

Proanthocyanidins in barley and malt analyzed by pressurized liquid extraction, solid-phase extraction and HPLC



Dissertation

**Proanthocyanidins in barley and malt analyzed  
by pressurized liquid extraction, solid-phase  
extraction and HPLC**

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## *Contents*

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## Abstract

Aim of this work is to develop a convenient method for the determination of proanthocyanidins in barley and malt. In a second step this method is applied to 61 barley and malt samples of different varieties, proveniences and growing years.

In the brewing industry proanthocyanidins are of special interest. Main activities of the proanthocyanidins are related to undesired formation of chill haze and to the positively valued augmentation of the antioxidative capacity of beer. The detailed mechanisms are still under discussion. It is clear, that the positive and negative effects of proanthocyanidins depend on their quantity and quality. So determination of the sum of proanthocyanidins does not give sufficient information to discuss their action.

Selective analysis of proanthocyanidins is time and labor consuming. Especially sample preparation requires a lot of manual work. Thus, this work presents a fully automated and therefore fast and reliable method for sample preparation of barley and malt followed by HPLC-UV.

The here described method bases on extraction using pressurized liquid extraction (PLE). Essentially it is a static solid/liquid extraction with high pressure and eventually high temperature in stainless steel extraction cells. Using the *Accelerated Solvent Extractor* (ASE) by *Dionex*, up to 24 samples in a series can be extracted automatically.

The second step of sample preparation is clean-up by solid-phase extraction (SPE). For the first time, commercially available polyamide cartridges are used for proanthocyanidins. SPE is accomplished automatically by a liquid handling robot, the *Automated Sample Preparation with Extraction Cartridges*-device (ASPEC) by ABIMED and Gilson. The ASPEC takes the extracts from the ASE and carries out the complete SPE procedure. The resulting solution is ready to inject into the HPLC, that separates and quantifies six proanthocyanidins and catechin in one run of 90 min.

Sample extraction and extract clean-up are coupled online. This coupling was developed by ABIMED and Dionex and is tested and established under real laboratory conditions for the first time. Within 24 hours 16 samples can be analyzed, about 6 hours of manual work is needed. Recovery of the overall method is 70-91 %, reproducibility is 2.3-6.4 %.

With this method 61 barley and malt samples of the growing years 1998–2001 from four locations including summer and winter barley varieties are analyzed. The annual and local variation of absolute contents of proanthocyanidins appears to interfere varietal differences, so differentiation between the samples is not possible. The ratio of several pairs of proanthocyanidins (the relative quantitative polyphenolic fingerprint) is characteristic for the variety and can be used to control authenticity.

In addition, the here presented method is supposed to be applicable to samples taken during the brewing process and to other food samples. Two examples are given: monitoring beer filtration and analyzing proanthocyanidins in the seeds of the açai fruit from northern Brazil. Since proanthocyanidins are discussed to have positive effects on health, there is a market for functional food with naturally high or enriched content of proanthocyanidins. Hence it is necessary to control such products.

## Zusammenfassung

Ziel dieser Arbeit ist, eine leicht durchführbare Methode zur selektiven Bestimmung von Proanthocyanidinen in Gerste und Malz zu entwickeln. Als zweiter Schritt wird diese Methode auf 61 Gersten- und Malzproben verschiedener Sorten, Anbauorte und Jahrgänge angewandt.

Im Brauereiwesen spielen die Proanthocyanidine eine wichtige Rolle. Die wichtigsten Eigenschaften der Proanthocyanidine sind die Bildung von unerwünschten Kälte-trübungen und die positiv bewertete Erhöhung der antioxidativen Kapazität des Bieres. Die einzelnen Reaktionen und genauen Mechanismen sind noch Gegenstand wissenschaftlicher Diskussion. Fest steht, daß die positiven und negativen Effekte der Proanthocyanidine von ihrer Art und Menge abhängen. Daher bringt die Bestimmung von Summenparametern keine ausreichende Information, um die Wirkungen zu diskutieren.

Die selektive Analyse von Proanthocyanidinen ist sowohl zeit- als auch arbeitsaufwendig - vor allem die Probenvorbereitung erfordert viel Handarbeit. Hier wird eine vollautomatische und daher schnelle und zuverlässige Methode für die Probenvorbereitung von Gerste und Malz zur anschließenden HPLC-UV-Analyse vorgestellt.

Die hier beschriebene Methode basiert auf der Extraktion mittels beschleunigter Lösemittelextraktion (*pressurized liquid extraction (PLE)*). Im wesentlichen ist dies eine statische fest/flüssig-Extraktion unter hohem Druck und ggf. hoher Temperatur in Edelstahlzellen. Mit dem *Accelerated Solvent Extractor (ASE)* von *Dionex* können bis zu 24 Proben in Serie automatisch extrahiert werden.

Der zweite Schritt der Probenvorbereitung ist die Aufreinigung mit Festphasenextraktion (*solid-phase extraction (SPE)*). Zum ersten Mal werden kommerziell erhältliche Polyamid-Kartuschen zur Aufreinigung von Proanthocyanidinen eingesetzt. Die SPE wird vollautomatisch von einem Pipettierroboter, dem *Automated Sample Preparation with Extraction Cartridges*-Gerät (ASPEC) von ABIMED und *Gilson*, durchgeführt. Der ASPEC übernimmt die Extrakte von der ASE und führt die komplette SPE durch. Die resultierende Lösung kann direkt in die HPLC injiziert werden, die sechs Proanthocyanidine und Catechin innerhalb eines Laufes von 90 min trennt.

Die Probenextraktion und -aufreinigung sind online gekoppelt. Diese Kopplung wurde von ABIMED und Dionex entwickelt und wird zum ersten Mal unter

realen Labor-Bedingungen getestet und eingesetzt. Innerhalb von 24 Stunden können 16 Proben analysiert werden, dabei sind etwa 6 Stunden Personaleinsatz erforderlich. Die Wiederfindung der gesamten Methode beträgt 70-91 %, die Reproduzierbarkeit 2.3-6.4 %.

Mit dieser Methode wurden 61 Gersten- und Malzsorten, darunter Sommer- und Wintergersten der Jahrgänge 1998-2001 von vier Anbauorten analysiert. Die jährlichen und lokalen Unterschiede überlagern die Sortenunterschiede, so daß keine Differenzierung zwischen den Proben möglich ist. Das Verhältnis bestimmter Paare von Proanthocyanidinen (der relative quantitative polyphenolische Fingerabdruck) ist jedoch charakteristisch für die Gerstensorte und kann zur Authentizitätskontrolle genutzt werden.

Außerdem kann die hier vorgestellte Methode auch eingesetzt werden, um Proben aus dem Brauprozess oder andere Lebensmittel zu untersuchen. Zwei Beispiele sind angeführt: die Überwachung der Bierfiltration und die Analyse von Samen der nordbrasilianischen Açaí-Frucht. Da Proanthocyanidine als gesundheitsförderlich diskutiert werden, gibt es einen Markt für Lebensmittel, die natürlicherweise viele Proanthocyanidine enthalten oder damit angereichert sind. Daraus ergibt sich die Notwendigkeit, solche Produkte zu überprüfen.

## Publications

Parts of this work have been published earlier:

### Papers

LICHTENTHÄLER R, RODRIGUES RB , ZIMMERMANN BF, PAPAGIANNPOULOS M, FABRICIUS H, ALMEIDA O, MAIA JGS AND MARX F: *Total Oxidant Scavenging Capacity of Euterpe oleracea* MART. (*Açaí*) Seeds J Agric Food Chem (submitted)

ZIMMERMANN B, PAPAGIANNPOULOS M, MELLENTIN A, KRAPPE M, MAIO M, GALENSA R (2002): *Coupling of ASE, ASPEC and HPLC - Automated Determination of Proanthocyanidins in Malt* G.I.T. Laboratory Journal Europe, 4 175-177

PAPAGIANNPOULOS M, ZIMMERMANN B, MELLENTIN A, KRAPPE M, MAIO G AND GALENSA R (2002): *Online coupling of pressurized liquid extraction, solid-phase extraction and high-performance liquid chromatography for automated analysis of proanthocyanidins in malt* J Chromatogr A 958 9-16

MELLENTIN A, PAPAGIANNPOULOS M, KINITZ C, ZIMMERMANN B AND GALENSA R (2001): *Bedeutung und Analytik von Polyphenolen im Brauprozess* Lebensmittelchemie 55 143-144

PAPAGIANNPOULOS M, ZIMMERMANN B, MELLENTIN A, KRAPPE M, MAIO G AND GALENSA R (2001): *ASE-ASPEC-HPLC Kopplung - Automatisierte Bestimmung von Proanthocyanidinen in Malz* GIT 9/01 950-952

ZIMMERMANN B, PAPAGIANNPOULOS M, MELLENTIN A AND GALENSA R (2001): *Online-Kopplung von ASE, SPE und HPLC am Beispiel der Polyphenolanalytik in Malz* Lebensmittelchemie 55 66-67

## Posters

RODRIGUES RB, LICHTENTHÄLER R, PAPAGIANNPOULOS M, ZIMMERMANN B, FABRICIUS H, MAIA JGS, ALMEIDA O AND MARX F: *Evaluation of the total Oxidant Scavenging Capacity of Açai Seeds (Euterpe oleracae MART.)*

Lebensmittelchemikertag, Bonn, 13–15<sup>th</sup> September 2004

abstract see: Lebensmittelchemie 59 2004 37

ZIMMERMANN B, PAPAGIANNPOULOS M AND GALENSA R: *Convenience, Sensitivity and Selectivity: How to Analyze Proanthocyanidins by Online Coupled Sample Preparation and HPLC with Coulometric Electrode Array Detection*

24<sup>th</sup> Symposium on Chromatography, Leipzig (Germany), 15–20<sup>th</sup> September 2002

ZIMMERMANN B AND GALENSA R: *Wie charakteristisch ist das Proanthocyanidinspektrum in Braugerste und Malz? – Jahrgangs- und Sortenunterschiede*

Lebensmittelchemikertag, Frankfurt (Germany), 9–11<sup>th</sup> September 2002

abstract see: Lebensmittelchemie 57 2003 30

ZIMMERMANN B AND GALENSA R: *Convenient, fast and selective analysis of proanthocyanidins is possible*

26<sup>th</sup> International Symposium on High Performance Liquid Phase Separations and Related Techniques, Montréal (Canada), 2–7<sup>th</sup> July 2002

ZIMMERMANN B, PAPAGIANNPOULOS M, MELLENTHIN A AND GALENSA R: *Extraktion von Polyphenolen aus Getreide mit der ASE*

ASE/HPLC user symposium, Trier (Germany), 27<sup>th</sup> February 2002

ZIMMERMANN B, FRIEDRICH W AND GALENSA R: *Proanthocyanidine in Braugerste und Malz: Analytik einer Polyphenolklasse mittels ASE, SPE und HPLC-CEAD*

Lebensmittelchemikertag, Braunschweig (Germany), 10–12<sup>th</sup> September 2001

abstract see: Lebensmittelchemie 55 2001 158–159

PAPAGIANNPOULOS M, ZIMMERMANN B, MELLENTHIN A AND GALENSA R: *Online Sample Preparation and HPLC Analysis of Solid Samples*

25<sup>th</sup> International Symposium on High Performance Liquid Phase Separations and Related Techniques, Maastricht (The Netherlands), 17–22<sup>th</sup> June 2001



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## Oral Presentations

*Malze und Proanthocyanidine: Polyphenole automatisch analysieren*

Regionaltagung Süd-West und Bayern of the Lebensmittelchemischen Gesellschaft, Würzburg (Germany), 9<sup>th</sup> March 2004

abstract see: Lebensmittelchemie 58 2004 91

*Die ASE-ASPEC-HPLC-Kopplung: Probieren geht über Studieren*

ABIMED user symposium, Hamburg (Germany), 5<sup>th</sup> April 2001

*Online-Kopplung von ASE, SPE und HPLC am Beispiel der Polyphenolanalytik in Malz oder Warum espresso besser schmeckt als Filterkaffee*

Regionaltagung NRW of the Lebensmittelchemische Gesellschaft, Paderborn (Germany), 15<sup>th</sup> March 2001

abstract see: Lebensmittelchemie 55 2001 66-67

*Die ASE-ASPEC-Kopplung in der lebensmittelchemischen Methodenentwicklung*

ABIMED user symposium, Frankfurt a.M. (Germany), 31<sup>th</sup> October 2000



# 1 Introduction

### 1.1 Aim of this Work

Brewing turned from handcraft to industry. Once the art of the craftsman based on tradition and experience of generations. Nowadays the engineer relies on science. Technical problems during production and poor quality of the product are subject to systematic investigation [75]. Two open questions are flavor stability and chill haze during storage [11, 12, 13, 16, 17, 31, 86, 87, 88].

Many efforts have been made to elucidate the formation of chill haze. It is caused by precipitation of proteins with proanthocyanidins [76, 83, 121, 122, 123, 124]. This knowledge lead brewers to remove either protein by silica hydrogel (SHG) or polyphenols by polyvinylpolypyrrolidone (PVPP) - a very costly step. Others choose malts containing low amounts of proanthocyanidins, and even new barley varieties, that are virtually proanthocyanidin-free are grown [59, 104].

Beers brewed in this way show excellent colloidal stability - and a rapid decrease in flavor quality after bottling. This flavor alteration is called staling. Staling is caused by various unsaturated carbonyl compounds formed by radicals and catalyzed by metal ions [62, 132].

Formation of these undesired compounds depends on the oxidative status of the beer during mashing and wort boiling [88, 91] and once again we are talking about polyphenols. Polyphenols, among them also proanthocyanidins, are supposed to be the most powerful antioxidants and radical scavengers in beer and act as chelating agents for the catalyzing metal ions [10, 42, 46, 134, 135]. So proanthocyanidins deal with both problems: in case of flavor stability in a positive way, in case of chill haze as cause of the problem.

The sources of proanthocyanidins are hops and malt, the latter accounting for 80% of the polyphenols in beer [58, 30], if not for 100% when only CO<sub>2</sub> hop extract is used. So knowledge about the malt employed in brewery allows predicting the quality of the finished beer. Mikyška et al. [88] summarizes: "The stabilizing effect also depended on the particular quality of the raw materials ... It is possible to produce beers with long shelf life and improved flavor stability, by using raw materials rich in polyphenols."

For better understanding of the role of proanthocyanidins in this context, it is indispensable to know the exact content of single proanthocyanidins in the raw materials and their balance during brewing. Nevertheless most of the publications in this field present sum parameters as "tannoids", "total polyphenols", or "total flavanols" instead of values for single proanthocyanidins. This is due to the time and labor intense methods to determine proanthocyanidins.

It is the aim of this work to develop a fully automated and therefore fast and reliable method for determination of proanthocyanidins. In a second step this

new method is applied to barley and malt of different cultivar, proveniences, and growing years to survey variability of proanthocyanidins in the most important beer ingredient. The polyphenolic pattern is characteristic for plants and can be used to control authenticity [21, 22, 41, 53, 102].

In addition this method is supposed to be applicable to samples taken during the brewing process and to other food samples as proven for beer and açai seed extracts. There is a market for functional food with naturally high or enriched content of proanthocyanidins, because they are discussed to have positive effects on health [9, 34, 56, 78, 95, 118, 119, 120]. Hence it is necessary to control such products.

This work continues the project B58 of the *Wissenschaftsförderung der deutschen Brauwirtschaft*. In the first part of this project, Wolfgang Friedrich developed a sample preparation procedure and a HPLC method for the analysis of proanthocyanidins in barley and malt. His method and results [36] define the starting point for this work.

### 1.2 Structure of Proanthocyanidins

Proanthocyanidins are oligo- and polymers of the flavan-3-ols (+)-catechin, (+)-gallocatechin, (-)-epicatechin, and (-)-epigallocatechin (see figure 1.1); other monomers e.g. afzelechin can be found rarely [34].

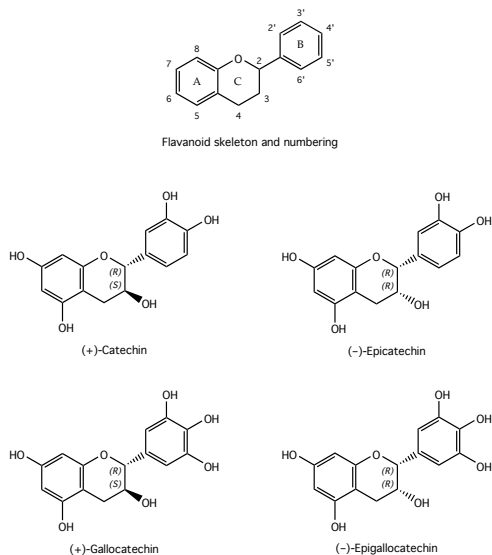


Figure 1.1: Monomers of the proanthocyanidins

They are linked to each other by single C<sub>4</sub>-C<sub>8</sub> or C<sub>6</sub>-C<sub>8</sub> bonds to build oligo- (2-8 monomers) or polymers (more than 8 monomers). The monomers can also be linked by two bonds between two monomers.

### 1.3 Nomenclature of Proanthocyanidins

Nomenclature of proanthocyanidins is quite confusing, since there are different systems and a lot of trivial names. Among the most popular synonyms for proanthocyanidins are: procyanidins, anthocyanogenes, catechins, tannins,

condensed tannins, tanninogenes, tannoids. These names sometimes are even used for other compounds than proanthocyanidins or as an umbrella term for a group of polyphenols including proanthocyanidins.

In this work the nomenclature after PORTER [54, 105, 106] is used. In short (see also figure 1.2):

- The linkage between the monomers and the configuration of the linking, newly asymmetric atom is named analogously to the linkage in polysaccharides, e.g.  $4\alpha-8$ .
- Single linked proanthocyanidins are called B-type, double linked ones are called A-type.
- To order the monomers within B-type proanthocyanidins, the monomer with just one interflavanoid bond at  $C_4$  is considered as the top of the molecule (T-unit). A monomer with a bond at  $C_4$  and a second bond to another monomer at  $C_6$  or  $C_8$  is in the middle of the molecule (M-unit). If all three positions has a bond to different monomers, it is called J-unit (this nomenclature is not officially explained; let's say "jammed"). A monomer with just one bond at  $C_6$  or  $C_8$  is called base (B-unit).

In addition, in this work some popular trivial names for certain compounds are used. These names are *procyanidin* and a code, if all monomers has two OH-groups at ring B (like catechin) respectively *prodelphinidin* and a code, if at least one of the monomers has three OH-groups at ring B (like gallocatechin). Furthermore trimeric proanthocyanidins with exclusively  $4\alpha-8$ -bonds are abbreviated in an intuitive manner. See table 1.1 for an overview over all proanthocyanidins occurring in this work. In figure 1.2 a proanthocyanidin trimer is shown.

Name after PORTER [54, 105, 106]	Trivial name	Abbreviation
Catechin-( $4\alpha-8$ )-catechin	Procyanidin B <sub>3</sub>	ProC B <sub>3</sub>
Gallocatechin-( $4\alpha-8$ )-catechin	Prodelphinidin B <sub>3</sub>	ProD B <sub>3</sub>
Catechin-( $4\alpha-8$ )-catechin-( $4\alpha-8$ )-catechin	Procyanidin C <sub>2</sub>	C-C-C
Gallocatechin-( $4\alpha-8$ )-catechin-( $4\alpha-8$ )-catechin	Prodelphinidin c <sub>2</sub>	GC-C-C
Gallocatechin-( $4\alpha-8$ )-gallocatechin-( $4\alpha-8$ )-catechin	Prodelphinidin C <sub>2</sub>	GC-GC-C
Catechin-( $4\alpha-8$ )-gallocatechin-( $4\alpha-8$ )-catechin	Prodelphinidin c <sub>2</sub>	C-GC-C

Table 1.1: Trivial names and abbreviations used for proanthocyanidins

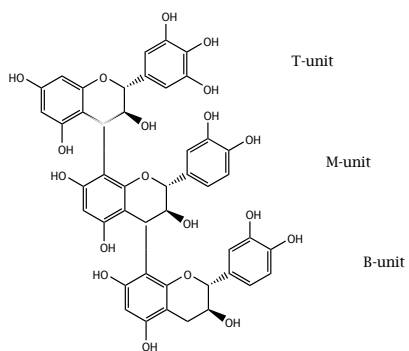


Figure 1.2: A proanthocyanidin trimer: gallocatechin-(4 $\alpha$ -8)-catechin-(4 $\alpha$ -8)-catechin, also known as prodelfinidin c2, or GC-C-C



## 1.4 Analysis of Proanthocyanidins

In literature you can find as many papers about the analysis of polyphenols as there are polyphenolic compounds in nature. The methods differ widely for the various subgroups of polyphenolics and for different kinds of samples.

So, here a short overview is given for the analysis of proanthocyanidins with a particular regard to samples related to barley, malt and beer. Methods including pre- or post-column derivatization are not mentioned. They can give structural information and enhance sensitivity, but complicate the analytical procedure. Details can be found at [111, 112, 129].

### 1.4.1 Sample Preparation

#### 1.4.1.1 Extraction

##### Conventional Extraction

The first obligatory step of analysis of solid samples is the extraction. Conventional solid/liquid extraction is often used. The high number of OH-groups in the proanthocyanidins leads to mixtures of water and polar organic solvents as extraction solvents. As mentioned by [116] hydrogen bonds between matrix molecules and the proanthocyanidins has to be broken.

The most common extraction solvent is a mixture of acetone and water in proportions of 50 + 50 to 80 + 20 [37, 45, 67, 85, 86, 90, 92, 107, 115, 136, 141], sometimes supported by ultrasonic or an ultraturrax blender [48, 68, 138] or modified by acetic acid [48, 70] to avoid degradation of polyphenols by high pH. Other authors prefer methanol with water in different proportions [32, 90, 127], in some cases acidified [6, 27], or ethanol/water [100]. Alcohols give good results for other flavanoids, but poor extraction yields for proanthocyanidins.

Some papers show results of different extraction solvent mixtures [28, 36, 84] agreeing, that acetone + water, 75 + 25, v + v is best. Pure solvents are not used, except for ethyl acetate in [99] applied to dealcoholized wine, so in fact there is also water present. To prevent degradation, high temperature is not applied and sometimes antioxidants as ascorbic acid or sulfur dioxide are added or extraction is carried out under nitrogen atmosphere [6, 45, 85, 90, 138].

##### Pressurized Liquid Extraction

A relatively new alternative technique to the conventional solid/liquid extraction is pressurized liquid extraction (PLE), also known as pressurized fluid extraction, pressurized solvent extraction, and accelerated solvent extraction. Essentially

it is a static solid/liquid extraction with high pressure and eventually high temperature in stainless steel extraction cells.

Applying high pressure allows the extraction solvent penetrating the samples thoroughly. Furthermore it keeps the extraction solvent liquid at elevated temperature. In the whole system air can be replaced by nitrogen. High temperature lowers the solvents viscosity and surface tension, so sample penetration is facilitated and diffusion of analyte molecules is accelerated. Both parameters lead to a faster, i.e. more efficient extraction [23,61,103,110,125].

At higher temperature polarity of water decreases and solubility of non polar compounds in water increases [50,89]. Hence extraction solvent mixtures has to be optimized depending on temperature. An overview over the key parameters of PLE can be found at [97].

Up to now, *Dionex* is the only manufacturer of PLE apparatus, marketed as *Accelerated Solvent Extractor (ASE)*. The model ASE200, that was used for this work, can apply pressures up to 200bar and temperatures up to 200°C. Up to four solvents can be mixed automatically. It can extract up to 24 samples automatically in series. The ASE is explained and some applications are given in [57,108,109].

