of 300 °C during positive ESI-ionization and a voltage of 180 V. Source voltage was maintained at 4 kV at a current of 100 µA. The tube lens was adjusted to 110 V. Nitrogen was used as sheath, auxiliary, and sweep gas at a flow of 45, 10, and 3 arb. units, respectively. Collision-induced dissociation spectra were obtained at 35 eV using helium as the collision gas.

2.6 UHPLC-DAD quantification of anthocyanins

Anthocyanin quantification was performed on a Nexera UFLC system (Shimadzu, Kyoto, Japan) equipped with two high pressure gradient pumps LC-30AD, a degasser DGU-20A5R, an autosampling unit SIL-30AD (cooled to 10 °C, injecting 2µL), a column oven CTO-20AC (at 40 °C), and a diode array detector SPD-M20A. The column, gradient and solvent were the same as for the identification. Anthocyanins were detected at 520 nm and quantified as malvidin 3-*O*-glucoside equivalents by external calibration. The mean standard deviations of all triplicate determinations ranged from 1 to 10 % and were therefore considered acceptable for repeatable and precise quantification (**supporting information, Table 1 & 2**). Total anthocyanin content was calculated as the sum of individual anthocyanins.

2.7 Statistics

For statistical and discriminant analysis the software XLSTAT was used. An ANOVA with Dunn-Sidak post-hoc test due to heterogeneity of variances was performed to determine significant differences. The level of significance was defined as $p \leq 0.05$.

3 Results and discussion

Since accelerated solvent extraction and ultrasound-assisted extraction have overcome most problems of conventional extraction methods like Soxhlet and maceration, they are of increasing importance for the extraction of bioactive compounds like polyphenols for further analysis (Azmir et al., 2013). UAE is based on the principle of cavitation, which means the production, growth, and collapse of bubbles in the extraction solvent and sample material (Doktycz & Suslick, 1990). In contrast, ASE utilizes high pressure in order to keep solvents liquid above their boiling point (Richter et al., 1996), which facilitates the extractability of the analytes. Among the broad range of extraction techniques, UAE and also ASE were extensively reviewed by Azmir et al. According to these authors, both methods are applied for the extraction of a broad range of different polyphenols. In particular, the extraction of anthocyanins from berry fruits was shown by Monrad et al. and Santos et al. (Monrad et al., 2010; Santos et al., 2012)

The extraction conditions chosen in the present study are based on applications of ASE and UAE reported for polyphenol extraction. Briefly, both polar (Arapitsas et al., 2008; Lätti et al., 2008) and unpolar (Müller et al., 2012; Nicoue et al., 2007) solvents had been applied for the extraction of anthocyanins. Furthermore, acidified solvents were used due to the higher anthocyanin stability and extractability at low pH values (Kalt et al., 2000). Accordingly, two acidified extraction solvents with different polarities were applied in this study. Preliminary work had revealed no significant influence of the extraction time on the target analytes during ASE and thus the extraction time for UAE was adjusted to the total time of the ASE methods (approx. 25 min). Besides the extraction of lyophilized berry material, also the application of fresh berries had previously been reported and was therefore investigated as well using ultrasound assisted extraction as the manufacturer suggested applying only dry sample materials in ASE. As reference extraction conditions the application of UAE with 80 % methanol on fresh berries was chosen, with the purpose to retain the anthocyanin profile as natural as possible by using unprocessed berries. Moreover, sonication baths are more common than accelerated solvent extractors and the majority of publications showed that less polar solvents are most efficient for the anthocyanin extraction.

For fast and reliable quantification of anthocyanins, a UHPLC-DAD method was developed which is capable of separating 15 individual anthocyanins from bilberry fruits within 20 minutes and requires only 8 mL of solvent per chromatographic run. Conventional HPLC

methods are either not suitable for the separation of all anthocyanins present in bilberries or require more time and solvent (Müller et al., 2012; Juadjur & Winterhalter, 2012; Prior et al., 2001). In addition, so far only few UHPLC-DAD methods have been reported which show similar separation capabilities (Díaz-García et al., 2013; Yamamoto et al., 2013).

3.1 Anthocyanin content of bilberry and lowbush blueberry in UAE and ASE extracts

The 15 anthocyanins were individually quantified after application of two different extraction techniques and extraction solvents in order to determine the influence of the extraction technique (ultrasound assisted extraction *vs.* accelerated solvent extraction) and solvent composition (20 % *vs.* 80 % acidified methanol) on the yields. The anthocyanins were identified according to their absorption spectra (Faria et al., 2005), order of elution (Müller et al., 2012) (**Figure 2-1**), and by UHPLC-MS experiments. For this purpose masses and fragmentations of anthocyanins previously reported were used (Wu & Prior, 2005). For bilberries, the contents of

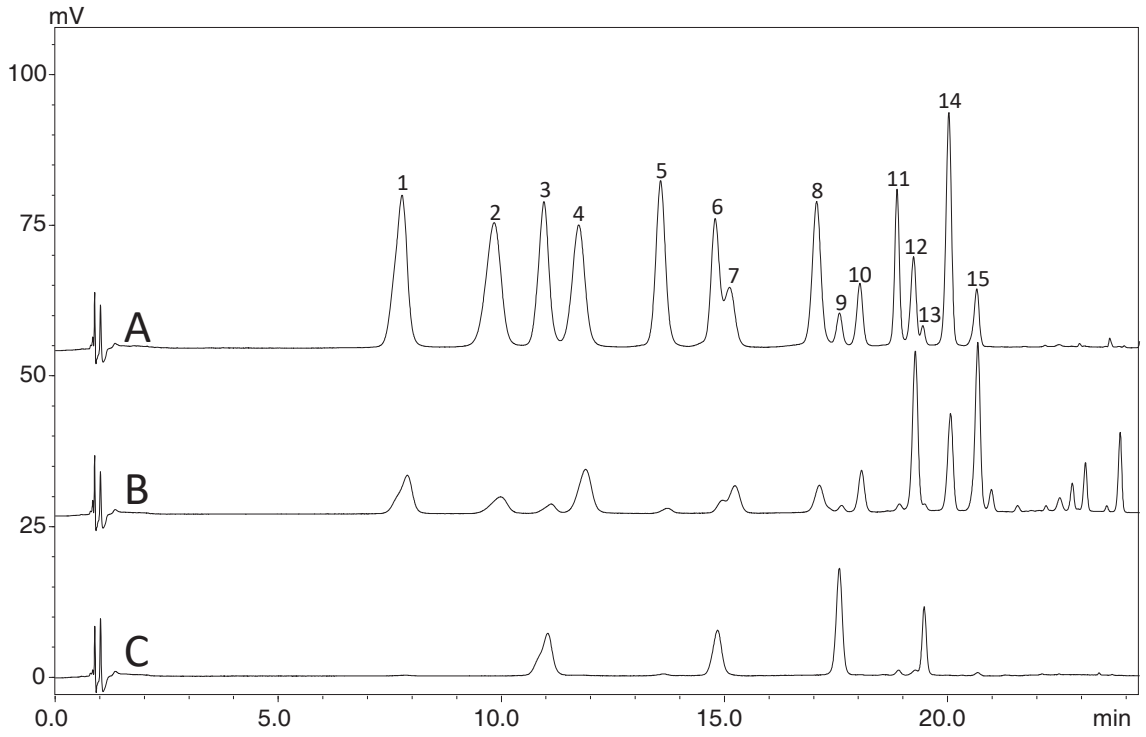

Figure 2-1: UHPLC-DAD chromatogram (520 nm) of the fruit extracts under reference conditions (80 % methanol, fresh berries, UAE): **A**: bilberry, **B**: lowbush blueberry and **C**: American cranberry; 1: dp-3-gal, 2: dp-3-glu, 3: cy-3-gal, 4: dp-3-ara, 5: cy-3-glu, 6: pt-3-gal, 7: cy-3-ara, 8: pt-3-glu, 9: pn-3-gal, 10: pt-3-ara, 11: pn-3-glu, 12: mv-3-gal, 13: pn-3-ara, 14: mv-3-glu, 15: mv-3-ara

total anthocyanins ranged from 15.9 mg/g DW to 200.1 mg/g DW and from 4.5 mg/g DW to 26.2 mg/g DW for lowbush blueberries, respectively (**supporting information, Table 1**). These results are in accordance with previously published concentrations (Lätti et al., 2008; Nicoue et al., 2007; Dinkova et al., 2014).

The wide range of concentrations are related to differing extraction conditions. For bilberry the total amount of anthocyanins using 80 % methanol as extraction solvent is significantly higher than the corresponding amounts after application of 20 % methanol. For lyophilized berries, ASE yields significantly higher anthocyanin amount than the corresponding UAE method. As expected, the anthocyanin contents of lowbush blueberries are considerably lower than those of bilberries. The total anthocyanin contents after extraction of lyophilized berries with 20 % methanol and 80 % methanol are about two-fold higher using ASE compared with UAE, respectively. Regarding the extraction solvent, the extraction with 80 % methanol leads to higher total anthocyanin amounts than the respective extraction with 20 % methanol.

3.2 Differences in anthocyanin profiles of bilberries

Anthocyanin profiles indicated by the percentage of each compound contributing to the sum of all anthocyanins were used to compare the different extraction methods (**supporting information, Table 3**). In order to interrelate those methods, correlation coefficients considering the whole anthocyanin profile of all 15 anthocyanins were calculated leading to high correlations between 0.995 and 0.976. To visualize how the different extraction conditions influence the individual anthocyanins, spider web plots were used. They efficiently reveal the effects of different extraction solvents, techniques and materials. **Figure 2-2** illustrates these effects for all individual anthocyanins in bilberry, whereby the aglycones are arranged according to their polarity (dp > cy > pt > pn > mv). It is obvious that the anthocyanins are not affected equally. Although the overall profiles are similar as reflected in good correlation coefficients and the variation of the extraction methods show only little effect especially on less polar anthocyanins, more polar anthocyanins show significant variances. Apparently, the delphinidin glycosides as well as cyanidin glucoside and arabinoside are affected most. In particular, the application of 80 % methanol leads to higher delphinidin glycoside proportions whereas cyanidin glycoside proportions are lower compared with proportions after the usage of 20 % methanol as extraction solvent. Besides these compounds, which are altered considerably in proportions, other anthocyanins are not subjected to method induced profile variations. Especially cyanidin and malvidin galactosides as well as petunidin and peonidin arabinosides

are not affected significantly by any extraction conditions. It is interesting to note that comparing the results of different extraction conditions with the reference method (80 % methanol, fresh berries, UAE) a glycoside-type derived pattern can be found. Almost all extraction conditions lead to higher proportions of anthocyanin glucosides compared with the reference methods, whereas arabinosides and galactosides tend to show lower relative amounts (**supporting information, Table 3**).

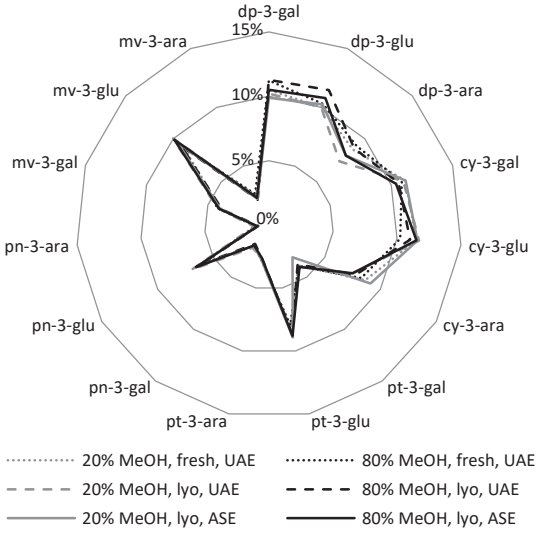

Figure 2-2: Anthocyanin proportions of bilberry after different extraction methods. UAE: ultrasound assisted extraction, ASE: accelerated solvent extraction, fresh: fresh berries, lyo: lyophilized berries

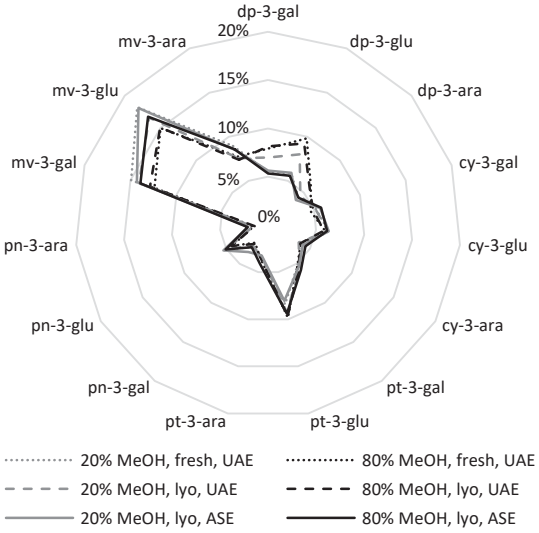

Figure 2-3: Anthocyanin proportions of lowbush blueberry after different extraction methods. UAE: ultrasound assisted extraction, ASE: accelerated solvent extraction, fresh: fresh berries, lyo: lyophilized berries

Obviously, the changes in the anthocyanin profile as affected by the extraction method are inconsistent and show different variabilities. Moreover, a trend toward greater variabilities in terms of more polar anthocyanins of bilberries can be observed. Consequently, the differing patterns exhibit that profile changes are caused by different extraction techniques (ASE vs. UAE) as well as by different solvent composition (20 % vs. 80 % methanol).

3.3 Differences in anthocyanin profiles of lowbush blueberries

The anthocyanin profiles of lowbush blueberries were used to interrelate the effects of all six extraction methods. They showed good correlations between 0.906 and 0.998 which means that the anthocyanin profiles are very similar. However, the variabilities of all individual anthocyanins are not homogeneous, meaning that all anthocyanins are individually affected (**Figure 2-3**). While dp glycosides reveal significantly higher proportions after UAE compared to ASE, all other anthocyanins are either not affected or show lower proportions. Regarding equal extraction techniques with different solvents, it can be stated that in terms of ASE the pt glucoside proportion is significantly higher and mv glucoside significantly lower when 80 % methanol is used instead of 20 % methanol. In contrast, the application of 80 % methanol in UAE leads to higher dp glycoside and lower mv glycoside proportions than the usage of 20 % methanol. The extraction of fresh berry material shows the same trend to a greater extent considering the extraction solvent effects. These results reveal that individual anthocyanins are differently affected by different extraction methods. Apparently, dp as the most polar and mv as the most abundant aglycones are affected most in lowbush blueberries as the variability between all extraction methods reveals. In contrast proportions of all other aglycones seem to be much more robust against method variations (**supporting information, Table 3**).

Interestingly, the individual differences of lowbush blueberry anthocyanins are not in line with those of the individual differences determined for bilberry anthocyanins. Obviously, profile variations due to differing extraction methods are plant depending and not generally consistent and, thus, not predictable.

3.4 Anthocyanin contents of American cranberry in UAE and ASE extracts

Six major anthocyanins in American cranberry were identified by their absorption spectra, order of elution, and characteristic fragmentation patterns (Wu & Prior, 2005). They were individually quantified after application of the two extraction methods and solvents. The contents of total anthocyanins range from 1.51 – 2.20 mg/g DW, which is in accordance with the results described by Pappas and Schaich (Pappas & Schaich, 2009) (**supporting information, Table 2**).

3.5 Differences in anthocyanin profiles of American cranberry after ASE and UAE

The application of ASE and UAE with different extraction solvents results in very similar anthocyanin yields whereas slightly higher contents were obtained via ASE and 80 % methanol.

Even the anthocyanin profiles are very similar indicated by very high correlation coefficients between 0.994 and 0.997 (**Figure 2-4**). Nevertheless, the post-hoc analysis shows significant differences due to very low standard deviations of each method. When 80 % methanol is used

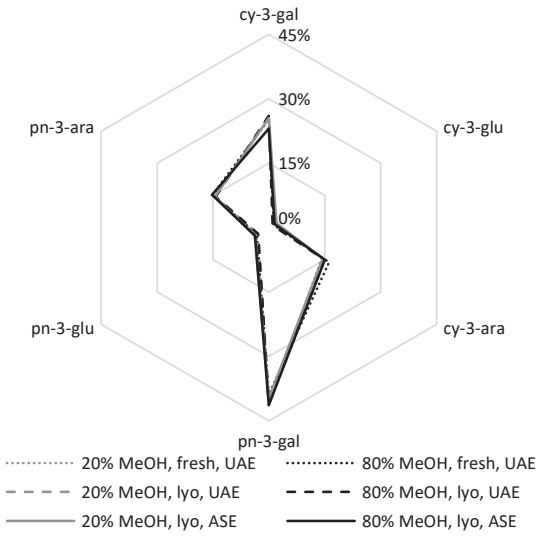

Figure 2-4: Anthocyanin proportions of American cranberry after different extraction methods. UAE: ultrasound assisted extraction, ASE: accelerated solvent extraction, fresh: fresh berries, lyo: lyophilized berries

instead of 20 % methanol, a significant decrease in the cy galactoside proportion during ASE, and an increased cy arabinoside proportion via UAE can be observed. These differences can only be noticed for fresh fruits. The anthocyanin profile of lyophilized berry material is not affected by extraction solvent using UAE. Therefore, the effects of the investigated extraction methods on the anthocyanin profile of American cranberry seem to be negligible.

3.6 Differences in anthocyanin profiles of bilberries and lowbush blueberries from various origins

Since authenticity data is often based on specific profiles of individual compounds, the acquisition of the corresponding data has to be reproducible and should ensure a good comparability. Several studies investigated whether there are typical fingerprints according to varieties (Müller et al., 2012), geographical origins (Primetta et al., 2013; Åkerström et al., 2010; Rieger et al., 2008), and climatic conditions (Jaakola et al., 2004). Some of these authors showed that botanical differences and external factors could be correlated to ratios of certain individual compounds. It could be of great interest to interrelate such findings performing a meta-analysis. However, the comparison might be critical, since different extraction methods were applied. In order to classify the profile variations induced by different extraction methods, 21 bilberry (*V. myrtillus*) cultivars from Germany, Poland, and Ukraine as well as 5 lowbush blueberry (*V. angustifolium*) cultivars from Canada were analyzed. The average total anthocyanin content in bilberries from Germany, Poland, and Ukraine was 6.0 ± 0.3 mg/g FW, 6.4 ± 0.8 mg/g FW, and 5.2 ± 0.6 mg/g FW, respectively. Canadian lowbush blueberries had a total anthocyanin content of 1.5 ± 0.2 mg/g FW. In order to obtain comparable anthocyanin profiles this set of authentic samples was extracted with the reference method applying UAE with 80 % methanol on fresh berries (**supporting information, Table 4**). For evaluation of the results, linear discriminant analysis (LDA) was performed. The LDA subdivides the multivariate space in as many subspaces as previously classes (CA, DE, PL, UA) were defined. The resulting orthogonal discriminant functions are linear combinations with a maximized between-class variance and a minimized within-class variance. **Figure 2-5** shows the result of a two dimensional LDA of the authentic data set. It is obvious, that samples from Canada, related to the species *V. angustifolium*, can be easily discriminated along F1 (explaining 98.08 % of the total variances) from the group of European samples, related to the species *V. myrtillus*. However, the samples from Germany, Poland, and the Ukraine cannot be separated well. A slight differentiation along F2 can be observed, but the inter-class variances are too high, leading to overlapping confidence ellipses. As can be seen in the **supporting information in Table 4** dp and cy glycosides show the greatest variations, while pt glycosides reveal the most consistent proportions. This may be of interest if authenticity markers shall be chosen from the anthocyanin profile. Compounds that are incorporated in constant amounts should be preferred to those which are constrained to natural fluctuations in order to obtain robust authenticity

information. In addition, high variances between samples of the same species but different growing conditions prove that geological factors as well as climate conditions apparently influence the biosynthesis of anthocyanins and thus the profiles. However, the results obtained from the data-set analyzed also shows that anthropogenic borders do need not to go along with these differences. Therefore, a differentiation of European samples solely based on the anthocyanin profile is barely reasonable.

