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**Phenolic acids and antioxidative capacity on ancient wheat
namely einkorn (*T. monococcum* ssp.), emmer
(*T. turgidum* ssp.) and spelt wheat (*T. aestivum* ssp. *spelta*)
and on germinated bread wheat
(*T. aestivum* ssp. *aestivum*)**

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From Glauchau

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Phenolic compounds:

Are they healthy?

Are they present in wheat?

Have they always been present in wheat?

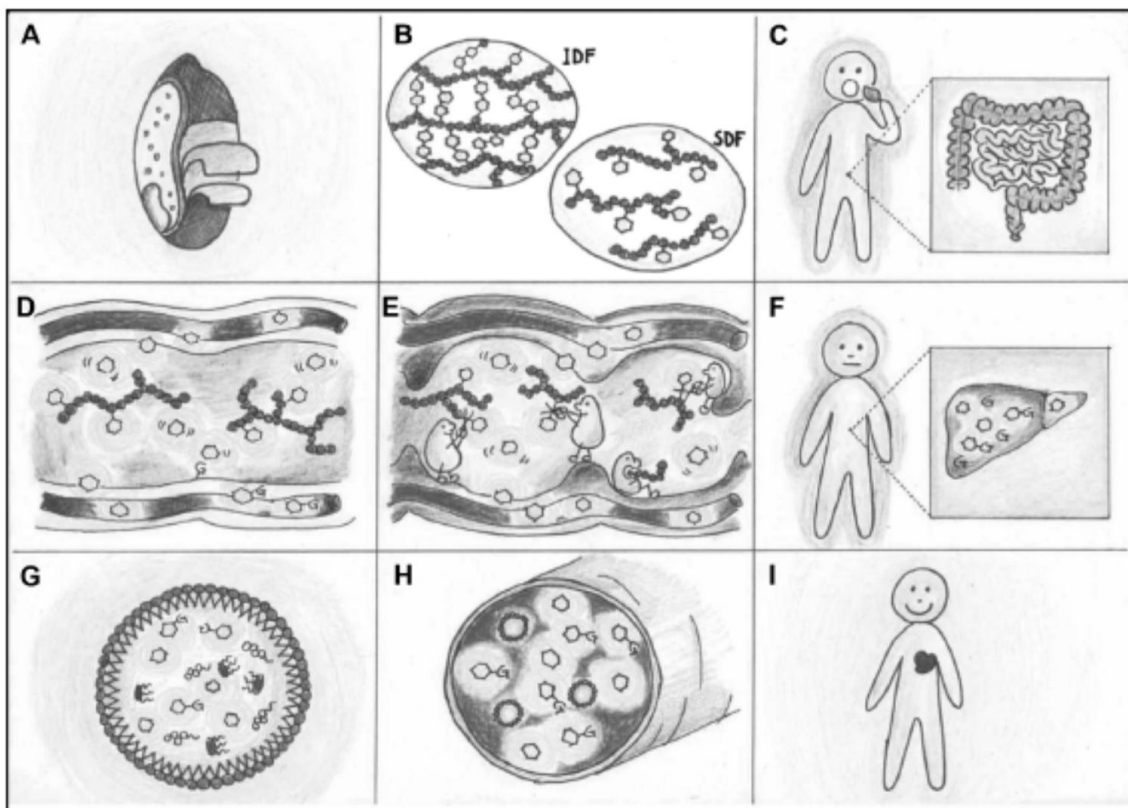


Fig. 0-1:Health benefits by whole grain consumption (VITAGLIONE et al., 2008)

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Abbreviations

AAPH	2,2'-azobis(2-methylpropionamidine)dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
AUC	Area under curve
CA	Caffeic acid
CCC	Chlorcholin chloride
CHD	Coronary heart disease
CRC	Colorectal cancer
CVD	Cardio vascular disease
DAD	Diode array detector
DPPH	Diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimetry
ET	Electron transfer
FA	Ferulic acid
FAE	Ferulic acid equivalent
FCR	Folin-Ciocalteu reagent
FN	Falling number
FRAP	Ferric ion reducing antioxidant parameter
GAE	Gallic acid equivalent
GI	Gluten index
GIT	Gastro intestinal tract
GSH	Glutathione
HAT	Hydrogen atom transfer
HLW	Hectoliter weight
HPLC	High performance liquid chromatography
KIHD	Kuopio Ischaemic Heart Disease
LA	Linoleic acid
MDA	Malondialdehyde
OIT	Oxidation induction temperature
ORAC	Oxygen radical absorbance capacity
PA	Phenolic acid
PAL	Phenylalanine ammonia lyase
PC	Phenolic compound