PLE was first used in environmental analysis and still the most publications about PLE talk about this field. The non-environmental applications are summarized in [33]. There is an increasing number of papers about extraction of polyphenols in food using PLE [7,8,19,61,93,94,96,97,98,103,117,143].

Among them one of the earliest papers [98] presents our first results for the application of PLE to proanthocyanidins in malt. These results, obtained by team-work mainly with Menelaos Papagiannopoulos, build the base for this work and further method variations [74,87,96,97,143]. Especially the online coupling of PLE and SPE and the main SPE settings reappear in this work.

PLE is compared to conventional solid/liquid extraction with many different solvent mixtures for extraction of barley by [19]. However for PLE only ethanol/water, 4/1, v/v is used. In the resulting extracts single polyphenols are not analyzed, but sum parameters are measured. So these results are hardly to compare with HPLC results.

Two proanthocyanidins and other phenolics are determined by [7,8] in apple after PLE extraction with methanol/water mixtures. Despite of higher extraction yields at 60°C and 75% methanol in water, they recommend 40°C and pure methanol as optimal (2 cycles of 5 min extraction).

Comprehensive studies to the extraction of anthocyanins in grape skin are conducted by [61]. Many acidified solvents and solvent mixtures as well as temperatures are checked and compared to Soxhlet extraction at different tem-

peratures. The overall best conditions are: 0.1% HCl in 60% methanol, pH 2.3 at 60 °C, 3x5 min. There are slight differences for the various groups of anthocyanins (acylated anthocyanins, monoglucosides) due to their different polarity.

Interestingly the same recovery of total anthocyanins is found at 80–100 °C for acidified water and 60 °C for acidified 60% methanol. The levels of total phenolics and anthocyanins in the Soxhlet extracts are 2–3.4-fold lower than those obtained by PLE.

A comparison is done by [103] extracting the monomers catechin and epicatechin from grape seeds by magnetic stirring, ultrasound-assisted extraction, and PLE applying water, methanol, ethanol, and ethyl acetate. No surprise that methanol as the most polar organic solvent gives best results; solvent mixtures are not tested. PLE yields highest recoveries among the tested extraction procedures.

When employing PLE the extraction of catechins is increased from 100 °C up to the range of 160–180 °C and then diminishes, also drastically, up to the maximum temperature tested, 200 °C. This behavior is attributed to the superposition of two different effects due to the increase of the temperature. The first of these effects is the greater facility of extraction at higher temperatures, due to the weakening of the bonds between the catechins and the matrix. The second effect, in this case of contrary consequences, is the degradation of these compounds at high temperatures.

In the extractions that are performed for only 5 min, the recoveries obtained at temperatures between 150 and 180 °C are appreciably higher than those obtained when the duration of the extraction is 10 min. It is thought that at those temperatures, the effect of the degradation overrides the effect of increased extraction from the sample.

Grape skin and grape seed are extracted with pure methanol by [93] testing extraction temperatures from 50 to 150 °C. The highest temperature gives best extraction yield for all tested phenolics, but proanthocyanidins are not determined.

Again grapes are the samples for the same research group [94], determining polyphenols, but no proanthocyanidins. As extraction solvents only pure solvents (water, ethanol, diethyl ether, ethyl acetate, and methanol) are examined. As usual best results are obtained with methanol.

In this paper an in-line PLE-SPE method is presented: The solid phase (LiChrolut EN, a polar modified RP-18 phase) is placed into the extraction cell just below the sample. A two-stage extraction-clean-up method is designed: the first stage is run using water at 40 °C. During this stage the phenolics shall be transferred

from the sample to the solid-phase material. Subsequently methanol at 100 °C is used to rinse out the retained compounds and to extract further polyphenols from the sample, which were not fully transferred to the solid phase in the first step.

Carob products are extracted by [97]. Water, acetone, methanol, water plus acetone, and water plus methanol in different compositions as well as different temperature and time settings are tested. The extracts are subjected to HPLC-MS to analyze the phenolics, among them a trimeric proanthocyanidin. The PLE parameters found to be optimal are: acetone plus water, 50+50, v+v; 60 °C, 5 min, 2 cycles.

Isoflavones from soybeans are extracted by means of PLE by [117]. Several extraction solvents (methanol, and ethanol, 30–80% in water and water), temperatures (60–200 °C), as well as the sample size (0.05–0.5 g) and extraction time (5–10 min) were studied for the optimization of the extraction protocol. The optimized extraction conditions are: 0.1 g of sample, 100 °C, 3 x 7 min, and ethanol 70% as extracting solvent. At temperatures above 150 °C the level of isoflavones in the extract decreases in favor of the aglucones, suggesting the break of the glycosidic linkage in the extraction cell.

### 1.4.1.2 Clean-Up

Extraction selectivity is often poor, so matrix compounds interfering analyte peaks in the chromatogram has to be removed. Beside that, due to the small amounts of proanthocyanidins in most foods, concentrations of analytes are often too low for direct analysis. So preconcentration is required.

### Liquid-Liquid Extraction

Some authors carry out a liquid-liquid extraction using ethyl acetate [45,86,141], or petroleum ether [45, 67, 127]. Anyway, analyte losses may occur, so the necessity of such a step should be proofed.

### Column Chromatography

It is rather popular to apply a column chromatography to the crude extracts prior to analysis. Sephadex LH-20 is a common stationary phase [48, 68, 86, 116, 136]. This technique allows separating the proanthocyanidins by degree of polymerization and to work on a preparative scale. A recent review about size exclusion chromatography and related techniques (e.g. high speed counter current chromatography) for separation of proanthocyanidins can be found at [140].

### **Solid-Phase Extraction**

Solid-phase extraction (SPE) is in theory basically a fast column chromatography. Among the stationary phases polyamide is the most widely used [36, 37, 40, 90, 96, 97, 98, 115, 127, 143]. As eluents acetone + water, 75 + 25 [90], pure methanol followed by DMF [36, 37, 115], pure methanol followed by acetone + water, 7 + 3 [127], acetonitrile + water, 30 + 70, followed by acetone + water, 75 + 25 [40], DMF + water, 85 + 15, v + v [96, 97, 98, 143] is applied.

Others use octadecyl [51, 101, 130, 136] or polar modified octadecyl [70] material, that is widely used for other phenolics, also for proanthocyanidins, but don't give values for recovery of the proanthocyanidins.

Kieselguhr is used by [67] for purification of a *Cistus* extract. The eluate is then applied to a RP SPE-cartridge. First the flavan-3-ols are eluted by 20% methanol, in a second fraction other flavanoids are eluted by pure methanol.

SPE of proanthocyanidins based on polyamide was carried out for the first time on commercially available disposable cartridges by our research group [98, 142]. All other authors use bulk-polyamide in self-packed glass columns.

#### **1.4.1.3 Further Concentration**

Whatever sample extraction or preparation method is applied (see 1.4.1.2), the volume of the resulting sample solution is often too high, and concentration of analytes in the resulting solution too low. So all authors mentioned above (except our workgroup) evaporate a part or all the solvent on a rotary evaporator. This step includes the risk of degradation, when applying high temperature, and can hardly be automated.

### **1.4.2 Analysis**

#### **1.4.2.1 HPLC**

##### **Chromatography**

High-performance liquid chromatography is the method of choice for almost all researchers to determine the single proanthocyanidins. Most use RP-18 stationary phases [32, 37, 39, 40, 45, 90, 127, 130, 136, 139], in some cases modified by polar endcapping [49, 96, 97, 98, 143]. The polar groups on the stationary phase increase the retention time of the relatively polar proanthocyanidins. Different conventional and polar modified RP-columns are compared by [63, 64, 138].

Also normal phase (NP) chromatography is suitable for proanthocyanidins. Not only sample preparation can be done by NP chromatography (see 1.4.1.2), but also separation for analysis [48, 51, 70, 140].

A new approach is the use of monolithic columns. In this way 17 monomeric compounds of wine (hydroxybenzoic acids, hydroxycinnamic acids, hydroxycinnamyltartaric acids, flavanol, flavonol and stilbenes; no proanthocyanidins) are separated in 35 min by [25].

Polyphenols in apple including procyanidin B2 and nine unknown dimers and trimers are separated on a monolithic RP-18 column by [26]. 29 phenolics can be determined within 21 minutes of HPLC run-time when detecting at 280, 320, and 350 nm.

### Detection

The far most used technique for detection of proanthocyanidins is UV-detection at 280 nm. The UV-spectra of the various proanthocyanidins are well known, but among each other very similar. So UV-spectra are fine to determine *whether* a peak is a proanthocyanidin or not, but one can't determine, *which* proanthocyanidin is eluted.

Mass spectrometry (MS) gives information on the structure of the analytes and is widely used [35, 37, 38, 39, 47, 48, 49, 63, 96, 97, 139]. In case of proanthocyanidins one can determine the order of the monomers, but information about their linkage or stereochemistry, e.g. to distinguish catechin and epicatechin as monomers, can not be obtained [37, 38]. Common interfaces are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI).

A rather new technique is matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). A broad range of oligomers up to heptamers can be identified in grape seeds [141] with high resolution. The MALDI-TOF-MS technique is not yet ready for quantification. In sorghum an unheard variety of proanthocyanidins and glucosylated proanthocyanidins up to nonamers is found by MALDI-TOF-MS [68].

Electrochemical detection is seldom used. Amperometric detection is more common than coulometric detection although better sensitivity and selectivity of the latter [2, 3, 20, 36, 79, 128]. Coulometric electrodes can be combined to a series of electrodes, known as coulometric electrode array detection (CEAD). CEAD gives hydrodynamic voltammograms, that allows identifying of proanthocyanidins. As for UV-detection (see above) a peak can be identified as proanthocyanidin, but the voltammograms of all proanthocyanidins are rather similar. A typical hydrodynamic voltammogram can be seen in figure 1.3.

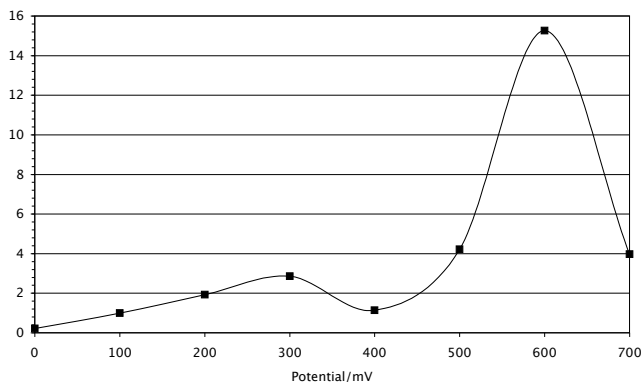


Figure 1.3: A typical CEAD voltammogram (procyanidin B<sub>3</sub>), normalized at 100 mV.

A table of publications dealing with phenolics and CEAD can be found at [36]. Some more recent papers are [1, 20]. In the field of brewing [77, 81, 86, 136] use amperometric detection while [18, 36, 98, 143] use CEAD.

#### 1.4.2.2 Other Techniques

Micellar electrokinetic chromatography (MEKC) is used to determine proanthocyanidins in *Cistus* and various food samples. 17 polyphenols of *Cistus* including 13 proanthocyanidins are separated simultaneously in less than seven minutes by [67].

Within a run-time of five minutes three proanthocyanidins, catechin, epicatechin, and *cis* and *trans* *p*-coumaric acid from various food samples are separated by [27] also applying MEKC.

When using chiral molecules in the buffer for MEKC, polyphenols can be separated according to their stereochemistry, i.e. even enantiomers are separated. This technique is applied to lemons and oranges [43, 44]. For polyphenols in the field of brewing work is in progress [65, 66].

Thin layer chromatography is only of historical interest [14, 82].

### 1.5 Proanthocyanidins and Brewing

As other flavanoids proanthocyanidins are present in many plants, hence in many foods. The most widely consumed tannin-like polymers are found in tea and wine. In both beverages the proanthocyanidins increase during fermentation and (in case of wine) storage. Cereals don't contain any proanthocyanidins except for sorghum and barley, the latter the main (and beside wheat the only) cereal used for german beer. Occurrence, intake, bioavailability and possible health effects of phenolics in general and proanthocyanidins in particular are reviewed in [34, 78, 95, 118, 119, 120].

In the brewing industry proanthocyanidins are of special interest, because they influence the properties of beer despite of their low concentrations. Main activities of the proanthocyanidins are the linkage of proteins to form haze and the contribution to the antioxidative capacity (among other phenolics and sulfur dioxide). So far all researchers agree. The detailed effects and mechanisms are still under discussion, so related papers are numerous and sometimes controversial.

Haze and flavor stability of beer depends on quantity and quality of phenolics, especially of proanthocyanidins. State of the research is summarized in [12, 13, 16, 17, 31, 36, 46, 59, 86, 87, 121, 122, 123, 131, 133]. Recent papers contribute the following aspects to the discussion:

The effects of general process variables of brewing were examined by [76]. Colloidal shelf life increased in direct proportion to the dosage of polyvinylpyrrolidone (PVPP) and silica hydrogel (SHG). As expected, total polyphenols and sensitive proteins decrease with dosage of PVPP and SHG respectively. But the concentration of haze precursors is not linearly related to the haze development potential.

Storage for up to three days at 4 °C before filtration confers additional stability on beer, but increasing the storage time to five days provides no further effect. Rapid assays (alcohol-chill haze, sensitive proteins, tannoids, total polyphenols, total flavanols; after European Brewing Convention (EBC)) are found to be poor predictors for colloidal shelf life. Fresh beers with equal results in the rapid assays differ substantially in shelf life. Flavor stability is not tested; single proanthocyanidins are not determined.

The phenolic content in alcohol free beer is shown to be lower than in standard beers. If the stability of those beers differs, is not reported [5, 15].

Sensory measurement of aged beer depending on PVPP and SO<sub>2</sub> treatment is the subject of [24]. In the US addition of SO<sub>2</sub> is allowed by law, but has to be labeled, if its presence is greater than 10 mg/L. At levels below the labeling limit



there is a slight positive effect on flavor stability. It is supposed that SO<sub>2</sub> exerts its protective effect as antioxidant rather than as agent that binds the carbonyls responsible for the aged characteristics. Treatment of beer with PVPP has no impact on flavor stability. The German beer purity law prohibits the addition of SO<sub>2</sub>. If its concentration due to naturally present in the raw materials or formation during fermentation exceeds 10 mg/L, the beer can not be sold.

A simple and rapid spectrometric assay (total reactive antioxidant potential (TRAP)), for measuring the antioxidative capacity is developed by [10] and applied to beer and wort as well as to standard compounds. Catechin and ferulic acid are found to be the most powerful antioxidants in beer (proanthocyanidins are not tested). Beers with higher TRAP values have better flavor stability. It is postulated that differences in antioxidant content of the malts are responsible for the differences in the TRAP values of wort and beer.

“Gallotannins” are added at the start of mashing by [4]. The beers from modified brewing are evaluated positively by the tasting panel when fresh (fullness of taste and mouthfeel) and when force aged. Chemical examination show a higher antioxidant potential, lower formation of carbonyls, e.g. trans-2-nonenal, and higher stability of cis-iso- $\alpha$ -acids. This proves the important antioxidative activity of polyphenols during mashing. The term “Gallotannins” is not defined in the paper. However, addition of “Gallotannins” is not permitted by the German beer purity law.

Comprehensive brewing trials are conducted by [88]. Hot sweet wort is treated with PVPP to reduce polyphenols. To this modified wort and to untreated wort hop pellets or virtually polyphenol-free CO<sub>2</sub> extract of the same hops are added. Thus the resulting four beers differ in polyphenol content and polyphenol sources. All beers are further examined with and without filtering of the fresh beer with PVPP, force aged and stored under real conditions (dark, 20 °C).

Haze stability and reducing activity of the beers behave as expected: the more polyphenols, the earlier the haze and the higher the reducing activity. Foaming properties are not affected by polyphenols. The sensory quality of all fresh beers are similar, but the samples with reduced polyphenol content tend to score a lower harsh character of bitterness. The most rapid decline in flavor quality (overall impression and staling) is observed in beer produced with hop extract and reduced malt polyphenols.

The PVPP-stabilized beers showed a slightly better flavor quality after force aging and storage. That indicates that the effect of PVPP treatment on flavor is either neutral if not slightly positive. The main protective effect of polyphenols on flavor stability is thought to be active during mashing and boiling and not in the final product.

## *1 Introduction*

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Beers are examined using common EBC and MEBAK sum parameters. Single polyphenols are not determined.

As a conclusion is said [88]: “The stabilizing effect also depended on the particular quality of the raw materials ... It is possible to produce beers with long shelf life and improved flavor stability, by using raw materials rich in polyphenols.”

## 2 Experimental

## 2.1 Materials

### 2.1.1 Samples

Barley (*Hordeum vulgare* L.) and malt samples were provided by the *Versuchs- und Lehranstalt für Brauerei in Berlin* (Berlin, Germany). 15 summer and 5 winter barleys and the corresponding malts of the growing years 1998–2001 from 3 locations (summer barley: Hadmersleben<sup>1</sup>, Rethmar<sup>2</sup> and Roggenstein<sup>3</sup>, winter barley: Hadmersleben, Roggenstein and Wetzze<sup>2</sup>).

Malt samples from the barley varieties Prisma and Caminant were kindly provided by Weissheimer Malz (Andernach, Germany).

### 2.1.2 Chemicals

#### 2.1.2.1 Solvents

Acetonitrile	Ultra Gradient HPLC Grade, Mallinckrot Baker (Deventer, The Netherlands)
Acetone	p.a., Merck (Darmstadt, Germany)
N,N-Dimethylformamide	p.a., Merck (Darmstadt, Germany)
Milli Q Water	provided by an Elgastat UHQ-II, Elga (Bucks, UK)

#### 2.1.2.2 Standard Compounds

(+)-Catechin	Hydrate, min. 98 %, Sigma (St. Louis, MO, USA)
Protocatechuic acid	H <sub>2</sub> O content 2.7 %, Sigma (St. Louis, MO, USA)

#### 2.1.2.3 Other Chemicals

Diatomaceous earth	Isolute HM-N, Separtis (Grenzach-Wyhlen, Germany)
o-Phosphoric Acid	p.a., 85 %, Roth (Karlsruhe, Germany)
Sodium dihydrogen phosphate	p.a., monohydrate, Merck, (Darmstadt, Germany)

<sup>1</sup>Saxony-Anhalt (Sachsen-Anhalt), Germany

<sup>2</sup>Lower Saxony (Niedersachsen), Germany

<sup>3</sup>Bavaria (Bayern), Germany

### 2.1.3 Consumables Supplies

SPE cartridges	Chromabond Polyamide 6 PA-F, 6 mL, 1000 mg, Macherey-Nagel (Düren, Germany)
Membrane filters	regenerated cellulose, RC58, 0.2 $\mu\text{m}$ , $\varnothing$ 50 mm, Schleicher & Schuell (Dassel, Germany)  PTFE, 0.2 $\mu\text{m}$ , $\varnothing$ 47 mm, Alltech (Deerfield, IL, USA), distributed by Restek (Bad Homburg v.d.H., Germany)
Filters for extraction cells	filter paper circles, $\varnothing$ 19.1 mm, Schleicher & Schuell (Dassel, Germany)

### 2.1.4 Milling

Ball mill MM Retsch (Haan, Germany)

### 2.1.5 Pressurized Liquid Extraction

ASE200 with Moduleware Rev. 3.10 and BIOS Rev. 30.00, 11 mL extraction cells, and Solvent Controller, Dionex (Idstein, Germany)

### 2.1.6 Automated Solid-Phase Extraction

ASPEC XLi with ASE-ASPEC-Kit, Syringe Pump 402 (25 mL and 1 mL syringe), and software 735 v4.03, Gilson (Paris, France), distributed by ABIMED (Langenfeld, Germany)

### 2.1.7 HPLC-UV- and -Electrochemical Detection

All devices by ESA (Chelmsford, MA, USA), if not otherwise indicated.

Pumps	580 Solvent Delivery Module
Degasser	Degasys DG-1310, Uniflows (Tokyo, Japan)
Mixer	M800, Kontron (Neufahrn, Germany)
Pulsation damper	Art. 14-0177

## 2 Experimental

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Autosampler	540 with 20 $\mu$ L injection loop and tray-cooling (set at 4 °C)
Column oven	Mistral, set at 30 °C, housing HPLC column and electrodes
Guard column	RP-18 Security Guard, 4 mm x 3 mm i. d., Phenomenex (Aschaffenburg, Germany)
Column	Aqua RP-18, 150 mm x 4.6 mm i. d., particle size 3 $\mu$ m, Phenomenex (Aschaffenburg, Germany)
UV detection	System Gold Scanning Detector 167 set at 280 nm, Beckman Coulter (Unterschleißheim, Germany) System Gold Diode Array Detector 168 set at 280 nm, UV spectra 210–400 nm, Beckman Coulter (Unterschleißheim, Germany) Gold 7.11 software, Beckman Coulter (Unterschleißheim, Germany) Analog interface for CoulArray
Electrochemical detection	CoulArray 5600 with 8 electrodes set at 0–700 mV in steps of 100 mV, maintained at 30 °C CoulArrayWin software v1.02
Eluents	A: 0.02 mol/L NaH <sub>2</sub> PO <sub>4</sub> set at pH 3.4 with phosphoric acid, filtered through regenerated cellulose membrane filters (see 2.1.3) B: acetonitrile + 0.1 mol/L NaH <sub>2</sub> PO <sub>4</sub> set at pH 3.0 with phosphoric acid, 2 + 1, v + v, filtered through PTFE membrane filters (see 2.1.3)

### 2.1.8 HPLC-MS

Summit; all devices by Dionex (Germering, Germany), if not otherwise indicated.

Pump	P-580A HPG
Degasser	Degasys DG-1310, Uniflows (Tokyo, Japan)
Autosampler	ASI-100 T

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Injection	5 $\mu$ L for the eluate, 20 $\mu$ L for the fractions
Column oven	STH-585 set at 35 °C
Guard column	RP-18 Security Guard, 4 mm x 2 mm i. d., Phenomenex (Aschaffenburg, Germany)
Column	Aqua RP-18, 150 mm x 2 mm i. d., particle size 3 $\mu$ m, Phenomenex (Aschaffenburg, Germany)
UV detection	UVD-340S UV/Vis detector set at 280 nm, equipped with a capillary cell Chromeleon Software package version 6.20 Build 531
Eluents	A: 1%, v+v, acetic acid in water B: 1%, v+v, acetic acid in acetonitrile
Gradient	0 min: 0% B; 40 min: 20% B; 41 min: 100% B; 50 min: 100% B; 51 min: 0% B; 60 min: 0% B
MS	LCQ classic ion-trap mass spectrometer, Thermo Finnigan (Dreieich, Germany) electrospray interface metal needle kit addition of 100 $\mu$ L/min methanol by a System Gold Programmable Solvent Module 116 (Beckman, Unterschleißheim, Germany) to the HPLC flow before entering the ion source Xcalibur Software version 1.2
	source voltage                    4.0 kV (negative mode)
	sheath gas flow                 90
	auxiliary gas flow             5
	capillary voltage               -40 V
	capillary temperature        310 °C
	first octapole offset          +3,8 V
	interoctapole lens voltage   +30.0 V
	second octapole offset       +10.2 V
	ion trap DC offset            +10.2 V

## 2.2 Optimized Methods

### 2.2.1 Pressurized Liquid Extraction

The optimized PLE parameters are shown in table 2.1

sample amount	3 g
diatomaceous earth	1.8 g
solvent	acetone + H <sub>2</sub> O, 40 + 60, v + v
temperature	80 °C
pressure	100 MPa
preheat time	5 min
static extraction time	7 min
number of cycles	2
purge volume	100 %
purge time with N <sub>2</sub>	60 sec

Table 2.1: Optimized PLE parameters

### 2.2.2 Automated Solid-Phase Extraction

The optimized SPE parameters are shown in table 2.2. The detailed ASPEC settings for this method can be found in appendix B and figure B.1.

dilution of the extract	40 mL H <sub>2</sub> O
mixing of the diluted extract	24 mL in liquid mode
loading of the extract onto the cartridge	40 mL (i.e. quantitatively)
washing	8 mL H <sub>2</sub> O
pre-eluting	1 mL DMF 85 % (to be discarded)
eluting	2.5 mL DMF 85 %

Table 2.2: Optimized SPE parameters. For detailed settings of the ASPEC software see appendix B and figure B.1.