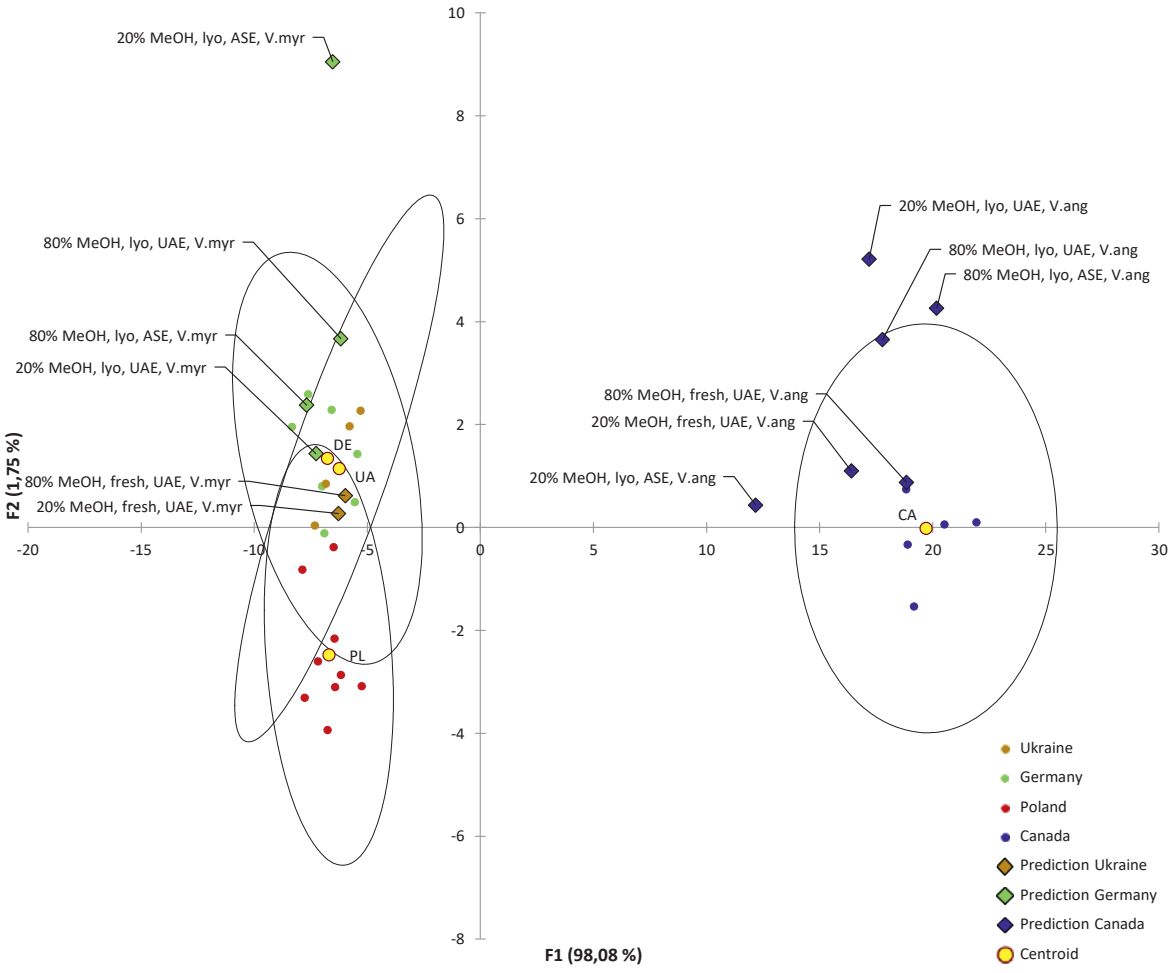

Figure 2-5: LDA of bilberry and lowbush blueberry samples from different origins extracted under reference conditions (80 % methanol, fresh berries, UAE) and prediction of samples extracted under various extraction conditions. UAE: ultrasound assisted extraction, ASE: accelerated solvent extraction, fresh: fresh berries, lyo: lyophilized berries

3.7 Extraction method induced profile variations vs. natural variations

As above-mentioned, anthocyanin profiles are subjected to considerable variations due to different extraction conditions. In order to assess the relevance of these variations, the results of the application of different extraction methods have to be evaluated in the context of naturally occurring between-species differences and inter-species variances. As the different extraction methods were applied to a Ukrainian *V. myrtillus* and a Canadian *V. angustifolium* sample, the anthocyanin profiles obtained were assigned to one of the classes of the discriminant analysis derived from the authentic data set using the prediction of the discriminant function. It becomes apparent that samples extracted under reference conditions are assigned correctly and are located close to the centroid of the respective class, which validates the discriminant function. Anthocyanin profiles of samples extracted under non-reference conditions, however vary widely, indicating considerable method effects. In terms of the predicted Ukrainian samples, only those samples extracted from fresh berries are assigned to the correct origin. Here it has to be stressed that the calculated discriminant function is not capable of differentiating the different European origins but allocate only samples to the specie *V. myrtillus* or *V. angustifolium*. The method dependent variations are so predominent that some predictions even fall out of the respective area of confidence, meaning that they cannot be assigned to one of the classes with a probability greater than 95 %. Especially samples extracted under conditions, which differ a lot from the reference conditions show greater distances from the centroids they are supposed to be assigned to. Nevertheless, all predictions regarding the specie assignment are correct. This is because a discriminant analysis only enables a ‘hard’ classification, meaning a predicted sample must be assigned to the most probable class independent from how low the probability is.

4 Conclusion

In conclusion, the current study addresses one of the crucial problems regarding the comparability of authenticity data sets. Different techniques and solvents applied for the extraction of bilberry, lowbush blueberry and American cranberry anthocyanins resulted in considerably altered yields and peak ratios. In particular, peak proportions of more polar compounds like dp and cy were affected by the applied extraction conditions, whereas other compounds like pt glycosides are only marginally influenced.

Comparing the differences caused by the extraction method with the natural differences within a set of 26 bilberry and lowbush blueberry samples of different origins revealed that profile variations induced by the extraction method are in a similar scale to profile variations due to geographic and climatic differences. LDA could not differentiate between bilberry samples of different European origins, but prediction models allocated differently extracted samples outside of the area of confidence. Application of different methods for the extraction could lead to a false allocation of samples. On the one hand, samples with a very similar genuine profile could be considered as different. On the other hand, actually different samples could erroneously be allocated as similar.

Possible correlations between anthocyanin profiles and individual origins may not be accounted for by assumed intrinsic profile differences but rather reasoned on disparate sample preparations. Furthermore, the results for the different fruits are not completely consistent regarding the resulting profile differences after ASE and UAE and hence do not inevitably demonstrate systematic differences between the investigated extraction methods but rather illustrate plant specific differences. In view of these results, a cautious review not only of the analytical methods but also of the sample preparation applied, in particular extraction methods, is a prerequisite for the reliable comparison of analytical data in general and authenticity data in particular.

Acknowledgments

The authors gratefully thank Haus Rabenhorst O. Lauffs GmbH & Co. KG, Rudolf Wild GmbH &Co. KG, and Faethe Labor GmbH for providing the berry material.

Supporting information

Table 1. Anthocyanin concentrations[a] in bilberry (*V. myrtillus*) and lowbush blueberry (*V. angustifolium*) after various extraction conditions.

	solvent[b]	technique[c]	type[d]	dp-3-gal	dp-3-glu	dp-3-ara	cy-3-gal	cy-3-glu	cy-3-ara	pt-3-gal	pt-3-glu	pt-3-ara	pn-3-gal	pn-3-glu	pn-3-ara	mv-3-gal	mv-3-glu	mv-3-ara	sum
V. myrtillus	20	UAE	fresh	390.2 ± 39.19	370.09 ± 32.9	423.63 ± 55.25	314.51 ± 18.88	438.65 ± 54.42	315.22 ± 29.79	146.59 ± 17.79	294.84 ± 25.84	73.46 ± 9.43	99.15 ± 7.24	248.13 ± 31.88	147.24 ± 21.04	35.11 ± 2.63	331.46 ± 35.51	91.27 ± 8.54	16944.51
		ASE	lyo	575.29 ± 36.07	595.95 ± 37.59	466.37 ± 27.85	649.83 ± 0.8	675.97 ± 6.16	529.39 ± 1.59	176.73 ± 7.92	519.83 ± 6.22	157.65 ± 2.12	111.16 ± 2.53	374.49 ± 2.11	48.49 ± 1.58	228.33 ± 1.9	554.61 ± 1.07	125.06 ± 0.38	5789.13
		UAE	lyo	163.82 ± 4.14	160.29 ± 5.11	117.9 ± 2.11	176.41 ± 2.05	188.35 ± 2.95	120.28 ± 2.44	63.5 ± 1.79	136.89 ± 2.82	41.13 ± 1.14	33.39 ± 0.49	110.74 ± 0.95	17.04 ± 0.74	66.61 ± 0.87	160.99 ± 2.71	36.96 ± 0.53	1594.32
	80	UAE	fresh	493.14 ± 26.03	452.49 ± 25.54	476.86 ± 33.88	403.61 ± 34.23	450.91 ± 22.72	364.06 ± 40.65	171.61 ± 10.9	349.85 ± 17.88	86.3 ± 9.5	122.72 ± 12.97	275.39 ± 27.15	183.95 ± 16.32	44.01 ± 7.12	402.67 ± 29.14	115.54 ± 15.79	20013.07
		ASE	lyo	1167.2 ± 95.09	1198.92 ± 96.2	892.23 ± 68.9	1152.82 ± 90.22	1281.62 ± 103.24	830.29 ± 64.36	444.23 ± 38.18	978.61 ± 82.29	287.92 ± 21.1	213.9 ± 16.2	726.53 ± 57.33	97.93 ± 12.92	448.32 ± 26.38	1100.22 ± 84.59	252.86 ± 18.63	11073.61
		UAE	lyo	608.76 ± 14.67	618.53 ± 12.24	480.8 ± 11.42	571.75 ± 12.46	594.63 ± 14.64	418.46 ± 19.01	202.46 ± 22.55	466.62 ± 11.02	142.01 ± 2.73	96.62 ± 1.88	328.35 ± 6.88	43.3 ± 4.81	204.48 ± 0.95	493.9 ± 9.82	115.32 ± 2.15	5385.99
V. angustifolium	20	UAE	fresh	23.67 ± 3.52	25.29 ± 4.43	22.59 ± 2.4	18.32 ± 3.42	27.31 ± 3.01	17.73 ± 3.5	22.43 ± 2.79	36.35 ± 6.62	12.18 ± 1.37	14.35 ± 2.79	22.45 ± 2.98	64.57 ± 6.55	7.46 ± 1.39	78.92 ± 12.3	38.66 ± 5.84	1969.3
		ASE	lyo	52.97 ± 1.68	55.5 ± 1.29	53.14 ± 2.23	38.14 ± 1.67	58.7 ± 0.58	40.07 ± 1.15	50.63 ± 1.3	75.64 ± 0.91	32.6 ± 0.42	30.29 ± 0.7	49.64 ± 0.79	134.17 ± 1.79	20.13 ± 0.55	170.43 ± 1.3	79.36 ± 0.21	941.4
		UAE	lyo	31.87 ± 1.23	36.01 ± 1.94	23.54 ± 0.6	20.76 ± 0.5	27.69 ± 1.67	16.56 ± 0.42	25.13 ± 1.45	41.63 ± 2.19	12.22 ± 0.59	15.03 ± 0.57	22.64 ± 0.19	63.83 ± 1.93	6.95 ± 0.25	75.87 ± 2.21	34.54 ± 0.96	454.3
	80	UAE	fresh	45.42 ± 3.28	56.46 ± 0.27	26.62 ± 1.91	34.15 ± 2.24	34.21 ± 1.21	22.27 ± 1.34	29.82 ± 2.65	56.52 ± 0.23	13.25 ± 1.09	20.78 ± 1.99	25 ± 0.96	71.21 ± 7.41	8.64 ± 0.41	86.2 ± 4.95	44.44 ± 3.72	2619.5
		ASE	lyo	62.05 ± 1.08	63.71 ± 3.77	71.97 ± 2.74	47.28 ± 3.89	81.49 ± 3.52	58.52 ± 1.02	73.78 ± 3.94	121.25 ± 6.33	36.37 ± 0.07	50.84 ± 1.29	64.28 ± 3.58	180.82 ± 7.78	26.03 ± 1.31	221.88 ± 7.92	111.41 ± 4.37	1271.7
		UAE	lyo	38.12 ± 26.98	64.56 ± 0.43	34.29 ± 0.62	40.53 ± 0.13	40.75 ± 0.16	27.06 ± 0.04	38.35 ± 0.9	67.95 ± 0.44	17.14 ± 0.35	25.08 ± 0.4	30.83 ± 0.59	90.2 ± 0.78	9.82 ± 0.04	105.48 ± 0.72	51.83 ± 0.51	682.0

[a] Values are expressed in mg/100g DW. [b] percent methanol in extraction solvent, [c] UAE: ultrasound assisted extraction, ASE: accelerated solvent extraction, [d] lyo: lyophilized berries, fresh: fresh thawed berries

Table 2. Anthocyanin concentrations[a] in cranberry (*V. macrocarpon*) after various extraction conditions.

	solvent[b]	technique[c]	type[d]	dp-3-gal	dp-3-glu	dp-3-ara	cy-3-gal	cy-3-glu	cy-3-ara	pt-3-gal	pt-3-glu	pt-3-ara	pn-3-gal	pn-3-glu	pn-3-ara	mv-3-gal	mv-3-glu	mv-3-ara	sum
V. macrocarpon	20	UAE	fresh				55.74 ± 0.15	2.86 ± 0.03	31.24 ± 0.55				91.07 ± 2.48	7.4 ± 0.09	31.93 ± 1.21				220.25
		ASE	lyo				39.69 ± 2.19	3.12 ± 0.12	22.45 ± 1.24				61.91 ± 3.74	5.87 ± 0.25	22.48 ± 1.52				155.51
		UAE	lyo				39.44 ± 0.44	1.72 ± 0.03	22.48 ± 0.31				61.59 ± 0.79	4.51 ± 0.05	21.66 ± 0.24				151.4
	80	UAE	fresh				35.97 ± 1.82	1.92 ± 0.01	23.02 ± 1.72				55.39 ± 5.88	4.52 ± 0.47	21.58 ± 2.7				142.40
		ASE	lyo				40.19 ± 5.41	2.68 ± 0.1	26.15 ± 3.68				72.07 ± 10.8	6.57 ± 0.61	26.61 ± 3.75				174.27
		UAE	lyo				44.18 ± 0.18	1.66 ± 0.03	25.26 ± 0.16				69.54 ± 0.48	4.82 ± 0.04	24.35 ± 0.13				169.8

[a] Values are expressed in mg/100g DW. [b] percent methanol in extraction solvent, [c] UAE: ultrasound assisted extraction, ASE: accelerated solvent extraction, [d] lyo: lyophilized berries, fresh: fresh thawed berries

Table 3. Anthocyanin proportions[a] in bilberry, lowbush blueberry and cranberry after various extraction conditions.

	extraction method			anthocyanin														
fruit	solvent[b]	technique[c]	type[d]	dp-3-gal	dp-3-glu	dp-3-ara	cy-3-gal	cy-3-glu	cy-3-ara	pt-3-gal	pt-3-glu	pt-3-ara	pn-3-gal	pn-3-glu	pn-3-ara	mv-3-gal	mv-3-glu	mv-3-ara
V. myrtillus	20	UAE	fresh	10.5 ab	10.1 b	8.8 ab	11.2 a	11.6 ab	8.6 ab	3.9 a	8 bc	2.8 a	1.9 ab	6.5 ab	1 a	3.9 a	8.9 c	2.5 ab
		ASE	lyo	9.9 b	10.3 ab	8 bc	11.2 a	11.7 ab	9.1 a	3.1 b	9 a	2.7 a	1.9 ab	6.5 ab	0.8 a	3.9 a	9.6 b	2.2 bc
		UAE	lyo	10.3 b	10.1 b	7.4 c	11.1 a	11.8 a	7.5 b	4 a	8.6 ab	2.6 a	2.1 a	6.9 a	1.1 a	4.2 a	10.1 a	2.3 abc
	80	UAE	fresh	11.2 a	10.3 ab	9.2 a	10.9 a	10.3 c	8.3 ab	3.9 a	8 c	2.8 a	2 ab	6.3 ab	1 a	4.2 a	9.2 c	2.6 a
		ASE	lyo	10.5 ab	10.8 ab	8.1 bc	10.4 a	11.6 ab	7.5 b	4 a	8.8 a	2.6 a	1.9 ab	6.6 ab	0.9 a	4.1 a	9.9 ab	2.3 abc
		UAE	lyo	11.3 a	11.5 a	8.9 ab	10.6 a	11 b	7.8 b	3.8 a	8.7 a	2.6 a	1.8 b	6.1 b	0.8 a	3.8 a	9.2 c	2.1 c
V. angustifolium	20	UAE	fresh	5.5 c	5.8 c	4.2 b	5.7 a	6.3 a	5.2 ab	4.1 ab	8.4 bc	3.3 b	2.8 b	5.2 a	1.7 b	15 a	18.2 a	8.9 a
		ASE	lyo	5.7 c	5.9 c	4 b	5.7 a	6.3 a	5.4 ab	4.2 ab	8 c	3.2 b	3.5 a	5.3 a	2.1 a	14.3 a	18 a	8.4 abc
		UAE	lyo	7 b	7.9 b	4.5 b	5.3 ab	6.1 a	5.5 ab	3.7 b	9.2 abc	3.3 b	2.7 bc	5 ab	1.6 b	14.1 ab	16.7 abc	7.6 c
	80	UAE	fresh	7.9 a	9.8 a	5.9 a	5.2 b	6 a	5.2 ab	3.9 ab	9.9 a	3.6 ab	2.3 c	4.4 c	1.5 b	12.4 c	15 c	7.7 c
		ASE	lyo	5.2 c	5.4 c	4 b	4.8 bc	6.4 a	5.7 a	4.5 a	9.4 ab	4.1 a	2.9 b	5 ab	2.1 a	13.9 abc	16.9 ab	8.6 ab
		UAE	lyo	7.3 ab	9.3 ab	5.7 a	4.6 c	6.1 a	5.1 b	4 ab	9.8 a	3.6 ab	2.3 c	4.5 bc	1.6 b	12.5 bc	15.5 bc	7.8 bc
V. macrocarpon	20	UAE	fresh				25.2 a	1.4 bc	14.5 b				40.8 a	3.4 b	14.7 a			
		ASE	lyo				25.5 a	2 a	14.4 b				39.8 ab	3.8 a	14.4 a			
		UAE	lyo				26 a	1.1 c	14.8 b				40.7 ab	3 bc	14.3 a			
	80	UAE	fresh				25.4 a	1.4 bc	16.2 a				38.8 b	3.2 bc	15.1 a			
		ASE	lyo				23.2 b	1.7 ab	15 b				41 a	3.9 a	15.3 a			
		UAE	lyo				26 a	1 c	14.9 b				41 a	2.8 c	14.3 a			

[a] Values are expressed as % of total anthocyanins. Values sharing the same letter in columns were not significantly different ($p > 0.05$) for each fruit. [b] percent methanol in extraction solvent, [c] UAE: ultrasound assisted extraction, ASE: accelerated solvent extraction, [d] lyo: lyophilized berries, fresh: fresh thawed berries

Table 4. Anthocyanin proportions[a] in bilberry from Germany (DE), Poland (PL), and Ukraine (UA) and lowbush blueberry from Canada (CA).

