DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

# Selected Bioactive Compounds in Cereals and Cereal Products – Their Role and Analysis by Chromatographic Techniques

**JUHA-MATTI PIHLAVA**

**Food Chemistry and Food Development**
**Department of Biochemistry**

DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU
Food Chemistry

# Selected Bioactive Compounds in Cereals and Cereal Products − Their Role and Analysis by Chromatographic Techniques

JUHA-MATTI PIHLAVA



**Food Chemistry and Food Development**
**Department of Biochemistry**

**TURKU, FINLAND – 2017**

Food Chemistry and Food Development
Department of Biochemistry
University of Turku, Finland

Supervised by
Principal Scientist Pirjo Mattila, Ph.D.
Bio-based Business and Industry
Natural Resources Institute Finland (Luke)
Jokioinen, Finland

Professor emeritus Rainer Huopalahti, Ph.D
Department of Biochemistry
University of Turku
Turku, Finland

Professor emeritus Heikki Kallio, Ph.D.
Department of Biochemistry
University of Turku
Turku, Finland

Reviewed by
Adjunct Professor Pekka Lehtonen, Ph.D.
Department of Chemistry
University of Helsinki
Helsinki, Finland

Professor Rikard Landberg, Ph.D.
Department of Biology and Biological Engineering
Food and Nutrition Science
Chalmers University of Technology
Gothenburg, Sweden

Opponent
Professor Charles Brennan, Ph.D.
Department of Wine, Food and Molecular Biosciences
Lincoln University
Christchurch, New Zealand

Research director
Professor Baoru Yang, Ph.D.
Department of Biochemistry
University of Turku
Turku, Finland

The originality of this dissertation has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service

ISBN 978-951-29-6889-3 (PRINT)
ISBN 978-951-29-6890-9 (PDF)
ISSN 2323-9395 (print)
ISSN 2323-9409 (pdf)
Painosalama Oy – Turku, Finland 2017

*In memory of my grandparents – mamma and pappa*.

# TABLE OF CONTENTS

# ABSTRACT

Plants, including cereal plants, defend themselves against abiotic and biotic stress factors by various means. In addition to physical barriers there are a number of chemical defense systems in order to protect growing parts or seeds of plants against pathogens and herbivores. These protective chemicals are classified as secondary metabolites, and some of them, such as phenolic acids, lignans and flavonoids, are found in all major cereal crops. On the other hand, some of these defense chemicals are cereal-specific, such as hordatines in barley and avenanthramides in oats. Some, such as alkylresorcinols, are found in barley, wheat and rye, while benzoxazinoids are present only in wheat and rye. Interestingly, when the grains of cereals are used as food, these phytochemicals may act as health promoting, bioactive, compounds in humans.

The main objective of this study was to gain new insight into certain less studied phytochemicals, e.g. flavonoids, benzoxazinoids and phenolamides such as hordatines, in cereals and cereal products. More basic research was carried out related to the content of avenanthramides, alkylresorcinols and lignans. In barley and beer, a number of phenolamides, namely hordatines, their agmatine precursors and their glycosides, were tentatively identified. The variation of these phenolamides and their sugar conjugates turned out to be surprisingly high. The content of hordatines in different beers was found to correlate positively with the alcohol content, although some non- or low-alcoholic beers could also contain relatively high concentrations of hordatines. Also, a new and interesting finding in beer was the spermidines conjugated with one or two hydroxycinnamoyl acids. A number of phenolamides, namely conjugates of spermidine, putrescine and agmatine, were also found in rye and rye products, and most of these were reported for the first time in this cereal. As a consecutive and supplementing work to our earlier study on rye milling fractions, a new study was conducted with a special focus on identification of flavonoids, lignans and benzoxazinoids, in addition to phenolamides. Based on the results of this work, the chemical diversity of the flavonoids in rye was found to be large, although the concentration of total flavonoids was relatively low. Flavonoids, phenolamides, lignans and benzoxazinoids were found also in rye products. Their presence in rye crisp breads, sourdough breads, malts and in a traditional Finnish food, *mämmi*, indicates that these compounds withstand relatively harsh conditions of various food processing operations. Probably the most intriguing finding was, however, that various benzoxazinoids can be found in wheat and rye beers and that even a non- or low-alcoholic wheat beer can be a relatively good source of dietary benzoxazinoids.

# SUOMENKIELINEN ABSTRAKTI

Kasvit, viljakasvit mukaan lukien, puolustautuvat abioottisia ja biottisia stressitekijöitä vastaan monin tavoin. Fysikaalisia suojamuureja vahvistavat lukuisat kemialliset puolustusjärjestelmät, jotka suojaavat kasvin kasvavia osia tai siemeniä kasvitauteja tai kasvinsyöjiä vastaan. Nämä suojaavat kemikaalit luokitellaan sekundaarimetaboliiteiksi, ja joitakin niitä, kuten fenolisia happoja, lignaaneja ja flavonoideja, löytyy kaikissa viljakasveista. Toisaalta jotkin näistä yhdisteistä ovat tyypillisiä vain tietyille viljoille, kuten hordatiinit ohralle ja avenantramidit kauralle. Joitakin yhdisteistä kuten alkyyliresorsinoleja esiintyy ohrassa, vehnässä ja rukiissa ja bentsoksatsinoideja vehnässä ja rukiissa. Käytettäessä viljaa ruokana näillä yhdisteillä voi olla terveyttä edistäviä vaikutuksia.

Tutkimuksen tavoitteena oli lisätä tietoa viljojen ja viljatuotteiden vähemmän tutkituista pienkomponenteista, flavonoideista, bentsoksatsinoideista ja fenolamideista kuten hordatiineista. Lisäksi määritettiin myös avenantramidien, alkyyliresorsinolien ja lignaanien pitoisuuksia viljoissa ja viljatuotteissa. Ohrasta ja oluesta tunnistettiin alustavasti hordatiineja, niiden agmatiiniprekursoreita sekä glykosideja. Näiden yhdisteiden aglykoninen sekä niiden glykosidien lukumäärä osoittautui yllättävän korkeaksi. Hordatiinien kokonaispitoisuus oluissa oli verrannollinen alkoholipitoisuuteen, joskin suhteellisen suuria hordatiinipitoisuuksisa mitattiin myös alkoholittomista tai matala-alkoholisista oluista. Uutena mielenkiintoisena löydöksenä olivat oluessa olevat spermidiinit, joihin oli konjugoitunut yksi tai kaksi hydroksikanelihappoa. Fenolamideja, spermidiinin, putreskiinin ja agmatiinin fenolihappokonjugaatteja havaittiin myös rukiista ja ruistuotteista. Suurinta osaa näistä yhdisteistä ei ole aikaisemmin raportoitu löytyvän rukiista. Aikaisempaa rukiin myllyjakeita koskevaa työtämme täydennettiin uudella tutkimuksella, jossa keskityttiin erityisesti flavonoidien, lignaanien ja bentsoksatsinoidien tunnistamiseen edellä mainittujen fenolamidien lisäksi. Etenkin flavonoidien monimuotoisuus rukiissa oli suuri, vaikkakin niiden kokonaispitoisuus oli pieni. Koska näitä bioaktiivisia yhdisteitä havaittiin myös ruisnäkkileivistä, tuoreleivistä, maltaista sekä mämmistä, voidaan päätellä ko. yhdisteiden ainakin osin kestävän suhteellisen kovia ruuanvalmistusolosuhteita. Ehkä mielenkiintoisimpana löydöksenä oli kuitenkin vehnä- ja ruisoluiden bentsoksatsinoidit, joiden määrä alkoholittomissa tai matala-alkoholisissa vehnäoluissa voi olla merkittävä.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AAPO | 2-acetylamino-3H-phenoxazin-3-one |
| AAMPO | 2-acetylamino-7-methoxy-3H-phenoxazin-3-one |
| ABV | alcohol per volume |
| ADC | arginine decarboxylase |
| AP | 2-aminophenol |
| APO | 2-amino-3H-phenoxazin-3-one |
| AMPO | 2-amino-7-methoxy-3H-phenoxazin-3-one |
| ASE | accelerated solvent extractor |
| BOA | 1,4-benzoxazin-3-one |
| CAED | CoulArray electrochemical detector |
| CRP | plasma C-reactive protein |
| CSE | common solvent extraction |
| DAD | diode array detector |
| DNA | deoxyribonucleic acid |
| DON | deoxynivalenol |
| DIBOA | 2,4-dihydroxy-1,4-benzoxazin-3-one |
| DIMBOA | 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one |
| $DIM_2BOA$ | 2,4-dihydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one |
| DPPH | 2,2-diphenyl-1-picrylhydrazine |
| DiFA | dehydrodimer of ferulic acid aka diferulic acid |
| dw | dry weight |
| ELSD | evaporative light scattering detector |
| FHB | Fusarium head blight |
| FID | flame ionization detector |
| FRAP | ferric reducing antioxidant power |
| FT | Fourier transform |
| fw | fresh weight |
| GC | gas chromatography |
| -Glc | glucoside |
| GPx | glutathione peroxidase |
| $H_2O_2$ | hydrogen peroxide |
| HDMBOA | 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one |
| $HDM_2BOA$ | 2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one |
| -Hex | hexoside (used in cases when hexose residue has not been identified as e.g. glucose) |
| HHT | Hydroxycinnamoyl CoA: hydroxyanthranilate N-hydroxy-cinnamoyl transferase |
| HORAC | hydroxyl radical absorbance capacity |
| HPAA | N-(2-hydroxyphenyl)-acetamide |

| | |
|---|---|
| HPLC | high performance liquid chromatography |
| HPMA | N-(2-hydroxyphenyl)malonamic acid |
| HMPMA | N-(2-hydroxy-4-methoxyphenyl)malonamic acid |
| HMBOA | 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one |
| HTP | hydrothermal process |
| ICR | ion cyclotron resonance |
| IT | ion trap |
| LC | liquid chromatography |
| LLE | liquid-liquid extraction |
| LPH | luminal laccase phloridzin hydrolase |
| MALDI | matrix assisted laser desorption ionization |
| MBOA | 6-methoxy-1,4-benzoxazin-3-one |
| MDA | malondialdehyde |
| MS | mass spectrometry |
| $MS^E$ | tandem mass spectrometry with ramped energy (Waters) |
| $MS^n$ | mass spectrometry to the nth (ion or Orbi trap equipment) |
| MS/MS | tandem mass spectrometry (triple quadrupole equipment) |
| MWE | microwave-assisted extraction |
| NIV | nivalenol |
| NORAC | peroxynitrite radical absorbance capacity |
| ORAC | oxygen radical absorbance capacity |
| PDA | photodiode array detector |
| QTOF | quadrupole time-of-flight |
| SDG | secoisolariciresinol diglucoside |
| SOAC | singlet oxygen absorbance capacity |
| SORAC | superoxide radical absorbance capacity |
| SPE | solid phase extraction |
| TLC | thin layer chromatography |
| TRIMBOA | 2,4,7-trihydroxy-8-methoxy-1,4-benzoxazin-3-one |
| UHPLC | ultra-high performance liquid chromatography |
| UHT | ultra high temperature |
| UPLC™ | ultraperformance liquid chromatography ™ (Waters) |
| UV | ultraviolet (detector) |

Abbreviations of chemicals listed in tables in alphabetical order:
Aac = Acetate, ACE = acetone, ACN = acetonitrile, aq = aqueous; water phase, BuOH = butanol, $CHCl_3$ = chloroform, $CH_2Cl_2$ = dichloromethane, DE = diethyl ether, EA = ethyl acetate, EtOH = ethanol, Hex = hexane, HOAc = acetic acid, IPA = isopropanol, LiAC = lithium acetate, MeOH = methanol, P = phosphate, PE = petroleum ether, PSA = pentasulfonic acid, TFA = trifluoroacetic acid, TOL = toluene

# LIST OF ORIGINAL PUBLICATIONS

I. Mattila, P.; Pihlava, JM.; Hellstöm, J. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *J. Agric. Food Chem*. **2005,** *53,* 8290–8295.

II. Pihlava, JM; Nordlund, E.; Heiniö, R-L.; Hietaniemi, V.; Lehtinen, P.; Poutanen, K. Phenolic compounds in wholegrain rye and its fractions. *J. Food Comp. Anal.* **2015**, *38*, 89-97

III. Pihlava, JM. Identification of hordatines and other phenolamides in barley (*Hordeum vulgare*) and beer by UPLC-QTOF-MS. *J. Cereal Sci*. **2014**, *60*, 645-652

IV. Pihlava, JM; Kurtelius, T.; Hurme, T. Total hordatine content in different types of beers. *Journal of the Institute of Brewing,* **2016,** *122*, 212-217.

V. Pihlava, JM; Kurtelius, T. Determination of benzoxazinoids in wheat and rye beers by HPLC-DAD and UPLC-QTOF MS. *Food Chem.* **2016**, *204,* 400-408.

VI. Pihlava J.M.; Hellström, J.; Kurtelius, T.; Mattila, P. Flavonoids, anthocyanins, phenolamines, benzoxazinoids, lignans and alkylresorcinols in rye (*Secale cereale*) and some rye products. Submitted.

# 1 INTRODUCTION

The history of mankind over the last 10 000 years and the development of civilizations are tightly woven with agriculture and cultivation of grain cereals. By favoring the growth of plants which would provide a non-perishable and storable, food for humans, or feed for domesticated animals, humankind has actively affected the evolution of cereal grains and also *vice-versa*. The domestication of cereals is still on-going, but nowadays due to scientific understanding of genetics, the plant breeding process could be considered as more predictable and directed to specific attributes.

Besides providing the seeds primarily for ensuring the survival of plants genetic material and secondly for human consumption, cereal plants are utilized as a source of nutrition and site of growth and reproduction by many pathogenic fungi and herbivores. In the response to the antagonistic users, plants have developed various defense strategies, which have been more or less cognizantly favored by humans when selecting the grains from the healthiest plants producing seeds with high harvest yields.

The first physical defense barrier for pathogen attack is the cell wall, which is further protected with inhibiting proteins to block the degrading enzymes of attacking microbes (Lagaert *et al*. 2009, Bellincampi *et al*. 2014) and/or peptides with various activities against insects and microbes (Marmiroli & Maestri 2014). In case physical barriers are penetrated, a plant´s innate immune system is rapidly activated, which leads to complex defense actions. Part of the defense arsenal is the small molecules produced by the cereal plants against plant pathogens and/or herbivores. Benzoxazinoids, hordatines and aveanthramides are examples of these molecules that are considered in greater depth in this literature review. Even below the ground, the plant is actively affecting and modifying the soil conditions and soil microbiota by roots exudates (Baetz & Martinoia 2014).

Despite the effectiveness of plant natural defense systems, pathogens and herbivorous insects have also evolved ways to overcome these barriers. There are a number of economically important plant diseases, which could lead to partial or total loss of harvest, to low-quality grains or even to infected, toxic grains not-usable as food or feed.

By modern agricultural means including tillage practices, use of good-quality coated seeds, fertilizing to meet plant nutrient needs, crop rotation, precise use of natural/synthetic plant protection substances (i.e. pesticides) during the plant´s growth, and proper post-harvest handling of the harvest, farmers were and are able to ensure good yields of cereal grains. However, unpredictable abiotic factors are always a threat to the harvest: frost at the

wrong time of plant development, drought, too much rain, rains preventing harvesting, low-quality grains due rains, etc. (Martin *et al*. 2006).

Besides providing energy as food, cereal grains can also affect human health either positively by minor components, such as avenanthramides, vitamins, sterols, phenolic compounds and in-organic compounds, or *via* gut microbes by fiber components (β-glucan, insoluble fiber, etc.), or negatively when too much energy in-take is in the form of products consisting of refined cereal flour (endosperm flour). Nowadays usage of whole-grain products is strongly recommended, not so much because of bioactive components, but for increasing intake of fibers and because of the favorable changes in gut microbiota (Belobrajdic & Bird 2013, Costabile *et al*. 2008). Cereal grains are an important part of Finnish diet through various breads, porridges and alcoholic beverages. Rye is important in forms of sourdough breads, oat in porridge and barley in beer. Of course barley is an important raw material for distilled spirits, but their functional properties in humans have nothing to do with the minor or fiber components of barley grains.

Unfortunately, cereal storage proteins (glutenin in wheat, secalin in rye and hordein in barley) can cause Celiac disease (CD) in some humans, which is an autoimmune disease characterized by aberrant inflammatory response leading in the worst cases to severe changes in small intestine function. However, pure oat (storage protein avenin) is usually tolerated by CD patients. Although in this case careful selection of the right oat cultivars is also important (Malalgoda & Simsek 2017). It is also possible to make reduced gluten/gluten-free beers (gluten < 20 ppm or <10) by the selection of the right barley cultivar for malting, and by a specific mashing process or by enzymatic treatment. In very recent years awareness of fermentable oligo-, di- and monosaccharides and polyols i.e. FODMAP –carbohydrates, has arisen, because these compounds can be problematic for people suffering from irritable bowel syndrome (Biesiekierski *et al*. 2011). Luckily, the amount of FODMAP carbohydrates can be reduced by certain lactobacilli during sourdough fermentation, and result in a low-FODMAP rye bread (Laatikainen *et al*. 2016)

This thesis focuses on selected bioactive compounds found in cereals using the approach presented in **Figure 1**. The literature review covers these selected and somewhat less-known bioactive compounds typical to oats, rye and barley, namely avenanthramides, benzoxazinoids and hordatines, respectively, from their biosynthesis and roles in plants to possible health effects in humans. Alkylresorcinols, which are found in larger amounts in rye and wheat, in smaller amounts in barley and being absent in oats, are briefly discussed as well as flavonoids in cereals. And although phenolic acids and lignans form a very important group of the secondary metabolites in cereals, they are also only briefly covered. When ever possible, examples of commercial applications and

patents relating to these compounds have been included. Finally, a few examples of the analytical methodology concerning each of these compound groups are presented.

The experimental part of this thesis includes articles and one submitted manuscript covering quantitation or identification of selected biochemicals of oats, barley, wheat and rye in raw materials as well as in some food products or beverages.

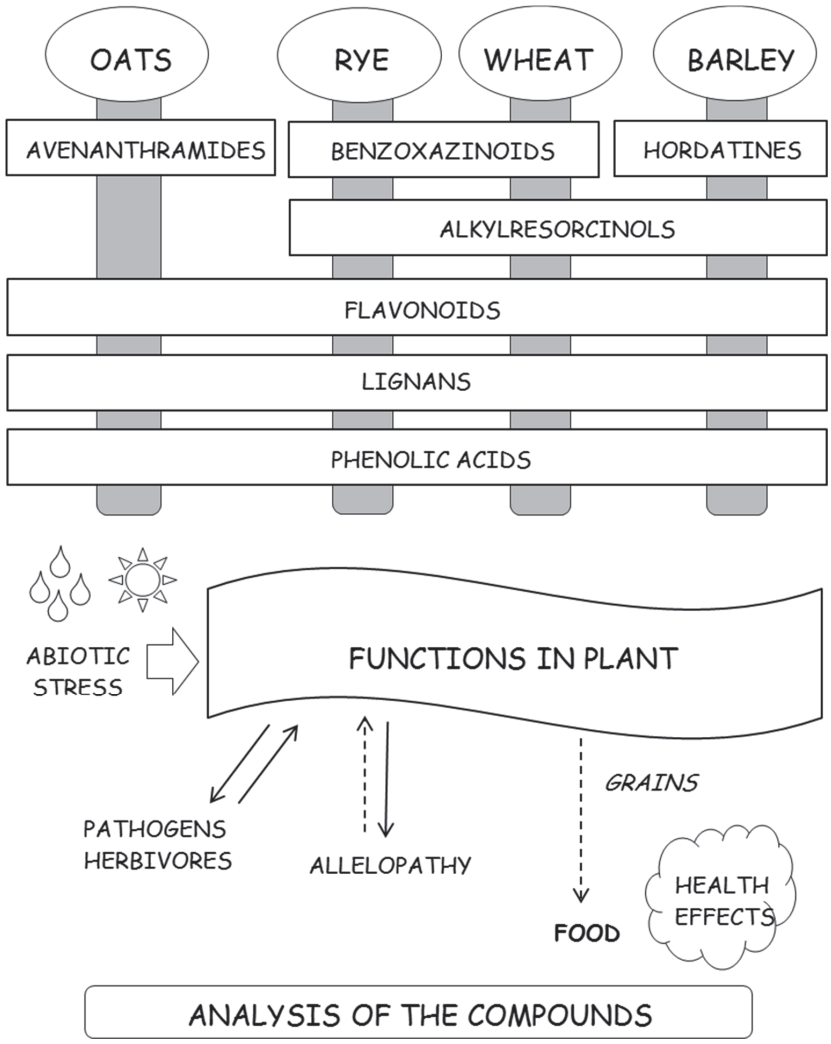



**Figure 1.** In the literature review selected bioactive compounds in cereals will be discussed from the view point of their role in plant, their role as dietary constituents and subsequently their possible health effects in humans. Analytical aspects relating to these compounds will also be briefly considered.

# 2 REVIEW OF THE LITERATURE

## 2.1 Phenolic acids in cereals

### 2.1.1 Background

Phenolic acids are divided in benzoic and hydroxylcinnamic acid derivatives (**Figure 2**). Based on their solubilities and stabilities of conjugates towards alkali or acidic treatments, phenolic acids have been further divided into groups of free, soluble esterified, soluble glycosidic and insoluble bound phenolic acids. The most common free phenolic acids in cereals are ferulic, sinapic, *p*-coumaric, 2-hydroxycinnamic, 2,4-dihydroxybenzoic, caffeic, syringic and vanillic acid (Li *et al*. 2008, Nyström *et al*. 2008, Weidner *et al*. 1999). Sinapic, ferulic, *p*-coumaric, caffeic, gentisic, syringic, 2-hydroxycinnamic, 2,4-dihydroxybenzoic and 4-hydroxybenzoic acid are the main soluble conjugated phenolic acids (Nyström *et al*. 2008, Weidner *et al*. 1999). In cereals phenolic acids can be conjugated to phytosterols by ester bonds (Hakala *et al*. 2002, Nyström *et al*. 2007), to flavonoid glycosides by acylation (Moheb *et al*. 2011, Wojakowska *et al*. 2013), to sugars by glycosidic bonds (Callipo *et al*. 2010, Quifer-Rada *et al*. 2015) and to quinic acid by ester bonds (Quifer-Rada *et al*. 2015). Soluble phenolic acid-polyamine conjugates, i.e. phenolamides, are discussed further in chapter 2.7. (page 69).

Ferulic, sinapic, *p*-coumaric, vanillic, 2,4-dihydroxybenzoic, 4-hydroxy-benzoic acid and 2-hydroxycinnamic acid are the most common bound phenolic acids (Nyström *et al.* 2008, Andreasen *et al*. 2000b). Bound phenolic acids also includes a special group of phenolic acids, namely dehydrodimers of ferulic acid (DiFA): 8-O-4′-DiFA, 5,5′-DiFA, 8,5′-DiFA, 8,5′-DiFA benzofuran form (Bf), 8,8′-DiFA, and 8,8′-DiFA aryl form (Andreasen *et al*. 2000a and 2000b, Renger & Steinhart 2000). However, the complexity of dehydrodimers of phenolic acids is most likely much wider than previously thought (Callipo *et al*. 2010) extending even to dehydrotrimers (Dobberstein & Bunzel 2010, Pedersen *et al.* 2015).

Phenolic acids have important functions as plant cell wall components binding proteins and/or polysaccharides by ester bonds or by ether bonds when incorporated into lignin structure (Buranov & Mazza 2008, Vanholme *et al*. 2010, Provan *et al*. 1994, Renger & Steinhart 2000, Barron *et al*. 2007). Accumulation of ferulic acid synthesis at a certain point after anthesis has been linked to *Fusarium* resistance of wheat cultivars (McKeehen *et al*. 1999).

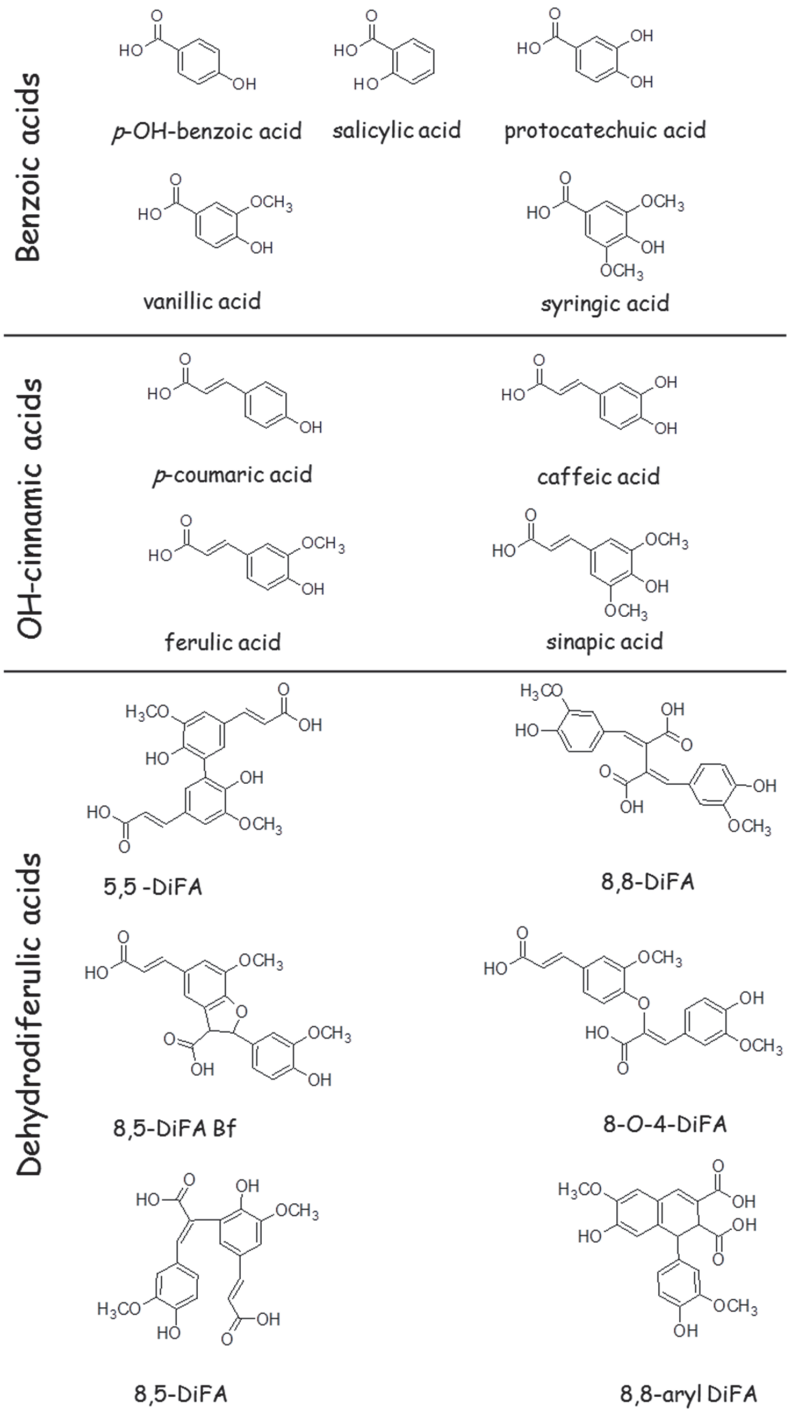



**Figure 2.** Examples of benzoic acids, hydroxycinnamic acids (OH-cinnamic acids) and dehydrodiferulic acids.

The tendency of polysaccharide-bound ferulic acid, to form covalently bonded dehydrodimers by peroxidases has been considered to be an important part of plant defense systems against pests and diseases (Santiago & Malvar 2010). Various phenolic acids (Boutigny *et al*. 2009, Ponts *et al*. 2011), as well as the wheat bran extract containing mainly ferulic acid and its dehydrodimers (Boutigny *et al*. 2010), has been found to inhibit *in-vitro* type B tricothecene biosynthesis in *Fusarium* by repressing the expression of the *Tri* gene. However, Boutigny *et al*. (2010) stated that mono- or dimeric phenolic acids alone do not explain the inhibitory effect of wheat bran extract. In maize, chlorogenic acid (i.e. ester of caffeic acid and quinic acid) has been reported to inhibit fumonisin production in *Fusarium verticillioides* (Atanasova-Penichon *et al*. 2014).

An interesting recent finding regarding the color of black hulled oats revealed that the dark pigment, melanin, consists of a homopolymer of *p*-coumaric acid. *p*-Coumaric acid is conjugated by C-C –linkages into three to nine monomer units, while tetrameric oligomer is the most dominant form (Varga *et al*. 2016).

### 2.1.2 Cereal phenolic acids in food

From the food technological point of view feroloylated arabinoxylans in cereals are of interest based on their gel-producing properties (Niño-Medina *et al*. 2010), their slower fermentability by human gut microbes (Snelders *et al*. 2014b) and their antioxidativity (Snelders *et al*. 2013). However, it appears that feroloylated arabinoxylans do not have great importance in bread making (Snelders *et al*. 2014a). In brewing, phenolic acids, especially *p*-coumaric and ferulic acid, are of importance because they are precursors of flavor active compounds such as 4-vinylphenol (4VP) and 4-vinylguaiacol (4VG). These volatile compounds can cause undesirable off-flavors in lagers, or create desirable, characteristic flavors in wheat and smoked beers (*rauchbier*) (Vanbeneden *et al*. 2008).

Soluble phenolic acids (free, soluble esterified and glycosylated forms) have been reported to contribute to the flavor (Heiniö *et al*. 2008) and antioxidant activity (Adom & Liu 2002) of cereal foods. However, most of the phenolic acids of cereals are in insoluble bound form (Adom & Liu 2002, Heiniö *et al*. 2008, Sosulski *et al*. 1982). Although in bound form, these phenolic acids can contribute to the antioxidative capacity of cereals *in vitro* (Gökmen *et al*. 2009, Liyanapathirana & Shahidi 2004), and be bioavailable along the GI-tract (Aura 2008, Hemery *et al*. 2010, Mateo Anson *et al*. 2009, Vitaglione *et al*. 2008).

The contribution of the “breads and cereals” –group to the dietary intake of phenolic acids in Finnish adults was second highest (12.3 %), while coffee was

the main source (67.9 %) (Ovaskainen *et al*. 2008). Similar results have been presented by Zamora-Ros *et al*. (2013), although the contribution of coffee to the phenolic acid intake was evaluated to be higher, namely 80.7 %, and contribution of cereal and cereal products lower, 8.7 %, in north Europe. Despite the slight differences, the outcome from both studies is that cereals and cereal products are important sources of phenolic acids at least in the northern European diet. Phenolic acids in rye have recently been reviewed by Aura (2014) and in oats by Collins (2011).

### 2.1.3 Analysis of phenolic acids

Phenolic compounds have been traditionally analyzed by liquid chromatography (LC) methods. Gas chromatographic (GC) analysis of phenolic acids is more tedious and involves extra sample preparation steps, because silylation is required to make the compounds less polar and more volatile for GC analysis.

Considering the analysis of phenolic acids in cereals, it must be taken into account that only part of the phenolic acid pool is in readily soluble and extractable form. Also, in the readily extractable form, only a part of the compounds are in free form and the rest are present as esters or glycosidic conjugates.

Extensive sample preparation methods are required when analyzing insoluble bound phenolic acid fractions (Sosulski *et al*. 1982). Mild alkali hydrolysis conditions can cleave ester bonds (Renger & Steinhart 2000), but harsher conditions, such as 170 °C, 1 h, 4 M NaOH (Renger & Steinhart 2000) or by microwave-assisted extraction (MWE) 170 °C, 90 sec, 4 M NaOH (Provan *et al*. 1994) are needed to liberate ether bound phenolic acids from the polysaccharide lignin structure. Acid hydrolysis has been used for cleavage of glycosidic bonds (Weidner *et al*. 1999, Heiniö *et al*. 2008, Mattila & Kumpulainen 2002).

Examples of the analysis of phenolic acids are listed in **Table 1**.

**Table 1.** Examples of the analysis of phenolic acids. Abbreviations are listed in pages iii-iv.

| **PHENOLIC ACIDS**<br>**Sample extraction**<br>***soluble forms*** | **Sample preparation** | **Equipment** | **Column & mobile phase (LC) or Column (GC)** | **Reference** |
|---|---|---|---|---|
| MeOH:Acetone:$H_2O$<br>(2:2:1) | free: LLE from acidic aq to DE:EA (1:1)<br>esterified: aq 2 M NaOH 4 h, LLE using DE:EA<br>glycosidic: aq ca 1 M HCl 1 h 90 °C, LLE using DE:EA | HPLC-DAD | NovaPak C-18<br>4 µm, 3.9 *150 mm<br>P-buffer (pH 2.4) - MeOH | Heiniö *et al*. 2008 |
| 80 % EtOH | free: LLE using EA | HPLC-DAD | Discovery Amide C-16<br>5 µm, 4.6*250 mm<br>ACN-$H_20$ 2 % acetic acid | Li *et al*. 2008<br>Nyström *et al*. 2008 |
| 80 % MeOH 80 C, 15 min | free: LLE from acidic aq to DE<br>esterified: aq freeze-dried, 2 M NaOH 4 h, LLE using DE<br>glycosidic: aq ca 3 M HCl 1 h 100 °C, LLE using DE | HPLC-UV | LiChrosphere 100 RP-18<br>5 µm, 4*250 mm<br>$H_2O$-ACN-acetic acid<br>(88:10:2) | Weidner *et al*. 1999 |
| MeOH (24 h* 7) | clean-up: silica column eluted with EA-Hex; evaporation; silylation by TMSA | GC-FID<br>GC-MS | SPB-1<br>30 m*0.25 mm, $d_f$ 0.25 µm | Xing & White 1997 |

| (…PHENOLIC ACIDS) Sample extraction *soluble forms* | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| 1 mM HCl (15 min) | centrifugation, LLE using DE, evaporation of DE, silylation with MSTFA | GC-ITD MS | DB-5 MSITD<br>30 m*0.25 mm i.d., $d_f$ 0.25 µm | Wu *et al*. 1999 |

| PHENOLIC ACIDS Sample extraction *bound forms* | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| | esterified 1. step: 2 M NaOH, 25 °C, 1 h, LLE of the acidified aq using EA<br><br>esterified 2. step: re-saponification of the solids as above | HPLC-PDA | LiCroCART 100 C-18<br>5 µm, 4*250 mm<br>P-buffer (pH 2.15) - MeOH | Andreasen *et al*. 2000a<br>Andreasen *et al*. 2000b |
| | 2 M NaOH, 20 °C, 4 h, SPE cleanup | LC-ESI-Q TRAP | Targa C-18 100<br>5 µm, 2.1 *150 mm<br>ACN-H20 + 0.1 % formic acid | Callipo *et al*. 2010 |
| | 2 M NaOH, 20 °C, 4 h, LLE using EA | HPLC-DAD | NovaPak C-18<br>4 µm, 3.9 *150 mm<br>P-buffer (pH 2.4) - MeOH | Heiniö *et al*. 2008 |

| (…PHENOLIC ACIDS) Sample extraction *bound forms* | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| | 2 M NaOH, 20 °C, 4 h, LLE using EA | HPLC-DAD | Discovery Amide C-16<br>5 µm, 4.6*250 mm<br>ACN-H20 2 % acetic acid | Li *et al*. 2008<br>Nyström *et al*. 2008 |
| | esterified: 1 M NaOH, r-temp, 24 h, SPE cleanup<br>esters+ethers: 4 M NaOH, 170 °C, 1 h, SPE cleanup | HPLC-PDA | Nucleosil 120-5 C18<br>5 µm, 4*250 mm<br>MeOH-aq TFA | Renger & Steinhart 2000 |
| | soluble phenolics removed by 80 % EtOH<br>esterified: 1 M NaOH, r-temp, time (na)<br>esters+ethers: MWE 4 M NaOH, 170 °C, 90 s, LLE using EA | HPLC-UV | Hichrom ODS<br>5 µm, 4.6*100 mm<br>$H_2O$-acetic acid-n-BuOH<br>983:12:5 | Provan *et al*. 1994 |

## 2.2 Alkylresorcinols in cereals

### 2.2.1 Background

Alkylresorcinols are 1,3-dihydroxy-5-alkylbenzene derivatives with an odd-numbered ($C_{15}$-$C_{27}$) alkyl chain attached at position 5 of the benzene ring and they can be described as phenolic lipids (Stasiuk & Kozubek 2010) (**Figure 3**). Among cereals they are found in rye, wheat, triticale and barley, in order of total alkylresorcinol contents. Besides the major saturated $C_{15:0}$, $C_{17:0}$, $C_{19:0}$, $C_{21:0}$, $C_{23:0}$ and $C_{25:0}$ substituted resorcinols, monoenoic forms of $C_{17:1}-C_{25:1}$, dienoic forms of $C_{17:2}-C_{23:2}$ and oxygenated forms of monoenoics of $C_{17:1}-C_{23:1}$ can be found in rye and some of them also in wheat (Knödler *et al*. 2008). The relation of alkylresorcinol homologs differ in cereals, in rye the homolog C19:0 is the most abundant where as in wheat it is the C21:0 -homologue. Rye and wheat can be compared also with the ratio C17:0 /C21:0, which is in rye approximately 1 and in wheat around 0.1 (Ross *et al*. 2003).

Alkylresorcinols can be enriched in bran fractions by milling processes (Heiniö *et al*. 2008, Liukkonen *et al*. 2003, Ross *et al*. 2003, Ross *et al*. 2001), although by milling, the pure botanical fractions cannot be produced. In barley seeds alkylresorcinols have been found in seed´s epicuticular wax (Garcia *et al*. 1997). In rye leaves alkylresorcinols were found in the intracuticular wax layer (Ji & Jetter 2008), which is corroborated by the findings of Landberg *et al*. (2008) that in wheat and rye kernels the alkylresorcinols are located in the outer cuticle of testa/inner cuticle of the pericarp.

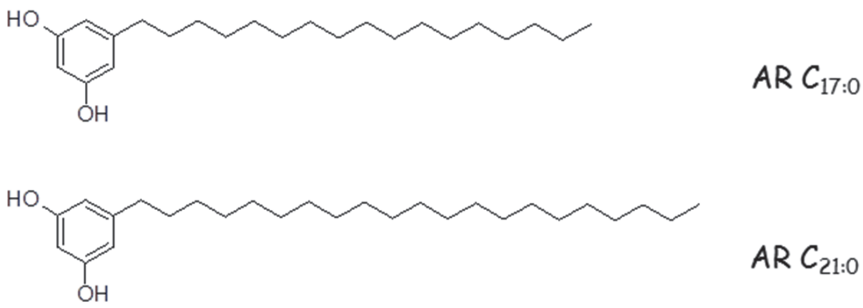



**Figure 3**. Examples of alkylresorcinol (AR) structures in cereals.

Alkylresorcinols have antifungal activity against a number of fungi, as reviewed by Stasiuk and Kozubek (2010). Resistance of barley seeds against pathogenic fungi *Aspergillus niger* and *Penicillium crysogenum* has been attributed to alkylresorcinols (Garcia *et al*. 1997). Recently, it was reported that rye bran alkylresorcinols could inhibit growth of *Penicillium expansum* and *Neofabraea perennans*, which cause post-harvest diseases in apples (Dey *et al*. 2013, Tahir *et al*. 2014).

### 2.2.2 Alkylresorcinols in foods

In the human diet whole-grain wheat and rye are the main sources of alkylresorcinols. These compounds in rye have received considerable interest during the last 10 years because of their possible contribution to positive health effects (Bondia-Pons *et al*. 2009, Ross *et al*. 2004), but also as a biomarkers of intake of whole-grain rye (Andersson *et al*. 2011, Chen *et al*. 2004b, Landberg *et al*. 2014a, Ross *et al*. 2012, Ross 2012, Söderholm *et al*. 2009). Alkylresorcinols are absorbed and metabolized in humans (Ross *et al*. 2004), but also stored as such in adipose tissue and liberated again from there (Jansson *et al*. 2010).

Content of alkylresorcinols in Swedish cereal food products has been reported by Menzel *et al*. (2012), in Norwegian flours and breads by Andersson *et al*. (2010), in Polish cereal products by Kulawinek *et al*. (2008) and in Latvian and Finnish breads by Meija *et al*. (2013). These studies show that whole-grain rye and wheat breads can be good sources of alkylresorcinols. Examples of the content of alkylresorcinols in rye products are given in the Results and Discussion sections.

Compared to other bioactive compounds, such as benzoxazinoids and avenanthramides, the malting process with an extensive germination step (6 d, 18 °C, 48 % moisture; pilsner malt drying, final temp 83°C) did not cause any clear effects on the alkylresorcinol content in four rye, three wheat and three barley cultivars (Pihlava *et al*. 2008) (**Figure 4**).

Health effects of alkylresorcinols are currently not well known. However, there are some indications that alkylresorcinols could affect the properties of the biological membranes and limit e.g. LDL oxidation (Bondia-Pons *et al*. 2009, Stasiuk and Kozubek 2010). There is indication that alkylresorcinols suppress the synthesis of testosterone and estradiol, and thus could be used in the chemoprevention of prostate and breast cancer (Oskarsson & Andersson 2016). Recently, Landberg *et al*. (2014b) reviewed the occurrence, pharmacokinetics and bioavailability of alkylresorcinols in rye as well as their use as biomarkers (Landerg *et al*. 2014a).

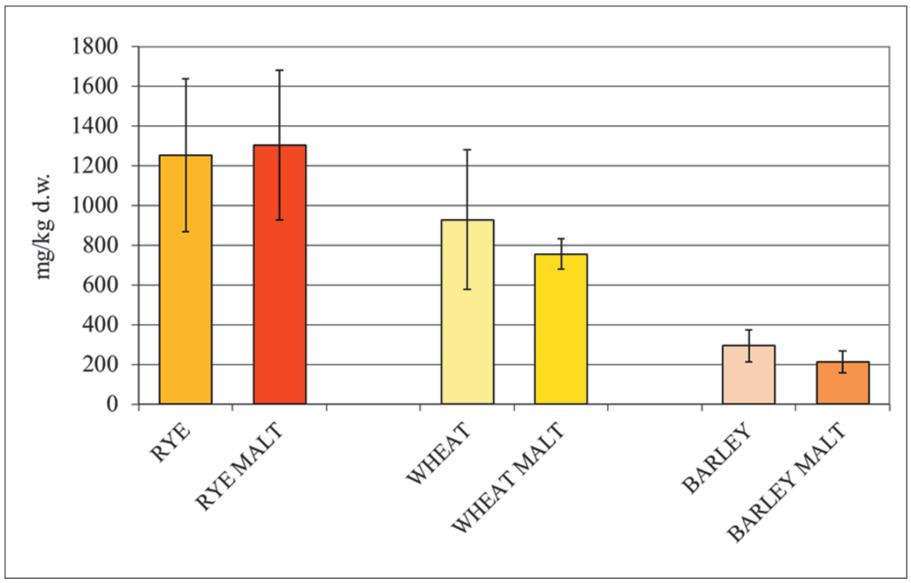



**Figure 4.** Total alkylresorcinol contents (mg/kg dw.) in four rye cultivars, three wheat cultivars, three barley cultivars and their corresponding malts (germination 6 d, 18 °C; pilsner malt drying, final temp 83°C) (Pihlava *et al*. 2008).

### 2.2.3 Analysis of alkylresorcinols

Alkylresorcinols are extractable by ethyl acetate, acetone and methanol in cereal raw material. However, hot IPA-$H_2O$ (3:1) extraction is more efficient for extracting alkylresorcinols from bread, because alkylrecorsinols are being trapped in starch-lipid complexes (Ross *et al*. 2003). Both LC and GC have been used in determination of alkylresorcinols. Alkylresorcinols can be analyzed without silylation by GC (Andersson *et al*. 2010, Landberg *et al*. 2008, Menzel *et al*. 2012), although silylation has also been used (Ji & Jetter 2008, Ross *et al*. 2001, 2003).

Reverse phase LC-$MS^n$ has been applied for alkylresorcinol profiling in rye, wheat, spelt and barley (Knödler *et al*. 2008). Due to the relative nonpolar nature of alkylresorcinols, atmospheric pressure chemical ionization (APCI) is preferred interface over the electrospray ionization (ESI) interface. APCI was also used in normal phase UPLC-MS/MS for analysis of alkylresorcinols in plasma (Ross *et al*. 2010) and by reverse phase mode in wheat breads (Geng *et al*. 2016). On the other hand, Koistinen *et al*. (2016) identified alkylresorcinols in rye bran samples by UHPLC-QTOF MS with ESI operated in negative mode.

Ross (2012) compared fluorescence, ultraviolet and CoulArray electrochemical (CAED) detectors attached to ultra-high pressure liquid chromatography in alkylresorcinol analysis of cereals. CAED was the most sensitive detector, followed by the fluorescence detector. The UV -detector was the least sensitive one and was also the least selective, resulting in overestimation of alkylresorcinol concentrations.

Examples of the analysis of alkylresorcinols are listed in **Table 2.**

**Table 2.** Examples of the analysis of alkylresorcinols. Abbreviations are listed in pages iii-iv.

| ALKYLRESORCINOLS<br>Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC)<br>or Column (GC) | Reference |
|---|---|---|---|---|
| $CHCl_3$ | elution in TLC: 1.step $CH_2Cl_2$-MeOH (97:3) and 2. step Toluene-PE (1:4) | TLC-scanning densitometer | visualization with Fast Blue B | Garcia *et al*. 1997 |
| n-ButOH saturated with $H_2O$<br>40 min sonication | centrifugation | UHPLC-APCI-LTQ Orbitrap | Poroshell 120EC-C18<br>2.7 µm, 2.1*150 mm<br>$H_20$-MeOH + 0.1 % formic acid | Geng *et al*. 2016 |
| $CHCl_3$: MeOH (2:1) | cleanup by TLC with $CHCl_3$-EA (85:15) as solvent, bands scraped off and extracted with $CHCl_3$; silylation | GC-MS, GC-FID | HP-1<br>30 m*0.32 mm i.d. | Ji & Jetter 2008 |
| DE | evaporation of supernatant; cleanup by Oasis MAX SPE; silylation | GC-MS | TR-5<br>15 m*0.25 mm i.d. | Jansson *et al*. 2010 |
| methanol, 19 h | evaporation of supernatant | HPLC-DAD | NovaPak C-18<br>4 µm, 3.9 *150 mm<br>P-buffer (pH 2.4)- MeOH | Heiniö *et al*. 2008<br>Liukkonen *et al*. 2003 |
| EA 1 h | evaporation of supernatant; to $CHCl_3$; cleanup by polyamide CC-6 SPE | HPLC-DAD-APCI-ITD MS | Aqua C-18<br>3 µm, 3 *150 mm<br>MeOH-$H_2O$ | Knödler *et al*. 2008 |

| (…ALKYLRESORCINOLS) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| plasma: deproteinization, extraction with DE | evaporation of supernatant; cleanup by Oasis MAX SPE; silylation | GC-MS | TR-5 15 m*0.25 mm i.d. | Landberg *et al.* 2009 Andersson *et al.* 2011 |
| Acetone 48 h breads: hot IPA-$H_2O$ (3:1) 5 h | filtration; evaporation of filtrate | HPLC-PDA | XTerra C18 5 µm, 4.6 *250 mm MeOH-$H_2O$ | Kulawinek *et al.* 2008 |
| hot IPA | evaporation of supernatant, (silylation ?) | GC-MS | BP-5 12.5 m*0.22 mm i.d. | Meija *et al.* 2012 |
| EA 24 h<br><br>breads: hot IPA-$H_2O$ (3:1) 5 h | evaporation of supernatant, silylation<br><br>evaporation of supernatant | GC-FID | BP-5 30 m*0.33 mm i.d. | Ross *et al.* 2001 Ross *et al.* 2003 Menzel *et al.* 2012 Andersson *et al.* 2010 Landberg *et al.* 2008 |
| EA 24 h<br><br>breads: hot IPA-$H_2O$ (3:1) 5 h | evaporation of supernatant | UHPLC-DAD, -FLD, -CAED | Kinetex 1.7 µm, 2.0 *150 mm DAD/FLD: MeOH/H2O(89:11)- MeOH/H2O (99:1) CAED: MeOH/H2O/5M Aac (89:10:1)- MeOH/5M Aac (99:1) | Ross 2012 |

| (…ALKYLRESORCINOLS) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| foods: hot IPA-H2O (3:1) 5 h | evaporation of supernatant | HPLC-CAED | Zorbax Extend C18<br>3.5 µm, 3.0 *150 mm<br>MeOH/H2O/5M Aac (89:10:1)-<br>MeOH/5M Aac (99:1) | Ross *et al.* 2012 |
| plasma: enzymatic hydrolysis | LLE with DE; evaporation of DE | HPLC-CAED | Inertsil ODS-3<br>3 µm, 3.0 *150 mm<br>50 mM P-buffer(pH 2.3)/MeOH<br>(90:10)-P-buffer/MeOH/ACN<br>(40:40:20) | Söderholm *et al.* 2009 |

## 2.3 Lignans in cereals

### 2.3.1 Background

Lignans are diphenolic compounds widely distributed in the plant kingdom and also found in cereals (**Figure 5**). Although the biological role of lignans is not yet clear, it is generally assumed that they act as plant defense compounds. For example pinoresinol and secoisolariciresinol have been shown to inhibit *in-vitro* growth and tricothecene biosynthesis in five *Fusarium graminearum* strains, on which pinoresinol was more effective on producers of DON and secoisolariciresinol more effective on NIV producers (Kulik *et al*. 2014).