### 2.2.3 HPLC

The optimized HPLC gradient parameters are shown in table 2.3.

time/min	% B	event
0	0	
8		autosampler inject
10		file start
10	0	
15	8	
35	8	
50	12	
60	30	
65	100	
75		file stop
75	100	
80	0	
85	0	

Table 2.3: Optimized HPLC gradient parameters.

Further HPLC settings:

Eluents           A: 0.02 mol/L  $\text{NaH}_2\text{PO}_4$  set at pH 3.4 with phosphoric acid  
                       B: acetonitrile + 0.1 mol/L  $\text{NaH}_2\text{PO}_4$  set at pH 3.0 with phosphoric acid, 2 + 1, v + v

Electrodes       set at 0 to 700 mV in steps of 100 mV

UV wavelength 280 nm

## 2.3 Statistical Analysis

For statistical analysis SPSS 12.0.1 (SPSS Inc., Chicago, Illinois, USA) is applied.



### **3 Results**

### 3.1 Milling<sup>1</sup>

As reported by [36] a ball mill provides fine ground and homogenous barley and malt flour. 8 minutes of grounding at an amplitude of 100% was found to be sufficient for an optimal extraction yield (see figure 3.1, detailed data see table C.1).

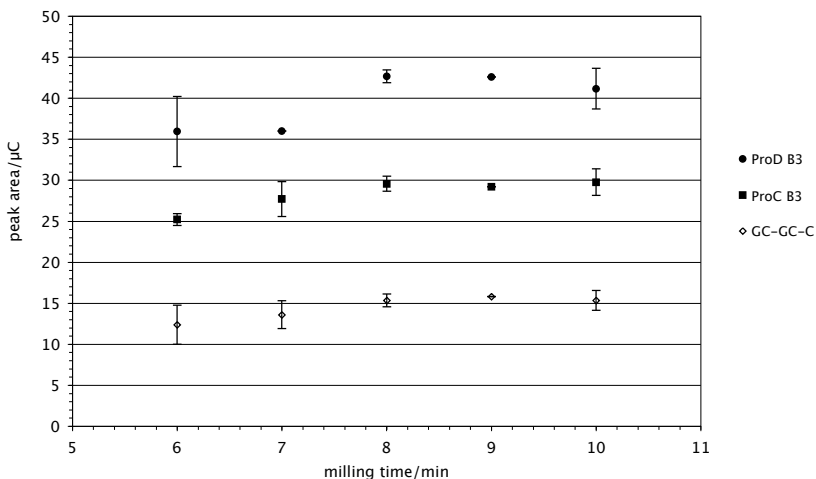


Figure 3.1: Milling time: peak area of selected analytes in the extracts depending on the milling time of the barley grains. For extraction the optimized method (see table 2.1) was applied.

In contrast to the manual extraction according to [36] cooling the milling cells with liquid nitrogen doesn't show a significant effect on extraction yield, although the resulting flour is apparently much finer. This proves the better penetration of the sample particles by the extraction solvent applying high pressure (see section 1.4.1.1).

For optimizing milling time barley was used, since it is much harder than the more brittle malt grains.

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<sup>1</sup>dedicated to Hanna

## 3.2 Extraction

### 3.2.1 Preparing of the Extraction Cells

A paper filter circle is placed at the bottom of the extraction cell to prevent plugging of the frits and the subsequent capillaries by sample particles.

At high temperature in presence of water the starch in the samples tends to agglutinate and to obstruct the solvent flow through the extraction cell. This can be evaded by mixing the sample with diatomaceous earth prior to fill the extraction cell. 2 g of sample and 2 g of diatomaceous earth or 3 g of sample and 1.8 g of diatomaceous earth fill an 11 mL extraction cell virtually completely. Diatomaceous earth does not affect the extraction yield.

At the same time diatomaceous earth is used to fill up the cell completely in order to avoid death volume. Death volume in the extraction cell results in higher extract volume, i.e. more diluted analytes in the extract.

Extracting a series of cells filled with 1, 2, 3, and 4 g of malt gives a linear correlation between peak area of the analytes and amount of sample from 1 to 3 g, while 4 g of malt can not be extracted completely (see figure 3.2, detailed data see table C.2).

### 3.2.2 Pressure

As mentioned in section 3.1 the applied pressure plays an important role to facilitate penetration of sample particles by the extraction solvent. Furthermore it keeps the extraction solvent liquid at elevated temperature. So it is indispensable, but there is no difference between extraction at 100 or 200 bar.

### 3.2.3 Temperature and Extraction Solvent Mixture

The main factor influencing the solid/liquid extraction is the extraction solvent mixture. Proanthocyanidins have polar and non polar groups, so polar organic solvents mixed with water show best results. For manual extraction at room temperature and atmospheric pressure a mixture of acetone and water, 75 + 25, v + v, is optimal, whereas the more toxic mixtures of DMF and methanol with water show poorer results [28,36].

The efficiency of a solvent mixture depends on the temperature: at higher temperature polarity of water decreases and solubility of non polar compounds in water increases [50,89]. So a smaller percentage of acetone in the acetone/water mixture for extraction at elevated temperature in respect to the

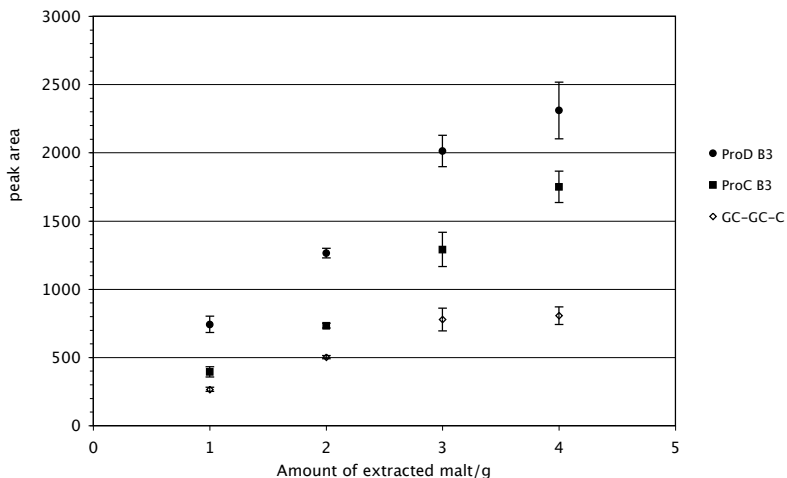


Figure 3.2: Amount of malt: peak area of selected analytes in the extracts depending on the extracted amount of malt (peak area in arbitrary units).

manual extraction at room temperature is expected to give the highest extraction yield. In fact, instead of 75 Vol% acetone in water for the manual extraction 40 Vol% at 80 °C is best in PLE.

Figures 3.3 and 3.4 show the extraction yield for the two major analytes at 40, 60 and 80 °C applying various acetone + water mixtures as extraction solvent. The other analytes behave in the same way (detailed data see tables C.3 and C.4).

Too high temperature ( $T \geq 100$  °C) seems to cause decomposition of the analytes. An extraction temperature of 80 °C was found to be optimal when acetone + water, 40 + 60, v + v is used.

The highest extraction yield is achieved with 80 °C and acetone + water, 40 + 60, v + v.

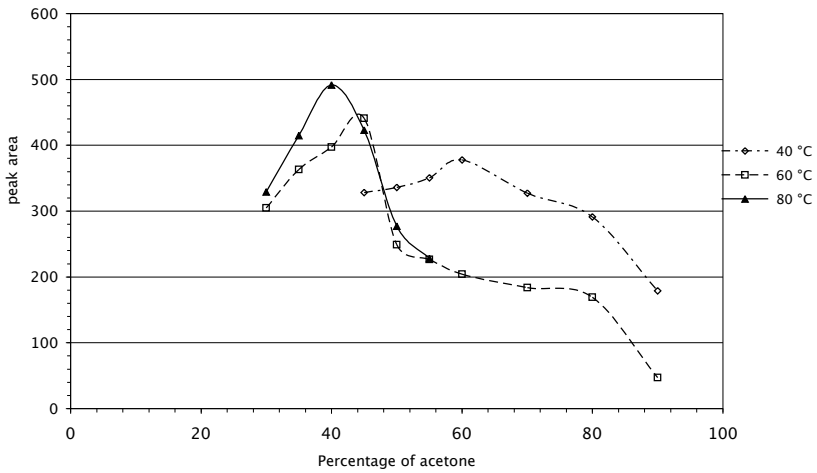


Figure 3.3: Extraction temperature and solvent: peak area of prodelphinidin B<sub>3</sub> in the extracts depending on the extraction solvent mixture at 40, 60 and 80 °C (peak area in arbitrary units).

### 3 Results

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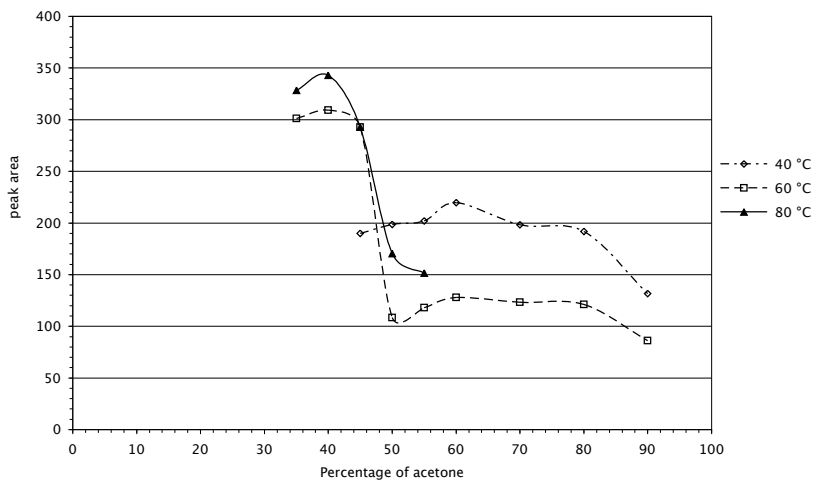


Figure 3.4: Extraction temperature and solvent: peak area of procyanidin B3 in the extracts depending on the extraction solvent mixture at 40, 60 and 80 °C (peak area in arbitrary units).



### 3.2.4 Multiple Extraction

According to Nernst's law on distribution an extraction can never be complete. In practical work multiple extraction steps lead to exhaustive extraction or at least to a sufficient recovery. Figure 3.5 shows the extraction yields of the first, second, third, and fourth extraction of the same sample. Two extraction steps are sufficient for an extraction yield greater than 95% for all analytes. The ASE defines multiple extraction steps as *cycles*.

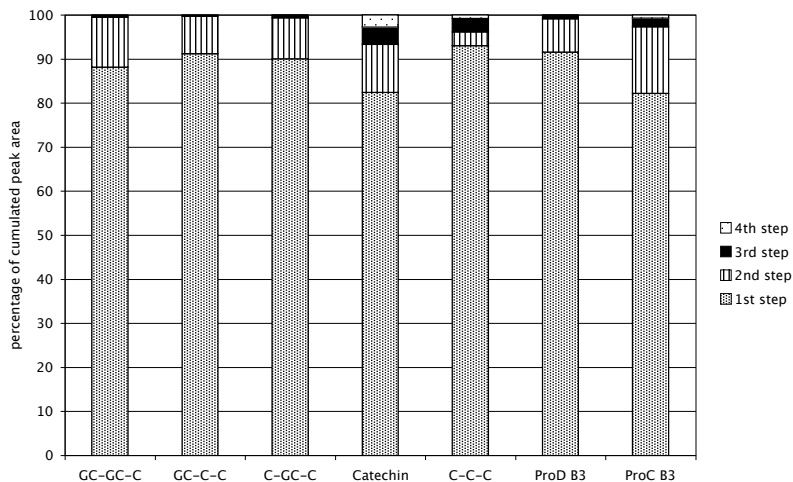


Figure 3.5: Multiple extraction: cumulated peak area of different analytes in the extracts depending on multiple extraction steps (extracted with acetone+water, 45+55, v+v, at 80°C) (sum of four extraction steps = 100).

### 3.2.5 Extraction Time and Multiple Extraction

PLE is a static extraction. The extraction cell is filled with the extraction solvent and then maintained at the desired pressure and temperature for a defined time. A too short extraction time doesn't allow the solvent penetrating the sample

### 3 Results

particles thoroughly and the analytes to diffuse in the solvent. Excessive extraction time may lead to analyte decomposition, specially at high temperature, and makes one loose time at work.

Figure 3.6 shows the extraction yield depending on extraction time and number of extraction steps (detailed data see table C.5). Two extraction steps of 7 min give best results.

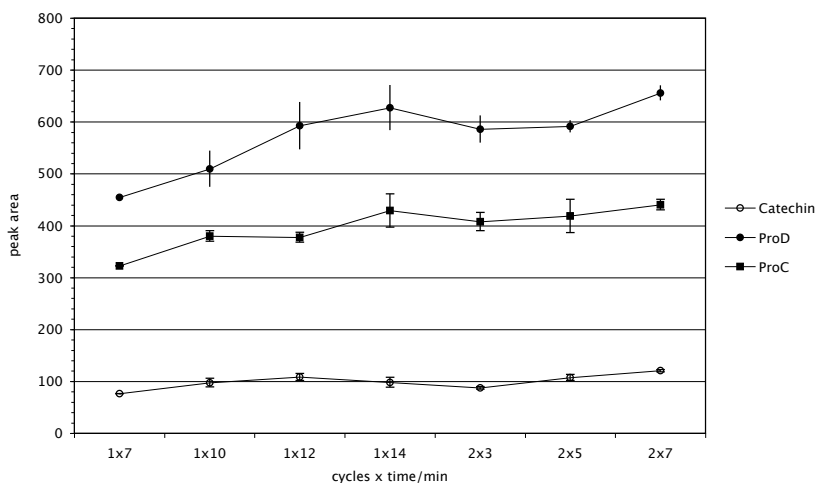


Figure 3.6: Extraction time and multiple extraction: peak area of selected analytes in the extracts depending on extraction time and multiple extraction steps (extracted with acetone + water, 45 + 55, v + v, at 80 °C); “1x7” means: 1 extraction cycle of 7 min. (peak area in arbitrary units)

### 3.3 Coupling of PLE and Automated SPE

The ASE and the ASPEC are built to work as stand-alone instruments. Installing the ASE-ASPEC-Kit they can be coupled, i.e. the ASPEC sample needle can access the ASE extract vial. So the just produced extract can be handled by the ASPEC with all his options as diluting, mixing, or loading on a SPE cartridge. This coupling was developed by ABIMED and Dionex and is tested and established under real laboratory conditions for the first time.

In fact solid/liquid extraction is not compatible with SPE: for solid/liquid extraction an optimal solvent mixture has to be chosen to extract the analytes quantitatively. If this extract was loaded on a SPE cartridge, the analytes would break through due to better solubility in the solvent than adsorption to the SPE filling material.

The classical way to solve this incompatibility is to evaporate the organic portion of the extract. This step has to be carried out manually and can not take part of a fully automated sample preparation procedure. But instead of eliminate the organic part completely it is sufficient to reduce it by diluting the extract with water.

As shown in table 3.1 after dilution to a percentage of acetone of 20% the analyte breakthrough is negligible.

Acetone	40% (not diluted)	30%	25%	20%
GC-GC-C	21.4 ± 1.7	5.4 ± 0.9	0.2 ± 2.7	1.6 ± 0.9
GC-C-C	18.9 ± 4.3	3.9 ± 0.6	0.3 ± 3.5	3.9 ± 0.2
C-GC-C	30.7 ± 12.0	10.7 ± 1.2	10.3 ± 3.2	6.4 ± 4.6
C-C-C	29.7 ± 17.0	9.7 ± 0.7	1.6 ± 2.9	2.2 ± 8.9
ProD B3	57.7 ± 7.8	32.6 ± 0.6	10.7 ± 2.1	12.7 ± 4.4
ProC B3	59.0 ± 11.9	37.1 ± 3.3	13.7 2.9	11.9 ± 6.1

Table 3.1: SPE breakthrough: percentage ( $\pm$  standard deviation) of analytes, that breaks through, loading the SPE cartridge depending on the percentage of acetone in the extract.

### 3.4 Solid-Phase Extraction

#### 3.4.1 Loading and Eluting

For loading refer also to section 3.3. The so called *Multi Collect* procedure allows optimizing elution quickly using the ASPEC. In this mode the ASPEC elutes the analytes from the cartridge in defined fractions e.g. of 2 mL. Thus a fine elution profile is obtained.

To determine the optimal elution solvent the loaded SPE cartridge was eluted with different acetone/water and DMF/water mixtures in fractions of 2 mL. Figures 3.7 and 3.8 show the elution power of solvent mixtures for the two major analytes. DMF + water, 85 + 15, v+v, elutes over 95 % of the analyte within the first two fractions.

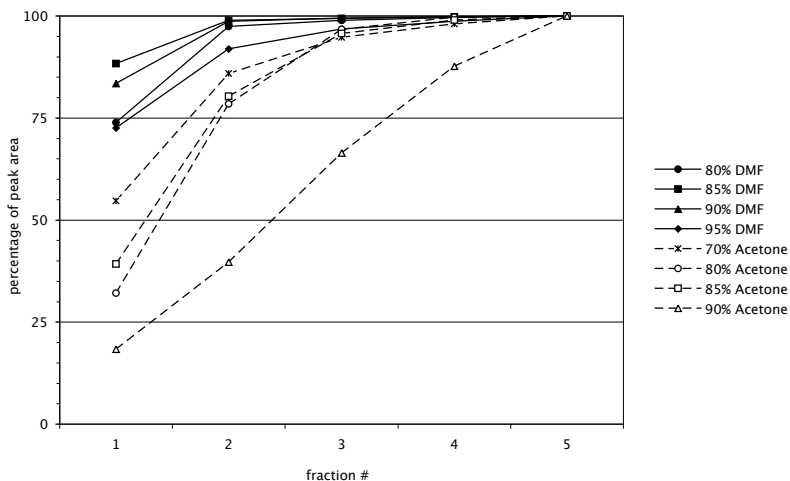


Figure 3.7: Elution profile: cumulated percentage of peak areas of prodeldphinidin B<sub>3</sub> in the elution fractions of 2 mL using different solvent mixtures.

In a finer elution profile of 1 mL fractions the first elution step of 1 mL of DMF + water (85 + 15, v+v) doesn't contain any analytes, likewise in the fourth

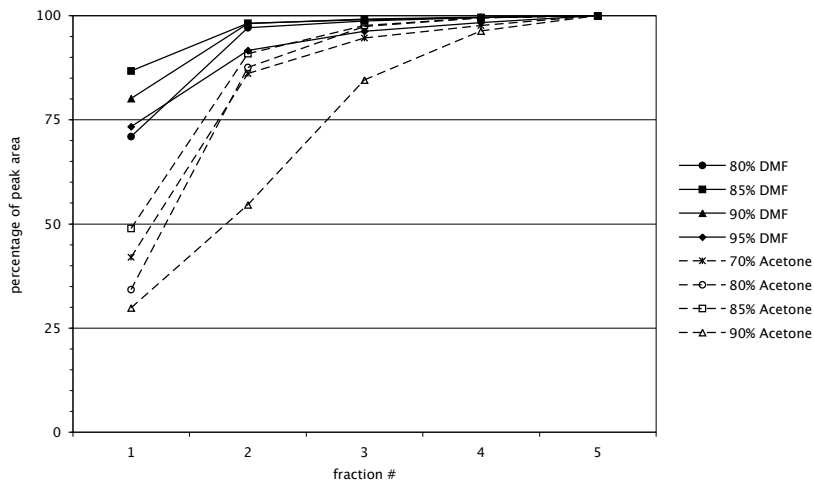


Figure 3.8: Elution profile: cumulated percentage of peak areas of procyanidin B3 in the elution fractions of 2 mL using different solvent mixtures.

and all following milliliters. Hence only the second and the third milliliter are collected, adding a further 0.5 mL to get sure.

In addition all solutions from the cartridge can be collected to obtain a complete SPE profile. Table 3.2 shows the elution profile for all SPE steps. Figure 3.9 illustrates the elution profile, so one can see the removed matrix peaks in the load and wash fractions.

The optimized SPE parameters are shown in table 2.2. The detailed ASPEC settings for this method can be found in appendix B and figure B.1.

### 3.4.2 Differences between Lots

The polyamide SPE cartridges can differ widely between lots. Every lot of PA-cartridges has to be checked prior to use.

A key parameter is permeability, because the ASPEC uses high pressure to push liquids through the cartridge (in contrast to many other SPE techniques that uses vacuum to suck liquids through the cartridge). Therefore Macherey-

### 3 Results

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	load0	load1	wash0	load2	wash1	elute0	elute1	elute2	elute3
GC-GC-C	0.04	1.91	2.20	1.10	0.11	0	93.79	0.60	0.25
GC-C-C	0	2.38	1.45	1.05	0	0	93.79	0.96	0.37
C-GC-C	0.92	2.39	1.71	2.10	0.32	0	91.57	0.70	0.29
Catechin	0	1.19	3.43	9.07	1.15	0.16	84.85	0.15	0
ProD B3	2.26	4.47	1.63	4.95	4.16	0.01	81.72	0.57	0.23
ProC B3	0.48	2.71	1.46	11.83	2.98	0	79.79	0.55	0.21

Table 3.2: SPE profile: percentage of analytes in the different SPE fractions (load0 = first load volume; load1 = second load volume; wash0 = wash step between loading; load2 = third load volume; wash1 = wash step after loading; elute0-3 = elute steps of 1 mL, except elute1 of 2.5 mL).

Nagel developed so called PA-F-cartridges with more pervious polyamide filling than the one of the standard cartridge.

One of the used lots showed a different elution profile, so the first milliliter of the eluate already contained analytes and could not be discarded. Using some lots recovery is poorer than optimal and can not be used for analysis.

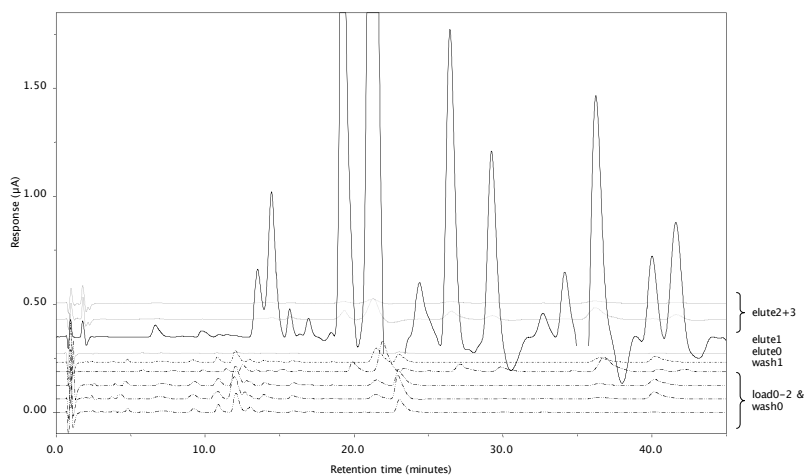


Figure 3.9: SPE profile: chromatograms of the single SPE fractions (load0-2 = load volumes; wash0 = wash step between loading; wash1 = wash step after loading; elute0 = elute step of 1 mL, elute2 = elute step of 2.5 mL; elute2-3 = elute steps of 1 mL). For peak naming refer to table 1.1.