		anthocyanin											
sample	vintage	dp-3-gal	dp-3-glu	dp-3-ara	cy-3-gal	cy-3-glu	cy-3-ara	pt-3-gal	pt-3-glu	pt-3-ara	pn-3-gal	pn-3-glu	pn-3-ara	mv-3-gal	mv-3-glu	mv-3-ara
CA1	2010	7.1 c	9.2 b	5.5 b	4.3 a	5.8 a	3.7 a	4.8 b	9.4 a	3.4 a	2.3 a	5.1 a	1.5 ab	13.6 a	16 a	8.2 a
CA2	2009	9.4 ab	10 ab	6 ab	4.5 a	4.9 ab	3.2 a	5.6 ab	9.2 a	3.2 a	2.3 ab	4.4 bc	1.2 ab	13.6 a	15.8 a	6.8 a
CA3	unknown	7.2 bc	9.3 b	5.7 ab	4.9 a	6 a	3.9 a	5 ab	9.8 a	3.7 a	2.4 a	4.9 ab	1.7 a	12.3 a	15.5 a	7.8 a
CA4	2012	10.2 a	12.1 a	6.7 a	4.3 a	5.1 ab	3.1 a	5.4 ab	10.4 a	3.4 a	1.8 b	3.7 c	1.1 b	11.4 a	14.7 a	6.6 a
CA5	2009	9.4 ab	9.5 b	5.8 ab	4.2 a	4.5 b	3.1 a	5.8 a	9 a	3.3 a	2.4 a	4.3 bc	1.4 ab	14.8 a	15.4 a	7 a
DE1	2014	14.1 ab	12.9 b	9.9 cd	9.7 ab	8.8 c	6.7 b	4.3 ab	9 bc	2.6 cd	1.4 b	5.2 bc	0.6 bc	4.1 b	2 bc	9.4 bc
DE2	2014	14.4 ab	11.6 b	12.5 ab	10.5 a	8.7 c	8.4 a	4.2 ab	7.7 d	3 ab	1.4 b	5.8 ab	0.7 abc	3.5 c	2.3 ab	7.2 d
DE3	2014	13.7 ab	13.3 b	11 bc	10.4 a	10.9 b	8.5 a	3 d	8.3 cd	2.4 d	1.2 bc	4.6 c	0.6 bc	2.9 d	1.6 d	7.5 d
DE4	2012	10 c	12.2 b	8.7 d	9.9 ab	13.4 a	8.9 a	3.1 cd	9.3 bc	2.6 cd	1.4 b	4.5 c	0.8 ab	3.2 cd	1.9 c	8.7 cd
DE5	2014	12.9 b	12.5 b	8.9 d	8.4 b	8.6 c	6.1 b	4.7 a	9.8 ab	2.7 bc	1.6 ab	4 cd	0.8 ab	4.7 a	2.3 a	10.9 a
DE6	2014	10.9 c	12 b	8.9 d	11.2 a	12.5 ab	9.2 a	2.8 d	8.3 cd	2.4 d	1.9 a	3 d	1 a	2.9 d	1.8 cd	7.5 d
DE7	2012	14.9 a	16.5 a	13 a	5.7 c	6.5 d	5.4 b	3.7 bc	10.7 a	3.1 a	0.8 c	6.8 a	0.5 c	3.5 c	2.4 a	10.5 ab
PL1	2014	12.6 abc	11.9 b	9.5 b	9.1 a	9.2 ab	7.6 a	4 a	9.1 a	2.8 a	1.5 a	5.1 a	0.8 ab	4.2 a	10.1 a	2.2 ab
PL2	2014	15.7 a	14.4 ab	12.6 a	7.8 a	7.4 b	6.4 a	4.1 a	9.5 a	2.9 a	0.9 cd	3.2 c	0.5 c	3.5 b	9 ab	2.1 ab
PL3	2014	14.7 ab	14 ab	9.8 ab	9 a	9.2 ab	6.3 a	4.3 a	9.5 a	2.4 a	1.1 abcd	4.2 abc	0.5 bc	3.6 ab	9.3 ab	1.8 b
PL4	2014	14.7 ab	14.1 ab	10.7 ab	9.2 a	9.5 ab	7.2 a	3.5 a	9.2 a	2.6 a	1.1 bcd	3.9 abc	0.6 bc	3.1 b	8.6 b	2.3 ab
PL5	2012	11.1 c	11.8 b	9.7 ab	9.8 a	11.4 a	8.2 a	3.7 a	8.8 a	2.7 a	1.4 ab	5.6 a	0.9 a	3.3 b	9.3 ab	1.8 ab
PL6	2013	12.5 bc	13 ab	9.9 ab	9.1 a	9.6 ab	7.6 a	3.8 a	9.1 a	2.7 a	1.4 ab	4.9 ab	0.8 ab	3.6 ab	9.7 ab	2.5 a
PL7	2013	14.4 ab	13.5 ab	10.5 ab	9 a	8.9 ab	6.9 a	3.9 a	9.1 a	2.6 a	1.1 bcd	4 abc	0.5 bc	3.6 ab	9.7 ab	2 ab
PL8	2013	15.6 ab	13.8 ab	10.5 ab	10 a	9.1 ab	7.2 a	4.2 a	8.8 a	2.6 a	1.3 abc	3.9 abc	0.6 abc	3.4 b	7.4 c	1.9 ab
PL9	2014	15.8 a	15 a	10.7 ab	8.5 a	8.6 ab	6.1 a	3.8 a	9.7 a	2.4 a	0.9 d	3.4 bc	0.4 c	3.3 b	9.6 ab	1.9 ab
UA1	2013	13.4 ab	14.2 ab	11.4 ab	9.2 bc	10.4 ab	7.5 a	3.5 a	9.3 ab	2.8 a	1.1 cd	4.2 bc	0.6 bc	2.8 bc	7.8 b	2 b
UA2	2013	11.2 c	10.3 d	9.2 c	10.8 a	10.3 ab	8.3 a	3.9 a	8 c	2.8 a	2 a	6.3 a	1 a	4.2 a	9.1 a	2.6 a
UA3	2010	12 bc	13.3 b	9.9 bc	9.9 ab	11.4 a	8.2 a	3.5 a	9.1 ab	2.6 a	1.3 bc	5.1 ab	0.8 bc	2.9 bc	8 ab	1.9 b
UA4	2012	11.9 bc	11.9 c	9.6 c	10.7 a	11.5 a	8.6 a	3.6 a	8.5 bc	2.7 a	1.6 b	5.5 a	0.8 ab	3.2 b	7.9 b	2 b
UA5	unknown	14.3 a	15.3 a	12.3 a	8.1 c	9.4 b	7.4 a	3.5 a	9.4 a	2.7 a	1 d	3.8 c	0.5 c	2.7 c	7.9 b	1.8 b
mean CA		8.7 b	10 b	5.9 b	4.5 b	5.3 b	3.4 b	5.3 a	9.5 a	3.4 a	2.2 a	4.5 a	1.4 a	13.1 a	15.5 a	7.3 a
mean DE		13 a	13 a	10.4 a	9.4 a	9.9 a	7.6 a	3.7 b	9 a	2.7 b	1.4 b	4.8 a	0.7 b	3.6 b	8.8 b	2 b
mean PL		14.1 a	13.5 a	10.4 a	9.1 a	9.2 a	7.1 a	3.9 b	9.2 a	2.6 b	1.2 b	4.3 a	0.6 b	3.5 b	9.2 b	2 b
mean UA		12.6 a	13 a	10.5 a	9.7 a	10.6 a	8 a	3.6 b	8.9 a	2.7 b	1.4 b	5 a	0.8 b	3.2 b	8.1 b	2 b

[a] Values are expressed as % of total anthocyanins. Values sharing the same letter in columns were not significantly different ($p > 0.05$) for each fruit.

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Chapter 3

Influence of common and excessive enzymatic treatment on juice yield and anthocyanin contents and profile during bilberry (*Vaccinium myrtillus* L.) juice production

Treatment with cell wall degrading enzymes is an important step during juice production to enhance juice yield and the amount of value adding compounds like polyphenols. Enzymatic side activities may lead to unintended alterations of the polyphenol profile. We determined the effects of enzyme treatment on juice yield and contents and profile of anthocyanins using four commercial pectinolytic and two cellulolytic enzymes during bilberry juice production. While enzyme dosage at commercial level (0.5 nkat/g) caused only small increases in juice yield but considerably higher anthocyanin yields, significant changes in the anthocyanin profile could be observed, which were related to the glycoside type as well as to the aglycone. Application of excessive enzyme dosage (10 nkat/g) significantly improved both juice yield and total anthocyanin content. Extractability of anthocyanins seems to be more relevant for profile changes during juice processing when usual enzyme dosages are applied, whereas excessive dosages lead to changes caused by enzymatic side activities.

Keywords: Bilberry, *Vaccinium myrtillus* L., juice, enzyme treatment, anthocyanin profile

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1 Introduction

Berries containing high amounts of phenolic compounds like anthocyanins, phenolic acids, and flavonols are growing in popularity because of the value adding effect of these substances (Kähkönen et al., 2003). In particular, bilberries have been reported to be a rich source of polyphenols and especially anthocyanins (Kähkönen et al., 2003; Häkkinen et al., 1999; Müller et al., 2012). Due to their appealing color and sweet taste (Laaksonen et al., 2010), they are not only eaten fresh but also processed into juices and jams (Howard et al., 2010). Anthocyanins constitute one of the most important groups of polyphenols in bilberries (Može et al., 2011). The aglycones delphinidin (dp), cyanidin (cy), petunidin (pt), peonidin (pn), and malvidin (mv) occur as 3-*O*-glycosides with attached glucose (glu), galactose (gal), and arabinose (ara) moieties, respectively. The resulting profile includes 15 different anthocyanins. This complex fingerprint has frequently been used for authentication of bilberry products because they represent high valued crops and adulteration is a common problem (Fügel et al., 2005; Penman et al., 2006; Filip et al., 2012; Primetta et al., 2013; Gardana et al., 2014). Variations of the anthocyanin profile are not exclusively the result of fraudulent practices but also occur inevitably due to technological influences during the production process (González-Neves et al., 2016; Skrede et al., 2000; Grassin & Coutel, 2009; Viljanen et al., 2014). One of the main processing steps which may greatly affect the anthocyanin profile is enzymatic treatment. Application of enzymes has several implications during fruit juice processing. Enzymes reduce the viscosity and thus increase juice yields and expedite production (Grassin & Coutel, 2009; Sandri et al., 2013). They cause disruption of the cell wall network, enhancing the extractability of polyphenols, which are predominantly associated with the fruit peel (Acosta-Estrada et al., 2014; Landbo & Meyer, 2001). Pectinases, cellulases, hemicellulases, and amylases have been reported to be suitable for enzymatic treatment and the release of polyphenols (Landbo & Meyer, 2001). Two important representatives of pectinases are polygalacturonases, which hydrolyze α-1,4-glycosidic linkages of the polygalacturonic acid backbone, and pectin lyases, which lead to the cleavage of α-1,4-glycosidic bonds with introduction of an unsaturated moiety at the non-reducing end (Kashyap et al., 2001). Both enzymes show endo- and exo-activities leading to random or sequential degradation, respectively (Kashyap et al., 2001). Cellulases include three different hydrolytic enzymes, that is, an endo-(l,4)-β-D-glucanase activity that randomly cleaves β-linkages of amorphous cellulose, an exo-(l,4)-β-D-glucanase activity, which releases sequentially cellobiose from either the reducing or the non-reducing end of cellulose, and a β-glucosidase activity that releases glucose from cellobiose (Bhat & Bhat,

1997). However, the most frequently applied preparations in commercial berry processing are pectinolytic enzymes (Grassin & Coutel, 2009). The great variety of commercial enzyme preparations is required because of the complex diversity of plant cell walls, which are composed of cellulose, hemicellulose, and pectin as their main polysaccharides (Albersheim et al., 1996; Thakur et al., 1997; Yadav et al., 2009). The structure of pectin is very diverse as it includes acidic polymers like homogalacturonans and rhamnogalacturonans with neutral polymers like arabinans, galactans and arabinogalactans attached (Albersheim et al., 1996). Besides the numerous positive effects of enzymatic treatment, adverse effects may also be observed. A considerable loss of anthocyanins during fruit processing has been reported, which was attributed to side activities of the enzyme preparations (Huang, 1955; Wightman & Wrolstad, 1996; Buchert et al., 2005; Koponen et al., 2008a). β-Glucosidase-, β-galactosidase- or α-L-arabinosidase side activities have been discussed in this context (Landbo & Meyer, 2001). However, a pure β-glucosidase had only little influence on the anthocyanins of elderberries, indicating that an anthocyanin specific β-glucosidase seems to be responsible for the observed degradation (Pricelius et al., 2009). Moreover, the application of enzymes may lead to secondary effects. Since pectin has been reported to interact with anthocyanins (Fernandes et al., 2014), it is reasonable that interaction of pectin degradation products with anthocyanins might also occur.

Accordingly, the application of technological processes such as enzymatic treatment may lead to various contrary effects. On one side, they preserve or enhance the amount of value adding compounds in the final products by facilitating their extractability. On the other side, they may also cause considerable losses of these compounds due to the side effects mentioned above.

The objective of this study was to characterize the changes in the anthocyanin profile caused by enzymatic side activities during bilberry juice production in order to obtain a better understanding of the underlying mechanisms. Since most published studies demonstrate the effects of extremely high enzyme dosages, the difference between the effect of commercially common dosages and excessive dosages were of particular interest as there is only little information about profile changes at industrially relevant dosages.

2 Materials and methods

2.1 Samples

Bilberries (*Vaccinium myrtillus* L.) were purchased in July 2014 from Stahl GmbH (Bodenwöhr-Blechhammer, Germany) and stored at -20 °C until further use.

2.2 Juice processing

For inactivation of polyphenoloxidase (PPO), 500 g batches of berries were placed in plastic bags, thawed overnight at 8 °C, and blanched afterwards for five minutes at 80 °C in a shaking water bath. Subsequently, they were cooled in an ice bath and pooled again. In 200 g batches, they were stored at -20 °C until juice processing. After mashing the berries with a stomacher for 6 seconds, the enzyme preparations shown in **Table 3-1** were added at two dosage levels (0.5 nkat/g and 10 nkat/g). Incubation was performed in a shaking water bath (60 rpm, 50 °C) for 120 min. Samples were placed in a hot water bath at 95 °C for 2 min to inactivate the enzymes and subsequently cooled at 5 °C in an ice bath. Juice pressing was performed in 3 cycles with a Para Press (Paul Arauner GmbH und Co. KG, Kitzingen, Germany) by applying a pressure of 3 bar for 10 seconds each time. Juice and pomace were stored at -20 °C for further analysis.

Table 3-1. Enzyme preparations used for the production of bilberry juices

Name	Abbreviation	Provider	Main activity*	Temp. opt.*
Pectinex BE XXL	PBXXL	Novozymes A/S *(Bagsvaerd, Denmark)*	Pectin-lyase	50 °C
Rohament CL	RCL	AB Enzymes GmbH (*Darmstadt, Germany)*	Cellulase	60 °C
Rohapect PTE 100	RPTE100	AB Enzymes GmbH (*Darmstadt, Germany)*	Pectin-Lyase (endo)	30 °C
Rohapect 10 L	R10L	AB Enzymes GmbH (*Darmstadt, Germany)*	Pectinase, polygalacturonase	50 °C
Vegazym HC	VHC	Erbslöh AG *(Geisenheim, Germany)*	Cellulase	-
Vinozym Ultra FCE	VinoUFC	Novozymes A/S *(Bagsvaerd, Denmark)*	Polygalacturonase	-

*Main activities and temperature optima according to manufacturer's information

2.3 Chemicals and standards

Ultrapure water was obtained from a Synergy purification system (Millipore, Molsheim, France). HPLC grade acetonitrile, acetic acid, and potassium sodium tartrate tetrahydrate were from VWR (Mannheim, Germany). Formic acid (≥ 98 %), 3,5-dinitrosalicylic acid, and D(+)-glucose monohydrate were purchased from Sigma-Aldrich (St. Louis, USA). Methanol was from Th. Geyer (Renningen, Germany) and regenerated cellulose filters (Chromafil RC-20/15 MS) were supplied by Macherey-Nagel (Düren, Germany). Malvidin 3-*O*-glucoside (≥ 87 %) was obtained from Phytoplan (Heidelberg, Germany). Pectin (Pectin Classic AU-L 024/10) was purchased from Herbstreith & Fox GmbH (Werder, Germany), D(+)-galacturonic acid from Fluka Analytical (Steinheim, Germany), and cellobiose and sulfuric acid from AppliChem GmbH (Darmstadt, Germany)

2.4 Extraction and quantification of anthocyanins

Fresh berries and pomace samples were extracted according to a method described by Koponen et al. with minor modifications (Koponen et al., 2008b). The sample (30 g) was homogenized with 100 mL extraction solvent A (water/methanol/hydrochloric acid, 78/20/2, v/v/v) using an Ultra Turrax T18 basic (IKA-Werke GmbH & CO. KG, Staufen, Germany) for 2 min. Subsequently, 15 mL of solvent A was added to 3 g homogenate and after 1 min of shaking, the sample was centrifuged for 10 min at 3941 *g* (Zentrifuge, Thermo Fisher Scientific, Schwerte, Germany). After 2 repetitions, the pellet was extracted with extraction solvent B (water/methanol/hydrochloric acid, 49/50/1, v/v/v). The combined supernatants were made up to 50 mL with water. Anthocyanin quantification was performed as reported by Heffels et al. (Heffels et al., 2015) Juices were injected directly after membrane filtration. Anthocyanins were detected at 520 nm and quantified as mv-3-glu equivalents by external calibration (range: 1.18 – 471.37 mg/L). The total anthocyanin content was calculated as the sum of individual anthocyanins.

2.5 Polygalacturonase activity

For the quantification of reducing sugars released from pectin, 450 µL of 0.1 % pectin suspension in sodium citrate buffer (pH 4) and 50 µL of 10 % aqueous enzyme solution were incubated for 5 min at 50 °C in a shaking water bath. The reaction was terminated by the addition of 750 µL of dinitrosalicylic acid (DNS) reagent containing 10 g/L 3,5-dinitrosalicylic acid, 30 g/L potassium sodium tartrate tetrahydrate, and 16 g/L sodium hydroxide in water.

After boiling for 5 min, cooling in iced water, and centrifugation for 5 min at 10947 *g*, the absorption was measured at 540 nm (Genesys 6, Thermo Fisher Scientific, Schwerte, Germany). Polygalacturonase activity was expressed as D(+)-galacturonic acid equivalents released during the enzymatic reaction (Pricelius et al., 2009; Bailey et al., 1992; Negrulescu et al., 2012; Miller, 1959; Wood et al., 2012).

2.6 β-Glucosidase activity

The amount of glucose released due to enzymatic treatment of cellobiose was quantified in order to determine the β-glucosidase activity. A reaction mixture containing 100 µL of 10 % aqueous enzyme solution and 900 µL of cellobiose solution (4 g/L) in sodium citrate buffer (pH 4) was incubated for 30 min at 50 °C in a shaking water bath. Enzyme activity was stopped by placing the reaction mixture in a hot water bath (95 °C) for 5 min and afterwards in an iced water bath for 5 min. After 5 min of centrifugation at 10947 *g*, the mixtures were kept at -20 °C until quantification of glucose via HPLC (Sadana et al., 1980). Quantification was conducted using a Shimadzu LC-6A pump, a Waters 717plus autosampler (injecting 10 µL), a Waters column thermostat Jetstream 2 plus (at 60 °C), and a Waters 2414 refraction index detector. The column was a Nucleogel ION 300OA (Macherey-Nagel, Düren, Germany). Sulfuric acid (2.5 mM) was delivered at an isocratic flow of 0.3 mL/min for 60 min.

2.7 Statistics

For statistical analysis and principal component analysis (PCA), the XLSTAT software was used. An ANOVA was performed to determine significant differences. The level of significance was defined as $p \leq 0.05$.

3 Results and discussion

3.1 Enzyme activity

The polygalacturonase activity of all enzyme preparations investigated ranged from 1304 to 4593 nkat/mL. **Table 3-2** shows the polygalacturonase activities ranked in ascending order. All enzyme preparations, which are labeled as polygalacturonases by the manufacturers, showed medium to high polygalacturonase activities. A comparison with literature data is difficult because of differing enzyme preparations, differing assays, or the usage of uncommon units to describe enzyme activity. While a polygalacturonase activity of 1230 nkat/mL was reported for Pectinex BE XXL (Puupponen-Pimiä et al., 2008), our analysis revealed an activity of 3254 nkat/mL. Similar differences are reported in literature. Although applying the same assay, Pextinex BE 3-L revealed an activity of 4900 nkal/mL in a study of Buchert et al. (Buchert et al., 2005) and an activity of 11950 nkat/mL in a study of Puuponen-Pimiä et al. (Puupponen-Pimiä et al., 2008).