Abbreviations

PCA	p-Coumaric acid
PGR	Plant growth regulator
RCS	Reactive chlorine species
RK	Rotkorn
RMCD	Randomly methylated β -cyclodextrin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS	Reactive species
SA	Sinapic acid
SDSS	Sodium dodecyl sulphate sedimentation
SFA	Saturated fatty acid
SOD	Superoxide dismutase
SYA	Syringic acid
TAL	Tyrosine ammonia lyase
TE	Trolox equivalent
TGW	Thousand grain weight
TKW	Thousand kernel weight
TP	Total phenolic
TPA	Total phenolic acid
TPC	Total phenolic content
VA	Vanillic acid
VSMC	Vascular smooth muscle cells

1 Introduction

Cereals like wheat, barley, rye or maize are staple foods contributing basic nutrients such as carbohydrates, proteins, dietary fibers, vitamins and minerals. Furthermore, health beneficial components such as secondary plant metabolites complete the composition of cereals. Wheat (*Triticum aestivum* L. ssp. *aestivum*) is one of the most important agricultural commodities worldwide with 670 million tons in 2009 and it is constantly rising (USDA, 2010). The first domesticated forms of wheat appeared about 10,000 BC (Stone Age), wild species of einkorn or emmer were taken into cultivation (TANNO and WILLCOX, 2006, FELDMAN, 2001, NESBITT and SAMUEL, 1995). At first only hulled wheat species with low grain yields and non-threshable grain appeared while during evolutionary development and cultivation free-threshing forms had come into view.

Since ancient wheat species such as einkorn (*T. monococcum* L.), emmer (*T. dicoccum* L.) and spelt (*T. aestivum* L. ssp. *spelta*) are more and more interesting in a balanced diet as an alternative for the widely distributed and used bread wheat the ingredients and their composition are of importance in order to know if they are really health beneficial. Interest in a higher variety in nutrition grew in some countries. For example, the harvested area of spelt rose from 2003 to 2009 more than 30 % in Germany (STATISTISCHES BUNDESAMT 2010).

Due to an enhanced health consciousness during the last years, natural antioxidants gained substantial attention. In this regard phenolic compounds such as phenolic acids or flavonoids in wheat have been coming into the focus of research. Dietary intake of fruits, vegetables and whole wheat is associated with positive effects due to phenolic compounds which are antioxidative, anticarcinogenic, antimicrobial and lower the risk of cardiovascular diseases (CVD) (KNEKT et al. 2002, PETERSON et al., 2003, SESSO et al. 2003, ARTS and HOLLMAN, 2005, GRAF et al. 2005, DURACKOVA, 2010, GONZALEZ and RIBOLI, 2010, RODRÍGUEZ VAQUERO et al., 2010). Two different kinds of phenolic acids (PA) can be found in wheat namely hydroxycinnamic and hydroxybenzoic acids with ferulic acid (FA) as the abundant phenolic acid (ADOM and LIU, 2002, ADOM et al., 2003, ZHOU et al., 2004b).

1 Introduction

Most phenolic compounds are bound to cell walls and mainly can be found in the bran and germ fraction indicating that whole wheat flours provide greater overall physiological effects and higher health benefits than refined wheat flours (ZHOU et al., 2004a, ADOM et al., 2005, RONDINI et al., 2004, VITAGLIONE et al., 2008). Whole grain wheat products reduce cardiovascular diseases, coronary artery diseases and coronary heart diseases (JENSEN et al., 2004, ERKKILÄ et al., 2005, HE et al., 2010). Products of ancient wheat such as spelt flour and emmer bread are often produced as whole grain products. Referring to the findings about whole grain products ancient wheat products seem to contribute health beneficial effects as well. On the one hand because of their cereal fiber fraction and on the other hand because of contained phenolic acids.