## 3.5 HPLC

### 3.5.1 HPLC-UV-CEAD

Due to their high polarity proanthocyanidins don't retain very well on RP-phases. Polar modified C-18 columns as the here used *Aqua* (see section 2.1.7) are more suitable than standard C-18 phases. Nevertheless the non-polar portion of the eluent has to rise slowly to separate all proanthocyanidins (see table 2.3). Figure 3.10 shows typical chromatograms of a malt eluate.

CEAD is much more sensitive and selective than UV-detection [20, 36, 128], so preconcentration of the ASE extract is dispensable. Method development for this work bases on these unique qualities of CEAD. Otherwise it is much harder - if not impossible - to analyze the crude extracts or the various SPE fractions. SPE is required for sufficient analyte concentration for UV detection.

There are no standard compounds commercially available to calibrate the CEAD. The UV detector instead can be calibrated using the proanthocyanidin monomer catechin [36, 69, 71]: the molar absorption of a proanthocyanidin dimer is twice as high as the one of catechin; for a trimer threefold higher than for catechin and so on.

The hydrodynamic voltammograms recorded by a coulometric electrode array detector allow proofing the identity of detected compounds. The voltammograms of proanthocyanidins are rather similar, but it is possible to distinguish, whether a detected compound is a proanthocyanidin or not. The voltammograms of the here detected peaks can be found at section C.3. Details about hydrodynamic voltammograms are explained in [29, 36].

### 3.5.2 HPLC-UV-MS<sup>n</sup>

An ion-trap mass spectrometer coupled to HPLC gives detailed information about molecular masses and fragmentation to confirm peak identity and purity. When injecting 50  $\mu\text{L}$  of a malt eluate of SPE in the HPLC system used for determination of proanthocyanidins (obviously by-passing the CEAD), fractions of 800  $\mu\text{L}$  (one minute of elution time) can be collected and then injected to the HPLC-UV-MS<sup>n</sup>-system.

The supposed peak identity suggested by elution order and peak pattern similar to that of [36] is confirmed by MS<sup>n</sup>-data. The fragmentation pattern is the same. Detailed mass values can be found in table C.6.

Additionally some more proanthocyanidins are found in the eluate: the dimer GC-GC, the trimer GC-GC-GC, several tetramers. These are partly coeluting with



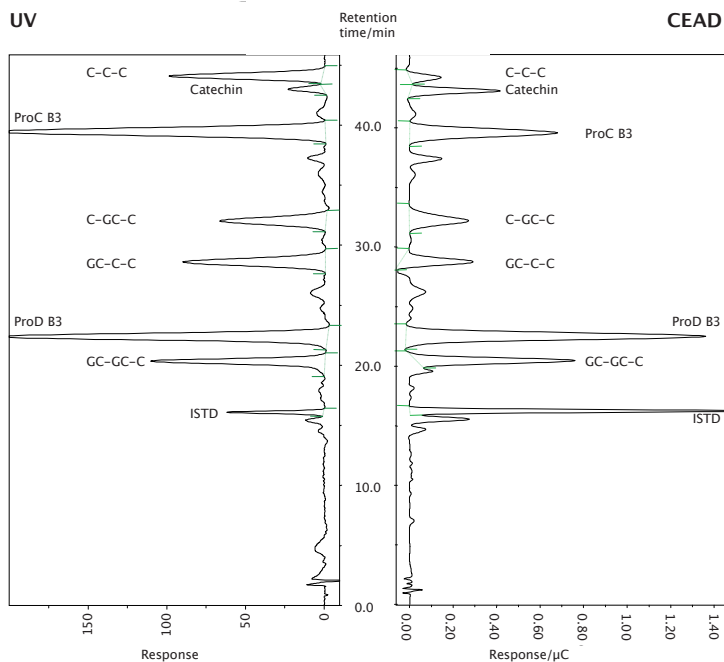


Figure 3.10: Typical uv (280 nm, left) and electrochemical (110 mV, right) trace (for peak labels refer to table 1.1).

the quantified proanthocyanidins, but MS-peak intensity allows estimating the amount of these neglected compounds to be more than one to two orders of magnitudes lower than the considered analytes. Their mass data is shown in tables C.6 and C.7. The overlaid chromatograms of the single ions are shown in figure 3.11, the single ion chromatograms can be found in section C.4.

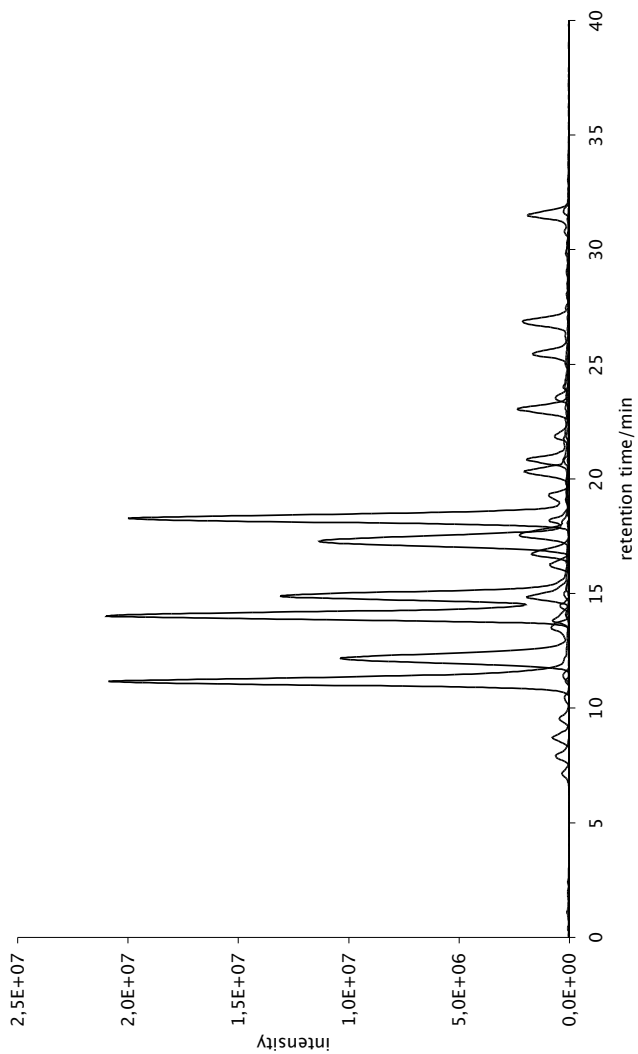


Figure 3.11: Mass traces of proanthocyanidins.

### 3.6 Reproducibility and Recovery

Due to the lack of available standards the usual methods to determine recovery and reproducibility can not be applied. In fact none of the interesting proanthocyanidins can be found on the market in sufficient purity and sufficient amounts for reasonable prices.

Recovery and reproducibility can be estimated as follows:

#### 3.6.1 Extraction

Extracting the same sample four times with the optimized extraction method can be considered as exhaustive. Hence the sum of peak areas of every analyte in these four extracts equals the total amount in the sample, supposing that there is no degradation during extraction. The peak area found in normal extracts can then be calculated as a percentage of the prior calculated total amount of this peak in the sample. This percentage corresponds therefore to the recovery of extraction.

Table 3.3 shows recovery and reproducibility of extraction (n=6).

	GC-GC-C	GC-C-C	C-GC-C	Catechin	C-C-C	ProD B3	ProC B3
rec.	99.47	99.69	99.36	93.29	96.16	99.14	97.29
repr.	2.75	1.33	3.52	2.45	0.53	3.09	4.65

Table 3.3: Estimated recovery (*rec.*, in percent) and reproducibility (*repr.*) of extraction.

#### 3.6.2 Clean-Up

Taking advantage of the high sensitivity of the CEAD the peak areas before and after SPE can be compared and thus the recovery estimated. Table 3.4 shows the estimated recovery of detected analytes of the ASE-extract in the SPE-eluate (n=6).

	GC-GC-C	GC-C-C	C-GC-C	Catechin	C-C-C	ProD B3	ProC B3
rec.	79.65	71.48	70.79	97.66	91.38	74.56	84.92
repr.	8.69	8.58	7.22	2.79	9.50	5.30	8.87

Table 3.4: Estimated recovery (*rec.*, in percent) and reproducibility (*repr.*) of SPE.

### 3 Results

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	GC-GC-C	GC-C-C	C-GC-C	Catechin	C-C-C	ProD B3	ProC B3
rec.	79.23	71.26	70.33	91.10	87.87	73.92	82.61
repr.	4.89	3.38	5.04	2.62	2.25	4.05	6.42

Table 3.5: Estimated recovery (*rec.*, in percent) and reproducibility (*repr.*) of the overall method.

#### 3.6.3 Overall Method

Knowing the recovery and reproducibility of the single steps of the analytical procedure, the overall recovery and reproducibility can be calculated (see table 3.5). N.B.: These results are estimated and can only be exactly determined with pure standard substances, that have not been available.

### 3.7 Content of Proanthocyanidins in Barley and Malt

For content of proanthocyanidins of all analyzed barley and malt samples see figure 3.12, detailed data see tables C.9, C.12, C.13, and C.14.

For content of proanthocyanidins of all analyzed barley samples see figure 3.13, detailed data see tables C.10, and C.12.

For content of proanthocyanidins of all analyzed malt samples see figure 3.14, detailed data see tables C.11, C.13, and C.14.

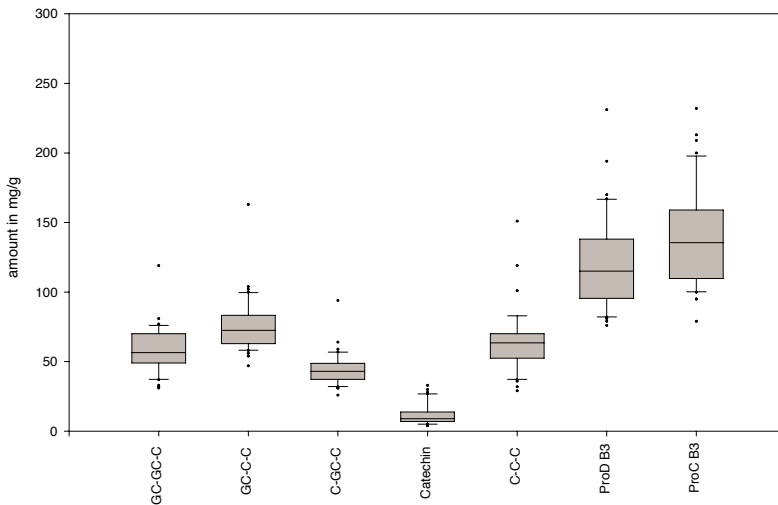


Figure 3.12: Content of proanthocyanidins in mg/kg fresh weight of all analyzed barley and malt samples. Legend: box: 25<sup>th</sup> and 75<sup>th</sup> percentile; horizontal line in the box: median; whiskers: 10<sup>th</sup> and 90<sup>th</sup> percentile; points: extreme values.

### 3 Results

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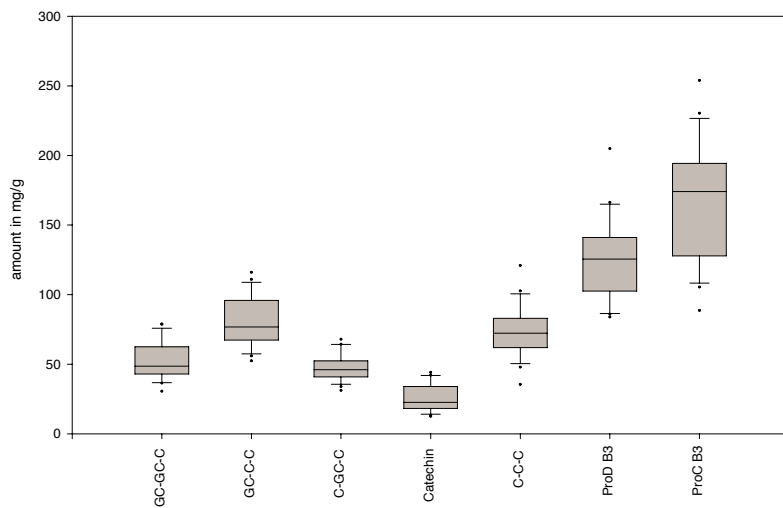


Figure 3.13: Content of proanthocyanidins in mg/kg fresh weight of all analyzed barley samples. Legend: box: 25<sup>th</sup> and 75<sup>th</sup> percentile; horizontal line in the box: median; whiskers: 10<sup>th</sup> and 90<sup>th</sup> percentile; points: extreme values.

### 3.7 Content of Proanthocyanidins in Barley and Malt

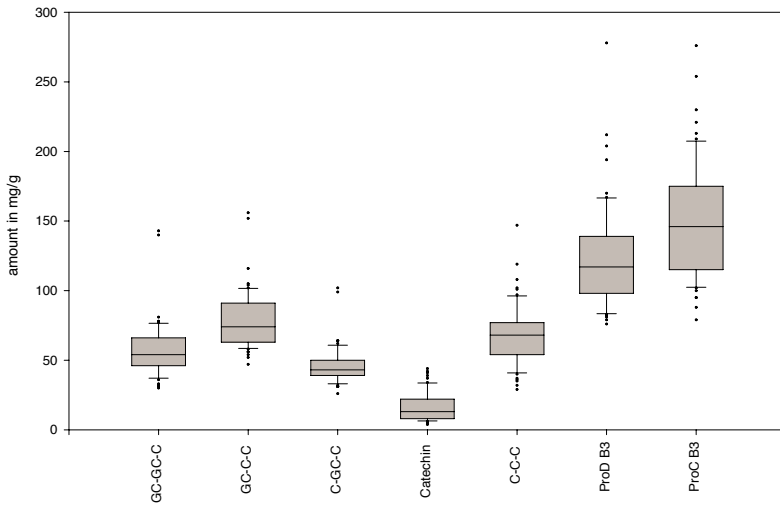


Figure 3.14: Content of proanthocyanidins in mg/kg fresh weight of all analyzed malt samples. Legend: box: 25<sup>th</sup> and 75<sup>th</sup> percentile; horizontal line in the box: median; whiskers: 10<sup>th</sup> and 90<sup>th</sup> percentile; points: extreme values.

### 3.8 Further Applications

The HPLC method developed for SPE eluates of barley and malt extracts (see section 2.2.3) is applied to two other kinds of samples: beer and extracts of the seeds of açai (*Euterpe oleracea* MART.).

#### 3.8.1 Beer

To assay the efficiency of filtration of beer with PVPP 20 hL of beer are filtered in the pilot scale brewery of Beck & Co. (Bremen, Germany) adding a dosage of 10 g/hL of PVPP. The proanthocyanidins are analyzed before and after filtering.

High sensitivity and selectivity of the CEAD allow injecting the beer samples without further sample treatment in the HPLC. The peak areas of the interesting polyphenol signals can be determined and compared in the incoming and the outgoing beer. Filter efficiency can be surveyed in course of filtration (see figure 3.15).

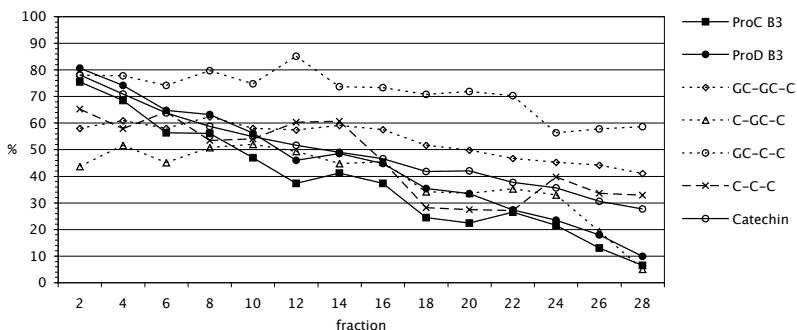


Figure 3.15: Efficiency of beer filtration: percentage of retained polyphenols relative to unfiltered beer (peak names see table 1.1).

At the start of filtration 19–26% of the dimers procyanidin B<sub>3</sub> and prodelphinidin B<sub>3</sub>, and 36–56% of the trimers (beside GC-C-C: 22%) are found in the filtered beer. That means, two fifths of the dimers are retained, while only two third to half of the trimers are adsorbed by the filter.

During filtration the percentage of all proanthocyanidins increases in the filtrate, i.e. filter efficiency decreases; at the end of the filtration the dimers and



C-GC-C are retained by 5-10%, so most of them is still present in the filtered beer. C-C-C and GC-GC-C as well as catechin show adsorption rates of 25-41%. Only GC-C-C has an adsorption rate over 50%.

In short: filter efficiency for the trimers is an average of 61% in the beginning and 34% at the end of filtration. The dimers, the main proanthocyanidins in beer, are well retained (over three fourth) at the start, while in the end virtually nothing is adsorbed (see figure 3.16).

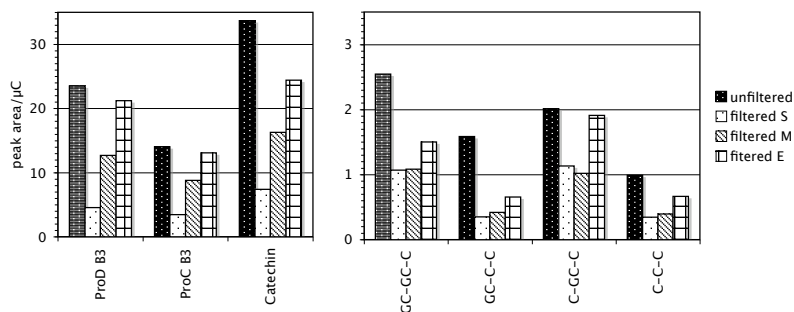


Figure 3.16: Course of beer filtration: peak areas of proanthocyanidins and catechin in the unfiltered beer at the start (S), in the middle (M) and in the end (E) of filtration (peak names see table 1.1.)

### 3.8.2 Açai Seed Extracts

The workgroup of Friedhelm Marx, Roberta Rodrigues and Ramona Lichtenhäler examines various fruits of the amazonian area, among them açai (*Euterpe oleracea* MART.) [72, 73, 74, 113]. For quantification of the polyphenols in açai seeds previously identified by HPLC-MS<sup>n</sup> the methanolic açai seed extracts are separated by the here developed HPLC method with a modified gradient (see section 2.2.3 and table 3.6) and quantified by UV detection.

Quantification of epicatechin is not possible due to interfering with other UV active compounds. However, from the comparatively small peak area of that multi compound peak it can be concluded that the epicatechin content in all extracts is in the lower percent region. The concentrations of the other

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time/min	% B	event
0	0	
8		autosampler inject
10		file start
10	0	
15	8	
35	10	
50	12	
75	35	
90	100	
90		file stop
95	100	
100	0	
105	0	

---

Table 3.6: Modified HPLC gradient for açai seed extracts.

polyphenols in the different seed extracts are summarized in table 3.7, a typical CEAD chromatogram is shown in figure 3.17.

In all seed extracts, small amounts of protocatechuic acid and epicatechin and high amounts of oligomeric proanthocyanidins (dimers up to pentamers) were detected. All of the five quantified oligomers occur in similar concentrations with slightly higher values for the compounds with higher condensation degrees. The differences in the polyphenol content of the four extracts fit together with their ranking of antioxidant activities. Thus the identified proanthocyanidins are thought to be among the decisive antioxidants of açai seeds.

harvest season	white açai high	purple açai high	purple açai low	purple açai high
pca	10.6	13.9	31.5	84.3
C-C	775.2	485.2	420.2	247.4
C-C-C	768.5	471.8	275.7	122.2
C-C-C-C	1002.3	638.3	484.3	144.7
C-C-C-C-C	766.0	476.3	366.9	64.6
C-C-C-C-C	665.3	446.9	342.6	19.7
$\Sigma$	3988.0	2532.0	1921.0	683.0

Table 3.7: Content of proanthocyanidins in açai seed extracts in mg/L of two kinds of açai (white and purple) from high and low harvest seasons. In this case, C stands for catechin or epicatechin (pca = procatechuic acid).

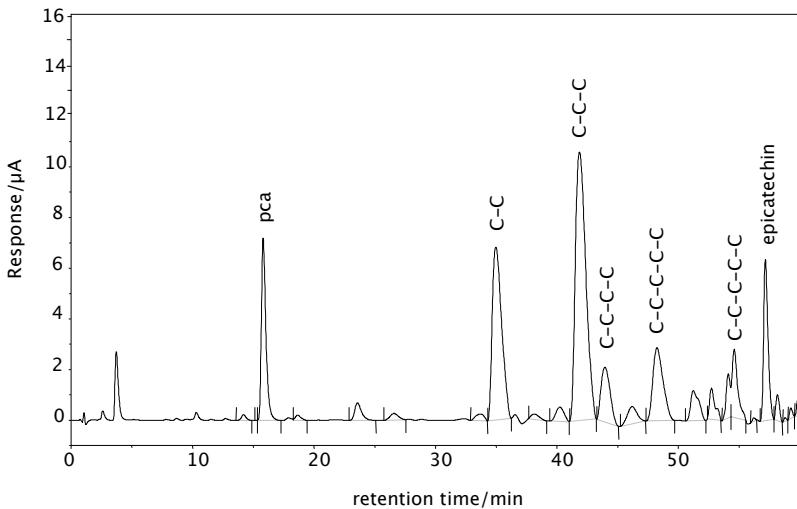


Figure 3.17: Typical açai seed extract CEAD chromatogram. In this case, C stands for catechin or epicatechin (pca = procatechuic acid).



## 4 Discussion

## 4.1 Methods and Technology

### 4.1.1 Milling

Due to better sample penetration at high temperature and high pressure while extracting (see section 1.4.1.1), cooling the milling cells with liquid nitrogen is not required. The resulting barley and malt flour is coarser than the flour obtained with cooling, but there is no significant difference in extraction yields.

### 4.1.2 Extraction

For the first time pressurized liquid extraction is applied to malt and barley to extract proanthocyanidins. Other authors extracted also polyphenols from food: [7, 8] in apple, two proanthocyanidins; [97] in carob products, one proanthocyanidin; [61, 93, 94, 103] in grape skin; [117] in soybeans. There is one very recent paper about extraction of barley [19], but single polyphenols are not determined, so these results are hardly to compare with HPLC results. Furthermore the influence of the extraction solvent mixture is not examined.

There is only one paper comparing different extraction solvent mixtures at various temperatures [97]. The authors conclude for the extraction of carob kibbles: "Extraction time and temperature are of significant influence if a nonoptimal solvent composition is used. If an optimal solvent composition is chosen, however, temperature and time are of marginal influence." This statement contrasts to the results shown in section 3.2.3. There is a slight difference between extraction yield at 60 and 80 °C, but a huge gap to the results of 40 °C, although the optimal extraction solvent mixture is used. Two hypothesis to explain this disagreement: 1. carob kibbles are ground under cooling with liquid nitrogen, so they may be easier to penetrate; 2. carob kibbles don't contain any starch, that depending on temperature, can agglutinate during extraction or be affected by enzymes present in the sample. However similar conditions are found to be optimal: acetone + water, 50 + 50, v + v; 60 °C, 5 min, 2 cycles.

For the extraction of grape skin two temperatures and two extraction solvents (pure water and 60% methanol in water) are assayed [61]. These are not so many variations, but a tendency is observable: the more polar solvent (water) shows better extraction yields at high temperature (120-140 °C), while 40-60 °C are optimal for the methanol/water mixture.

The other mentioned articles [8, 93, 94, 103] don't compare extraction solvent mixtures at different temperatures, although the optimal extraction solvent

mixture for a given temperature is not optimal for another temperature. So their methods must be considered as not readily optimized.