Table 3-2. Polygalacturonase (PG) and β-glucosidase (β-Glu) activities of commercial enzyme preparations

Name	PG [nkat/mL]	β-Glu [nkat/mL]
Rohapect PTE 100	1304	15
Rohament CL	1511	339
Pectinex BE XXL	3254	557
Vinozym Ultra FCE	3604	514
Rohapect 10 L	3664	920
Vegazym HC	4593	607

The β-glucosidase activity ranged from 15 to 920 nkat/mL. As shown in **Table 3-2**, the difference between the β-glucosidase activities of the enzyme preparations investigated are more pronounced than those between their polygalacturonase activities. Moreover, the β-glucosidase activity is considerably lower than the polygalacturonase activity, reflecting that the β-glucosidase should be regarded as a side activity in the investigated enzyme preparations. It was reported that glycosidases are generally associated with pectinolytic enzymes (Pricelius et al., 2009; Bloor, 2001). Whereas the high β-glucosidase activity of Vegazym HC is reasonable because the group of cellulases encompasses cellobiases (Bhat & Bhat, 1997;

Bruchmann & Fauveau, 2010), the considerably lower β-glucosidase activity of Rohament CL is remarkable. Although both enzymes are labeled cellulases, differing β-glucosidase activities can be explained by the microbial origin of the enzymes, which derive from different microbial strains (*Trichoderma longibrachiatum* vs. *T. reseei*). Therefore, the three cellulase main activities endo-l,4-β-D-glucanase, exo-l,4-β-D-glucanase, and β-glucosidase may be differently expressed for both enzyme preparations.

For the production of a juice with improved polyphenol contents, a high polygalacturonase activity is necessary to degrade the cell wall, which in turn leads to an increased release of polyphenols and galacturonic acid (Buchert et al., 2005). Polygalacturonase and β-glucosidase activities of the enzymes investigated show a high correlation of 0.685, suggesting that enzymes with a high polygalacturonase activity also have a marked β-glucosidase activity. This needs to be considered in juice production because a pronounced β-glucosidase activity might lead to unintended degradation of anthocyanins (Buchert et al., 2005; Pricelius et al., 2009).

3.2 Effect of enzyme-assisted processing of bilberries on juice yield and anthocyanin contents

Bilberries were processed applying six commercial preparations which represent the enzyme activities polygalacturonase, pectin lyase, and cellulase. All enzymes were used at two dosage

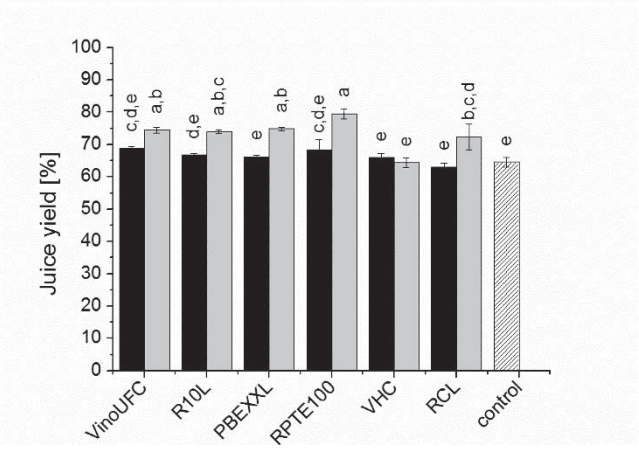

Figure 3-1: Effects of enzyme preparation and dosage on juice yield during bilberry juice processing. *Black bar*: 0.5 nkat/g, *grey bar*: 10 nkat/g. Bars with different letters are significantly different at $p \leq 0.05$ (n=2)

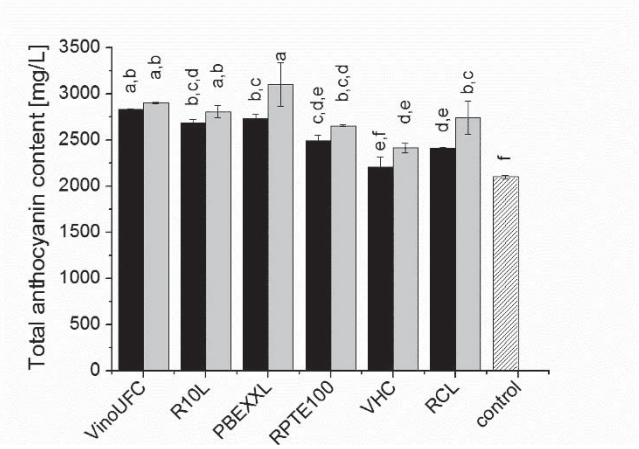

Figure 3-2: Effects of enzyme preparation and dosage on the total anthocyanin content during bilberry juice processing. *Black bar*: 0.5 nkat/g, *grey bar*: 10 nkat/g. Bars with different letters are significantly different at $p \leq 0.05$ (n=2)

levels (0.5 nkat/g and 10 nkat/g) based on their polygalacturonase activity. The lower level of 0.5 nkat/g represents the dosage recommended by the manufacturer, whereas the higher level of 10 nkat/g can be considered an excessive dosage similar to those reported in literature (Buchert et al., 2005; Puupponen-Pimiä et al., 2008; Laaksonen et al., 2012). In order to characterize the resulting juices, both juice yield (**Figure 3-1**) and total anthocyanin content (**Figure 3-2**) were determined**.**

Enzyme dosages of 0.5 nkat/g had only little effect on the juice yield compared with a reference juice produced without the addition of any enzyme (**Figure 3-1**). The largest increase in juice yield (5 %) was achieved by the application of VinoUFC and RPTE100. However, the various enzyme preparations did not lead to significantly different juice yields at a dosage of 0.5 nkat/g.

A higher dosage of 10 nkat/g resulted in significantly higher juice yields compared with the control juice for all enzyme preparations except for VHC. Polygalacturonases and pectin lyase PBEXXL led to an increase of about 10 % and pectin lyase RPTE100 increased the juice yield about 17 %. The cellulase VHC did not affect the juice yield, but application of RCL increased the yield by 10 %. These findings are in accordance with Koponen et al., who observed a significantly increased bilberry juice yield applying similar enzyme preparations at a level of 1 nkat/g without further increases at 10 and 100 nkat/g (Koponen et al., 2008a). Buchert et al. reported similar increasing bilberry juice yields at a dosage of 1000 nkat/g, noting that the maximum yield was already obtained at dosages below 50 nkat/g (Buchert et al., 2005). Similar increases in juice yield due to maceration with pectinolytic enzymes were reported for other fruits like black currant (Laaksonen et al., 2012; Bagger-Jørgensen & Meyer, 2004; Landbo & Meyer, 2004), elder berry (Landbo et al., 2007), and plum (Mieszczakowska-Frąc et al., 2012). However, the dosages used significantly exceed the commonly applied levels.

Although enzyme dosages of 0.5 nkat/g did not increase the juice yield significantly, a pronounced effect on the total anthocyanin content of the corresponding juices was observed. Polygalacturonases and pectin lyase PBEXXL increased the amount of anthocyanins between 23.0 % - 29.3 % compared to the control juice (**Figure 3-2**). Pectin lyase RPTE100 and cellulase RCL increased the anthocyanin content by about 10.1 – 13.9 %, whereas VHC had no significant impact on the anthocyanins content compared to the control juice. Applying 10 nkat/g of the enzyme preparations resulted in an even more pronounced increase in total anthocyanins. At the higher dosage, polygalacturonases and pectin lyase PBEXXL showed the largest effects in comparison to the control juice with increases of 28.3 - 41.9 %, whereas pectin lyase RPTE100 and the cellulases tended to lead to smaller increases of 10.4 % - 25.3 %. Juices

elaborated with enzymes generally showed higher anthocyanin yields than the control juice. Similar increases in anthocyanin and flavonol yields during the production of bilberry juice (Koponen et al., 2008a; Puupponen-Pimiä et al., 2008), elderberry juice (Landbo et al., 2007), and black currant juice (Landbo & Meyer, 2004) and concentrate (Pap et al., 2010) were reported previously, when various polygalacturonases and pectin lyases above dosages used in industrial production were applied. Cellulases were reported to enhance cell wall degradation of black currant pomace, thereby facilitating the release of anthocyanins (Kapasakalidis et al., 2009). Yet, there are reports about decreased anthocyanin contents during blueberry (Brownmiller et al., 2008; Skrede et al., 2000), lingonberry (Viljanen et al., 2014), and black currant (Mieszczakowska-Frąc et al., 2012) juice processing. Glycosidase side activities of the enzyme preparations applied and prolonged treatments were supposed to be responsible for the anthocyanin losses (Koponen et al., 2008a; Buchert et al., 2005).

As shown in **Figure 3-3**, anthocyanins present in bilberry were almost entirely recovered in the respective juice and pomace. The summarized recovery ranged from 81 % to 94.9 % and from 84.5 % to 96.2 % for 0.5 nkat/g and 10 nkat/g, respectively. Loss of anthocyanins due to physical removal of the anthocyanin rich skins during pressing is the main reason for the lack

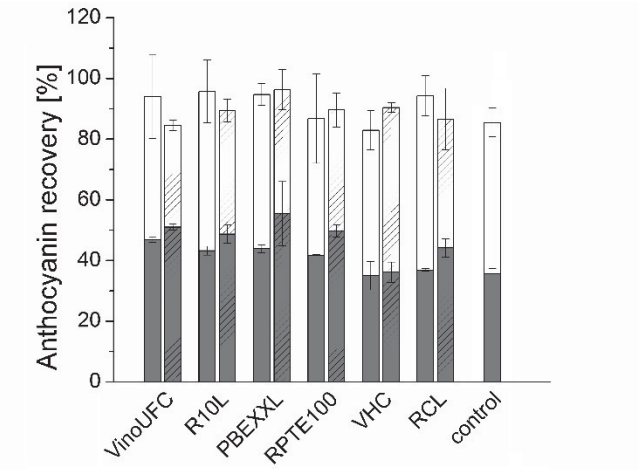

Figure 3-3: Effects of enzyme preparation and dosage on the anthocyanin recovery from raw bilberries and distribution between juice and pomace during bilberry juice processing. *Gray bar* juice, 0.5 nkat/g; *gray stripped bar* juice, 10 nkat/g; *white bar* pomace, 0.5 nkat/g; w*hite stripped bar* pomace, 10 nkat/g

of anthocyanins in comparison to the fresh berry (Mieszczakowska-Frąc et al., 2012), which showed an anthocyanin content of 4.2 mg/g fresh weight. The applied enzyme dosage had no significant effect on the total anthocyanin recovery. But apparently, the recovery into the juice was slightly higher after treatment with 10 nkat/g. The present study revealed no decreases in the anthocyanin yield compared with the control juice although enzymes with considerable β-glucosidase activities were applied. It can therefore be assumed that the β-glucosidase activity is not directly responsible for anthocyanin degradation. Furthermore, the enhanced release of anthocyanins due to enzymatic treatment may outweigh an anthocyanin loss. It has to be considered that besides immediate interactions between enzyme and anthocyanins, secondary effects may occur as well. It is conceivable that besides pectin also pectin degradation products interact with anthocyanins resulting in a loss of individual anthocyanins. All discussed effects will lead to an alteration of the anthocyanin profile during juice processing.

3.3 Principal component analysis

In order to reveal the underlying mechanisms of anthocyanin profile changes during juice processing due to enzymatic treatment a principal component analysis was performed. This technique enables the reduction of numerous variables to a few principal components while preserving as much variance of experimental data as possible. Thereby, the influences of different enzyme preparations regarding the individual anthocyanins become more apparent. The analysis included the anthocyanin profile of fresh bilberry fruits, the juices produced thereof, and the corresponding pomaces (see **Table 1** and **2** of the Supporting Information). The latter were included because they contain the remaining anthocyanins, which were not extracted into the juices and therefore complete the anthocyanin profile.

The statistical model obtained for the lower enzyme dosage of 0.5 nkat/g describes 91.9 % (F1: 76.0 %, F2: 15.9 %) of the total variance of the anthocyanin profile. As shown in **Figure 3-4**, the fresh fruit, the juices, and the pomaces are well separated into three distinct groups by PCA. While the fresh fruit is characterized by the highest portions of cy-arabinoside and galactosides of cy, dp, and pt, the juices show increased portions of mv- and pn-glycosides. Pomaces show the highest portions of dp-glycosides as well as pt-galactoside and -arabinoside. This distribution shows the basic change in composition of the anthocyanin profile occurring in bilberry fruits during juice production. While the genuine anthocyanin profile differs a lot from the profile of corresponding juices and pomaces, the distribution of the latter two is less

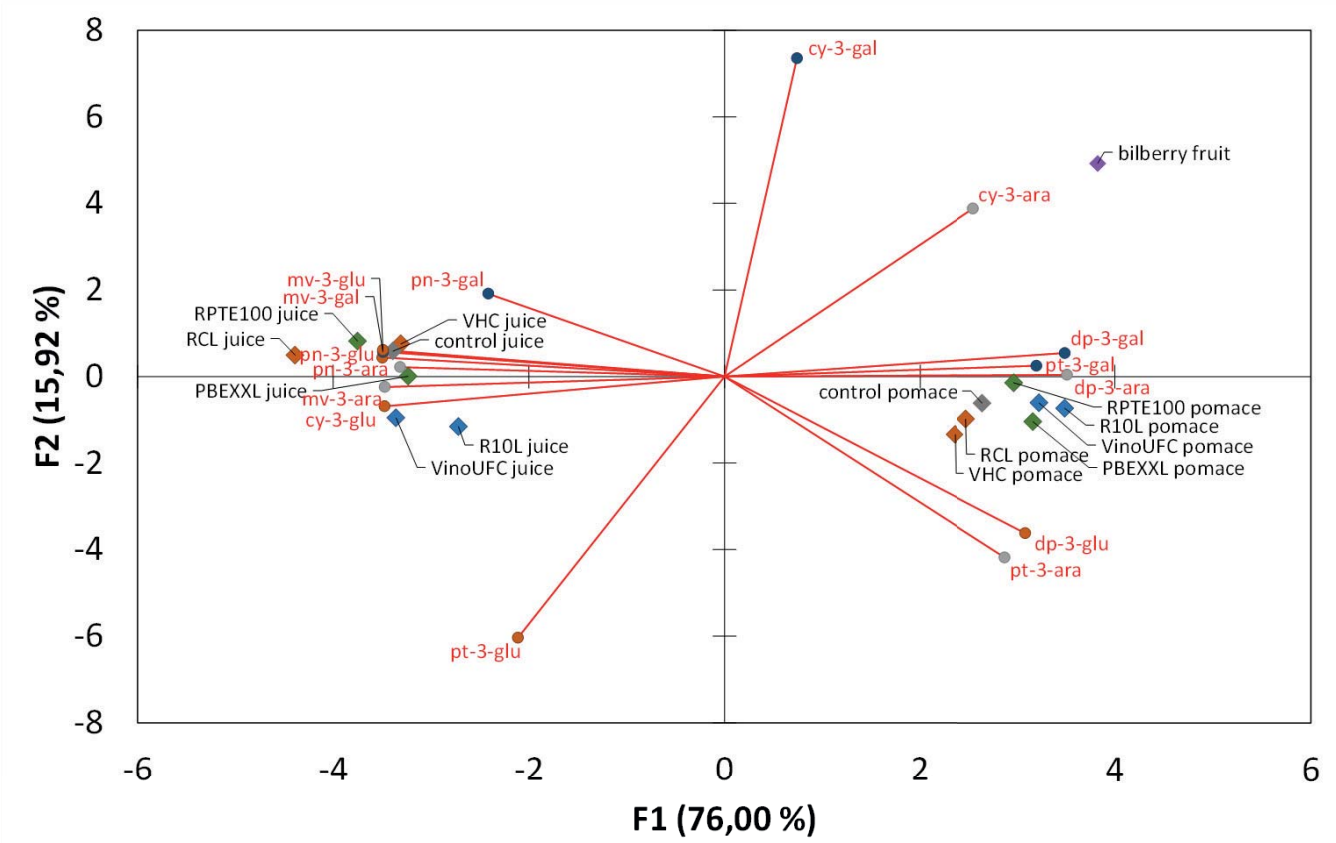

Figure 3-4: PCA of anthocyanin profiles during bilberry juice processing applying various enzymes at a dosage level of 0.5 nkat/g. ●: galactosides, ●: arabinosides, ●: glucosides, ♦: polygalacturonases, ♦: pectin lyases, ♦: cellulases

pronounced. Thus, profile differences between juices produced with different enzyme preparations seem to be small. This is supported by the position of the control sample.

As the control juice is prepared without any pectinolytic enzyme, the changes in the anthocyanin profile compared with the fresh fruit must be attributed to non-enzymatic effects. Anthocyanins show an uneven extractability (Heffels et al., 2015) and it has been reported that thermal treatments like blanching, boiling and steaming affect the anthocyanin contents of red cabbage (Volden et al., 2008), black raspberry (Hager et al., 2008), blueberry (Lee et al., 2002; Srivastava et al., 2007), strawberry, and blackberry (Patras et al., 2009) products. Thus, the narrow allocation of juices to the control juice leads to the assumption that the anthocyanin profiles are affected by differences in extractability and thermal stability rather than by enzymatic degradation. Nevertheless, there are differences in the anthocyanin profiles, which can be attributed to the applied enzymes.

Both juices processed using polygalacturonases (R10L and VinoUFC) are negatively correlated with F2, whereas all other enzyme treated juices as well as the control juice are positively correlated. Polygalacturonases lead to changes of the anthocyanin profile that are different from those caused by the application of pectin lyases or cellulases. Juices made using R10L and VinoUFC correlate negatively with F2 and all factor loadings of galactosides show a positive correlation with F2. This indicates the reduction of the galactoside portions in these juices compared to juices produced with pectin lyases and cellulases. It can be assumed that the polygalacturonase activity causes cleavage of the anthocyanin galactosides. Viljanen et al. observed similar effects applying a pectinase at 100 nkat/g on lingonberries. They found the main anthocyanin cy-3-gal to be very sensitive to pectinase treatment because approximately 60 % was degraded, whereas cy-3-glu and cy-3-ara were not affected by enzymatic treatment (Viljanen et al., 2014).

In the present study, the application of polygalacturonases at low dosages only led to decreased portions of cy-3-gal indicated by its highly positive correlation with F2 and decreased portions of dp-3-gal and pt-3-gal indicated by their highly positive correlation with F1. However, pn-3-gal and mv-3-gal showed increased portions as they correlate highly negatively with F1. This inhomogeneous distribution of galactosidic factor loadings contradicts the above-mentioned assumption of an exclusively glycoside related enzymatic degradation. Previous studies on blueberries demonstrated the alteration of the anthocyanin profile during maceration to be dependent on the aglycone (Skrede et al., 2000; Lee et al., 2002).

Skrede et al. ranked the anthocyanins in the following order of decreasing stability during blueberry juice production: mv > cy > pn > pt > dp. Likewise, Lee et al. found increased malvidin portions accompanied by decreasing delphinidin and petunidin portions during blueberry juice processing (Lee et al., 2002; Skrede et al., 2000). These findings are in line with the present results. At a dosage of 0.5 nkat/g all juices show decreased portions of dp- and pt-glycosides and increased proportions of mv- and pn-glycosides irrespective of their sugar moiety. Both effects occurred during juice processing at an enzyme dosage of 0.5 nkat/g, and thus it cannot be differentiated whether changes in anthocyanin profile are based on aglycone or glycoside depending effects.

Juices obtained with the aid of the cellulases RCL and VHC and the pectin lyase RPTE100 are quite different from those produced using polygalacturonases. They show higher ratios of cy-3-gal as well as mv- and pn-3-gal because they are associated with higher values for F2 and lower values for F1. The distinct position along F2 indicates a reduced portion of glucosides,

as pt-3-glu and dp-3-glu are negatively associated with F2. Yet, a clear decrease in all glucoside portions cannot be derived, due to the highly negative correlation of the glucosides of pn, mv, and cy with F1, while the juices show almost no separation along F1. Hence, a clear association of the cellulases with the degradation of the respective glucosides cannot be deduced either.