Additionally, wheat sprouts provide potential phenolic compounds to promote human health (KAHLIL and MANSOUR, 1995, PRODANOV et al., 1997, YANG et al., 2001, ARORA et al. 2010, TIAN et al., 2010). With regard to health benefits it could be desirable to grind sprouted grain although production disadvantages might occur. Sprouting reduces the starchy endosperm through enzymatic degradation processes which is negative for processing. A starchy endosperm is needed in order to produce high-quality flours and other high-quality grain products.

In this regard it is important to determine phenolic acids and their composition in ancient whole wheat flours as well as in sprouted whole wheat flours. The results might supply information for the selection of specific species or the modulation of milling technologies to enhance cereal health benefits.

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2 Objectives

Wheat is one of the world's staple foods and has got a very high nutritive value. During domestication and evolutionary development many different species have arisen. While the so called bread wheat (*Triticum aestivum* L. ssp. *aestivum*) with its manifold cultivars and its properties such as free-threshing property, high content of carbohydrates, high protein and gluten content is of importance for flour processing and baking products, ancient wheat like einkorn (*T. monococcum* L.), emmer (*T. dicoccum* L.) and spelt (*T. aestivum* L. ssp. *spelt*) arouse interest in order to a varied diet. Furthermore, secondary plant metabolites like phenolic acids with their antioxidative and additional health beneficial aspects play an important role when talking about enhanced health awareness.

Information about phenolic acids in cereals including common wheat were available but methods and contents of phenolic acids were widely ranging. Only little is known about the concentration, composition and antioxidative capacity of phenolic acids in ancient wheat. Thus, the aim of this study was to quantitatively investigate the composition of present phenolic acids, and their variation in ancient wheat like einkorn, emmer and spelt. Since oxidative stress produces radicals leading to cell damage and other irreversible injuries in the human body the antioxidative efficiency of phenolic acids was of value.

Experiment 1 determined the concentration and composition of phenolic acids in einkorn, emmer and spelt derived from Gießen. Since phenolic acids were present in different types of bonds three fractions were analyzed: free phenolic acids, soluble conjugated (e.g. esterified to sugars) and bound phenolic acids (e.g. esterified to cell walls). Additionally, the antioxidative capacity measured by Folin-Ciocalteu assay and ORAC assay was determined to get a better knowledge about the nutritionally positive and health affecting potential in einkorn, emmer and spelt grown in Germany.

Until today little information were available about the effect of nitrogen fertilization and germination of the caryopses on the content of phenolic acids. This lead to a second experiment with the aim to investigate phenolic acids, their composition and their variation in three different wheat cultivars grown in Germany in dependency on

2 Objectives

sprouting time and nitrogen fertilization. Sprouts are considered to be health beneficial, even more than the cereal grain itself hence information about sprouting and fertilization are needed.

Spelt wheat is considered as ancient wheat because of its hulledness and the matter of fact that it is the progenitor of the today known bread wheat. It is a low grain yield species which grows under modest environmentally conditions. As a consequence of its properties it is usually used for organic production where no synthetic fertilizers or pesticides are allowed. Today many products of spelt wheat can be found in Germany. The aim of experiment 3 was to specify the concentration and composition of phenolic acids and the antioxidative capacity according to Folin-Ciocalteu and ORAC assay in spelt grown in Germany in dependence of species and site.

3 Literature review

3.1 Characterization of the genus *Triticum*

3.1.1 Domestication

Wheat (*Triticum aestivum* L. ssp. *aestivum*) as it is known and used today passed through a long time of domestication. About 10,000 BC (Stone Age) the first wild species were taken into cultivation and were changed morphologically during the centuries (TANNO and WILLCOX, 2006). Hulledness is one of the few morphological attributes which is still present for some cultivated wheat species nowadays. E.g. cultivated emmer, einkorn and spelt wheat are showing this attribute (FELDMAN, 2001, NESBITT and SAMUEL, 1995). The identification of wild progenitors of wheat and the geographical site of domestication confronted scientists with challenges and is partly known for only 100-150 years, now. Figures 3-1 and 3-2 display the evolutionary development of wheat and origin of ancient wheat with still some open questions.