The sample/solvent ratio for manual extraction is usually around 1:10 [58]. The value for this PLE method is about 1:4 (3 g of sample/ca. 12 mL of extract volume), so the analyte concentration in the extract is three times higher than in conventional extracts. This fact has a strong influence on the further sample handling: before SPE no further concentration by solvent evaporation is required, a time consuming step that is hardly to automatize.

#### 4.1.3 Solid-Phase Extraction

For the first time SPE of proanthocyanidins is carried out with commercially available disposable cartridges. The key step to couple PLE and SPE is to dilute the extracts by water to diminish the organic portion. Otherwise the analytes would break through. The classical way to diminish the organic portion by evaporation would be hardly to automatize.

The elution of the analytes from the SPE cartridge is optimized by testing solvent mixtures of DMF and acetone respectively with water. DMF+water, 85+15, v+v turns out to have the highest elution power, i.e. the volume needed for quantitative elution is minimal compared to other solvent mixtures.

Others use self-packed polyamide columns and other eluents, requiring a greater volume for elution: 100 mL methanol and 100 mL DMF [115], 120 mL methanol and 40 mL DMF [36, 37], 50 mL acetone+water, 75+25, v+v [40, 90] or 14 mL methanol and 20 mL acetone+water, 7+3 [127]. Thus the solvents has to be evaporated to reduce volume for further analysis. Using DMF+water, 85+15, v+v instead, 2.5 mL are sufficient for elution. The volume of the eluate is about 15-60 times smaller, consequently concentration of analytes is higher. The eluate can be injected in the HPLC immediately.

#### 4.1.4 HPLC

Based on a former method [36, 37] the HPLC method is enhanced. The smaller particle size (3  $\mu\text{m}$  instead of 4  $\mu\text{m}$ ) and the special kind of column (*Aqua* with polar endcapping) allows separating the six interesting proanthocyanidins and catechin in one single run of 85 min, instead of three runs of 71 min as in the former method.

A significant faster separation seems to be possible only by other techniques than classical HPLC as HPLC on monolithic columns [25, 26] or electrophoresis [27, 67]. Up to now these methods are not applied to beer-related samples.

#### 4.1.5 Method Validation

Method validation is Achilles' heel in analysis of proanthocyanidins. The lack of standards impedes application of usual procedures for method validation, because they all base on the ratio between known ideal values and measured actual values.

##### Reproducibility

In this case reproducibility can be determined by analyzing a well homogenized barley or malt flour several times and calculating standard deviation. This equals reproducibility of the overall method, hence including all steps of the analytical methodology: extraction, clean-up and HPLC.

Considering the multi-step sample preparation, values for reproducibility of 2,3% (C-C-C) to 6,4% (procyanidin B<sub>3</sub>) are very low. In literature only few statistical data can be found. Average reproducibility of 8% [45] and 17% [136], but no values for single compounds are given in two papers. Two other authors from our workgroup present detailed data, another author gives results for catechin only (see table 4.1, where these values are also compared to those of this work).

	Roeder [114]	Friedrich [36]	Wollersen [138]	here
GC-GC-C	40.0	4.9	n. d.	4.9
GC-C-C	17.9	5.2	n. d.	3.4
C-GC-C	19.5	5.2	n. d.	5.0
C-C-C	23.0	5.1	n. d.	2.3
Catechin	n. d.	5.6	7.1	2.6
ProD B <sub>3</sub>	12.0	3.8	n. d.	4.1
ProC B <sub>3</sub>	11.0	4.3	n. d.	6.4

Table 4.1: Comparison of reproducibility of the determination of proanthocyanidins and catechin in three earlier publications and this work.

The here presented method shows an excellent reproducibility, in most case even better than in the previous work of [36]. This is due to the automatization of all analytical steps besides the filling of the extraction cells. Hence reproducibility is virtually independent of the personnel deployed, while the very good values of reproducibility of [36] can only be reached by an experienced analyzer.



### Recovery

For a precise determination of recovery, a reference sample with a known content of the analytes is needed. If such a sample is not available, a sample spiked with pure reference substances can be used instead. If neither reference samples nor reference compounds are available, the following way can be chosen:

When extracting the same sample four times with the optimized extraction method, the analytes can be found quantitatively in the extract, supposing that there is no degradation during extraction. The peak area of every analyte found in normal (i.e. non repetitive) extracts can then be calculated as a percentage the total peak area in the repetitive extract. This percentage corresponds to the recovery of extraction.

A similar approach allows estimating the recovery of SPE: Taking advantage of the high sensitivity of the CEAD the peak areas before and after SPE can be compared and thus the recovery calculated. Precondition is the absence of interfering peaks in the crude extract before SPE, facilitated by the high selectivity of the CEAD. Recovery of the overall method can then be calculated from the values of the single steps.

In literature only few statistical data about recovery of proanthocyanidins can be found. After extraction and purification on LH-20 [48] find over 80% of the proanthocyanidins, while [16] find 85% after SPE on polyamide with a colorimetric assay. More specific values of [36, 114] and [138] are compared in table 4.2 with the herein published values.

	Roeder [114]	Friedrich [36]	Wollersen [138]	here
GC-GC-C	46.0	n. d.	n. d.	79.2
GC-C-C	49.5	n. d.	n. d.	71.3
C-GC-C	55.4	n. d.	n. d.	70.3
C-C-C	28.0	n. d.	n. d.	87.9
Catechin	n. d.	87.0	90.1	91.1
ProD B3	59.6	84.1	n. d.	73.9
ProC B3	64.7	n. d.	n. d.	82.6

Table 4.2: Comparison of recovery of the determination of proanthocyanidins and catechin in three earlier publications and this work.

The estimated recovery of the here presented method is as high as for other published values for catechin and prodelphinidin B<sub>3</sub>. For the other analytes there is no published data, except for the very low values of [114]. However the here presented recoveries are in an acceptable range.

#### 4.1.6 Time and Labor

Aim of this work is to present a procedure for the determination of proanthocyanidins, which requires less time and work than previous published methods. Normally no comments are made on the time needed for the whole analytical process. But from the description in the materials and method-part of the papers can be deduced, how much work is to be conducted. The main steps, that require much manual work and so a lot of time are:

solid/liquid extraction	agitating samples in solvent filtering or decanting the extract concentrating the extract (e.g. using a rotary evaporator)
clean-up	manual liquid/liquid extraction column chromatography self packing of columns concentrating the solution (e.g. using a rotary evaporator)

All these steps are avoided in the here presented method. Partly they are obsolete due to new techniques as the PLE, partly due to enhancement of established methods as SPE. The remaining necessary steps to fulfill are automatized, so manual labor is minimized.

So up to 16 samples can be analyzed within 24 hours. About 6 hours of manual work for milling of the samples, filling the extraction cells, feeding the apparatus with solvents and SPE cartridges and transferring the eluates to the HPLC-autosampler are needed. This is rather simple work, thus even unexperienced personal can reach the given values for reproducibility without a training period. Programming of the ASE and ASPEC software instead, requires well instructed coworkers.

## 4.2 Proanthocyanidins in Barley and Malt

### 4.2.1 Absolute Contents

Here collected data is rather complex: five parameters (barley/malt, summer/winter barley, varieties, proveniences, growing years) and seven depending variables (six proanthocyanidins and catechin). This results in a multi-dimensional coordinate system.

The principle component analysis (PCA) reduces this system to a smaller number of dimensions. Variables that correlate are combined to new factors, variables that don't contribute to the differentiation of samples are excluded. A reduction to two factors leads to easily interpretable diagrams (scatterplots). Groups of samples that are distinguishable show up as discrete accumulations in the diagram.

Here, catechin and GC-GC-C can be excluded. The remaining variables can be combined to two factors. The resulting scatterplots can be found at section C.6.

Differentiation of malt and barley of winter and summer varieties is reported by [36]. 15 summer and 5 winter barley varieties of two proveniences each (one common) of the year 1998 are examined. Only one more paper with results of more than one growing year can be found in literature [60]. It is shown that the percentage of different flavanoids varies depending rather on the variety than on the year. All other articles found dealing with differences of flavanoids in barley and malt present data from only one year and one provenience [77,80,84]. In all cases differences between cultivar are found.

The here presented data give the widest overview. The findings of [36], that the subgroups of summer barley, winter barley, malt of summer barley and malt of winter barley can be distinguished, are partly confirmed: the malt of summer barley samples stand close together in the scatterplot (see figure 4.1), the winter barley samples too (with one outlier), whereas the two other groups (summer barley and malt of winter barley) spread over a wide area. So the tendency shown by [36] is still visible, but the annual variation disturbs the formation of the same clear subgroups.

Concerning the other parameters (variety, year, provenience) no subgroups of the analyzed barley and malt samples emerge (see scatterplots in section C.6). Thus it has to be deducted, that annual differences are greater than differences caused by the other factors.

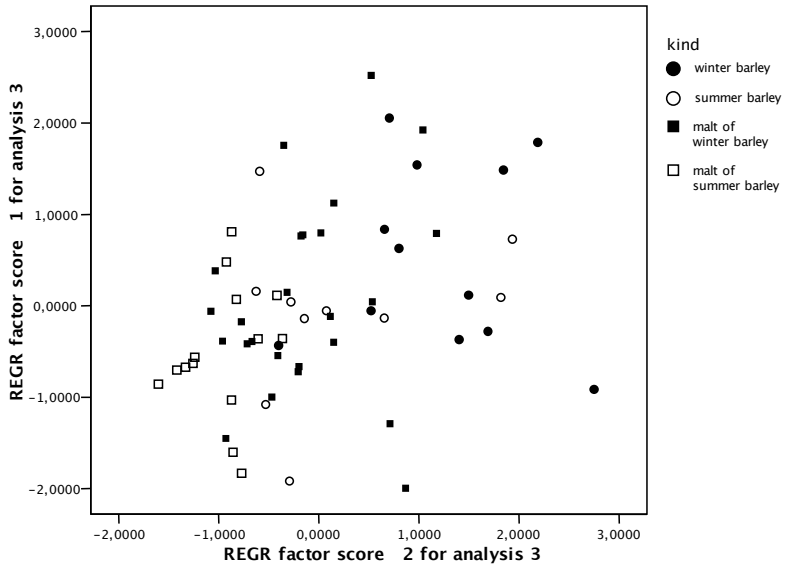


Figure 4.1: Scatterplot summer barley/winter barley/malt of summer barley/  
malt of winter barley

### 4.2.2 Ratio of Proanthocyanidins

The absolute content of polyphenols in plants can differ widely even within the same species. Formation of polyphenols is induced by UV rays [55,126]: the higher the radiation, the higher the content of polyphenols. Biosynthesis of polyphenols has one precursor for all other produced polyphenols [52]. Synthesis of the subsequently formed polyphenols is also enzyme controlled, so the kind of produced compounds is genetically determined and their ratio is presumably constant. So every plant has its characteristic polyphenolic fingerprint. This fingerprint allows proofing the authenticity of food of plant origin [31].

Often different plant species can be distinguished by their *qualitative* polyphenolic fingerprint, i.e. the occurrence or lack of single polyphenols. In some cases certain polyphenols occur in a species at a distinct level, so the species can be identified by their *absolute quantitative* polyphenolic fingerprint, i.e. one can define an upper or lower limit for a polyphenol. On the other hand, the differentiation between cultivars of the same species is – in some cases – possible by the *relative quantitative* polyphenolic fingerprint, i.e. the ratio of one polyphenol to another.

This has been shown by the working group of Galensa for the first time: the variety and the provenience of orange juice [21,22,53] can be determined by the relative quantitative polyphenolic fingerprint, and also the ratio of mixed juices and an adulteration by pulp-wash; likewise the origin of marjoram (*Origanum majorana* L.) and peppers (*Capsicum annum* L.) [31,102] can be determined by the relative quantitative polyphenolic fingerprint. Furthermore the differentiation of Darjeeling and non-Darjeeling tea is possible [137].

The consideration of peak ratios instead of absolute amounts of a compound facilitates the methodology of analysis: systematic errors don't influence the results; standard compounds for calibration – for polyphenols rarely available – are superfluous; and in this special case it is supposed, that instead of UV detection one can use CEAD and therefore SPE is obsolete.

For the here analyzed barleys and malts the ratio of all possible pairs of analytes are calculated. When comparing the means of the ratios of all varieties (both barleys and malts of all years and proveniences) by means of one-way ANOVA (analysis of variance) 14 peak ratios show significant differences for groups of varieties or single varieties (significance level  $\alpha=0.5$ ), see figure 4.2, detailed data see tables C.15 and C.17/C.18. Considering only malt samples (of all years and proveniences) 15 peak ratios show significant differences between the varieties, see tables C.16 and C.19/C.20.

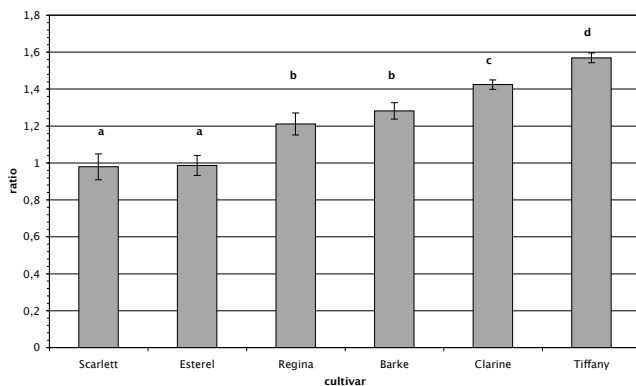


Figure 4.2: Ratio procyanidin B<sub>3</sub>/prodelphinidin B<sub>3</sub>: mean and standard deviation; significantly different ratios are labeled with different letters.

That means, that one can identify the variety of malt by analyzing the proanthocyanidins and catechin, because the peak ratios are characteristic for the variety, independent of growing year and provenience, at least for the six varieties grown in Germany as shown here. As proven for orange juice [21, 22] it is supposed, that the percentage of varieties in malt blends can be calculated. The database has to be expanded, if one wants to check further varieties and samples from other places.

## 5 Conclusion

### **Methodology**

The here presented method for the analysis of proanthocyanidins has been proven to be suitable for barley and malt: recovery is acceptable and due to the high degree of automatization and the use of pre-packed SPE cartridges values for reproducibility are below the values given in other publications. Time and labor consumption is lower than for other published methods. In short: it is a fast and reliable method.

### **Application in brewery**

This method is a tool to examine raw materials even under the conditions of production in the brewery. It is applicable for monitoring under routine conditions. Furthermore the method is thought to be able to analyze other samples taken during the brewing process to survey the content of proanthocyanidins at various steps of production. As an example the method is applied to control the filtering process.

### **Analyzed samples**

The analysis of a large number of barley and malt samples gives an overview over the range of proanthocyanidins in the most important raw material for brewing. Annual, local and varietal differences interfere, so that the content of proanthocyanidins in a given sample is not predictable. This underlines the necessity to analyze every badge of malt. The ratio of several pairs of proanthocyanidins (the relative quantitative polyphenolic fingerprint) is characteristic for the variety and can be used to control authenticity.

### **Applications outside the brewhouse**

Proanthocyanidins are discussed to have positive effects on health. There is a market for functional food with naturally high or enriched content of proanthocyanidins. These products has to be examined, i.e. the claimed content has to be checked. The here presented method is supposed to be applicable to various food samples as proven for açai seed extracts.



## Appendix



## **Appendix A**

### **Abbreviations**

For peak names refer to table 1.1.

**ANOVA** analysis of variance

**ASE** accelerated solvent extractor

**ASPEC** automated sample preparation with extraction cartridges

**C** catechin

**CEAD** coulometric electrode array detection

**DMF** N,N-dimethylformamide

**EBC** European Brewing Convention

**GC** galocatechin

**HPLC** high-performance liquid chromatography

**i. d.** inside diameter

**ID** identification number

**ISTD** internal standard

**MALDI-TOF-MS** matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

**MEBAK** Mitteleuropäische Brautechnische Analysenkommission e. V.

**MEKC** micellar electrokinetic chromatography

**MS** mass spectrometry

**n. d.** not determined

## *Appendix A Abbreviations*

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<b>NP</b>	normal phase
<b>PA</b>	polyamide
<b>PCA</b>	protocatechuic acid
<b>Ph</b>	phloroglucin
<b>PLE</b>	pressurized liquid extraction
<b>PVPP</b>	polyvinylpolypyrrolidone
<b>ProC</b>	procyanidin
<b>ProD</b>	prodelphinidin
<b>rec</b>	recovery
<b>repr</b>	reproducibility
<b>RP</b>	reversed phase
<b>SHG</b>	silica hydrogel
<b>SPE</b>	solid-phase extraction
<b>TRAP</b>	total reactive antioxidant potential
<b>UK</b>	United Kingdom
<b>US</b>	United States of America
<b>UV</b>	ultraviolet
<b>v</b>	volume

## **Appendix B**

### **Detailed ASPEC Settings**

## Appendix B Detailed ASPEC Settings

---

```
Condition
Solvent:
- Select = From_Reservoir
- Volume = 8 ml
- Disp Flow Rate = 6 ml/min

AIR PUSH:
- Method = Using_Syringe
- Air Push Volume = 2 ml
- Air Push Disp Flow Rate = 6 ml/min

SOLVENT:
- Extra Volume = 0 mul
- Pressure Equilibration Time = 0.1 min

AIR PUSH:
- Asp Flow Rate = 40 ml/min
- Air Pressure Equilibration Time = 0.1 min

RINSE:
off

AIR GAP:
- Volume = 38 mul
- Flow Rate = 1 ml/min

Wait
Wait For = Contact
Contact Module Name = CONTACT
Contact Number = 1
State = On

Hold On Pause = False

Rinse Needle
SOLVENT:
- Select = From_Reservoir

RINSE:
- Inside Volume = 3000 mul
- Outside Volume = 0 mul

RINSE:
- Inside Flow Rate = 60 ml/min
- Outside Flow Rate = 60 ml/min
- Depth = 80 mm

Ase Dispense
SOURCE:
- From = From_Reservoir

DESTINATION:
- Into = Result
- RESULTS[0] = 1
  RESULTS[1]:
  - Name = ase
  - Volume = 20000 mul

- Disp Flow Rate = 120 ml/min
- Multiple Use = True

RINSE:
off

Equilibration Time = 0 s

Ase Mix
Mixing Zone = ase
Multiple Use = True
Nb Of Cycles = 1
Asp Flow Rate = 40 ml/min
Mode = Liquid
Extra Volume = 0 mul
Aspirating Height = 0 mm
Steps[0] = 1

Steps[1]:
- Volume = 24000 mul
- Disp Flow Rate = 120 ml/min
- Height = 100 mm

AIR GAP:
- Volume = 38 mul
- Flow Rate = 0.3 ml/min

RINSE:
off

Equilibration Time = 0 s

Ase Load
SOURCE:
- Select = From_Tray
- Name = ase
- Volume = 17 ml
- Disp Flow Rate = 3 ml/min
- Asp Flow Rate = 40 ml/min
- Multiple Use = True

AIR PUSH:
- Method = Using_Syringe
- Air Push Volume = 2 ml
- Air Push Disp Flow Rate = 6 ml/min

SOURCE:
- Extra Volume = 0 mul
- Pressure Equilibration Time = 0.5 min

AIR PUSH:
- Asp Flow Rate = 6 ml/min
- Air Pressure Equilibration Time = 0.5 min

RINSE:
off
```

---

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

Ase Load

SOURCE:  
- Select = From\_Tray  
- Name = ase  
- Volume = 17 ml  
- Disp Flow Rate = 3 ml/min  
- Asp Flow Rate = 40 ml/min  
- Multiple Use = True

AIR PUSH:

- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 6 ml/min

SOURCE:

- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.5 min

AIR PUSH:

- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.5 min

RINSE:

off

AIR GAP:

- Volume = 38 µl  
- Flow Rate = 1 ml/min

Add To Dec

ADDED SOLUTION:  
- Select = From\_Reservoir  
- Volume = 4 ml  
- Disp Flow Rate = 3 ml/min

AIR PUSH:

- Method = Using\_Syringe  
- Air Push Volume = 3 ml  
- Air Push Disp Flow Rate = 2 ml/min

ADDED SOLUTION:

- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:

- Asp Flow Rate = 30 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:

off

AIR GAP:

- Volume = 38 µl  
- Flow Rate = 1 ml/min

Ase Load

SOURCE:  
- Select = From\_Tray  
- Name = ase  
- Volume = 10 ml  
- Disp Flow Rate = 2 ml/min  
- Asp Flow Rate = 30 ml/min  
- Multiple Use = True

AIR PUSH:

- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

SOURCE:

- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.5 min

AIR PUSH:

- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.5 min

RINSE:

off

AIR GAP:

- Volume = 38 µl  
- Flow Rate = 1 ml/min

Wash

Solvent:

- Select = From\_Reservoir  
- Volume = 4 ml  
- Disp Flow Rate = 2 ml/min

AIR PUSH:

- Method = Using\_Syringe  
- Air Push Volume = 3 ml  
- Air Push Disp Flow Rate = 2 ml/min

SOLVENT:

- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:

- Asp Flow Rate = 20 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:

off

AIR GAP:

- Volume = 38 µl  
- Flow Rate = 1 ml/min

## Appendix B Detailed ASPEC Settings

---

Wash  
Solvent:  
- Select = From\_Reservoir  
- Volume = 4 ml  
- Disp Flow Rate = 2 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 3 ml  
- Air Push Disp Flow Rate = 2 ml/min

SOLVENT:  
- Extra Volume = 0 ml  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 20 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 ml  
- Flow Rate = 1 ml/min

Rinse Needle  
SOLVENT:  
- Select = From\_Reservoir

RINSE:  
- Inside Volume = 2000 ml  
- Outside Volume = 3000 ml

RINSE:  
- Inside Flow Rate = 60 ml/min  
- Outside Flow Rate = 60 ml/min  
- Depth = 80 mm

Wash  
Solvent:  
- Select = From\_Tray  
- Name = LM1  
- Volume = 1 ml  
- Disp Flow Rate = 2 ml/min  
- Asp Flow Rate = 3 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

SOLVENT:  
- Extra Volume = 50 ml  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min

- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 ml  
- Flow Rate = 1 ml/min

Wash  
Solvent:  
- Select = From\_Reservoir  
- Volume = 0.001 ml  
- Disp Flow Rate = 6 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

SOLVENT:  
- Extra Volume = 0 ml  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
- Inside Volume = 500 ml  
- Inside Flow Rate = 60 ml/min  
- Outside Volume = 1000 ml  
- Outside Flow Rate = 60 ml/min  
- Depth = 80 mm

AIR GAP:  
- Volume = 38 ml  
- Flow Rate = 1 ml/min

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Tray  
- Name = LM1  
- Volume = 1 ml  
- Disp Flow Rate = 1.5 ml/min  
- Asp Flow Rate = 8 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = False



---

SOLVENT:  
- Extra Volume = 50 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Reservoir  
- Volume = 0.001 ml  
- Disp Flow Rate = 3 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = False

SOLVENT:  
- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Reservoir  
- Volume = 0.001 ml  
- Disp Flow Rate = 3 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = False

SOLVENT:  
- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

Set Electrical Contact  
Contact Number = 4  
State = PULSE  
Pulse Duration = 1.8 s

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Tray  
- Name = LM1  
- Volume = 1.5 ml  
- Disp Flow Rate = 1.5 ml/min  
- Asp Flow Rate = 8 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = False

SOLVENT:  
- Extra Volume = 50 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

## Appendix B Detailed ASPEC Settings

---

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Reservoir  
- Volume = 0.001 ml  
- Disp Flow Rate = 3 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = False

SOLVENT:  
- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Reservoir  
- Volume = 0.001 ml  
- Disp Flow Rate = 3 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = False