The juice produced with the aid of the pectin lyase PBEXXL was expected to be allocated close to the juices produced using the other pectin lyase. However, it takes an intermediate position between polygalacturonases and pectin lyases. Thus, the main activity of this lyase is accompanied by a strong polygalacturonase side activity. Puupponen-Pimiä et al. reported similar strong side activities of PBEXXL (Puupponen-Pimiä et al., 2008).

The pomaces can well be distinguished from their corresponding juices along the axis of F1. This separation predominantly reflects an inhomogeneous release of anthocyanin from the fruit during juice processing, resulting in considerably different anthocyanin profiles of juices and the remaining pomaces.

Dosages of 10 nkat/g are significantly above those typically applied in commercial processes. As reported in numerous previous studies, the influence of enzymatic treatment on the anthocyanin profile applying high dosages is more distinct. The PCA shown in **Figure 3-5** represents 93.6 % of the total variance of the original data. The principal component F1 accounts for 58.8 % and F2 accounts for 34.7 %. Similar to the results obtained at lower dosage, the fresh fruit, juices, and pomaces are well separated into three groups. The variance of the juices is considerably more pronounced, implying a greater influence of the enzyme preparations on the anthocyanin profiles. A distinct separation of groups along the axis of F1 can be made, which separates the juices from the corresponding pomaces and the fresh fruit. The fruit and pomaces are associated with positive values for F1, whereas the juices are negatively associated with F1.

Juices prepared with the aid of polygalacturonases align with positive values for F2, whereas juices obtained with cellulase treatment are correlated negatively with F2. Although all juices are negatively correlated with F1, the extent of negative correlation is less for cellulases than for polygalacturonases. This implies a stronger alteration of the profile due to polygalacturonases activity compared with the fresh fruit. Considering the loadings of variables, similar trends like those for lower dosage can be observed, yet to a greater extent.

All galactosides show reduced portions in juices produced with polygalacturonases because the loadings of galactosides are associated with positive values for F1 and negative values for F2.

Only mv-3-gal is correlated negatively with both F1 and F2. This supports the above-mentioned assumption that the profile alteration due to the application of enzymes is glycoside related.

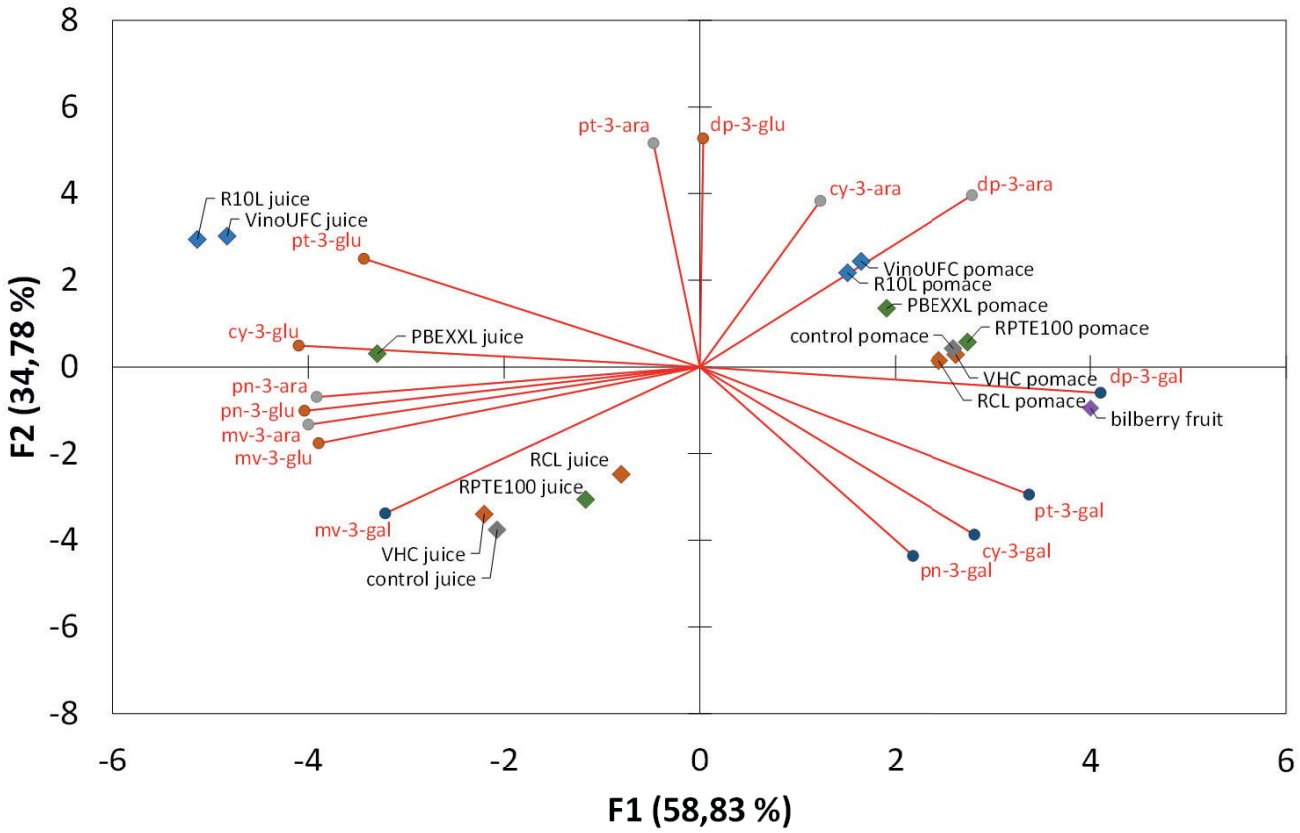

Figure 3-5: PCA of anthocyanin profiles during juice processing applying various enzymes at a dosage level of 10 nkat/g. ●: galactosides, ●: arabinosides, ●: glucosides, ♦: polygalacturonases, ♦: pectin lyases, ♦: cellulases

The glucosides are highly correlated with negative values for F1 except for dp-3-glu, which shows a highly positive correlation with F2. Because juices produced using cellulases are associated with higher values for F1 and lower values for F2 compared with juices obtained with the aid of polygalacturonases, they contain higher proportions of galactosides and lower proportions of glucosides. A degrading effect of cellulases on the glucosidic bonds is conceivable and further substantiates the above-mentioned hypothesis of a glycoside related degradation. Nevertheless, it has to be stressed that effects for cellulases are less pronounced than for polygalacturonases.

The differences between the two pectin lyases PBEXXL and RPTE100 are even more apparent at a dosage level of 10 nkat/g compared to the differences which have been observed at a dosage of 0.5 nkat/g. While the juice produced using RPTE100 shows changes in the profile similar to

those of the juices prepared with the aid of cellulases, the application of PBEXXL leads to an intermediate allocation between polygalacturonases and cellulases. This kind of profile changes confirms the strong polygalacturonase side activity of the pectin lyase PBEXXL. Interestingly, the non-treated control juice is associated with the juices after treatment with cellulases and the pectin lyase RPTE100. As the control juice indicates the changes in the anthocyanin profile due to non-enzymatic effects, the distance to all other juices represents the degree of the enzymatic influences. This implies that the effects due to polygalacturonases and the pectin lyase PBEXXL are considerably higher compared with those of the other enzymes.

The above discussed trends can also be observed for the corresponding pomaces. The control pomace is closely related to the pomaces obtained after treatment with cellulases and the pectin lyase RPTE100. They are characterized by reduced glucoside proportions compared with the pomaces resulting from treatment with polygalacturonase. The latter, in turn, show decreased proportions of galactosides. The pomace obtained after treatment with pectin lyase PBEXXL is allocated between the pomaces of polygalacturonase treated berries and those after application of pectin lyase RPTE100 and cellulases, similar to the distribution in the group of juices.

Authentication aspects

The addition of pectinolytic enzymes and cellulases during mash treatment at industrially common dosages (0.5 nkat/g) considerably influences the anthocyanin profile of bilberry juices. The degree of these variations needs to be considered because these profiles are frequently used for authenticity control. As it was discussed above, also non-enzymatic effects or secondary effects are relevant. The changes in anthocyanin profiles of closely related *Vaccinium* species caused by the different extractability of the anthocyanins was recently demonstrated (Heffels et al., 2015). The natural differences in anthocyanin profiles exceed the variations caused by industrial juice processing and therefore do not affect authenticity studies. In contrast, excessive enzyme dosages result in more pronounced differences, which may be relevant for authentication.

4 Conclusion

The anthocyanin profile of bilberries is altered during juice processing. The type and extent of alteration is affected by the enzyme activity and dosage. At a dosage level of 0.5 nkat/g commonly used in industrial processing, polygalacturonases and cellulases show a preferred degradation of galactosides and glucosides, respectively. At the same time, all juices, including the non-treated control juice show a decreased proportion of dp- and pt-glycosides. Thus, no conclusion can be made whether juice processing alters the anthocyanin profile depending on the aglycone or the glycoside moiety. However, the slight differences between profiles of the control juice and juices produced with the aid of enzymes suggest that non-enzymatic effects predominate. Extractability and thermal stability of anthocyanins are more relevant for changes in anthocyanins during juice processing at an industrially common dosage level of enzymes than the action of the enzyme itself. The well-reported hypothesis of glycoside dependent anthocyanin degradation during juice processing is supported by the results of the application of enzymes exceeding common dosages (10 nkat/g). In this case, alterations regarding the anthocyanin profiles are more pronounced. Juices obtained with the aid of polygalacturonases and cellulases show clearly decreased galactoside and glucoside amounts, respectively. However, it should be noted that these effects only occur by overdosing pectinolytic enzymes and cellulases, as shown in the present study as well as in literature. The anthocyanin profile is altered during juice production due to various effects. The positive influence on the extractability of anthocyanins seems to exceed the degrading effects of the added enzyme. However, the degradation of cell walls might lead to secondary effects which influence the anthocyanin profile. The reported interactions of phenolic compounds with cell wall polysaccharides may also occur with fragments of the latter. The complex variety of cell wall degradation products and their effects on polyphenols necessitate a prudent application of enzymes regarding dosage and main activity during the processing of different fruits.

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Chapter 4

Profiling of iridoid glycosides in *Vaccinium* species by UHPLC-MS

The iridoid profile of four *Vaccinium* species was investigated using UHPLC-MS to obtain further information about this group of species for phytochemical characterization. Fruits of bog bilberry (*Vaccinium uliginosum* L.) showed 14 different iridoid glycosides with a total amount of 20 mg/kg fresh weight (FW), whereas bilberry (*Vaccinium myrtillus* L.) contained 11 iridoid glycosides and a total amount of 127 mg/kg FW. Highbush blueberry (*Vaccinium corymbosum* L.) and lowbush blueberry (*Vaccinium angustifolium* L.) contained none of the investigated iridoid glycosides. Among the different iridoids, the isomers scandoside and deacetylasperulosidic acid as well as a dihydro derivative thereof were described for the first time in the Ericaceae family. The *p*-coumaroyl isomers of scandoside, deacetylasperulosidic acid and dihydromonotropein are reported for the first time in *V. myrtillus* and *V. uliginosum*. Monotropein and its *p*-coumaroyl isomers were found for the first time in *V. uliginosum*. The comparison of iridoid profiles in bilberry fruit and juice samples revealed constant proportions throughout the juice processing. Quantification and profile determination of iridoids may be used for species differentiation and thus for authentication purposes.

Keywords: *Vaccinium* species; iridoids; profiling; authentication; UHPLC-MS

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1 Introduction

Iridoids are a widespread group of monoterpenes encompassing over 1200 different compounds (Boros & Stermitz, 1990; Dinda et al., 2007; Dinda et al., 2009; Dinda et al., 2011; El-Naggar & Beal, 1980). In plants, they have protective functions, while several physiological effects such as antimicrobial, analgesic, immunomodulatory or antioxidant activities have been reported as well (Hänsel et al., 2010). In natural medicine, they have often been applied as tonics, sedatives, diuretics, antidiabetics, and for wound healing (Dinda et al., 2011).

Most iridoids show a glycosylated lactol ring, which stabilizes the structure and increases the hydrophilicity compared to other monoterpenes (Hänsel et al., 2010; Lindner et al., 2014). Glucose is usually attached as the sugar moiety. Iridoids occur in all plant organs from the roots to the leaves and seeds (Rebeiz et al., 2010; Seigler, 1999). Although they are present in a wide range of botanical families (Dinda et al., 2007), they are particularly common in Rubiaceae, where they account for the major part of secondary metabolites in the subfamilies Ixoroideae and Rubioideae (Martins & Nunez, 2015).

Monotropein **1** is a widespread representative of the iridoid class, which predominantly occurs in glycosylated form (**Figure 4-1**) (Asada et al., 2013; Nagatoshi et al., 2011). It was found in several plants used in natural medicine such as bog rosemary (*Andromeda polifolia* L.) (Chung et al., 1980), morinda roots (*Morinda citrifolia* L.) (Shin et al., 2013) and cleaver (*Galium melanantherum*) (Tzakou et al., 2007), but also in edible plants and derived products like juices of American cranberry (*Vaccinium macrocarpon*), small Cranberry (*Vaccinium oxycoccus*),

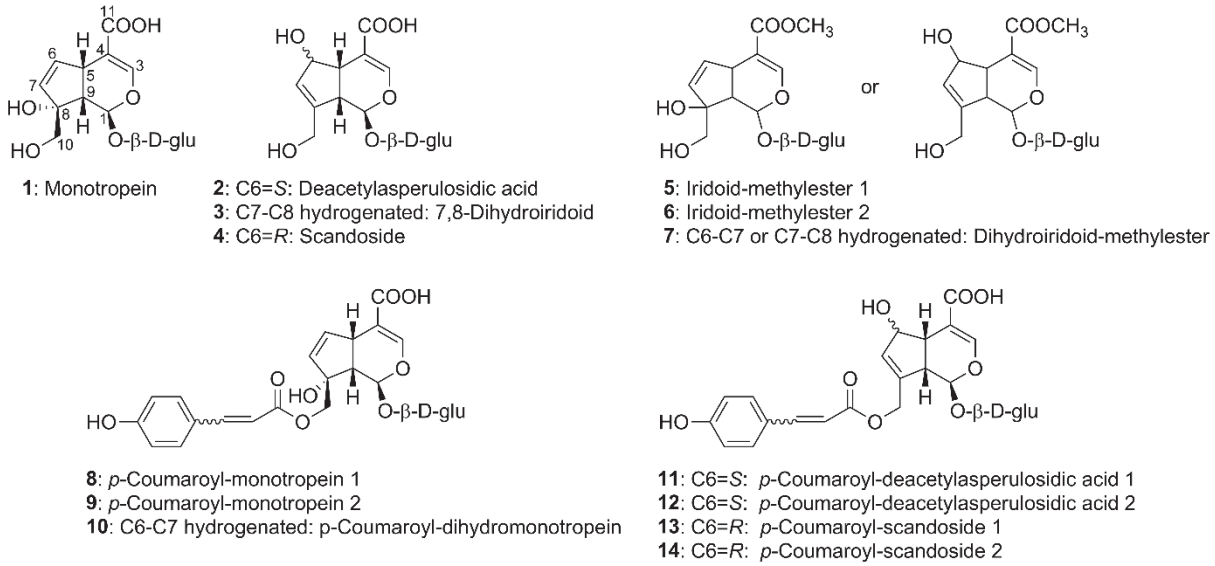

Figure 4-1: Iridoid glucosides detected in *Vaccinium myrtillus* and *Vaccinium uliginosum*.

lingonberry (*Vaccinium vitis-idaea*), and bilberry (*Vaccinium myrtillus*) (Jensen et al., 2002). Numerous derivatives of monotropein have been found in various plants. The 6,7-dihydromonotropein derivative was detected in all above mentioned berries except bilberry (Jensen et al., 2002). *p*-Cumaroyl monotropein **8** and **9** (**Figure 4-1**) and their derivatives were detected in bog rosemary (Chung et al., 1980). The respective reduced compound *p*-coumaroyl dihydromonotropein **10** (**Figure 4-1**) was found in American cranberry (Turner et al., 2007). Most of the above-mentioned plants belong to the botanical genus *Vaccinium* of the Ericaceae family. Many species of the genus *Vaccinium* are well known for their high content of secondary plant metabolites. The phytochemistry is dominated by a great amount and variety of polyphenols (Howard & Hager, 2007). Especially anthocyanins are characteristic for many *Vaccinium* fruits as they are responsible for the typical red or blue colored berry fruits (Kähkönen et al., 2003). They are associated with multiple health benefits which render these fruits to be attractive candidates for a health-conscious nutrition (Yousuf et al., 2016). Berry fruits of *Vaccinium* species are consumed fresh, dried, processed to jams and juices or as additives to other foods (Fügel et al., 2005). As some fruits of this genus command high prices, they are common subjects of authenticity debates (Filip et al., 2012; Fügel et al., 2005; Gardana et al., 2014; Penman et al., 2006; Primetta et al., 2013). In this context, profiles and quantities of secondary plant metabolites have frequently been used as authenticity markers. The significance of differences in these profiles may be explained by phytochemical considerations. Evolutionary processes may influence the metabolism of plants, leading to changes in the structure and proportions of certain compounds. These processes lead to phytochemical differences and may allow the assessment of authenticity among closely related plants based on their secondary plant metabolites. Iridoid synthesis follows the biosynthetic pathway of the monoterpene synthesis via the cytosolic mevalonic acid (MVA) pathway or the plastidial 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Hänsel et al., 2010; Rebeiz et al., 2010). The resulting monoterpenes isopentenyl diphosphate and dimethylallyl diphosphate react via a head-to-tail addition to form geranylpyrophosphate (Heldt & Piechulla, 2015), from which 10-oxogeranial is synthesized (Sun et al., 2012). The latter may cyclize via Michael addition or a Hetero-Diels-Alder reaction to form iridodial (open ring) and nepetalactol (closed ring) (Geu-Flores et al., 2012; Lindner et al., 2014). These two precursors may form various iridoids via two different routes. Route I yields secoiridoids and terpenoid indole alkaloids, whereas route II leads to decarboxylated carbocyclic iridoids (Sun et al., 2012). Evolutionary changes in the biosynthesis may lead to changes in the structure and proportions of the final iridoids. The

resulting phytochemical differences, from a food analytical point of view, may help understand the metabolism of plants and, might be the basis of modern authenticity verification.

The present study covers the fruits bilberry (*Vaccinium myrtillus*), highbush blueberry (*Vaccinium corymbosum*), lowbush blueberry (*Vaccinium angustifolium*), and bog bilberry (*Vaccinium uliginosum*). The qualitative and quantitative profiles of iridoids in these species were investigated by UHPLC-MS^n in order to expand the basis for their phytochemical characterization.

2 Materials and methods

2.1 Samples

Samples (berries, juices, and concentrates) were provided by Eckes-Granini Deutschland GmbH (Nieder-Olm, Germany), Haus Rabenhorst O. Lauffs GmbH & Co. KG (Unkel, Germany), Rudolf Wild GmbH & Co. KG (Heidelberg, Germany), SGF International e.V. (Nieder-Olm, Germany) or were manually collected (**table 4-1**). All samples were stored deep frozen at -20 °C until extraction.

Table 4-1. Composition of the sample set in terms of origin and species.

	V. angustifolium	*V. corymbosum*		*V. myrtillus*		*V. uliginosum*
	fruit	fruit	juice	fruit	juice	fruit
Austria	-	-	-	-	1[c]	-
Belgium	-	-	-	1	-	-
Germany	-	3	-	7	-	-
Poland	-	-	-	9	2[j,c]	2
Romania	-	-	-	1	1[j]	-
Serbia	-	-	-	2	-	-
Ukraine	-	-	-	5	2[c]	-
Belarus	-	-	-	2	-	-
Canada	5	-	-	-	-	-
USA	1	1	1[c]	-	-	-
Argentina	-	1	-	-	-	-
Chile	-	1	1[c]	-	-	-
Peru	-	1	-	-	-	-
Morocco	-	1	-	-	-	-
Spain	-	2	-	-	-	-
unknown	-	1	-	2	2[c]	-

Numbers indicate the number of samples of the specific origin and species, [c]: concentrate, [j]: juice.