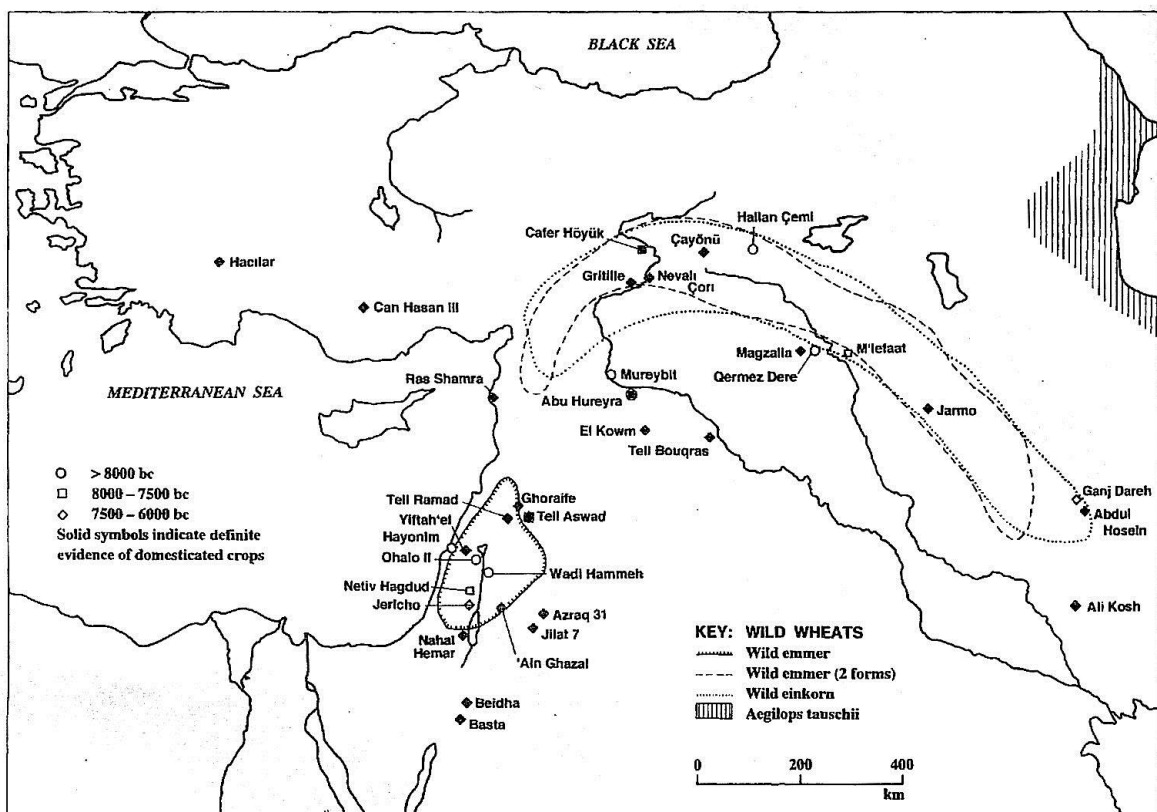


Fig. 3-1: Origin of ancient wheat (NESBIT, 2001)

Cultivated wheat originated from different diploid and tetraploid wild and cultivated progenitors. Wild einkorn (*T. monococcum* L. ssp. *aegilopoides* = *T. boeiticum* Boiss.)

3 Literature review

was discovered in Turkey, Greece, Bulgaria, southern Serbia, southwestern Syria, southeastern Lebanon, northern Iraq and western Iran as the progenitor of the cultivated diploid wheat *T. monococcum* L. ssp. *monococcum* (FELDMAN, 2001). A second wild einkorn was discovered in Armenia, northern Lebanon, southeastern Turkey and southwestern Iran called *T. urartu* Tum. ex. Gandil. At first, this wild einkorn was not recognized as a progenitor of cultivated einkorn other than *T. monococcum* L. ssp. *aegilopoides* (JAASKA, 1997). Later, studies showed that *T. urartu* Tum. ex. Gandil. could have been the pollen donor of wild emmer (KILIAN et al., 2007) (Fig. 3-2).