SOLVENT:  
- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Reservoir  
- Volume = 0.001 ml  
- Disp Flow Rate = 3 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = False

SOLVENT:  
- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

Elute  
Collect Name = Elution

Solvent:  
- Select = From\_Reservoir  
- Volume = 0.001 ml  
- Disp Flow Rate = 3 ml/min

AIR PUSH:  
- Method = Using\_Syringe  
- Air Push Volume = 2 ml  
- Air Push Disp Flow Rate = 1.5 ml/min

Reset Mobile Rack = True

SOLVENT:  
- Extra Volume = 0 µl  
- Pressure Equilibration Time = 0.1 min

AIR PUSH:  
- Asp Flow Rate = 6 ml/min  
- Air Pressure Equilibration Time = 0.1 min

RINSE:  
off

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

---

Dispense  
SOURCE:  
- From = From\_Tray  
- Name = ISTD E  
- Multiple Use = True  
- Extra Volume = 50 µl  
- Flow Rate = 3 ml/min  
AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

- Depth = 80 mm  
Equilibration Time = 0 s

DESTINATION:  
- Into = Result  
- RESULTS[0] = 1  
RESULTS[1]:  
- Name = Elution  
- Volume = 300 µl  
- Disp Flow Rate = 3 ml/min

RINSE:  
- Inside Volume = 1500 µl  
- Inside Flow Rate = 60 ml/min  
- Outside Volume = 2000 µl  
- Outside Flow Rate = 60 ml/min  
- Depth = 80 mm

Equilibration Time = 0 s

Mix And Transfer  
MIX:  
- Zone Name = Elution  
- Nb Of Cycles = 1  
- Asp Flow Rate = 6 ml/min  
- Mode = Liquid  
- Extra Volume = 0 µl  
- Aspirating Height = 0 mm  
- Steps[0] = 1  
Steps[1]:  
- Volume = 2000 µl  
- Disp Flow Rate = 180 ml/min  
- Height = 0 mm

TRANSFER:  
- Result Name = ASVials1  
- Volume = 1000 µl

AIR GAP:  
- Volume = 38 µl  
- Flow Rate = 1 ml/min

TRANSFER:  
- Asp Flow Rate = 3 ml/min  
- Disp Flow Rate = 3 ml/min

RINSE:  
- Inside Volume = 2000 µl  
- Inside Flow Rate = 60 ml/min  
- Outside Volume = 6000 µl  
- Outside Flow Rate = 60 ml/min

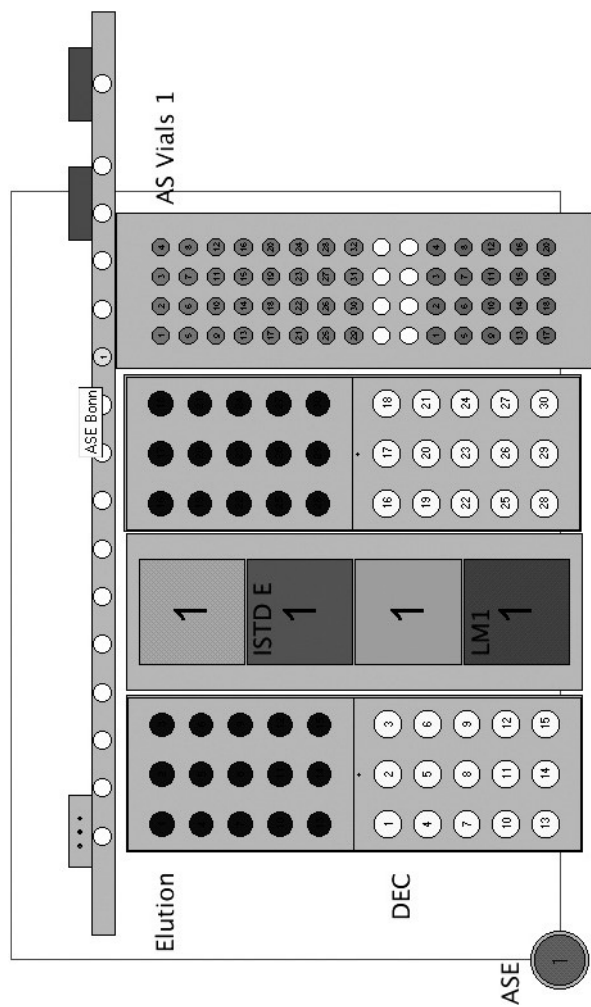


Figure B.1: ASPEC tray with zone names

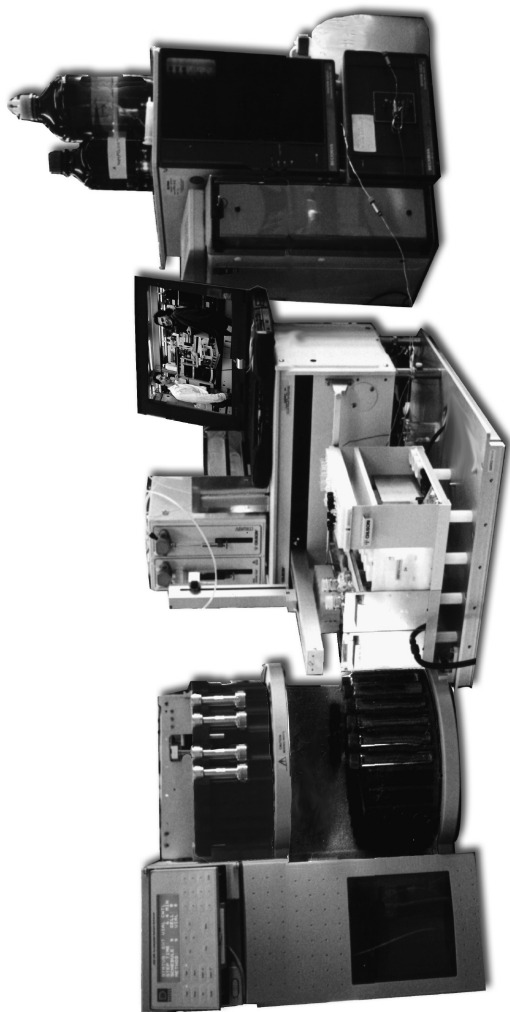


Figure B.2: The hardware setup: coupling of PLE, automated SPE and HPLC (from left to right: ASE 200, ASPEC Xli, HPLC).



## Appendix C

### Detailed Data Sheets

#### C.1 Milling

time	6	±	7	±	8	±	9	10	±
GC-GC-C	12.4	2.4	13.6	1.7	15.4	0.8	15.8	15.4	1.2
GC-C-C	12.5	1.7	13.7	1.0	15.6	0.8	15.5	15.0	1.0
C-GC-C	9.1	1.5	9.5	0.4	10.9	0.6	10.4	10.5	0.7
Catechin	8.4	0.5	8.8	0.1	9.2	0.3	9.1	9.0	1.2
C-C-C	6.2	0.3	6.7	0.0	7.3	0.2	7.2	6.4	0.5
ProD B <sub>3</sub>	35.9	4.3	36.0	0.0	42.7	0.8	42.6	41.2	2.5
ProC B <sub>3</sub>	25.2	0.7	27.7	2.1	29.6	0.9	29.2	29.8	1.6

Table C.1: Milling time: peak area of different analytes in the extracts depending on the milling time of the barley grains. For extraction the optimized method (see table 2.1) was applied. Time in min, peak area in  $\mu\text{C} \pm$  standard deviation.

#### C.2 Extraction

amount	1	±	2	±	3	±	4	±
GC-GC-C	265.3	15.4	502.7	11.5	777.8	83.2	806.3	7.9
GC-C-C	259.8	5.9	562.6	16.9	865.9	8.5	1122.5	5.6
C-GC-C	110.6	0.3	192.9	11.1	313.8	13.1	516.9	3.1
Catechin	21.9	1.5	49.9	5.1	104.2	2.3	117.1	16.8
ProD B <sub>3</sub>	741.6	59.7	1263.7	35.7	2012.4	115.0	2309.5	9.0
ProC B <sub>3</sub>	394.4	37.9	732.8	18.3	1290.5	125.5	1750.4	6.6

Table C.2: Amount of malt: peak area of different analytes in the extracts depending on the extracted amount of malt in g (peak area in arbitrary units  $\pm$  standard deviation).

<b>40°C</b>		30	35	40	45	50	±			
GC-GC-C	nd	nd	nd	nd	62,5	9,1	73,1 11,4			
GC-C-C	nd	nd	nd	nd	81,2	8,2	84,7 5,6			
C-GC-C	nd	nd	nd	nd	48,6	2,6	53,2 2,4			
Catechin	nd	nd	nd	nd	25,2	2,4	26,4 1,8			
C-C-C	nd	nd	nd	nd	35,6	2,9	34,7 2,7			
ProD B3	nd	nd	nd	nd	328,2	22,8	336,0 22,2			
ProC B3	nd	nd	nd	nd	190,0	9,7	198,6 7,7			
<b>60°C</b>										
	30	±	35	±	40	±	45	±	50	±
GC-GC-C	44,5	7,0	62,3	5,3	61,7	5,2	44,3	0,5	29,3	2,6
GC-C-C	60,6	7,2	81,7	6,8	86,2	7,6	80,8	6,5	73,7	6,5
C-GC-C	45,3	4,9	56,2	5,1	55,0	3,6	63,3	6,7	22,4	6,9
Catechin	52,4	5,0	52,1	2,4	68,1	4,2	54,6	5,2	53,4	3,6
C-C-C	54,7	11,3	78,3	6,8	77,3	4,1	78,0	5,6	20,2	4,0
ProD B3	304,7	9,8	363,4	3,6	397,3	8,5	441,1	7,9	378,0	6,0
ProC B3	225,2	4,6	301,1	4,9	309,3	6,4	292,8	7,5	108,4	2,5
<b>80°C</b>										
	30	±	35	±	40	±	45	±	50	±
GC-GC-C	47,0	0,6	65,1	1,0	65,8	3,7	39,8	2,7	nd	nd
GC-C-C	63,0	0,0	83,2	4,9	100,2	5,7	56,7	3,8	nd	nd
C-GC-C	44,8	0,3	58,6	1,7	57,3	3,8	36,4	2,7	nd	nd
Catechin	76,0	4,6	92,6	2,9	102,4	5,8	92,1	0,7	nd	nd
C-C-C	26,8	0,7	32,3	2,5	56,9	3,1	16,4	1,1	nd	nd
ProD B3	329,1	25,3	377,1	26,4	446,8	24,4	252,1	18,2	nd	nd
ProC B3	276,0	20,4	328,3	18,7	343,0	15,3	170,4	13,1	nd	nd

Table C.3: Extraction temperature and solvent: peak area of the analytes in the extracts depending on extraction temperature and solvent (peak area in arbitrary units  $\pm$  standard deviation; solvent: Vol% of acetone in water from 30 to 50%, to be continued on next page).



<b>40 °C</b>		55	±	60	±	70	±	80	±	90	±
GC-GC-C	92,8	3,2	82,6	7,9	75,6	2,7	55,9	4,7	19,3	2,4	
GC-C-C	84,6	5,9	88,4	1,9	73,6	4,9	65,6	4,0	32,5	2,3	
C-GC-C	51,3	3,1	55,9	2,1	44,9	3,4	38,1	1,7	18,4	2,0	
Catechin	22,9	0,4	23,8	1,1	19,1	1,8	15,2	0,4	4,0	0,4	
C-C-C	36,8	0,8	36,3	1,4	33,0	1,2	32,8	1,5	23,6	2,2	
ProD B3	350,5	21,3	378,0	16,3	327,4	17,7	291,1	28,3	179,0	24,3	
ProC B3	202,0	1,6	219,8	2,5	198,3	6,5	191,8	11,3	131,6	15,0	
<b>60 °C</b>		55	±	60	±	70	±	80	±	90	±
GC-GC-C	nd	28,0	2,8	34,2	0,8	45,5	4,1	3,1	3,0		
GC-C-C	nd	41,1	4,2	36,4	0,1	35,3	2,4	5,0	4,2		
C-GC-C	nd	28,5	3,9	25,5	0,1	26,1	5,0	5,7	3,9		
Catechin	nd	28,1	5,0	32,8	0,1	27,9	1,6	25,0	7,1		
C-C-C	nd	12,6	1,6	13,7	0,4	13,1	2,2	7,8	4,4		
ProD B3	nd	204,1	5,3	184,0	3,0	169,3	6,2	47,0	5,6		
ProC B3	nd	128,0	4,1	123,4	2,6	121,1	2,9	86,1	7,4		
<b>80 °C</b>		55	±	60	±	70	±	80	±	90	±
GC-GC-C	32,1	4,9	nd	nd	nd	nd	nd	nd	nd	nd	
GC-C-C	45,3	4,3	nd	nd	nd	nd	nd	nd	nd	nd	
C-GC-C	29,7	2,4	nd	nd	nd	nd	nd	nd	nd	nd	
Catechin	42,4	2,5	nd	nd	nd	nd	nd	nd	nd	nd	
C-C-C	13,7	1,3	nd	nd	nd	nd	nd	nd	nd	nd	
ProD B3	227,9	18,8	nd	nd	nd	nd	nd	nd	nd	nd	
ProC B3	151,4	14,3	nd	nd	nd	nd	nd	nd	nd	nd	

Table C.4: Extraction temperature and solvent (continued): peak area of the analytes in the extracts depending on extraction temperature and solvent (peak area in arbitrary units  $\pm$  standard deviation; solvent: Vol% of acetone in water from 55 to 90%).

	1 x 7	1 x 10	±	1 x 12	±	1 x 14	±	2 x 3	±	2 x 5	±	2 x 7	±
GC-GC-C	68.6	78.7	6.7	75.2	6.5	123.0	6.7	119.6	10.4	108.7	8.3	103.8	5.0
GC-C-C	126.2	140.6	6.8	133.4	11.9	185.7	14.0	160.9	17.9	185.4	2.0	189.4	8.8
C-GC-C	83.7	73.6	5.2	74.5	8.0	130.9	4.5	117.2	2.0	121.0	0.2	121.8	11.2
C	76.6	97.5	8.4	108.3	6.8	98.1	9.6	87.7	2.3	107.6	5.6	120.7	2.3
C-C-C	101.1	109.0	7.2	84.8	7.9	158.6	12.6	144.7	12.2	159.8	3.4	146.8	1.2
ProD B3	454.8	509.8	34.7	592.8	45.8	627.6	43.3	586.2	26.2	591.6	11.7	655.9	14.5
ProC B3	322.8	380.3	10.2	377.7	9.4	429.2	32.1	407.8	17.5	418.8	31.9	440.7	10.0

Table C.5: Extraction time and multiple extraction: peak area of the analytes in the extracts depending on extraction time and multiple extraction steps; "1 x 7" means: 1 extraction cycle of 7 min. Extracted with acetone + water, 45 + 55, v + v, at 80 °C (peak area in arbitrary units ± standard deviation).

### C.3 Hydrodynamic Voltammograms

The voltammograms are normalized at 100 mV. All voltammograms are recorded in a malt sample and not in standard solutions, except for the last one (catechin).

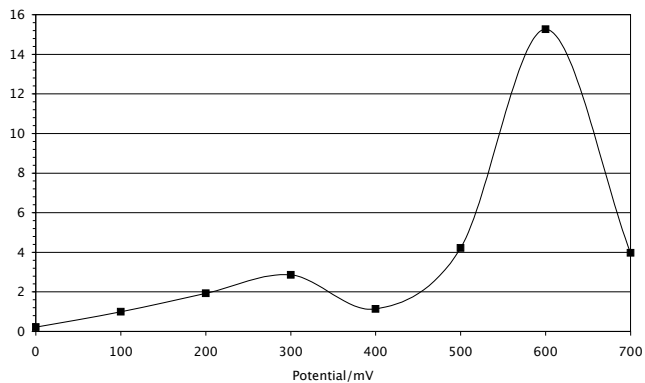


Figure C.1: Hydrodynamic voltammogram of procyanidin B<sub>3</sub>.

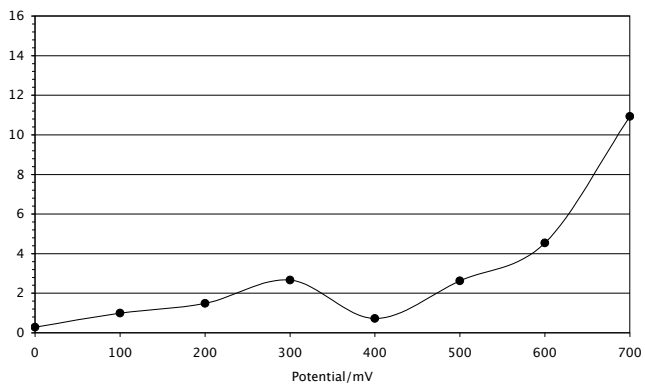


Figure C.2: Hydrodynamic voltammogram of prodelphinidin B3.

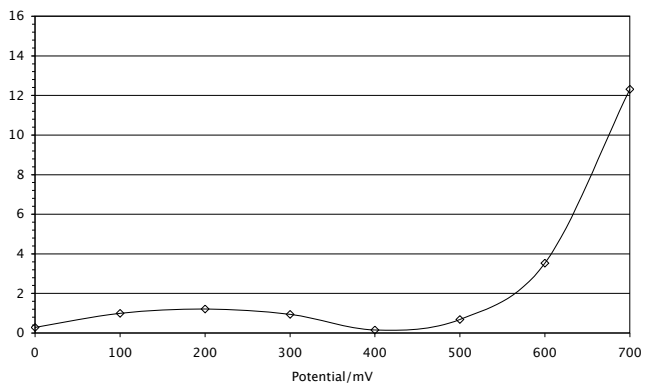


Figure C.3: Hydrodynamic voltammogram of GC-GC-C.

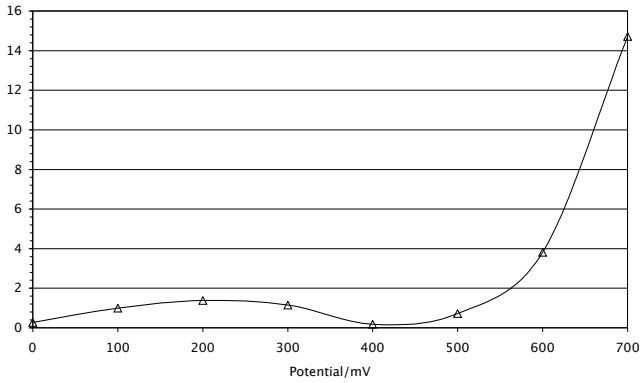


Figure C.4: Hydrodynamic voltammogram of GC-C-C.

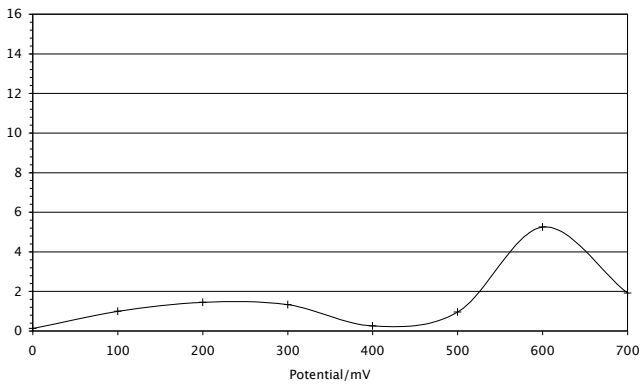


Figure C.5: Hydrodynamic voltammogram of C-GC-C.

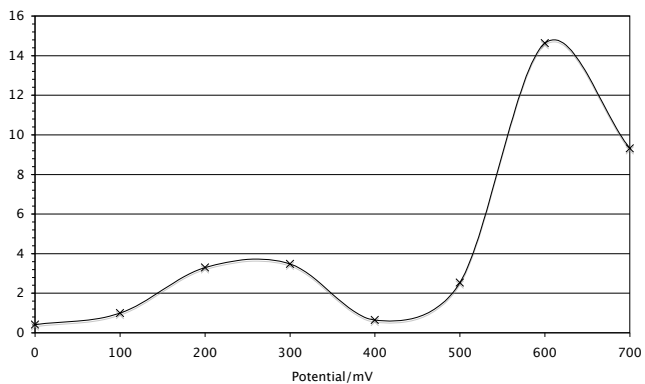


Figure C.6: Hydrodynamic voltammogram of C-C-C.

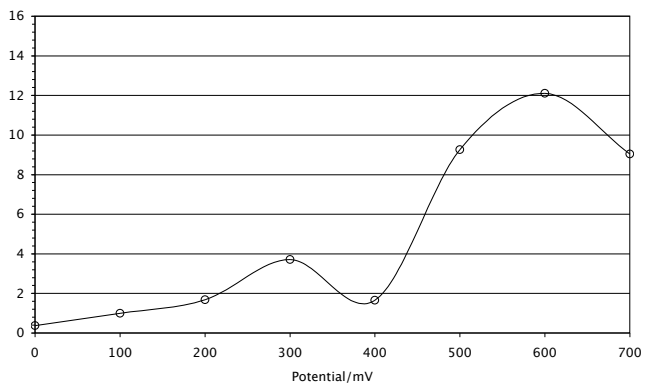


Figure C.7: Hydrodynamic voltammogram of catechin.

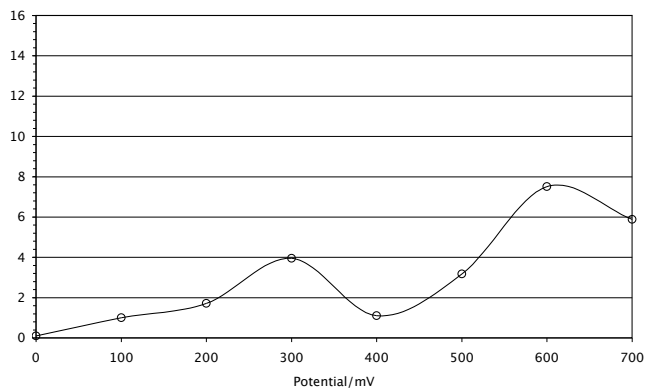


Figure C.8: Hydrodynamic voltammogram of a catechin standard.

### C.4 MS Data

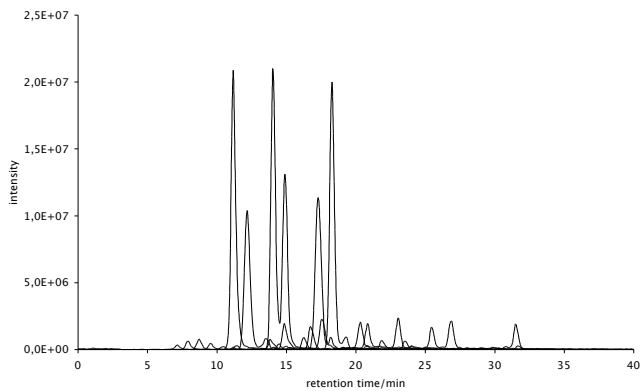


Figure C.9: Overlaid mass traces of proanthocyanidins.