2.2 **Chemicals and standards**

Ultrapure water was obtained from a Synergy purification system (Millipore, Molsheim, France). HPLC grade acetonitrile, acetic acid, and hydrochloric acid were from VWR (Mannheim, Germany). Formic acid (≥ 98 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol was from Th. Geyer (Renningen, Germany), and regenerated cellulose filters (Chromafil RC-20/15 MS) and SPE cartridges (Chromabond HR-XC, 500 mg, 3 mL) were supplied by Macherey-Nagel (Düren, Germany). Deacetylasperulosidic acid standard was

provided by the Department of Botany and Biodiversity Research of the University of Vienna and monotropein standard was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). *Galium spurium* leaves were obtained from herbarium specimen *W. Till 110593* collected near Tribuswinkel, Lower Austria, Austria, and preserved in the Herbarium WU, University of Vienna (voucher number WU 0062288). Detailed information and a digital image of the specimens are accessible at http://www.herbarium.univie.ac.at.

2.3 Extraction

Fresh berries samples (50 g) were homogenized with 100 mL extraction solvent (methanol/water/glacial acetic acid, 80/15/5, v/v/v) using an Ultra Turrax T18 basic (IKA-Werke GmbH & CO. KG, Staufen, Germany). After sonication for 10 minutes, samples were centrifuged for 10 min at 3941 *g* (Zentrifuge, Thermo Fisher Scientific, Schwerte, Germany). Pellets were extracted again with 50 mL extraction solvent. The supernatants were combined and the organic solvent was evaporated under vacuum. The resulting extract was made up to 100 mL with water. Frozen juices and concentrates were diluted with distilled water to 1°Brix.

2.4 Solid-phase extraction (SPE)

SPE was carried out on multimode cartridges Chromabond HR-XC consisting of a strong acidic benzosulfonic acid cation exchanger based on polysterene-divinyl-benzene according to a previously reported extraction process (Jungfer et al., 2012). First, the cartridges were conditioned with 5 mL of methanol and 5 mL of distilled water. The cartridges were loaded with 20 mL extract, subsequently washed with 40 mL of 0.1 M HCl, and treated with 3 mL of methanol for the elution of analytes. After evaporation to dryness under nitrogen, the samples were dissolved with 2 mL distilled water for further analysis.

2.5 UHPLC-MS identification and quantification of iridoids

The identification and quantification of iridoids was conducted on a Waters Aquity I-Class system (Milford, MA, USA) coupled with a LTQ-XL ion trap mass spectrometer (Thermo Scientific, Inc., Braunschweig, Germany). The column was a Kinetex C-18, 1.7 µm particle size (150 × 2.1 mm) (Phenomenex, Inc., Aschaffenburg, Germany) equipped with a security guard cartridge of the same material (2.1 × 5 mm, 1.7 µm). The following gradient was used at a flow rate of 0.4 mL/min: 0 min, 0 % B; 20 min, 25 % B; 25 min, 90 % B; 31 min, 100 % B; 35 min,

100 % B; 36 min, 0 % B; 40 min, 0 % B, where eluent A was water/formic acid (99.9/0.1, v/v) and eluent B was acetonitrile/formic acid (99.9/0.1, v/v). The conditions of the mass spectrometer were as follows: capillary was set at a temperature of 300 °C in negative electrospray ionization (ESI) mode and was operated at a voltage of -12 V. The source voltage was maintained at 3.5 kV at a current of 100 µA. The tube lens was adjusted to -64 V. Nitrogen was used as the sheath, auxiliary, and sweep gas at a flow of 80, 50, and 1 arbitrary units, respectively. Collision-induced dissociation spectra were obtained at 35 eV using helium as the collision gas. All compounds were quantified as monotropein equivalents by external calibration (1-500 mg/L). The calibration curve showed a regression of R^2=0.993.

3 Results and discussion

In total, 14 iridoid glucosides were either unambiguously identified or tentatively characterized in the four *Vaccinium* species (**Table 4-1**). UHPLC-MS² chromatograms of *Vaccinium* extracts are shown in **Figure 4-2**. *Vaccinium angustifolium* and *Vaccinium corymbosum* showed none of the iridoid glucosides found in *Vaccinium myrtillus* and *Vaccinium uliginosum*. All identified compounds are listed in **Table 4-2** with the respective proportions of product ions.

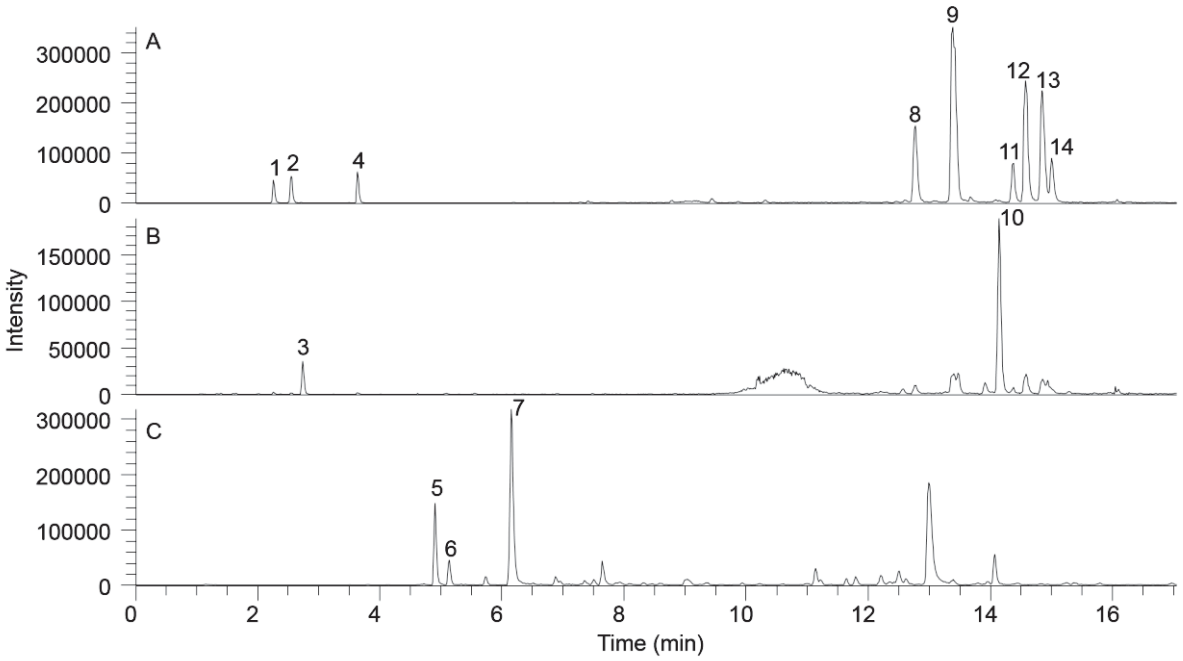

Figure 4-2: Extracted ion chromatogram of iridoids in **A**: bilberry (*Vaccinium myrtillus*) mass traces m/z 389 + 435 + 535 + 581, **B**: bilberry (*Vaccinium myrtillus*) mass traces m/z 391 + 437 + 537 + 583, and **C**: bog bilberry (*Vaccinium uliginosum*) m/z 403 + 449 + 405 + 451. For compound names see **table 4-2**.

Compound **1** eluted at a retention time of 2.24 minutes as formate adduct ($[M+formate]^-$, *m/z* 435) and the MS^2 experiment revealed its molecular ion after a loss of 46 Da ($[M-H]^-$, *m/z* 389). A product ion corresponding to a loss of 162 Da after a further MS^3 experiment was detected (*m/z* 227), suggesting the loss of a hexose (**Figure 4-3**). Further secessions of 44 Da and 18 Da (*m/z* 371, *m/z* 345, and *m/z* 327) can be attributed to the loss of CO_2 and water, respectively. Both mass differences occur in various consecutive steps, pointing out that the respective substituents are independently attached to the core structure. A comparison with a monotropein standard confirmed the identity of compound **1**, since the monotropein standard coelutes with compound **1** and shows the same fragmentation pattern. This is supported by previous findings

of monotropein in juice of American and European cranberries (*V. macrocarpon* and *V. oxycoccos*), lingonberries (*V. vitis-idaea*), and bilberries (*V. myrtillus*) (Jensen et al., 2002).

Compounds **2** and **4** showed similar fragmentation patterns as compound **1** albeit with slightly different abundance of the product ions (**Table 4-2**). It can be assumed that compounds **2** and **4** are isomers of monotropein. The most abundant product ion of compound **1** at *m/z* 191 indicates the loss of a hexose and two distinct water molecules (**Figure 4-3**). The structure of compound **1** presents vicinal hydroxyl groups at the positions C-8 and C-10 of monotropein, which promotes the loss of a second water moiety by forming an epoxy ring during fragmentation. This additional loss of a water molecule was not observed for compounds **2** and **4**, since the corresponding signal at *m/z* 191 is very low. The signals at *m/z* 227, which

Table 4-2. UHPLC-MS^n based identification of iridroids in *V. uliginosum* and *V. myrtillus*.

Comp.	Name	Rt (min)	$[M+Formate]^-$ (*m/z*)	$[M-H]^-$ (*m/z*)	Product ions (*m/z*)
1	Monotropein	2.24	435 (100)	389 (10)	371 (6), 345 (7), 327 (4), 227 (87), 209 (10), 191 (100), 183 (7), 165 (55), 147 (42)
2	Deacetylasperulosidic acid	2.51	435 (100)	389 (34)	371 (2), 345 (0), 327 (0), 227 (49), 209 (100), 191 (4), 183 (60), 165 (35), 147 (4)
3	7,8-Dihyroiridoid	2.74	437 (100)	391 (5)	373 (10), 347 (30), 329 (7), 229 (100), 211 (20), 185 (25), 167 (57), 149 (3)
4	Scandoside	3.64	435 (100)	389 (70)	371 (2), 345 (0), 327 (0), 227 (84), 209 (61), 191 (7), 183 (100), 165 (38), 147 (8)
5	Iridoid-methylester 1	4.90	449 (100)	403 (2)	371 (1), 241 (100), 223 (4), 209 (0.5), 191 (2)
6	Iridoid-methylester 2	5.15	449 (100)	403 (0.5)	371 (2), 241 (100), 223 (3), 209 (0.5), 191 (1)
7	Dihydroiridoid-methylester	6.15	451 (100)	405 (2)	387 (0.5), 373 (4), 243 (100), 225 (3), 211 (0.5)
8	*p*-Coumaroyl-monotropein derivative 1	12.76	581 (100)	535 (22)	491 (29), 373 (55), 371 (100), 355 (2), 329 (74), 311 (16), 209 (4), 191 (35), 165(13), 163 (32)
9	*p*-Coumaroyl-monotropein derivative 2	13.40	581 (100)	535 (6)	491 (19), 373 (53), 371 (100), 355 (2), 329 (57), 311 (11), 209 (4), 191 (25), 165(7), 163 (31)
10	*p*-Coumaroyl-6,7-dihydromonotropein	14.14	583 (100)	537 (6)	493 (36), 375(24), 373 (100), 357 (3), 331 (70), 313 (11), 211 (3), 193 (6), 167 (2), 163 (20)
11	*p*-Coumaroyl-deacetylasperulosidic acid derivative 1	14.36	581 (22)	535 (100)	491 (25), 373 (44), 371 (11), 355 (100), 329 (98), 311 (10), 209 (1), 191 (5), 165 (2), 163 (13)
12	*p*-Coumaroyl-deacetylasperulosidic acid derivative 2	14.57	581 (100)	535 (25)	491 (22), 373 (39), 371 (12), 355 (100), 329 (96), 311 (13), 209 (1), 191 (7), 165 (2), 163 (12)
13	*p*-Coumaroyl-scandoside derivative 1	14.84	581 (100)	535 (24)	491 (100), 373 (6), 371 (5), 355 (73), 329 (20), 311 (25), 209 (1), 191 (2), 165 (2), 163 (17)
14	*p*-Coumaroyl-scandoside derivative 2	15.00	581 (40)	535 (100)	491 (100), 373 (5), 371 (6), 355 (74), 329 (17), 311 (33), 209 (1), 191 (1), 165 (2), 163 (18)

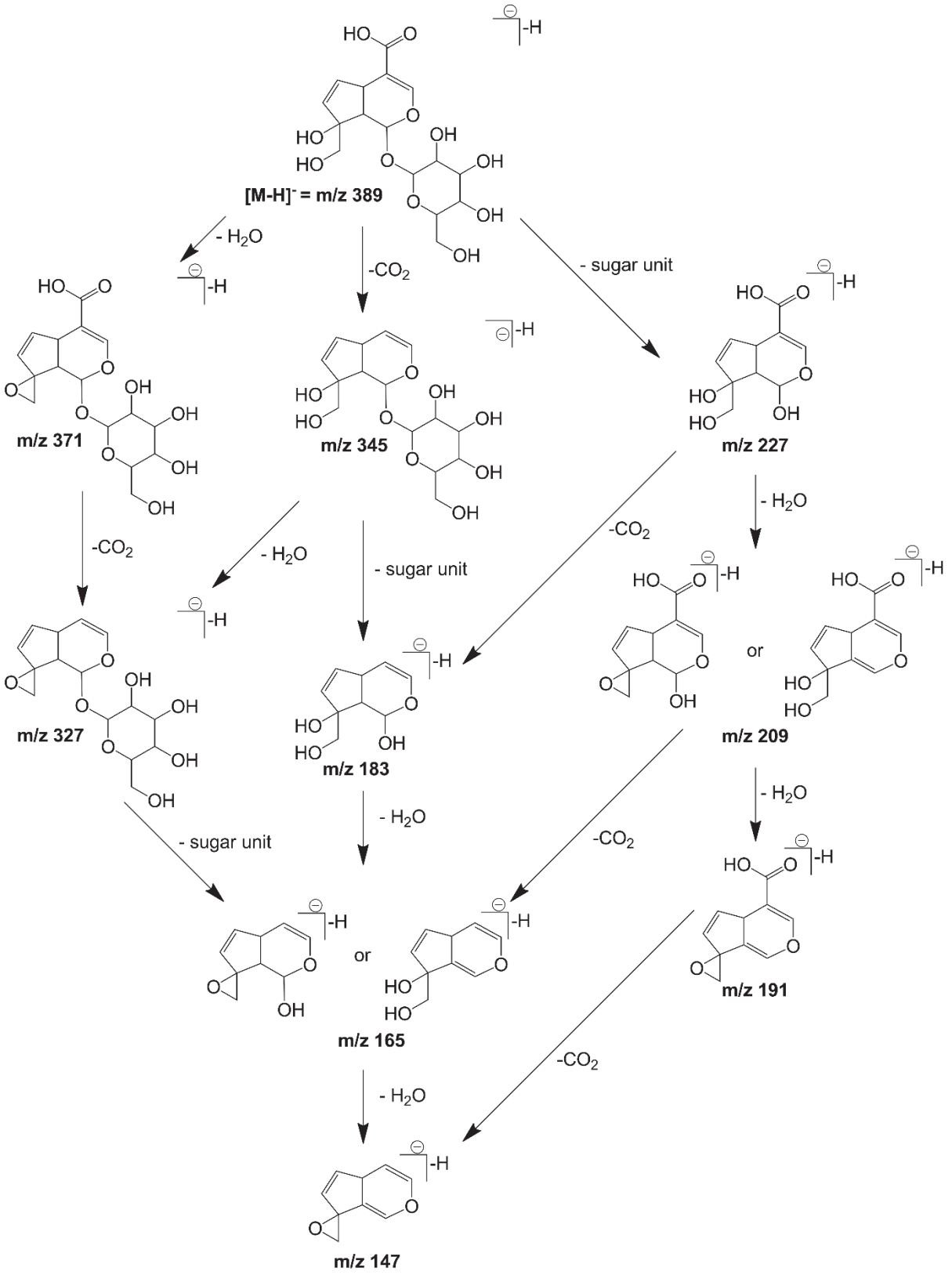

Figure 4-3: Suggested fragmentation pathway of compound 1 (Monotropein).

corresponds to the loss of a hexose, and at *m/z* 209, which is caused by the loss of a hexose and one single water molecule, respectively, are dominating.

It can be assumed that the position of the hydroxyl group, which is responsible for the second loss of water in compound **1,** must be different in compounds **2** and **4**. We hypothesized that the fragmentation patterns observed can be explained by the structures of scandoside and deacetylasperulosidic acid. As these iridoids are known to be present in *Galium spurium* (Dalrae & Dae, 2012), a comparison with an extract of this species was performed. The signals of scandoside and deacetylasperulosidic acid in *Galium spurium* coincided in respect of retention time, UV-absorption spectra, and fragmentation pattern with compound **2** and **4**. The assignment was conducted by a comparison with a deacetylasperulosidic acid standard which showed the same retention time, UV-absorption spectra and fragmentation pattern as compound **2**, and accordingly compound **4** can be identified as scandoside. Scandoside and deacetylasperulosidic acid are well known from various Rubiaceae species as *Galium spurium* L. (Dalrae & Dae, 2012), *Paederia scandens* (Wu et al., 2013), *Morinda citrifolia* L. (Kamiya et al., 2008), and *Ronabea emetica* (Berger et al., 2011) but are described here for the first time in fruits of Ericaceae species. Scandoside was previously reported in leaves of *Rhododendron latoucheae* and buds of *Vaccinium dunalianum* which are also part of the Ericaceae family (Fan et al., 2001; Zhao et al., 2008).

Compound **3** had a parent ion of *m/z* 391 and showed similar fragmentation products as compounds **1**, **2**, and **4** with a shift of 2 Da to higher *m/z* ratios. This leads to the assumption that one double bond is hydrogenated compared to the structure of monotropein or its isomers. The absence of a fragment ion with *m/z* 193, which would indicate a second loss of water similar to the fragment ion *m/z* 191 for monotropein and the dominance of fragment ion *m/z* 211 and *m/z* 229 similar to the fragment ion *m/z* 209 and *m/z* 227 for scandoside and deacetylasperulosidic acid, indicate that compound **3** is a hydrogenated derivative of scandoside or deacetylasperulosidic acid rather than 6,7-dihydromonotropein. This is supported by the fact that 6,7-dihydromonotropein was found in cranberries (*V. macrocarpon*) and lingonberries (*V. vitis-idaea*) but could not be detected in bilberries (*V. myrtillus*) (Jensen et al., 2002). However, a comparison with standard substances is necessary for final confirmation. To our knowledge, such a reference compound is not commercially available. It would be the first time that a dihydro derivative of scandoside or deacetylasperulosidic was described in Ericaceae. A similar compound, 6-β-hydroxyadoxosidic acid, was previously described in *Castillerja integra* (Scrophulariaceae) (Mead & Stermitz, 1993).

Compounds **5**, **6**, and **7** were quantifiable only in *V. uliginosum* samples. The molecular ions of compounds **5** and **6** with *m/z* 403 showed a loss of 32 Da (*m/z* 371), indicating the presence of a methoxy substituent. The base peak during fragmentation arose from a neutral loss of a hexose with 162 Da (*m/z* 241). Additional losses of water (*m/*z 223) and a methoxy unit (*m/*z 191) were also observed. Compound **7** showed the same ion pattern with a shift of 2 Da to higher masses. For all three compounds the fragment ion due to the loss a hexose (*m/z* 241 and *m/z* 243) is dominating. Based on the present fragmentation patterns, compounds **5** and **6** can tentatively be identified as the methyl ester of an iridoid glycoside and compound **7** as a dihydroiridoid glycoside methyl ester. Methyl esters of monotropein and dihydromonotropein were reported in Fouquieriaceae (Jensen & Nielsen, 1982) and the methyl ester of dihydromonotropein was also found in Ericaceae plants *Craibiodendron henryi* (Huang et al., 2005) and *V. uliginosum* (Kim et al., 2015).