Typical lignans in cereals are secoisolariciresinol, matairesinol, pinoresinol, lariciresinol and syringaresinol (Smeds *et al*. 2007). Number of other lignans has been reported in cereals such as 7-hydroxymatairesinol, medioresinol, cyclolariciresinol, oxomatairesinol, α-conidendrin and todolactol A (Smeds *et al*. 2007), buddlenols C-E, hedyotisol A, methoxyhedyotisol A, hydroxymedioresinol and hydroxysyringaresinol (Hanhineva *et al*. 2012). Based on results using different sample preparation methods, Smeds *et al*. (2007) concluded that most of the lignans are linked to lignin structures, although soluble free and esterified forms, especially of syringaresinol, are also present in cereals. Contrary to cereals, in flaxseed, which is the richest foodborne source of lignans, lignan secoisolariciresinol is present as diglucoside units esterified via 3-hydroxy-3-methyl-glutaric acid (HMGA) to polymers (Kamal-Eldin *et al*. 2001), whereas in trees considerable amounts of lignans are in free form (Willför *et al*. 2006).

Like other phenolic compounds, lignans are enriched in the bran fractions of the grains (Liukkonen *et al*. 2003, Heiniö *et al*. 2008, Glitsø *et al*. 2000). The one exception is the 4,4′-dihydroxy-3,3′-dimethoxy b-truxinic acid, a lignan containing dicarboxylic acid-cyclobutane -structure, esterified to sucrose (aka tase), which was found in oat groats (101–150 mg/kg) but not in hulls (Dimerg *et al*. 2001a). Since cyclobutane structures are readily formed by light-mediated dimerization of hydroxycinnamoyl sugar esters, some doubt whether these were actual secondary metabolites or just artifacts, has been presented (Collins 2011). However, cyclobutanedicarboxylic acid structures have been found in a number of other plants as well (Krauze-Baranowska 2002).

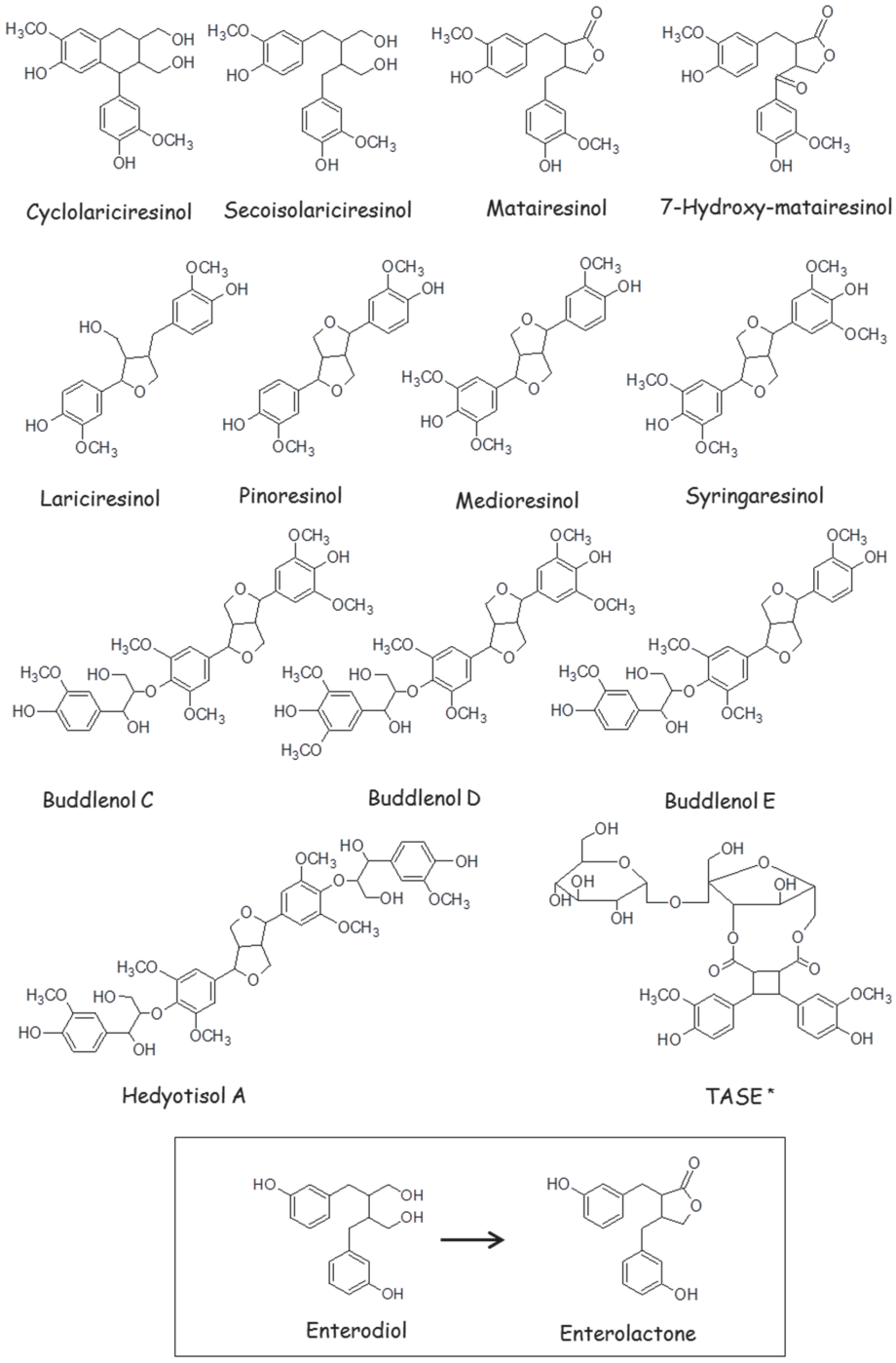



**Figure 5.** Example of lignans in cereals and mammalian lignans, enterodiol and enterolactone. * TASE = 4,4´-dihydroxy-3,3´-dimethoxy b-truxinic acid sucrose ester is a lignan found in oats.

### 2.3.2 Cereal lignans in food

As ingested plant lignans, secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, 7-hydroxymatairesinol and syringaresinol, are completely or partly converted to mammalian lignans or enterolignans, namely enterolactone and enterodiol, by colon microbiota, which are then absorbed and metabolized (Heinonen *et al*. 2001, Adlercreutz 2007, Landete 2012). Besides enterolactone and enterodiol, also matairesinol, lariciresinol and cyclolariciresinol have been detected in serum samples indicating that plant lignans can also be absorbed as intact aglycons (Smeds *et al*. 2006). Bolvig *et al*. (2016) came to a conclusion that part of the plant lignans are indeed absorbed in the small intestine, but they are also rapidly cleared from the body. Epidemiological studies indicate that there is an inverse association with increased enterolignan levels and risk of breast, colon and prostate cancer (Adlercreutz 2007, Landete 2012). Bioavailability of rye lignans has been reviewed by Peñalvo et al. (2014).

Intake of lignans, which are converted to enterolignans, was evaluated to be 1.08, 1.77 and 1.45 mg/d in Finland, Sweden and Denmark, respectively. Cereals and cereal products contributed 26-43 % of the total intake of lignans (Tetens *et al*. 2013). Smeds *et al*. (2007) listed cereals in the order of total lignan content: rye, wheat, triticale, oat and barley.

### 2.3.3 Analysis of lignans

Traditionally lignans have been analyzed by GC-MS and only recently has LC-MS/MS gained popularity in lignan analysis. Various aspects of the lignans analysis have been reviewed by Willför *et al*. (2006). Choosing an appropriate sample preparation method is important in lignan analysis, especially since the common lignans in cereals are not in readily extractable form. Using alkali and/or acid hydrolysis, more lignan structures could be detected compared to the sample extracts made by accelerated solvent extraction (ASE) (Smeds *et al*. 2007). However, recently some new lignans in readily extractable form have been identified by Hanhineva *et al*. (2012).

As a curiosity, flaxseed lignan, secoisolariciresinol diglucoside (SDG), is in polymeric form and can be conveniently analyzed by HPLC-UV after alkaline hydrolysis (Eliasson *et al*. 2003, Willför *et al*. 2006).

Examples of the analysis of lignans are listed in **Table 3.**

**Table 3**. Examples of the analysis of lignans. Abbreviations are listed in pages iii-iv.

| **LIGNANS**<br>**Sample extraction** | **Sample preparation** | **Equipment** | **Column & mobile phase (LC)**<br>**or Column (GC)** | **Reference** |
|---|---|---|---|---|
| methanol | evaporation of supernatant | HPLC-DAD | Hypersil ODS<br>5 µm, 4.0 *125 mm<br>P-buffer (5 % ACN) - ACN | Dimerg *et al*. 2001 |
| (fat-free with Hex), 2 M NaOH 1h | adjustment of pH to 3 with $H_2SO_4$; centrifugation; addition of 95 % EtOH; centrifugation | HPLC-DAD | Econosil RP C18<br>5 µm, 4.6 *250 mm<br>P-buffer (pH 2.8, 5 % ACN) - ACN | Eliasson *et al*. 2003 |
| enzymatic treatment + acid hydrolysis | LLE with DE-EA; evaporation of DE-EA; clean-up by DEAE and QAE columns, silylation | GC-MS | BP-1<br>12 m*0.22 mm i.d., $d_f$ 0.25 | Glitsø *et al*. 2000<br>Heiniö *et al*. 2008 |
| 75 % MetOH-0.1 % formic acid | centrifugation | UPLC-QTOF MS | BEH C-18<br>5 µm, 2.1 *125 mm<br>$H_20$ (5 % ACN)- ACN + 0.1 % formic acid | Hanhineva *et al*. 2012 |
| fecal slurry: DE | evaporation of DE extract; cleanup by Lipidex 5000 SPE; silylation | GC-MS | BP-1<br>12 m*0.22 mm i.d., $d_f$ 0.25 | Heinonen *et al*. 2001 |

| (…LIGNANS) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| 70 % MeOH + 0.3 M NaOH 1 h | pH adjustment, centrifugation, enzymatic treatment, C18 SPE, clean-up by DEAE column, silylation | GC-MS | BP-1 12 m*0.22 mm i.d., $d_f$ 0.25 | Peñalvo *et al*. 2005 |
| serum: enzymatic treatment 19 h | cleanup by Oasis HLB SPE | HPLC-MS-MS | Hypersil BDS 3 µm, 2.0 *125 mm $H_2O$ (1 % IPA)- MeOH:$H_2O$ (0.01 % IPA) + 0.1 % formic acid | Smeds *et al*. 2006 |
| ASE: hexane; acetone; acetone-$H_20$ (7:3) | evaporation of extract, enzymatic treatment, LLE with EA | HPLC-MS-MS | Zorbax SB-C8 3.5 µm, 2.1 *100 mm $H_2O$(1% IPA + 0.1 % HOAc)-MeOH/ACN (1:1) $H_2O$(1% IPA + 0.05% TFA)-MeOH/ACN (1:1) | Smeds *et al*. 2007 |
| 70 % MeOH (0.3 M NaOH) 1 h 60 °C | centrifugation; aliquot to enzymatic treatment; LLE with EA | HPLC-MS-MS | Zorbax SB-C8 3.5 µm, 2.1 *100 mm $H_2O$(1% IPA + 0.1 % HOAc )-MeOH/ACN (1:1) | Smeds *et al*. 2009 |

## 2.4 Flavonoids in cereals

### 2.4.1 Background

Flavonoids are ubiquitously distributed in the plant kingdom. Flavonoid aglycons can be divided into several subgroups such as flavones (e.g. apigenin, luteolin, tricin, tangeritine), flavonols (e.g. quercetin, kaempferol, myricetin, isorhamnetin), flavanonols (e.g. taxifolin), flavanons (e.g. hesperitin, eriodictyol, naringenin), flavan-3-ols (e.g. catechin, epicatechin), proanthocyanidin oligomers, isoflavons (e.g. genistein, daidzein, glycitein) and anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin). Examples of the chemical structures of flavonoids are presented in **Figure 6** and in the **Figure 23** in the Results and Discussion (p.106).

Flavonoids play central roles in a number of processes such as plant-pathogen interactions, pollination, seed development, photoprotection and allelopathy. Their biosynthesis can be induced as a result of biotic or abiotic stress (Hernández *et al*. 2009, Moheb *et al*. 2013a). An example of biotic stress is the induced production of C-glycosylflavones (sugar O-conjugates of isoswertisin, isovitexin and iso-orientin) in roots and shoots of oats after attack by plant-parasitic nematodes. Concentrations of these flavonoids were also increased by application of the wound hormone methyl jasmonate (Soriano *et al*. 2004). In maize leaves, the amount of rutin (quercetin-rutinoside) and kaempferol rutinoside was increased after attack by the caterpillar *Spodoptera littoralis* (Marti *et al*. 2013). A number of flavonoids has been identified as resistance related constitutive metabolites in barley varieties, which were more resistant to Fusarium head blight (FHB) caused by *Fusarium graminearum* (Bollina *et al*. 2011). Certain flavonoids have also been found to correlate to multiple quality traits in malting barley (Heuberger *et al*. 2014).

Flavonoids are normally present as O-glycosidic conjugates in berries, fruits and vegetables. In cereals such as maize, wheat and rice, flavonoids are also accumulated as C-glycosidic forms (Brazier-Hicks *et al*. 2009). In wheat leaves or grains, besides flavonoid O-glycosides and C-glycosides, combinations of O,C-diglycosides and C,C-diglycosides have been reported (Cavaliere *et al*. 2005, Dinelli *et al*. 2011, Feng *et al*. 2008, Kamiyama & Shibamoto 2012, Ma *et al*. 2014, Moheb *et al*. 2011, Wojakowska *et al*. 2013). The chemical diversity of flavonoid conjugates is further broadened with acylation of sugars by phenolic and simple organic acids (Wojakowska *et al*. 2013). Mixed O,C-

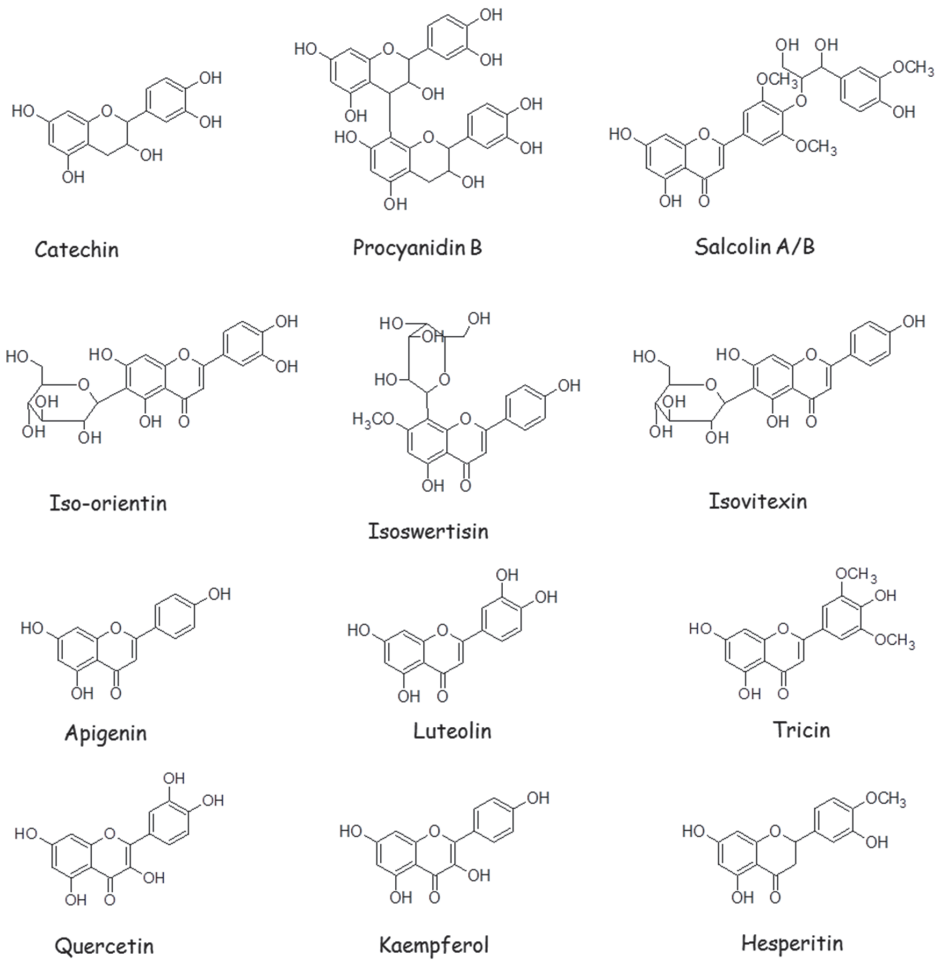



**Figure 6.** Examples of flavonoids. Catechin belongs to flavan-3-ols and procyanidin B is a dimer of catechin. Salcolin A/B is a flavonolignan. Iso-orientin, isoswertisin and isovitexin are C-glucosides: glucose attached to the flavonoid structure by C-C –bond. In iso-orientin and isovitexin glucose is attached to the C6-position, while in isoswertisin it is attached to the C8-position. Apigenin, luteolin and tricin are flavones, quercetin and kaempferol are flavonols. Hesperitin belongs to flavanons and it has not been reported in cereals.

glycosides has been reported also from barley leaves (Nørbæk *et al*. 2000, Nørbæk *et al*. 2003). Luteolin O-glycosides and isovitexin O-glycosides have been found in rye primary leaves (Schulz *et al*. 1985, Dellamonica *et al*. 1983).

A special class of polyphenols, namely flavonolignans, were isolated from green parts of oats (aka oat herb). All of these three compounds were tricin derivatives. Salcolin A and B had been previously found in three other plants, while the third compound was a novel one (i.e.(-) -(5S,6S)-5,6-dihydro-3,8,10-trihydroxy-5-(4-hydroxy-3-methoxyphenyl)-6-hydroxymethyl-2,4-dimethoxy-7H-benzo[c]xanthen-7-one) composed of a tricin and coniferyl alcohol moiety (Wenzig *et al*. 2005).

Extensive review of flavonoids in oats has been done by Collins (2011).

### 2.4.2 Cereal flavonoids in food

Tricin, which belongs to the flavone-group, has been found in wheat bran at concentrations of 33–45 mg/kg dw. In the non-edible husks, the concentration of tricin was 408–772 mg/kg dw, corresponding 33–45 % of the detected phenolic compounds (Moheb *et al*. 2013b). Skoglund (2008) reported tricin in oat hull samples at concentrations of 10–41 mg/kg dw (n=28), but in most cases (104 samples) concentration of tricin was below 10 mg/kg. Tricin was not found in oat groats.

Flavan-3-ols and proanthocyanidins in barley and barley malts are of interest because they can form unwanted turbidity or haze together with certain proteins in beers (Steiner *et al.* 2010) and also participate in discoloration of other barley food products (Kohyama *et al.* 2009). Besides monomeric flavan-3-ols((+)-catechin and (-)-epicatechin), dimeric forms, such as prodelphinidin B3 and procyanidin B3, as well as trimeric forms are typical to barley (Goupy *et al*. 1999, Dvorakova *et al*. 2008). In nine barley varieties the total flavan-3-ol content as (+)-catechin equivalents were 12–225 mg/kg. In the same study, the total content of unspecified flavonols in barley varieties was 11–67 mg/kg as rutin equivalents (Goupy *et al*. 1999). In flours from three pearled (6 %) barley cultivars, the total flavan-3-ol concentration was 854–1094 mg/kg as (+)-catechin equivalents. In coarse (40 % of the pearled barley) and fine (60 %) fractions produced by air classification from the barley flour, the total flavan-3-ol concentration was 1380–1721 mg/kg and 495–728 mg/kg, respectively, as (+)-catechin equivalents (Verardo *et al*. 2011). Considering the flavan-3-ol content as absolute amounts, 63–70 % of the flavan-3-ols were distributed to the coarse fraction. With the development of analytical methods, it has been found that barley contains also tetra and pentamers of flavan-3-ols, i.e. proanthocyanidins (Hellström *et al*. 2009, Verardo *et al*. 2015). According to

Verardo *et al*. (2015) the distribution of the total flavan-3-ols in 14 barley samples was as follows: monomers 6–12 %, dimers 33–37 %, trimers 46 %, tetramers 7–9 % and pentamers 2 %. Of the total proathocynidins roughly 1/3 is in unextractable form (Hellström *et al*. 2009).

Apigenin-C-glycosides, as well as sinapoyl derivatives of apigenin-C-diglycosides, were identified in durum and soft wheat by HPLC-MS. The total amount of flavonoids was 50–300 mg/kg expressed as apigenin-7-O-glucoside (Heimler *et al*. 2010). In a purple wheat variety, the total anthocyanin content was 491 mg/kg, of which cyanidin sugar conjugates formed a major part (42 %), pelargonidin glycosides 13 %, delphinidin 11 %, malvidin 10 %, petunidin 8 % and peonidin glycosides 6 % (Hosseinian *et al*. 2008). In the blue wheat variety 'Purendo' the total anthocyanin content was 148 mg/kg, with delphinidin and cyanidin being the major aglycons (Abdel-Aal *et al*. 2014).

Zieliński *et al*. (2007) and Michalska *et al*. (2007) reported total flavonoid contents of 290–307 mg/kg and 431 mg/kg as catechin equivalent in whole-grain rye, using spectrophotometric methods. In coarse and fine rye bran fractions, the flavonoid content was 1910 and 2390 mg/kg, respectively, as quercetin equivalent (Ivanišova *et al*. 2012). Michalska *et al*. (2007) reported the distribution of flavonoids into the bran fraction (30 % yield of the wholegrain) being 46 %. The anthocyanins in the pericarp of purple-seeded rye varieties were identified as cyanidin and peonidin 3-glycosides and their acylated derivatives (Dedio *et al*. 1972). In green-seeded rye varieties, the color is located in the aleurone layer and is due to delphinidin 3-rutinoside (Dedio *et al*. 1969).

Total flavonoid content as quercetin of three barley, four oat, four rye and three wheat cultivars is presented in **Figure 7**. Although the concentrations are quite small and the number of cultivars included is limited, it appears that there could be cultivar-related differences in the flavonoid content. The amount of major proanthocyanins and catechins as catechin equivalent in the barley cultivars was 339–440 mg/kg (Pihlava *et al*. 2010).

There is increasing evidence that dietary flavonoids can reduce the risk of cancers, cardiovascular disease, type II diabetes and neurodegerenative diseases (Del Rio *et al*. 2013, Rodriguez-Mateos *et al*. 2014). The main sources of anthocyanins and flavonoids in our diet are fruits, berries and vegetables. Also, the contribution of tea to flavonoid intake is important (Ovaskainen *et al*. 2008). Common cereals have not been considered as important sources of dietary flavonoids.

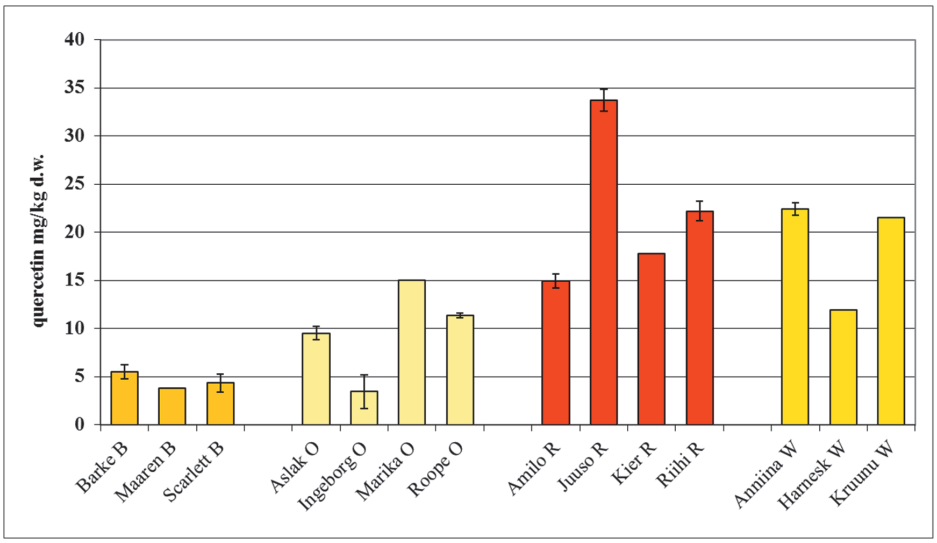



**Figure 7**. Total flavonoid content in barley (B), oats (O), rye (R) and wheat (W) cultivars as quercetin equivalents (mg/kg) (Pihlava *et al*. 2010). In the case of barley, the proanthocyanidins were not included.

### 2.4.3 Analysis of flavonoids

Due to their polar nature, flavonoids are exclusively analyzed by LC. Flavonoids with their various glycosides set a great challenge for their identification and in practice a triple quadrupole MS, QTOF or ion-trap MS is needed for this work. Fragmentation of flavonoid glycosides by multi-stage mass spectrometry has been presented by Vukics & Guttman (2010). QTOF and ion-trap MS has been compared in the analysis of C-glycosidic flavonoid isomers (Waridel *et al*. 2001).

Quantification of flavonoids is also challenging. Acid hydrolysis is an often-used sample preparation method, since analysis of only aglycons of flavonoids will simplify the quantitation.

Due to the poor UV-characteristics of flavan-3-ols, these compounds are best analyzed by liquid chromatography combined with fluorescence detection. Mass spectrometry is used as a confirmation technique.

Examples of the analysis of flavonoids are listed in **Table 4**.

**Table 4**. Examples of the analysis of flavonoids. Abbreviations are listed in pages iii – iv. (na) = information not given

| **FLAVONOIDS**<br>**Sample extraction** | **Sample preparation** | **Equipment** | **Column & mobile phase (LC)** | **Reference** |
|---|---|---|---|---|
| comparison of CSE, MAE and ASE | (centrifugation in CSE); evaporation | HPLC-PDA<br>HPLC-IT MS | Zorbax SB-C18<br>3.5 µm, 4.6 *75 mm<br>6 % formic acid-MeOH | Abdel-Aal *et al*. 2014 |
| 65 % MeOH 15 min (sonication) | centrifugation | LC-LTQ-Orbitrap | Jupiter C18<br>5 µm, 0.5 *100 mm<br>2.5 mM ammonium acetate-MeOH | Bollina *et al*. 2011 |
| 70 % MeOH | C18 SPE; Oasis HLB SPE | LC-QLIT MS | Alltima C18<br>5 µm, 2.1 *250 mm<br>$H_2O$- ACN +<br>0.1 % formic acid | Cavaliere *et al*. 2005 |
| ACE:MeOH: $H_2O$ (2:2:1) | Discovery DPA-6S SPE | HPLC-DAD-FLD | Luna Si<br>5 µm, 4.6 *250 mm<br>$CH_2Cl_2$/MeOH/$H_2O$/HOAc-<br>$CH_2Cl_2$/MeOH/$H_2O$/HOAc<br>(41/7/1/1 : 5/43/1/1)<br>Inertsil ODS-3<br>3 µm, 4.0 *150 mm<br>50 mM P-buffer ( pH 2.5) -ACN | Hellström *et al*. 2009 |

| (…FLAVONOIDS)<br>Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| hot MeOH-$H_2O$ (85:15) | concentration to aq; LLE with hexane; aq phase hydrolyzed with 2 M HCl 95 °C 0.5 h; LLE with EA, evaporation of EA<br>concentration to aq; LLE with hexane; aq phase analyzed | HPLC-DAD, LC-MSMS, LC-QTOF MS | Zorbax SB-C18<br>3.5 µm, 2.1 *30 mm<br>0.1 % formic acid-MeOH<br>Polaris 5-C18-A<br>5 µm, 4.6 *150 mm<br>1 % HOAc-40 % MeOH | Moheb *et al.* 2011<br>Moheb *et al.* 2013a<br>Moheb *et al.* 2013b |
| 50 % ACN 1 h | evaporation; fractionation by Amberlite XAD-7; clean up of certain fractions by prep HPLC | HPLC-UV | Develosil ODS-HG-5<br>(na) µm, 4.6*250 mm<br>H2O/ACN (42:8)-ACN/$H_2O$ (1:1) | Nørbæk *et al.* 2000 |
| 40 % ACN (pH 3) 20 h | | HPLC-UV | LiChrosper C18<br>(na) µm, 4.0*250 mm<br>$H_2O$-ACN (pH 3) | Nørbæk *et al.* 2003 |
| MeOH:$H_2O$ (4:1) | fractionation by polyamide CC; prep TLC; clean up by Sephadex LH-20 | HPLC-UV | LiChrosorb RP-8<br>µm (na), 4.0*250 mm<br>1 % $H_3PO_4$-ACN | Shulz *et al.* 1985 |
| 80 % EtOH 50 °C 20 min | centrifugation; evaporation | HPLC-DAD | Zorbax-SB-C18<br>5 µm, 4.6 *30 mm<br>10 mM P-buffer/ACN (95:5, pH 2.8) -ACN | Skoglund 2008 |

| (…FLAVONOIDS) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| MeOH 48 h | extract: addition of $H_2O$; LLE with hexane (48 h); evaporation of aq phase; addition of $H_2O$; LLE with BuOH; evaporation of BuOH | HPLC-UV<br><br>LC-MS | Sperisorb ODS-2<br>5 µm, 4.6 *150 mm<br>$H_2O$-MeOH<br><br>Synergi Hydro-RP<br>5 µm, 4.6 *150 mm<br>$H_2O$-$H_2O$/ACN(16:84) +<br>5 % formic acid | Soriano *et al*. 2004 |
| 80 % MeOH (*vs*. 80 % EtOH *vs*. 80 % Acetone) | centrifugation; evaporation | HPLC-DAD-MSD | Luna C18<br>5 µm, 3.0 *250 mm<br>1% HOAc- 1% HOAc/ACN (60:40) | Verardo *et al*. 2011 |
| ACE:$H_2O$ (4:1) | centrifugation; evaporation | HPLC-FLD-MSD | Develosil Diol<br>5 µm, 4.6 * 250 mm<br>Eluents | Verardo *et al*. 2015 |
| Soxhlet extraction: PE, $CH_2Cl_2$ | evaporation of $CH_2Cl_2$; fractionation by Sephadex LH-20 (EA as eluent); C18 CC; semi-prep HPLC; finally Sephadex LH-20 | semi-prep HPLC | LiChroCart RP-18<br>7 um, 10 *250 mm<br>ACN-$H_2O$/TFA (20:80) | Wenzig *et al*. 2005 |

| (…FLAVONOIDS) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| Soxhlet extraction: PE, $CH_2Cl_2$ | evaporation of $CH_2Cl_2$; fractionation by Sephadex LH-20 (EA as eluent); C18 CC; semi-prep HPLC; finally Sephadex LH-20 | semi-prep HPLC | LiChroCart RP-18<br>7 um, 10 *250 mm<br>ACN-$H_2O$/TFA (20:80) | Wenzig *et al*. 2005 |
| 80 % MeOH 30 min | centrifugation; evaporation; cleanup by C18 SPE | UPLC-QTOF MS | Poroshell 120 EC-C18<br>2.7 um, 2.1 *100 mm<br>$H_2O$-ACN + 0.5 % formic acid | Wojakowska *et al*. 2013 |
| 80 % MeOH | centrifugation; dilution with H2O; addition of 5 % NaNO2; addition of10 % AlCl3*6 $H_2O$; addition of 1 M NaOH | spectrophotometer | 510 nm as catechin | Zieliński *et al*. 2007<br>Michalska *et al*. 2007 |

## 2.5 Benzoxazinoids in rye, wheat, triticale and maize

### 2.5.1 Background

Benzoxazinoids are plant defense compounds found of the major agricultural crops in maize (*Zea maize*), wheat (*Triticum aestivum*), triticale (x *Triticosecale*) and rye (*Secale cereale* L.) but not in oats (*Avena sativa*) or barley (*Hordeum vulgare*). However, they are found in some wild *Hordeum* species, such as *H. brachyantherum*, *H. flexuosum*, *H. lechleri* and *H. roshevitzii* (Grün *et al*. 2005). Benzoxazinoids are hetero-aromatic compounds and they can be divided into three chemical groups, namely hydroxamic acids (e.g. DIBOA, DIMBOA, $DIM_2BOA$ and HDMBOA), lactams (HBOA and HMBOA) and benzoxazolinones (BOA and MBOA) (**Figure 8**). Hydroxamic acids and lactams are also present in plant cells as glycosides.

### 2.5.2 Biosynthesis of benzoxazinoids

The presence of DIBOA and its glucoside in rye and DIMBOA and its glucoside in maize and wheat was first reported over 50 years ago (Hietala & Virtanen 1960, Virtanen & Hietala 1955, Virtanen & Hietala 1960, Virtanen *et al*. 1956, Wahlroos & Virtanen 1959). Biosynthesis of benzoxazinoids has been widely studied in maize. Briefly, the biosynthesis starts from the indole-3-glyserolphophate originating from the biosynthesis of tryptophan linking primary metabolism to secondary metabolism. In the first step enzyme Bx1 (indole-3-glycerolphosphate lyase) produce indole in the chloroplast, the following four oxidative steps in microsomes in the endoplasmic reticulum involves cytochrome P450 monooxygenases (Bx2−Bx5) leading to DIBOA. In the next step DIBOA is glucosylated at the 2-position by UDP-glucosyltransferases (Bx8/9). DIBOA glucoside is then methoxylated to DIMBOA in two steps, requiring oxoglutarate-dependent dioxygenase (Bx6) and methyltransferase (Bx7) (Niemeyer 2009, Frey *et al*. 2009). In maize DIMBOA-Glc can be further converted to HDMBOA-Glc by 4-O-methyltrasferase (Glauser *et al*. 2011) and via TRIMBOA-Glc (Bx13) to $DIM_2BOA$-Glc (Bx7) and finally to $HDM_2BOA$-Glc (Bx14) (Handrick *et al*. 2016) (**Figure 9**).

DIBOA and DIMBOA are stored in the vacuoles as in less-toxic glucosidic forms, while the specific glucosidases are stored in the plastids (Niemeyer 2009, Frey *et al*. 2009, Schullehner *et al*. 2008). Ahmad *et al*. (2011) proposed recently that in maize DIMBOA-Glc and HDMBOA-Glc accumulate in the apoplast.

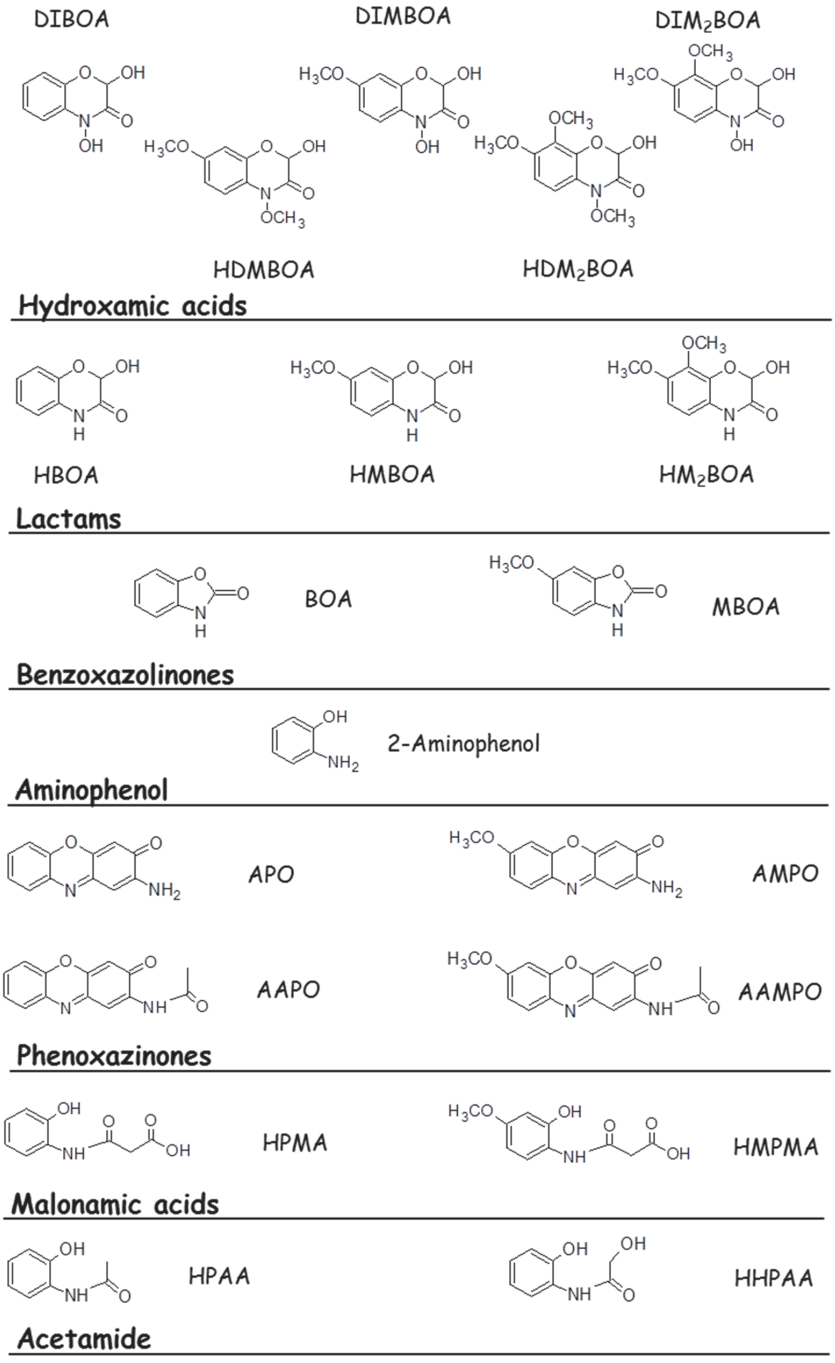



**Figure 8**. Various benzoxazinoids and conversion products of BOA and MBOA (adopted from Fomsgaard *et al*. 2004). More benzoxazinoid structures are presented in **Figure 9**.

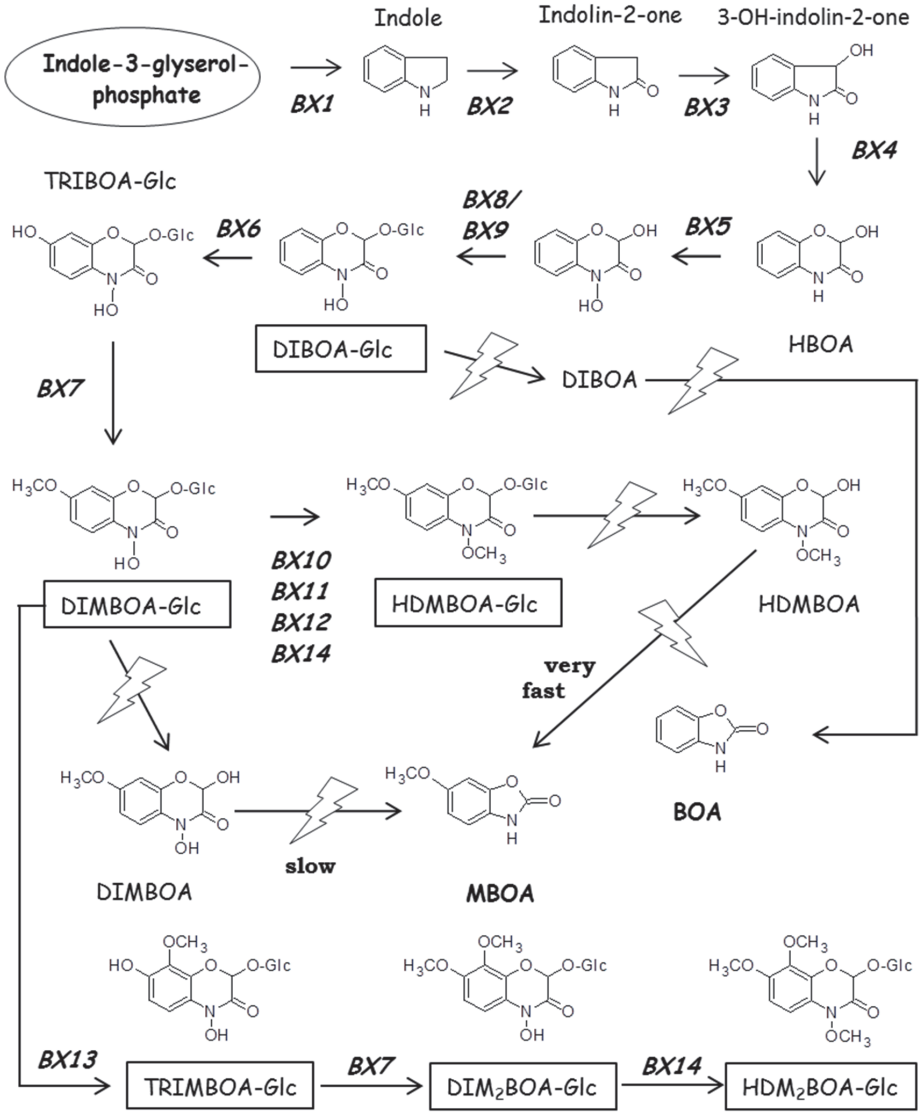



**Figure 9**. Biosynthesis of benzoxazinoids and formation of their breakdown products BOA and MBOA. Bx 1−9 are enzymes taking part in biosynthesis. Flash-signs indicate decomposition routes (Constructed from various sources. The lowest line is adopted from Handrick *et al*. 2016).

Emergence of hydroxamic acid glucosides in wheat occurs fast and reaches the maximum 12−48 h after germination. The aglycons, DIBOA and DIMBOA, appear soon after that. When reaching the autotrophic growth stage, the amount of bezoxazinoids had decreased to an almost negligible level. The event was not affected by induction of pathogens or wounding of the plant (Nakagawa *et al*. 1995). Similarly, the concentration of DIMBOA- and $DIM_2BOA$- glucoside in maize was highest two days after germination and after that decreased rapidly in roots and aerial parts to the final sampling of 20 days after germination. However, the total amount of benzoxazinoids per plant increased during the first 10 days after germination. HDMBOA glucoside was found to be the major benzoxazinoid in the roots and the main form in older (20-day-old) maize leaves (Cambier *et al*. 2000).

Hydroxamic acids are produced in varying rations in different crop plants, e.g. the main hydroxamic acid in maize is DIMBOA, with lesser amounts of DIBOA and $DIM_2BOA$ and in rye the main hydroxamic acid is DIBOA, with small amounts of DIMBOA. DIMBOA is also the main hydroxamic acid in wheat (Niemeyer 2009, Frey *et al*. 2009). Benzoxazinoid levels vary depending on tissue localization in the plant (roots and leaves) and age of the plant. Also the amounts and ratios of benzoxazinoids may vary in shoots and roots (Nakawa *et al*. 1995). It is not yet clear how the lactam HMBOA is fitted to the biosynthesis of benzoxazinoids (Meihls *et al*. 2013).

### 2.5.3 Benzoxazinoids as defense compounds

Examples of the transformation and breakdown products of benzoxazinoids are presented in **Figure 9**. Upon rupture of the tissue structure, benzoxazinoid glucosides are hydrolyzed to aglycons, which are then spontaneously and rapidly decomposed to BOA and MBOA. Benzoxazinoids can be released from a plant to soil either passively from vegetative residues (as in agriculture, by mulching or tilling of wheat or rye shoots) or actively by root exudation (Schulz *et al*. 2013). In soil, benzoxazolinones are degraded by microbial activity to the corresponding 2-aminophenols, which may then dimerize yielding aminophenoxazines (APO and AMPO). APO and AMPO are toxic to bacteria and fungi, but some microbes are able to detoxify them by acylation to AAPO and AAMPO, respectively (Schulz *et al*. 2013). Benzoxazinoids can affect the rhizospehere and soil microbial community structure by favoring those bacteria and fungi that can tolerate/consume/decompose exudated benzoxazinoids (Chen *et al*. 2010).

BOA can suppress germination and growth of seedlings of other plant species. However, some plants, most of them considered as weeds, have

adapted and developed defense by which the absorbed BOA can be detoxified via 6-hydroxylation and subsequent O-glucosylation or via glucoside carbamate pathway (Schulz *et al*. 2012). Interestingly, oat roots can detoxify BOA also via formation of N-glucoside, 3-β-D-glucopyranosyl-BOA (Wieland *et al*. 1998). Characteristic to BOA-sensitive plants is the accumulation of highly toxic 6-hydroxylated BOA.

The allelopathic activity of benzoxazinoids may also be expressed by the further decomposition products, especially APO, which has shown high inhibitory effect against growth of number of studied plants (Schulz *et al*. 2013).

Antifungal activity of BOA on *Fusarium nivale* was first reported by Virtanen and Hietala (1955) and inhibition of MBOA to the growth of a range of fungi (e.g. *Penicillium*, *Aspergillus*), yeasts (*Torula*) and bacteria (*E.coli*, *Staphylococcus*) in 0.04 % concentration by Virtanen *et al*. (1956). Antifungal activity of DIMBOA, MBOA and BOA against snow mold (*Fusarium nivale*) was reported by Wahlroos and Virtanen (1959). Simpson *et al*. (2000) studied the host preference of *Microdochium nivale* var. *nivale* and var. *majus* on wheat, rye and oats and explained the observed strong selective advantage of var. *nivale* over var. *majus* on rye as a result from the sensitivity of var. *majus* to BOA. The pathogenic fungi *Gaeumannomyces graminis*, which causes take-all disease in barley and wheat, was found to be more sensitive to DIBOA than to DIMBOA (Wilkes *et al*. 1999). However, some maize- and wheat-infecting fungi, such as *Fusarium verticillioides (F. moniliforme), F. subglutinans F. crookwellense* or *F. graminaerum*, can detoxify MBOA and BOA by actively metabolizing them via 2-amino-5-methoxyphenol and 2-amino-phenol into nontoxic HMPMA and HPMA, respectively (Yue *et al*. 1998, Glenn & Bacon 2009 and references within). To make things even more complicated, maize varieties that are bred for high DIMBOA content may alter the fungal endophyte community and actually favor the BOA-resistant, toxins producing *Fusarium* species (Saunders & Kohn 2009).

Ahmad *et al*. (2011) proposed recently, that in maize DIMBOA-Glc and HDMBOA-Glc accumulate into the apoplast, where they act as defense against aphids and fungal pathogens. Insect feeding induces enzymatic conversion of DIMBOA-Glc to HDMBOA-Glc by 4-O-methyltrasferase. Nonenzymatic breakdown of the HDMBOA aglycon is faster than the breakdown of DIMBOA aglycon (Meihls *et al*. 2013 and references within). Besides a direct toxic effect against aphids, DIMBOA can induce callose formation, which is another defense compound against aphids (Meihls *et al*. 2013 and references within). Glauser *et al*. (2011) studied benzoxazinoid induction and detoxification in maize during caterpillar attack. They also found accumulation of HDMBOA-Glc as the first step in response to herbivory. In the second step,

aglycon HDMBOA is released enzymatically in disrupted tissues. Some chewing herbivores, such as caterpillars, like the specialist *Spodoptera frugiperda*, can detoxify DIMBOA by keeping it in glycosylated form either by its own highly active glucose transferase or by inhibiting the β-glucosidases of the ingested plant material (Glauser *et al*. 2011). Recently it was shown that the detoxification involved stereoselective reglucosylation of DIMBOA from (2*R*)-epimer form into (2*S*)-epimer. The detoxification process of DIMBOA aglycone took place in the gut cells of *S. frugiperda* by UPD-glucosyltransferase (Wouters *et al*. 2014). In addition, *S. frugiperda* is able to convert MBOA to the non-toxic and stable MBOA-N-Glc form enzymatically in the gut (Maag *et al*. 2014). The more generalist caterpillar *S. littoralis*, is not able to make these transformations, and when feeding on plants with higher DIMBOA content, it grew less than larvae on the control diet (Glauser *et al*. 2011). The distribution of benzoxazinoids is different in old and young maize leaves and leads *S. frugiperda* to prefer and grow better on young maize leaves and *S. littoralis* to prefer and grow better on old leaves (Köhler *et al*. 2015).

Glauser *et al*. (2011) speculated that the development of HDMBOA-Glc in plants has been a novel adaptation against DIMBOA resistant herbivorous insects. In maize leaves herbivore attack (*S. littoralis*) induced metabolites including benzoxazinoids HDMBOA-Glc and $HDM_2BOA$-Glc (Marti *et al*. 2013). Most of the induced metabolites remained localized in the leaves, so, if the metabolites were found in roots, their concentration was not affected by the attack. Interestingly, after *S. littoralis* attack, vascular sap and root exudates contained more DIMBOA-Glc, HMBOA-Glc and HDMBOA-Glc than control plants. The question of why benzoxazinoids were not induced in roots, but found in larger quantities in root exudates remained unanswered (Marti *et al*. 2013).