Wild emmer (*T. turgidum* ssp. *dicoccoides* (Korn. ex Asch. Graebn.) Thell.) was discovered as the progenitor of cultivated tetraploid wheat such as cultivated emmer (*T. turgidum* ssp. *dicoccon*) or durum wheat (*T. turgidum* ssp. *durum*). It was found in southeastern Lebanon, Israel, Jordan and Syria. There are evidences that wild emmer is about 360,000 years old (DVORAK and AKHUNOV, 2005). *T. timopheevii* (Zhuk.) is another two-grained cultivated wheat of this region (AARONSON, 1910, FELDMAN, 2001, NESBITT and SAMUEL, 1995). Also for emmer there is a second wild ancestor *T. timopheevii* ssp. *armeniicum* (Jakubz.) (JAASKA, 1997).

Geographically the origin of ancient wheat species with low grain yields and non-threshable grain is the Fertile Crescent (southwest Asia). There have been assumptions, that this was also the area of domestication (Fig. 3-1) (FELDMAN, 2001, NESBITT and SAMUEL, 1995). Wild emmer still can be found in this area where two races have been recognized, the western Palestine and the central-eastern Turkish-Iraqi race (LUO et al., 2007, ÖZKAN et al., 2005). Recent studies found out that the present domesticated emmer must be domesticated from wild emmer lines of southeast Turkey (ÖZKAN et al., 2010). Those wild ancient wheat were domesticated in these regions first and spread into Europe, Near East and Africa. A domestication model for emmer couldn't be found yet (NESBIT and SAMUEL, 1995, HEUN et al., 1997, ÖZKAN et al., 2010).

In eastern Turkey and western Iran *Aegilops tauschii* Coss. (= *Ae. squarrosa* L.) grew as a wild weed containing the D-genome with the character of free-threshing, tough rachis and high protein (AYKUT TONK et al., 2010). DVORAK and AKHUNOV (2005) found out that the divergence time of *T. urartu* Tum. ex. Gandil. and *Ae. tauschii*

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Coss. is 2.7 million years. *Ae. tauschii* Coss. and *T. turgidum* ssp. *dicoccon* built the hexaploid hulled wheat *T. aestivum* ssp. *spelta* by hybridization which was proven by MCFADDEN and SEARS (1946). There is still some uncertainty concerning the area and date of domestication (NESBIT and SAMUEL, 1995). This first bread wheat was still hulled (glumes are tightly to the grain) and had to undergo some mutations to form a free-threshing bread wheat (SIMONS et al., 2006, DUBCOVSKY and DVORAK, 2007, SANG, 2009). Bread wheat as a hexaploid cereal did not develop as a natural population but existed in cultivation and was chosen by farmers because of its better properties (SHEWRY, 2009).