Compounds **8**, **9**, **11**, **12**, **13**, and **14** occurred as formate adducts at *m/z* 581 rather than as their molecular ion at *m/z* 535. Compounds **8**, **9**, **12**, and **13** revealed the molecular ions after a MS^2 experiment, whereas compounds **11** and **14** primarily occurred as molecular ions without previous fragmentation. All molecular ions showed a loss of 44 Da (*m/z* 491) indicating the release of CO_2, and a neutral loss of 162 Da (*m/z* 373) suggesting the presence of a hexose. The fragment ion at *m/z* 163 and the neutral loss of 164 Da (*m/z* 371) from the molecular ion indicate the presence of a coumaroyl unit. The direct loss of the mentioned fragments from the molecular ion indicates that they are individually attached to the iridoid backbone of the molecule. This is supported by the fragment ions with *m/z* 355, 329, and 311, which can be attributed to the loss of a hexose and H_2O, a hexose and CO_2, or a hexose and both H_2O and CO_2, respectively. The fragment ions with *m/z* 209, 191, and 165 occur due to the loss of a hexose and coumaric acid, whereby the latter two show an additional loss of H_2O and CO_2, respectively. The fragmentation pattern described was previously reported for *p*-coumaroyl-monotropein in bilberries (*V. myrtillus*) and lingonberries (*V. vitis-idaea*) (Hokkanen et al., 2009).

The proportions of fragment ions of compounds **8**, **9**, **11**, **12**, **13**, and **14** show remarkable differences and can be categorized into three groups of two compounds. The release of coumaric acid with a neutral loss of 164 Da seems to be favored for compounds **8** and **9** since the fragment ion at *m/z* 371 shows the most intense signal. The relatively high signal at *m/z* 191 occurs due to the loss of a hexose and water additionally to the loss of coumaric acid. A similar structural effect as described above for compound **1** facilitates the release of coumaric acid. Hokkanen et al. (2009) explained the loss of coumaric acid from *p*-coumaroyl-monotropein by

the formation of an epoxy ring between the hydroxyl group at position C-8 and the C-10 atom stabilizing the resulting fragment. In contrast to compounds **8** and **9**, signals of the fragment ions at *m/z* 371 and *m/z* 191 showed low intensities for compounds **11**, **12**, **13**, and **14**, indicating a structural difference at position C-8. Accordingly, the structures of compounds **11**, **12**, **13**, and **14** appear to support the loss of coumaric acid less than those of compounds **8** and **9**, which suggests the lack of a hydroxyl group at position C-8. This points to the presence of conformational isomers of *p*-coumaroyl-monotropein such as *p*-coumaroyl-scandoside and *p*-coumaroyl-deacetylasperulosidic acid. The latter two diastereomers were reported previously in *Craibiodendron henryi*, which belongs to the Ericaceae family (Huang et al., 2005). The occurrence of three sets of two signals showing identical fragmentation patterns reflect the presence of *cis*- and *trans*-isomers of the coumaroyl-iridoids, however, an assignment is not possible based on the MS spectra. Based on the identification of compounds **1**, **3**, and **4** as monotropein, deacetylasperulosidic acid, and scandoside, respectively, the same order of elution is assumed for compounds **8**, **9**, **11**, **12**, **13**, and **14**. Thus, compounds **8** and **9** are tentatively characterized as *p*-coumaroyl-monotropein derivative 1 and *p*-coumaroyl-monotropein derivative 2, compounds **11** and **12** as *p*-coumaroyl-deacetylasperulosidic acid derivative 1 and *p*-coumaroyl-deacetylasperulosidic acid derivative 2, and compounds **13** and **14** as *p*-coumaroyl-scandoside derivative 1 and *p*-coumaroyl-scandoside derivative 2, respectively.

Compound **10** shows a molecular ion with *m/z* 537 and a predominating formate adduct with *m/z* 583. Thus, the molecular mass is 2 Da higher compared to *p*-coumaroyl-monotropein. Further similarities are the neutral losses of 44 Da (*m/z* 493) and 162 (*m/z* 375) indicating the presence of a carboxylic acid and a hexose, respectively. The fragment ion at *m/z* 163 and the neutral loss of 164 Da (*m/z* 373) suggests a coumaroyl subunit. Further fragmentation products are similar to that of *p*-coumaroyl-monotropein, whereas the aglycone unit shows a mass increment of 2 Da. This compound may therefore be assigned to a *p*-coumaroyl-dihydromonotropein as it was previously found in cranberries (*V. macrocarpon*) (Turner et al., 2007).

The iridoid profile in the four *Vaccinium* species showed significant differences. While the iridoids were absent in *V. angustifolium* and *V. corymbosum* samples, *V. myrtillus* contained notable amounts of 11 iridoid glucosides. *V. uliginosum* contained 14 iridoid glucosides (**table 4-3**).

Table 4-3. Quantification of iridoids in *Vaccinium* species (mg/kg FW as monotropein equivalents for fruits and mg/L as monotropein equivalents for juices).

Comp.	Name	*V. myrtillus* fruit	*V. myrtillus* juice	*V. uliginosum*
1	Monotropein	4.4 ± 1.2	54.0 ± 34.0	0.8 ± 0.1
2	Deacetylasperulosidic acid	1.6 ± 0.9	42.7 ± 16.0	0.2 ± 0.1
3	7,8-Dihyroiridoid	2.7 ± 0.8	52.5 ± 23.7	0.6 ± 0.2
4	Scandoside	0.6 ± 0.4	16.1 ± 5.8	0.7 ± 0.1
5	Iridoid-methylester 1	n.q.	n.q.	5.9 ± 2
6	Iridoid-methylester 2	n.q.	n.q.	1.5 ± 0.9
7	Dihydroiridoid-methylester	n.q.	n.q.	21.2 ± 2.3
8	*p*-Coumaroyl-monotropein derivative 1	22.6 ± 5.2	300.8 ± 195.7	6.2 ± 1.2
9	*p*-Coumaroyl-monotropein derivative 2	55.0 ± 12.8	841.3 ± 439.3	4.6 ± 1.2
10	*p*-Coumaroyl-6,7-dihydromonotropein	3.0 ± 1.3	57.2 ± 26.2	0.8 ± 0.3
11	*p*-Coumaroyl-deacetylasperulosidic acid derivative 1	15.0 ± 4.8	294.8 ± 104.9	0.4 ± 0.1
12	*p*-Coumaroyl-deacetylasperulosidic acid derivative 2	8.3 ± 3.2	203.1 ± 69.3	0.4 ± 0.1
13	*p*-Coumaroyl-scandoside derivative 1	2.7 ± 1.3	48.5 ± 26.0	1.3 ± 0.5
14	*p*-Coumaroyl-scandoside derivative 2	10.8 ± 4.7	147.0 ± 85.1	4.3 ± 1.4

n.q.: not quantifiable, no iridoids were detected in *V. angustifolium* and *V. corymbosum*.

The mean total sum of iridoids in *V. myrtillus* was 127 mg/ kg FW, which is about 6-fold higher than the sum of iridoids in *V. uliginosum* samples (20 mg/ kg FW). Except for compound **4**, the content of each iridoid was significantly higher in *V. myrtillus* samples compared with the amounts in *V. uliginosum* samples. Considerable differences in the iridoid profiles of the two species were observed. The proportion of coumaroyl derivatives of the total sum of all iridoids was higher in *V. myrtillus*, whereas methylated iridoids were only present in *V. uliginosum*. The methylated iridoids account for the main proportion of the total iridoids in *V.* uliginosum. However, no differences in the proportion of monotropein, scandoside, and deacetylasperulosidic acid were detected. Also, no differences in the iridoid profiles were found between juices and berry fruits. Thus, it appears that processing affects all iridoids to the same extent. Both fruit and juice samples contain the *p*-coumaroyl-monotropein derivative 2 as the predominant iridoid glucoside with a mean proportion of 43.4 % and 40.9 %, respectively.

Both species (*V. angustifolium* and *V. corymbosum*) that contain no iridoids belong to the section C*yanococcus*. The absence of iridoids reflects the close genetic relationship of these

species. The species *V. myrtillus* (section *Myrtillus*) and *V. uliginosum* (section *Vaccinium*) show various iridoids and the different amounts may be attributed to their affiliation to different sections. The differences indicate the modulation of the plant metabolism during the evolutionary process and therefore allow a chemotaxonomic classification.

4 Conclusion

In the species *V. uliginosum* and *V. myrtillus*, 14 and 11 different iridoids, respectively, were found, whereas *V. corymbosum* and *V. angustifolium* did not contain any of the iridoid compounds. To our knowledge, this is the first time that the isomers scandoside and deacetylasperulosidic acid as well as a dihydro derivative thereof were described in the Ericaceae family. In addition, the *p*-coumaroyl isomers of scandoside, deacetylasperulosidic, and dihydromonotropein are described for the first time in *V. myrtillus* and *V. uliginosum*. The presence of monotropein and its coumaroyl isomers was confirmed for *V. myrtillus* and described for the first time in *V. uliginosum*. Knowledge of the occurrence of the investigated compounds in various *Vaccinium* species may be helpful for further chemotaxonomic considerations and authentication purposes. Identification of compounds exclusively present in either species would further substantiate authentication analyses. Investigation of the effects of processing on the stability of iridoids is necessary as only those compounds are valid authentication markers that are not affected during juice processing. The present sample set with independent juice and berry fruit samples shows a constant iridoid profile in both types of samples (juices and berries), indicating a general applicability of iridoids as authentication marker compounds. The investigation of corresponding fruit and juice samples will give further information on the mass consistency of iridoids, which would be interesting in terms of juice quality aspects.

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Chapter 5

Concluding remarks

In times of globalized food markets and sometimes long supply chains, concerns about the authenticity and quality of foodstuff are growing and numerous food scandals have impaired the confidence of consumers in the past. Thus, the demand for reliable authentication analysis is increasing, as reflected by the numerous scientific studies dealing with this topic. The present work focused on the authentication process of fruits of the genus *Vaccinium* L. of the Ericaceae family. Since there are various studies proposing possible authentication techniques for these fruits, the principal comparability of different authentication methods was investigated. For this purpose, the influence on authentication marker compounds caused by different extraction methods and enzymatic treatments during juice processing was examined. Finally, by profiling the substance class of iridoids, the knowledge of the phytochemical profile of *Vaccinium* L. species was extended.

1 Impact on anthocyanin profiles caused by extraction methods

Various methods are applied in order to profile reference materials and determine possible marker compounds for authentication and quality control. To obtain a comprehensive overview of the results, a meta-analysis of published investigations is necessary. Such a meta-analysis could provide a dataset of marker compounds, which can be applied as a reference database for future authenticity assessments without necessitating the acquisition of new reference material for each analysis. As a prerequisite, it has to be validated whether the outcome of the various authenticity studies shows sufficient comparability. The present study addresses this crucial problem regarding the comparability of authenticity data sets. For this purpose, accelerated solvent extraction and ultrasound assisted extraction were applied using different extraction solvents. The fruits of bilberry, lowbush blueberry, and American cranberry showed considerable differences in yields and peak ratios of anthocyanins. In particular, peak

proportions of more polar compounds like delphinidin and cyanidin glycosides were affected by the extraction conditions applied, whereas other compounds like petunidin and malvidin glycosides were only marginally influenced. The alteration of the profile of lowbush blueberries followed a different pattern. The most polar anthocyanins containing the aglycone delphinidin and the least polar anthocyanins, which are the glycosides of malvidin, were affected most. Finally, the application of different extraction methods on American cranberries did not affect the anthocyanin profile at all. The very divergent results for different fruits indicate that the alteration of the anthocyanin composition is not necessarily predictable due to the applied extraction technique but rather depends on the plant material. It is presumable that specific differences between the investigated plants cause the observed varying effects. One of the most distinguishing features might be the structure of cell walls. It is well known that the composition of cellulose, hemicellulose, pectin, and other components varies for different berries (Hilz et al., 2005). The associated differences in physical properties might be an issue concerning the efficiency of an applied method of extraction. It has been shown that the structure and nanostructure of epidermal cell walls respond differently to blanching, freezing-thawing processes and ultrasound treatment (Fava et al., 2006). Thus, it is very likely that differently composed cell walls are affected differently by one extraction method. Besides cell wall differences between fruits of different species, various types of cell structures can be found in one berry of one species. The structure of epidermal cells being part of the exocarp differs compared with the structure of cells forming the mesocarp. An uneven distribution of anthocyanins throughout the berry fruit would result in different anthocyanin profiles in fruit extracts, depending on the efficiency of the extraction method dealing with cells of the mesocarp and the exocarp. Comparing the anthocyanin compositions of fruit extracts of different species, the distribution of anthocyanins in the plant material has to be considered as well. Bilberries, for example, contain anthocyanins in the mesocarp and exocarp, whereas the anthocyanins of lowbush blueberry are only located in the exocarp. After applying various extraction techniques, the resulting anthocyanin profiles are in these cases depending on the distribution of anthocyanins in the fruit and the structure of cells containing the anthocyanins. It would be interesting to conduct further research in order to ascertain which plant specific differences in terms of distribution of anthocyanins and structure of cell walls influence the extractability of anthocyanins. In view of a meta-analysis of authenticity data, the comparison of studies using different extraction methods seems to be unreasonable. An attempt to identify factors which compensate the altering effects of different extraction methods appears to be very

ambitious due to the multiple aspects influencing this phenomenon. Thus, a reliable comparison of authenticity data is only possible using one constant method of extraction.

A comparison of changes induced by extraction methods and natural variations of anthocyanin profiles due to species, geographic and climatic differences was conducted aiming at a relative classification of the profile differences. For this purpose, a linear discriminant analysis was performed on the basis of 26 bilberry and lowbush blueberry samples of different origins. The analysis showed that the different species can be well separated from each other on the basis of their anthocyanin profiles. However, a distinction between bilberry samples from Germany, Ukraine, and Poland cannot be made as these countries are geographically located very closely and underlie similar climatic conditions. As a result, the anthocyanin profiles are similarly affected, which renders a proper differentiation impossible. The prediction model classified the samples extracted with different methods correctly in terms of their species. However, the distribution of sample extracts made with different extraction methods exceeded the naturally occurring differences of the reference fruits. Various sample extracts were allocated outside the respective area of confidence, which means that the assignment is correct with a probability of less than 95 %. Based on the distribution of respective samples in the LDA, it becomes apparent that the processing technique prior to extraction, like lyophilizing and grinding, affects the anthocyanin profile more than the selection of different extraction solvents. The prediction model allocates lyophilized samples more distant from the expected position than samples extracted from fresh berries. Thus, grinding lyophilized berries to a fine powder facilitates the extraction of certain anthocyanins differently than mashing fresh berries during extraction.

2 Impact of enzymatic treatment during juice processing

Besides the analysis of fresh berry material, the authenticity of products derived from these fruits has to be assessed as well. The cell structures of plant material are impacted by technological process steps which can incidentally affect plant compounds. The present work discusses the differences in the anthocyanin profiles after juice processing with commercially relevant and excessive dosages of various common enzyme preparations.

2.1 Determination of enzyme activity

In order to apply defined and comparable enzyme dosages, the polygalacturonase and β-glucosidase activities were measured. Correlating enzyme activities stated in the present study with literature data as well as a meta-analysis of published enzyme activities revealed that

activity tests underlie considerable variations and make a proper comparison difficult. Possible explanations are the usage of various units expressing the activity of enzymes and differences in the activity assays applied. The determination of polygalacturonase activity, for example, is often based on the degradation of pectin. The same enzyme can show different activities for pectin from different sources because the structure of pectin varies depending on the source of pectin. As the present dissertation investigates the enzymatic influences on the authentication process of berries from an analytical point of view, the technological aspect of enzyme activities and their measurement will not be discussed in detail as it would go beyond the scope of this study. However, the knowledge about enzyme activities is essential for the optimization of juice processing as the proper selection of enzymes is crucial for obtaining the requested viscosity, haze, and amount of value adding compounds at a maximum yield.

2.2 Influences of enzymatic treatment on juice yield and anthocyanin content

From a producer point of view, the juice yield is one of the most important markers for the efficiency of production. Juice production on a laboratory scale, which was performed in the present dissertation, lacked a significant increase in juice yield when applying enzymes at a commercially relevant scale (0.5 nkat/g). This might be ascribed to the fact that juice pressing conditions differed from those at industrial production sites. However, an excessive dosage (10 nkat/g) revealed a significant increase for most enzymes, reflecting an enzymatic degradation of the cell wall network and a lowering of the viscosity. Another marker for an efficient production is the content of value adding compounds. In terms of bilberries investigated in the present study, these compounds are polyphenols and anthocyanins in particular. Although showing no increase in juice yield, the efficacy of enzymes at a low dosage (0.5 nkat/g) is reflected by the significant increase in the anthocyanin content compared to the anthocyanin content of the control juice. A higher dosage (10 nkat/g) leads to a further increase. As the present study was designed to investigate the changes in anthocyanin profiles due to enzymatic treatment, no effort was put on the maximization of juice yield and content. Several studies were published in the past covering this topic (Buchert et al., 2005; Landbo et al., 2007). When comparing the obtained juice yields and anthocyanin contents with literature data, it has to be considered that the ambient juice processing conditions in the present work were maintained equal for all enzyme preparations. The applied time-temperature regimen was a consensus between the various optimum conditions for a maximum enzyme activity. The obtained juice yields and anthocyanin contents may not reflect the potential of the applied enzyme preparations but the advantage of this consensus is the fact that the effects of enzymes

were the only source of profile differences between the various juices. All other ambient conditions like duration and temperature of processing affected all varieties equally, which renders an exclusive investigation of enzymatic influences possible.

2.3 Changes in anthocyanin profiles due to enzymatic treatment

A considerable alteration of the anthocyanin profile of bilberries was observed during juice processing depending on the dosage of applied enzymes. Preparation of a control juice without adding enzyme preparations during processing led to a variation of the anthocyanin profile based on the differences in extractability and thermal stability of anthocyanins. Adding a low dosage (0.5 nkat/g) of enzyme preparations to the fruit mash resulted in juices with anthocyanin profiles similar to that of the control juice. However, profiles were not identical but showed little differences, which did not follow a glycoside or aglycone depending degradation. Thus, a straight enzymatic degradation of anthocyanins is very unlikely as it was expected to result in equal degradation rates for the same type of glycosides or aglycones. The lack of such systematics indicates the occurrence of secondary effects influencing the anthocyanin profile. It is conceivable that fragments of the cell wall polysaccharides interact with anthocyanins which would influence the respective detectable anthocyanin content. Further studies need to investigate the phenomena of interaction between polyphenols and cell wall degradation products.

Such interactions might also occur at enzymatic treatment with a higher dosage, although they appear to be overlaid by straight enzymatic effects. The hypothesis of glycoside-dependent anthocyanin degradation during juice processing seems to be supported by the results of the application of enzyme preparations exceeding common dosages (10 nkat/g). Beside the fundamental profile alteration due to extractability and thermal stability of anthocyanins, the differences between anthocyanin profiles of juices produced with the aid of different enzyme preparations is much more pronounced compared to those after using low dosages. Juices produced with enzyme preparations containing primarily cellulases tend to show lower glucoside proportions and the addition of preparations declared as polygalacturonases result in a high correlation with lower proportions of galactosides. However, a straight degradation of anthocyanins due to cellulase or polygalacturonase activity of enzyme preparations is questionable. For juices treated with cellulases, deviation of the anthocyanin profile compared to the profile of the control juice is less pronounced after application of a preparation with a high β-glucosidase activity compared with the one showing a low activity. Thus, a correlation

between β-glucosidase activity and anthocyanin glucoside degradation appears unreliable. In terms of the correlation between a polygalacturonase activity and anthocyanin galactosides degradation it has to be noted that polygalacturonases degrade pectin, which contains α-D-linkages, whereas the sugar moieties of anthocyanins are bound to the aglycone via β-D-linkages. Steric differences between these binding types render the induced fit theory impossible and it can be assumed that the polygalacturonase does not cause a straight anthocyanin degradation. Nevertheless, certain correlations can be observed. The most likely theory therefore is the presence of enzymatic side activities in the applied enzyme preparations. The latter do not present purified isolates of individual enzymes but rather present the enzymatic set up of microorganisms due to their way of production (Alimardani-Theuil et al., 2011). As microorganisms produce enzymes in order to metabolize carbon sources, they necessitate various enzymes due to the complexity of the available carbon source. It has been reported that the pectinolytic enzyme setup of the fungus *Aspergillus niger* is dependent on the available substrate composition (Martens-Uzunova & Schaap, 2009). Beside pectinases that attack the backbone of pectin, there are further accessory enzymes dealing with the various side chains. Such accessory enzymes encompass also β-galactosidases, which might have led to the observed side activity of applied polygalacturonase preparations (Benoit et al., 2012).