An interesting sidetrack regarding the role of benzoxazinoids related to a bit larger herbivores, is the effect of MBOA as a chemical triggering reproduction of montane meadow moles (*Microtus montanus*) (Sanders *et al*. 1981, Berger *et al*. 1981). The MBOA in these cases would originate from the benzoxazinoids in fresh growth of grasses and sedges. Also reproduction of other small rodents has been found to be affected by MBOA, whereas larger mammals (e.g. goats and ponies) were not sensitive to it (Martin *et al*. 2008 and the references within).

### 2.5.4 Benzoxazinoid contents in cereals and food

Only recently has the presence of benzoxazinoids been acknowledged in the human diet. It had been a general belief that benzoxazinoids were not present in dormant grain seeds, but emerged upon germination. Benzoxazinoid content in rye grains (64 mg/kg as DIBOA aglycon) was first reported by Katina *et al*. (2007). But only after Pedersen *et al*. (2011) had published a detailed, compound-specific report on the content of benzoxazinoids in rye flours and rye bread, more attention was paid to these neglected compounds as bioactive constituents in our diet. The next major step was taken when Hanhineva *et al*. (2011) expanded the view of the chemical diversity of benzoxazinoids, by reporting DIBOA-, DIMBOA- and HBOA- diglucoside in rye bran. Tanwir *et al*. (2013) provided quantitative data on benzoxazinoid content in wheat and rye fractions. In rye the benzoxazinoids were concentrated in the bran fraction and the main form was DIBOA-diglucoside. The total amount of DIBOA calculated as aglycon, was in whole-grain rye 32.19 mg/kg and in rye bran 148.27 mg/kg (**Table 5)**. Based on Tanwir *et al*. (2013), of the total aglycon DIBOA content, ca. 87% is in the form of dihexoside, 9% is in the form of monohexoside and 4 % in the form of aglycon in rye grain; the contents in rye bran were ca. 94%, 5% and 1% and in rye fine flour ca. 77%, 22% and 3%, respectively. The average content of DIBOA in rye samples in harvest years 2001(n=22) and 2002 (n=79) was 66 and 82 mg/kg, respectively (Hanhineva *et al*. 2014b). The total DIBOA content in whole-grain wheat was only 1.4 mg/kg and the highest content was found in the germ fraction (40.6 mg/kg) followed by fine bran and pollard flour (aleurone layer) (**Table 6**).

Liukkonen *et al*. (2007) studied the effects of germination conditions on various phytochemicals in rye. The content of benzoxazinoids increased to the final germination time (7 days), but the highest increase was seen in the first three days. After germination and drying, benzoxazinoid content was found to be 25 and 42 times higher in rootlets compared to germinated and non-germinated grains, respectively (Liukkonen *et al*. 2007). Extensive germination (6 d, 18 °C, 48 % moisture) followed by a typical pilsner malt drying process (final temperature 83 °C), resulted 46–102 times increase of benzoxazinoid content in wheat and 6.3–8,9 times increase in rye compared to the starting material (Pihlava *et al*. 2008) (**Figures 10** and **11** respectively).

In 2009 Fomsgaard *et al*. patented the method "Use of benzoxazinoids-containing cereal grain products for health-improving purposes", which was later published by Pedersen *et al*. (2011). Briefly, benzoxazinoid content in rye or wheat grains was increased by a simple four days germination process at room temperature. The germinated grains were then dried in the oven (50 °C) and milled into flour. By this hydrothermal process (HTP), the amount of total

**Table 5**. Benzoxazinoids (mg/kg) in rye samples (Tanwir *et al*. 2013, Pedersen *et al*. 2011, Katina *et al*. 2007). na= compound not included in MS/MS analysis. Values in parentheses are from the original publication. Values *not* in parentheses are calculated as aglycons. HTP=hydrothermal processing (i.e. malting).

| | **Tanwir *et al.* 2013** | | | **Pedersen *et al.* 2011** | | | | **Katina *et al.* 2007** | |
|---|---|---|---|---|---|---|---|---|---|
| | Evolo whole-grain mg/kg | Bran mg/kg | Fine flour mg/kg | Picasso mg/kg | HTP Picasso mg/kg | Svedjerug mg/kg | HTP Svedjerug mg/kg | Amilo mg/kg | Amilo germin. mg/kg |
| **Benzoxazolinones** | | | | | | | | | |
| BOA | 0.41 | 0.54 | 0.67 | 3.01 | 73.02 | 2.17 | 49.34 | | |
| MBOA | 0.08 | 0.20 | 0.11 | 0.11 | 38.70 | 0.20 | 27.88 | | |
| **Lactams** | | | | | | | | | |
| HBOA | 0.17 | 0.29 | 0.20 | 0.26 | 26.90 | 0.34 | 19.52 | | |
| HBOA glc | 0.88 (1.76) | 1.88 (3.74) | 0.38 (0.75) | 4.08 (8.13) | 13.72 (27.30) | 1.86 (3.71) | 8.82 (17.55) | | |
| HBOA 2*glc | 2.15 (6.41) | 7.04 (20.98) | 0.98 (2.92) | na | | na | na | | |
| HMBOA | 0.07 | 0.08 | 0.08 | | 2.13 | | 1.75 | | |
| HMBOA glc | 0.09 (0.17) | 0.27 (0.49) | 0.08 (0.16) | 0.14 (0.25) | 2.52 (4.63) | 0.19 (0.36) | 2.13 (3.92) | | |
| **Hydroxamic acids** | | | | | | | | | |
| DIBOA | 1.16 | 1.07 | 0.51 | 1.86 | 681.45 | 3.71 | 397.2 | | |
| DIBOA glc | 3.02 (5.73) | 7.11 (13.50) | 4.14 (7.87) | 56.50 (107.35) | 188.28 (357.73) | 18.43 (35.02) | 84.96 (161.42) | | |
| DIBOA 2*glc | 28.01 (78.44) | 140.10 (392.27) | 15.34 (42.96) | na | na | na | na | | |
| DIMBOA | 0.20 | 0.19 | 0.19 | nd | 11.76 | nd | 6.72 | | |
| DIMBOA glc | 0.09 (0.16) | 0.14 (0.25) | 0.13 (0.23) | 0.50 (0.89) | 23.75 (42.04) | 0.92 (1.64) | 18.28 (32.364) | | |
| **Sum as DIBOA aglycon** | 32.19 | 148.27 | 20.00 | 58.36 | 869.73 | 22.14 | 482.16 | 64 | 530 |
| **Sum as DIMBOA aglycon** | 0.29 | 0.33 | 0.33 | 0.50 | 23.75 | 0.92 | 18.28 | | |

**Table 6.** Benzoxazinoids (mg/kg) in whole- grain wheat and its milling fractions (Tanwir *et al*. 2013). Values in parentheses are from the original publication. Values *not* in parentheses are calculated as aglycons. Semolina are wheat middlings [*Finnish* manna], pollard is a mixture of fine bran and a small amount of flour.

| | Whole-grain wheat mg/kg | Fine flour mg/kg | Semolina mg/kg | Pollard flour mg/kg | Germ mg/kg | Fine bran mg/kg | Coarse bran mg/kg |
|---|---|---|---|---|---|---|---|
| **Benzoxazolinones** | | | | | | | |
| BOA | 0.087 | 0.078 | 0.069 | 0.093 | 0.155 | 0.145 | 0.072 |
| MBOA | 0.074 | 0.075 | 0.079 | 0.079 | 0.095 | 0.086 | 0.072 |
| **Lactams** | | | | | | | |
| HBOA | 0.002 | 0.001 | 0.004 | 0.049 | 0.131 | 0.042 | 0.015 |
| HBOA glc | 0.03 (0.064) | 0.03 (0.067) | 0.03 (0.064) | 0.25 (0.491) | 0.79 (1.582) | 0.21 (0.421) | 0.05 (0.109) |
| HBOA 2*glc | 0.16 (0.468) | 0.03 (0.098) | 0.03 (0.081) | 1.08 (3.217) | 4.34 (12.937) | 1.00 (2.969) | 021 (0.617) |
| HMBOA | 0.065 | 0.065 | 0.067 | 0.072 | 0.074 | 0.068 | 0.065 |
| HMBOA glc | 0.07 (0.124) | 0.07 (0.125) | 0.07 (0.127) | 0.08 (0.151) | 0.09 (0.174) | 0.08 (0.144) | 0.07 (0.134) |
| **Hydroxamic acids** | | | | | | | |
| DIBOA | 0.14 | 0.134 | 0.141 | 0.208 | 2.241 | 0.204 | 0.165 |
| DIBOA glc | 0.07 (0.124) | 0.07 (0.131) | 0.06 (0.12) | 1.06 (2.013) | 4.47 (8.499) | 1.00 (1.906) | 0.26 (0.500) |
| DIBOA 2*glc | 1.18 (3.29) | 0.21 (0.591) | 0.07 (0.205) | 9.58 (26.811) | 33.92 (94.967) | 10.12 (28.34) | 1.57 (4.399) |
| DIMBOA | 0.224 | 0.188 | 0.214 | 0.094 | 0.197 | 0.189 | 0.092 |
| DIMBOA glc | 0.05 (0.094) | 0.05 (0.095) | 0.05 (0.096) | 0.07 (0.123) | 0.10 (0.169) | 0.07 (0.129) | 0.06 (0.099) |
| **Sum DIBOA aglycon** | 1.38 | 0.41 | 0.28 | 10.84 | 40.63 | 11.33 | 2.00 |
| **Sum DIMBOA aglycon** | 0.28 | 0.24 | 0.27 | 0.16 | 0.29 | 0.26 | 0.15 |

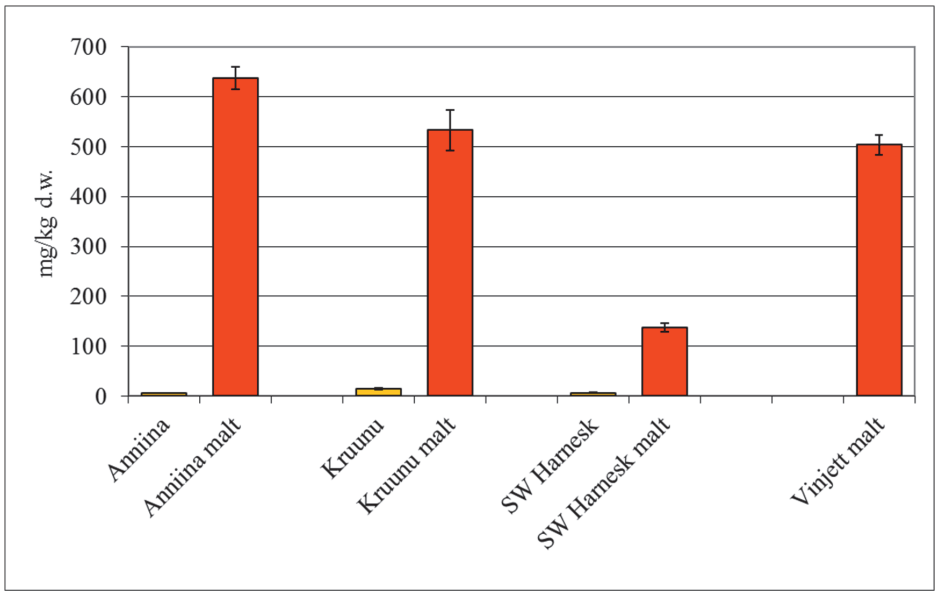



**Figure 10**. The effect of malting (germination 6 d, 18 °C; pilsner malt drying, final temp 83°C) on benzoxazinoid content in four wheat cultivars (Pihlava *et al*. 2008).

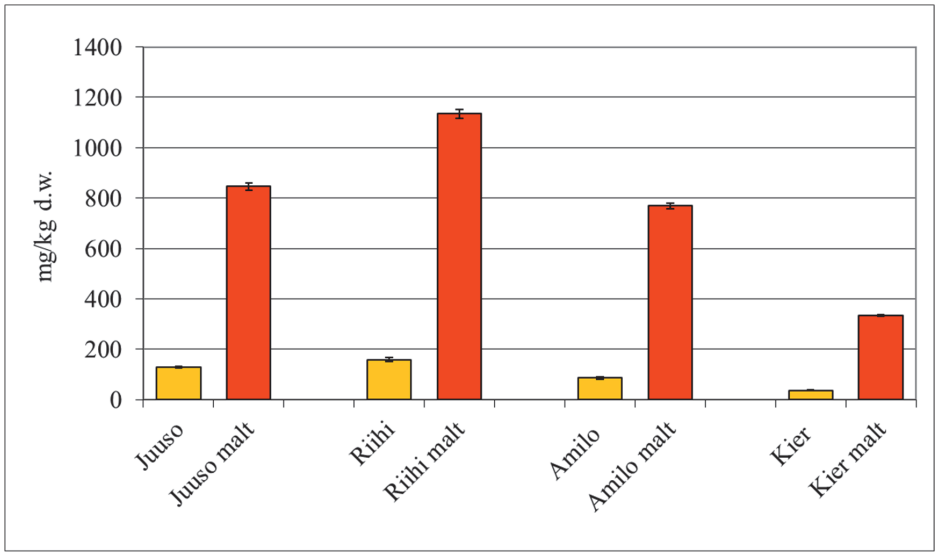



**Figure 11**. The effect of malting (germination 6 d, 18 °C; pilsner malt drying, final temp 83°C) on benzoxazinoid content in four rye cultivars (Pihlava *et al*. 2008).

DIBOA increased in ‘Picasso’ and ‘Svedjerug’ rye cultivars 15 and 22 times higher compared to the starting material (**Table 5**). An even more dramatic increase of benzoxazinoids was noted in the wheat cultivar ‘Kamut’, in which the DIBOA-form increased from practically zero to 329.66 mg/kg and similarly DIMBOA aglycon from zero to 56.12 mg/kg by HTP (**Table 6**) (Pedersen *et al*. 2011). Katina *et al*. (2007) reported ca. eight times higher benzoxazinoid concentrations in ‘Amilo’ rye after germination (6 days at 18 °C) and drying at 50 °C (25 h). Sourdough fermentation of the germinated rye flour resulted an increase of 23–26 % in benzoxazinoid content with two starter types, and a 20–62 % decrase with three other starter types. In native rye, benzoxazinoid content after sourdough fermentation was increased with all five starters 9–45 % (**Table 5**) (Katina *et al*. 2007). Benzoxazinoids also withstood the baking process well and were found in breads (Pedersen *et al*. 2011). Savolainen *et al*. (2015) reported that during the fermentation (12 or 24 h) of wheat bran, glycosylated benzoxazinoids are completely degraded to HHPAA and BOA and several unknown metabolites. However, Koistinen *et al*. (2016) reported glycosidic forms of benzoxazinoids in sourdough breads, so complete degradation may not occur at least in the case of rye.

Beckmann *et al*. (2013) reported that the DIBOA-dihexose decomposed during the fermentation and baking to BOA, DIBOA-glucoside and HPAA (N-(2-hydroxyphenyl)-acetamide) in commercial whole-grain sourdough rye bread (in order of signal intensity). Hanhineva *et al*. (2014a) reported DIBOA-hex, DIBOA-dihex, BOA, HBOA-Dihex, HBOA-hex and HPAA in commercial whole-grain sourdough rye bread, as in the order of peak intensity in arbitrary units. In wheat bread enriched with rye bran, the benzoxazinoids were in order of signal intensity DIBOA-hex, DIBOA-dihex, BOA/HHPAA, HPAA, HBOA-Dihex and HBOA-hex. The differences were assumed to have originated by different pH and different microbial composition in the fermentation process. No quantitative data for these compounds is available, only signal intensity.

BOA and MBOA have also been found in wheat malt beers by Pothou *et al*. (2013) and Manoukian *et al*. (2016).

### 2.5.5 Metabolism of dietary benzoxazinoids in mammals

Adhikari *et al*. (2012a) studied the uptake, distribution and metabolism of benzoxazinoids in pig. For pigs fed a rye diet the primary benzoxazinoid in 3 h postprandial plasma was HBOA-Glc (80−93 % of the total benzoxazinoid content in the plasma) derived from DIBOA-Glc-hex, HBOA-Glc-hex and DIBOA-Glc. Adhikari *et al*. (2012a) concluded that DIBOA-Glc-hex and HBOA-Glc-hex were deglycosylated (losing one hexose unit), and absorbed as HBOA-Glc after reduction of DIBOA to HBOA. It was not clear where the deglycolyzation took place, by luminal laccase phloridzin hydrolase (LPH) before absorption or cytosolic β-glucosidase after absorption or by intestinal microbes. HBOA-Glc was also found in urine. In feces the main metabolite was HBOA.

Further studies by Adhikari *et al*. (2012b) using rats showed also that the main benzoxazinoid in plasma was HBOA-Glc (68 %) and the other two metabolites detected were HBOA (appr. 16 %) and DIBOA-Glc (appr. 16 %). Glucuronides of HBOA and DIBOA were detected in plasma. Of the ingested benzoxazinoids 25 % were excreted in urine within 24 h, and the main metabolites were HBOA-Glc (55 % of the total metabolites), DIBOA-Glc (40%) and BOA (3 %). Benzoxazinoids excreted in feces was less than 0.7 % of the ingested amount. Rats metabolized benzoxazinoids faster than pigs. In humans, HBOA-Glc and DIBOA-Glc were the only benzoxazinoids detected in plasma after rye bread and rye bun ingestion (Adhikari *et al*. 2013). These were also the main forms excreted in urine, although a number of other benzoxazinoids were also detected. Glucuronides of HBOA and DIBOA were detected in plasma and urine. DIBOA sulfate was detected in plasma and urine and HBOA sulfate in urine as an indication of Phase II metabolism. It was concluded that the appearance of HBOA-Glc in plasma after 1 h of ingestion is due to the intestinal epithelial reductase activity, continuous increase of HBOA-Glc due to the microbial reductase activity and absorption in the lower intestinal tract (Adhikari *et al*. 2013). Benzoxazinoid concentration in the plasma peaked ca. three hours after eating, whereas benzoxazinoids in urine were found even at 36 h after consuming a meal (Jensen *et al*. 2017). Also in this study the predominant benzoxazinoid in plasma and urine was HBOA-Glc. BOA was found to be rapidly eliminated from the body, not found in plasma, but being one of the major urinary benzoxazinoids (Jensen *et al*. 2017).

In another study, the metabolites found in human urine after consumption of sourdough whole-grain rye bread were HBOA glucuronide, HPAA sulfate, HHPAA glucuronide and HHPAA sulfate (Beckmann *et al*. 2013).

Hanhineva *et al*. 2014a did not find HBOA glucose or DIBOA glucose from human plasma samples, but instead detected HPAA and HHPAA sulfate

conjugates, which were not included in the targeted MS/MS study by Adhikari *et al*. (2013). Missing HBOA glucose or DIBOA glucose in plasma was explained by higher sensitivity of the targeted MS/MS method.

HHPAA sulfate was more abundant in the plasma samples collected after ingestion of whole-grain sourdough rye bread than after ingestion of wheat bread enriched with rye bran. Since the HHPAA was not detected in whole-grain rye bread and HHPAA sulfate appeared in plasma after 1 h and increased to 2 h, it was concluded that the formation of DIBOA precursor must occur in the upper intestinal tract or once metabolized in the liver. It is also possible that there are more, yet unidentified, phenylacetamide derivatives (Hanhineva *et al*. 2014a).

Bondia-Pons *et al*. (2013) compared changes of metabolites in urine after whole-grain rye bread and whole-grain wheat bread using non-targeted profiling by UPLC-QTOF MS. Of the benzoxazinoids, the main differences in urine were the higher intensities of 2-aminophenol sulfate and DIBOA sulfate after intake of whole-grain rye bread.

Garcia-Aloy *et al*. (2015) compared urine samples from groups of non-consumers of bread, white-bread consumers and whole-grain bread consumers by nontargeted HPLC-QTOF MS. Of the bread consumption biomarker, compounds belonging to benzoxazinoids were HMBOA glucuronide, HMBOA, HBOA glycoside, 2-aminophenol sulfate, HPAA glucuronide, HHPAA and HPAA.

Possible health effects of dietary benzoxazinoids are not currently known. Buchmann *et al*. (2007) reported aneugenic effects of DIMBOA and DIBOA on human-derived liver cells (HepG2). This was considered of interest, since only a few naturally occurring compounds, such as vinca alkaloids and colchicine, have been found to have aneugenic properties, and DIMBOA and DIBOA were the first ones found in plant parts considered as edible and safe, i.e. wheat shoots. Prinz *et al*. (2010) expressed their concern about the increasing intake of benzoxazinoids from wheat seedlings or wheatgrass juices. In worst case, consumption of 100 g (as fresh weight) of three day-old wheat seedlings would lead to an intake of 629 mg of benzoxazinoids (as aglycons). However, the articles by Buchmann *et al*. (2007) and Prinz *et al*. (2010) were written before it was known that benzoxazinoids are found in whole-grain rye or wheat breads and have in fact been part of our diet for ages. Of course the acute intake of benzoxazinoid would be much smaller by consumption of breads than by sprouts.

Recently, Adhikari *et al*. (2015) reviewed the putative health effects of benzoxazinoids and their conversion products (such as APO). Possible health effects included anti-inflammatory, anticancer, antimicrobial, and central nervous system and reproductive system stimulatory effects. Preliminary

results by Steffensen *et al*. (2016) indicated that there is an inverse correlation between the concentrations of benzoxazinoids and malignant tissue from prostatectomies, but more research is needed before any definite conclusions about the anticarcinogenity of benzoxazinoids can be made.

Although limited data has so far been available on metabolism and possible negative or positive effects of benzoxazinoids in humans, at least one product is available based on maize leaf benzoxazinoids. Unigen's (Fremont, CA, USA) Maizinol™ is claimed to affect mood health by a proprietary compound (6-MBOA aka MBOA) known to enhance serotonin levels. Relating to this product, there are two patent applications, which includes the major natural benzoxazinoids, namely "Novel compounds for use as antidepressants, aphrodisiacs and adjunctive therepies in humans" by Rosenfeld *et al*. (2001) and "Novel compounds for use in weight loss and appetite suppression in humans" by Rosenfeld & Forsberg (2006). Benzoxazinoids, and especially MBOA, were also key-compounds in the patent "Methods for inducing anti-anxiety and calming effects in animals and humans." by Shelby *et al*. (2006).

### 2.5.6 Analysis of benzoxazinoids

Due to the polar nature of benzoxazinoids, LC would be the first choice in the analysis of these compounds as seen in **Table 7**.

However, in a few studies GC has been used. DIMBOA was analyzed after silylation by GC-MS/MS (Wu *et al*. 1999). In a large study, DIBOA was analyzed from a rye plants at their flag leaf stage by GC-FID (Brooks *et al*. 2012). Benzoxazinoids were extracted with dilute ethanol, which would allow enzymatic decomposition of benzoxazinoid glucosides during the 1 h incubation. DIBOA aglycon and DIBOA-glucoside required silylation before analysis by GC (Brooks *et al*. 2012, Finney *et al*. 2005).

Pothou *et al*. (2013) identified BOA as one of the volatile compounds in wheat beer by GC-MS. Samples were not silylated before analysis.

Quantification of these compounds is difficult, because to this point no commercial standards except for BOA and MBOA have been available.

**Table 7**. Examples of the analysis of benzoxazinoids. Abbreviations are listed in pages iii–iv. (na) = information not given.

| **BENZOXAZINOIDS**<br>**Sample extraction** | **Sample preparation** | **Equipment** | **Column & mobile phase (LC)**<br>**or Column (GC)** | **Reference** |
|---|---|---|---|---|
| MeOH (2% HOAc) | centrifugation | HPLC-DAD | BetaSil C18<br>5 µm, 4.6 *250 mm<br>$H_2O$-MeOH/IPA/HOAc<br>(95:5:0.025) | Ahmad *et al*. 2011 |
| bread: $CHCl_3$/MeOH/$H_2O$ (1:1:1) | after homogenization<br>addition of $CHCl_3$/MeOH/$H_2O$<br>(1:2.5:1); centrifugation;<br>evaporation | FIE-LTQ MS<br>(flow infusion) | - | Beckmann *et al*. 2013 |
| urine: as such | dilution with $H_2O$ | UPLC-QTOF MS | Acquity BEH C18<br>1.7 µm, 2.1 *100 mm<br>$H_2O$-ACN /acetone (80:20)<br>+ 0.1% formic acid | Bondia-Pons *et al*. 2013 |
| EtOH/$H_2O$ (1:10)<br>+ $H_2O$ | filtration; add. $H_2O$<br>incubation for 1 h, LLE with<br>EA; centrifugation; evaporation<br>of EA; silylation | GC-FID | DB-5<br>30 m*0.32 mm ID, $d_f$ 1.5 µm | Brooks *et al*. 2012<br>Finney *et al*. 2005 |
| isolation: boiling MeOH | filtration; evaporation; clean<br>up with SepPak C18 SPE;<br>purific. by prep-HPLC | prep-HPLC-UV | Econosphere C18<br>10 µm, 10*250 mm<br>$H_2O$-MeOH | Cambier *et al*. 2000 |

| (…BENZOXAZINOIDS) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| urine: as such | centrifugation; dilution with $H_2O$ | HPLC-QTOF MS | Luna C18(2) 5 µm, 2.0*50 mm $H_2O$-ACN + 0.1 % formic acid | Garcia-Aloy *et al.* 2014 |
| metabolomic analysis: IPA<br><br>BXs: MeOH/$H_2O$ (1:1) + 0.5 % formic acid | centrifugation; evaporation, clean up with SepPak C18 SPE<br><br>centrifugation | UPLC-PDA -QTOF MS<br><br>UPLC-QTOF MS | Acquity BEH C18 1.7 µm, 2.1 *50 mm $H_2O$-ACN + 0.1 % formic acid | Glauser *et al.* 2011 |
| (biotrasformation of BOA) | | agar plug TLC | silica-coated sheet with UV indicator (254 nm) TOL/EA/formic acid (50:40:10) | Glenn & Bacon 2009 |
| 75 % MeOH + 0.1% formic acid | centrifugation (crude extract) | UPLC-PDA -QTOF MS | Acquity BEH C18 1.7 µm, 2.1 *100 mm $H_2O$/ACN (95:5)-ACN + 0.1% formic acid | Hanhineva *et al.* 2011 |
| plasma: as such<br>bread: 80 % MeOH 15 min | addition of ACN; centrifugation | UHPLC-QTOF MS | Eclipse XDB-C18 1.8 µm, 2.1 *100 mm H2O-MeOH + 0.1 % formic acid | Hanhineva *et al.* 2014a |
| methanol, 19 h | evaporation of supernatant | HPLC-DAD | NovaPak C-18 4 µm, 3.9 *150 mm P-buffer - MeOH | Katina *et al.* 2007<br>Liukkonen *et al.* 2007 |

| (…BENZOXAZINOIDS)<br>Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC)<br>or Column (GC) | Reference |
|---|---|---|---|---|
| MeOH/$H_2O$/formic acid<br>(50:49.5:0.5) | centrifugation | UPLC-PDA<br>-QTOF MS | Acquity BEH C18<br>1.7 µm, 2.1 *50 mm<br>H2O-ACN + 0.05% formic acid | Marti *et al*. 2013<br>Meihls *et al*. 2013<br>Maag *et al*. 2014 |
| MeOH (2% HOAc)<br>50 °C 10 min | filtration | HPLC-UV | Wakosil-II-5 C18 HG<br>5 µm, 6.0*100 mm<br>$H_2O$/MeOH (78:22) +<br>2 % HOAc | Nakagawa *et al*. 1995 |
| ASE: MeOH/$H_2O$/HOAc<br>(80:19:1)<br><br>plasma: as such<br><br>urine: as such | <br><br>clean up by Oasis C18 SPE<br><br>clean up by Oasis C8 SPE | HPLC-MSMS | Synergi Polar RP-80A<br>4 µm, 2.0 *250 mm<br>$H_2O$/ACN (92:7)-ACN/$H_2O$<br>(78:22) + 20 mM HOAc | Pedersen *et al*. 2011<br>Tanwir *et al*. 2013<br><br>Adhikari *et al*. 2013<br>Adhikari *et al*. 2012a<br>Adhikari *et al*. 2012b |
| beer: as such | XAD-4 column, elution of<br>the compounds by DE:pentane<br>(1:1), evaporation | GC-MS | β-DEX sm<br>30 m*0.25mm ID, $d_f$ 0.25µm<br>HP-5<br>30 m*0.25mm ID, df 0.25µm | Pothou *et al*. 2013 |

| (…BENZOXAZINOIDS) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) or Column (GC) | Reference |
|---|---|---|---|---|
| wheat sprouts: addition of $H_2O$ | endo-enzymatic hydrolysis; LLE with DE; evaporation of DE | HPLC-DAD -ELSD | LiChrosphere C18 5 µm, 4.0*250 mm H2O/HOAc (90:10)-MeOH | Prinz *et al*. 2010 |
| 70 % MeOH | centrifugation | HPLC-DAD | ODS RP C18 (na) µm, (na) *(na) mm | Schulz *et al*. 2012 |
| 10 % MeOH, 30 min | filtration; centrifugation; evaporation | HPLC-DAD | Ultrasphere ODS RP-18 (na) µm, (na) *(na) mm 0.1 % TFA-MeOH 0.2 % TFA-ACN | Wieland *et al*. 1998 |
| $H_2O$ | filtration; adjustment to pH 3; centrifugation; LLE with DE | HPLC-UV | Zorbax SB C18 (na) µm, 4.6*150 mm $H_2O$/MeOH/ P-acid (59:40:1) | Wilkes *et al*. 1999 |
| MeOH/$H_2O$ (1:1) + 0.5 % formic acid | centrifugation | HPLC-MSMS, HPLC-ITD MS, direct infusion ESI-QOrbitrap | XDB C18 1.8 µm, 4.6 *50 mm Nucleodur Sphinx RP 5 µm, 4.6 *250 mm $H_2O$-ACN + 0.05% formic acid | Wouters *et al*. 2014 |
| 1 mM HCl (15 min) | centrifugation, LLE using DE, evaporation of DE, silylation with MSTFA | GC-ITD MS | DB-5 MSITD 30 m*0.25 mm i.d., $d_f$ 0.25 µm | Wu *et al*. 1999 |

## 2.6 Avenanthramides in oats

### 2.6.1 Background

Avenanthramides are phenolic compounds typical to oats (*Avena sativa*) and are not found in other cereals. Pioneering work in the identification of avenanthramide structures has been done by Collins (1983, 1989) and Collins *et al*. (1991), although avenalumin structures in crown-rust infected oat leaves had been already reported in the early 1980´s (Mayama *et al*. 1981). There are two ways of naming avenanthramides, by the alphabetical system of Collins (1989), or by the more systematic way of Dimberg and co-workers (Bratt *et al*. 2003). The latter system is used in this thesis.

Avenanthramides can be divided chemically into two main groups. The first one, Type I, consists of avenanthramides with an anthranilic acid part and hydroxycinnamoyl (coumaroyl, caffeoyl or feruloyl) part. The second group, Type II, consists of avenanthramides with an anthranilic acid part and avenalumoyl part (corresponding structures to coumaroyl, caffeoyl or feruloyl). Chemical diversity of avenanthramides is further expanded by hydroxylations or methoxylation of the anthranilic acid part (**Figure 12**). Although the number of possible avenanthramides is high, three of them, namely 2p, 2f and 2c, are the most common ones in oat groats. Three new avenanthramide structures containing 4,5-dihydroanthranilic acid moiety (avenanthramides 5p,c and f in **Figure 12**), was recently characterized by Ishihara *et al*. (2014). An extensive review of avenanthramides in oats has been made by Collins (2011).

### 2.6.2 Biosynthesis of avenanthramides

Avenanthramides are classified as phytoalexins, i.e. they are anti-fungal defense compounds produced by plants upon pathogen attack. Most of the studies have been focused on avenanthramide production on crown rust infection cause by *Puccinia coronata*. The key enzyme in the biosynthesis of avenanthramides is HHT (Hydroxycinnamoyl CoA: hydroxyanthranilate N-hydroxycinnamoyl transferase, EC 2.3.1), which catalyze the N-acylation of anthranilic acid by hydroxycinnamoyl-CoA thioester. Biosynthesis of avenanthramides with hydroxyavenalumoyl structure includes extra steps for elongation of the side chain of *p*-coumaroyl-CoA by malonyl-CoA (Ishihara *et al*. 1999a, Ishihara *et al*. 1999b, Matsukawa *et al*. 2000, Yang *et al*. 2004).

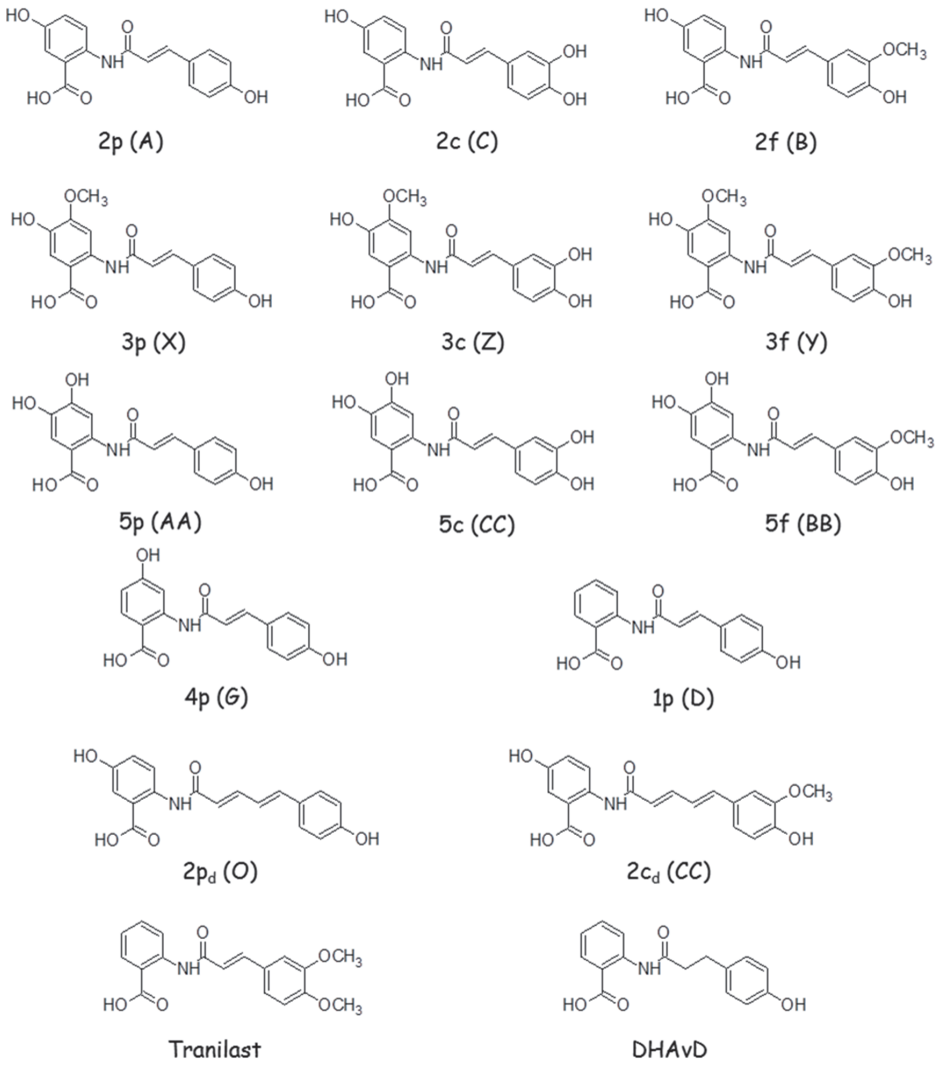



**Figure 12.** Examples of avenanthramide structures. Letters in parentheses are nomenclature used by Collins (2011). Letter "d" as a subscript in avenanthramides $2p_d$ and $2c_d$ indicate avenalumoyl structure (i.e. with conjugated diene). Tranilast is a synthetic methyl ester of avenanthramide 1f (E). Another synthetic avenanthramide is dihydroavenanthramide D (DHAvD).

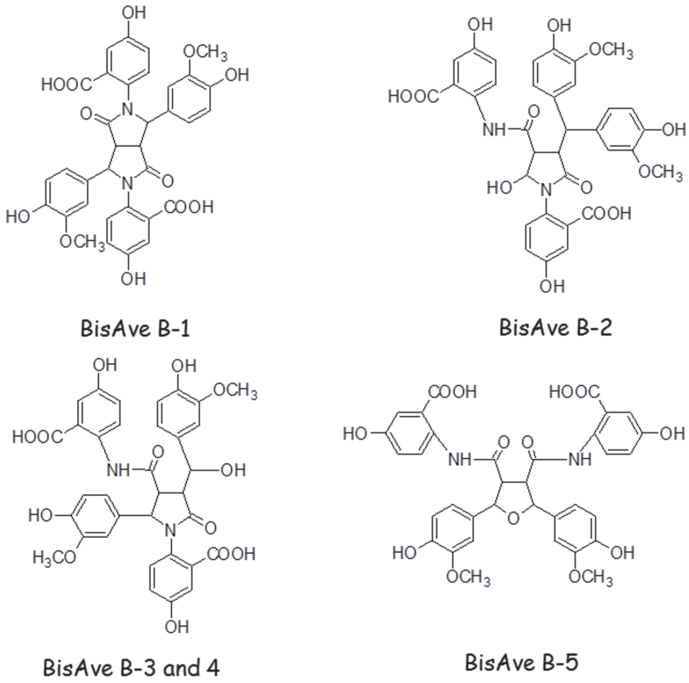



**Figure 13.** Examples of bisavenanthramides originating from avenanthramide 2f (B) (adopted from Okazaki *et al*. 2004 and 2007) Stereochemistry of the structures is not shown; BisAve B-3 and 4 are isomers of the same compound.

Avenanthramides can further dimerize to bisavenanthramides by action of peroxidase (Okazaki *et al*. 2004 and 2007) and be incorporated into cell walls (Okazaki *et al*. 2004). Bisavenanthramide structures originating from avenanthramide 2f by peroxidation are presented in **Figure 13**. In fact, bisavenanthramides belong to the chemical group of lignanamides (Okazaki *et al*. 2007).

Avenanthramide production in oat plants can be triggered with addition of elicitors such as benzothiadiazole (BTH) and 2,6-dichloroisonicotinic acid (INA) (Ren & Wise 2013) and penta-*N*-acetylchitopentaose (Izumi *et al*. 2009).

Izumi *et al*. (2009) studied elicitor-triggered production of avenanthramides at the cellular level. It appeared that some cells acted as hypersensitive responders (HR), accumulating avenanthramides fast followed by degradation of chloroplasts and leading to programmed cell death. Avenanthramides produced by HR cells and adjascent cells are also transported to the other parts of the infected tissues (Uchihashi *et al.* 2011).

A low amount of avenanthramides (1.55 mg/kg dw) was found in control (non-elicitor treated) oat plants at three leaf stages (Wise 2011). Application of elicitor BTH in field conditions as well as in greenhouse conditions was studied later by Wise *et al.* (2016). As a conclusion, there was no demonstrable correlation between avenanthramide levels and crown rust infection, although BHT treatment did enhance rust resistance and avenanthramide production. The authors doubted the practicality of the use of this elicitor without deeper understanding of the various factors affecting the activation process of plant defense systems.

Peterson & Dimberg (2008) studied the concentrations of avenanthramides during oat plant development. Avenanthramides were detected in oat spikelets 3− 5 d after heading and their concentrations were increasing towards maturation. Avenanthramides 2p and 2f were the main forms in the early development stage, while 2c appeared later and it was concluded that it is formed in the grains. Avenanthramide 2p was the main form in oat leaves from just-before heading until the leaves were no longer green. Avenanthramide 2c was present only in trace amounts. Interestingly, there was no correlation between avenanthramide concentration in oat leaves and grains. In addition, the differences between cultivars were substantial (Peterson & Dimberg 2008).

Spikelets of oat genotypes being resistant to crown rust had in general a higher content of avenanthramides than in spikelets of susceptible genotypes. However, in the leaves avenanthramides were not associated with resistance (Dimberg & Peterson 2009). Avenanthramides have also been detected in roots of oats, and based on the delayed response on elicitor tests, it was concluded that they might be transported to roots from leaves (Wise 2011).

### 2.6.3 Avenanthramides in oat kernels or groats and the effect of processing

For human consumption the hulls are removed from oat kernels and resulting oat groats are used. Normal commercial processing also involves heat treatment, either before or after hulling to inactivate lipolytic enzymes and thus enhance the stability of oat products. Avenanthramides are found both in oat groats and at lower concentrations in hulls (Collins 1989, Bryngelsson *et al*. 2002b, Emmons & Peterson 1999). In three Swedish oat varieties the concentration of avenanthramide 2f was 21–43 mg/kg, 2c 28–62 mg/kg and 2p 25–47 mg/kg (Dimberg *et al*. 1996). Somewhat lower concentrations were reported later by Bryngelsson *et al*. (2002a) in the groats of seven Swedish oat varieties, namely avenanthramide 2f 1–6 mg/kg, 2c 3–8 mg/kg and 2p 4–7 mg/kg. In three U.S. cultivars the concentration of avenanthramide 2f was 22–

42 mg/kg, 2c 37–66 mg/kg and 2p 12–23 mg/kg in groats (Emmons & Peterson 2001). The avenanthramide concentrations differ among oat cultivars, but the growing environment can have a stronger effect on variation (Emmons & Peterson 2001, Peterson *et al*. 2005). The difference between minimum and maximum value of total avenanthramide content in oat cultivars can be over 20 times, i.e. a minimum of 10 mg/kg and a maximum of 208 mg/kg (Li *et al*. 2016). Similarly, Antonini *et al*. (2016) found ca. 20 times difference between the minimum and maximum content of total avenanthramides in 15 naked and 15 common oat varieties. The average content of avenanthramides was three times higher in the naked oat cultivars compared to the common oat cultivars. Analysis of 137 accessions of oats revealed remarkably high concentrations of total avenanthramides e.g. in the cultivar 'Jaak' 1100–2700 mg/kg and in *A. strigosa* even up to 4100 mg/kg (Redaelli *et al*. 2016). Observed variations of total avenanthramide content in oats cultivated in different experiment sites could not be explained by different weather conditions (Redaelli *et al*. 2016). According to Li *et al*. (2017) the environmental factors had a greater effect on avenanthramide content than genetic, or genetic x environment. The mean content of total avenanthramides was 89 mg/kg with a range of 22–471 mg/kg in 39 oat genotypes grown in four locations in China.

Based on the pearling studies by Peterson *et al*. (2001), avenanthramides are localized in the outer parts, namely subaleurone, aleurone and pericarp, of the grain. However, the distribution of avenanthramides was not as clear as in case of ferulic and *p*-coumaric acid. According to Dimberg *et al*. (1993) oat bran fraction contained more avenanthramide(s) than endosperm fractions. Interestingly, no difference in avenanthramide concentration between outer and inner endosperm fraction, created by commercial milling, was noted. Hitayezu *et al*. (2015) reported the sum of avenanthramides 2c, 2f and 2p being in the medium and fine bran milling fractions 2.4 times higher than in the whole-grain oat flour. On the other hand, Li *et al.* (2016) reported slightly lower avenanthramide concentrations in the oat low bran flour *vs*. the bran, and both being somewhat lower compared to the starting material.

The heat treatment, which is usually done as part of commercial oat processing, caused only a slight reduction (20 %) in the concentrations of avenanthramides 2f and 2c, but in case of 2p the reduction was higher (Dimberg *et al*. 1996). Avenanthramides are also stable at pH 2 even after heat treatments (3 h, 95–98 °C water bath). At neutral pH, the heat treatment reduced the concentration of 2f roughly 30 % and 80 % of 2c. At pH 12 avenanthramides 2p and 2f were stable, but avenanthramide 2c was completely lost (Dimberg *et al*. 2001). Avenanthramides were found to withstand processing, such as baking (bread, tea-cake and muffin) and boiling (macaroni and fresh pasta) and in fact, there was an increase of avenanthramide

concentrations in all products except in fresh pasta. In another study, steaming and flaking oat groats reduced the concentration of the 2p form, but did not affect the concentration of 2f and 2c. Autoclaving of oat grains and drum drying of milled rolled oats reduced concentrations of all three avenanthramides (Bryngelsson *et al*. 2002a). Steeping (i.e. soaking in water) of oat groats had a favorable effect on avenanthramide concentrations. There was a 50 % increase of avenanthramide concentrations by e.g. 8 h steeping at 20 °C or 6 h at 40 °C (Bryngelsson *et al*. 2003). Cooking of oat porridge has resulted in somewhat conflicting results. Making the porridge from the oats containing lower avenanthramide content (20 mg/kg dw) resulted in a bit higher total avenanthramide content (36 mg/kg dw), whereas starting from oats with higher avenanthramides content (150 or 208 mg/kg dw) resulted in lower amounts (117 and 85 mg/kg dw respectively). The lower amounts mainly resulted from the degradation of avenanthramide 2c. Kaur *et al*. (2017) studied the effect of ultra high temperature (UHT) processing on liquids containing oat particles 1− 10 %. UHT treatment appeared to decrease the total avenanthramide content 43− 50 % compared to the unprocessed sample. Interestingly, when the UHT processed samples were stored at 22 °C for 2, 4, 8 or 12 weeks, the concentration of total avenanthramides increased so that in the end of the experiment it was 2.5−3.8 times higher compared to the unprocessed control sample. However, storage temperature 40 °C was not as favorable to the total avenanthramide content as 22 °C. The results, especially in the lower storage temperature, would indicate that considerable part of the avenanthramides are bound to the cell wall structures and can be gradually liberated during the storage.

Germination increases the avenanthramide levels (Matsukawa *et al*. 2000, Bryngelsson *et al*. 2003, Pihlava *et al*. 2008, Pihlava & Oksman-Caldentey 2001, Skoglund *et al*. 2008). Pihlava & Oksman-Caldentey (2001) reported a 3.8–5 times increase of avenanthramide content in oat groats after germination and heat treatments. Similar processing using the naked oat cultivar ‘Lisbeth’ resulted 5–5.5 times higher avenanthramide content compared to the starting material. However, exact germination parameters were not reported. Skoglund *et al*. (2008) studied the steeping and germination processes in more detail using three oat cultivars. An increase of avenanthramides was noted in the first sampling point (3–5 h to the moisture content of 42 %) of the steeping phase and was less in the second and third sampling points of the steeping. After steeping and germination processes, the content of avenanthramides 2f, 2p and 3f increased in all three cultivars, but avenanthramide 2c increased slightly only in one. The most remarkable increase was noted in the content of avenanthramide 3f. Xu *et al*. (2009) found that steeping of naked oats increased

the total avenanthramide content only by ca. 4 %, while in the end of the germination process (48 h) the increase was almost 50 %.

Pihlava *et al*. (2008) studied the effect of extensive germination (6 d, 18 °C, 48 % moisture) combined to pilsner malt drying using four oat cultivars (as kernels). As a result of the process avenanthramide contents were increased from the original values 1.8–4.5 times in three cultivars (**Figure 14**). Interestingly, the most notable effect was in content of type II avenanthramides, containing the anthranilic acid-avenalumoyl structures (**Figure 15**). Besides 2c, 2f and 2p, structures of other avenanthramides were not specified. For some unknown reason, there was practically no change in the content of avenanthramides in the oat cultivar 'Marika' after the experiment.

Collins & Burrows (2010) patented a novel method for increasing concentration of avenanthramides in oats. The method is based on enhanced dormancy and false-malting. Briefly, the method begins by heating step 1 (32 °C/72 h), during which the moisture content of the seed reduced to ca. 3 %. Step 1 was followed by heating step 2 (70 °C/144 h), in order to destroy seed borne molds and bacterial spores. The seeds were then cooled and steeped anaerobically at 32 °C for 18 h. After steeping the seeds were dried for four hours and then surface sterilized with sodium hypochlorite treatment and finally subjected to the malting process (e.g. 4 days at 30 °C). When the grains in secondary dormancy were malted, actual germination will not occur and thus the term "false malting" is used. As a result of the process, exceptionally high avenanthramide contents can be reached especially using the naked -oat variety 'Satake' as a raw material (Collins & Burrows 2010).

Recently, Carder *et al*. (2014) applied a patent "Method of processing oats to achieve oats with an increased avenanthramide content". Briefly, the method consists of mixing whole oat flour with sugar, maltodextrin, tocopherols (as antioxidants) and α-amylase. Water is finally added to bring the moisture content to 20–40 % and the mixture is applied to an extruder, with carefully controlled temperatures so that starch is gelatinized and in the end α-amylase is deactivated. The resulting product is then dried and granulated or milled. The resulting flour has a water solubility index about five times higher than the starting material and a modest 22 % higher total avenanthramide content compared to the starting material. In an adjoining patent by Chatel *et al*. (2014), the resulting flour was further agglomerated leading to highly dispersible whole-grain oat flour.