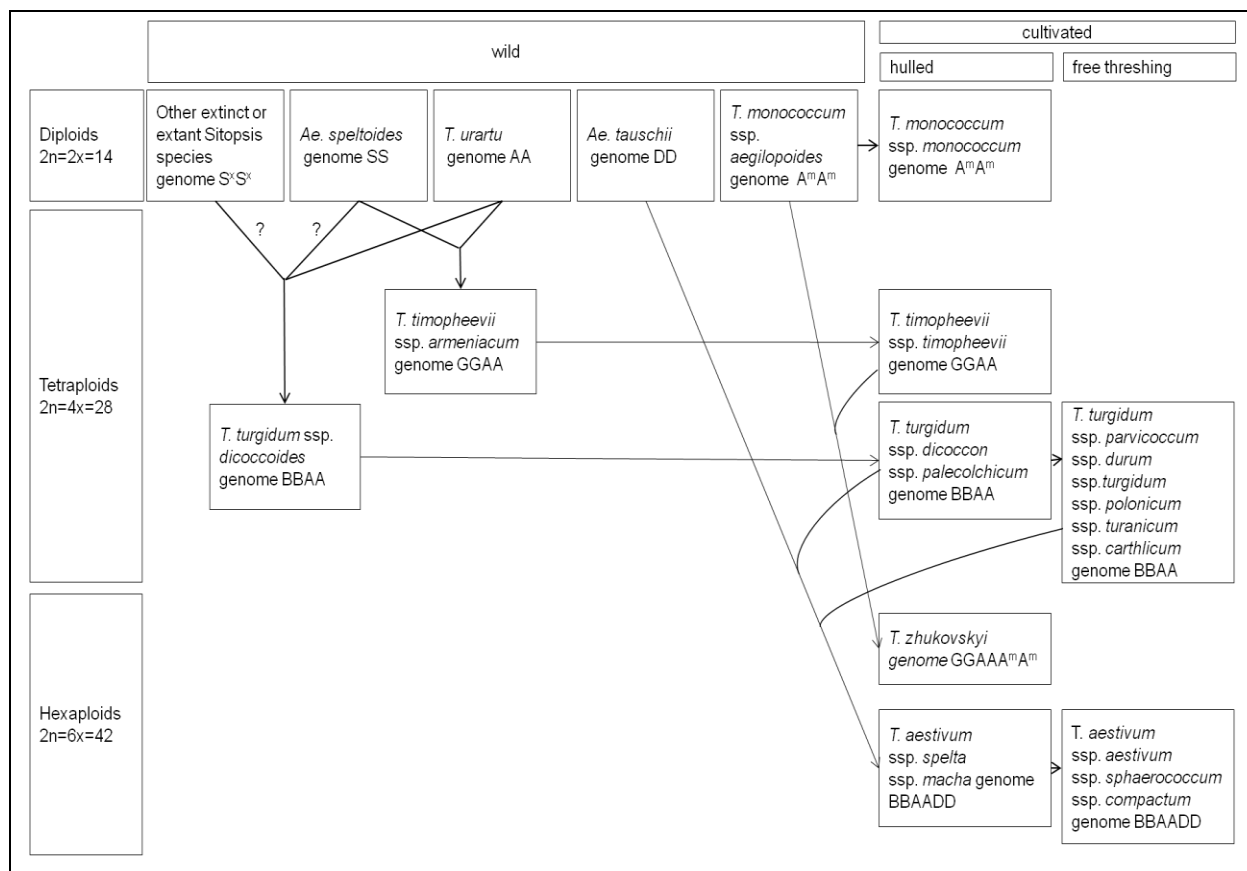


Fig. 3-2: Evolutionary relationship of wheat (modified by FELDMAN, 2001)

3.1.2 Taxonomy of wheat

Wheat consists in the head of a spike with spikelets situated on a rachis which is the extension of the stem. The spikelet is covered with glumes and around the kernel there is the lemma and palea, which are the hulls of the kernel. Einkorn, emmer and spelt are hulled and thresh with the whole spikelet whereas *T. aestivum* ssp. *aestivum* is free threshing and the kernel is situated loose in the glumes

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(STALLKNECHT et al., 1996). Wheat (*Triticum* sp.) belongs to the class of *Monokotyledonae* and to the family of *Poaceae*. Einkorn, emmer, spelt and bread wheat are different species based on sets of seven chromosome pairs ($x=7$) (FELDMAN and MILLET, 2001).

T. monococcum L. ssp. *aegilopoides* is a single grain cereal (one grain per flower) and *T. urartu* Tum. ex. Gandil. is a two grain cereal (two grains per flower). Both wild einkorn have a brittle rachis and the spikelet brakes as soon as it is fully ripened (FELDMAN and MILLET, 2001). The cultivated form of einkorn is less breakable and the spikelet stays intact. Harsh environments and poor soils haven't been a problem for wild einkorn, but the kernels are very small. Einkorn is a diploid wheat with the genome type AA ($2n=2x=14$ chromosomes) and was probably cultivated in the Karacadag mountains (south-east Turkey) (STALLKNECHT et al., 1996, HEUN et al., 1997).