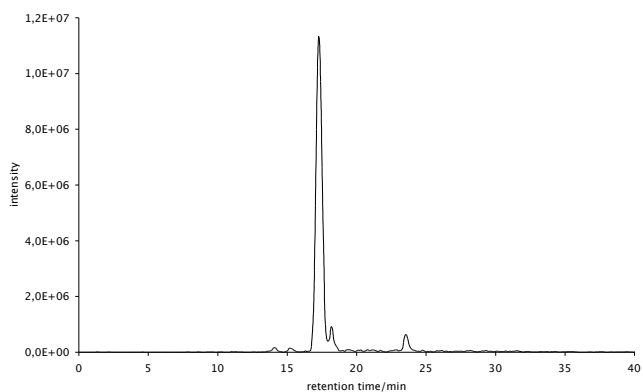


Figure C.10: Mass trace of a proanthocyanidin with  $m/s = 577$

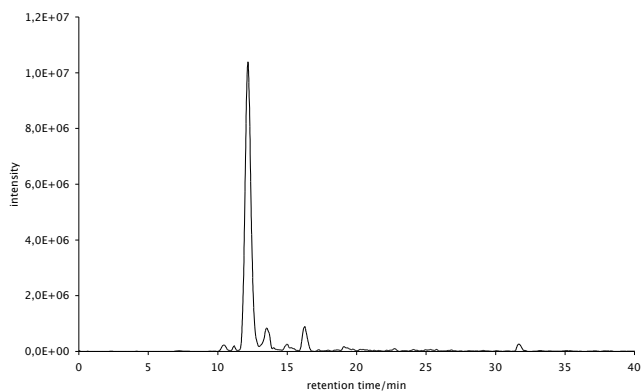


Figure C.11: Mass trace of a proanthocyanidin with  $m/s = 593$

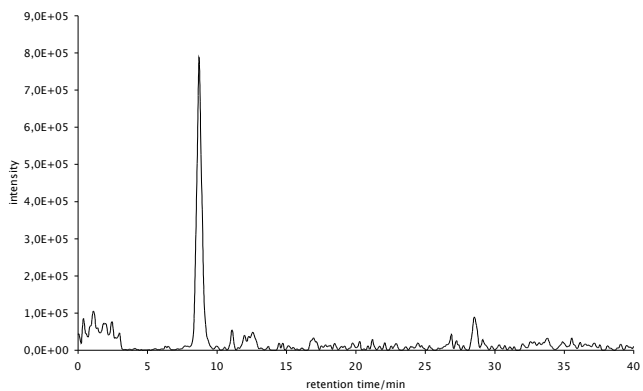


Figure C.12: Mass trace of a proanthocyanidin with  $m/s = 609$

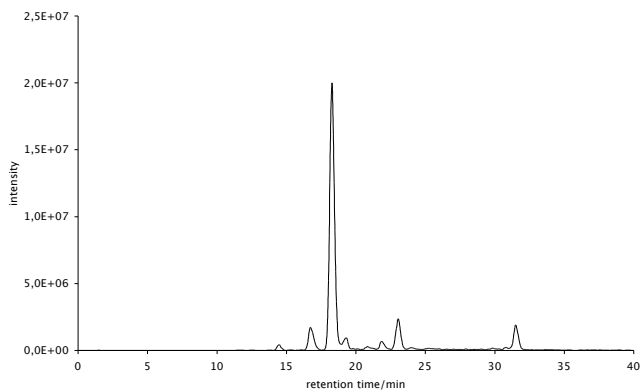


Figure C.13: Mass trace of a proanthocyanidin with  $m/s = 865$

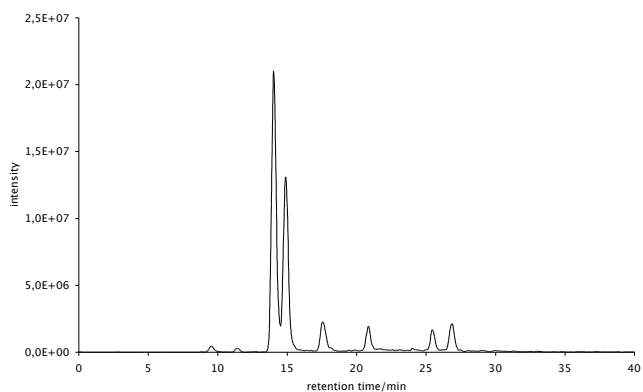


Figure C.14: Mass trace of a proanthocyanidin with  $m/s = 881$

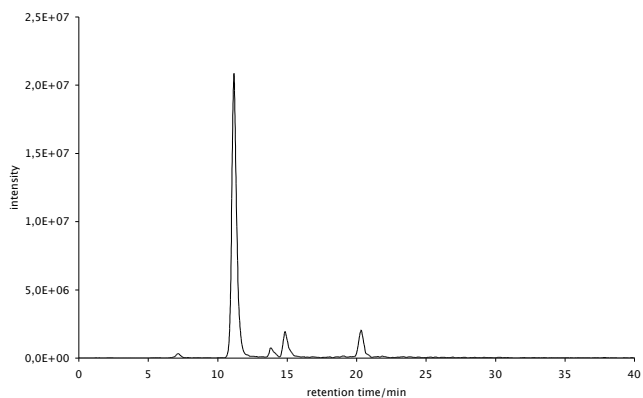


Figure C.15: Mass trace of a proanthocyanidin with  $m/s = 897$

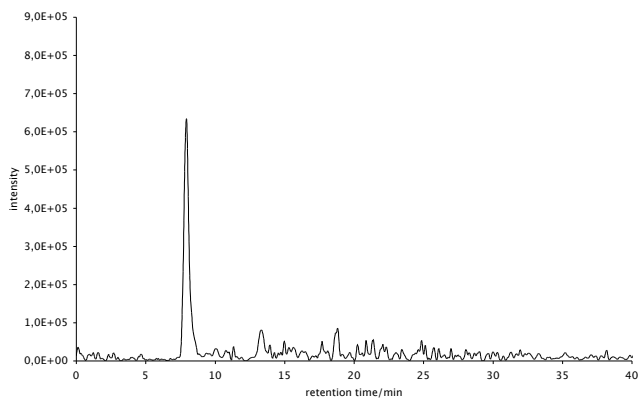


Figure C.16: Mass trace of a proanthocyanidin with  $m/s = 913$

	M-H	M-H <sub>2</sub> O-H	R <sub>T</sub>	R <sub>T</sub> -H <sub>2</sub> O	M-M <sub>B</sub> -3H	M-M <sub>T</sub> -H	R <sub>M</sub>	M <sub>T</sub> -3H	M <sub>B</sub> -H	M <sub>T</sub> -Ph	other
GC-GC-C	<b>897</b>										
MS <sup>2</sup> of 897	879	729	<b>711</b>	607	593	729	303	289	289	771	
MS <sup>3</sup> of 593						425		289			
GC-C-C	<b>881</b>										
MS <sup>2</sup> of 881	863	729	<b>695</b>	591	577	729	303	289	289	755	
MS <sup>3</sup> of 577	559					425		289		<b>451</b>	
C-GC-C	<b>881</b>										
MS <sup>2</sup> of 881	863	729	<b>407</b>	591	593	713	287	289	289		
MS <sup>3</sup> of 593								289			
C-C-C	<b>865</b>										
MS <sup>2</sup> of 865	847	713		575	577	713	287	289	289	<b>695</b>	
MS <sup>3</sup> of 577								289		<b>451</b>	
Catechin	<b>289</b>										
MS <sup>2</sup> of 289											<b>245</b>
ProD B3	<b>593</b>										
MS <sup>2</sup> of 593	575	<b>425</b>	407				303	289	289	467	
ProC B3	<b>577</b>										
MS <sup>2</sup> of 577	559	<b>425</b>	407				287	289	289	451	

Table C.6: Mass spectrometric data of the quantified proanthocyanidins and catechin. The most abundant ion is in bold face. (M=pseudomolecular ion, R=retro Diels-Alder product, T = top unit, M = middle unit, B=base unit, Ph = phloroglucin)

GC-GC	M-H	M-H <sub>2</sub> O-H	R <sub>T</sub>	R <sub>T</sub> -H <sub>2</sub> O	M-M <sub>B</sub> -3H	M-M <sub>T</sub> -H	R <sub>M</sub>	M <sub>T</sub> -3H	M <sub>B</sub> -H	M <sub>T</sub> -Ph
MS <sup>2</sup> of 609	<b>609</b>	591	441	<b>423</b>				303	305	483
GC-GC-GC	<b>913</b>									
MS <sup>2</sup> of 913		895		<b>727</b>						787
MS <sup>3</sup> of 609				<b>423</b>	591					
C-GC	<b>593</b>									
MS <sup>2</sup> of 593		575		<b>423</b>				287	305	

Table C.7: Mass spectrometric data of minor proanthocyanidins. The most abundant ion is in bold face. (M = pseudomolecular ion, R = retro Diels-Alder product, T = top unit, M = middle unit, B = base unit, Ph = phloroglucin)

mass	composition	number
1201	(GC) <sub>3</sub> C	1
1185	(GC) <sub>2</sub> C <sub>2</sub>	2
1169	(GC) <sub>2</sub> C <sub>3</sub>	3
1153	C <sub>4</sub>	1

Table C.8: Mass spectrometric data minor tetrameric proanthocyanidins. The order of the monomers is not known.

**C.5 Content of Proanthocyanidins**

	Mean	Max	Percentiles					
			90th	75th	Median	25th	10th	min
GC-GC-C	55.3	81.7	71.6	65.8	54.4	46.3	37.6	30.6
GC-C-C	75.8	116.1	100.1	82.4	73.2	63.4	59.4	47.2
C-GC-C	44.7	64.8	57.0	50.1	43.4	39.2	33.9	26.1
Catechin	17.2	44.1	33.4	22.6	13.4	8.5	7.3	4.1
C-C-C	66.2	119.4	83.7	75.9	67.8	54.3	42.3	29.6
ProD B <sub>3</sub>	120.1	205.0	160.6	134.8	115.6	98.9	84.0	76.7
ProC B <sub>3</sub>	146.1	230.4	195.1	173.3	145.3	115.3	103.6	79.5

Table C.9: Content of determined proanthocyanidins in mg/kg fresh weight of all analyzed barley and malt samples (overview).

	Mean	Max	Percentiles					
			90th	75th	Median	25th	10th	min
GC-GC-C	50.3	78.9	70.2	59.0	48.4	42.5	36.6	20.8
GC-C-C	76.0	116.1	101.4	86.8	73.2	66.4	56.7	28.3
C-GC-C	45.7	64.4	61.3	50.9	45.7	40.5	34.8	15.1
Catechin	25.5	44.1	41.2	33.0	22.4	17.2	15.5	12.6
C-C-C	70.5	102.7	94.5	80.7	70.3	60.5	49.1	28.9
ProD B <sub>3</sub>	118.8	205.0	145.6	132.3	121.0	100.7	86.2	42.7
ProC B <sub>3</sub>	158.1	230.4	203.6	193.2	169.1	121.7	106.9	49.8

Table C.10: Content of determined proanthocyanidins in mg/kg fresh weight of all analyzed barley samples (overview).

*Appendix C Detailed Data Sheets*

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	Mean	Max	Percentiles					
			90th	75th	Median	25th	10th	min
GC-GC-C	56.5	81.7	72.1	67.8	56.8	49.5	37.2	26.0
GC-C-C	73.3	104.3	94.2	81.5	71.4	63.0	58.5	29.4
C-GC-C	42.7	64.8	51.9	48.4	42.9	37.8	32.7	16.9
Catechin	12.2	33.4	25.5	13.5	9.7	7.7	6.1	4.1
C-C-C	61.9	119.4	83.2	69.9	63.3	50.9	37.1	29.6
ProD B <sub>3</sub>	117.0	194.1	160.9	135.0	114.0	94.1	82.3	45.6
ProC B <sub>3</sub>	134.2	213.3	173.7	156.4	133.4	109.0	99.6	40.4

Table C.11: Content of determined proanthocyanidins in mg/kg fresh weight of all analyzed malt samples (overview).



C.5 Content of Proanthocyanidins

ID	Year	Prov.	Cultivar	Kind	GC-GC-C	GC-C-C	C-GC-C	Catechin	C-C-C	ProD B3	ProC B3
6121	1998	h	Barke	sg	47.2	68.1	40.9	23.7	81.2	111.1	152.1
6262	1999	h	Barke	sg	48.4	77.3	52.4	22.6	59.1	86.1	105.5
6152	1999	r	Barke	sg	46.3	62.5	50.2	18.2	68.3	90.0	127.8
8546	2001	h	Barke	sg	54.6	73.2	46.1	39.1	77.2	129.4	174.1
10646	2001	R	Barke	sg	55.5	76.8	51.7	37.5	90.1	141.0	195.1
6107	1998	h	Scarlett	sg	62.5	82.0	50.1	15.4	58.8	110.1	115.6
6064	1998	r	Scarlett	sg	42.0	52.5	31.2	20.8	35.5	86.8	88.7
6248	1999	h	Scarlett	sg	63.2	76.8	48.5	20.4	54.2	145.7	143.6
6138	1999	r	Scarlett	sg	41.2	59.4	38.0	13.3	47.9	98.9	115.3
10647	2001	R	Scarlett	sg	78.9	95.8	64.0	12.6	72.3	133.7	149.6
6099	1998	h	Clarine	wg	48.6	101.6	43.7	16.1	102.7	129.7	230.4
6056	1998	w	Clarine	wg	43.9	82.4	40.1	18.5	95.6	125.5	192.7
6233	1999	h	Clarine	wg	49.1	91.1	47.3	22.4	82.9	106.4	175.9
6184	1999	w	Clarine	wg	71.3	116.1	57.3	27.6	97.5	130.8	169.1
6231	1999	h	Esterel	wg	65.9	100.8	64.4	42.1	70.3	166.3	194.3
6182	1999	w	Esterel	wg	78.8	105.7	62.3	41.7	80.1	205.0	221.1
6234	1999	h	Regina	wg	43.5	71.1	40.1	16.1	66.0	84.0	112.4
6185	1999	w	Regina	wg	48.7	68.5	42.4	32.1	61.9	145.0	176.6
6104	1998	h	Tiffany	wg	36.5	67.3	45.7	29.8	79.1	114.6	193.7
6061	1998	w	Tiffany	wg	30.6	56.1	34.0	44.1	68.7	121.0	205.8
6239	1999	h	Tiffany	wg	37.0	69.7	42.8	21.2	75.9	102.5	159.3
6190	1999	w	Tiffany	wg	42.9	65.5	43.3	34.0	67.8	125.8	187.5

Table C.12: Content of determined proanthocyanidins in mg/kg fresh weight of all analyzed barley samples (ID=identification number, prov.=provenience, h=Hadmersleben, r=Rethmar, w=Wetze; sg = summer barley, wg = winter barley).

ID	Year	Prov.	Cultivar	Kind	GC-GC-C	GC-C-C	C-GC-C	Catechin	C-C-C	ProD B3	ProC B3
6531	1998	h	Barke	sg	50.1	65.5	43.4	13.3	70.3	82.4	109.0
7046	1999	h	Barke	sg	54.4	69.7	50.3	13.4	69.5	112.5	133.2
7547	1999	r	Barke	sg	49.8	60.1	42.9	4.8	59.4	79.4	95.8
10758	2000	R	Barke	sg	50.2	63.4	42.3	4.1	54.1	76.7	95.2
11352	2001	h	Barke	sg	53.8	63.4	45.9	9.4	54.3	131.0	156.7
11424	2001	R	Barke	sg	71.6	78.1	51.5	7.4	73.9	106.4	127.5
6517	1998	h	Scarlett	sg	77.3	91.7	55.9	8.5	59.8	149.0	141.7
6500	1998	r	Scarlett	sg	54.3	58.7	33.9	10.2	29.6	117.8	106.0
7032	1999	h	Scarlett	sg	71.0	71.7	43.0	5.0	45.0	127.1	112.7
7533	1999	r	Scarlett	sg	71.6	69.1	44.0	6.5	45.3	118.5	102.9
10163	2000	h	Scarlett	sg	49.9	47.2	31.2	8.3	37.2	115.6	112.4
10759	2000	R	Scarlett	sg	65.8	71.1	40.5	5.3	42.3	86.2	79.5
11354	2001	h	Scarlett	sg	60.8	62.8	39.3	8.5	40.3	132.9	117.6
11425	2001	R	Scarlett	sg	70.3	76.4	49.8	7.7	54.2	139.4	138.2

Table C.13: Content of determined proanthocyanidins in mg/kg fresh weight of all analyzed malt samples (ID = identification number, prov. = provenience, h = Hadmersleben, r = Rethmar, R = Roggenstein, w = Wetzze; sg = summer barley, wg = winter barley (to be continued on next page)).

C.5 Content of Proanthocyanidins

ID	Year	Prov.	Cultivar	Kind	GC-GC-C	GC-C-C	C-GC-C	Catechin	C-C-C	ProD B3	ProC B3
6277	1998	h	Clarine	wg	40.1	63.1	33.2	8.2	63.0	108.3	160.7
6268	1998	w	Clarine	wg	31.7	62.7	35.8	7.4	69.8	83.1	148.6
7569	1999	h	Clarine	wg	60.0	91.7	46.4	13.4	77.7	134.8	173.3
7017	1999	w	Clarine	wg	48.7	78.8	39.2	18.1	69.4	111.9	145.3
10732	2000	h	Clarine	wg	58.3	80.0	44.4	9.8	63.7	87.4	100.1
11049	2000	R	Clarine	wg	59.8	104.3	59.8	14.6	119.4	135.4	213.3
7232	2001	h	Clarine	wg	56.8	79.4	38.2	12.7	58.6	99.9	109.1
7256	2001	R	Clarine	wg	71.2	100.1	48.2	17.2	83.7	143.6	167.2
7567	1999	h	Esterel	wg	71.1	91.7	48.9	25.3	69.3	194.1	209.4
7015	1999	w	Esterel	wg	81.7	94.0	50.3	15.4	68.2	159.9	156.3
10730	2000	h	Esterel	wg	66.9	63.6	37.5	30.5	36.3	163.1	130.2
11047	2000	R	Esterel	wg	76.6	102.8	64.8	28.1	83.7	170.8	200.1
7230	2001	h	Esterel	wg	63.7	60.8	26.1	33.4	32.1	160.6	115.8
7254	2001	R	Esterel	wg	76.4	84.7	47.7	27.1	52.8	167.0	148.6
7570	1999	h	Regina	wg	53.5	74.9	41.9	7.4	61.8	110.6	133.5
7018	1999	w	Regina	wg	58.0	81.5	43.0	12.3	68.1	123.3	146.6
10733	2000	h	Regina	wg	43.1	56.8	32.8	8.5	42.9	98.0	103.6
11050	2000	R	Regina	wg	55.9	96.0	57.0	10.0	101.4	110.9	166.0
7233	2001	h	Regina	wg	56.8	79.3	37.9	9.6	60.6	89.1	104.6
7257	2001	R	Regina	wg	59.7	81.5	47.1	7.3	75.9	115.5	116.3
6273	1998	w	Tiffany	wg	32.5	54.3	31.8	6.2	65.8	84.0	143.3
7575	1999	h	Tiffany	wg	33.8	63.3	38.4	13.6	70.9	95.8	163.5
7023	1999	w	Tiffany	wg	37.6	62.7	35.8	10.8	64.3	108.7	149.5
7231	2001	h	Tiffany	wg	42.2	67.4	41.1	10.8	67.5	81.1	117.7
7255	2001	R	Tiffany	wg	47.7	77.5	50.3	8.7	83.2	123.1	177.8

Table C.14: Content of determined proanthocyanidins in mg/kg fresh weight of all analyzed malt samples (ID = identification number, prov. = provenience, h = Hadmersleben, r = Rethmar, R = Roggenstein, w = Wetzze; sg = summer barley, wg = winter barley (continued)).

## **C.6 Scatterplots**

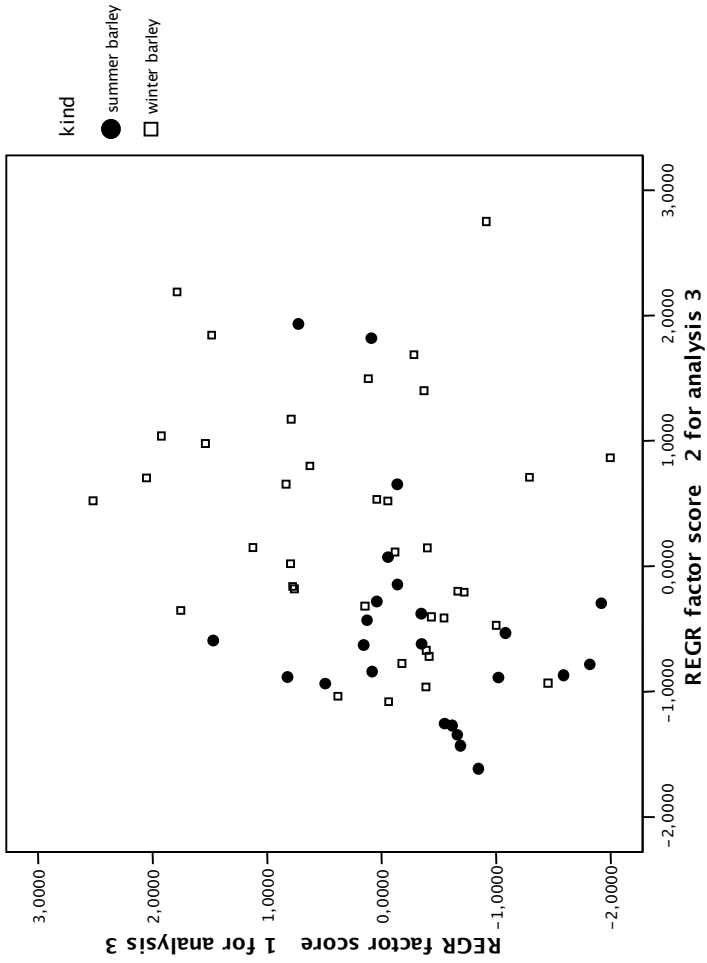


Figure C.17: Scatterplot summer/winter barley

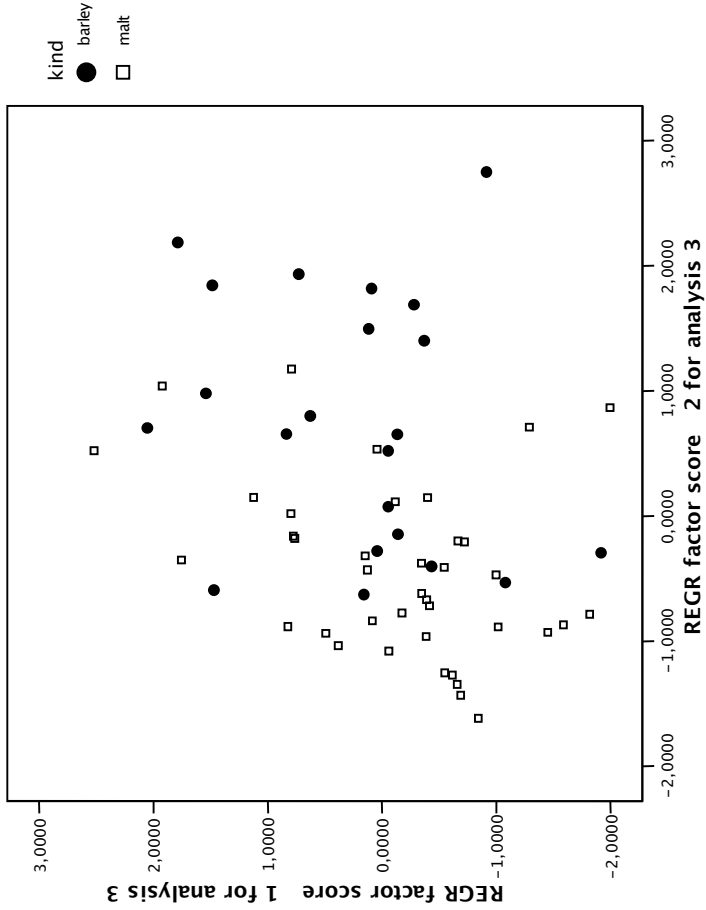


Figure C.18: Scatterplot barley/malt

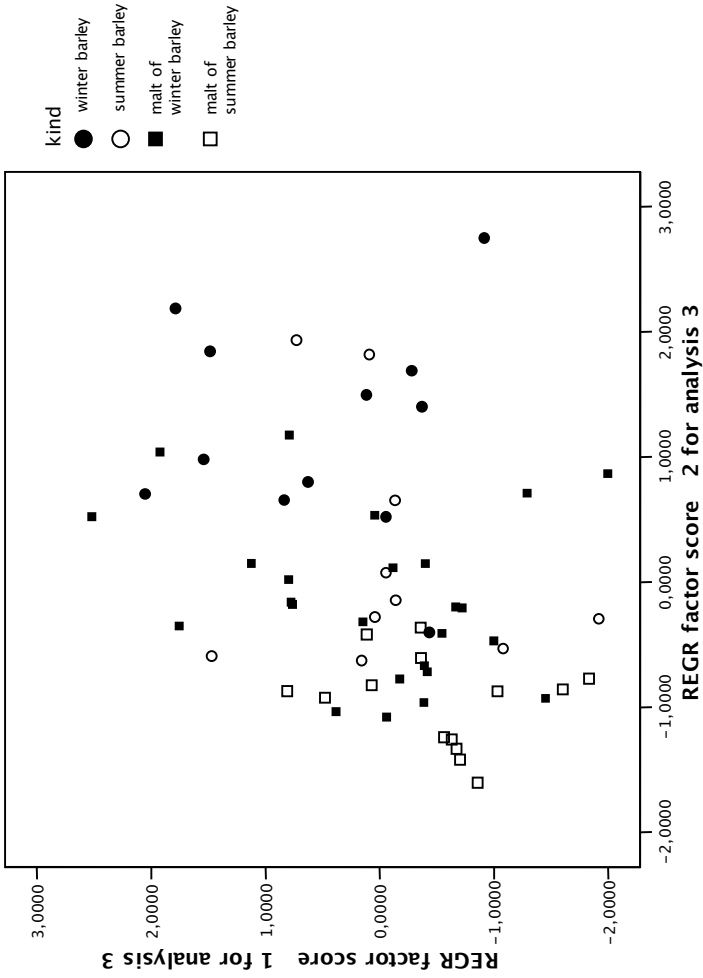


Figure C.19: Scatterplot summer barley/winter barley/malt of summer barley/malt of winter barley