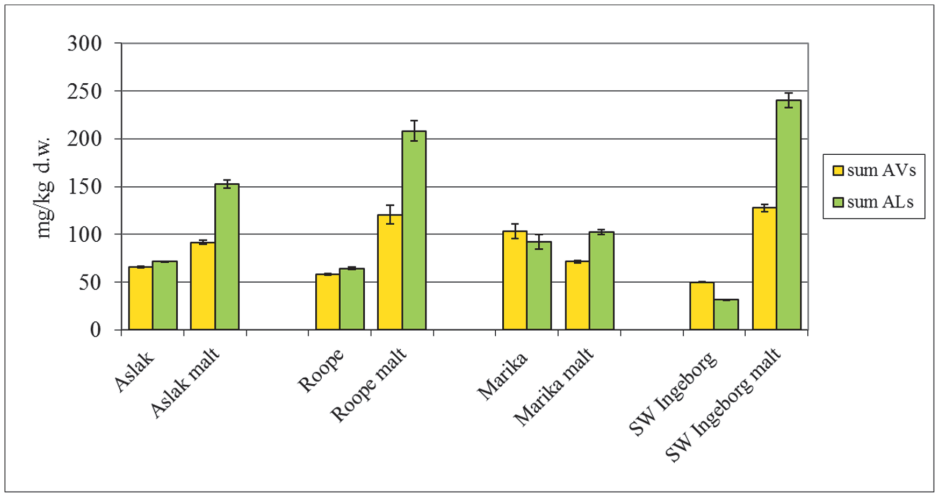



**Figure 14.** Sum amounts (mg/kg dw) of Type I avenanthramides (AV) and Type II avenanthramides (AL) in four oat cultivars and their corresponding malts (germination 6 d, 18 °C; pilsner malt drying, final temp 83°C). Unknown avenanthramides were quantified as Tranilast (Pihlava *et al*. 2008).

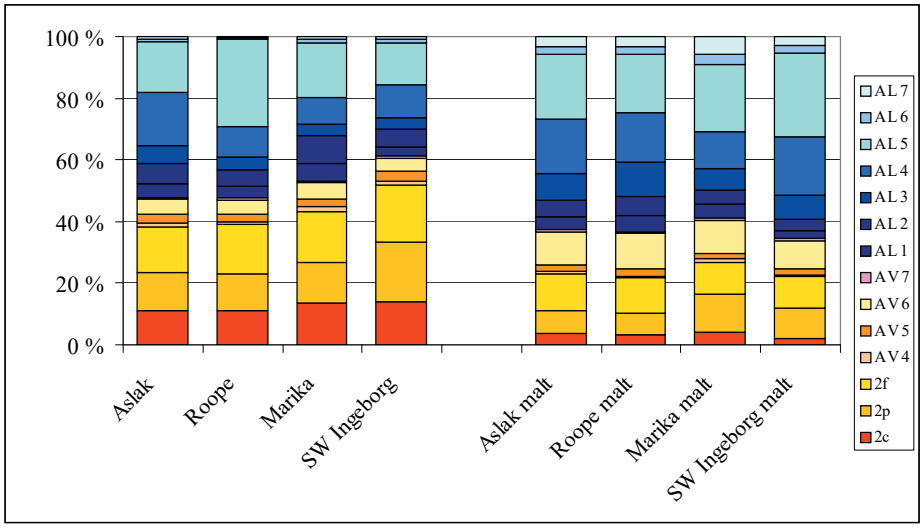



**Figure 15.** Relative content (%) of avenanthramides in four oat cultivars and their corresponding malts determined by HPLC-DAD. AV4− AV7 are unidentified Type I avenanthramides. AL1− AL7 are unidentified Type II avenanthramides with avenalumoyl structures (Pihlava *et al*. 2008).

### 2.6.4 Avenanthramides as dietary components

Yang *et al*. (2014) carried out an extensive study on antioxidant capacity of avenanthramides 2c, 2f and 2p against peroxyl radicals, hydroxyl radicals, superoxide anion, singlet oxygen and peroxynitrite. The tests used were ORAC, HORAC, SORAC, SOAC and NORAC, respectively. The total antioxidativity of 2c was about 1.5 times higher compared to 2f and 2p. These results were in-line with earlier results of higher antioxidant capacity of 2c over 2f and 2p in DPPH (Bratt *et al*. 2003, Lee-Manion *et al*. 2009, Peterson *et al*. 2002), β-carotene bleaching assays (Peterson *et al*. 2002) and FRAP assay (Lee-Manion *et al*. 2009). Although all avenanthramides typical to oats inhibited the formation of conjugated diene hydroperoxides in linoleic acid assay, their activities were considered only as intermediate. However, the antioxidant activity of avenanthramides persisted longer compared to α-tocopherol (Bratt *et al*. 2003). Avenanthramides also showed antigenotoxic effects in Comet assay with $H_2O_2$-stressed human adenocarcinoma colon cells (Lee-Manion *et al*. 2009). Interestingly, according to Chu *et al*. (2013) avenanthramide levels of the whole oat extracts did not correlate with the antioxidant capacity determined by H- and L-ORAC or the anti-inflammatory activity by NF-κB inhibition. Fu *et al*. (2015) proposed a novel mechanism by which the antioxidant activity of avenanthramides might be expressed. In this model avenanthramides activate potent cellular defense systems against oxidative stress by inducing the enzyme HO-1(heme oxygenase-1) expression in human kidney (HK-2) cells. The α,β-unsaturated carbonyl group in the structure of avenanthramides was proved to be crucial for their antioxidant activity. Future studies are clearly needed in this field.

Avenanthramides (2f and 2p) were first shown to be bioavailable in a hamster model, although the relative bioavailability of avenanthramides was much lower compared to phenolic acids. Plasma concentrations of avenanthramides were highest 40 min after administration of oat-bran phenol-rich powder and were eliminated by 120 min (Chen *et al*. 2004a). Bioavailability of avenanthramides was studied in more detail by Koening *et al*. (2011) using rats as a model. After administration of equal amounts of avenanthramides 2p (A), 2f (B) and 2c (C), the concentration of avenanthramides were measured in plasma, liver, heart and skeletal muscle and were found in various organs at various concentrations even after 12 hours of treatment (Koening *et al*. 2011). In humans avenanthramides 2p (A), 2f (B) and 2c (C) were detected in plasma 15 min after consuming a dose of an avenanthramide-enriched drink (Chen *et al*. 2007). Concentrations of avenanthramides in plasma peaked 1.5–2.3 hours after administration. The bioavailability of avenanthramide 2p was higher than that of 2f and 2c. A

single oral dose administration of avenanthramide mixture caused the concentration of plasma-reduced glutathione (GSH) to remain elevated even after 10 h compared to the GSH concentrations in the control group. Acute dosing of avenanthramides did not affect other measured markers of antioxidant capacity (GPx i.e. glutathione peroxidase and MDA i.e. malondialdehyde) or lipid peroxidation (LDL resistance to oxidation) (Chen *et al*. 2007).

Chronic avenanthramide supplementation for eight weeks reduced exercise-induced inflammation in postmenopausal women. High avenanthramide – intake reduced plasma inflammatory markers such as post- exercise CRP (plasma C-reactive protein) and neutrophil respiratory burst activity. High avenanthramide supplementation also suppressed pro-inflammatory cytokine production (Koening *et al*. 2014).

Liu *et al*. (2004) reported that an avenanthramide-enriched mixture inhibited the adhesion of monocytes to human aortic endothelial cell monolayers through inhibiting the expression of adhesion molecule expression. The avenanthramide mixture also decreased the production of proinflammatory cytokines and chemoattractant monocyte protein-1 in the test model. Since avenanthramides have anti-inflammatory and antiatherogenic activities, they might also modulate the inflammatory process associated with the development of atherosclerosis (Liu *et al*. 2004). Similarly, relating to the prevention of development of coronary heart disease, avenanthramide 2c was found to inhibit proliferation of vascular smooth muscle cells. The inhibitory effect was mediated by several key growth-regulatory proteins (Nie *et al*. 2006a, 2006b).

Guo *et al*. (2008) suggested that avenanthramides decrease the expression of endothelial proinflammatory cytokines at least partly through inhibition of NF-κB activation by inhibiting the phosphorylation of IKK and IκB, and by suppressing proteasome activity.

Based on *in-vitro* studies, Guo *et al.* (2010) concluded that consumption of oats and oat bran may reduce the risk of colon cancer not only because of their high dietary fiber content, but also due to avenanthramides, which can attenuate proliferation of colonic cancer cells. Later, Wang *et al*. (2012) found that avenanthramide 2p has proapoptotic effects and thus it could be a suitable candidate as a chemopreventive agent in further investigations.

Potential health effects of avenanthramides and their modes of action have been reviewed by Meydani (2009).

### 2.6.5 Avenanthramides as cosmeceutical components or drugs

Although the anti-inflammatory and anti-itching properties of oat extracts have been known for centuries, the phytochemicals mediating these activities have not been extensively studied. Sur *et al*. (2008) found that avenanthramides, e.g., inhibited the expression of many pro-inflammatory proteins and reduced the release of proinflammatory chemokines. Topically applied avenanthramides significantly inhibited neurogenic inflammation presumably by inhibiting the release of cytokines. Examples of commercial applications based on these properties of avenanthramides are Symrise AG´s (Holzminden, Germany) DragoCalm®, Johnson & Johnson´s (New Brunswick, NJ, USA) Aveeno® products and Sogeval´s (Irving, TX, USA) Duoxy Calm® products for cats and dogs.

Oat extracts with standardized concentrations of avenanthramides and other high-value oat products are produced by CEAPRO Inc. (Edmonton, Canada), which also has a portfolio of patents on processing technologies, composition and method-of-use such as "Oat extracts: refining, compositions and methods of use" (Redmond & Fielder 2004) and "Avenanthramide-containing compositions (Fielder *et al*. 2010). The patent application "Compositions for inhibiting or reducing inflammation of skin" by Magee *et al*. (2007) is based on the anti-inflammatory properties of avenanthramides combined with those of hydrocortisone.

Another example of commercial applications of avenanthramides is the synthetic antiallergenic/anti-inflammatory drug Tranilast (N-(3´,4´-dimethoxycinnamoyl)-anthranilic acid, i.e. a methyl ester of avenanthramide 1f) (**Figure 12**), which is approved for use in Japan and South Korea. Tranilast is also used against asthma, autoimmune diseases, and atopic and fibrotic pathologies (Rogosnitzky *et al*. 2012). Tranilast did not show antioxidative activity in DPPH and FRAP assays, but showed antigenotoxic effect in Comet assay (Lee-Manion *et al*. 2009) and may have potential in cancer prevention and/or treatment of cancer (Darakhshan & Ghanbari 2013, Izumi *et al*. 2010, Rogosnitzky *et al*. 2012, Subramaniam *et al*. 2011). Tranilast can also have an effect on insulin metabolism resistance (Tagushi *et al*. 2008). Some of the most recent patents regarding Tranilast or similar compounds are e.g."Methods of treating eye diseases associated with inflammation and vascular proliferation" by Kelly & Stapleton (2012), "Preventing or treating agent of retinal disease, containing tranilast" by Yoshida & Hirai (2011), "Use of tranilast and derivates thereof for the therapy of neurological conditions" by Schneider *et al*. (2009), "Tranilast as modulator of T cell functioning for use in the treatment of autoimmune diseases" by Steinman *et al*. (2010), "Combination therapy of

arthritis with tranilast" by Pearlman *et al*. (2010) and "Pharmaceutical composition for prevention of progress of intestinal constriction associated with Chron´s disease" by Oshitani (2010).

Recently, another synthetic avenanthramide, dihydroavenanthramide D (**Figure 12**), was introduced and is available from Symrise AG (Holzminden, Germany) as SymCalmin®, a 5 % solution of dihydroavenanthramide D. It has been used as one of the ingredients in, e.g., Dr. Sebagh´s Rose de Vie Serum Delicat, which is claimed to "restore, calm and heal skin that need help".

Dihydroavenanthramide D has been shown to have an antiphotoageing effect *in vitro* (Kim *et al*. 2013). The mechanism of action may be mediated by inhibition of reactive oxygen species (ROS) produced by UV-B irradiation and expression of metalloproteinases MMP-1 and MMP-3. Dihydroavenanthramide D has also been reported to protect pancreatic β-cells against cytokine toxicity (Lv *et al*. 2009). Destruction of β-cells and subsequent deficiency of insulin in type 1 diabetes is at least partially believed to be mediated by cytokines. Intraperitoneal application of dihydroavenanthramide D prior treatment with streptozotocin (STZ) in mice also showed protection against pancreatic islet damage. Based on the results Lv *et al*. (2009) concluded that dihydroavenanthramide D may be useful to preserve functional β-cell mass. In addition, Lee *et al*. (2011) reported that dihydroavenanthramide D inhibited human breast cancer cell invasion. Inhibition was mediated through suppression of metalloproteinase-9 (MMP-9), which is a key enzyme for degrading type IV collagen. Examples of patents relating to the dihydroavenanthramide D are "Mixtures compromising anthranilic acid amides and cooling agents as cosmetic and pharmaceutical compositions for alleviating itching" by Vielhaber & Schmaus (2006), "Mixtures compromising anthranilic acid amides and antidandruff agents as cosmetic and pharmaceutical compositions for alleviating itching" by Schmaus & Röding (2009) and "Anthranillic acid amides and derivatives thereof as cosmetic and pharmaceutical active compounds" by Schmaus *et al*. (2012).

### 2.6.6 Analysis of avenanthramides

Avenanthramides have traditionally been analyzed by HPLC-UV methods (e.g. Dimberg *et al*. 1993, 1996, 2001; Dimberg & Peterson 2009; Bryngelsson *et al*. 2002, 2003; Skoglund *et al*. 2008).

Jastrebova *et al*. (2006) described a selective and sensitive method for avenanthramide determinations by LC-MS (single quadrupole mass selective detector). LC-MS was also used in identification of avenanthramides 2a, 2f and 2p in oat milling fractions (Hitayezu *et al*. 2015) and in oat products (Xie *et al*. 2017). Nowadays, the most common avenanthramides are also commercially available, which makes the identification and quantitation easier and more reliable.

Examples of the analysis of avenanthramides are presented in **Table 8**. Various aspects concerning the analysis of avenanthramides are discussed in detail by Dimberg & Jastrebova (2009).

**Table 8**. Examples of the analysis of avenanthramides. Abbreviations are listed in pages iii − iv. (na) = information not given.

| **AVENANTHRAMIDES**<br>**Sample extraction** | **Sample preparation** | **Equipment** | **Column & mobile phase (LC)** | **Reference** |
|---|---|---|---|---|
| plasma: addition of vitamin C-EDTA | enzymatic (glucuronidase/sulfatase) treatment; addition of ACN; centrifugation; evaporation | HPLC-CAED | Zorbax ODS C18 3.5 µm, 4.6 *150 mm $H_2O$/ACN (90:10)-ACN/$H_2O$ (50:50) + 75 mM H3Cit/25 mM NH4OAc | Chen *et al*. 2007 |
| | | HPLC-CAED | Meta-250 C18 5 µm, 4.6 *250 mm 0.06 M PSA-0.1 M LiAC in MeOH/ACN/IPA (80:10:10) | Chen *et al*. 2004 |
| 80 % EtOH in 10 mM P- buffer (pH 2.8) | filtration; evaporation | HPLC-PDA | Luna C18 (na) µm, 4.6*250 mm $H_2O$/ACN (95:5)-ACN + 0.1% formic acid | Chu *et al*. 2013 |

| (…AVENANTHRAMI-DES) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| refluxing EtOH/$H_2O$/HOAc (80:19.9:0.1) sodium dithionite as antioxidant | percolation extraction; evaporation; clean up with Octyl Sepharose CL 4-B; evaporation; clean up with Sephadex LH-20; evaporation | HPLC-DAD | Hypersil ODS C18<br>5 µm, 4.6 *250 mm<br>MeOH-$H_2O$-5% HOAc<br>MeOH-$H_2O$-10 mM P-acid<br>StableBond C18<br>3.5 µm, 4.6 *150 mm<br>MeOH-$H_2O$-0.5% formic acid | Collins & Burrows 2010 |
| | | HPLC-DAD-IT MS | Hypersil ODS C18<br>5 µm, 4.6 *250 mm<br>MeOH-$H_2O$-5% HOAc | |
| | | HPLC-PDA | Hypersil ODS C18<br>3 µm, 4 *250 mm<br>MeOH-$H_2O$-10% HOAc | Liu *et al*. 2004 |
| 80 % EtOH | filtration; evaporation | HPLC-DAD | LiChrospher C18<br>5 µm, 4.0 *125 mm<br>P-buffer (pH 2.8) - ACN | Dimberg *et al*. 1993<br>Dimberg *et al*. 1996 |

| (…AVENANTHRAMI-DES)<br>Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| methanol | filtration/centrifugation;<br>evaporation | HPLC-DAD | Hypersil ODS C18<br>5 µm, 4.0 *125 mm<br>P-buffer (pH 2.8)/ACN (95:5)- CAN | Dimberg *et al.* 2001<br>Bryngelsson *et al.* 2002<br>Bryngelsson *et al.* 2003 |
| 80 % EtOH 50 °C 20 min | centrifugation; evaporation<br>( leaves:clean up by Bond Elut C18 SPE ) | | | Dimberg & Peterson 2009 |
| 80 % EtOH 20 min | centrifugation; evaporation | HPLC-DAD | uBondapak C18<br>10 µm, 4.6 *250 mm<br>$H_20$ (pH 2.8 with HOAc)-ACN | Emmons & Peterson 1999 |
| 80 % EtOH 50 °C 20 min | | | | Peterson *et al*. 2001<br>Emmons & Peterson 2001 |
| defattening with hexane 1 h<br>50 % MeOH 50 °C 2 h | centrifugation, pH to 2,<br>LLE with EA, evaporation | HPLC-DAD<br>HPLC-MS | Nova-Pak C18<br>4 µm, 3.9*150 mm<br>$H_2O$-MeOH + 1% acetic acid<br>$H_2O$-ACN + 1% acetic acid | Hitayezu *et al.* 2015 |
| methanol, 24 h | filtration | UPLC-PDA-MSMS | Acquity BEH C18<br>1.7 µm, 2.1 *50 mm<br>$H_2O$-ACN + 0.1 % formic acid | Ishihara *et al*. 2014 |

| (…AVENANTHRAMI-DES) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| 70 % MeOH | centrifugation; evaporation; clean up Oasis HLB SPE; evaporation | capillary HPLC -IT MS | Inertsil ODS-3 C18 3 µm, 0.3*250 mm $H_2O$/ACN (95:5)-ACN/$H_2O$ (95:5) + 0.1 % formic acid | Izumi *et al*. 2009 |
| single cell sampling | none; low diffuse sampling | nanoflow HPLC -IT MS | Nucleodur C18 Gravity 3 µm, 0.075*150 mm $H_2O$/ACN (95:5)-ACN/$H_2O$ (95:5) + 0.1 % formic acid | |
| 80 % EtOH | filtration | HPLC-UV-MS | Genesis C18 4 µm, 4.6 *150 mm 10 mM formic acid-ACN | Jastrebova *et al*. 2006 |
| plasma/tissue: addition of vitamin C-EDTA | enzymatic (glucuronidase/ sulfatase) treatment; addition of ACN; centrifugation; evaporation non-conjugated: addition of ACN;centrifugation; evaporation | HPLC-UV | Supelco C18 (na) µm, (na) *(na) mm H2O/ACN (95:5)-ACN + 0.1 % formic acid | Koening *et al*. 2011 |
| cookies: addition of vitamin C-EDTA + ACN | centrifugation; evaporation | HPLC-UV | Supelco C18 (na) µm, (na) *(na) mm $H_2O$/ACN (95:5)-ACN + 0.1 % formic acid | Koening *et al*. 2014 |

| (…AVENANTHRAMI-DES) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| 80 % MeOH + 0.2 % formic acid | evaporation; re-extraction with EA;evaporation | UPLC-PDA-MS | Aqcuity BEH C18<br>1.7 µm, 2.1*100 mm<br>$H_20$/ACN(95:5)-CAN + 0.2 % formic acid | Li *et al*. 2016 |
| methanol | centrifugation; evaporation | HPLC-UV | Wakosil II C18 HG<br>5 µm, 4.6*150 mm<br>0.5 % TFA-ACN | Matsukawa *et al*. 2000 |
| Hot methanol | (filtration; evaporation ?) fractionationing by Sephadex LH-20; purification by prep-TLC | $^{13}$C NMR,<br>$^{1}$H NMR<br>GC-MS as diacetate | | Mayama *et al*. 1981 |
| $H_2O$ | | HPLC-MS | Wakosil II C18 HG<br>3 µm, 4.6*150 mm<br>MeOH/$H_2O$/TFA | Okazaki *et al*. 2004 |
| 80 % EtOH 50 °C 20 min | centrifugation; evaporation | HPLC-DAD | Discovery HS C18<br>5 µm, 4.6*50 mm<br>$H_20$ (pH 2.8 with HOAc)-ACN | Peterson *et al*. 2005 |
| 80 % EtOH 50 °C 20 min | centrifugation; evaporation (leaves:clean up by Bond Elut C18 SPE) | HPLC-UV-MS | Genesis C18<br>4 µm, 4.6 *150 mm<br>10 mM formic acid-ACN | Peterson & Dimberg 2008 |

| (…AVENANTHRAMI-DES) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| 80 % MeOH 19 h | centrifugation; evaporation | HPLC-DAD | NovaPak C-18<br>4 µm, 3.9 *150 mm<br>P-buffer (pH 2.4) - MeOH | Pihlava & Oksman-Caldentey 2001<br>Pihlava *et al*. 2008 |
| 80 % EtOH 50 °C 20 min | centrifugation; evaporation | HPLC-DAD | Zorbax SB-C18<br>5 µm, 4.6 *30 mm<br>P-buffer (pH 2.8) /ACN (95:5)-ACN<br>Hypersil ODS C18<br>5 µm, 4.0 *125 mm<br>P-buffer (pH 2.8) /ACN (95:5)-ACN<br>Discovery C-18<br>5 µm, 4.6 *50 mm<br>P-buffer (pH 2.3) /ACN (95:5)-ACN (85:15) | Skoglund *et al*. 2008 |
| 80 % EtOH in 10 mM P-buffer (pH 2) | centrifugation; evaporation | HPLC-PDA | Discovery HS C18<br>5 µm, 4.6*50 mm<br>$H_2O$/ACN (95:5)-ACN +<br>0.1% formic acid | Wise 2011 |
| 80 % EtOH in 10 mM P-buffer (pH 2.8) | centrifugation; evaporation | HPLC-MS MS | Halo C18<br>2.7 µm, 3.2*50 mm<br>$H_2O$-MeOH + 0.1% formic acid | Xie *et al*. 2017 |

## 2.7 Polyamines and phenolamides in cereals

Polyamines putrescine, spermidine, spermine and agmatine are cationic compounds ubiquitously distributed in the plant kingdom. Polyamines occur in higher plants in free forms, but they also conjugate with small molecules and proteins. They have a critical role in plant development, in response to abiotic stress (e.g. temperature) and in defense systems against pathogens and herbivores (Alcázar *et al*. 2010, Alcázar *et al*. 2011, Bassard *et al*. 2010, Hussain *et al*. 2011, Kristensen *et al*. 2004, Kuznetsov & Shevyakova 2007).

Interestingly, polyamines (putrescine and agmatine) have been found to induce *in vitro* tricothecene deoxynivalenol (DON) production in *Fusarium graminearum* (Gardiner *et al*. 2009). FHB or scab in wheat is mostly caused by *Fusarium graminearum*¸ but also by other related *Fusaria* (*eg*. *F.culmorum*) (Gardiner *et al*. 2010). One of the roles of the produced mycotoxin, deoxynivalenol (DON), during the development of FHB is to allow the fungi to colonize and spread from the infection site, being the spikelets or the stem base of the plant (Ilgen *et al*. 2009, Winter *et al*. 2013). *F*. *culmorum* can also infect the stem base of wheat via soil. One the other hand, infected plant tissues can detoxify DON by glucosylating it to DON-3-Glc or even to glucosides containing four glucose units (Zachariasova *et al*. 2012). Glutathione (GSH) conjugate and several other metabolites of DON produced by wheat have recently been identified by Kluger *et al*. (2013).

Polyamines are natural dietary components in the human diet. Putrescine is the main polyamine in fruits and cheeses, whereas vegetables are rich in spermidine and meat products in spermine (Ali *et al*. 2011). Agmatine is usually found in fermented foods, such as beer (Galgano *et al*. 2012 and references within). Besides beer, free agmatine has been also found in pilsner malts and corresponding worts, although after fermentation of the wort the agmatine content was reduced 38−52 % (Halász *et al*. 1999). There are also a number of minor polyamines, such as the methylated form of agmatine. $N^6$-methylagmatine, presumably originating from the decarboxylation of N-methylarginine, has been found, e.g., in soybeans, kidney beans, peas and peanut (Matsusaki *et al*. 1990).

In the human body, the polyamine pool is maintained by endogenous biosynthesis, intestinal microbiota and exogenously from diet. The polyamines *per se*, and especially agmatine, also have a wide variety of biological activities in mammals. They are required for the growth, maintenance and function of normal cells (Piletz *et al*. 2013, Moinard *et al*. 2005).

When polyamines are conjugated with hydroxycinnamic acids, such as *p*-coumaric, ferulic and caffeic acid, they form a group called phenolamides, or

hydroxycinnamic acid amides. The number of hydroxycinnamic acid residues in the polyamine backbone is usually one to three (Bienz *et al*. 2005). Examples of the phenolamide structures are presented in **Figure 16**.

Conjugation reduces the polarity and hydrophility of the polyamine group and may favor translocation and storage of the phenolamides. The accumulation of phenolamides has been described as a stress response against biotic or abiotic factors (Bassard *et al*. 2010, Gaquerel *et al*. 2014, Kristensen *et al*. 2004, Moheb *et al*. 2011). It has also been suggested that due their photochemical properties, polyhydroxycinnamoylamides, which are particularly accumulated in pollen, might act as a photoprotectant of DNA (Bienz *et al*. 2005). Phenolamides are also usually found in plant flower parts (Bassard *et al*. 2010).

At this time there is no quantitative data available of the content of phenolamides in our diet or in different foods or food raw materials. Even of the best-known phenolamides, namely hordatines, only a very limited amount of information is available. Savolainen *et al*. (2014) reported various phenolamides in wheat bran and Wen *et al*. (2014) in maize kernels, but no quantitative data is available.

The two examples of the analysis of phenolamides will be presented together with hordatines in **Table 9**.

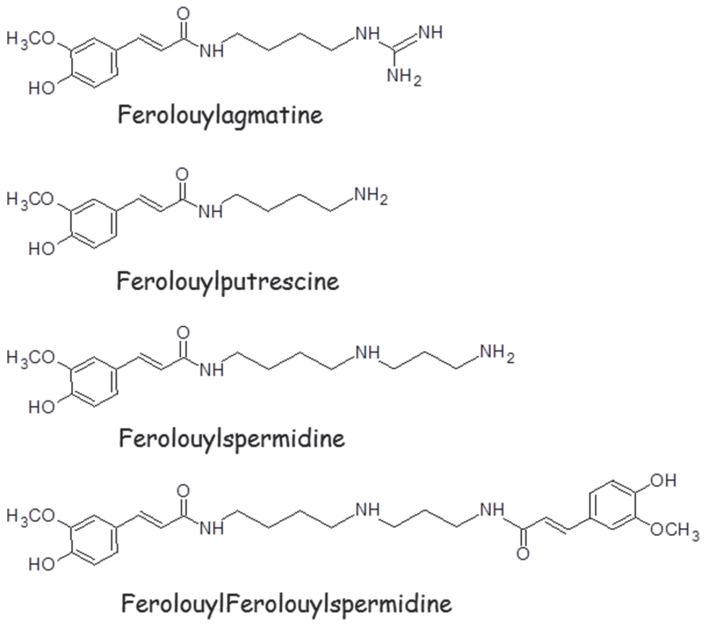



**Figure 16**. Examples of the phenolamides; ferulouylagmatine, feruloyl-putrescine, feruloylspermidine and feruloylferuloylspermidine aka. diferuloyl-spermidine.

## 2.8 Phenolamides in barley: Hordatines and hydroxycinnamoylagmatines

### 2.8.1 Background

Hordatines and their precursors, hydroxycinnamoylagmatines, are phenolamides typical to barley, although small amounts can also be found in wheat (Nomura *et al*. 2007).

Until recently, no other hordatine aglycon structures, besides hordatine A, a dimer of *p*-coumaroylagmatine, and hordatine B, a dimer of feruloylagmatine and *p*-coumaroylagmatine, has been reported since their discovery (Stoessl 1965, Stoessl 1967). The glycosylated forms of hordatine A and B have collectively been known as hordatine M (Stoessl 1966, Stoessl 1967). Gorzolka *et al*. (2014) reported a new hordatine structure, hordatine C, consisting of two feroloylagmatines, in germinated barley. They also reported a fourth hordatine aglycon structure, hordatine D, which consists of feruloyl- and sinapoylagmatine. In addition, Gorzolka *et al*. (2014) reported the hydroxyl-forms of hordatines A, B, C and D as well as their glycosylated and diglycosylated forms. Also Heuberger *et al*. (2014) reported glycosides of hordatine C (exact m/z not provided), hydroxy-hordatine B (759.37 m/z = hordatine B1), dihydroxy-hordatine B (775.363 m/z = hordatine B2) and dihydroxy-hordatine C (805.372 m/z = hordatine C2) from a large study of barley varieties and their corresponding malts. The most common hordatine structures are presented in **Figure 17** and more in detail later in **Figure 27** on p. 118 of the Results and Discussion section.

### 2.8.2 Biosynthesis of hordatines

The biosynthesis of hydroxycinnamoylagmatines and hordatines is a continuum for hydroxycinnamic acids from phenylpropanoid pathway and agmatine from arginine by ADC arginine decarboxylase. Putrescine, spermidine and spermine can further be formed enzymatically from agmatine.

In the first step of hordatine biosynthesis, agmatine coumaroyltransferase (ACT) catalyzes the formation of agmatine conjugates from *p*-coumaroyl-CoA and feruloyl-CoA. The hydroxycinnamoylagmatines are further oxidatively dimerized to hordatines (**Figure 17**).

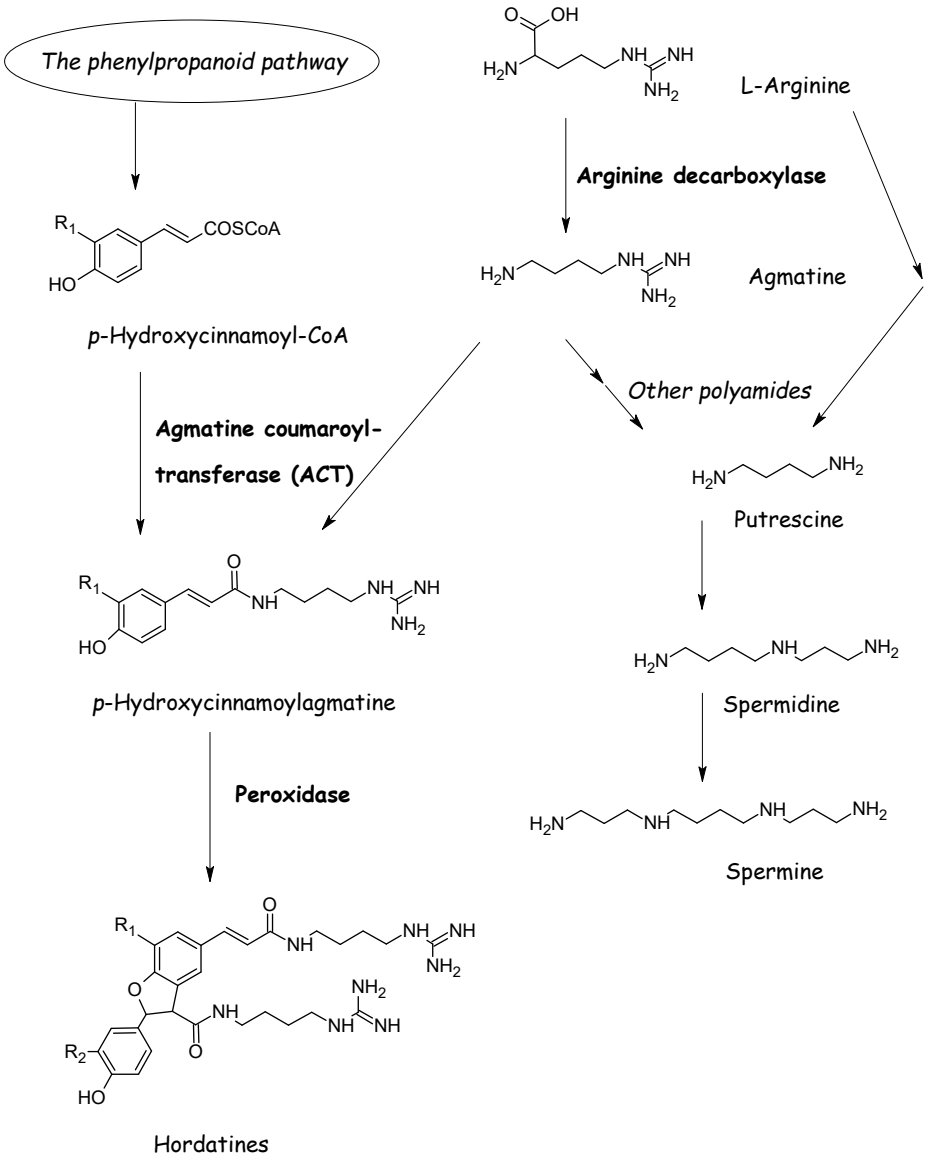



**Figure 17.** Biosynthesis of polyamines, *p*-hydroxycinnamic acid agmatines and hordatines (adopted from Kristensen *et al*. 2004 and Bassard *et al*. 2010). In monomeric forms $R_1$ = H, *p*-coumaroyl-CoA and *p*-coumaroylagmatine, and $R_1$ = $OCH_3$, feruloyl-CoA and feruloylagmatine. In hydroxycinnamoylagmatines glycosylation is via the OH-group in position 4 of the aromatic ring. For **hordatine A**, $R_1$ = $R_2$ = H, for **hordatine B**, $R_1$ = $OCH_3$ and $R_2$ = H (or $R_1$ = H and $R_2$ =$OCH_3$) and for **hordatine C**, $R_1$ = $OCH_3$ and $R_2$ = $OCH_3$. Reprinted from Pihlava 2014. *Journal of Cereal Science*, *60*, 645–652 with permission from Elsevier

Hydroxycinnamoylagmatines (and other hydroxycinnamoylamides) can also be conjugated to the cell wall material by peroxidase Prx7 and thus make it more resistant against plant pathogens (Kristensen *et al*. 1999, Kristensen *et al*. 2004). The concentration of hordatines and peroxidases are especially high in the coleoptiles, which are rapidly growing protective tissue that shields the emerging primary leaf (Stoessl 1967, Kristensen *et al*. 1999).

Concentration of coumaroylagmatine and hordatines were high in the barley shoots in the early stages (3−6 d) of germination but declined to 12 days after germination. Roots were found to contain less hordatines than shoots. In 12-day-old shoots, coumaroylagmatine and hordatines were more concentrated in stem than in leaves (Smith & Best 1978).

### 2.8.3 Hordatines against plant pathogens

Hordatines possess strong antifungal activities, i.e. inhibition of spore germination, against *Botrytis allii*, *Colletotrichum coccodes*, *Fusarium solani*, *Glomerra cingulate*, *Helminthosporium sativum* and *Monilinia fructicola* (Ludwig *et al*. 1960, Stoessl & Unwin 1969). Also, the precursors, *p*-coumaroylagmatine and hydroxylated *p*-coumaroylagmatine, possess antifungal activity against powdery mildew causing *Blumeria graminis hordei* (von Röpenack *et al*. 1998) and against snow mold causing *Monographella nivalis* (Jin *et al*. 2003). These precursors can act as preformed defense compounds in barley seedlings or as inducible accumulating defense chemicals in older plants after pathogen attack (Batchu *et al*. 2006, Kristensen *et al*. 2004). The accumulation of hordatines seems to be more a genetic property, rather than the result of direct influence of abiotic factors (Batchu *et al*. 2006).

### 2.8.4 Hordatines as dietary components

Possible health effects of hordatines or their metabolism in humans are not known at the moment. Based on their chemical structure it could be assumed that they could be antioxidative compounds.

Considering barley as part of our diet, hordatine A glucoside has been identified from unmalted barley by Kohyama & Ono (2013) at concentrations of 103 to 254 nmol/g dry weight (73–181 mg/kg dw). Based on the pearling tests, Kohyama & Ono (2013) concluded that hordatine A glucoside is localized in the aleurone layer of the grain. Gorzolka *et al*. (2014) used a novel MALDI MS imaging technique to study the localization of hordatines in germinated barley seeds and were able to identify eight hordatine aglycon structures and 12 glycosyl conjugates of hordatines. Interestingly, localization

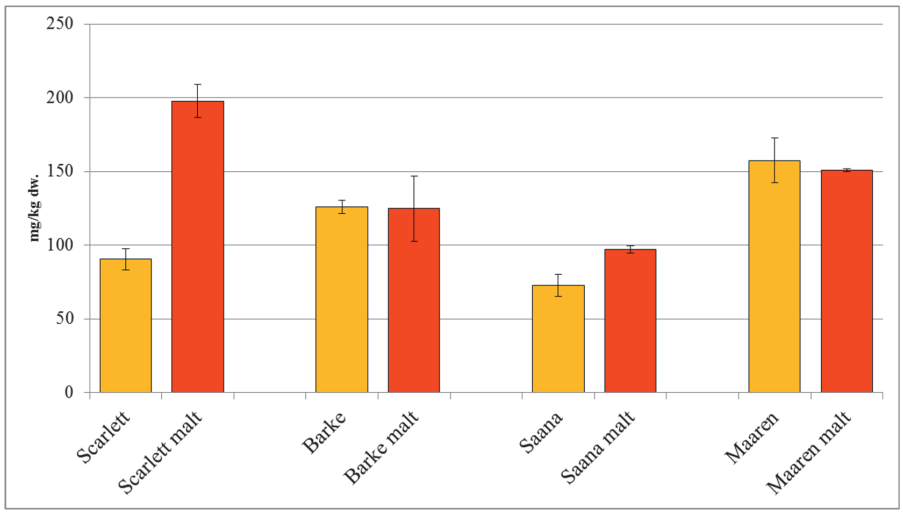



**Figure 18**. Effect of malting (germination 6 d, 18 °C; pilsner malt drying, final temp 83°C) on the total hordatine content (mg/kg dw) in four barley cultivars (Pihlava *et al*. 2008). Quantitation was first done by *p*-coumaric acid and then multiplying that using experimental response ratio 5.5.

of hordatines varies, e.g. the amount of hordatine A was highest in the shoots, while the hordatine B was highest in the roots of germinated grain. In ungerminated barley, Gorzolka *et al.* (2014) proposed the location of hordatines as being in the outer embryo tissue and in the interspace between the husk and the aleurone layer.

Pihlava *et al*. (2008) studied the effect of the malting process on the content of hordatines in four barley cultivars 'Scarlet', 'Barke', 'Saana', and 'Maaren'. In 'Barke' and 'Maaren' the malting process had no effect to the hordatine content while in 'Saana' and 'Scarlet' hordatine content increased 1.4 and 4.2 times, respectively, in their malts as compared to the native grains (**Figure 18**). Reason for the differences would require further studies.

Fujii *et al*. (2002) set up a study to investigate why certain compound congeners in beer stimulated gastrointestinal motility. The underlying mechanism was shown to be the ability of this congener to bind to muscarinic M3 receptors, which are present in smooth muscles of the upper gastrointestinal tract. Yokoo *et al*. (2004) were able to isolate these active components from beer and named them compounds A and B. These compounds were later identified and characterized as *cis*- and *trans* -isomers of hordatine A by Yamaji *et al*. (2007).

Furthermore, hordatine A also exhibits antagonist activity against the $\alpha_{1A}$ adrenoceptors, which are found in the central and peripheral nervous systems. $\alpha_{1A}$-Adrenoceptors play an important role in contraction of smooth muscle tissues in the cardiovascular system and lower urinary tract (Wakimoto *et al*. 2009). Based on the observed physiological effects, a patent application "Refreshment capable of stimulating movement of digestive tract" was launched by Suwa *et al*. (2004).

Besides the physiological effects, hordatines or their glycosides, can also cause astringent oral sensations and thus attribute to beer aftertaste. Compounds isolated from astringent fraction of water extract of barley malt acrospires, were identified as 4′-O-β-D-glucopyranosyl hordatine A, 4′-O-β-D-glucopyranosyl hordatine B, and 4′-O-β-D-maltosyl hordatine A (Kageyama *et al*. 2011) and later it was shown that these *trans*-cinnamoyl moieties containing hordatines had also corresponding *cis*-cinnamoyl forms, although being present as minor components (Kageyama *et al*. 2012). Interestingly, a patent was also applied as an oral cavity stimulating, acrid, substance (hordatines) by Kageyama *et al*. (2005). Ways for reducing hordatine levels in sprouted grains were also subjected to patent application by Suntory Ltd (Osaka, Japan) with Kageyama and Nakahara (2007) as inventors. The method presented lowering the amount of "oral cavity stimulating substance", e.g., hordatine A dihexoside, by acid or alkali hydrolysis, removal by adsorption, degradation by enzyme or removal by separation. Treatment of barley malt with subcritical water was found to reduce the amount of hordatine β-glycosides (a mixture of hordatine-β-D-glucopyranoside derivatives), resulting in beer with less astringency (Kageyama *et al*. 2013).

### 2.8.5 Analysis of hordatines

Being very polar compounds, the choice of analysis of hordatines is by LC. Quantitation of hordatines has been difficult due to the lack of commercial standards. Also the diversity of the chemical structures of hordatines (Gorzolka *et al*. 2014, Heuberger *et al*. 2014) will make the quantitation even more challenging.

Examples of the analysis of hordatines are listed in **Table 9**.

**Table 9**. Examples of the analysis of hydroxycinnamoylphenolamides and hordatines. Abbreviations are listed in pages iii – iv. (na) = information not given.

| **PHENOLAMIDES**<br>**Sample extraction** | **Sample preparation** | **Equipment** | **Column & mobile phase (LC)** | **Reference** |
|---|---|---|---|---|
| 80 % MeOH 30 min | centrifugation | UHPLC-PDA<br>-QTOF MS | Zorbax Eclipse XDB-C18<br>1.8 µm, 2.1 *100 mm<br>$H_2O$-MeOH + 0.1 % formic acid | Savolainen *et al*. 2014 |
| hydrophilic: 70 % MeOH,<br>19 h, 4 °C<br>lipophilic: 100 % MeOH,<br>19 h, 4 °C | | HPLC-QTRAP<br>MS or<br>HPLC-QTOF MS | Shim-pack VP-ODS C18<br>5 µm, 2.0*100 mm<br>$H_2O$-ACN + 0.04 % HOAc | Wen *et al*. 2014 |

| **HORDATINES**<br>**Sample extraction** | **Sample preparation** | **Equipment** | **Column & mobile phase (LC)** | **Reference** |
|---|---|---|---|---|
| MeOH-HOAc (98:2) 1 h | centrifugation | HPLC-UV<br><br><br><br>HPLC-MSMS | Nucleosil C18<br>5 µm, 4.0*250 mm<br>$H_2O$-ACN/$H_2O$ (99:2) + 0.1 % TFA<br><br>Supelco C18<br>(na) µm, 2.1*250 mm<br>$H_2O$-ACN/$H_2O$ (99:2)+0.1 % TFA | Batchu *et al*. 2006 |
| 80 % MeOH | centrifugation | UHPLC-QTOF MS<br>MALDI-TOF/TOF<br>MALDI-FT-ICR MS | BlueSpher C18<br>1.7 µm, 2.0*100 mm<br>$H_2O$-ACN + 0.1 % formic acid | Gorzolka *et al*. 2014 |

| (…HORDATINES) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| 70 % MeOH 2 h | centrifugation | UPLC-QTOF MS | Acquity T3<br>1.8 µm, 1.0*100 mm<br>$H_2O$-ACN + 0.1 % formic acid | Heuberger *et al*. 2014 |
| Acetone | filtration; evaporation; disolv. in $H_2O$; LLE with $CHCl_3$, EA and BuOH; BuOH evaporated; cleanup in C18 column | HPLC-UV | STR-ODS<br>(na) µm, 4.6*250 mm<br>0.05 % TFA-MeOH | Jin *et al*. 2003 |
| Acrospires: $H_2O$ 65 °C, 30 min | filtration; lyophilization; prep-HPLC | prep-HPLC-UV | Deverosil C-30 UG5<br>(na) µm, 10*250 mm<br>$H_2O$-ACN + 0.05 TFA | Kageyama *et al*. 2011<br>Kageyama *et al*. 2012 |
| 75 % Acetone 1 h | centrifugation; evaporation; clean-up by EDS-1 SPE | HPLC-DAD | TSKgel ODS-100 V<br>5 µm, 4.6*150 mm<br>20 mM ammonium P (pH 3.0)-ACN | Kohyama & Ono 2013 |
| MeOH-HOAc (98:2) | filtration | HPLC-MSMS | Mightysil RP-18 GP<br>3 µm, 2.0*150 mm<br>$H_20$ (0.01 % TFA)-ACN | Nomura *et al*. 2007 |
| MeOH,<br>19 h, room temp. | centrifugation; evaporation | HPLC-DAD | NovaPak C-18<br>4 µm, 3.9 *150 mm<br>P-buffer (pH 2.4) - MeOH | Pihlava *et al*. 2008 |

| (…HORDATINES) Sample extraction | Sample preparation | Equipment | Column & mobile phase (LC) | Reference |
|---|---|---|---|---|
| 17 M HOAc | filtration; evaporation | TLC-densitometer | 1.step with DE, 2. step with BuOH-HOAc-$H_2O$ (4:1:5) visualization with Sakaguchi reagent | Smith and Best 1978 |
| 70 % MeOH | | HPLC-UV<br><br>HPLC-UV<br><br>HPLC-MSMS | Hypersil BDS 5 µm, 4.6*250 mm 10mM ammoniun formate (pH 8) -MeOH/ACN (1:1)<br>Prodigy ODS-2 5 µm, 4.6*250 mm 10mM ammoniun formate (pH 3) -MeOH/ACN (1:1)<br>Nucleosil 100 C18 10 µm, 4.0*250 mm ACN/$H_2O$ (0.2 % HOAc) | von Röpenack *et al.* 1998 |
| Beer, as such | Concentration; lyophilization; fractionation by Amberlite XAD-2, CM-Sephadex C-25, Sephadex G-15; prep-HPLC | prep-HPLC-UV | ODS C18 (na) µm, 10*300 mm 0.1 M HCl/ACN (84:16) | Yokoo *et al.* 2004<br>Yamaji *et al.* 2007 |

## 2.9 General remarks regarding analytical methodology

In trecent years, there has been a growing trend in untargeted metabolomics analysis. As high resolution-mass spectrometry, either in the form of QTOF MS or Orbitrap MS, combined with HPLC or preferably with UHPLC have become more or less standard equipment in laboratories, new information on the vast diversity of secondary metabolites in cereals is emerging at an accelerating speed (Bollina *et al*. 2011, Bondia-Pons *et al*. 2013, Garcia-Aloy *et al*. 2014, Glauser *et al*. 2011, Heuberger *et al*. 2014, Kluger *et al*. 2013, Leoncini *et al*. 2012, Moheb *et al*. 2011, Savolainen *et al*. 2014, Wojakowska *et al*. 2013). In conjunction with the development of UHPLC-instruments, the advances in the analytical column technologies are of great importance.

However, the limitations of the MS techniques should be kept in mind. MS is not to be considered as a universal detector suitable for all compounds. Compounds to be analyzed have to form ions, otherwise they remain undetected. And although electrospray ionization (ESI) is the most commonly used technique, some compounds require atmospheric pressure chemical ionization (APCI) in order to become ionized. Also, depending on the chemical structure and elemental composition of the analyte, some compounds will be ionized easier in the positive mode than in the negative mode. Although crude sample extracts with minimum clean up steps are preferred, these may cause problems in the ionization process by enhancing or suppressing the ion formation of the analytes (Allwood & Goodacre 2010).

Sample preparation, starting from the representativeness of the sample, will still be a fundamental and the most critical step concerning the accuracy of results (Berg *et al*. 2013, Kim & Verporte 2010, Vuckovic 2012).

# 3 AIMS OF THE STUDY

Phytochemicals in cereals have been of interest because of their potential positive health effects when cereal grains are consumed as food. Although these minor compounds have been the subjects of numerous studies, knowledge about these compounds and their quantities in cereals and cereal products is far from complete.