Wild emmer, namely *T. turgidum* ssp. *dicoccoides* (Korn. ex Asch. Graebn.) Thell. is the first wild tetraploid wheat with the genome AABB ($2n=4x=28$ Chromosomes). It has got a brittle rachis whereas cultivated emmer shows a less fragile rachis. *T. turgidum* ssp. *dicoccon*, *T. timopheevii* ssp. *timopheevii* (Zhuk.) and *T. turgidum* ssp. *durum* (durum wheat) are cultivated species of emmer. Durum wheat is already free-threshing while the others are hulled wheat. Because of the BB genome emmer could grow in a wider range of environments, from high temperature and dry areas (200 m below sea level, Jordan Valley) to cold and humid areas (1600 m, Mt. Hermon) also different types of soils were no difficulty (STALLKNECHT et al., 1996, FELDMAN and MILLET, 2001).

The origin of spelt (*T. aestivum* ssp. *spelta*), as the third ancient wheat species, is difficult to define. It is assumend that there were two different regions of origin, the Iranian region and the southeastern European region. For sure is, that the diploid goatgrass *Aegilops tauschii* Coss. (= *A. squarrosa* L.) with the DD genome had to be present to build the hexaploid hulled wheat *T. aestivum* ssp. *spelta* (AABBDD) by hybridization with domesticated tetraploid wheat (hulled or free-threshing) (MCFADDEN AND SEARS, 1946, FELDMAN and MILLET, 2001). There is no wild hexaploid wheat but hulled and free-threshing forms (FELDMAN and MILLET, 2001, STALLKNECHT

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et al., 1996). The rachis of spelt is brittle (STALLKNECHT et al., 1996). In order to get free-threshing tetraploid and hexaploid wheat, genetic changes had to take place.

The so called Q gene on chromosome 5A donated by *T. urartu* is responsible for the free-threshing character and has pleiotropic effects on the rachis fragility, glume shape and tenacity, spike length and plant height (DVORAK et al., 1993, HEUN et al., 1997). The q allele is responsible for a spear-shape spike with a stretched out rachis, the so called speltoid spike. This spike is characterized by a tenacious glume, fragile rachis and nonfree-threshing seed (SIMONS et al., 2006). The dominant Q allele provides the characteristics: shorter wheat plants, compacter spikes, non-brittle rachis and free-threshing seeds (JANTASURIYARAT et al., 2004, SIMONS et al., 2006). For hexaploid wheat with the D genome another partly dominant gene is present leading to the nonfree-threshing character although the Q allele is dominant. This gene is called Tg (tenacious glumes) and is situated on the chromosome 2D donated by *Ae. tauschii*. In order to get free-threshing wheat tg has to be recessive and Q dominant (JANTASURIYARAT et al., 2004).

3.2 Secondary plant metabolites

Plant metabolites embrace more than 100,000 different substances and they are commonly divided into primary and secondary metabolites (RAVEN et al., 2006). Primary metabolites (macronutrients) such as carbohydrates, proteins and fats are essential nutrients for the human diet to provide energy for cellular functions. Although plant secondary metabolites or phytochemicals (Greek phyto=plant) build a wide range of chemically different substances and are ubiquitously present in plants they are not essential for humans (WATZL and LEITZMANN, 2005, YOUNG et al., 2005). In contrast to primary metabolites they are present in very small quantities with no specific functions in plants (PICHERSKY and GANG, 2000).

In former times phytochemicals were considered as antinutrients and functionless in plants (GROßKLAUS, 2000, RAVEN et al., 2006, TAIZ and ZEIGER, 2007). However, during the last years scientific knowledge about phytochemicals grew, their function and potential with regard to health beneficial effects became more interesting and important. Phytochemicals provide potential health effects such as the reducing

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capacity of cancer or coronary heart diseases (ARTS, et al., 2001, KNEKT, et al., 2002, SESSO et al., 2003, LEMARCHAND, 2000, ARTS, et al., 2001).

In plants they are biochemically active substances with the ability of defending plants against pathogens and herbivores, they are growth regulators and pigments to attract insects. Furthermore, they protect against different abiotic factors like heat, UV-light, nutrient and water deficiencies (RAVEN et al., 2006, TREUTTER, 2005). The appearance of phytochemicals is not allocated consistently, depending on the type of phytochemical. Mostly, they can be found in the vacuoles of flowers, fruits, seeds or the embryo of plants (RAVEN et al., 2006). Until today there is neither a standardized definition of the term secondary plant metabolites nor standardized criteria for phytochemicals (GROßKLAUS, 2000). Generally, primary metabolites are essential cell components and they are present in every plant cell while phytochemicals are produced only in certain tissues and development stages (MOHR and SCHOPFER, 1992). There are many different phytochemicals and an overview of the different groups of phytochemicals is displayed in figure 3-3. The most studied phytochemicals are carotenoids and phenolics. With regard to health beneficial effects the group of phenolic compounds has an enormous potential and are classified into five main groups: phenolic acids, flavonoids, stilbenes, coumarins and tannins (LIU, 2004).