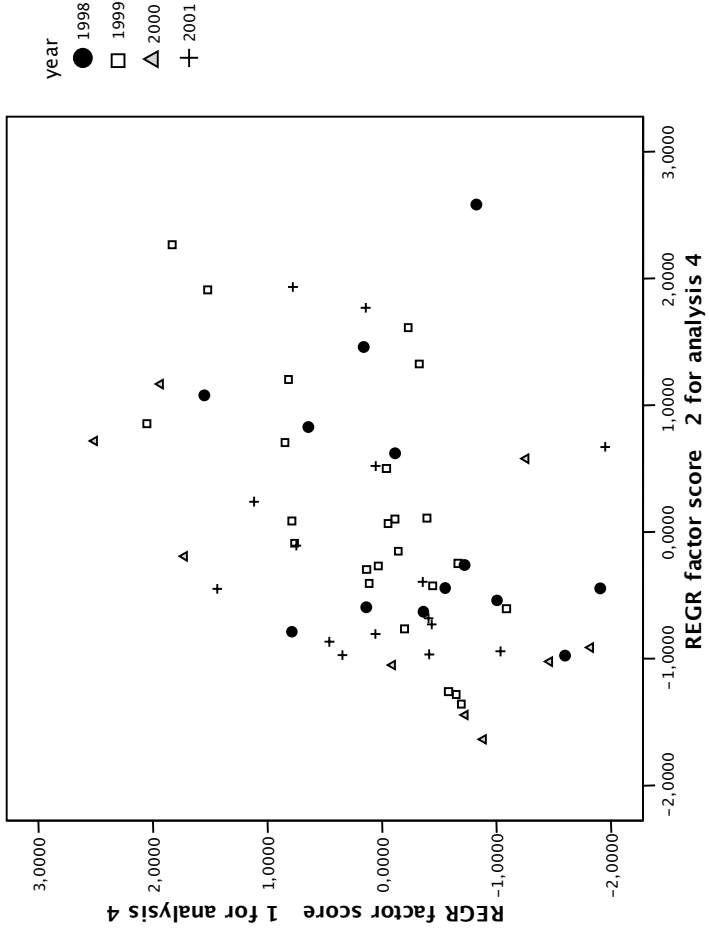


Figure C.20: Scatterplot growing year



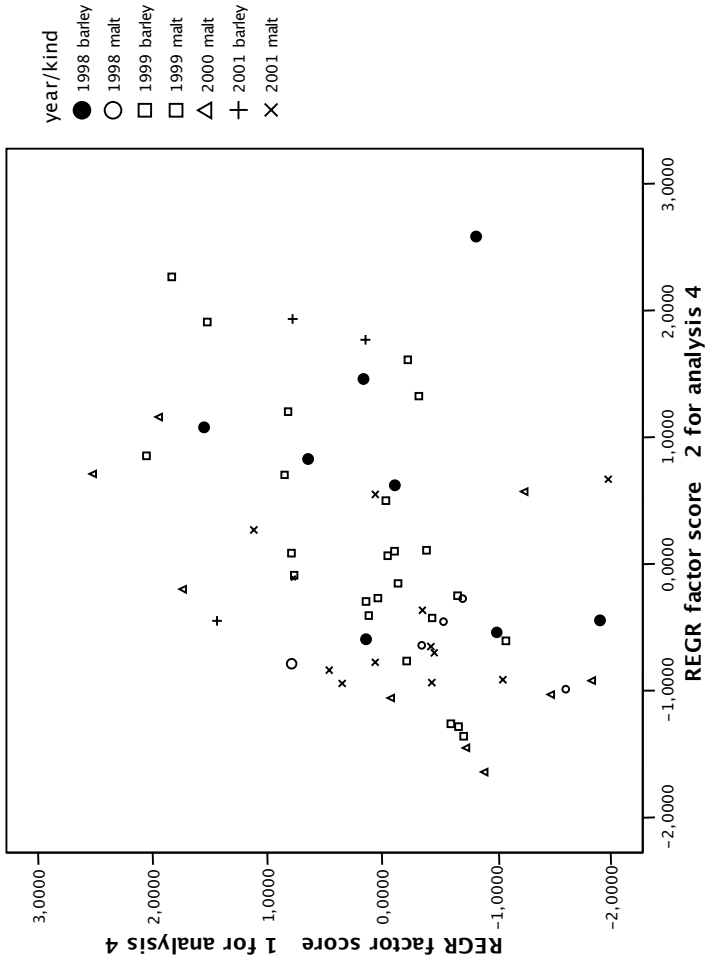


Figure C.21: Scatterplot growing year and barley/malt

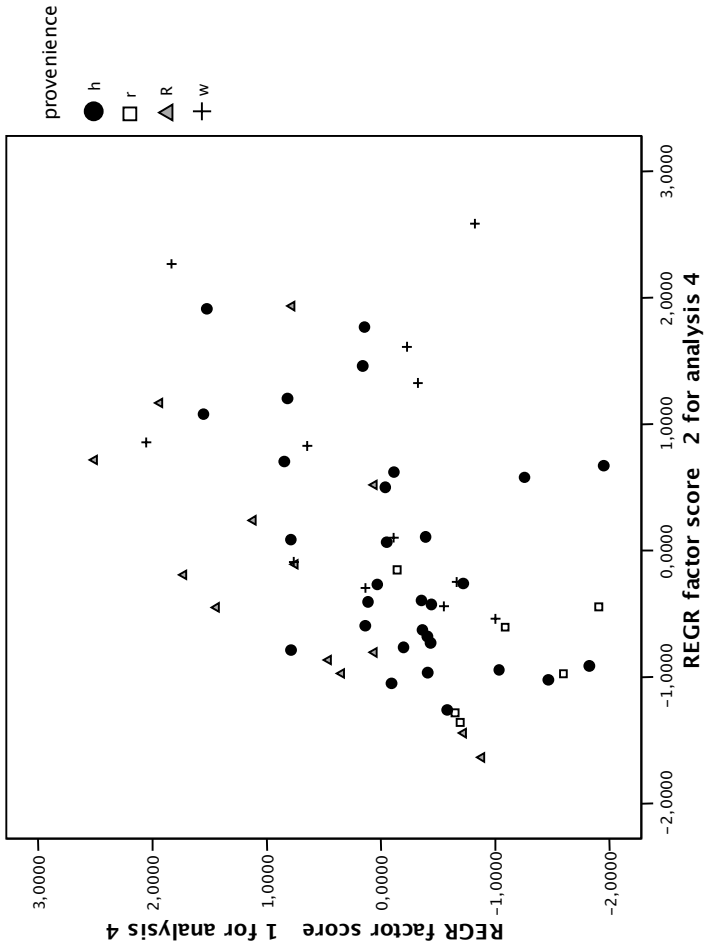


Figure C.22: Scatterplot provenience (h = Hadmersleben, r = Rethmar, R = Roggenstein, w = Wetze)

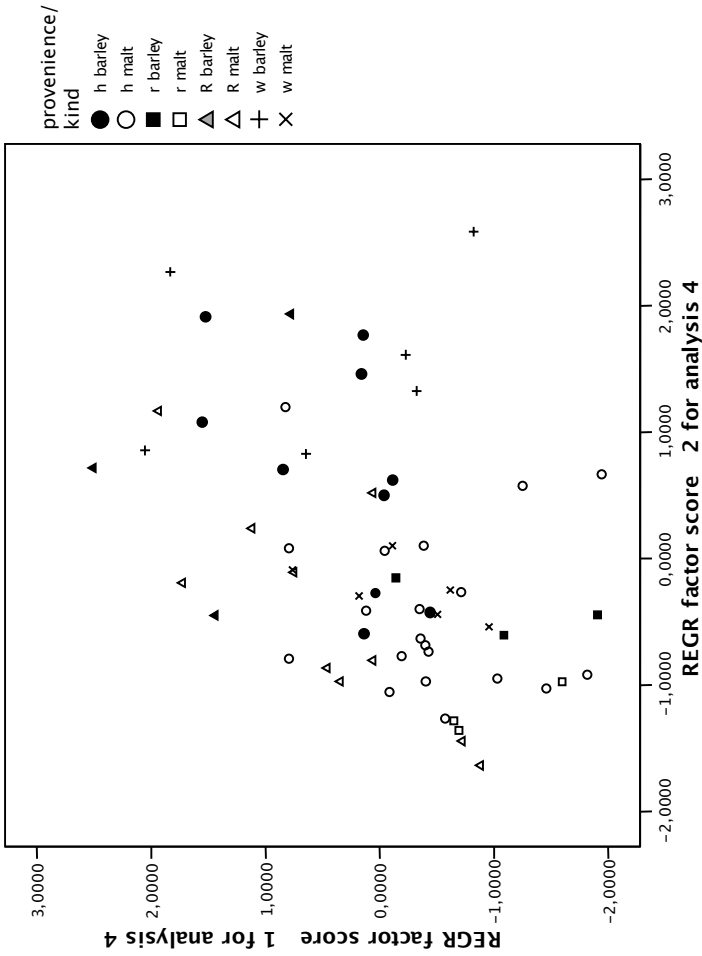


Figure C.23: Scatterplot growing year and barley/malt

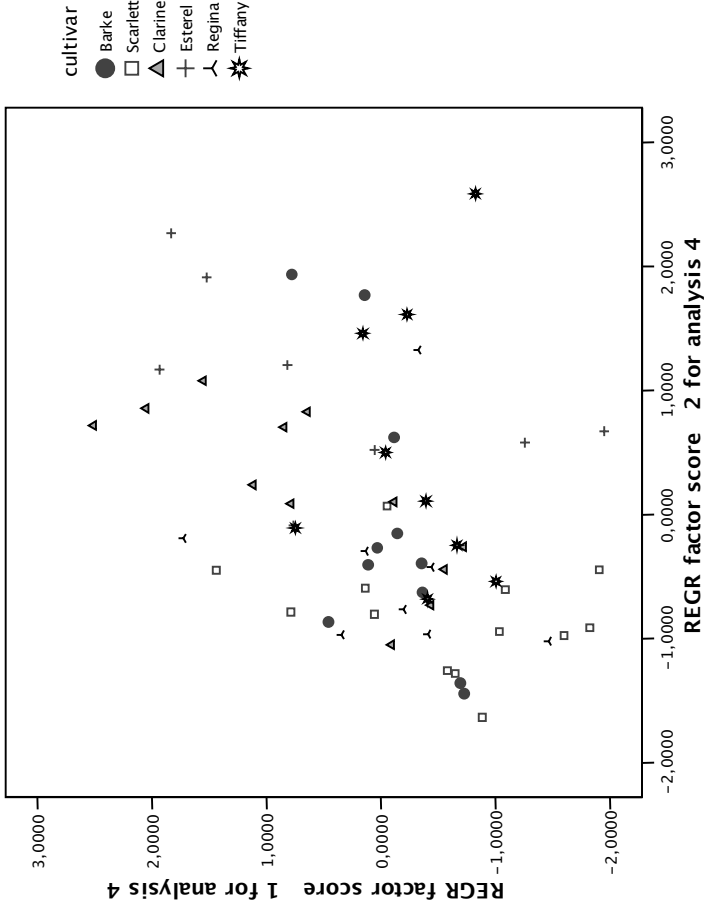


Figure C.24: Scatterplot cultivar

**C.7 Peak Ratios**

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	Scarlett	Esterel	Barke	Regina	Clarine	Tiffany
ProC B3/GC-GC-C	a	ab	ab	ab	b	c
ProC B3/GC-C-C	a	a	a	a	a	c
ProC B3/C-C-C	b	<b>c</b>	a	a	a	ab
ProC B3/ProD B3	a	a	b	b	<b>c</b>	<b>d</b>
ProD B3/GC-GC-C	a	a	a	a	a	<b>b</b>
ProD B3/GC-C-C	b	<b>c</b>	ab	ab	a	ab
ProD B3/C-GC-C	a	<b>b</b>	a	a	a	a
ProD B3/C-C-C	<b>b</b>	<b>c</b>	a	a	a	a
C-C-C/GC-GC-C	a	a	b	b	<b>c</b>	<b>d</b>
C-C-C/GC-C-C	a	a	c	b	bc	<b>d</b>
C-C-C/C-GC-C	a	a	b	b	c	c
C-C-C/Cat	b	<b>a</b>	b	b	b	b
C-GC-C/GC-GC-C	a	a	b	ab	b	c
C-GC-C/GC-C-C	c	b	<b>d</b>	b	<b>a</b>	c

Table C.15: Significant differences of peak ratios: different letters indicate significantly different means of a peak ratio, e.g. ProC B3/GC-GC-C (first row) of Clarine is significantly different from Tiffany, but not from Esterel, Barke and Regina. Letters in bold face indicate, that for this peak ratio this variety is significantly different from all other varieties (significance level  $\alpha = 0.5$ ).

	Scarlett	Esterel	Barke	Regina	Clarine	Tiffany
ProC B3/GC-GC-C	a	ab	ab	ab	b	<b>c</b>
ProC B3/Cat	a	<b>b</b>	a	a	a	a
ProC B3/ProD B3	a	a	b	b	b	<b>c</b>
ProD B3/GC-C-C	ab	b	a	a	a	a
ProD B3/C-GC-C	a	<b>b</b>	a	a	a	a
ProD B3/Cat	<b>c</b>	a	b	b	ab	ab
ProD B3/C-C-C	b	b	a	a	a	a
C-C-C/GC-GC-C	a	a	b	b	b	<b>c</b>
C-C-C/GC-C-C	a	a	b	b	b	<b>c</b>
C-C-C/Cat	b	<b>a</b>	b	b	b	b
Cat/GC-GC-C	a	<b>d</b>	ab	ab	bc	c
Cat/GC-C-C	a	<b>b</b>	a	a	a	a
Cat/C-GC-C	a	<b>b</b>	a	a	a	a
C-GC-C/GC-GC-C	a	a	b	b	b	<b>c</b>
C-GC-C/GC-C-C	c	ab	<b>d</b>	ab	<b>a</b>	bc

Table C.16: Significant differences of peak ratios of malt; explanation see table C.16.

	Scarlett	Esterel	Regina	Barke	Clarine	Tiffany
<b>ProC B3</b>						
GC-GC-C	1.931	2.367	2.551	2.546	3.267	4.494
±	0.305	0.200	0.179	0.366	0.111	0.183
GC-C-C	1.697	1.952	1.755	1.940	1.915	2.594
±	0.121	0.135	0.069	0.177	0.085	0.126
C-GC-C	2.726	3.509	3.108	2.852	3.775	4.186
±	0.254	0.184	0.198	0.289	0.121	0.205
Catechin	12.681	6.084	12.286	11.171	11.878	11.629
±	1.309	1.593	0.799	2.194	1.402	2.048
C-C-C	2.514	2.906	2.023	1.945	2.028	2.333
±	0.076	0.161	0.173	0.124	0.128	0.113
ProD B3	0.978	0.986	1.211	1.281	1.424	1.569
±	0.071	0.054	0.060	0.045	0.026	0.027
<b>ProD B3</b>						
GC-GC-C	1.966	2.399	2.109	1.976	2.245	2.845
±	0.121	0.144	0.090	0.180	0.088	0.120
GC-C-C	1.744	2.031	1.464	1.511	1.343	1.649
±	0.052	0.119	0.138	0.092	0.092	0.090
C-GC-C	2.806	3.708	2.595	2.220	2.649	2.661
±	0.121	0.173	0.410	0.151	0.140	0.141
Catechin	13.299	6.163	10.328	8.963	8.291	7.504
±	0.728	1.437	0.722	1.424	1.655	1.742
C-C-C	2.603	3.098	1.698	1.525	1.449	1.490
±	0.066	0.159	0.383	0.076	0.169	0.099
<b>C-C-C</b>						
GC-GC-C	0.779	0.840	1.284	1.313	1.595	1.913
±	0.125	0.094	0.083	0.085	0.046	0.071
GC-C-C	0.679	0.684	0.875	0.999	0.940	1.107
±	0.041	0.035	0.036	0.027	0.023	0.041
C-GC-C	1.092	1.218	1.557	1.471	1.848	1.789
±	0.084	0.050	0.054	0.056	0.027	0.078
Catechin	5.199	2.229	6.493	5.997	5.810	5.273
±	0.546	1.054	0.398	1.075	0.626	1.213
<b>Catechin</b>						
GC-GC-C	0.193	0.427	0.256	0.338	0.292	0.549
±	0.028	0.064	0.049	0.138	0.034	0.069
GC-C-C	0.163	0.359	0.176	0.249	0.174	0.314
±	0.014	0.045	0.044	0.076	0.025	0.048
C-GC-C	0.264	0.656	0.307	0.371	0.345	0.502
±	0.030	0.070	0.105	0.121	0.042	0.076

Table C.17: Peak ratios of barley and malt samples (part 1): ratio of the compound in bold face to the compounds in the left column ± standard deviation (to be continued in table C.18 on next page).

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	Scarlett	Esterel	Regina	Barke	Clarine	Tiffany
<b>C-GC-C</b>						
GC-GC-C	0.708	0.689	0.820	0.897	0.853	1.070
±	0.039	0.040	0.063	0.034	0.027	0.032
GC-C-C	0.621	0.563	0.562	0.684	0.510	0.621
±	0.012	0.015	0.024	0.012	0.009	0.016
<b>GC-C-C</b>						
GC-GC-C	0.890	0.849	0.692	0.769	0.609	0.583
±	0.024	0.022	0.052	0.015	0.030	0.024
<b>sum2</b>						
sum3	1.076	1.287	1.027	0.997	1.063	1.279
±	0.048	0.073	0.057	0.074	0.048	0.057

Table C.18: Peak ratios of barley and malt samples (part 2): ratio of the compound in bold face to the compounds in the left column ± standard deviation; sum2 = sum of all dimers, sum3 = sum of all trimers (continuation of table C.17 on previous page).

	Barke	Scarlett	Clarine	Esterel	Regina	Tiffany
<b>ProC B3</b>						
GC-GC-C	2,189	1,771	3,015	2,197	2,365	3,947
±	0,175	0,119	0,364	0,190	0,170	0,346
GC-C-C	1,796	1,698	1,874	1,933	1,647	2,330
±	0,146	0,130	0,159	0,090	0,089	0,159
C-GC-C	2,591	2,744	3,570	3,585	2,982	3,866
±	0,177	0,179	0,275	0,253	0,139	0,297
Catechin	15,850	15,682	12,981	6,459	14,297	16,096
±	2,362	1,258	1,684	1,036	1,210	2,426
C-C-C	1,908	2,637	2,027	2,954	1,937	2,138
±	0,202	0,185	0,105	0,232	0,144	0,105
ProD B3	1,225	0,922	1,354	0,940	1,189	1,537
±	0,021	0,016	0,086	0,070	0,070	0,070

Table C.19: Peak ratios of malt samples (part 1): ratio of the compound in bold face to the compounds in the left column ± standard deviation (to be continued in table C.20 on next page).



	Barke	Scarlett	Clarine	Esterel	Regina	Tiffany
<b>ProD B3</b>						
GC-GC-C	1.793	1.917	2.176	2.344	1.993	2.562
±	0.154	0.116	0.144	0.112	0.098	0.172
GC-C-C	1.472	1.839	1.377	2.109	1.402	1.517
±	0.132	0.129	0.065	0.171	0.093	0.087
C-GC-C	2.122	2.975	2.645	3.966	2.540	2.518
±	0.161	0.183	0.153	0.502	0.155	0.171
Cat	13.012	17.043	9.396	6.739	12.145	10.484
±	1.962	1.431	0.763	0.831	1.098	1.481
C-C-C	1.566	2.866	1.523	3.310	1.661	1.400
±	0.179	0.209	0.089	0.488	0.163	0.086
<b>C-C-C</b>						
GC-GC-C	1.166	0.676	1.474	0.773	1.247	1.835
±	0.063	0.028	0.151	0.097	0.120	0.096
GC-C-C	0.953	0.646	0.919	0.669	0.861	1.087
±	0.035	0.029	0.053	0.046	0.047	0.037
C-GC-C	1.382	1.046	1.748	1.228	1.559	1.801
±	0.060	0.031	0.070	0.068	0.063	0.078
Catechin	8.630	6.255	6.368	2.373	7.651	7.535
±	1.507	0.718	0.687	0.527	0.960	1.079
<b>Catechin</b>						
GC-GC-C	0.162	0.120	0.239	0.375	0.169	0.263
±	0.033	0.015	0.021	0.046	0.014	0.040
GC-C-C	0.131	0.115	0.152	0.344	0.119	0.154
±	0.024	0.015	0.013	0.059	0.011	0.019
C-GC-C	0.189	0.186	0.294	0.654	0.217	0.257
±	0.035	0.024	0.030	0.143	0.022	0.033
<b>C-GC-C</b>						
GC-GC-C	0.844	0.645	0.831	0.624	0.794	1.019
±	0.028	0.016	0.056	0.059	0.049	0.034
GC-C-C	0.691	0.616	0.524	0.547	0.552	0.605
±	0.013	0.012	0.013	0.028	0.017	0.013
<b>GC-C-C</b>						
GC-GC-C	0.822	0.958	0.643	0.898	0.702	0.595
±	0.023	0.025	0.027	0.053	0.025	0.016
<b>sum2</b>						
sum3	0.943	1.095	1.052	1.300	0.979	1.170
±	0.081	0.070	0.062	0.075	0.053	0.068

Table C.20: Peak ratios of malt samples (part 2): ratio of the compound in bold face to the compounds in the left column ± standard deviation; sum2 = sum of all dimers, sum3 = sum of all trimers (continuation of table C.19 on previous page).



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