The main objective of this thesis was to gain new insight into certain less-studied phytochemicals in cereals and cereal products, especially rye and barley. These phytochemicals were: alkylresorcinols, avenanthramides, benzoxazinoids, flavonoids, hordatines, lignans, phenolamides and phenolic acids.

The specific aims of the thesis were:

1. To develop and validate methods for the determination of avenanthramides, benzoxazinoids, hordatines, flavonoids, phenolic acids and alkylresorcinols in cereal samples by liquid chromatography (studies I, II, IV and V).
2. To provide quantitative data of the content of the bioactive compounds in cereals and cereal products (studies I, II, IV−VI).
3. To identify novel phenolic and/or phenolic-like compounds in cereals and cereal products by UPLC-QTOF MS (studies III, V and VI).

# 4 MATERIALS AND METHODS

This section is a summary of the materials and methods used in the present study and detailed information is described in original publications (I−VI). A schematic outline of this work, i.e. cereals studied, analytes, and chromatographic techniques, is presented in **Figure 19**.

## 4.1 Samples

Cereal products were purchased from local grocery stores and homogenized as described in details in original publications I and VI. Especially in Study I, special attention was given to build up representative pooled samples of commercially available cereal products.

Preparation of the rye fractions in Study II is presented in **Figure 20** and in more detail by Nordlund *et al*. (2013). Medium-coarse whole-grain rye flour (R1 in Study II) and whole-grain rye and milling fractions in Study VI, were from Fazer Mylly (Lahti, Finland).

In Study III, the malting type barley variety 'SW Makof' and waxy type barley variety 'Cinnamon' were from the field trials (Hauho, Finland).

Beers (n= 208) for the studies III−V were purchased from local grocery and alcohol stores (Alko Inc., Helsinki, Finland). Tap rye ale was obtained from Plevna brew pub (Tampere, Finland).

## 4.2 Sample preparation methods

### 4.2.1 Avenanthramides

The extraction of avenanthramides used in Study I was based on the method by Bryngelsson *et al*. (2002a) with slight modifications. Briefly, a milled sample (5.0 g) was extracted twice with 80 % methanol for 30 min using a magnetic stirrer. The samples were centrifuged and supernatants were evaporated to dryness in a rotary evaporator. Samples were redissolved in methanol (2.0 ml), filtered through a PTFE membrane filter (Pall Corporation, Port Washington, NY, USA) and analyzed by HPLC.

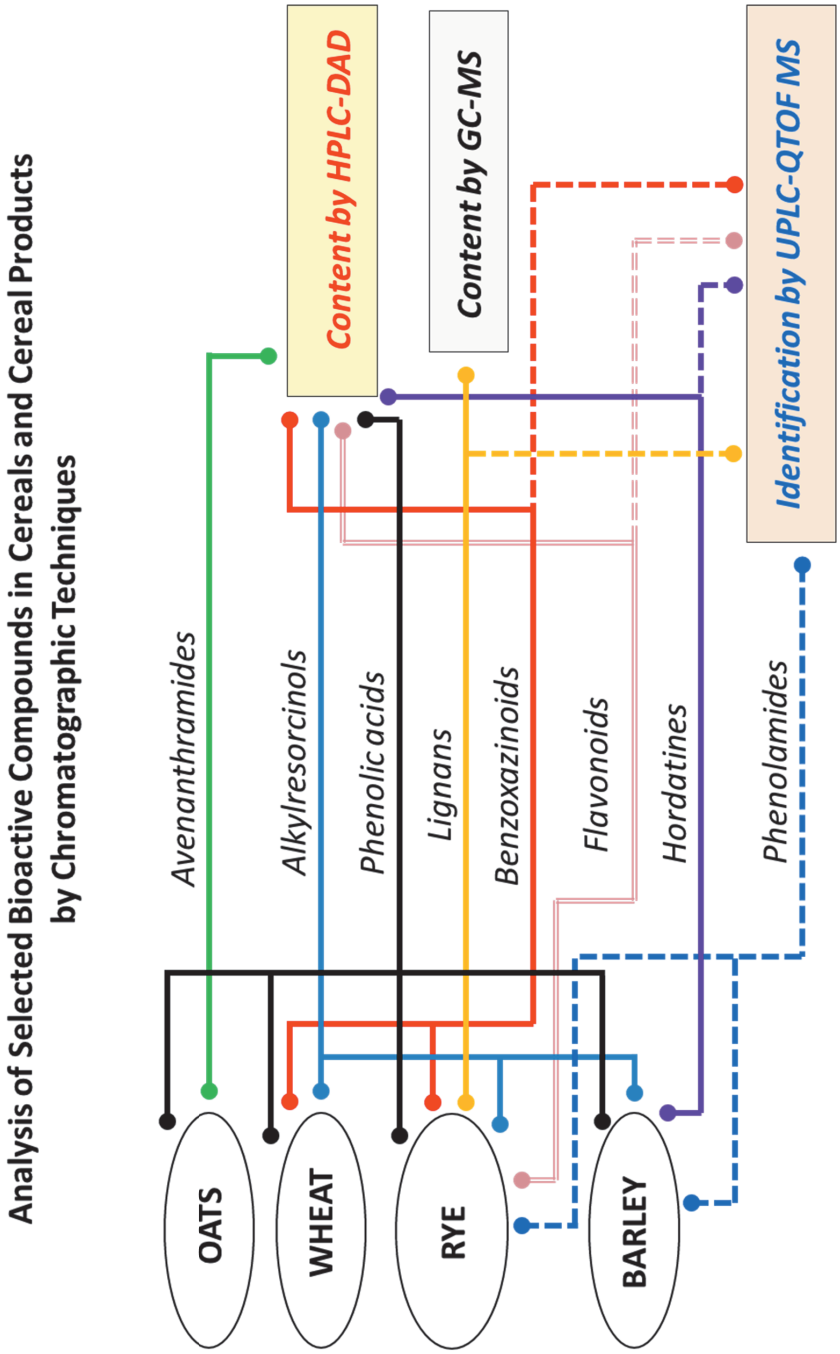



**Figure 19.** Outline of the cereals, analytes and chromatographic techniques. Dashed line indicates qualitative analysis.

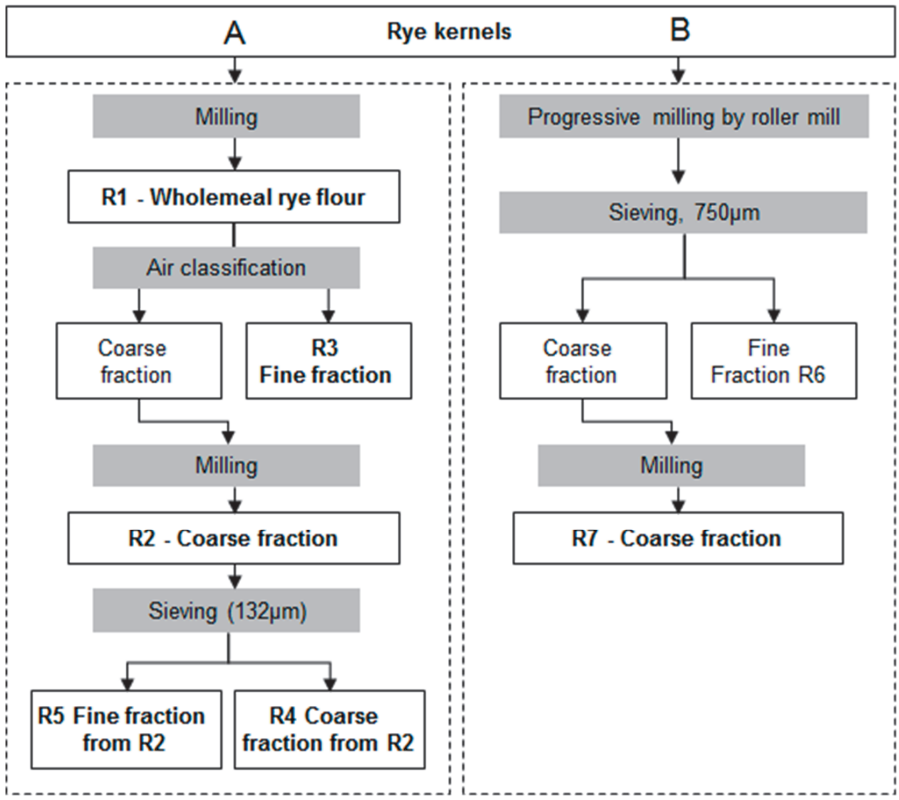



**Figure 20.** Mechanical processing of rye kernels for fractionation of rye. Consecutive milling and air classification process of rye flour R1 to produce rye fractions R2, R3, R4, R5 (A) and progressive milling and sieving of rye kernels to produce rye bran R7 (B). Reprinted from Nordlund *et al*. 2013. *Food Research international*, *54*, 48–56 with permission from Elsevier.

### 4.2.2 Alkylresorcinols, benzoxazinoids and flavonoids

In studies I, II and VI alkylresorcinols were extracted from the milled samples (2.5 g or 5.0 g) by methanol (50 ml) with magnetic stirring overnight. Samples were centrifuged and the remaining solids were briefly mixed with methanol (50 ml) and centrifuged again. Combined supernatants were evaporated to dryness in a rotary evaporator and redissolved in methanol (1.0 ml or 2.0 ml depending on the sample amount). Samples were filtered and analyzed by HPLC.

### 4.2.3 Flavonoid aglycons and anthocyanins

In Study VI, for the acid hydrolysis of flavonoid sugar conjugates to aglycons, 100 g of milled sample was extracted with 1 l of 80 % methanol containing 0.5 % formic acid, overnight. The sample was centrifuged (1500 rpm 15 min) and supernatant was transferred to a rotary evaporation bottle. Extraction was repeated with 100 % methanol. Combined supernatants were evaporated to near dryness in a rotary evaporator. Sample volume was adjusted to 40 ml with 80% methanol and 30 ml was transferred to an Erlenmeyer flask. Next 10 ml methanol and 10 ml of 6 M HCl was added and the sample was refluxed at 90 °C for 1 h as described by Hertog *et al*. (1992). A cooled sample was filtered through a paper into volumetric flask and made up to 100 ml with methanol. The sample was sonicated for 5 min and ca. 2 ml of the sample was filtered through a 0.45 um membrane filter for the HPLC analysis. Anthocyanins were analyzed by HPLC-DAD from the remaining 10 ml extract not subjected to the hydrolysis.

### 4.2.4 Hordatines

In Study III, hordatines were extracted from the milled barley sample (2.5 g) with 80 % methanol (100 ml) with magnetic stirring overnight. After extraction, the sample was centrifuged (1500 rpm 10 min) and the supernatant was transferred to a rotary evaporation bottle. The remaining solid residue was mixed with methanol and centrifuged. The combined supernatants were evaporated to dryness in a rotary evaporator. The sample was dissolved in methanol (2.0 ml) and filtered through a 0.20 um PTFE membrane filter into an autosampler vial to be analyzed by HPLC and UPLC-QTOF.

### 4.2.5 Lignans

In Study II, the extraction and clean-up method used in lignan analysis was based on the method of Peñalvo *et al.* (2005). Briefly, analytes were extracted with 70 % methanol containing 0.3 M NaOH, and after enzymatic hydrolysis cleaned-up with C-18 and ion exchange resin. As a modification to the method of Peñalvo *et al*. (2005), 4,4´-dichlorobenxhydrol was used as an internal standard. Also, the silylation of the lignans differed as it was done according to the method developed in our laboratory: the dried sample was dissolved in anhydrous pyridine (0.05 ml) and Sylon BTZ (0.200 ml) was added. Silylation was accomplished by incubation at 70 °C for 0.5 h. After the silylation, toluene

(1.0 ml) and injection standard PCB 53 were added and the excess of silylating agent and pyridine was removed by addition of water (2-3 ml). Washed organic phase was transferred into an autosampler vial and analyzed by GC-MS.

### 4.2.6 Phenolic acids

The method used in Study I was based on Mattila & Kumpulainen (2002). Briefly, phenolic acids were determined as free fraction, and a bound fraction liberated by alkali and acid treatments.

The sample preparation method for comprehensive phenolic acid determination used in Study II, is outlined in **Figure 21**. The method is based on methods described by Hatcher and Kruger (1997), Heiniö *et al*. (2008) and Provan *et al*. (1994). The method presented by Heiniö *et al*. (2008) was further modified by extending the OSI alkali treatment from four hours to overnight extraction. Briefly, the phenolic acids were divided into fractions of organic solvent soluble (OSS) free, esterified and glycosidic, and organic solvent insoluble (OSI) esterified and β-aryl etherified phenolic acids. Also, the amount of OSI phenolic acids esterified to water extractable fibers was determined.

All phenolic acids were analyzed by HPLC as their aglyconic forms.

### 4.2.7 Beer samples: hordatines and benzoxazinoids

For the analysis of hordatines in studies III and IV and benzoxazinoids in Study V, frozen beer samples were thawed and an aliquot was filtered into an autosampler vial through a 0.20 um PTFE membrane filter if subjected to UPLC-QTOF MS analysis or a 0.45 um membrane filter for the analysis by HPLC-DAD.

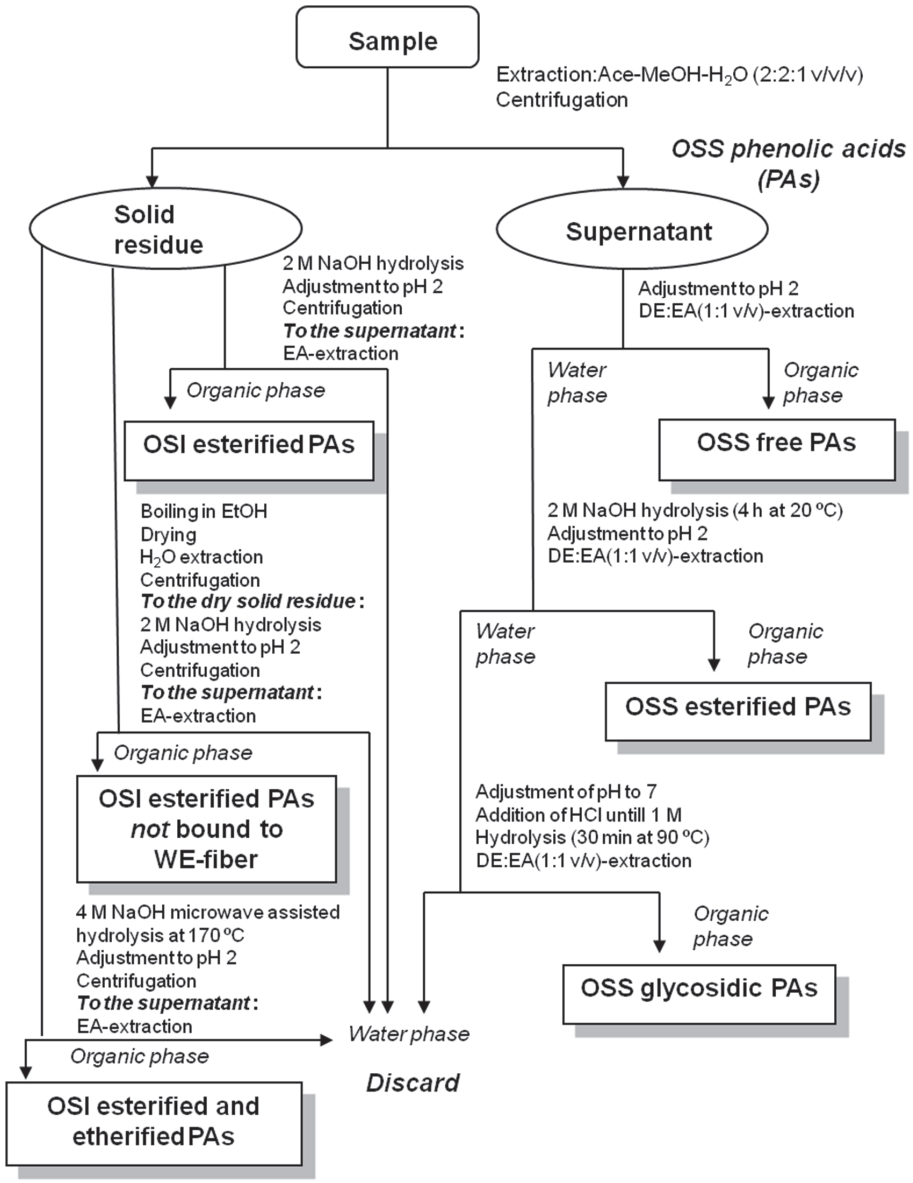



**Figure 21**. Outline of the methods used for determination of organic solvent soluble (OSS) free, esterified and glycosidic phenolic acids (PAs), organic solvent in-soluble (OSI) esterified, OSI-esterified phenolic acids not bound to water-extractable (WE) fiber and OSI-esterified and etherified phenolic acids. The organic phases from liquid-liquid extractions were evaporated to dryness and the samples were redissolved in methanol and filtered prior analysis by HPLC-DAD. II. Reprinted from Pihlava *et al*. 2015. *Journal of Food Composition and Analysis*, *38*, 89–97 with permission from Elsevier.

## 4.3 Chromatographic methods

### 4.3.1 HPLC-DAD analysis

In the studies I, II, IV, V and VI, alkylresorcinols, avenanthramides, benzoxazinoids (DIBOA aglycon and glycosides, BOA and MBOA), flavonoids, hordatines and phenolic acids were identified and quantified using an Agilent 1100 Series high-performance liquid chromatography equipped with a diode array detector (Agilent, Waldbronn, Germany). The HPLC pumps, autosampler, column oven, and diode array system were monitored and controlled by the ChemStation computer program. The analytical column was Nova Pak C18 (150 * 3.9 mm i.d., 4 μm, Waters, Milford, MA, USA) at 35 °C. The mobile phase was a gradient of 0.05 M phosphate buffer at pH 2.4 (A) and methanol (B) at 0.9 ml/min. For the analysis of phenolic acids the gradient was 5−60% B within 50 min, followed by 60−90% B in 6 min. Alkylresorcinols, benzoxazinoids, flavonoids and hordatines were analyzed using the gradient program as follows: 5−60% B within 50 min, 60−90% B in 6 min, hold at 90% for 12 min, and finally to 100% B within 32 min. Hordatines and benzoxazinoids in beer samples were analyzed using the method for phenolic acids. Injection volumes were 10 or 25 ul.

For identification purposes, UV/VIS -spectra were recorded at 190-600 nm. The compounds were quantitated as follows:

- *Alkylresorcinols* were quantified at 280 nm.
- *Avenanthramides* were quantitated at the wavelength of 350 nm. Usually, only three major avenanthramides, 2c, 2p and 2 f, were detected. Unidentified avenanthramides were quantified as Tranilast (Pihlava *et al*. 2008).
- *Benzoxazinoids*, mainly unspecified sugar conjugates of DIBOA and in some cases DIMBOA as well as the breakdown product BOA and MBOA, were quantified at 280 nm. Usually in rye samples, two closely eluting compounds with distinctive UV-spectra of DIBOA were seen (Figure 3 in Publication II). These two compounds were quantified separately as DIBOA aglycon (II) or as a sum of these (IV and VI).
- *Flavonoids* (II, VI) were tentatively identified from the chromatogram at 370 nm (Figure 4 in Publication II) based on their typical UV-spectra similar to flavonols, flavones or flavanones and were quantified as sum of quercetin equivalents.
- *Hordatines* were quantified at 280 nm. Usually several peaks with typical UV-spectra of hordatines were detected (see Figure 2 in Publication IV), these were quantified separately, but presented as summed amount.

- The wavelengths used for the quantification of *phenolic acids* in Study II were as follows: 280 nm for caffeic acid, *p*-coumaric acid, ferulic acid, 3,4- dihydroxy-benzaldehyde; 254 nm for *p*-hydroxy-benzoic acid; 270 nm for vanillic acid and 350 nm for sinapic acid. Ferulic acid dehydrodimers were tentatively identified as 8-0-4′-DiFA, 5-5′-DiFA and 8-5′-DiFA benzofuran form (Bf) based on the literature (Andreasen *et al*. 2000a and 2000b) and quantitated at 280 nm using response factors presented by Waldron *et al*. (1996). The amount of ferulic acid dehydrodimers is presented as the sum of these compounds (II). Analytical conditions used for the determination of phenolic acids in Study I, are described in detail in the original publication.

### 4.3.2 GC-MS analysis

In Study II, lignans were analyzed by a gas-chromatograph equipped with a mass selective detector (Hewlett-Packard 5890 II GC-5971 MSD, Palo Alto, CA, USA) using the selected ion monitoring technique. The analytical column used was ZB-1ms (25 m, 0.25 mm, 0.25 µm $d_f$, Phenomenex, Torrance, CA, USA) with helium as a carrier gas set to a constant flow of 0.9 ml/min. The analytical parameters were as follows: injector temperature 250 °C, oven temperature program: 85 °C (1.40 min), 85 to 150 °C at 15 °C/min, 150 to 210 °C at 3 °C/min, 210 to 280 °C at 20 °C/min and hold at 280 °C for 30 min and gc-ms-transferline temperature 280 °C. The sample was injected (2 ul) in a splitless mode to a deactivated double-gooseneck liner with purge-off time 1.40 min. The *m/z* target ions and qualification ions (in parentheses) were as follows: hydroxymatairesinol 297 (298, 179), isolariciresinol 455 (527,209), lariciresinol 223 (179, 209), pinoresinol 223 (209, 194), matairesinol 209 (179, 502), secoisolariciresinol 209 (261,179), syringaresinol 239 (224, 253), internal standard 4,4′-dichloro benzhydrol 235 (237, 177) and PCB 53 292 (220, 290).

### 4.3.3 UPLC-QTOF MS analysis

In studies III, IV and VI, Acquity UPLC I-Class system was coupled to a Xevo G2 QTOF mass spectrometer (Waters, Milford, MA, USA). Compounds were separated on a Waters Acquity BEH C18 (1.7 µm, 2.1 mm * 150 mm) column using a gradient of 0.1 % formic acid in water (A) and of 0.1 % formic acid in acetonitrile (B). The gradient programme was as follows: 2−60 % of B in 24 min, 60−100 % of B in 24−31 min, held at 100 % of B for 2 min, 100−2 % in 1

min and held at 2 % of B for 4 min. The mobile-phase flow rate was 0.55 ml/min. Temperature of the column oven was 45 °C. Sample injection volume was one or two µl.

The UPLC was connected to the mass spectrometer via an electrospray interface (ESI), using the capillary voltage of +0.5 kV and -1. Lower capillary voltages compared to the default value ± 3 kV have been recommended by Waters when higher eluent flow rates, e.g. 0.5 ml/min, are used. The sampling cone was set to 35 V and extraction cone to 4 V. The cone and desolvation nitrogen gas flows were 15 and 990 l/h, respectively. The desolvation temperature was 550 °C. Source temperature was 150 °C. Argon was used as the collision gas.

The mass spectrometer was operated in high-resolution negative or positive ion mode. Sample analysis was done by data independent acquisition ($MS^E$) centroid data mode in a full scan m/z 50-1200 with 0.2 sec scan time. Acquisition of mass spectral data was accomplished in two continuous functions. In the first function, precursor data was collected using the constant low collision energy of 4 V in MS-mode and in the second, $MS^E$, function, the precursor ions were further fragmented using high collision energy ramped from 15 to 40 eV. Leucine enkephalin ($C_{28}H_{37}N_5O_7$; m/z 554.2615 in negative and m/z 556.2771 in positive mode) was used as a reference lock-mass compound with automatic mass correction enabled. The system was operated by Waters MarkerLynx 4.1 software.

## 4.4 Statistical analysis

In Study IV, the statistical analysis of beer comparison was conducted by Senior Specialist Timo Hurme (M.Sc. Statistics) using SAS Statictical Analysis System (version 9.4, SAS Insitute Inc., Cary, North Carolina, USA). The total hordatine contents in individual beers were analyzed using an unequal slopes analysis-of-covariance model, where the mean of the total hordatine content of different beer types was compared to the alcohol content (alcohol by volume, ABV) of the beers. Concentrations of the total hordatine contents were also normalized by their alcohol content, i.e. divided by the ABV. The normalized total hordatine contents of individual beers were analyzed using a one-way analysis of variance model, where the mean normalized total hordatine contents of different beer types were compared. Square root transformation was applied to the response variable in order to meet the model assumptions. The statistical models were fitted using the GLM procedures of SAS 9.4 and the model assumptions were checked using appropriate graphs.

## 4.5 Method validation and quality assurance/control

Laboratory work in Jokioinen was done complying with the standard EN ISO/IEC 17025. The laboratories (T024) of Natural Resources Institute Finland (Luke) are accredited by the FINAS Finnish Accreditation Service (Helsinki, Finland), as they also were in the former MTT Agrifood Research Finland. However, it should be noted that of the methods used in these studies, only the ones used for proximate composition were accredited.

Validation of the methods was done as comprehensive way as possible, although in many cases lack of commercial reference compounds set limits to, e.g, recovery tests. In these cases, factors such as extraction time and/or different extraction solvents were tested in order to end up with the most quantitative (accuracy) and reproduciple (precision) methods. Inter-laboratory comparisons were proven to be (at least few years ago) practically impossible. Validation included also estimations of limit of detection and quantitation, determination of linearity and range and evaluation of ruggedness and robustness.

Expanded uncertainty (k=2) of the methods was 30 % for alkylresorcinols, avenanthramides, benzoxazinoids, flavonoids, hordatines and phenolic acids and 40 % for lignans.

All chemical analyses with quantitative results were performed at least in duplicate. Mean values of results were accepted if their difference was < 20 %. Each sample batch contained a certain mixture of QA/QC -standards in order to verify the performance of the analytical instrument. In addition, the 5971 mass selective detector used in lignan analysis was checked on a regular basis with perfluorotributylamine (PFTBA). Correctness of the calibration and detector of the Xevo G2 QTOF MS were checked on a regular basis by using the automated IntelliStart$^{TM}$ system with sodium formate and leucine enkephalin.

In-house reference materials were used whenever possible.

# 5 RESULTS AND DISCUSSION

## 5.1 Development of HPLC-DAD –method (I, III-VI)

The HPLC-method described for the analysis of flavonoid aglycons by Hertog *et al*. (1992) was used as a basis for method development for alkylresorcinols, benzoxazinoids, hordatines and phenolic acids. Separation of the compounds was done in a reverse-phase mode with a Waters Nova-Pak C18 column. Methanol was preferred as a second eluent with phosphate buffer (pH 2.4) because it is less toxic and more economic to use than acetonitrile. The gradient program of eluents was similar for all analytes, except in the cases when alkylresorcinols were included in the analysis. For the alkylresorcinols, the eluent programme included an extra gradient step ending in 100 % methanol. The extended method took over 90 min (plus the equilibrium time between the analysis), which is clearly a drawback and sometimes is the limiting step in sample throughput. The HPLC-method has over the years proven to be cost-effective, with a low system down-time. For example changing of analytical columns or mobile phases has been able to be kept minimal**.** The downside for using a more traditional analytical column is that it cannot provide a similar resolution as the new column types with smaller particle size (3 μm) and/or with the solid core particles and smaller inner diameter (e.g. 3 mm).

## 5.2 Avenanthramides in oats (I)

Avenanthramides were analyzed from two oat flake samples and one oat bran sample. Total avenanthramide content (sum of 2c, 2f and 2p) in oat flakes and precooked oat flakes were similar, 27 and 26 mg/kg, respectively. Thus, a portion of oat flakes (40 g), in the form of porridge, would provide approximately 1 mg of avenanthramides. These values are in line with other published total avenanthramide contents, such as in two Swedish oat cultivars 32.1–64.2 mg/kg (Bratt *et al*. 2002), in seven Swedish oat cultivars 7.7–18.3 mg/kg (Bryngelsson *et al*. 2002b), in four Turkish genotypes 13.5–36.2 mg/kg (Dokuyucu *et al*. 2003), in 33 US oat genotypes 3.6–16.0 mg/kg (Peterson *et al*. 2005) and in oat flakes and flours 5.6–24.4 mg/kg (Bryngelsson *et al*. 2002). However, clearly higher total avenanthramide values have also been reported, such as in three US oat cultivars 71.3–126.8 mg/kg (Emmons & Peterson 2001), in four naked oat cultivars 82.5–343.5 mg/kg (Bratt *et al*. 2002), in three Swedish oat cultivars 74–152 mg/kg (Dimberg *et al*. 1996), in ten US oat

cultivars 9.9–207.6 mg/kg (Li *et al*. 2016), and in 39 Chinese oat genotypes 22.1–471.2 mg/kg (Li *et al*. 2017).

Total avenanthramide content in oat bran was about half of that found in oat flakes, i.e. 13 mg/kg. However, direct comparison of the total avenanthramide content in the bran and flake samples should be done cautiously, since the oat raw material is most likely different in these cases. Avenanthramides are localized in the outer parts of the grain. Based on the results presented by Hitayezu *et al*. (2017) the enrichment factor for total avenanthramides in whole-grain oats *vs*. fine bran milling fraction would be 2.4. The enrichment factor calculated from the results of Peterson *et al*. (2001) was 3.5 for total avenanthramides using abrasive milling that removed 14 % of the groat weight.

## 5.3 Alkylresorcinols in rye, wheat and barley (I, II and VI)

The content of alkylresorcinols in cereal samples (Study I) and rye fractions (studies II and VI) is presented in **Table 10**. As expected, alkylresorcinols were not found in oat products, rice, millet or maize, but were present in rye, wheat and barley products. The highest amounts of alkylresorcinols were found in rye and wheat brans. The content of alkylresorcinols in whole-grain rye, rye bran and fine flour (Table 2) were similar to Andersson *et al*. (2010), Heiniö *et al*. 2008, Kulawinek *et al*. 2008, Menzel *et al*. (2012) and Ross *et al*. (2001, 2003, 2012). The total content of alkylresorcinols in rye bran was clearly higher in Study I (3930 mg/kg without C19:1 and unknown AR) than in study II and VI (1433 mg/kg and 2916 mg/kg, respectively).

The content of alkylresorcinols in rye products as mg/kg in dry weight (DW) and fresh weight (FW) are presented in **Table 11**. In soft rye bread samples the total content of alkylresorcinols was 872 mg/kg dw in Study I and 640–904 mg/kg dw in Study VI. These results are in line with Meija *et al*. (2013) (652–841 mg/kg dw) and somewhat higher than reported by Kulawinek *et al*. (2008) (300–614 mg/kg dw) and Chen *et al*. (2004) (197–686 mg/kg dw). The total content of alkylresorcinols in crisp bread was 868 mg/kg and in sourdough crisp bread 618 mg/kg, which are similar to the values in Andersson *et al*. (2010) (759–950 mg/kg dw), Ross *et al*. (2003) (886–1007 mg/kg dw), Chen *et al*. (2004) (490–804 mg/kg dw) and Menzel *et al*. (2012) (415–1182 mg/kg fw). Alkylresorcinol content of malted rye samples and mämmi were comparable to the bread samples on a dry weight basis (**Table 11**).

If the fresh weight results are converted to serving sizes, then a one slice (28 g) of soft rye bread would provide 14–18 mg; one slice (15 g) of crisp bread 8–12 mg; one tablespoon (10 g) of rye malt flour 9 mg and one serving (100 g) of mämmi 40 mg of alkylresorcinols.

The C17:0/C21:0 ratio was 0.9–1.4 in rye samples (**Table 10**) and in rye products 0.9–1.4 (**Table 11**). Andersson *et al*. (2010) reported C17:0/C21:0 ratio of 0.8–0.9 in 14 whole-grain rye flours, Kulawinek *et al.* (2008) reported ratios of 1.23–1.89 in ten rye samples and Meija *et al*. (2012) found C17:0/C21:0 in ratio of 1.2–1.3 in four Finnish rye breads.

The higher C17:0/C21:0 ratio, especially in Study I, could be a result that the response factors calculated based on the calibration curve of olivetol (5-pentylresorcinol) were used in quantitation of individual alkylresorcinols or due to co-eluting compounds with C17:0.

Hot 1-propanol/water (3:1 v/v) is generally recommended for extraction of alkylresorcinols in bread samples, since it is able to extract lipids from stach-lipid complexes (Ross *et al*. 2003). Method comparison using hot 1-propanol extraction and our standard methanol extraction is something to be done in the future.

**Table 10.** Alkylresorcinols in cereal samples (mg/kg). Abbreviations nd= not detected; R1−R5 and R7 see Figure 20.

| Sample | C17:0 mg/kg | C19:1 mg/kg | C19:0 mg/kg | C21:0 mg/kg | unknown mg/kg | C23:0 mg/kg | C25:0 mg/kg | **AR sum mg/kg** | **C17:0 to C21:0 ratio** |
|---|---|---|---|---|---|---|---|---|---|
| **Study I** | | | | | | | | | |
| Whole-grain rye, flour | 240 | 33 | 260 | 180 | 34 | 110 | 70 | **927** | 1.3 |
| Whole-grain rye, flour, organic | 270 | 38 | 280 | 190 | 40 | 120 | 70 | **1008** | 1.4 |
| Rye bran | 1100 | 130 | 1300 | 850 | 48 | 420 | 260 | **4108** | 1.3 |
| **Study II** | | | | | | | | | |
| Whole-grain rye flour R1 | 183 | | 231 | 207 | | 99 | 89 | **809** | 0.9 |
| Coarse fraction R2 | 173 | | 227 | 185 | | 94 | 101 | **781** | 0.9 |
| Endosperm R3 | 93 | | 109 | 99 | | 55 | 54 | **410** | 1.0 |
| Fine fraction R5 (from R2) | 255 | | 275 | 191 | | 99 | 81 | **901** | 1.3 |
| Bran fraction R4 (from R2) | 367 | | 450 | 329 | | 157 | 156 | **1459** | 1.1 |
| Bran R7 Prog.milling | 394 | | 440 | 296 | | 218 | 85 | **1433** | 1.3 |
| **Study VI** | | | | | | | | | |
| Whole-grain rye flour | 177 | | 226 | 137 | | 72 | 50 | **663** | 1.3 |
| Fine flour, endosperm | 36 | | 53 | 29 | | 19 | 18 | **155** | 1.3 |
| Bran | 750 | | 994 | 685 | | 312 | 176 | **2916** | 1.1 |
| **Study I** | | | | | | | | | |
| Whole-grain wheat, flour | 71 | 28 | 242 | 293 | 30 | 70 | 23 | **759** | 0.2 |
| Wheat, white flour | nd | 13 | 11 | 16 | 6.5 | nd | nd | **47** | - |
| Wheat, white flour, organic | nd | 13 | 10 | 16 | 5.0 | nd | nd | **44** | - |
| Wheat bran | 260 | 72 | 950 | 1500 | 33 | 310 | 100 | **3225** | 0.2 |
| Whole-grain barley, flour | 7.0 | nd | 15 | nd | nd | 10 | nd | **32** | - |

**Table 11.** Alkylresorcinols in rye products (mg/kg). Abbreviations: nd= not detected, DW=dry weight, FW=fresh weight.

| Sample | C17:0 mg/kg | C19:1 mg/kg | C19:0 mg/kg | C21:0 mg/kg | unknown mg/kg | C23:0 mg/kg | C25:0 mg/kg | **AR sum mg/kg** | **C17:0 to C21:0 ratio** | serving size (g) | AR per serving mg |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Study I** | | | | | | | | | | | |
| WG soft rye bread, DW | 250 | 38 | 250 | 166 | 33 | 87 | 48 | **872** | 1.5 | | |
| *WG soft rye bread, FW* | *150* | *23* | *150* | *100* | *20* | *52* | *29* | ***524*** | 1.5 | 28 | 15 |
| **Study VI** | | | | | | | | | | | |
| WG soft rye bread 1, DW | 219 | | 261 | 168 | | 98 | 42 | **788** | 1.3 | | |
| *WG soft rye bread 1, FW* | *156* | | *186* | *120* | | *70* | *30* | ***562*** | | 28 | 16 |
| WG soft rye bread 1, DW | 159 | | 196 | 155 | | 80 | 49 | **640** | 1.0 | | |
| *WG soft rye bread 2, FW* | *123* | | *152* | *120* | | *62* | *38* | ***495*** | | 28 | 14 |
| WG soft rye bread 1, DW | 190 | | 246 | 201 | | 114 | 58 | **809** | 0.9 | | |
| *WG soft rye bread 3, FW* | *140* | | *182* | *149* | | *84* | *43* | ***598*** | | 28 | 17 |
| WG soft rye bread 1, DW | 223 | | 249 | 244 | | 108 | 80 | **904** | 0.9 | | |
| *WG soft rye bread 4, FW* | *156* | | *174* | *170* | | *75* | *56* | ***631*** | | 28 | 18 |
| Crisp bread DW | 215 | | 260 | 218 | | 114 | 60 | **868** | 1.0 | | |
| *Crisp bread FW* | *193* | | *233* | *196* | | *102* | *54* | ***779*** | | 15 | 12 |
| Crisp bread, sourdough, DW | 149 | | 212 | 144 | | 70 | 44 | **618** | 1.0 | | |
| *Crisp bread, sourdough, FW* | *132* | | *188* | *127* | | *62* | *39* | ***547*** | | 15 | 8 |
| Rye malt flour, DW | 215 | | 301 | 217 | | 106 | 74 | **914** | 1.0 | | |
| *Rye malt flour, FW* | *200* | | *280* | *202* | | *99* | *69* | ***851*** | | 10 | 9 |
| Coarse rye malt, DW | 138 | | 184 | 144 | | 67 | 42 | **574** | 1.0 | | |
| *Coarse rye malt, FW* | *128* | | *170* | *133* | | *62* | *39* | ***531*** | | - | - |
| Mämmi, DW | 281 | | 336 | 247 | | 108 | nd | **972** | 1.1 | | |
| *Mämmi, FW* | *116* | | *138* | *102* | | *44* | *nd* | ***400*** | | 100 | 40 |

## 5.4 Lignans in rye (II and VI)

### 5.4.1 Determination of lignans in rye by GC-MS (II)

The total amount of lignans in wholegrain rye R1 (Figure 20) was 11.39 mg/kg, which was lower than reported before by Peñalvo *et al*. (2005) (18.9 mg/kg), Heiniö *et al*. (2008) (22.7 mg/kg) and Smeds *et al*. (2009) (25–67 mg/kg). The difference was mainly due to the lower content of syringaresinol. However, the contents of secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, isolariciresinol and hydroxymatairesinol were comparable with the references mentioned.

Of the rye grain´s (R1) absolute total lignan amount, 57 % was found in the bran R4 and 41 % in the fine fraction R5. Endosperm fraction R3 contained the least amount of lignans. The results about the distribution of lignans in the bran and endosperm rich fractions are consistent with the results reported by Liukkonen *et al*. (2003), Heiniö *et al*. (2008) and Glitsø *et al*. (2000). Again, the contents of other lignans except syringaresinol were comparable with the values presented by Kamal-Eldin *et al*. (2009) (total amount of lignans 58–70 mg/kg) in commercial rye brans. Concentrations of individual lignans in rye fractions can be found in Table 2 of original Publication II.

### 5.4.2 Identification of lignans by UPLC-QTOF MS (VI)

In Study VI, mass spectral data was searched for a number of lignans based on various literature sources, such as Hanhineva *et al*. (2012), Gauthier *et al*. (2015) and Smeds *et al*. (2007). The extraction method was much simpler in Study VI than in Study II, and it thus would include only the readily extactable forms of lignans. Lignans which were found are as follows: buddlenol C, buddlenol D, buddlenol E, hedyotisol A, methoxyhedyotisol A, seco-isolariciresinol, syringaresinol and hydroxymatairesinol. In addition, hexosides of secoisolariciresinol, syringaresinol and hydroxymatairesinol were found, as well as the dihexoside form of syringaresinol (Table 3 in Publication VI). Interestingly, the response of buddlenol C–E, hedyotisol A and methoxy-hedyotisol A in our mass spectrometric system was better in positive mode and as sodium adducts. On the other hand, secoisolariciresinol, secoisolariciresinol-glucoside, syringaresinol and hydroxymatairesinol were observed only in negative mode (Table 3 in Publication VI). Of the found lignans, buddlenol C–E, hedyotisol A and syringaresinol hexoside appear to withstand various food processing methods best, since these were present in all rye products.

## 5.5 Phenolic acids (I, II, VI)

### 5.5.1 Determination of the total amount of phenolic acids in cereals (I)

Results of total, soluble and bound, phenolic acid content in rye, wheat, barley and oat products are presented in **Figure 22**.Whole-grain flours of rye and wheat provided approximately the same amount of phenolic acids (1518 and 1496 mg/kg dw, respectively), and whole-grain oats and barley roughly three times less than that of rye and wheat (518 and 498 mg/kg dw, respectively). Ferulic acid formed approximately 65 % of the total phenolic acids in rye and wheat, while in barley and oats it was about 55 %.

As a comparison, a slice of rye bread (28 g) would provide 21 mg of phenolic acids, while the amount in the same serving size of white wheat bread would be 3 mg (calculated from the results in Table 1 of the original Publication I).

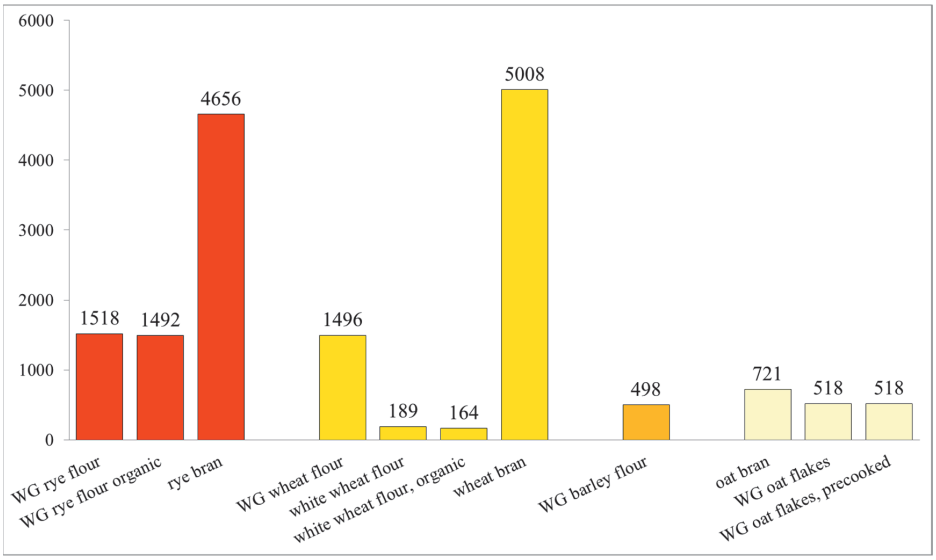



**Figure 22**.Total phenolic acids (soluble and bound) in cereal flours (mg/kg dw). Abbreviation WG= whole-grain. Processed results from Table 1 in original Publication I.

### 5.5.2 Determination of phenolic acids in rye (II)

Description relating to the various forms of phenolic acids is often vague and sometimes simply confusing. For example, the terms “bound” and “conjugated” can be mixed easily. Therefore, in study II a systematic nomenclature of various phenolic acid fractions was developed and proposed for wider use for clarity reasons.

Briefly, fractions are first divided into two groups based on their extractability with an organic solvent mixture. The extractable fraction is called “OSS” (organic solvent soluble) and the non-extractable is “OSI” (organic solvent in-soluble). OSS was further divided into free phenolic acids, alkali-labile phenolic acid conjugates (i.e. esterified) and acid-labile phenolic acid conjugates (i.e. glycosidic). OSI was divided into phenolic acids esterified into cell walls, phenolic acids esterified into a water-extractable (WE) dietary fiber component and phenolic acids etherified into lignin structure. Although giving lot of information, the sample preparation protocol is very tedious and admittedly not suitable for the analysis of a large number of samples. Comparison of the results presented in the literature is difficult due to the variability of the used sample preparation methods. In addition, there could still be some phenolic acid fractions, which should be considered when developing a new and even better method for the analysis of phenolic acids. For example, we found out (unpublished results) that additional heating step at the end of saponification would indeed liberate more phenolic acids as mentioned by Martens (2002).

Results of the phenolic acids in rye and its fractions are presented in **tables 12** and **13**. Results of the individual phenolic acids can be found from the original article. Total phenolic acid content in wholegrain rye flour (R1, Figure 20) and rye brans R4 and R7 were quite similar to the values presented in Study I, despite the differences in sample preparation method. Compared to Nyström *et al*. (2008) the total phenolic acid content was clearly higher in Study II, but the OSS free and conjugated fractions were similar. A major part of the phenolic acid pool in rye is in the OSI esterified and etherified groups (**Table 13**). Of the OSI ferulic acids, ca. 15% was bound to water-extractable (WE) fiber in whole-grain rye R1, i.e. expressed in absolute value, the amount of WE-OSI ferulic acid was 97 mg/kg. The amount of water soluble pentosan in R1 was determined earlier to be 15 g/kg (Nordlund *et al*. 2013), so the amount of ferulic acid in water extractable fiber was 0.65 %, which is twice as high as the 0.35% reported by Figueroa-Espinoza *et al*. (2002). This could indicate the presence of other, yet unknown, forms of water-soluble complexes containing ferulic acid. WE-OSI ferulic acid was found in the bran R4 (291 mg/kg; 1.7 % in water soluble pentosan) and R7 (206 mg/kg; 1.4 % in water soluble

pentosan), but not in the fine endosperm-rich fractions R3 and R5. This is consistent with the fact that arabinoxylans are enriched in the pericarp/testa fraction of rye, and in this fraction the absolute amount of water-extractable arabinoxylans is highest (5.5 g/100 g), followed by the aleurone layer (4.6 g/100 g) and endosperm (2.2 g/100 g) (Glitsø & Bach Knudsen 1999). Although the sample preparation method was totally different to that used in Publication I, the total amount of phenolic acids in rye flours and bran were quite comparable.

Results by HPLC analysis support rather well those made earlier by Folin-Ciocalteu method (Nordlund *et al*. 2013), although no statistical analysis to support this observation was done. However, based on the results presented in **Table 14**, it can be also concluded that Folin-Ciocalteu gives an estimation of phenolic compounds at a quite rough level. It is well-known that the Folin-Ciocalteu is a somewhat unspecific test, and usually a more specific test, such as Fast Blue BB should also be used if the liquid chromatographic method is not available.

**Table 12.** Phenolic acid groups (mg/kg) in rye fractions. See Figures 20 and 21 for abbreviations.

| Group | Whole-grain R1 | Milling fractions R2 | R3 | R5 | Bran R4 | Bran R7 |
|---|---|---|---|---|---|---|
| OSS free | 10.9 | 13.3 | 6.2 | 7.7 | 19.5 | 27.1 |
| OSS esterified | 136.7 | 173.4 | 40.5 | 115.2 | 266.7 | 300.7 |
| OSS glycosidic | 45.3 | 41.5 | 15.0 | 31.8 | 70.6 | 79.5 |
| OSI esterified | 927.3 | 1360.9 | 396.9 | 548.0 | 2411.1 | 2330.4 |
| *monomers* | *725.2* | *1124.3* | *210.5* | *372.8* | *2105.3* | *1995.0* |
| *dimers* | *202.1* | *236.7* | *186.4* | *175.1* | *305.8* | *335.4* |
| OSI etherified | 576.8 | 413.7 | 327.0 | 411.4 | 1362.7 | 847.4 |
| *monomers* | *272.7* | *128.7* | *39.8* | *140.7* | *876.1* | *415.8* |
| *dimers* | *304.1* | *285.0* | *287.1* | *270.7* | *486.6* | *431.5* |
| **Total (mg/kg)** | **1646.2** | **2002.8** | **785.6** | **1114.1** | **4130.6** | **3585.1** |

**Table 13.** Distribution (%) of phenolic acid into groups in rye fractions. See Figures 20 and 21 for abbreviations.

| Group | Whole-grain R1 | Milling fractions R2 | R3 | R5 | Bran R4 | Bran R7 |
|---|---|---|---|---|---|---|
| OSS free | 0.6 | 0.7 | 0.8 | 0.7 | 0.5 | 0.8 |
| OSS esterified | 8.1 | 8.7 | 5.2 | 10.3 | 6.5 | 8.4 |
| OSS glycosidic | 2.7 | 2.1 | 1.9 | 2.9 | 1.7 | 2.2 |
| OSI esterified | 54.6 | 67.9 | 50.5 | 49.2 | 58.4 | 65.0 |
| OSI etherified | 34.0 | 20.7 | 41.6 | 36.9 | 33.0 | 23.6 |
| **Total (%)** | **100.0** | **100.0** | **100.0** | **100.0** | **100.0** | **100.0** |

**Table 14.** Organic solvent soluble (OSS) fraction of phenolic and phenolic-like compounds and total phenolic compounds (OSS all + lignans + OSI esterified phenolic acids) in rye fractions (mg/kg) and the corresponding results by the Folin-Ciocalteu method (* as gallic acid equivalents GAE mg/kg) (adopted from Nordlund *et al*. 2013). See Figures 20 and 21 for abbreviations.

| Group | Whole grain | Milling fractions | | | | |
|---|---|---|---|---|---|---|
| | **R1** | **R2** | **R3** | **R5** | **Bran R4** | **Bran R7** |
| OSS phenolic acids | 193 | 228 | 62 | 155 | 357 | 407 |
| Flavonoids | 30 | 36 | 7 | 27 | 45 | 58 |
| Alkylresorcinols | 809 | 781 | 410 | 901 | 1459 | 1433 |
| Benzoxazinoids | 161 | 173 | 55 | 151 | 232 | 293 |
| **Sum of OSS compounds (mg/kg)** | **1193** | **1218** | **534** | **1234** | **2093** | **2191** |
| F-C free phenolics (GAE mg/kg)* | 800 | 1000 | 400 | 800 | 1300 | 1600 |
| *HPLC vs. F-C ratio* | *0.67* | *0.82* | *0.75* | *0.65* | *0.62* | *0.73* |
| **OSS all + lignans + OSI esterified** | **2131** | **2157** | **1465** | **2172** | **3042** | **3139** |
| F-C total phenolics (GAE mg/kg)* | 1700 | 2000 | 800 | 1200 | 3700 | 3500 |
| *HPLC+GC-MS vs. F-C ratio* | *0.80* | *0.93* | *0.55* | *0.55* | *1.22* | *1.12* |

## 5.6 Flavonoids and anthocyanins in rye (II, VI)

### 5.6.1 Determination of flavonoids and anthocyanins by HPLC-DAD (II)

Results of flavonoid and anthocyanin content in rye samples are presented in **Table 15**. Total flavonoid content in whole-grain rye flour was in studies II and VI 30 and 46 mg/kg, respectively, experessed as quercetin equivalents. Our earlier study showed that the average amount of flavonoids in four rye cultivars was 22 mg/kg dw on average (minimum value being 15 and maximum 34 mg/kg), quantitated as quercetin (Pihlava *et al*. 2010). These values are lower than those reported by Zieliński *et al*. (2007) and Michalska *et al*. (2007) 290−307 mg/kg and 431 mg/kg as catechin equivalent respectively. Slightly lower total flavonoid contents, 67− 75 mg/kg as catechin equivalent, in four rye genotypes, was reported by Žilić *et al*. (2011). In coarse and fine rye bran fractions, the flavonoid content was 1910 and 2390 mg/kg, respectively, as quercetin equivalent (Ivanišova *et al*. 2012).The main reason for the difference may be the different approaches of determination, namely liquid chromatographic *vs*. spectrophotometric method. Our results corroborated the results of Ivanišova *et al*. (2012), that flavonoids are enriched in the rye bran.