It becomes apparent that the application of enzymes during juice processing goes along with the advantages of increasing juice yield and total anthocyanin content. Yet, disadvantages regarding quality and authenticity aspects have to be considered as well. For producers and consumers, the loss of individual anthocyanins, which contribute to the value adding compounds of berry products, causes a reduced quality. This aspect is overlaid by a higher increase in the total anthocyanin content compared with the decrease in individual anthocyanins. Still, the assessment of the authenticity of berry products is affected as it relies on the quantitative anthocyanin profile. Unknown alterations of this profile during juice processing do not allow a reliable authentication on the basis of berry fruit reference material representing the natural anthocyanin profile. The present dissertation showed that straight enzymatic effects caused by ingredients of commercial enzyme preparations as well as secondary effects caused by interactions between polyphenols and degradation products have to be considered when explaining the alteration of anthocyanin profiles. Thus, further investigations have to focus on both, the composition of enzyme preparations and the interactions of cell wall degradation products with phenolic compounds representing potential marker compounds for authentication purposes. The enzyme preparations can be assumed to be very complicated as they comprise the complex enzymatic setup of potentially multiple

microbial organisms. Thus, various accessory enzymes might lead to various side activities of the enzymatic preparations. In order to understand the interactions of degradation products and polyphenols, the complexity and diversity of cell wall structures have to be considered. A single enzyme preparation might cause different effects when applied to different fruits as the resulting degradation products can differ. It is necessary to expand the knowledge about side activities of enzyme preparations and secondary effects of cell wall degradation products in order to enable a proper authenticity assessment of enzymatically treated berry fruit products.

3 Iridoid profile of *Vaccinium* L. species

The assessment of berry fruit authenticity is often limited to the evaluation of the anthocyanin profile. The present dissertation profiled the, for *Vaccinium* L. species, so far underreported substance class of iridoids by UHPLC-MS^n in order to extend the phytochemical knowledge about these species. The species *V. uliginosum* and *V. myrtillus* showed 14 and 11 different iridoids, respectively, whereas *V. corymbosum* and *V. angustifolium* did not contain any of these iridoid compounds. It was possible to confirm the presence of all so far known iridoids for the respective species. Moreover, various iridoids are reported for the first time for the investigated species or even for the whole Ericaceae family. Several of these iridoids can be rated as minor compounds due to their low concentrations. A sensitive method for analysis is crucial for a proper detection of these compounds. In this context, the most challenging aspect is the high anthocyanin content when analyzing *Vaccinium* L. species. The high amount of anthocyanins consumes most of the separation efficiency during the liquid chromatographic separation and suppresses the ionization of minor compounds during the following mass spectrometric detection. The usage of an unpolar solid phase extraction cartridge equipped with the functional surface of a cation exchanger for sample preparation overcomes this challenge. It renders the detection of iridoid minor compounds possible by removing the interfering anthocyanins.

Further characterization of the phytochemical profile can be used for ongoing chemotaxonomic considerations. The knowledge about presence, or absence, of particular iridoids allows a reliable distinction of closely related *Vaccinium* species. The iridoids present potent marker compounds as they do not only differ in terms of their quantitative profile but also in terms of their qualitative profile. The presented method of iridoid profiling can therefore be used for the characterization of numerous other *Vaccinium* species. The United States Department of Agriculture lists about 200 *Vaccinium* species but a proper knowledge about their iridoid profile lacks for the most of them (USDA et al., 2015). One reason for the multitude of species is the

hybridization and introgression occurring among wild and cultivated species (Hancock et al., 2008). The combination of genetic material causes an evolution of taxonomic groups as new species with new properties arise (Whitney et al., 2010). Beside changes in the morphology, generation of qualitative and quantitative variation in the secondary plant metabolites occurs as well (Orains, 2000; Caseys et al., 2015). These phytochemical variations are not limited to different species but also exist for different varieties of a single species. In terms of the genus *Vaccinium* L., the two commercially most important species are *Vaccinium corymbosum* and *Vaccinium macrocarpon*. Both show multiple varieties which have been generated via hybridization in order to match particular climatic conditions or extend the seasonal availability. It has been shown that the phytochemical profile of the *Vaccinium corymbosum* varieties differ in case of individual flavonoid compounds (Rodriguez-Mateos et al., 2012; Gavrilova et al., 2011). It would be important to conduct a systematic analysis of these varieties in terms of their iridoid profile. The present dissertation reports the absence of iridoids in *Vaccinium corymbosum* only for one variety, but it is conceivable that some varieties contain iridoids via introgression. Additionally, the authentication of species might be difficult if an unknown variety is analyzed which shows a suspicious flavonoid profile due to the introgression of the respective alleles influencing the flavonoid synthesis. Inconclusive results of authentication could be secured via the complementary information about the iridoid profile. Apart from this it has to be stressed that the fruits of *Vaccinium corymbosum* may contain some iridoids which were not detected in the present investigations. In view of authentication analysis, for example, it would be very valuable to identify compounds which are exclusively present in one species, as it would easily render the detection of blended species possible. In general, a wider knowledge of the phytochemical profile can support the chemotaxonomic classification and discriminability.

However, the applicability of iridoids as authentication marker compounds has to be validated in respect of their processing stability. Storage stability tests have already indicated a degradation of certain iridoid glycosides (Gonda et al., 2012), but the degradation properties of iridoid glycosides discussed in the present dissertation have not been investigated so far. Especially the processing stability is of great importance, as it has been shown above for anthocyanins. Thus, not only a profiling of further *Vaccinium* species for their iridoid compounds but also their stability during heat, pressure and enzymatic treatment should be subjects of further examinations. For this purpose, fruits and their corresponding juices should be investigated in view of mass consistency of iridoids.

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Summary

This thesis covered three challenging aspects of berry fruit authentication regarding the profile of secondary plant metabolites. The constantly growing importance of proper authenticity analysis in the food sector is a result of a globalized food market and its long channels of trade. The necessity of multiple middlemen causes an increased risk for fraudulent practices in view of gaining higher profits. Especially berry fruits and their products are exposed to this risk as they belong to a group of high value products demanding high prices. Blending with less valuable and cheaper fruits is a common practice to increase profits. Beside a deliberate deception, confusion about the identity of species can arise due to cultural or regional differences in the nomenclature of species. Food authentication has to create clarity in all these cases by identifying species, origins, or production and processing methods. In terms of berry fruits, the identification often focuses on the characteristic secondary plant metabolites in recent approaches. These metabolites can deliver typical fingerprints which allow the identification of individual species. However, this approach has to be critically reviewed as alterations of these fingerprints influence the authenticity assessments. Some effects which may cause such alterations are discussed in the present dissertation.

Firstly, the present dissertation considered the influence of different extraction methods on the quantitative profile of anthocyanins, which represent a characteristic class of secondary plant metabolites for various berry fruits. The application of accelerated solvent extraction and ultra sound assisted extraction combined with two different extraction solvents containing 80 % and 20 % acidified methanol revealed variations in the anthocyanin profile of fresh and lyophilized bilberries and lowbush blueberries. The glucosides, galactosides, and arabinosides of the most polar anthocyanidins delphinidin and cyanidin showed the largest variations for bilberries. In terms of the lowbush blueberry, the aglycone delphinidin and malvidin were affected most. The effects on the anthocyanin profiles of American cranberry samples were negligible. A classification of the profile variations for bilberries and lowbush blueberries was conducted by the application of a prediction model obtained from a linear discriminant analysis (LDA). In total, 26 authentic bilberry and lowbush blueberry samples from various origins were used as objects for the LDA. The quantitative profile of 15 anthocyanins was applied as variables. Samples extracted under similar conditions to the authentic samples were positioned closely to the centroid of the respective cluster in the LDA. Deviating extraction methods lead to a pronounced scatter, which was comparable to the natural profile variations due to geographical

or climatic differences. However, the discriminability of the species bilberry and lowbush blueberry is not affected by the application of different extraction methods.

Secondly, the alteration of the anthocyanin profile due to enzymatic treatment during juice processing was investigated. Six cell wall degrading enzyme preparations which are commercially used were applied representing the main activities polygalacturonase, pectin lyase, and cellulase. The application of enzyme preparations at two dosage levels according to the polygalacturonase activity affected the juice yield, total anthocyanin content, and the anthocyanin profile differently. In comparison to a control juice which was produced without the aid of an enzyme preparation, an enzymatic treatment at a commercial level (0.5 nkat/g) resulted in a slightly increased juice yield and a considerably higher anthocyanin content. In terms of an excessive dosage (10 nkat/g), both the juice yield and the anthocyanin content increased significantly. Juices and pomaces showed a recovery of anthocyanins contained in bilberry fruits that ranged from 81 to 94.9 % and from 84.5 to 96.2 % for 0.5 nkat/g and 10 nkat/g, respectively. Changes in the anthocyanin profile were analyzed by application of a principal component analysis (PCA). Six enzymatically prepared juices and respective pomaces, a control juice and its pomace as well as a fresh bilberry fruit were used for this purpose. The quantitative profile of 15 anthocyanins were applied as variables. PCA revealed that the anthocyanin profiles of juices and pomaces after the usage of a common enzyme dosage differ significantly from the profile of fresh berries and show a slight deviation from the controls. In view of the high recovery of anthocyanins, this could be attributed to the uneven extractability of anthocyanins during juice processing. Within the groups of juices and pomaces, differences were small and could not be associated with an aglycone- or glycoside-dependent degradation. Thus, it was concluded that the profile variations are probably results of secondary effects like interactions between anthocyanins and cell wall degradation products rather than caused by an immediate enzymatic degradation. The application of an excessive dosage resulted in distinct differences within the groups of juices and pomaces. Samples treated with cellulases showed the lowest glucoside proportions and the usage of polygalacturonases caused the lowest proportions of galactosides. As polygalacturonases hydrolyze the α-1,4-galactosidic backbone of pectin, it can be assumed that a strong β-galactosidic side activity is present in the respective enzyme preparations, which degrades the galactosidic anthocyanins.

Thirdly, the knowledge about the phytochemical profile of various *Vaccinium* L. species was extended by characterizing so far unknown iridoid compounds. The low amounts of iridoids demand a proper sample preparation in order to ensure a high sensitivity. For this purpose, a

solid phase extraction was carried out using strong cation exchanger cartridges based on a polystyrene-divinylbenzene surface. The removal of predominant anthocyanins via the cation exchanger facilitated the detection of minor compounds like iridoid glycosides, which was conducted by LC-MS in negative electrospray ionization mode. Using this analytical procedure, 14 different iridoid glycosides were characterized in the fruits of bog bilberry (*Vaccinium uliginosum* L.) with a total amount of 20 mg/kg fresh weight (FW). The fruits of bilberry (*Vaccinium myrtillus* L.) contained 11 iridoid glycosides at a total amount of 127 mg/kg FW. None of the investigated iridoid glycosides were found in highbush blueberry (*Vaccinium corymbosum* L.) and lowbush blueberry (*Vaccinium angustifolium* L.). As only few studies have covered the phytochemical profile of iridoids in *Vaccinium* L. species so far, some of the investigated compounds were reported for the first time in these species. The *p*-coumaroyl isomers of scandoside, deacetylasperulosidic acid, and dihydromonotropein, for example, are reported for the first time in bilberries and bog bilberries. Monotropein and its *p*-coumaroyl isomers were found for the first time in bog bilberries. Moreover, scandoside and deacetylasperulosidic acid as well as their dihydro derivatives were described for the first time in the Ericaceae family.

Zusammenfassung

Diese Dissertation beschäftigt sich mit drei herausfordernden Aspekten bei der Authentifizierung von Beerenfrüchten in Bezug auf das Profil sekundärer Pflanzenstoffe. Die wachsende Bedeutung von geeigneten Authentifizierungsmethoden im Lebensmittelbereich hängt mit einem globalisierten Lebensmittelmarkt und den langen Handelswegen zusammen. Die Notwendigkeit von zahlreichen Zwischenhändlern führt zu einem erhöhten Risiko von Verfälschungen mit der Absicht, größere Gewinne zu erzielen. Insbesondere Beerenfrüchte sowie daraus hergestellte Produkte unterliegen diesem Risiko, da sie zu hohen Preisen gehandelt werden. Ein Verschnitt mit minderwertigen und günstigeren Produkten ist eine verbreitete Vorgehensweise, um den Profit zu steigern. Neben einer solch vorsätzlichen Täuschung können auch Missverständnisse bezüglich der Identität von Beerenfrüchten entstehen, da einzelne Spezies einer kulturell oder regional unterschiedlichen Nomenklatur unterliegen. Die Aufgabe von Authentizitätsprüfungen ist es daher, Klarheit in Bezug auf die Spezies, Herkunft und Verarbeitung der Früchte zu schaffen. Aktuelle Ansätze verfolgen dabei die Untersuchung der Profile sekundärer Pflanzenstoffe, da diese oft einen typischen Fingerabdruck darstellen, der Schlüsse auf die jeweilige Spezies zulässt. Jedoch muss dabei beachtet werden, dass Veränderungen dieser Fingerabdrücke die Authentizitätsbewertungen beeinflussen können. Einige Effekte, die solche Veränderungen verursachen können, werden in der vorliegenden Arbeit behandelt.

Zunächst wurden die Einflüsse von verschiedenen Extraktionsmethoden auf das quantitative Profil von Anthocyanen, welche zu den charakteristischen sekundären Pflanzenstoffen von Beerenfrüchten zählen, untersucht. Der Einsatz der beschleunigten Lösemittelextraktion sowie der ultraschallunterstützen Extraktion in Verbindung mit angesäuertem 20 %igem bzw. 80 %igem Methanol führte zu Profilveränderungen bei frischen und gefriergetrockneten Heidelbeeren und Blaubeeren. Die Glukoside, Galaktoside und Arabinoside der polarsten Anthocyanidine Delphinidin und Cyanidin wurden in der Heidelbeere am stärksten beeinflusst. Bei der Blaubeere unterlagen die Aglykone Delphinidin und Malvidin den größten Veränderungen. Effekte bei der Amerikanischen Cranberry wurden nicht festgestellt. Eine Einschätzung der Größenordnung der Veränderungen erfolgte über Anwendung eines Vorhersagemodells einer linearen Diskriminanzanalyse. Dazu wurden 26 authentische Heidelbeeren und Blaubeeren als Beobachtungen und das Profil ihrer 15 Anthocyane als Variablen herangezogen. Proben, die mit den gleichen Methoden extrahiert wurden wie die

authentischen Proben, wurden nahe der Zentroiden der entsprechenden Cluster zugeordnet. Abweichende Extraktionsmethoden führten zu einer deutlichen Streuung, welche in ihrer Ausprägung den natürlichen Schwankungen durch geographische oder klimatische Gegebenheiten entsprechen. Eine grundsätzliche Unterscheidbarkeit von Heidelbeeren und Blaubeeren war jedoch dennoch gegeben.

Weiterhin wurde der Einfluss von Enzympräparaten auf das Anthocyanprofil während der Saftherstellung untersucht. Dazu wurden sechs kommerzielle Enzympräparate zum Zellwandabbau mit den Hauptaktivitäten Polygalakturonase, Pektinlyase und Cellulase verwendet. Die Präparate wurden abhängig von ihrer Polygalakturonaseaktivität in zwei Konzentrationen dosiert und beeinflussten die Saftausbeute, den Anthocyangehalt und das Anthocyanprofil unterschiedlich stark. Im Vergleich zu einem Kontrollsaft, der ohne Enzymbehandlung hergestellt wurde, hatte eine industriell übliche Dosage (0,5 nkat/g) nur eine geringe Steigerung der Saftausbeute bei deutlich erhöhten Anthocyangehalten bewirkt. Eine Überdosierung (10 nkat/g) führte zu einer signifikanten Steigerung von Saftausbeute und Anthocyankonzentration. Die Wiederfindung der Anthocyane in der Summe aus Saft und Trester lag zwischen 81 und 94,9 % (0,5 nkat/g) beziehungsweise zwischen 84.5 und 96,2 % (10 nkat/g). Veränderungen im Anthocyanprofil wurden mittels Hauptkomponentenanalyse (HKA) untersucht. Als Beobachtungen dienten sechs Säfte und ihre Trester nach Anwendung eines Enzympräparates, ein Kontrollsaft und sein Trester ohne Enzymbehandlung sowie eine unverarbeitete Heidelbeere. Die quantitativen Profile der 15 enthaltenen Anthocyane wurden als Variablen genutzt. Es zeigte sich, dass die Anthocyanprofile der mit einer industriell üblichen Enzymdosis hergestellten Säfte und Trester sich signifikant vom Profil der unbehandelten Heidelbeere und nur leicht von den Kontrollen unterscheiden. Mit Blick auf die hohen Wiederfindungsraten der Anthocyane konnte dies auf eine ungleichmäßige Extrahierbarkeit der Anthocyane während der Saftherstellung zurückgeführt werden. Innerhalb der Gruppe der Säfte und Trester waren die Unterschiede nur sehr gering, sodass kein aglykon- oder zuckerabhängiger Abbau festgestellt werden konnte. Anstatt eines direkten enzymatischen Abbaus von Anthocyanen wurde gefolgert, dass sekundäre Effekte durch Wechselwirkungen zwischen Anthocyanen und Abbauprodukten der Zellwände zu den Profilunterschieden geführt haben. Die Verwendung von extrem hohen Enzymdosen verursachte deutliche Profilunterschiede innerhalb der Gruppen der Säfte und Trester. Cellulasen führten zu deutlich erniedrigten Glukosidanteilen und Polygalakturonasen zu niedrigen Galaktosidanteilen. Da Polygalakturonasen die Hydrolyse des α-1,4-galaktosidischen Pektingrundgerüstes

verursachen, wurde angenommen, dass eine β-galaktosidische Seitenaktivität der Grund für den Abbau von Anthocyangalaktosiden war.

Abschließend wurde das Wissen um das phytochemische Profil einiger *Vaccinium* L. Spezies erweitert, indem in diesen Früchten bislang unbekannte Iridoide charakterisiert wurden. Die niedrigen Gehalte an Iridoiden erforderten eine geeignete Probenvorbereitung, um eine ausreichende Empfindlichkeit zu gewährleisten. Dazu wurde eine Multimodekartusche aus einem Polystyrol-Divinylbenzol-Copolymer und einem starken Kationenaustauscher zur Festphasenextraktion eingesetzt. Die Entfernung der dominierenden Anthocyane erleichterte die Detektion der Iridoide als Minorkomponenten mittels LC-MS und negativer Elektrospray-Ionisation. In Rauschbeeren (*Vaccinium uliginosum* L.) wurden 14 verschiedene Iridoidglykoside mit einem Gehalt von 20 mg/kg Frischgewicht (FG) charakterisiert. Heidelbeeren (*Vaccinium myrtillus* L.) enthielten 11 dieser Iridoidglykoside in einer Konzentration von 127 mg/kg FG. Die Kulturblaubeere (*Vaccinium corymbosum* L.) sowie die wilde Blaubeere (*Vaccinium angustifolium* L.) zeigten keine dieser Iridoidglykoside. Da das phytochemische Profil der Iridoide für diese Spezies weitgehend unerforscht war, ist die Kenntnis von einigen der beschriebenen Verbindungen neu. Die *p*-Cumaroylisomere von Scandosid, Desacetylasperulosidsäure und Dihydromonotropein wurden im Rahmen der vorliegenden Arbeit erstmals in Heidelbeeren und Rauschbeeren gefunden. Monotropein und seine *p*-Cumaroylisomere wurden erstmals in Rauschbeeren beschrieben. Für die gesamte Familie der Ericaceae wurde mit der vorliegenden Arbeit erstmals die Existenz von Scandoside und Deacetylasperulosidsäure sowie deren Dihydroderivate beschrieben.

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