The total flavonoid content was low in three bread samples ca. 6 mg/kg dw and below the limit of quantitation (< 5 mg/kg) in one rye bread, both crisp breads and malted rye samples. In mämmi the total flavonoid content was higher, namely 20 mg/kg dw, due to the flavonoids in Seville orange zest (**Table 1** in original Publication VI).

The total amount of anthocyanidins in whole-grain rye flour was 1.8 mg/kg dw and the main anthocyanidin was delphinidin and the main anthocyanin being delphinidine-3-rutinoside. Like other flavonoids, anthocyanins are also enriched in the rye bran. Total anthocyanin content (3.6 mg/kg dw) in whole-grain rye flour was lower than in purple wheat (491 mg/kg) (Hosseinian *et al*. 2008) or in blue wheat (148 mg/kg) (Abdel-Aal *et al*. 2014). However, much more research would be needed regarding, e.g. cultivar variation of flavonoids and anthocyanins in rye. This kind of information could be of use while promoting health-related benefits of rye to consumers.

**Table 15.** Flavonoids, anthocyanins and benzoxazinoids in rye (mg/kg dw). See Figure 20 for abbreviations.

| | **Flavonoids** | **Flavonoids** | **Anthocyanidins (aglycons)** | | | | **Benzoxazinoids** | |
|---|---|---|---|---|---|---|---|---|
| | sum as quercetin | hydrol., sum as quercetin | Delphinidins | Cyanidins | Petunidins | **sum** | DiBOA | BOA mg/kg dw |
| **Sample** | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | **mg/kg** | mg/kg dw | |
| **Study II** | | | | | | | | |
| Whole-grain flour R1 | 30 | | | | | | 161 | |
| Coarse fraction R2 | 36 | | | | | | 173 | |
| Endosperm R3 | 7 | | | | | | 55 | |
| Fine fraction R5 (from R2) | 27 | | | | | | 151 | |
| Bran fraction R4 (from R2) | 45 | | | | | | 232 | |
| Bran R7 Prog.milling | 58 | | | | | | 293 | |
| | | | | | | | | |
| **Study VI** | | | | | | | | |
| Whole-grain rye flour | | 46 | 1.6 | 0.1 | 0.1 | **1.8** | 75 | |
| Fine flour, endosperm | | nd | 0.23 | 0.02 | 0.01 | **0.3** | 22 | 9 |
| Bran | | 181 | 13.4 | 1.1 | 0.5 | **15.0** | 265 | |

### 5.6.2 Identification of flavonoids and anthocyanins by UPLC-QTOF MS (VI)

Study VI was a consecutive and supplementing work to Study II, with more effort put into identification of flavonoids in rye milling fractions and rye products. Eleven flavonoid aglycon structures were tentatively identified from the $MS^E$ data in rye bran and whole-grain flour, namely one flavan-3-ol (catechin), one flavanone (naringenin), three flavones (chrysoeriol, luteolin and tricin) and five flavonols (kaempferol, laricitrin, quercetin, rhamnetin and syringetin). In addition, dihydrotricin, which has been earlier identified in barley (Nakano *et al*. 2011), was found in rye milling fractions and products. Examples of the structures of these flavonoids are presented in **Figure 23**.

With the exception of catechin, all of these flavonoid aglycones, as well as the additional two dihydroxy-tetramethoxyflavones and myricetin, were found from the extract of the acid-hydrolyzed bran sample. Luteolin, chrysoeriol and tricin were also present as aglycons in rye milling fractions (Table 3 in Publication VI). Most of the flavonoids were present as the disaccharide, rutinoside (rhamnosyl-glucose), conjugates in rye samples (Table 3 in Publication VI). Because it was not possible to determine the exact positions of the sugar conjugates in the flavonoid structures, the naming of the flavonoid glycosides was done by listing the tentative combination of possible sugar conjugates. Rye also contained anthocyanins, which were tentatively identified by QTOF MS analysis.

Flavonoids were first identified from the rye bran sample and then these compounds were checked from the fine flour and in rye products. Although the origin of the whole-grain rye raw material was most likely different in breads, malts and mämmi, the same flavonoids were found in most cases. Based on this preliminary study, preparation processes of crisp bread, sourdough crisp bread and soft sourdough bread, could lead to minor and in some cases severe losses of flavonoids. However, it seems that flavonoids can withstand long cooking (mashing) and baking times as seen in the case of mämmi. Flavonoids were found also in the light-colored, malted rye flour, while many of these were not detected from the considerably darker, coarsely ground rye malt. This could be due to the longer kilning time of the coarse rye malt.

The flavanone hesperitin and its sugar conjugate hesperidin, hesperitin-7-rutinoside, and narigenin´s rutinoside conjugate, narirutin, were found in mämmi, but these compounds originated from the Seville orange zest (Pellati *et al*. 2004) used as flavoring in mämmi.

The following flavonoid C-glycosides were found in rye bran and whole-grain flour: luteolin C–hexoside (aka orientin/iso-orietin), luteolin C–pentoside C–hexoside, unknown luteolin C–diglycosidic derivative, chrysoeriol C–hexoside, chrysoeriol C–hexoside C–hexoside, apigenin C–hexoside (aka vitexin/isovitexin), apigenin C–hexoside C–hexoside (aka vicenin 2), apigenin C–pentoside C–pentoside, apigenin C–pentoside C–hexoside (aka schaftoside/isoschaftoside) and apigenin C–hexoside–O–hexoside (Table 3 in the original Publication VI).

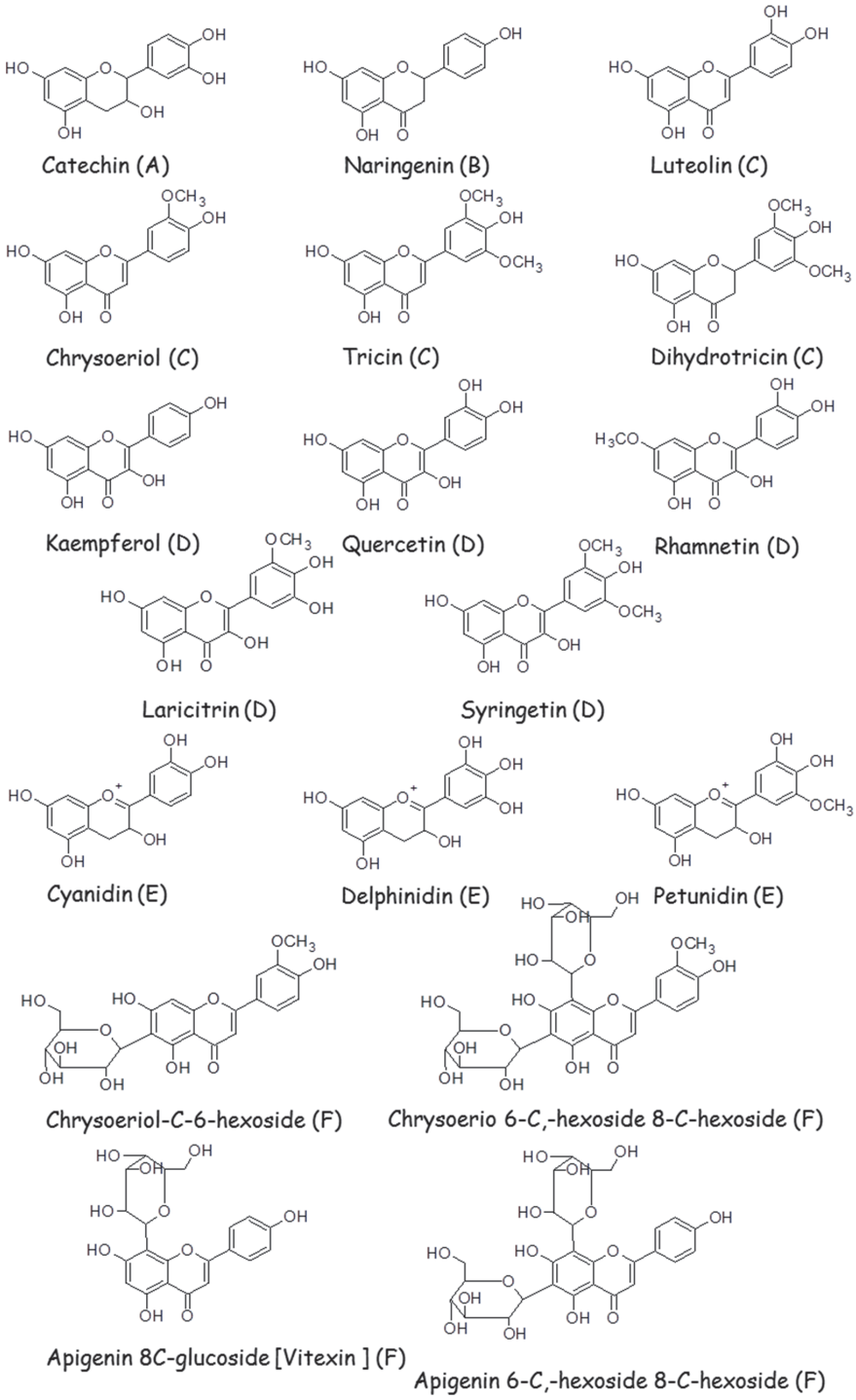



**Figure 23**. Examples of the chemical structures of flavan-3-ol (A), flavanone (B), flavones (C), flavonols (D), anthocyanidins (E) and flavone C-glycosides (F) found in rye.

## 5.7 Benzoxazinoids of rye and wheat (II, IV, VI)

### 5.7.1 Determination of benzoxazinoids in rye and its milling fractions by HPLC-DAD (II, VI)

Results of total benzoxazinoids in rye samples are presented in **Table 14.** The main benzoxazinoid in rye is DIBOA and it is mainly present as diglycoside (Hanhineva *et al*. 2011, Pedersen *et al*. 2011, Tanwir *et al*. 2013), which was also quantitated by HPLC-DAD in studies II and IV. Quantitation was done as DIBOA aglycon, with an assumption that the sugar units do not affect the molar absorptivity of the aglycon part. This is a justified assumption based on our experiences with flavonoid aglycons and their sugar conjugates, such as quercetin and quercetin-3-O-rutinoside, i.e. rutin.

The amount of DIBOA in whole-grain rye flour in studies II and VI was 161 and 75 mg/kg, respectively. These results are similar to those presented by Katina *et al*. (2007) and Hanhineva *et al*. (2014b). Benzoxazinoids are enriched in the bran fraction, which corroborates earlier studies such as Tanwir *et al*. (2013).

In four commercial soft sourdough rye breads the concentration of DIBOA was 50–97 mg/kg dw and BOA 12–23 mg/kg dw (**Table 16**). For comparison, in the study by Jensen *et al*. (2017), the commercial Danish rye bread (Multikerne rugbrød, which, in addition to rye, contained also barley malt and wheat) contained DIBOA, calculated as aglycon, 64.6 mg/kg dw and BOA 6.2 mg/kg dw; the whole-grain buns (Mørk multikerne bolle which, in addition to wheat, contained also rye and oats) contained DIBOA 27.8 mg/kg dw and BOA 0.2 mg/kg dw and the sunflower buns (Solsikke klapper, which, in addition to wheat, contained also rye and barley malt) contained DIBOA 21.5 mg/kg dw and BOA 1.0 mg/kg dw. In the rye bread 9.3 % of DIBOA was present in free form, 61.0 % as monoglucoside and 29.7 % as diglucoside; in the whole-grain buns 3.1 % in free form, 37.4 % as monoglucoside and 59.5 % as diglucoside and in the sunflower buns 5.9 % in free form, 45.6 % as monoglucoside and 48.5 % as diglucoside (calculated from Jensen *et al*. 2017). Pedersen *et al*. (2011) reported the concentrations of BOA in two breads: the bread containing 1/3 wheat flour and 2/3 rye flour had 22.0–27.9 mg/kg dw and the other bread containing 1/3 wheat flour, 1/3 rye flour and 1/3 malted rye flour had 82.5–115.4 mg/kg dw. In theory, as all-rye breads these would correspond to BOA concentrations 33.0–41.9 mg/kg and 123.8–173.11 mg/kg. Unfortunately, the DIBOA diglucoside was not included in the work of Pedersen *et al*. (2011), so only BOA values can be used for comparison here. Interestingly, rye crisp bread and sourdough rye crisp bread contained lower amounts of DIBOA (35 and 26 mg/kg dw, respectively), but roughly the same amounts of BOA (30 and 18 mg/kg dw, respectively) than the soft sourdough rye breads in dry weight.

Clearly higher benzoxazinoid contents were found in malted rye flour (DIBOA 266 mg/kg dw; BOA 156 mg/kg dw) and mämmi (DIBOA 266 mg/kg dw; BOA 156 mg/kg dw), as was expected, because it is known that the content of benzoxazinoids increases during germination of rye grains (Katina *et al*. 2007, Pedersen *et al*. 2011). The decomposition product of DIBOA, namely BOA, was found in all rye products in this study (**Table 16**). The results in **Table 16** are also presented per serving size, in which case the organic mämmi would provide the highest dose of benzoxazinoids. Rye breads and rye malt flour would provide 5–10 times less benzoxazinoids per serving size than organic mämmi. However, organic mämmi is rarely consumed daily, or as often as rye breads, so it can be assumed that all-rye breads are likely to provide most of the dietary benzoxazinoids in Finland. One tablespoon of malted rye flour could also be added to porridge or cold cereals, and it would easily supplement the intake of benzoxazinoids.

### 5.7.2 Determination of benzoxazinoids in wheat and rye beers by HPLC-DAD (V)

Of the 32 wheat beers, 14 samples had the DIBOA content below 2 mg/l. Variation of the concentration of DIBOA in the remaining 18 wheat beers was high, ranging from 3.0 mg/l to 20.6 mg/l. DIBOA was also found in two non-/low-alcoholic beers at the concentrations of 6.8 and 12.3 mg/l. The decomposition product BOA was detected in 15 beer samples and MBOA in two (**Table 1** in Publication V). The average concentration of benzoxazinoids as DIBOA aglycon in wheat beers was 5.9 mg/l, which would mean that a bottle of beer (0.33 l) would contain approximately 2 mg of benzoxazinoids. However, wheat beers are usually sold in 0.5 l bottles, so the amount of DIBOA in these would be 3 mg per serving (**Table 16**). One of the most interesting results was from beer ID 12 (Austrian ale, ABV 4.7 %), in which spelt, einkorn wheat and emmer-wheat (as malts ?) were also used in addition to barley malts. The beer in question had 10.2 mg/l DIBOA, 9.6 mg/l BOA and 10.5 mg/l MBOA. Also of special interest is the concentration of DIBOA in beer pair ID 10 and ID 156 (English ale, ABV 4.5 %). The DIBOA content in the organic wheat beer (ID 156) was 21.9 mg/l and in “ordinary” wheat beer 8.4 mg/l, however this could be just co-incidence, but it would be interesting to study more deeply if agricultural practices would affect the contents of these compounds.

In case of rye beers, DIBOA was found in all four beers ranging from 5.6 to 31.6 mg/l. The decomposition product BOA was not detected (< 3 mg/l) (**Table 1** in Publication V). The average concentration of DIBOA in rye beer was 17.6

mg/l and thus a bottle of rye beer would contain roughly 6 mg of benzoxazinoids (**Table 16**).

Compared to the serving sizes, one or two slices of soft sourdough rye bread would provide an equal amount of DIBOA as one bottle of wheat beer. In case of a bottle of rye beer, six to eight slices of soft rye bread would be needed for an equal amount of DIBOA.

Considering the possible positive health effects of benzoxazinoids, it would be of importance to determine how to maximize the transfer of benzoxazinoids from wheat or rye malts into wort and consequently into beer. More research would be needed to study also how storage time affects the decomposition of benzoxazinoids into BOA and/or MBOA.

**Table 16.** Benzoxazinoids in rye products (mg/kg) and in wheat and rye beers (mg/L). DW=dry weight, *FW=fresh weight*. The absolute amount of DIBOA and BOA per serving size in fresh weight is given as mg.

| | **Benzoxazinoids** | | | Amount per serving | |
|---|---|---|---|---|---|
| **Sample** | DIBOA sum mg/kg | BOA mg/kg | serving size (g) | DIBOA sum mg | BOA mg |
| **Study VI** | | | | | |
| WG soft rye bread 1, DW | 67 | 20 | | | |
| *WG soft rye bread 1, FW* | *48* | *14* | 28 | 1.3 | 0.4 |
| WG soft rye bread 2, DW | 54 | 12 | | | |
| *WG soft rye bread 2, FW* | *42* | *9* | 28 | 1.2 | 0.3 |
| WG soft rye bread 3, DW | 98 | 23 | | | |
| *WG soft rye bread 3, FW* | *72* | *17* | 28 | 2.0 | 0.5 |
| WG soft rye bread 4, DW | 50 | 18 | | | |
| *WG soft rye bread 4, FW* | *35* | *13* | 28 | 1.0 | 0.4 |
| Crisp bread DW | 35 | 30 | | | |
| *Crisp bread FW* | *31* | *27* | 15 | 0.5 | 0.8 |
| Crisp bread, sourdough, DW | 26 | 18 | | | |
| *Crisp bread, sourdough, FW* | *23* | *16* | 15 | 0.3 | 0.4 |
| Rye malt flour, DW | 266 | 156 | | | |
| *Rye malt flour, FW* | *248* | *145* | 10 | 2.5 | 1.5 |
| Mämmi, DW | 283 | 163 | | | |
| *Mämmi, FW* | *117* | *67* | 100 | 11.7 | 6.7 |
| **Study V** | | | | | |
| Wheat beers, FW | *5.9* | *3.1* | 0.33 l | 1.9 | 1.0 |
| | | | 0.5 l | 3.0 | 1.6 |
| Rye beers, FW | *17.6* | | 0.33 l | 5.8 | |

### 5.7.3 Identification of benzoxazinoids by UPLC-QTOF MS (IV, VI)

With the mobile phase used and the analytical conditions chosen, the ionization of benzoxazinoids appeared to be better in negative than in positive mode. In negative mode $[M-H]^-$ ions were of good intensity, while in positive mode $[M+Na]^+$ ions were more intense than $[M+H]^+$ ions. Sodium adducts did not produce informative fragment ions in $MS^E$ mode.

In Study V, the MS data was filtered manually using exact masses ($[M-H]^-$ ± 25 mDa) of a number of possible hydroxamic acids, lactams and benzoxazolinones as well as potential microbial transformation products, and creating ion chromatograms. The $[M-H]^-$ ion in the mass spectrum of the peaks in these chromatograms were then checked using the Elemental Composition function. For confirmation purposes presence of $[M+H]^+$ ions of BOA and MBOA and for all other benzoxazinoids sodium adduct ($[M+Na]^+$) ions were checked from the positive mass spectrum data. In our analytical system the intensity of $[M+H]^+$ ion was poor compared to the $[M+Na]^+$ ion and could not be used in Elemental Composition evaluation. The same strategy was applied also in Study VI, although in this case the work was easier than in Study V.

Tentative identification of benzoxazinoids in wheat and rye containing beers by QTOF MS, revealed that benzoxazinoids can be conjugated with three or four hexose units (Study V). Besides these previously unknown glycosides, the whole idea that wheat or rye beer could contain benzoxazinoids was completely new, although, the decomposition products BOA and MBOA had been reported earlier in wheat beers by Pothou *et al*. (2013) and Manoukian *et al*. (2016).

In addition to the benzoxazinoids reported earlier, a new benzoxazinoid, which had not been earlier reported in wheat or rye, was proposed, namely DHBOA (2,7-dihydroxy-1,4-benzoxazin-3-one). This benzoxazinoid has been previously reported in aerial parts of yellow archangel (*Laminum galeobdolon*) by Alipieva *et al*. (2003) and in maize by Köhler *et al*. (2014), but at that time not in wheat or rye. Interestingly, de Bruijn *et al*. (2016) also reported DHBOA in elicited and germinated wheat in a publication received and accepted roughly a half year later than Study V had been accepted in a different journal.

Also based on the chromatographic behavior, a completely new benzoxazinoid structure, which would be the corresponding lactam form (HMBOA) of DIMBOA with an additional hydroxyl group attached preferably to the carbon in position eight, was proposed and named in Publication V as DHMBOA (2,8-dihydroxy-7 methoxy-1,4-benzoxazin-3-one). This compound was first found in the ion chromatograms ($[M-H]^-$ 372.0931 m/z and $[M+Na]^+$ 396.0907 m/z) of DIMBOA-hexoside, as an early eluting peak at 3.45 min was present in all wheat beers studied and was assumed to be the DHMBOA-

hexoside. However, this kind of benzoxazinoid has not been reported in the literature before and more studies would be needed for complete identification.

Benzoxazinoids were also of interest in rye and rye products (Study VI). Malted rye products showed a very complex benzoxazinoid profile compared to the whole-grain rye. It is worth noting that even harsh processing conditions do not decompose benzoxazinoids completely. Unfortunately, due to the lack of funding, the effect of processing on the benzoxazinoid content could not be studied. The diversity of benzoxazinoid sugar conjugates was found to be larger than expected on the basis of previous reports (Tanwir *et al*. 2013, Pedersen *et al*. 2011, Hanhineva *et al*. 2011). Of the lactams, HBOA-dihexoside and hexoside, HMBOA-dihexoside and of the hydroxamic acids, DIBOA-dihexoside and hexoside and DIMBOA-hexoside and of the benzoxazolinones BOA and MBOA have been reported in rye by Tanwir *et al*. (2013). In Study VI, also DIBOA-tetrahexoside and a number of DIBOA-trihexoside and DIBOA-dihexoside isomers, were tentatively identified. Especially malted rye contained many, presumably isomeric forms of di- and trihexosides. In malted rye flour, coarse rye malt and mämmi, but not in, e.g. rye bran, the same early eluting benzoxazinoids were observed as in wheat and rye beers, and proposed to be the hexoside and dihexoside of DHBOA (2-O-β-D-glucopyranosyl-7-hydroxy-2H-1,4-benzoxazin-3(4H)-one). Benzoxazinoids were found in all rye products. This was expected since earlier studies indicate that benzoxazinoids can withstand baking or boiling, although considerable changes in benzoxazinoid content and profile may occur (Pedersen *et al*. 2011, Tanwir *et al*. 2013), especially in the case of sourdough processing (Hanhineva *et al*. 2014, Savolainen *et al*. 2015). Also, decomposition products HPAA (N-2(2-hydroxyphenyl)acetamide and HHPAA (2-hydroxy-N-2(2-hydroxy-phenyl)acetamide) were tentatively identified from certain rye products (Table 3 in Publication VI). These have been previously reported as a result of sourdough fermentation by Hanhineva *et al*. (2014) and Savolainen *et al*. (2015).

As a closely related compound to benzoxazinoids, mono-, di- and triglucosides of aminophenol were tentatively identified in rye milling samples, but not in any rye product (Table 3 in Publication VI). The fragment of the aminophenol aglycon ($C6H10NO^+$ m/z 110.0607) was observed in the $MS^E$ spectra of these glucosides, but more studies would be needed to fully identify these compounds.

## 5.8 Phenolamides in barley and rye (III, VI)

### 5.8.1 Determination of total hordatine content of beers by HPLC-DAD (IV)

Study IV presented the total hordatine content in 208 beers. To avoid possible ranking of the beers based on their hordatine content, it was chosen to hide the beer names behind ID numbers. This was also justified because variations of hordatine contents in beers would be expected to mostly result from the variation of hordatine content in barley malts.

Due to the lack of commercial standards, quantitation of hordatines was done with *p*-coumaric acid, because it is commercially readily available and it is one of the building blocks in hordatine structures. Thus, the results of total hordatine content are expressed as mg/l *p*-coumaric acid equivalent (PCAE). One to six peaks showing typical UV-spectra of hordatines were seen in the chromatograms of the beer samples (Figure 2 in original Publication IV). Based on Study II, it was assumed that the major peaks would be hordatines A, B and C and their hexosides.

The average total hordatine content of all analyzed beers (n=208) was 5.6 ± 3.1 mg/l PCAE. Hordatine content in different beer groups is presented in **Table 17**. Hordatines were found in all beer samples except in three ales and in one wheat beer (limit of detection 0.1 mg/l PCAE). The highest hordatine contents, 18.7 and 17.7 mg/l PCAE, were found in ale ID 68 (Belgium, 6.6 % ABV) and barley wine ID 134 (USA, 10.2% ABV) respectively. One of the most interesting results was the quite high total hordatine content, 14.6 mg/l, in sahti (ID 103, 8 % ABV), which is a traditional Finnish malt beverage. Unfortunately, only one sahti sample was included in this study. There was no statistical difference on the average concentrations of hordatines between different types of beers, when the non-alcoholic (NA) beer group was excluded.

The results of the total hordatine contents in relation to the ABVs of all beers, except the non-alcoholic beers (NA), are presented in **Figure 24**. Total hordatine contents of ales (ALE), lagers (LA), stouts and porters (SP) and miscellaneous beers (MISC) correlates positively with the ABV. This is expected with the all malt beers, since to obtain higher ABV beers, more malt is needed to provide a sufficient amount of fermentable sugars. Also, the phenolic compounds extracted from the malts are in more concentrated form, i.e. less diluted with water, in stronger beers. In wheat beers (WB) the correlation with ABV was not clear and was different compared to other beer groups, which is most likely due to the different amounts of barley malts used in the production of these beers.

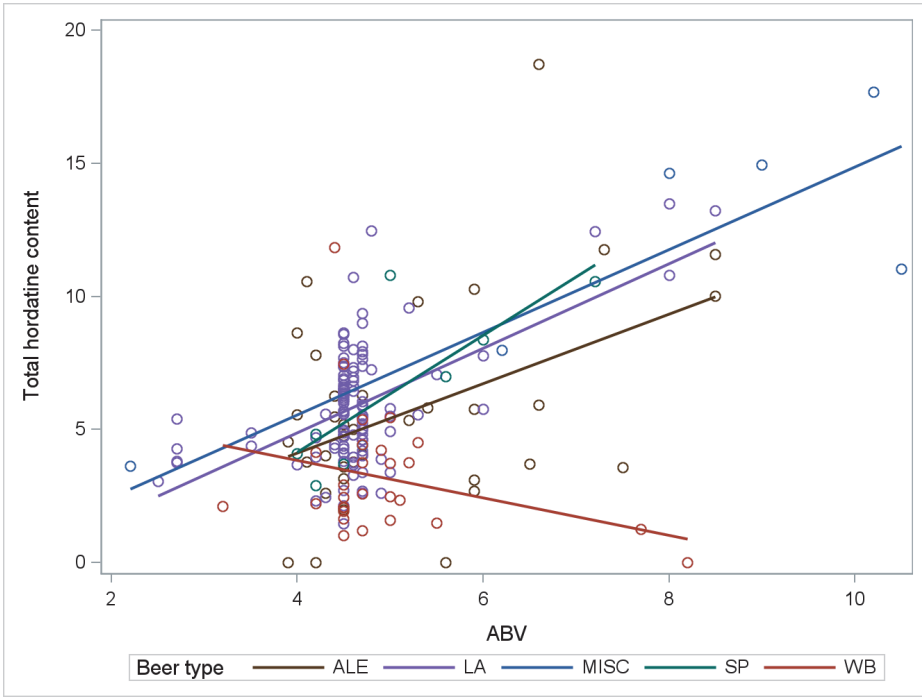



**Figure 24.** Total hordatine content (mg/l PCAE) in beers (non- or low alcoholic beers were excluded < 0.5 ABV) in correlation to ABV. Abbreviations: ALE= ale, LA=lager, MISC= miscellaneous, SP=stouts and porters and WB=wheat beers. IV. Reprinted from Pihlava *et al*. 2016. *Journal of the Institute of Brewing*, 122, 212–217 with permission from John Wiley and Sons.

Year to year variation of total hordatine content in the same brand of beers purchased in 2009 and 2011 can be seen in **Figure 25**. Of the 18 pairs of beers, the total hordatine content in seven beer pairs differed more than 20 %. Also of special interest is the hordatine content in wheat beer pair ID 10 and ID 156 (conventional and organic beer, respectively, UK, ABV 4.5 %). Contrary to the case with DIBOA, the total hordatine content in these wheat beers was almost identical: 2.1 mg/l in organic wheat beer and 1.9 mg/l in "conventional" one (Supporting Information Table S1 of the original Publication III).

It was assumed that a simpler way to compare the results could be achieved by normalization of the total hordatine content by ABV. It was also assumed that substituting barley malts with rice, corn, barley starch or other source of fermentable sugars, would lead to a lower normalized hordatine content in the beers, although this was not especially studied here.

**Table 17.** Total hordatine content (mg/l PCAE) in beers. Total hordatine content of the beers normalized by alcohol content (mg/l PCAE $ABV^{-1}$) are marked in *italic*. Normalization was not done for non-alcoholic beers (ABV < 0.5, n=13).

| | Average | Stdev | Min | Max | n |
|---|---|---|---|---|---|
| **All beers** | **5.6** | **3.1** | **0** | **18.7** | **208** |
| | *1.2* | *0.5* | *0* | *8.1* | 195 |
| Ales (ALE) | **5.7** | **3.7** | **0** | **18.7** | **38** |
| | *1.1* | *0.6* | *0* | *2.8* | |
| Lagers (LA) | **5.9** | **2.2** | **1.5** | **13.5** | **114** |
| | *1.3* | *0.4* | *0.3* | *2.6* | |
| Miscellaneous beers (MISC) | **10.6** | **5.5** | **3.6** | **17.7** | **7** |
| | *1.4* | *0.4* | *0.9* | *1.8* | |
| Stouts and porters (SP) | **6.5** | **3.1** | **2.9** | **10.8** | **8** |
| | *1.2* | *0.5* | *0.7* | *2.2* | |
| Wheat beers (WB) | **3.2** | **2.3** | **0** | **11.8** | **33** |
| | *0.7* | *0.5* | *0* | *2.7* | 28 |
| Non-alcoholic beer (NA) | **5.2** | **2.9** | **0.6** | **8.1** | **8** |

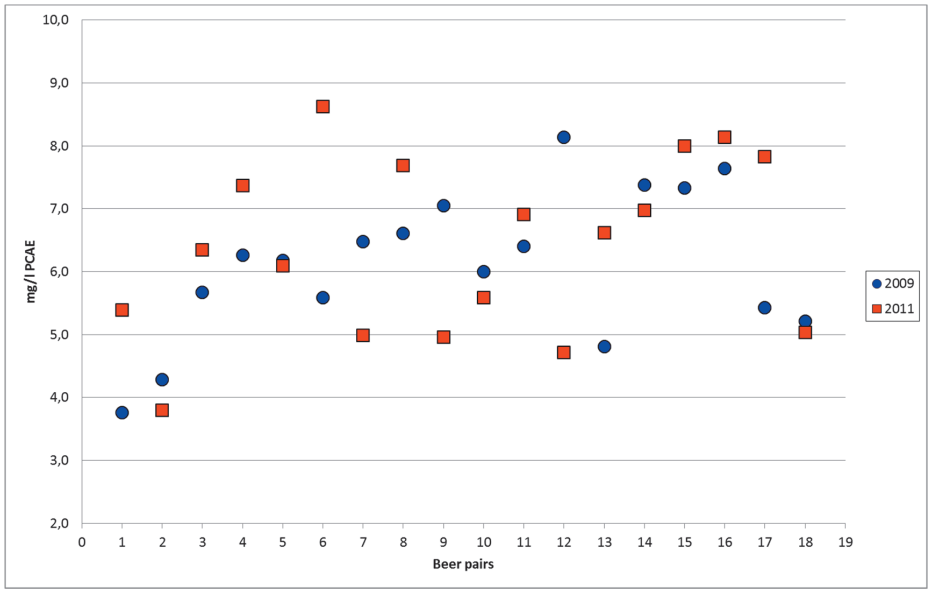



**Figure 25**. Variation of total hordatine content (mg/l PCAE) in the same 18 Finnish beer brands in 2009 and 2011. In the beer pairs one and two the ABV is 2.7, and in the rest the ABV is 4.5−4.7 %. Reprocessed data is from the Supporting Information Table S1 of Publication IV.

The normalized average total hordatine content of all analyzed beers excluding non-alcoholic beers (n=195) was 1.2 ± 0.5 mg/l PCAE. Normalized hordatine content in different beer groups is presented in **Table 17**. No statistical differences were found between different beer types or styles of normalized hordatine contents, when non-alcoholic (NA) beers were excluded.

The highest normalized hordatine contents, 2.8 and 2.7 mg/l PCAE, were found in ale ID 68 (Belgium, 6.6 % ABV) and in ale ID 147 (USA, 4.4 % ABV), respectively. While lower normalized hordatine content was found, e.g. in a globally known US lager beer (ID 18, Supplemental information of the original publication), in which 1/3 of the barley malt has been replaced by rice, the hordatine content was still 1.0 mg/l PCAE and actually about the same as in many all-barley lagers. This could indicate variations in the hordatine content of barley malts and possibly different extraction recoveries of hordatines due to the differences in the mashing process variables. However, this would also require more in-depth research with the knowledge of raw materials and details of the processing.

Perhaps the most striking result of Study IV was that the variation of total hordatine content in non-alcoholic beers was quite high, namely from 0.6 to 8.1 mg/l PCAE and in NA wheat beers from 1.6 to 5.7 mg/l PCAE (figures 2 and 3 and in the original Publication III Supporting Information Table S1). Also in this case, more research would be needed in order to understand how higher hordatine concentrations in non-alcoholic beers could be achieved.

Based on the results by Kohyama & Ono (2013) the hordatine A content in beer was estimated to be 9.6–23.8 mg/l (see Results and Discussion in the original Publication IV). If it is further assumed that at least hordatine B would be present at the same concentration, then the beer would contain hordatines roughly 19–48 mg/l. By multiplying the average concentration of total hordatine content (mg/l PCAE) in all beer (**Table 17**) by the response factor 5.5 obtained from our unpublished work, the total hordatine content would be approximately 30 mg/l, and the maximum concentration would be ca. 100 mg/l. Thus, on the average a bottle of beer would provide 10 mg of hordatine aglycons. However, it must be emphasized that this is a very crude estimation and would require more research using proper reference compounds.

Based on our unpublished preliminary studies, it was found out that 1 kg of barley malt contained as much hordatines as in a resulting 0.5 kg dw of brewers spent grain. When also in the corresponding amount of wort (10 l) the absolute amount of hordatines was three times the amount in malt, and the sum of the absolute amount of hordatines in brewers spent grain and wort was four times higher than in 1 kg of malt: this would indicate that the extraction method used does not remove the hordatines quantitatively from the barley malt matrix. It is likely that the mashing process opens and loosens the cell wall structures and

makes the remaining hordatines in brewer´s spent grain easier to extract compared to the starting material. But in any case, brewer´s spent grain contains considerable amounts of hordatines. More research also on this matter would be warranted.

In a small-scale follow-up study it was also found out that hordatines are chemically very stable compounds: in a beer brewed over 20 years ago (Falcon Gammel Brygd, Stockholm, Sweden, ABV 4.5 %) the total hordatine content was still 5 mg/l PCAE. The chromatogram at the wavelength of 280 nm of this beer, showing three hordatine peaks and their UV-spectra, is presented in **Figure 26**. Another example of the chromatogram is presented in Figure 2 of the Publication IV.

This was the first publication in which the total hordatine contents, even at semiquantitative levels, had been published.

### 5.8.2 Identification of hordatines and other phenolamides in barley and beer by UPLC-QTOF MS (III)

In Study III, a number of possible hordatine structures, their phenolic agmatine precursors, their glycosides as well as other phenolamides were searched from the positive ion MS-data with the exact mass ± 25 mDa. $[M+H]^+$ ions of the possible compound candidates were then subjected to Elemental Composition. The molecular structures of certain compounds were further processed using the MassFragment function of MassLynx, in order to assign the structural composition of the major fragment ions in the $MS^E$ spectrum. Additional confirmation of the hordatines, in addition to $[M+H]^+$ ion, was the presence of $[M+2H]^{+2}$ ion, which was easily distinguishable by the 0.504 amu (atomic mass unit) difference of isotopic peaks. Ion chromatograms from $MS^E$ data of hordatines also contained fragments typical to (i) agmatine ($C_5H_{12}N_3$ 114.1031 *m/z*) (ii) hydroxy-agmatine ($C_5H_{13}N_4$ 129.1140 *m/z*) or (iii) methyl-agmatine ($C_6H_{14}N_3$ 128.1188 *m/z*) structures (Figure 3 in the original Publication III and the Supplementary data).

Altogether 12 hordatine aglycons and almost 50 of their glycosides were reported in barley and beer. A systematic and simple way to name hordatine structures was proposed in Study III. Nomenclature was based on three hordatine basic structures: A, B and C. The structures containing one hydroxyagmatine residue were named A1, B1 and C1, and structures containing two hydroxyagmatine residues A2, B2 and C2. The structures are presented in **Figure 27.** In addition to these, $N^6$-methyl-hordatines A, B and C, were tentatively identified in barley samples for the first time (Table 2 in the original Publication III).

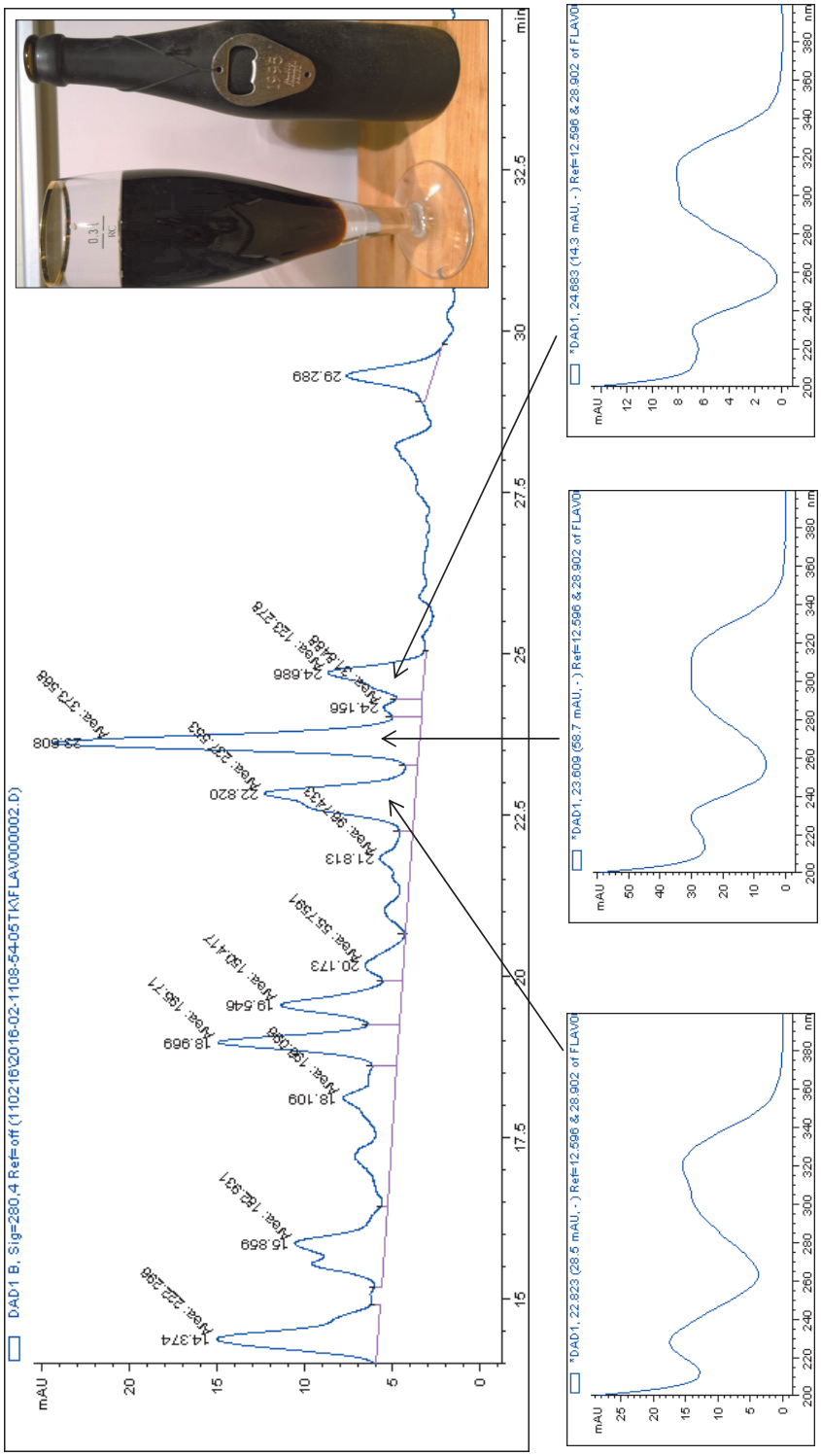
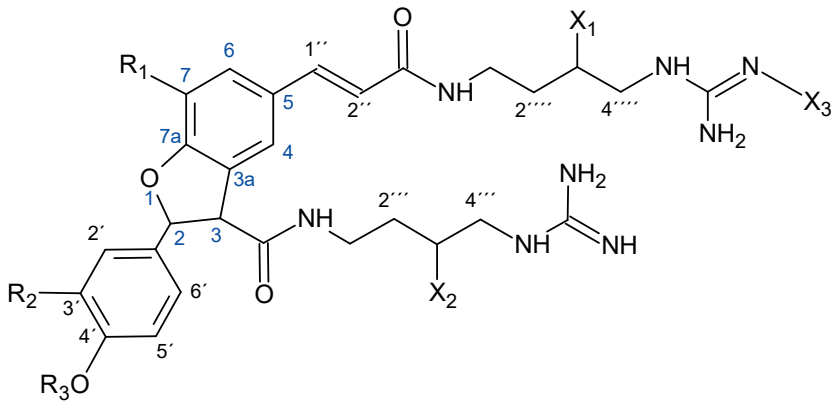



**Figure 26.** Chromatogam at the wavelength of 280 nm of a Swedish Falcon Gammel Brygd 1995 beer. The UV-spectra of three hordatines at 200–400 nm are presented below the chromatogram. The brew was normally aged three years before being released. The bottle was purchased approximately in 1998, opened, sampled and consumed at February 2016. The bottle had been stored in a cellar until opening.




| Hordatine | $R_1$ | $R_2$ | $R_3$ | $X_1$ | $X_2$ | $X_3$ |
|---|---|---|---|---|---|---|
| A | H | H | H / hex | H | H | H |
| B | $OCH_3$ | H | H /hex | H | H | H |
| C | $OCH_3$ | $OCH_3$ | H / hex | H | H | H |
| A1 | H | H | H / hex | OH | H | H |
| B1 | $OCH_3$ | H | H / hex | OH | H | H |
| C1 | $OCH_3$ | $OCH_3$ | H / hex | OH | H | H |
| A2 | H | H | H / hex | OH | OH | H |
| B2 | $OCH_3$ | H | H / hex | OH | OH | H |
| C2 | $OCH_3$ | $OCH_3$ | H / hex | OH | OH | H |
| Met- A | H | H | H / hex | H | H | $CH_3$ |
| Met- B | $OCH_3$ | H | H / hex | H | H | $CH_3$ |
| Met- C | $OCH_3$ | $OCH_3$ | H / hex | H | H | $CH_3$ |

**Figure 27.** Structures of hordatines found in barley and beer. In hordatine aglycons $R_3$ = H and in hexosides $R_3$ = 1–9*hex. Numbering of hordatine structure is adopted from Kageyama *et al*. 2011. Reprinted from Pihlava 2014. *Journal of Cereal Science*, *60*, 645–652 with permission from Elsevier.

A few weeks after the manuscript of Publication III had been submitted, the work of Gorzolka *et al*. (2014) was published on-line. Gorzolka *et al*. (2014) reported eight hordatine aglycon structures, namely hordatines A, B, C, A1, B1 and C1, as well as hordatine D (dimer of sinapoyl- and feruloylagmatine) and D1 as well as 12 glycosylated hordatines. Hordatines containing sinapoyl structures were not found in Study III.

Although the exact place of hydroxylation cannot be distinguished by the equipment used in the current work, the most likely place is the γ(3)-position of the agmatine structure (von Röpenack *et al*. 1998). The other possibility could have been a hypothetical agmatine analog of $N^{\omega}$ -hydroxy-L-arginine (NOHA).

However, since the $N^{\omega}$-hydroxy-agmatine structure has not been reported, while Hamana & Matsuzaki (1993) reported γ(3)-hydroxyagmatine in the seeds of leguminous plants, the most likely structure would also in this case be γ(3)-hydroxyagmatine.

Previously, hordatines with up to two hexose residues had been reported (Gorzolka et al. 2014). In Study III, hordatine A conjugated with up to nine hexose units and hordatine B with eight hexose units were tentatively identified in beer. Identification of these hordatinehexosides was based on the $[M+2H]^{+2}$ ion since the scan range was limited only up to 1200 m/z.

These finding can also give new perspective to the guestion of masked mycotoxins, where the degree of glucosylation of DON with up to four hexose units, has been reported by Zachariasova *et al*. (2012). It would be assumed, that compounds with a high number of hexoside residues will have limited solubility to commonly used extraction solvents, e.g. 80–100 % methanol or ethanol.

A new finding in this study was that the precursors of hordatines can be presents as glycosides, and that there are forms of $N^6$-methyl hydroxycinnamoylagmatines. The following agmatine conjugate precursors of hordatines were found in barley and beer: *p*-coumaroylagmatine and its hexoside, coumaroyl-OH-agmatine and its hexoside and feruloylagmatine. In addition, the following agmatines were found only in barley: feruloylagmatine-hexoside, feruloyl-OH-agmatine and its hexoside, feruloyl-$N^6$-methyl-agmatine and its hexoside. Of these agmatines, p-coumaroylagmatine, coumaroyl-OH-agmatine, feruloylagmatine and feruloyl-OH-agmatine had been reported by Gorzolka *et al*. (2014). Feruloyl-$N^6$-methyl-agmatine and all agmatine hexosides had not been reported before. On the other hand, sinapoylagmatine or sinapoylhydroxyagmatine were not found from the barley or beer samples analyzed in Study III. In addition to phenolic agmatines, coumaroyl-, feruloyl- and caffeoylspermidines; dicoumaroyl-, diferuloyl- and dicaffeoylspermidines as well as caffeoylferuloyl-, coumaroylcaffeoyl- and coumaroylferuloyl-spermidines, were found in barley and beer.

While there is much information on how free phenolic acids behave during the brewing process, phenolamides have basically been unknown compounds in beer so far. This could be considered as somewhat surprising, regarding how much the minor compounds in beer have been studied over the years.

### 5.8.3 Identification of phenolamides in rye by UPLC-QTOF MS (VI)

In Study VI, the identification work of phenolamides which was started in Study III with barley, was continued with rye. The number of possible (and impossible) phenolamides was searched first from the positive mode MS data as described in chapter 5.8.2. In this work, the MS data on negative mode was also checked and in many cases the corresponding $[M-H]^-$ ions were found, although peak intensities were in most cases lower compared to those in positive mode (Table 3 in Publication VI).

Phenolamides had been earlier reported in wheat bran by Savolainen *et al*. (2014) and in rye bran by Koistinen *et al*. (2016), but this was the first time that phenolamides had been reported in rye to this extent.

Interestingly, hydroxycinnamoylagmatines were found in rye, but contrary to barley, glycosylated forms of these compounds were not found, except for feruloylagmatine. Coumaroyl-, caffeoyl-, feruloyl- and sinapoylagmatines were present as two peaks (Table 3 in Publication VI). These could be isomeric forms of these compounds. Feruloyl-$N^6$-methylagmatine was present as a single peak only.

QTOF MS analysis also revealed coumaroyl-, caffeoyl-, feruloyl-, hydroxyferuloyl- and dimethoxycinnamoylputrescines as well as ten dihydroxycinnamoylspermidines (Table 3). Spermine conjugates were not found. Coumaroyl- and caffeoylspermedines were found only in the two crisp bread samples, but not in other rye samples. In addition, hydroxycinnamoylputrescines were present in all other samples but not in soft rye bread samples. Explanations for these findings could not be given based on the current knowledge. Phenolamides appear to be relatively stable towards pro-longed, low-temperature baking, as indicated by the presence of these compounds in mämmi.

It has been speculated by Heiniö *et al*. (2008) that the phenolic compounds could at least partly contribute to the perceived bitterness of rye bran. Similarly, it could be possible that some of the identified phenolamides might in fact affect the flavor of rye, but further studies would be needed to verify this.

# 6 SUMMARY AND CONCLUSION

This thesis brings new insight to the content and diversity of phenolic and phenolic-like compounds in cereals and cereal products. Besides being of academic interest, and justified as such, these new findings can proceed into new business opportunities; e.g. in the form of valorization of cereal side-streams into value-added products (food, feed or cosmeceuticals).

The aim of Study I was to determine the content of phenolic compounds in commercial grain products. The importance and need for this kind of work has been obvious since Publication I has become well cited over the years. With the current knowledge, it could be interesting to repeat the study. Results of Study I also show how poor a source of phenolic compounds are refined wheat flours compared to the whole-grain wheat flour and also how valuable sources of phenolic compounds rye and wheat brans can be.

Comprehensive analysis of phenolic acids in rye was developed by combining several sample preparation methods in Study II. A new, logical, naming of different phenolic acid fractions was developed in this study, which would help and clarify method descriptions, if this would be accepted in the scientific community.

In the Study III completely new hordatine structures were discovered in barley and beer and a rational nomenclature system for hordatines, containing either one or two hydroxyagmatine residues, was proposed. Content of total hordatines in different beer types was reported in Study IV.

The tentative identification of minor compounds in wheat and rye beers was continued in Study V. Several new benzoxazinoid compounds were found and reported for the first time. This work and the work started in Study II was finalized in Study VI, in which the focus was sifted back to rye and rye products.

This thesis shows also how versatile and cost-effective one HPLC-method can be. Without having to change analytical column or mobile phases, a large set of phenolic compounds ranging from polar to almost non-polar could be analyzed in a single HPLC run. Unfortunately, the lack of commercially available reference compounds became a limiting factor for quantitative results of, e.g. hordatines and benzoxazinoids.

Results regarding especially benzoxazinoids and hordatines could be utilized in the food and brewing industries for creating new and healthy food products enriched with these components. It should also be kept in mind that the traditional Finnish cereal products, such as various porridges, whole-grain rye breads, organic mämmi, rye malts and rye malt flour can, as such, be considered as “super-foods”.

# ACKNOWLEDGEMENTS

This work was carried out at the Natural Resources Institute Finland (Luke), (former MTT Agrifood Research Finland) in Jokioinen and in close collaboration with the Department of Biochemistry, University of Turku. Publication II was part of the project "Novel (bio)processing techniques for flavour design in plant-based foods — FLAVOUR DESIGN", funded by Tekes – the Finnish Funding Agency for Technology and Innovation, five Finnish companies, VTT and MTT. Research grants from the Olvi-foundation enabled publications III, IV and V, and contract analysis for Fazer Mills justified the research work leading to Publication VI. I would also like to emphasise that Figures 4, 7, 10, 11, 14, 15 and 18 presented in the "Review of the Literature" –part of this thesis, originated from the project "Terveyttä edistävät viljapohjaiset elintarvikejalosteet [Health promoting cereal based food products]" funded by Tekes and four Finnish companies. The Ministry of Agricultural and Forestry is acknowledged for financing a number of cereal-related projects, during which we have been able to develop and exercise our analytical expertise, which have also made this thesis possible.

I wish to express my deepest gratitude to my long-time supervisors, Dr. Pirjo Mattila and Professor Rainer Huopalahti for their patience for letting this thesis to simmer to the point that I can be pleased with. My warmest thanks go to Professor Heikki Kallio for ordering/telling me to stop simmering and Professor Baoru Yang for flexible and understanding attitude towards this work.

The official reviewers of this thesis, Adjunct Professor Pekka Lehtonen and Professor Rikard Landberg, are gratefully acknowledged for their valuable and constructive comments. Warm thanks with apologies, go also to Professor Vieno Piironen, for acting as an unofficial reviewer.

Over the years, I have had the privilege and honor to work with a number of bright scientists (a few of them now retired) within MTT and nowadays in Luke, in other research institutes and universities. During my career at MTT I was working in more or less in the central laboratory, being responsible for a number of chromatographic methods. I consider myself lucky to have had such a good vantage point to observe things – to see research needs from the perspective of plant production, plant protection and food research. Collaboration with experts of various fields has been exceptionally interesting, educational and rewarding. It would be unfair to name just few of the collegues I have had the opportunity to work with over the years, so I will just make a general salute to you all for making my days at work so unpredictable (in a good way!).

However, of the closest colleagues in Luke I would like to name M.Sc. Sari Rämö, whose exceptional meticulous attitude in her work I can just be envious. Dr. Veli Hietaniemi, my former boss, is acknowledged for being an encouraging and supportive person I could always count on, and who always seemed to have time to listen to my blabberings. Also, other co-workers in MTT/Luke are acknowledged for good and refreshing conversations and creating a good and jovial working atmosphere over the years. Carpooler Dr. Terhi Iso-Touru is thanked for her valuable comments and peer support. MTT/Luke is thanked for providing the expensive infrastructure needed for this kind of work: personnel, instrumentation and facilities.

I would like to emphasize the importance of a trustworthy technical staff, without of whom the analyses described in the enclosed publications would not have been possible. So, my special and warm thanks go to Laboratory Technicians Tuula Kurtelius (emerita), Marja-Terttu Wiisak, Seppo Nummela and Riitta Henriksson. It really has been a pleasure to work with you.

My sincere thanks also to co-authors: Dr. Raija-Liisa Heiniö, M.Sc. Jarkko Hellström, Dr. Veli Hietaniemi, M.Sc. Timo Hurme, Mrs. Tuula Kurtelius, Dr. Pekka Lehtinen, Dr. Pirjo Mattila, Dr. Emilia Nordlund and Academy Professor Kaisa Poutanen.

I would also like to point out the good collaboration we have had over the years with VTT in a number of research projects related to cereals and I hope to see this collaboration continue also in the future. Colleagues and laboratory visits abroad have had a deep impact on me and given new ideas. Although a month´s visit in 2011 to Dr. Inge S. Fomsgaard and her team´s laboratory in Flakkebjerg, Denmark, did not produce the planned publication, it certainly affected our work in MTT/Luke and on its part led to Publication V. The innovative Finnish cereal, malting and baking industry is something we can all be proud of, and I do hope that this thesis would offer something useful for them as well.

My deepest thanks go to my wife Helena and our son Lassi, for giving fulfillment and happiness in my life. Warm thanks also to my closest relatives: it has been important to have you around! And of course more distant relatives in Finland and USA are in my thoughts as well. And finally to a bunch of longtime friends, who have been more or less patiently waiting for the invitation to the promised party, please accept my apologies that this took so long.

Rusko, June 2017

# REFERENCES


Abdel–Aal, el–SM., Akhtar, H., Rabalski, I., Bryan, M. 2014. Accelerated, microwave–assisted, and conventional solvent extraction methods affect anthocyanin composition from colored grains. *J. Food Sci.* 79, 138–146.

Adhikari, K.B., Lærke, H., Mortensen, A., Fomsgaard, I.S. 2012a. Plasma and urine concentrations of bioactive dietary benzoxazinoids and their glucuronidated conjugates in rats fed a rye bread–based diet. *J. Agric. Food Chem.* 60, 11518–11524.

Adhikari, K.B., Laursen, BB., Gregersen, PL., Schnoor, HJ., Witten, M., Poulsen, LK., Jensen, BM., Fomsgaard, IS. 2013. Absorption and metabolic fate of bioactive dietary benzoxazinoids in humans. *Mol. Nutr. Food Res*. 57, 1847–1858

Adhikari, K.B., Laursen, B., Lærke, H., Fomsgaard, I.S. 2012b. Bioactive benzoxazinoids in rye bread are absorbed and metabolized in pigs. *J. Agric. Food Chem.* 60, 2497–2506.

Adhikari, K.B., Tanwir, F., Gregersen, P., Steffensen, S., Jensen, B., Poulsen, L., Nielsen. C., Høyer, S., Borre M., Fomsgaard, I.S. 2015. Benzoxazinoids: Cereal phytochemicals with putative therapeutic and health–protecting properties. *Mol. Nutr. Food Res.* 59, 1324–1338

Adlercreutz, H. 2007. Lignans and human health. *Crit. Rev. Clin. Lab. Sci.* 44,483–525

Adom, K.K., Liu, R.H. 2002. Antioxidant activity of grains. *J. Agric. Food Chem.*, 50,6182–6187.

Ahmad, S., Veyrat, N., Gordon–Weeks, R., Zhang, Y., Martin, J., Smart, L., Glauser, G., Erb, M., Flors, V., Frey, M., Ton, J. 2011. Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiol*. 157, 317–327.

Alcázar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P., Tiburcio, AF. 2010. Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta*. 231, 1237–1249

Alcázar, R., Cuevas, J., Planas, J., Zarza, X., Bortolotti, C., Carrasco, P., Salinas, J., Tiburcio, A., Altabella, T. 2011. Integration of polyamines in the cold acclimation response. *Plant Sci*. 180, 31–38.

Ali, MA., Poortvliet, E., Strömberg, R., Yngve, A. 2011. Polyamines: total daily intake in adolescents compared to the intake estimated from the Swedish Nutrition Recommendations Objectified (SNO). *Food Nutr. Res*. 55, 5455

Alipieva KI, Taskova RM, Evstatieva LN, Handjieva NV, Popov SS. 2003. Benzoxazinoids and iridoid glucosides from four Lamium species. *Phytochemistry,* 64, 1413–1417

Allwood, J.W., Goodacre, R. 2010. An introduction to liquid chromatography–mass spectrometry instrumentation applied in plant metabolomic analyses. *Phytochem Anal*. 21, 33–47

Andersson, A., Marklund, M., Diana, M., Landberg, R. 2011. Plasma alkylresorcinol concentrations correlate with whole grain wheat and rye intake and show moderate reproducibility over a 2– to 3–month period in free–living Swedish adults. *J. Nutr.*, 141,1712–1718.

Andersson, A., Åman, P., Wandel, M., Frølich, W. 2010. Alkylresorcinols in wheat and rye flour and bread. *J. Food Comp. Anal.*, 23, 794–801

Andreasen, M. F., Christensen, L., Meyer, A., Hansen, Å. 2000a. Ferulic acid dehydrodimers in rye (Secale cereale L.). *J. Cereal Sci.,* 31,303–307.

Andreasen, M.F., Christensen, L., Meyer, A., Hansen, Å. 2000b. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (Secale cereale L.) varieties. *J. Agric. Food Chem.*, 48, 2837–2842.

Antonini, E,, Lombardi, F., Alfieri, M., Diamantini , G., Redaelli, R., Ninfali, P. 2016. Nutritional characterization of naked and dehulled oat cultivar samples at harvest and after storage. *J. Cereal Sci.,*72, 46–53

Atanasova–Penichon,V., Bernillon, S., Marchegay, G., Lornac, A., Pinson–Gadais, L., Ponts, N., Zehraoui, E., Barreau, C., Richard–Forget, F. 2014. Bioguided isolation, characterization, and biotransformation by *Fusarium verticillioides* of maize kernel compounds that inhibit fumonisin production. *Mol. Plant Microbe Interact.* 27, 1148–1158

Aura, A.–M. 2008. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.*, 7, 407–429.

Aura, A.–M. 2014. Phenolic acids in rye. In: *Rye and Health*. Editors: Kaisa Poutanen, Per Åman. AACC International Inc., St. Paul, MN, USA. pp.109–119. ISBN 978–1–891127–81–6

Baetz,U., Martinoia, E. 2014. Root exudates: the hidden part of plant defense. *Trends Plant Sci*. 19, 90–98

Barron, C., Surget, A., Rouau, X. 2007. Relative amounts of tissues in mature wheat (*Triticum aestivum* L.) grain and their carbohydrate and phenolic acid composition. J. Cereal Sci., 45, 88–96

Bassard, J.–E., Ullmann, P., Bernier, F., Werck–Reichhart, D. 2010. Phenolamides: Bridging polyamines to the phenolic metabolism. *Phytochemistry* 71, 1808–1824.

Batchu, A.K., Zimmermann, D., Schulze–Lefert, P., Koprek, T. 2006. Correlation between hordatine accumulation, environmental factors and genetic diversity in wild barley (*Hordeum spontaneum* C. Koch) accessions from the Near East Fertile Crescent. *Genetica*, 127, 87–99.

Beckmann, M., Lloyd, A., Haldar, S., Seal, C., Brandt, K., Draper, J. 2013. Hydroxylated phenylacetamides derived from bioactive benzoxazinoids are bioavailable in humans after habitual consumption of whole grain sourdough rye bread. *Mol. Nutr. Food Res*. 57, 1859–1873

Bellincampi, D., Cervone, F., Lionetti, V. 2014. Plant cell wall dynamics and wall–related susceptibility in plant–pathogen interactions. *Front. Plant Sci*. 5:228. doi: 10.3389/fpls.2014.00228.

Belobrajdic, D.P., Bird, A. 2013. The potential role of phytochemicals in wholegrain cereals for the prevention of type–2 diabetes. *Nutr. J.* 12:62. doi: 10.1186/1475–2891–12–62.

Berg, M., Vanaerschot, M., Jankevics, A., Cuypers, B., Breitling, R., Dujardin, J. 2013. LC–MS metabolomics from study design to data–analysis – using a versatile pathogen as a test case. *Comput. Struct. Biotechnol. J*. 4, 1–8

Berger, PJ., Negus, N., Sanders, E., Gardner P. 1981. Chemical triggering of reproduction in *Microtus montanus*. *Science*. 214, 69–70

Bienz, S., Bisegger, P., Guggisberg, A., Hesse, M., 2005. Polyamine alkaloids. *Nat. Prod. Rep.*. 22, 647–658

Biesiekierski, J.R., Rosella, O., Rose, R., Liels, K., Barrett, J., Shepherd, S., Gibson, P., Muir, J.G. 2011. Quantification of fructans, galacto–oligosaccharides and other short–chain carbohydrates in processed grains and cereals. *J. Hum. Nutr. Diet.* 24, 154–176

Bollina, V., Kushalappa, A., Choo, T., Dion, Y., Rioux, S. 2011. Identification of metabolites related to mechanisms of resistance in barley against *Fusarium graminearum*, based on mass spectrometry. *Plant Mol. Biol*. 77, 355–370

Bolvig, A. K., Adlercreutz, H., Theil, P., Jørgensen, H., Bach Knudsen, K.E. 2016. Absorption of plant lignans from cereals in an experimental pig model. *Br. J. Nutr.* 115, 1711–1720.

Bondia–Pons, I., Aura, A.–M., Vuorela, S., Kolehmainen, M., Mykkänen, H., Poutanen, K. 2009. Rye phenolics in nutrition and health. *J. Cereal Sci.*, 49,323–336.

Bondia–Pons, I., Barri, T., Hanhineva, K., Juntunen, K., Dragsted, L., Mykkänen, H., Poutanen, K. 2013. UPLC–QTOF/MS metabolic profiling unveils urinary changes in humans after a whole grain rye versus refined wheat bread intervention. *Mol. Nutr. Food Res.* 57, 412–422

Boutigny, A. L., Atanasova–Penichon, V, Benet, M., Barreau, C., Richard–Forget, F. 2010. Natural phenolic acids from wheat bran inhibit *Fusarium culmorum* trichothecene biosynthesis in vitro by repressing *Tri* gene expression. *Eur. J. Plant Pathol.*, 127, 275–286

Boutigny, A. L., Barreau, C., Atanasova–Penichon, V., Verdal–Bonnin, M., Pinson–Gadais, L., Richard–Forget, F. 2009. Ferulic acid, an efficient inhibitor of type B trichothecene biosynthesis and *Tri* gene expression in *Fusarium* liquid cultures. *Mycol. Res*. 113, 746–753.

Bratt, K., Sunnerheim, K., Bryngelsson, S., Fagerlund, A., Engman, L., Andersson, R., Dimberg, L.H. 2003. Avenanthramides in oats (*Avena sativa* L.) and structure–antioxidant activity relationships. *J. Agric. Food Chem.* 51, 594–600.

Brazier–Hicks, M., Evans, K., Gershater, M., Puschmann, H., Steel, P., Edwards, R. 2009. The C–glycosylation of flavonoids in cereals. *J Biol Chem*. 284, 17926–17934.

Brooks, A., Danehower, D., Murphy, P., Reberg–Horton, C., Burton, J. 2012. Estimation of heritability of benzoxazinoid production in rye (*Secale cereale*) using gas chromatographic analysis. *Plant Breeding*, 131, 104–109

Bryngelsson, S., Dimberg, L.H., Kamal–Eldin, A. 2002a. Effects of commercial processing on levels of antioxidants in oats (*Avena sativa* L.). *J. Agric. Food Chem.* 50, 1890–1896

Bryngelsson, S., Ishihara, A., Dimberg, L.H, 2003. Levels of avenanthramides and activity of Hydroxycinnamoyl–CoA:Hydroxyanthranilate N–Hydroxy-cinnamoyl Transferase (HHT) in steeped or germinated oat samples. *Cereal Chem.*, 80, 356–360.

Bryngelsson, S., Mannerstedt–Fogelfors, B., Kamal–Eldin, A., Dimberg, L.H. 2002b. Lipids and antioxidants in groats and hulls of Swedish oats (*Avena sativa* L.). *J. Sci. Food Agric*. 82, 606–614

Buchmann, CA,, Nersesyan, A., Kopp, B., Schauberger, D., Darroudi, F., Grummt, T., Krupitza, G., Kundi, M., Schulte–Hermann, R., Knasmueller, S. 2007. Dihydroxy–7–methoxy–1,4–benzoxazin–3–one (DIMBOA) and 2,4–dihydroxy–1,4–benzoxazin–3–one (DIBOA), two naturally occurring benzoxazinones contained in sprouts of *Gramineae* are potent aneugens in human–derived liver cells (HepG2). *Cancer Lett*. 246, 290–299

Buranov, A., Mazza, G. 2008. Lignin in straw of herbaceous crops. *Ind. Crops Prod.*, 28, 237–259

Callipo, L., Cavaliere, C., Fuscoletti, V., Gubbiotti, R., Samperi, R., Laganà, A. 2010. Phenilpropanoate identification in young wheat plants by liquid chromatography/tandem mass spectrometry: monomeric and dimeric compounds. *J Mass Spectrom*. 45, 1026–1040.

Cambier, V., Hance, T., de Hoffmann, E. 2000. Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize. *Phytochemistry*. 53, 223–229.

Carder, G., Chatel, R., Chu, Y., Chung, Y., Lay, M., French, J., O´Shea, M., Van Klinken, B. 2014. Method of processin oats to achieve oats with an increased avananthramide content. WO2014150029 A1. Applicant: The Quaker Oats Company

Cavaliere, C., Foglia, P., Pastorini, E., Samperi, R., Laganà, A. 2005. Identification and mass spectrometric characterization of glycosylated flavonoids in Triticum durum plants by high–performance liquid chromatography with tandem mass spectrometry. *Rapid Commun. Mass Spectrom*. 19, 3143–3158.

Chatel, R., Carder, G., Chu, Y., Chung, Y., French, J., O´Shea, M., Van Klinken, B. 2014. Method of preparing highly dispersible whole grain flour with an increased avananthramide content. WO2014150029 A1. Applicant: The Quaker Oats Company

Chen, CY., Milbury, P., Collins, F.W, Blumberg, J. 2007. Avenanthramides are bioavailable and have antioxidant activity in humans after acute consumption of an enriched mixture from oats. *J. Nutr*. 137, 1375–1382.

Chen, C.Y., Milbury, P., Kwak, H., Collins, F.W, Samuel, P., Blumberg, J. 2004a. Avenanthramides and phenolic acids from oats are bioavailable and act synergistically with vitamin C to enhance hamster and human LDL resistance to oxidation. *J. Nutr*. 134, 1459–1466.

Chen, Y., Ross, A.B., Åman, P., Kamal-Eldin, A. 2004b. Alkylresorcinols as markers of whole grain wheat and rye in cereal products. J. Agric. Food Chem. 52, 8242-8246.

Chen, KJ., Zheng, Y., Kong, C., Zhang, S., Li, J., Liu, X. 2010. 2,4–Dihydroxy–7–methoxy–1,4–benzoxazin–3–one (DIMBOA) and 6–methoxy–benzoxazolin–2–one (MBOA) levels in the wheat rhizosphere and their effect on the soil microbial community structure. *J. Agric. Food Chem.* 58, 12710–12716.

Chu, YF., Wise, M., Gulvady, A., Chang, T., Kendra, D., van Klinken, J., Shi, Y., O'Shea, M. 2013. In vitro antioxidant capacity and anti–inflammatory activity of seven common oats. *Food Chem*. 139, 426–431

Collins, F.W., 1983. Oat phenolics: Isolation and structural elucidation of substituted benzoxazinones. *Cereal Food World* 28, 560.

Collins, F. W. 1989. Oat phenolics: avenanthramides, novel substituted N–cinnamoyl–anthranilate alkaloids from oat groats and hulls. *J. Agric*. *Food Chem*. 37, 60–66.

Collins, F.W. 2011. Oat phenolics: biochemistry and biological functionality. In *OATS: Chemistry and Technology*. Second Edition. Editors: Francis H. Webster, Peter J. Wood, AACC International Inc., St. Paul, MN, USA. ISBN: 978–1–891127–64–9

Collins, F.W. Burrows, V. 2010. Method for increasing concentration of avenanthramides in oats. WO2010108277

Collins, F.W., McLachlan, D., Blackwell, B. 1991. Oat phenolics: Avenalumic acids, a new group of bound phenolic acids from oat groats and hulls. *Cereal Chem.* 68, 184–189.

Costabile, A., Klinder, A., Fava, F., Napolitano, A., Fogliano, V., Leonard, C., Gibson, G., Tuohy, K. 2008. Whole–grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double–blind, placebo–controlled, crossover study. *Br. J. Nutr*. 99, 110–120

Darakhshan, S., Ghanbari, A. 2013. Tranilast enhances the anti–tumor effects of tamoxifen on human breast cancer cells in vitro. *J. Biomed. Sci*. 20:76. doi:10.1186/1423–0127–20–76

de Bruijn, W.,Vincken, J.P., Duran, K., Gruppen, H. 2016. Mass spectrometric characterization of benzoxazinoid glycosides from *Rhizopus*-elicited wheat (*Triticum aestivum*) Seedlings. *J. Agric. Food Chem.*, 64, 6267−6276

Dedio, W., Hill, R., Evans, L. 1972. Anthocyanins in the pericarb and coleoptiles of purple–seeded rye. *Can. J. Plant Sci.* 52, 981–983.

Dedio, W., Kaltsikes, P., Larter, E.. 1969. The anthocyanins of Secale cereale. *Phytochemistry* 8, 2531–2532.

Del Rio, D., Rodriguez–Mateos, A., Spencer, J., Tognolini, M., Borges, G., Crozier, A. 2013. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal*. 18, 1818–1892

Dellamonica, G., Meurer, B., Strack, D., Weissenböck, G. Chopin, J. 1983. Two isovitexin 2 ″ ‐ O ‐ glycosides from primary leaves of *Secale cereale*. *Phytochemistry*, 22, 2627 ‐ 2628

Dey, ES., Ahmadi–Afzadib, M., Nybomb, H., Tahir, I. 2013. Alkylresorcinols isolated from rye bran by supercritical fluid of carbon dioxide and suspended in a food–grade emulsion show activity against *Penicillium expansum* on apples. *Arch. Phytopathology Plant Protect.* 46, 105–119

Dimberg, L.H., Theander, O., Lingnert. H. 1993. Avenanthramides – a group of phenolic antioxidants in oats. *Cereal Chem*. 70, 637–641.

Dimberg, L.H. Molteberg, E., Solheim, R. Frølich, W. 1996. Variation in Oat Groats Due to Variety, Storage and Heat Treatment. I: Phenolic Compounds. *J. Cereal Sci.*, 24, 263–272

Dimberg, L.H., Andersson, R., Gohil, S., Bryngelsson, S., Lundgren, L. 2001a. Identification of a sucrose diester of a substituted beta–truxinic acid in oats. *Phytochemistry*, 56, 843–847.

Dimberg, L.H., Sunnerheim, K.., Sundberg, B., Walsh, K. 2001b. Stability of oat avenanthramides. *Cereal Chem.*. 78, 278–281

Dimberg, L.H., Jastrebova, J. 2009. Quantitative analysis of oat avenanthramides. In: HEALTHGRAIN methods: analysis of bioactive components in small grain cereals. Editors: Peter Shewry, Jane Ward. AACC International Inc., Minnesota, ISBN 978-1-891127-70-0. pp 113–127

Dimberg, L.H, Peterson, D. 2009. Phenols in spikelets and leaves of field–grown oats (*Avena sativa*) with different inherent resistance to crown rust (*Puccinia coronata* f. sp. *avenae*). *J. Sci. Food Agric.*, 89, 1815–1824.

Dinelli, G., Segura–Carretero, A., Di Silvestro, R., Marotti, I., Arráez–Román, D., Benedettelli, S., Ghiselli, L., Fernadez–Gutierrez, A. 2011. Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time–of–flight mass spectrometry. *J. Chromatogr. A*. 1218, 7670–7681.

Dobberstein, D., Bunzel, M. 2010. Separation and detection of cell wall–bound ferulic acid dehydrodimers and dehydrotrimers in cereals and other plant materials by reversed phase high–performance liquid chromatography with ultraviolet detection. *J. Agric. Food Chem.* 58, 8927–8935

Dokuyucu, T., Peterson, E., Akkaya, A. 2003. Contents of antioxidant compounds in Turkish oats: simple phenolics and avenanthramide concentrations. *Cereal Chem*. 80, 524–543

Dvorakova, M., Moreira, M., Dostalek, P., Skulilova, Z., Guido, L., Barros, A. 2008. Characterization of monomeric and oligomeric flavan–3–ols from barley and malt by liquid chromatography–ultraviolet detection–electrospray ionization mass spectrometry. *J Chromatogr A*. 1189, 398–405.

Eliasson, C., Kamal–Eldin, A., Andersson, R., Åman, P. 2003. High–performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. *J. Chromatogr. A*. 1012, 151–159

Emmons, C., Peterson, D. 1999. Antioxidant activity and phenolic contents of oat groats and hulls. *Cereal Chem.* 76, 902–209.

Emmons, C., Peterson. D. 2001. Antioxidant activity and phenolic content of oat as affected by cultivar and location. *Crop Sci.* 41, 1676–1681.

Feng, X., Jiang, D., Shan, Y., Dai, T., Dong, Y., Cao, W. 2008. New flavonoid–C–glycosides from Triticum aestivum. *Chem. Nat. Comp.* 44, 171–173

Fielder, D., Redmond, M., Cottrell, I. 2010. Avenanthramide–containing compositions. US 2010/0267662 A1. Applicant: Ceapro Inc.

Figueroa–Espinoza, MC., Poulsen, C., Borch Søe, J., Zargahi, M., Rouau, X. 2002. Enzymatic solubilization of arabinoxylans from isolated rye pentosans and rye flour by different endo–xylanases and other hydrolyzing enzymes. Effect of a fungal caccase on the flour extracts oxidative gelation. *J. Agric. Food Chem.*, 50,6473–6484

Finney, M.M., Danehower, D., Burton, J. 2005. Gas chromatographic method for the analysis of allelopathic natural products in rye (*Secale cereale* L.). *J. Chromatogr. A*, 1066, 249–253

Fomsgaard, I.S., Mortensen, A., Carlsen, S. 2004. Microbial transformation products of benzoxazolinone and benzoxazinone allelochemicals—a review. *Chemosphere*. 54, 1025–1038.

Fomsgaard, I.S., Mortensen, A., Holm, P. Gregersen, P. 2009. Use of benzoxazinoids–containing cereal grain products for health–improving purposes. WO 2009115093 A. Applicant: Aarhus Universitet.

Frey, M., Schullehner, K., Dick, R., Fiesselmann, A., Gierl, A. 2009. Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. Review. *Phytochemistry*. 70, 1645–1651

Fu, J., Zhu, Y., Yerke, A., Wise, M.L., Johnson, J., Chu, Y., Sang, S. 2015. Oat avenanthramides induce heme oxygenase–1 expression via Nrf2–mediated signaling in HK–2 cells. *Mol. Nutr. Food Res.* 59, 2471–2479

Fujii, W., Hori, H., Yokoo, Y., Suwa, Y., Nukaya, H., Taniyama, K., 2002. Beer congener stimulates gastrointestinal motility via the muscarinic acetylcholine receptors. *Alcohol. Clin. Exp. Res.*, 26, 677–681.

Galgano, F., Caruso, M., Condelli, N., Favati, F. 2012. Focused review: agmatine in fermented foods. *Front Microbiol*. 3, 199. doi: 10.3389/fmicb.2012.00199.

Gaquerel, E., Gulati, J., Baldwin, I. 2014. Revealing insect herbivory–induced phenolamide metabolism: from single genes to metabolic network plasticity analysis. *The Plant Journal*, 79, 679–692

García, S., García, C., Heinzen, H., Moyna, P. 1997. Chemical basis of the resistance of barley seeds to pathogenic fungi. *Phytochemistry*, 44, 415–418.

Garcia–Aloy, M., Llorach, R., Urpi–Sarda, M., Tulipani, S., Salas–Salvadó, J., Martínez González, M., Corella, D., Fitó, M.; Estruch, R., Serra–Majem, L., Andres–Lacueva, C. 2015. Nutrimetabolomics fingerprinting to identify biomarkers of bread exposure in a free–living population from the PREDIMED study cohort. *Metabolomics*, 11, 155–165

Gardiner, D.M., Kazan, K., Manners, J. 2009. Nutrient profiling reveals potent inducers of trichothecene biosynthesis in *Fusarium graminearum*. *Fungal Genet. Biol*. 46, 604–613

Gardiner, D.M., Kazan, K., Praud, S., Torney, F., Rusu, A., Manners, J. 2010. Early activation of wheat polyamine biosynthesis during Fusarium head blight implicates putrescine as an inducer of trichothecene mycotoxin production. *BMC Plant Biol*. 10, 289, doi:10.1186/1471-2229-10-289

Gauthier, L., Atanasova-Penichon, V., Chéreau, S., Richard-Forget, F. 2015. Metabolomics to decipher the chemicald of cereals against Fusarium graminearum and deoxynivalenol accumulation. *Int. J. Mol. Sci.* 16, 24839-24872

Geng, P., Harnly, J.M., Chen, P. 2016. Differentiation of bread made with whole grain and refined wheat (*T. aestivum*) flour using LC/MS-based chromatographic fingerprinting and chemometric approaches. *J. Food Comp. Anal*., 47, 92–100

Glauser, G., Marti, G., Villard, N., Doyen, G., Wolfender, J., Turlings, T., Erb, M. 2011. Induction and detoxification of maize 1,4–benzoxazin–3–ones by insect herbivores. *Plant J*. 68, 901–911.

Glenn, A.E., Bacon, C.W. 2009. FDB2 encodes a member of the arylamine N–acetyltransferase family and is necessary for biotransformation of benzoxazolinones by Fusarium verticillioides. *J. Appl. Microbiol.* 107, 657–671.

Glitsø, L.V., Bach Knudsen, K. E. 1999. Milling of wholegrain rye to obtain fractions with different dietary fibre characteristics. *J. Cereal Sci.*, 29,89–97.

Glitsø, L.V., Mazur,W., Adlercreutz, H., Wähälä, K., Mäkelä,T., Sandström, B., Bach Knudsen K.E. 2000. Intestinal metabolism of rye lignans in pigs. *British J. Nutr.*, 84,429–437.

Gorzolka, K., Bednarz, H., Niehaus, K., 2014. Detection and localization of novel hordatine–like compounds and glycosylated derivates of hordatines by imaging mass spectrometry of barley seeds. *Planta*, 239, 1321–1335.

Goupy, P., Hugues, M., Boivin, P., Amiot, M. 1999. Antioxidant composition and activity of barley (Hordeum vulgare) and malt extracts and of isolated phenolic compounds. *J. Sci. Food Agric.* 79, 1625–1634

Grün, S., Frey, M., Gierl, A. 2005. Evolution of the indole alkaloid biosynthesis in the genus Hordeum: distribution of gramine and DIBOA and isolation of the benzoxazinoid biosynthesis genes from *Hordeum lechleri*. *Phytochemistry*. 66, 1264–1272.

Guo, W., Nie, L., Wu, D., Wise, M., Collins F.W., Meydani, S., Meydani, M. 2010. Avenanthramides inhibit proliferation of human colon cancer cell lines in vitro. *Nutr. Cancer*. 62, 1007–1016.

Guo, W., Wise, M., Collins, F.W., Meydani, M. 2008. Avenanthramides, polyphenols from oats, inhibit IL–1beta–induced NF–kappaB activation in endothelial cells. *Free Radic. Biol. Med*. 44, 415–429.

Gökmen, V., Serpen, A., Fogliano, V. 2009. Direct measurement of the total antioxidant capacity of foods, the 'QUENCHER' approach. *Trends Food Sci. Tech.*, 20, 278–288

Halász, A., Baráth, A., Holzapfel, W. 1999. The biogenic amine content of beer; the effect of barley, malting and brewing on amine concentration. *Z. Lebensm. Unters. For. A*, 208, 418–423

Hakala, P., Lampi, A.M., Ollilainen, V., Werner, U., Murkovic, M., Wähälä, K., Karkola, S., Piironen, V. 2002. Steryl phenolic acid esters in cereals and their milling fractions. *J. Agric. Food Chem.* 50, 5300–5307

Hamana, K., Matsuzaki, S. 1993. Diamines, guanidinoamines, and their hydroxy derivatives in seeds and seedlings of leguminous plants. *Can. J. Bot.*, 71, 1381–1385

Handrick, V., Robert, C., Ahern, K., Zhou, S., Machado, R., Maag, D., Glauser, G., Fernandez-Penny, F., Chandran, J., Rodgers-Melnik, E., Schneider, B., Buckler, E., Boland, W., Gershenzon, J., Jander, G., Erb, M., Köllner, T. 2016. Biosynthesis of 8-O-Methylated Benzoxazinoid Defense Compounds in Maize. Plant Cell. 28, 1682-1700.

Hanhineva, K., Keski–Rahkonen, P., Lappi, J., Katina, K., Pekkinen, J., Savolainen, O., Timonen, O., Paananen, J., Mykkänen, H., Poutanen, K. 2014a. The postprandial plasma rye fingerprint includes benzoxazinoid–derived phenylacetamide sulfates. *J. Nutr*. 144, 1016–1022.

Hanhineva, K., Pihlava, J., Mykkänen, H., Poutanen, K. 2014b. Benzoxazinoids in rye and rye–derived products. In: *Rye and Health*. Editors: Kaisa Poutanen, Per Åman. AACC International Inc., St. Paul, MN, USA. pp.121–129. ISBN 978–1–891127–81–6

Hanhineva, K., Rogachev, I., Aura, A.M., Aharoni, A., Poutanen, K., Mykkänen, H. 2011. Qualitative characterization of benzoxazinoid derivatives in whole grain rye and wheat by LC–MS metabolite profiling. *J. Agric. Food Chem.* 59, 921–927.

Hanhineva, K., Rogachev, I., Aura, A.M., Aharoni, A., Poutanen, K., Mykkänen, H. 2012. Identification of novel lignans in the whole grain rye bran by non–targeted LC–MS metabolite profiling. *Metabolomics*, 8, 399–409.

Hatcher, D.W., Kruger, J.E., 1997. Simple phenolic acids in flours prepared from Canadian wheat: relationship to ash content, color, and polyphenol oxidase activity. *Cereal Chem.* 74, 337–343

Heinonen, S., Nurmi, T., Liukkonen, K., Poutanen, K., Wähälä, K., Deyama, T., Nishibe, S., Adlercreutz, H. 2001. In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. *J. Agric. Food Chem.* 49, 3178–3186.

Heiniö, R.–L., Liukkonen, K., Myllymäki, O., Pihlava, J.M., Adlercreutz, H., Heinonen, S., Poutanen, K. 2008. Quantities of phenolic compounds and their impacts on the perceived flavour attributes of rye grain. *J. Cereal Sci.*, 47,566–575.

Heimler, D., Vignolini, P., Isolani, L., Arfaioli, P., Ghiselli, L., Romani, A. 2010. Polyphenol content of modern and old varieties of *Triticum aestivum* L. and T. durum Desf. grains in two years of production. *J. Agric. Food Chem.* 58, 7329–7334

Hellström, J., Törrönen, A., Mattila, P. 2009. Proanthocyanidins in common food products of plant origin. *J. Agric. Food Chem.* 57, 7899–7906

Hemery, Y., Mateo Anson, N., Havenaar, R., Haenen G., Noort, M., Rouau, X. 2010. Dry–fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Res. Int.*, 43, 1429–1438

Hernández, I., Alegre, L., Van Breusegem, F., Munné–Bosch, S. 2009. How relevant are flavonoids as antioxidants in plants? *Trends Plant Sci*. 14, 125–132.

Hertog, M.G. Hollman, P., Venema, D. 1992. Optimalization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *J. Agric. Food Chem.* 40, 1591–1598

Heuberger, A., Broeckling, C., Kirkpatrick, K., Prenni, J. 2014. Application of nontargeted metabolite profiling to discover novel markers of quality traits in an advanced population of malting barley. *Plant Biotech. J.* 12, 147–160

Hietala, P., Virtanen, A.I. 1960. Precursors of benzoxazolinone in rye plants. 2. Precursor I, the glucoside. *Acta Chem. Scand*. 14, 502–504

Hitayezu, R., Baakdah, M., Kinnin, J., Henderson,K. Tsopmo, A. 2015. Antioxidant activity, avenanthramide and phenolic acid contents of oat milling fractions. *J. Cereal Sci.* 63, 35–40

Hosseinian, F., Li, W., Beta, T. 2008. Measurement of anthocyanins and other phytochemicals in purple wheat. *Food Chem.* 109, 916–924.

Hussain, S., Ali, M., Ahmad, M., Siddique, K. 2011. Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. *Biotechnol. Adv*. 29, 300–311.

Ilgen, P., Hadeler, B., Maier, F., Schäfer, W. 2009. Developing kernel and rachis node induce the trichothecene pathway of *Fusarium graminearum* during wheat head infection. *Mol. Plant Microbe Interact.* 22, 899–908.

Ishihara, A., Kojima, K., Fujita, T., Yamamoto, Y., Nakajima, H. 2014. New series of avenanthramides in oat seed. *Biosci. Biotechnol. Biochem*. 78, 1975–1983

Ishihara, A., Ohtsu, Y., Iwamura, H. 1999a. Biosynthesis of oat avenanthramide phytoalexins. *Phytochemistry*, 50, 237–242

Ishihara, A., Ohtsu, Y., Iwamura, H. 1999b. Induction of biosynthetic enzymes for avenanthramides in elicitor–treated oat leaves. *Planta* 208, 512–518

Ivanišova, E., Ondrejovič, M. and Šilhár, S. 2012. Antioxidant activity of milling fractions of selected cereals. *Nova Biotechnologica et Chimica*, 11, 45–56

Izumi, K., Mizokami, A., Shima, T., Narimoto, K., Sugimoto, K., Kobori, Y., Maeda, Y., Konaka, H., Koh, E., Namiki, M. 2010. Preliminary results of tranilast treatment for patients with advanced castration–resistant prostate cancer. *Anticancer Res*. 30, 3077–3081.

Izumi, Y., Kajiyama, S., Nakamura, R., Ishihara, A., Okazawa, A., Fukusaki, E., Kanematsu, Y., Kobayashi, A. 2009. High–resolution spatial and temporal analysis of phytoalexin production in oats. *Planta*, 229, 931–943.

Jansson, E., Landberg, R., Kamal–Eldin, A., Wolk, A., Vessby, B., Åman, P. 2010. Presence of alkylresorcinols, potential whole grain biomarkers, in human adipose tissue. *Br. J. Nutr*. 104, 633–636.

Jastrebova, J., Skoglund, M., Dimberg, L.H. 2006. Selective and sensitive LC–MS determination of avenanthramides in oats. *Chromatographia*, 63, 419–423.

Jensen, B., Adhikari, K., Schnoor, H., Juel-Berg, N., Fomsgaard, I.S., Poulsen, L. 2017. Quantitative analysis of absorption, metabolism, and excretion of benzoxazinoids in humans after the consumption of high and low‐benzoxazinoid diets with similar contents of cereal dietary fibres: a crossover study. *Eur. J. Nutr*. 56, 387–397.

Ji, X., Jetter, R. 2008. Very long chain alkylresorcinols accumulate in the intracuticular wax of rye (Secale cereale L.) leaves near the tissue surface. *Phytochemistry*, 69, 1197–207

Jin, S., Yoshida, M., Nakajoma, T., Murai, A. 2003. Accumulation of hydroxycinnamic acid amides in winter wheat under snow. *Biosci. Biotech., Biochem.* 67, 1245–1249.

Kageyama, N., Inui, T., Fukami, H., Komura, H. 2011. Elucidation of chemical structures of components responsible for beer aftertaste. *J. Am. Soc. Brew. Chem.* , 69, 255–259

Kageyama, N., Inui, T., Fukami, H., Komura, H., 2012. Structures in the hordatine family with cis–cinnamoyl moieties. *J. Am. Soc. Brew. Chem.* , 70, 133–136

Kageyama, N., Inui,T., Nakahara, K., Fukami, H. 2013. Beer aftertaste improved by reducing astringent substances in the barley malt with subcritical water treatment. *J. Am. Soc. Brew. Chem.* 71, 105–108

Kageyama, N., Takako, I., Koichi, N., Hajime, K. 2005. Oral cavity stimulating substance EP1834958 A1. Applicant: Suntory Ltd.

Kageyama, N., Nakahara, K. 2007. Method for reducing intraoral irritants of sprouted grains. WO2007072747. Applicant: Suntory Ltd.

Kamal–Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L., Åman, P. 2001. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3–hydroxy–3–methyl glutaric acid residues. *Phytochemistry*, 58, 587–590

Kamal–Eldin, A., Lærke, H., Knudsen, K., Lampi, A.–M., Piironen, V., Adlercreutz, H., Katina, K., Poutanen, K., Åman, P. 2009. Physical, microscopic and chemical characterisation of industrial rye and wheat brans from the Nordic countries. *Food Nutr. Res.* 53, 1–11

Kamiyama, M., Shibamoto, T. 2012. Flavonoids with potent antioxidant activity found in young green barley leaves. Review. *J. Agric. Food Chem.* 60, 6260–6267.

Katina, K., Liukkonen, K. Kaukovirta–Norja, A., Adlercreutz, H., Heinonen, S., Lampi, A.M., Pihlava, J.M., Poutanen, K. 2007. Fermentation–induced changes in the nutritional value of native or germinated rye. *J. Cereal Sci.*, 46, 348–355

Kaur, J., Whitson, A., Ashton, J., Katopo, L., Kasapis, S. 2016. Effect of ultra high temperature processing and storage conditions on phenolic acid, avenanthramide, free fatty acid and volatile profiles from Australian oat grains. *Bioact. Carbohydr. Diet. Fibre*, In press, https://doi.org/10.1016/j.bcdf.2016.09.002

Kelly, D., Stapleton, D. 2012. Methods of treating eye diseases associated with inflammation and vascular proliferation. WO2012068612 A1 Applicant: Fibrotech Therapeutics PTY

Kim, J.M., Noh, E., Kwon, K., Hwang, B., Hwang, J., You, Y., Kim, M., Lee, W., Lee J., Kim, H., Kim, J., Lee, Y. 2013. Dihydroavenanthramide D prevents UV–irradiated generation of reactive oxygen species and expression of matrix metalloproteinase–1 and –3 in human dermal fibroblasts. *Exp. Dermatol*. 22, 59–761.

Kim, H.K., Verpoorte, R. 2010. Sample preparation for plant metabolomics. *Phytochem. Anal.* 21, 4–13

Kluger, B., Bueschl, C., Lemmens, M., Berthiller, F., Häubl, G., Jaunecker, G., Adam, G., Krska, R., Schuhmacher, R. 2013. Stable isotopic labelling–assisted untargeted metabolic profiling reveals novel conjugates of the mycotoxin deoxynivalenol in wheat. *Anal. Bioanal. Chem*. 405, 5031–5036.

Knödler, M., Kaiser, A., Carle, R., Schieber, A. 2008. Profiling of alk(en)ylresorcinols in cereals by HPLC–DAD–APcI–MSn. *Anal. Bioanal. Chem*. 391, 221–228.

Koenig, R., Dickman, J., Kang, C., Zhang, T., Chu, Y., Ji, L. 2014. Avenanthramide supplementation attenuates exercise–induced inflammation in postmenopausal women. *Nutr. J.* 2014 Mar 19;13:21. doi: 10.1186/1475–2891–13–21.

Koenig, R., Dickman, J., Wise, M., Ji, L. 2011. Avenanthramides are bioavailable and accumulate in hepatic, cardiac, and skeletal muscle tissue following oral gavage in rats. *J. Agric. Food Chem.* 59, 6438–6443.

Kohyama, N., Ono, H., 2013. Hordatine A β–D–glucopyranoside from ungerminated barley grains. *J. Agric. Food Chem.*, 61, 1112–1116.

Kohyama, N., Fujita, M., Ono, H., Ohnishi–Kameyama, M., Matsunaka, H., Takayama, T., Murata, M. 2009. Effects of phenolic compounds on the browning of cooked barley. *J. Agric. Food Chem.* 57, 6402–6407

Koistinen, V., Katina, K., Nordlund, E., Poutanen, K., Hanhineva, K. 2016. Changes in the phytochemical profile of rye bran induced by enzymatic bioprocessing and sourdough fermentation. *Food Res. Int*. 89, 1106-1115.

Krauze–Baranowska, M. 2002. Truxillic and truxinic acids – occurrence in plant kingdom. *Acta Pol. Pharm*. 59, 403–410.

Kristensen, B., Burhenne, K., Rasmussen, S., 2004. Peroxidases and the metabolism of hydroxycinnamic acid amides in Poaceae. *Phytochemistry Rev.* 3, 127–140

Kristensen, B., Bloch, H., Rasmussen, S., 1999. Barley coleoptile peroxidases. Purification, molecular cloning, and induction by pathogens. *Plant Physiol.* 120, 501–512

Kulawinek, M., Jaromin, A., Kozubek, A., Zarnowski, R. 2008. Alkylresorcinols in selected Polish rye and wheat cereals and whole–grain cereal products. *J. Agric. Food Chem.* 56, 7236–7242

Kulik, T., Buśko, M., Pszczółkowska, A., Perkowski, J., Okorski, A. 2014. Plant lignans inhibit growth and trichothecene biosynthesis in *Fusarium graminearum*. *Lett. Appl. Microbiol*. 59, 99–107.

Kuznetsov, V., Shevyakova, N., 2007. Polyamides and stress tolerance of plants. *Plant Stress*, 1, 50–71

Köhler, A., Maag, D., Veyrat, N., Glauser, G., Wolfender, J.L., Turlings, T., Erb, M. 2015. Within–plant distribution of 1,4–benzoxazin–3–ones contributes to herbivore niche differentiation in maize. *Plant Cell Environ.* 38, 1081–1093

Laatikainen, R., Koskenpato, J., Hongisto, S.–M., Loponen, J., Poussa, T., Hillilä, M., Korpela, R. 2016. Randomised clinical trial: low–FODMAP rye bread vs. regular rye bread to relieve the symptoms of irritable bowel syndrome. *Aliment. Pharmacol. Ther*. 44, 460–470

Lagaert, S., Beliën, T., Volckaert, G. 2009. Plant cell walls: Protecting the barrier from degradation by microbial enzymes. *Semin. Cell Dev. Biol*. 20, 1064–1073

Landberg, R., Kamal–Eldin, A., Salmenkallio–Marttila, M., Rouau, X., Åman, P. 2008. Localization of alkylresorcinols in wheat, rye and barley kernels. *J. Cereal Sci.*, 48, 401–406

Landberg, R., Kamal–Eldin, A., Marklund, M. Adlercreutz, H., Åman, P. 2014a. Alkylresorcinols and their metabolited as biomarkers of whole–grain rye and wheat intake. In: *Rye and Health*. Editors: Kaisa Poutanen, Per Åman. AACC International Inc., St. Paul, MN, USA. pp.159–187. ISBN 978–1–891127–81–6

Landberg, R., Marklund, M. Andersson, A., Kamal–Eldin, A., Åman, P. 2014b. Alkylresorcinols in rye: occurance, pharmacokinetics, and bioavailability. In: *Rye and Health*. Editors: Kaisa Poutanen, Per Åman. AACC International Inc., St. Paul, MN, USA. pp.85–108. ISBN 978–1–891127–81–6

Landete, J.M. 2012. Plant and mammalian lignans: A review of source, intake, metabolism, intestinal bacteria and health. *Food Res. Int.*, 46, 410–424.

Lee, Y.R., Noh, E., Oh, H., Hur, H., Kim, J.M., Han, J., Hwang, J., Park, B., Park, J., Youn, H., Jung, S., Kim, B.S., Jung, J., Lee, S., Park, C., Kim, J.S. 2011. Dihydroavenanthramide D inhibits human breast cancer cell invasion through suppression of MMP–9 expression. *Biochem. Biophys. Res. Commun*. 405, 552–557.

Lee–Manion, A.M., Price, R., Strain, J., Dimberg, L.H., Sunnerheim, K., Welch, R. 2009. In vitro antioxidant activity and antigenotoxic effects of avenanthramides and related compounds. *J. Agric. Food Chem.* 57, 10619–10624.

Li, M., Koecher, K., Hansen, L., Ferruzzi, M. 2016. Phenolic recovery and bioaccessibility from milled and finished whole grain oat products. *Food Funct.* 7, 3370-3381.

Li, L., Shewry, P., Ward, J. 2008. Phenolic acids in wheat varieties in the HEALTHGRAIN Diversity Screen. *J. Agric. Food Chem*. 56, 9732–9739.

Li, X., Li, M., Ling, A., Hu, X., Ma, Z., Liu Liu, L., Li, Y. 2017. Effects of genotype and environment on avenanthramides and antioxidant activity of oats grown in northwestern China. *J. Cereal Sci.*, 48, 130–137

Liu, L., Zubik, L., Collins, F.W, Marko, M., Meydani, M. 2004. The antiatherogenic potential of oat phenolic compounds. *Atherosclerosis*, 175, 39–49.

Liukkonen, K.H., Katina, K., Kaukovirta–Norja, A., Lampi, A.M., Kariluoto, S., Piironen, V., Heinonen, S., Adlercreutz, H., Nurmi, A., Pihlava, J.M., Poutanen, K. 2007. Influence of germination conditions on the bioactivity of rye. pp. 229–239 in *Whole grains and health* Editors: Len Marquart, David.Jacobs, Jr., Graeme McIntosh, Kaisa Poutanen, Marla Reicks. Blackwell Publishing, Ames,IA, USA.

Liukkonen, K.H., Katina, K., Wilhelmson, A., Myllymäki, O., Lampi, A.M., Kariluoto, S., Piironen, V., Heinonen, S., Nurmi, T., Adlercreutz, H., Peltoketo, A., Pihlava, J.M., Hietaniemi V., Poutanen, K. 2003. Process–induced changes on bioactive compounds in wholegrain rye. *Proc. Nutr. Soc.*, 62,117–122

Liyanapathirana, C., Shahidi, F. 2004. Antioxidant activity of wheat extracts as affected by in vitro digestion. *BioFactors*, 21,325–328

Ludwig, R., Spencer, E., Unwin, C., 1960. An antifungal factor from barley of possible significance in disease resistance. *Can. J. Bot.*, 38, 21–29.

Lv, N., Song, M., Lee, Y., Choi, H., Kwon, K., Park, J., Park, B. 2009. Dihydro-avenanthramide D protects pancreatic beta–cells from cytokine and streptozotocin toxicity. *Biochem. Biophys. Res. Commun*. 387, 97–102.

Ma, D., Sun, D., Wang, C., Li, Y., Guo, T. 2014. Expression of flavonoid biosynthesis genes and accumulation of flavonoid in wheat leaves in response to drought stress. *Plant Physiol. Biochem.*, 80, 60–66

Maag, D., Dalvit, C., Thevenet, D., Köhler, A., Wouters, F., Vassão, D., Gershenzon, J., Wolfender, J, Turlings, T., Erb, M., Glauser, G. 2014. 3–β–d–Glucopyranosyl–6–methoxy–2–benzoxazolinone (MBOA–N–Glc) is an insect detoxification product of maize 1,4–benzoxazin–3–ones. *Phytochemistry*. 102, 97–105

Magee, L., Liebel, F., Southall, M. 2007. Compositions for inhibiting or reducing inflammation of skin. US 2007/0059390 A1.

Malalgoda, M., Simsek, S. 2017. Celiac disease and cereal proteins. *Food Hydrocolloids*, 68, 108-113

Manoukian, P., Melliou, E., Liouni, M., Magiatis, P. 2016. Identification and quantitation of benzoxazinoids in wheat malt beer by qNMR and GC–MS. *LWT - Food Sci. Technol.*, 65, 1133-1137

Marmiroli, N., Maestri, E. 2014. Plant peptides in defense and signaling. *Peptides*. 56, 30–44.

Martens, D. 2002. Identification of phenolic acid composition of alkali–extracted plants and soils. *Soil Sci. Soc. Am. J.* 66, 1240–1248

Marti, G., Erb, M., Boccard, J., Glauser, G., Doyen, G., Villard, N., Robert, C., Turlings, T., Rudaz, S., Wolfender, J. 2013. Metabolomics reveals herbivore–induced metabolites of resistance and susceptibility in maize leaves and roots. *Plant Cell Environ*. 36, 621–639.

Martin, J.H, Waldren, R. P., Stamp, D.L, 2006. Principles of Field Crop Production. 4th Edition. Pearson Prentince Hall, New Jersey, USA

Martin, L.B., Johnson, E., Hutch, C., Nelson, R. 2008. 6–MBOA affects testis size, but not delayed–type hypersensitivity, in white–footed mice (*Peromyscus leucopus*). *Comp. Biochem. Physiol. Mol. Integr. Physiol*. 149, 181–187.

Mateo Anson, N.M., Selinheimo, E., Havenaar, R., Aura, A.M., Mattila, I., Lehtinen, P., Bast, A., Poutanen, K., Haenen, G. 2009. Bioprocessing of wheat bran improves in vitro bioaccessibility and colonic metabolism of phenolic compounds. *J. Agric. Food Chem.* 57, 6148–6155.

Mattila, P., Kumpulainen, J. 2002. Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *J. Agric. Food Chem.* 50, 3660–3667.

Matsukawa, T., Isobe, T., Ishihara, A., Iwamura, H. 2000. Occurrence of avenanthramides and hydroxycinnamoyl–CoA:hydroxyanthranila-te N–hydroxycinnamoyltransferase activity in oat seeds. *Z. Naturforsch. C*. 55, 30–36.

Matsuzaki, S., Hamana,K., Isobe, K., 1990. Occurrence of $N^6$–methylagmatine in seeds of leguminous plants. *Phytochemistry*, 29, 1313–1315

Mayama, S., Tani, T., Ueno, T., Hirabayashi, K., Nakashima, T., Fukami, H., Mizuno, Y., Irie, H. 1981. Isolation and structure elucidation of genuine oat phytoalexin, avenalumin I. *Tetrahedron Lett*. 22, 2103–2106

McKeehen, J., Busch, R.., Fulcher, R. 1999. Evaluation of wheat (*Triticum aestivum* L.) phenolic acids during grain development and their contribution to Fusarium resistance. *J. Agric. Food Chem.* 47, 1476–1482.

Meihls, L.N., Handrick, V., Glauser, G., Barbier, H., Kaur, H., Haribal, M., Lipka, A., Gershenzon, J., Buckler, E., Erb, M., Köllner, T., Jander, G. 2013. Natural variation in maize aphid resistance is associated with 2,4–dihydroxy–7–methoxy–1,4–benzoxazin–3–one glucoside methyltransferase activity. *Plant Cell*. 25, 2341–2355.

Meija, L., Samaletdin, A., Koskela, A., Lejnieks, A., Lietuvietis, V., Adlercreutz, H. 2013. Alkylresorcinols in Latvian and Finnish breads. *Int. J. Food Sci. Nutr*. 64, 117–121.

Menzel, C., Kamal–Eldin, A., Marklund, M., Andersson, A., Åman, P., Landberg, R. 2012. Alkylresorcinols in Swedish cereal food products. *J. Food Comp. Anal.* 28, 119–125

Meydani, M. 2009. Potential health benefits of avenanthramides of oats. *Nutr. Rev*. 67, 731–735.

Michalska, A., Ceglińska, A. and Zieliński, H. 2007. Bioactive compounds in rye flours with different extraction rates. *Eur. Food Res. Tech.*, 225, 545–551.

Moheb, A., Ibrahim, R., Roy, R., Sarhan, F., 2011. Changes in wheat leaf phenolome in response to cold acclimation. *Phytochemistry*, 72, 2294–2307

Moheb, A., Agharbaoui, Z., Kanapathy, F., Ibrahim, R., Roy, R., Sarhan, F. 2013a. Tricin biosynthesis during growth of wheat under different abiotic stresses. *Plant Sci*. 2013 Mar;201–202:115–20.

Moheb, A., Grondin, M., Ibrahim, R., Roy, R., Sarhan, F. 2013b. Winter wheat hull (husk) is a valuable source for tricin, a potential selective cytotoxic agent. *Food Chem*. 138, 931–937.

Moinard, C., Cynober, L., de Bandt, J. 2005. Polyamines: metabolism and implications in human diseases. *Clin. Nutr.*, 24, 184–197

Nakagawa, E., Amano, T., Hirai, N., Iwamura, H. 1995. Non–induced cyclic hydroxamic acids in wheat during juvenile stage of growth. *Phytochemistry*, 38, 1349–1354

Nakano, H., Kawada, N., Yoshida, M., Ono, H., Iwaura, R., Tonooka, T. 2011. Isolation and identification of flavonoids accumulated in proanthocyanidin-free barley. *J. Agric. Food Chem*. 59, 9581–9587.

Nie, L., Wise, M., Peterson, D., Meydani, M. 2006a. Avenanthramide, a polyphenol from oats, inhibits vascular smooth muscle cell proliferation and enhances nitric oxide production. *Atherosclerosis*. 186, 260–266.

Nie, L., Wise, M., Peterson, D., Meydani, M. 2006b. Mechanism by which avenanthramide–c, a polyphenol of oats, blocks cell cycle progression in vascular smooth muscle cells. *Free Radic. Biol. Med*. 41, 702–708.

Niemeyer, H.M. 2009. Hydroxamic acids derived from 2–hydroxy–2H–1,4–benzoxazin–3(4H)–one: key defense chemicals of cereals. *J. Agric. Food Chem.* 57, 1677–1696

Niño–Medina, G., Carvajal–Millán, E., Rascon–Chu, A., Marquez–Escalante, J., Guerrero, V., Salas–Muñoz, E. 2010. Feruloylated arabinoxylans and arabinoxylan gels: structure, sources and applications. *Phytochemistry Rev.* 9, 111–120

Nordlund, E., Heiniö, R.L., Viljanen, K., Pihlava, J.M., Lehtinen, P. and Poutanen, K. 2013. Flavour and stability of rye grain fractions in relation to their chemical composition. *Food Res. Int.* 54, 48–56

Nomura, T., Ishizuka, A., Kishida, K., Islam, A., Endo,T., Iwamura, H., Ishihara, A., 2007. Chromosome arm location of the genes for the biosynthesis of hordatines in barley. *Genes Genet. Syst.*, 82, 455–464.

Nyström, L., Lampi, A.M.., Andersson, A., Kamal–Eldin, A., Gebruers, K., Courtin, C., Delcour, J., Li, L., Ward, J., Fra, A., Boros, D., Rakszegi, M., Bed, Z., Shewry, P., Piironen, V. 2008. Phytochemicals and dietary fiber components in rye varieties in the HEALTHGRAIN diversity screen. *J. Agric. Food Chem.*, 56, 9758–9766

Nyström, L., Paasonen, A., Lampi, A.M., Piironen, V. 2007. Total plant sterols, steryl ferulates and steryl glycosides in milling fractions of wheat and rye. *J. Cereal Sci.* 45, 106–115

Nørbæk, R., Aaboer, D., Bleeg, I., Christensen, B., Kondo, T., Brandt, K. 2003. Flavone C–glycoside, phenolic acid, and nitrogen contents in leaves of barley subject to organic fertilization treatments. *J. Agric. Food Chem*., 51, 809–813

Nørbæk, R., Brandt, K., Kondo, T. 2000. Identification of flavone C–glycosides including a new flavonoid chromophore from barley leaves (*Hordeum vulgare* L.) by improved NMR techniques. *J. Agric. Food Chem*., 48, 1703–1707

Okazaki Y., Ishizuka A, Ishihara A, Nishioka T, Iwamura H. 2007. New dimeric compounds of avenanthramide phytoalexin in oats. *J. Org. Chem*. 72, 3830–3839

Okazaki, Y., Isobe, T., Iwata, Y., Matsukawa, T., Matsuda, F., Miyagawa, H., Ishihara, A., Nishioka, T., Iwamura, H. 2004. Metabolism of avenanthramide phytoalexins in oats. *Plant J*. 39, 560–572.

Oshitani, N. 2010. Pharmaceutical composition for prevention of progress of intestinal constriction associated with Chron´s disease. US2010113597 A1

Oskarsson, A., Andersson, Å. 2016. Suppressed sex hormone biosynthesis by alkylresorcinols: a possible link to chemoprevention. *Nutr. Cancer*,68, 978–987.

Ovaskainen, M.L., Törrönen, R., Koponen, J., Sinkko, H., Hellström, J., Reinivuo, H., Mattila, P. 2008. Dietary intake and major food sources of polyphenols in finnish adults. *J. Nutr.*, 138, 562–566.

Pearlman, R., Jenkins, H., Serafini, T. 2010. Combination therapy of arthritis with tranilast. US2010158905 A1. Applicant: Nuon Therapeutics Inc.

Pedersen, M.B., Bunzel, M., Schäfer, J., Knudsen, K., Sørensen, J., Yu S., Lærke, H. 2015. Ferulic acid dehydrodimer and dehydrotrimer profiles of distiller's dried grains with solubles from different cereal species. *J. Agric. Food Chem*. 63, 2006–2012

Pedersen, H.A., Laursen, B., Mortensen, A., Fomsgaard, I.S. 2011. Bread from common cereal cultivars contains an important array of neglected bioactive benzoxazinoids. *Food Chem.*, 127, 1814–1820

Peñalvo, J., Hanhineva, K. Adlercreutz, H. 2014. Bioavailability of rye lignans and their relevance for human health. In: *Rye and Health*. Editors: Kaisa Poutanen, Per Åman. AACC International Inc., St. Paul, MN, USA. pp.71–84. ISBN 978–1–891127–81–6

Peñalvo, J., Haajanen, K.., Botting, N., Adlercreutz, H. 2005. Quantification of lignans in food using isotope dilution gas chromatography/mass spectrometry. *J. Agric. Food Chem.*, 53,9342–9347.

Pellati, F., Benvenuti, S., Melegari, M. 2004. High-performance liquid chromatography methods for the analysis of adrenergic amines and flavanones in *Citrus aurantium* L. var. *amara*. *Phytochem. Anal*. 15, 220-225.

Peterson, D., Hahn, M., Emmons, C. 2002. Oat avenanthramides exhibit antioxidant activities in vitro. *Food Chem.* 79, 473–478.

Peterson, D., Emmons, C., Hibbs, A. 2001. Phenolic antioxidants and antioxidant activity in pearling fractions of oat groats. *J. Cereal Sci*. 33(1):97–103.

Peterson, D. , Wesenberg, D., Burrup, D., Erickson, C. 2005. Relationships among agronomic traits and grain composition in oat genotypes grown in different environments. *Crop Sci.* 45, 1249–1255

Peterson, D. Dimberg, L.H. 2008. Avenanthramide concentrations and hydroxycinnamoyl–CoA:hydroxyanthra-nilate N–hydroxycinnamoyltransferase activities in developing oats. *J. Cereal Sci.*, 47, 101–108

Pihlava, J.M., Oksman–Caldentey, K.M. 2001. Effect of biotechnological processing on phenolic compounds and antioxidativity in oats. In: *Biologically–active Phytochemicals in Food: Analysis, Metabolism, Bioavailability and Function* Editors: W. Pfannhauser, G. R. Fenwick and S. Khokhar. Royal Society of Chemistry, Cambridge, UK. 2001. 616 pp. ISBN 0–85404–806–5

Pihlava, J.M., Reinikainen, P., Hietaniemi, V., Kaukovirta–Norja, A., 2008. Effect of malting on selected phytochemicals in wheat, rye, barley and oats. AACC International Annual Meeting September 21–24, 2008 Honolulu, Hawaii, USA. *Cereal Foods World* 53:A38 [abstract]

Pihlava, J.M., Reinikainen, P., Hietaniemi, V., Kaukovirta–Norja, A., 2010 Flavonoid content in barley, oat, rye and wheat cultivars. HEALTHGRAIN Conference 5.–7.5.2010 Lund, Sweden. Abstract book p. 75 [abstract]

Piletz, J., Aricioglu, F., Cheng, J., Fairbanks, C., Gilad, V., Haenisch, B., Halaris, A., Hong, S., Lee, J., *et al*. 2013. Agmatine: clinical applications after 100 years in translation. *Drug Discov. Today*, 18, 880–893

Ponts, N., Pinson–Gadais, L., Boutigny, A., Barreau, C., Richard–Forget, F. 2011. Cinnamic acids significantly affect *Fusarium graminearum* growth and *in vitro* production of type B trichothecenes. *Phytopathology.* 101, 929–934

Pothou, E., Melliou, E., Skaltsounis, A–L., Liouni, M., Magiatis, P. 2013. Investigation of volatile constituents of beer, using resin adsorption and GC/MS, and correlation of 2–(3H)–benzoxazolone with wheat malt. *J. Am. Soc. Brew. Chem.* ,71 (1), 35–40

Prinz, S., Schauberger, D., Bauer,I., Knasmueller, S., Kopp; B. 2010. Aneugenic 2,4–dihydroxy–7–methoxy–1,4–benzoxazin–3–one (DIMBOA) and 2,4–dihydroxy–1,4–benzoxazin–3–one (DIBOA) in sprouts of Triticum aestivum cultivars – A 'safety health food'? *Food Chem.* 121, 973–979

Provan, G., Scobbie, L., Chesson, A. 1994. Determination of phenolic acids in plant cell walls by microwave digestion. *J. Sci. Food Agric.*, 64,63–65

Quifer–Rada, P., Vallverdú–Queralt, A., Martínez–Huélamo, M., Chiva–Blanch, G., Jáuregui, O., Estruch, R., Lamuela–Raventós, R. 2015. A comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC–ESI–LTQ–Orbitrap–MS). *Food Chem.* 169, 336–343

Radaelli, R., Dimberg, L., Germeier, C., Berardo, N., locatelli, S., Guerrini, L. 2016. Variability of tocols, tocotrienols and avenanthramides contents in European oat germplasm. *Euphytica*, 207, 273–292.

Redmond, M., Fielder, D. 2004. Oat extracts: refining, compositions and methods of use. US 6,818,232 B1. Applicant: Ceapro Inc.

Ren, Y., Wise, M. 2013. Avenanthramide biosynthesis in oat cultivars treated with systemic acquired resistance elicitors. *Cereal Res. Commun.*41, 255–265

Renger, A., Steinhart, H. 2000. Ferulic acid dehydrodimers as structural elements in cereal dietary fiber. *Eur. Food Res. Tech.*, 211, 422–428

Rodriguez–Mateos, A., Vauzour, D., Krueger, C., Shanmuganayagam, D., Reed, J., Calani, L., Mena, P., Del Rio, D., Crozier, A. 2014. Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. *Arch. Toxicol*. 88, 1803–1853

Rogosnitzky, M., Danks, R., Kardash, E. 2012. Therapeutic potential of tranilast, an anti–allergy drug, in proliferative disorders. *Anticancer Res*. 2012 Jul;32(7):2471–8.

Rosenfeld, M., Berger, P., Negus, N., 2001. Novel compounds for use as antidepressants, aphrodisiacs and adjunctive therepies in humans. US20010053789 A1

Rosenfeld, M., Forsberg, S. 2006. Novel compounds for use in weight loss and appetite suppression in humans. US20060223796 A1

Ross, A., 2012. Present status and perspectives on the use of alkylresorcinols as biomarkers of wholegrain wheat and rye Intake. *J. Nutr. Metab.* doi,10.1155/2012/462967

Ross, A., Bourgeois, A., Macharia, H., Kochhar, S., Jebb, S., Brownlee, I., Seal, C. 2012. Plasma alkylresorcinols as a biomarker of whole–grain food consumption in a large population, results from the WHOLEheart Intervention Study. *Am. J. Clin. Nutr.*, 95, 204–211

Ross, A., Kamal-Eldin, A., Jung, C., Shepherd, M., Åman, P. 2001. Gas chromatographic analysis of alkylresorcinols in rye (Secale cereale L) grains. *J. Sci. Food Agric*. 81, 1405–1411.

Ross, A., Kamal–Eldin, A., Åman, P. 2004. Dietary alkylresorcinols, absorption, bioactivities, and possible use as biomarkers of whole–grain wheat– and rye–rich foods. *Nutr. Rev.* 62, 81–95

Ross, A., Redeuil, K., Vigo, M., Rezzi, S., Nagy, K. 2010. Quantification of alkylresorcinols in human plasma by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom*. 24, 554–560.

Ross, A., Shepherd, M., Schüpphaus, M., Sinclair, V., Alfaro, B., Kamal–Eldin, A. Åman, P. 2003. Alkylresorcinols in cereals and cereal products. *J. Agric. Food Chem.*, 51,4111–4118

Sanders, E., Gardner, P., Berger, P., Negus N. 1981. 6–Methoxybenzoxazolinone: a plant derivative that stimulates reproduction in *Microtus montanus*. *Science*. 214, 67–69

Santiago, R., Malvar, R. 2010. Role of dehydrodiferulates in maize resistance to pests and diseases. *Int. J. Mol. Sci.* 11, 691–703

SAS Institute Inc. 2011. SAS/STAT 9.3 User's Guide. SAS Institure Inc., Cary, NC, USA

Saunders, M., Kohn, L. 2009. Evidence for alteration of fungal endophyte community assembly by host defense compounds. *New Phytol*. 182, 229–238

Savolainen, O., Coda, R., Suomi, K., Katina, K., Juvonen, R., Hanhineva, K., Poutanen, K., 2014. The role of oxygen in the liquid fermentation of wheat bran. *Food Chem.*, 153, 424–431

Savolainen, O., Pekkinen, J., Katina, K., Poutanen, K., Hanhineva, K. 2015. Glycosylated benzoxazinoids are degraded during fermentation of wheat bran. *J. Agric. Food Chem.*, 63, 5943–5949

Schmaus, G., Röding, J. 2009. Mixtures compromising anthranilic acid amides and antidandruff agents as cosmetic and pharmaceutical compositions for alleviating itching. US2009/0226537 A1. Applicant: Symrise Gmbh & Co. KG.

Schmaus, G., Joppe, H., Herrmann, M., Sabater–Luntzel, C., Voessing, T. 2012. Anthranillic acid amides and derivatives thereof as cosmetic and pharmaceutical active compounds. US2012315233 A1. Applicant: Symrise AG.

Schneider, A., Moraru, A., Krueger, C., Laage, R., Pitzer, C. 2009. Use of tranilast and derivates thereof for the therapy of neurological conditions. WO2009024543 A1. Applicant: Sygnis Bioscience Gmbh & Co KG

Schulz, M., Marocco, A., Tabaglio, V., Macias, F., Molinillo, J. 2013. Benzoxazinoids in rye allelopathy – from discovery to application in sustainable weed control and organic farming. Review. *J. Chem. Ecol*. 39, 154–174.

Schulz, M., Marocco, A., Tabaglio, V. 2012. BOA detoxification of four summer weeds during germination and seedling growth. *J .Chem. Ecol*. 38, 933–946.

Schulz, M., Strack, D., Weissenböck, G., Markham, K., Dellamonica, G., Chopin, J. 1985. Two luteolin o–glucuronides from primary leaves of Secale cereale. *Phytochemistry*. 24,343–345

Schullehner, K., Dick, R., Vitzthum, F., Schwab, W., Brandt, W., Frey, M., Gierl, A. 2008. Benzoxazinoid biosynthesis in dicot plants. *Phytochemistry*. 69, 2668–2677

Shelby, N., Godfrey, M., Rosenfeld, M. 2006. Methods for inducing anti–anxiety and calming effects in animls and humans. WO2006017281 A1. Applicant: Seroctin Research & Technology Inc.

Simpson, D., Rezanoor, H., Parry, D., Nicholson, P. 2000. Evidence for differential host preference in *Microdochium nivale* var. *majus* and *Microdochium nivale* var. *nivale*. *Plant Pathol.* 49, 261–268

Skoglund, M. 2008. Phenolic compounds in oats. Diss. (sammanfattning/summary) Uppsala : Sveriges lantbruksuniv., Acta Universitatis agriculturae Sueciae, 1652–6880 ; 2008:2 [Doctoral thesis]

Skoglund, M., Peterson, D., Andersson, R., Nilsson,J., Dimberg, L. 2008. Avenanthramide content and related enzyme activities in oats as affected by steeping and germination. *J. Cereal Sci.*, 48, 294–303

Smeds, A., Eklund, P., Sjöholm, R., Willför S., Nishibe, S., Deyama, T., Holmbom, B. 2007. Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. *J. Agric. Food Chem.* 55, 1337–1346.

Smeds, A., Hakala, K., Hurmerinta, T., Kortela, L., Saarinen, N., Mäkelä, S. 2006. Determination of plant and enterolignans in human serum by high–performance liquid chromatography with tandem mass spectrometric detection. *J. Pharm. Biomed .Anal*. 41, 898–905

Smeds, A., Jauhiainen, L., Tuomola, E. and Peltonen–Sainio, P. 2009. Characterization of variation in the lignan content and composition of winter rye, spring wheat, and spring oat. *J. Agric. Food Chem.* 57, 5837–5842.

Smith, T., Best, G. 1978. Distribution of hordatines in barley. *Phytochemistry*, 17, 1093–1098.

Snelders, J., Dornez, E., Delcour, J., Courtin C. 2013. Ferulic Acid content and appearance determine the antioxidant capacity of arabinoxylanoligosaccharides. *J. Agric. Food Chem.* 61, 10173–10182

Snelders, J., Dornez, E., Delcour, J., Courtin C. 2014a. Impact of wheat bran derived arabinoxylanoligosaccharides and associated ferulic acid on dough and bread properties. *J. Agric. Food Chem.* 62, 7190–7199

Snelders, J., Olaerts, H., Dornez, E., Van de Wiele, T. Aura, A.M., Vanhaecke, L., Delcour, J., Courtin, C. 2014b. Structural features and feruloylation modulate the fermentability and evolution of antioxidant properties of arabinoxylanoligosaccharides during in vitro fermentation by human gut derived microbiota. *J. Funct. Foods*, 10, 1–12

Soriano, I., Asenstorfer, R., Schmidt, O., Riley, I. 2004. Inducible Flavone in Oats (*Avena sativa*) Is a Novel Defense Against Plant–Parasitic Nematodes. *Phytopathology*, 94, 1207–1214.

Sosulski, F., Krygier, K., Hogge, L. 1982. Free, esterified, and insoluble–bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *J. Agric. Food Chem.* 30, 337–340.

Stasiuk M., Kozubek A. 2010. Biological activity of phenolic lipids. *Cell. Mol. Life Sci*. 67, 841–860

Steffensen, S., Pedersen, H., Adhikari, K.B., Laursen, B., Jensen, C., Høyer,S., Borre,M., Pedersen,H., Borre, M., Edwards, D., Fomsgaard, I. S. 2016. Benzoxazinoids in prostate cancer patients after a rye-intensive diet: methods and initial results. *J. Agric. Food Chem.* 64, 8235−8245.

Steiner, E, Becker, T., Gastl, M. 2010. Turbidity and haze formation in beer — insights and overview. *J. Inst. Brew.* 116, 360–368

Steinman, L., Platten, M., Ho, P., Salley, M. 2010. Tranilast as modulator of T cell functioning for use in the treatment of autoimmune diseases. EP2253313 A1. Applicant: Univ. Leland Stanford Junior

Stoessl, A., 1965. The antifungal factors in barley–III. Isolation of p–coumaroylagmatine. *Phytochemistry.* 4, 973–976

Stoessl, A., 1966. The antifungal factors in barley – the constitutions of hordatines A and B. *Tetrahedron Lett.* 7, 2287–2292

Stoessl, A., 1967. The antifungal factors in barley. IV. Isolation, structure, and synthesis of the hordatines. *Can. J. Chem.* 45, 1745–1760.

Stoessl, A., Unwin, C., 1969. The antifungal factors in barley. V. Antifungal activity of the hordatines. *Can. J. Bot.*, 48, 465–470.

Subramaniam, V., Ace, O., Prud'homme, G., Jothy, S. 2011. Tranilast treatment decreases cell growth, migration and inhibits colony formation of human breast cancer cells. *Exp Mol Pathol*. 90, 116–122.

Sur, R., Nigam, A., Grote, D., Liebel, F., Southall, M. 2008. Avenanthramides, polyphenols from oats, exhibit anti–inflammatory and anti–itch activity. *Arch. Dermatol. Res*. 300, 569–574.

Suwa, Y., Fujii, W., Hisako, H., Yoshiaki, Y., Haruo, N., Kuniro, T. 2004. Refreshment capable of stimulating movement of digestive tract. WO2004002978. Applicant: Suntory Ltd.

Söderholm, P., Koskela, A., Lundin, J., Tikkanen, M., Adlercreutz, H. 2009. Plasma pharmacokinetics of alkylresorcinol metabolites, new candidate biomarkers for whole–grain rye and wheat intake. *Am. J. Clin. Nutr.* 90, 1167–1171.

Taguchi, S., Yamada, T., Oiso, Y.. Ozaki, N., Umeda, H., Mizutani, N. 2008. Tranilast inhibits glucose–induced insulin secretion from pancreatic beta–cells. *Horm. Metab. Res*. 40, 518–523. abstract only

Tahir, I., Ahmadi–Afzadi, M., Nybom, H., Dey, E. 2014. Rye bran alkylresorcinols inhibit growth of *Penicillium expansum* and *Neofabraea perennans in vitro* and *in vivo* on different apple cultivars. *Eur. J. Hortic. Sci*. 79, 218–225.

Tanwir, F., Fredholm, M., Gregersen, P., Fomsgaard, I.S. 2013. Comparison of the levels of bioactive benzoxazinoids in different wheat and rye fractions and the transformation of these compounds in homemade foods. *Food Chem*. 141, 444–450.

Tetens, I., Turrini, A., Tapanainen, H., Christensen, T., Lampe, J., Fagt, S., Håkansson, N., Lundquist, A., Hallund, J., Valsta, L.; Phytohealth WP1 working group. 2013. Dietary intake and main sources of plant lignans in five European countries. *Food Nutr Res*. 2013 Jun 11;57. doi: 10.3402/fnr.v57i0.19805.

Uchihashi, K., Nakayashiki, H., Okamura, K., Ishihara, A., Tosa, Y., Park, P., Mayama, S. 2011. In situ localization of avenanthramide A and its biosynthetic enzyme in oat leaves infected with the crown rust fungus, *Puccinia coronata* f. sp. *avenae*. *Physiol. Mol. Plant Pathol.* 76, 173–181

Vanbeneden, N., Van Roey, T., Willems, F., Delvaux, F., Delvaux, F.R. . 2008. Release of phenolic flavour precursors during wort production: Influence of process parameters and grist composition on ferulic acid release during brewing. *Food Chem.* 111, 83–91

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W. 2010. Lignin biosynthesis and structure. *Plant Physiol.* 153, 895–905

Verardo, V., Gómez–Caravaca, A., Marconi, E., Caboni, M. 2011. Air classification of barley flours to produce phenolic enriched ingredients: Comparative study among MEKC–UV, RP–HPLC–DAD–MS and spectrophotometric determinations. *LWT–Food Sci. Technol.* 44, 1555–1561

Verardo, V., Gómez–Caravaca, A., Marconi, E., Caboni, M. 2015. Analysis of oligomer proanthocyanidins in different barley genotypes using high–performance liquid chromatography–fluorescense detection–mass spectrometry and near–infrared methodologies *J. Agric. Food Chem.* 63, 4130–4137

Varga, M., Berkesi, O., Darula, Z., May, N., Palagyi, A. 2016. Structural characterization of allomelanin from black oat. *Phytochemistry*, 130, 313–320

Vielhaber, G., Schmaus, G. 2006. Mixtures compromising anthranilic acid amides and cooling agents as cosmetic and pharmaceutical compositions for alleviating itching. WO2006/134013 A1. Applicant: Symrise Gmbh & Co. KG.

Virtanen, A.I., Hietala, P., 1955. 2(3)–Benzoxazolinone, an antifusarium factor in rye seedlings. *Acta Chem. Scand*. 9, 1543–1544

Virtanen, A.I., Hietala, P., 1960. Precursors of benzoxazolinone in rye plants. 1. Precursor II, the aglycon. *Acta Chem. Scand*. 14, 499–502

Virtanen, A.I., Hietala, P., Wahlroos, Ö. 1956. An anti–fungal factor in maize and wheat plants. *Suomen Kemistilehti* B29, 143

Vitaglione, P., Napolitano, A. and Fogliano, V. 2008. Cereal dietary fibre, a natural functional ingredient to deliver phenolic compounds into the gut. *Trends Food Sci. Tech.*, 19,451–463

von Röpenack, E., Parr, A., Schulze–Lefert, P., 1998. Structural analyses and dynamics of soluble and cell wall–bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *J. Biol. Chem.* 273, 9013–9022

Vuckovic, D. 2012. Current trends and challenges in sample preparation for global metabolomics using liquid chromatography–mass spectrometry. *Anal. Bioanal. Chem.* 403, 1523–1248

Vukics, V., Guttman, A. 2010. Structural characterization of flavonoid glycosides by multi–stage mass spectrometry. *Mass Spectrom. Rev*. 29, 1–16

Wahlroos, Ö.,Virtanen A.I. 1959. The precursors of 6–methoxybenzoxazolinone in maize and wheat plants, their isolation and some of their properties. *Acta Chem. Scand*. 13, 1906–1908.

Wakimoto, T., Nitta, M., Kasahara, K., Chiba, T., Ye, Y., Tsuji, K., Kan, T., Nukaya, H., Ishiguro, M., Koike, M., Yokoo, Y., Suwa, Y. 2009. Structure–activity relationship study on alpha1 adrenergic receptor antagonists from beer. *Bioorg. Med. Chem. Lett.* 19, 5905–5908.

Wang, D., Wise, M., Li, F., Dey, M. 2012. Phytochemicals attenuating aberrant activation of β–catenin in cancer cells. *PLoS One*. 7(12):e50508. doi: 10.1371/journal.pone.0050508.

Waridel, P., Wolfender, J., Ndjoko, K., Hobby, K., Major, H., Hostettmann, K. 2001. Evaluation of quadrupole time–of–flight tandem mass spectrometry and ion–trap multiple–stage mass spectrometry for the differentiation of C–glycosidic flavonoid isomers. *J. Chromatogr. A*. 926, 29–41.

Weidner, S., Amarowicz, R., Karamać, M., Dąbrowski, G. 1999. Phenolic acids in caryopses of two cultivars of wheat, rye and triticale that display different resistance to pre–harvest sprouting. *Eur. Food Res. Tech.*, 210, 109–113

Wen, W., Li, D., Li, X., Gao, Y., Li, W., Li, H., Liu, J., Liu, H., Chen, W., Luo, J., Yan, J. 2014. Metabolome–based genome–wide association study of maize kernel leads to novel biochemical insights. *Nat Commun*. 5:3438. doi: 10.1038/ncomms4438

Wenzig, E., Kunert, O., Ferreira, D., Schmid, M., Schühly, W., Bauer, R., Hiermann, A. 2005. Flavonolignans from *Avena sativa*. *J Nat Prod*. 68, 289–292.

Wieland, I., Kluge, M., Schneider, B., Schmidt, J., Sicker, D., Schulz, M. 1998. 3–β–d–glucopyranosyl–benzoxazolin–2(3H)–one—A detoxification product of benzoxazolin–2(3H)–one in oat roots. *Phytochemistry*, 49, 719–722

Wilkes, M., Marshall, D., Copeland, L. 1999. Hydroxamic acids in cereal roots inhibit the growth of take–all. *Soil Biol. Biochem.* 31, 1831–1836

Willför, S., Smeds, A., Holmbom, B. 2006. Chromatographic analysis of lignans. *J. Chromatogr. A*. 1112, 64–77.

Winter, M., Koopmann, B., Döll, K., Karlovsky, P., Kropf, U., Schlüter, K., von Tiedemann, A. 2013. Mechanisms regulating grain contamination with trichothecenes translocated from the stem base of wheat (*Triticum aestivum)* infected with *Fusarium culmorum*. *Phytopathology*. 103, 682–689.

Wise, M. 2011. Effect of chemical systemic acquired resistance elicitors on avenanthramide biosynthesis in oat (*Avena sativa*). *J. Agric. Food Chem.* 59, 7028–7038

Wise, M., Vinje, M., Conley, S. 2016. Field application of benzothiadiazole (BTH) to oats (*Avena sativa*): effects on crown rust resistance and avenanthramide production. *Crop Sci*., 56, 1904–1913.

Wojakowska, A., Perkowski, J., Góral, T., Stobiecki, M. 2013. Structural characterization of flavonoid glycosides from leaves of wheat (*Triticum aestivum* L.) using LC/MS/MS profiling of the target compounds. *J. Mass. Spectrom*. 48, 329–339.

Wouters, F., Reichelt, M., Glauser, G., Bauer, E., Erb, M., Gershenzon, J., Vassão, D. 2014. Reglucosylation of the benzoxazinoid DIMBOA with inversion of stereochemical configuration is a detoxification strategy in Lepidopteran herbivores. *Angew. Chem. Int. Ed Engl*. 53, 11320–11324.

Wu, H., Haig, E., Pratley, J., Lemerle, D., An, M. 1999. Simultaneous determination of phenolic acids and 2,4–dihydroxy–7–methoxy–1,4–benzoxazin–3–one in wheat (*Triticum aestivum* L.) by gas chromatography–tandem mass spectrometry. *J. Chromatogr. A*. 864, 315–321

Xie, Z., Mui, T., Sintara, M., Ou, B., Johnson, J., Chu, Y., O'shea, M., Kasturi, P., Chen Y. 2017. Rapid quantitation of avenanthramides in oat-containing products by high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (HPLC-TQMS). *Food Chem*. 224, 280–288.

Xing, Y.–M., White, P. 1997. Identification and function of antioxidants from oat groats and hulls. *J.Am. Oil Chem. Soc.* 74, 303–307

Xu, J., Tian, C., Hu, Q., Luo, J., Wang, X., Tian, X. 2009. Dynamic changes in phenolic compounds and antioxidant activity in oats (*Avena nuda* L.) during steeping and germination. *J. Agric. Food Chem.* 57, 10392–10398.

Yamaji, N., Yokoo, Y., Iwashita, T., Nemoto, A., Koike, M., Suwa, Y., Wakimoto, T., Tsuji, K., Nukaya, H. 2007. Structural determination of two active compounds that bind to the muscarinic M3 receptor in beer. *Alcohol. Clin. Exp. Res.*, 31(1 Suppl), 9S–14S

Yang, J., Ou, B., Wise, M., Chu, Y. 2014. In vitro total antioxidant capacity and anti–inflammatory activity of three common oat–derived avenanthramides. *Food Chem.* 160, 338–345

Yang, Q., Trinh, H., Imai, S., Ishihara, A., Zhang, L., Nakayashiki, H., Tosa, Y., Mayama, S. 2004. Analysis of the involvement of hydroxyanthranilate hydroxycinnamoyltransferase and caffeoyl–CoA 3–O–methyltransferase in phytoalexin biosynthesis in oat. *Mol. Plant. Microbe Interact*. 17, 81–89.

Yue, Q., Bacon, C., Richardson, M.. 1998. Biotransformation of 2–benzoxazolinone and 6–methoxy–benzoxazolinone by *Fusarium moniliforme*. *Phytochemistry*. 48, 451–454.

Yokoo, Y., Fujii, W., Hori, H., Nagao, K., Suwa, Y., Taniyama, K., Tsuji, K., Yoshida, T., Nukaya, H. 2004. Isolation of stimulants of gastrointestinal motility in beer. *Alcohol. Clin. Exp. Res.* 28 (8 Suppl), 129S–133S

Yoshida, A., Hirai, S. 2011. Preventing or treating agent of retinal disease, containing tranilast. JP2011006406 A. Applicant: Santen Pharma Co Ltd

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., Hajslova, J. 2012. Deoxynivalenol oligoglycosides: new "masked" fusarium toxins occurring in malt, beer, and breadstuff. *J. Agric. Food Chem.* 60, 9280–9291.

Zamora–Ros, R., Rothwell, J., Scalbert, A., Knaze, V., *et al*. 2013. Dietary intakes and food sources of phenolic acids in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Br. J. Nutr*. 110, 1500–1511.

Zieliński, H., Ceglińska, A., Michalska, A. 2007. Antioxidant contents and properties as quality indices of rye cultivars. *Food Chem.*, 104,980–988

Žilić, S., Šukalović, V.H., Dodig, D., Maksimović, V., Maksimović, M., Basić, Z. 2011. Antioxidant activity of small grain cereals caused by phenolics and lipid soluble antioxidants. *J. Cereal Sci*. 54, 417–424.

# APPENDIX: ORIGINAL PUBLICATIONS

I. Reprinted with permission from *Journal of Agricultural and Food Chemistry* 2005, *53*, 8290–8295. Copyright 2005 American Chemical Society.

II. Reprinted from *Journal of Food Composition and Analysis*, 2015, *38*, 89–97 with permission from Elsevier.

III. Reprinted from *Journal of Cereal Science*, 2014, *60*, 645–652 with permission from Elsevier.

IV. Reprinted from Reprinted from *Journal of the Institute of Brewing*, 2016, *122*, 212–217 with permission from John Wiley and Sons.

V. Reprinted from *Food Chemistry,* 2016, *204*, 400–408 with permission from Elsevier.

VI. Submitted manuscript

## DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic herring flesh lipids. (Organic chemistry).
2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.
3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.
4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.
5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.
6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.
7. **PÄIVI LAAKSO (1992)** Triacylglycerols – approaching the molecular composition of natural mixtures.
8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.
9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.
10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: food use potential.
11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.
12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: estimation of optimal *Lactobacillus* GG concentrations.
13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.
14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.
15. **SUSANNA EEROLA (1997)** Biologically active amines: analytics, occurrence and formation in dry sausages.
16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.
17. **TUULA VESA (1997)** Symptoms of lactose intolerance: influence of milk composition, gastric emptying, and irritable bowel syndrome.
18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.
19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.
20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.
21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.
22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole – compounds associated with boar taint problem. (Biotechnology).
23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: studies on diagnosis, prevalence and nutritional management.
24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.
25. **BAORU YANG (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.
26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.
27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.
28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.
29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria x ananassa* Duch.) fruit.
30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota – a target for treatment in infant atopic eczema?
31. **TARJA ARO (2003)** Chemical composition of Baltic herring: effects of processing and storage on fatty acids, mineral elements and volatile compounds.
32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.
33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.
34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.
35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.
36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.
37. **FANDI FAWAZ ALI IBRAHIM (2006)** Lactic acid bacteria: an approach for heavy metal detoxification.
38. **JUKKA-PEKKA SUOMELA (2006)** Effects of dietary fat oxidation products and flavonols on lipoprotein oxidation.
39. **SAMPO LAHTINEN (2007)** New insights into the viability of probiotic bacteria.
40. **SASKA TUOMASJUKKA (2007)** Strategies for reducing postprandial triacylglycerolemia.

41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: studies on polydextrose, lactose and cocoa mass.
42. **RENATA ADAMI (2007)** Micronization of pharmaceuticals and food ingredients using supercritical fluid techniques.
43. **TEEMU HALTTUNEN (2008)** Removal of cadmium, lead and arsenic from water by lactic acid bacteria.
44. **SUSANNA ROKKA (2008)** Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections.
45. **ANU LÄHTEENMÄKI-UUTELA (2009)** Foodstuffs and medicines as legal categories in the EU and China. Functional foods as a borderline case. (Law).
46. **TARJA SUOMALAINEN (2009)** Characterizing *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 as a new probiotic combination: basic properties of JS and pilot *in vivo* assessment of the combination.
47. **HEIDI LESKINEN (2010)** Positional distribution of fatty acids in plant triacylglycerols: contributing factors and chromatographic/mass spectrometric analysis.
48. **TERHI POHJANHEIMO (2010)** Sensory and non-sensory factors behind the liking and choice of healthy food products.
49. **RIIKKA JÄRVINEN (2010)** Cuticular and suberin polymers of edible plants – analysis by gas chromatographic-mass spectrometric and solid state spectroscopic methods.
50. **HENNA-MARIA LEHTONEN (2010)** Berry polyphenol absorption and the effect of northern berries on metabolism, ectopic fat accumulation, and associated diseases.
51. **PASI KANKAANPÄÄ (2010)** Interactions between polyunsaturated fatty acids and probiotics.
52. **PETRA LARMO (2011)** The health effects of sea buckthorn berries and oil.
53. **HENNA RÖYTIÖ (2011)** Identifying and characterizing new ingredients *in vitro* for prebiotic and synbiotic use.
54. **RITVA REPO-CARRASCO-VALENCIA (2011)** Andean indigenous food crops: nutritional value and bioactive compounds.
55. **OSKAR LAAKSONEN (2011)** Astringent food compounds and their interactions with taste properties.
56. **ŁUKASZ MARCIN GRZEŚKOWIAK (2012)** Gut microbiota in early infancy: effect of environment, diet and probiotics.
57. **PENGZHAN LIU (2012)** Composition of hawthorn (*Crataegus* spp.) fruits and leaves and emblic leafflower (*Phyllanthus emblica*) fruits.
58. **HEIKKI ARO (2012)** Fractionation of hen egg and oat lipids with supercritical fluids. Chemical and functional properties of fractions.
59. **SOILI ALANNE (2012)** An infant with food allergy and eczema in the family – the mental and economic burden of caring.
60. **MARKO TARVAINEN (2013)** Analysis of lipid oxidation during digestion by liquid chromatography-mass spectrometric and nuclear magnetic resonance spectroscopic techniques.
61. **JIE ZHENG (2013)** Sugars, acids and phenolic compounds in currants and sea buckthorn in relation to the effects of environmental factors.
62. **SARI MÄKINEN (2014)** Production, isolation and characterization of bioactive peptides with antihypertensive properties from potato and rapeseed proteins.
63. **MIKA KAIMAINEN (2014)** Stability of natural colorants of plant origin.
64. **LOTTA NYLUND (2015)** Early life intestinal microbiota in health and in atopic eczema.
65. **JAAKKO HIIDENHOVI (2015)** Isolation and characterization of ovomucin – a bioactive agent of egg white.
66. **HANNA-LEENA HIETARANTA-LUOMA (2016)** Promoting healthy lifestyles with personalized, *APOE* genotype based health information: The effects on psychological-, health behavioral and clinical factors.
67. **VELI HIETANIEMI (2016)** The *Fusarium* mycotoxins in Finnish cereal grains: How to control and manage the risk.
68. **MAARIA KORTESNIEMI (2016)** NMR metabolomics of foods – Investigating the influence of origin on sea buckthorn berries, *Brassica* oilseeds and honey.
69. **JUHANI AAKKO (2016)** New insights into human gut microbiota development in early infancy: influence of diet, environment and mother's microbiota.
70. **WEI YANG (2017)** Effects of genetic and environmental factors on proanthocyanidins in sea buckthorn (*Hippophaë rhamnoides*) and flavonol glycosides in leaves of currants (*Ribes* spp.).
71. **LEENAMAIJA MÄKILÄ (2017)** Effect of processing technologies on phenolic compounds in berry products.
72. **JUHA-MATTI PIHLAVA (2017)** Selected bioactive compounds in cereals and cereal products – their role and analysis by chromatographic techniques

Turun yliopisto
University of Turku