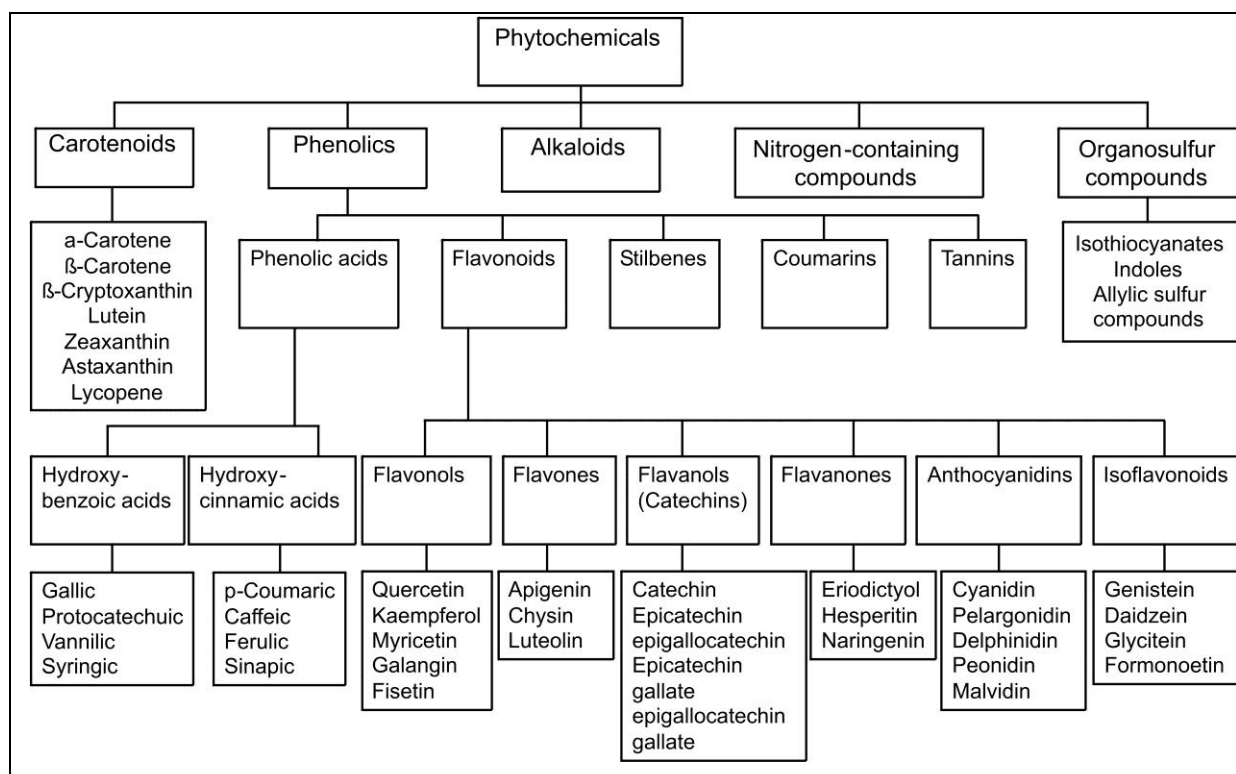


Fig. 3-3: Classification of dietary phytochemicals (modified by LIU, 2004)

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Phenolic compounds (phenylpropanoids) are build of an aromatic ring with one or more hydroxyl substituents and they are derived from phenylalanine, sometimes from tyrosine (HELDT, 2005) (Fig. 3-4). Compounds with two and more hydroxylic groups are called polyphenols. They are biosynthesized in different ways and represent a large variety of secondary compounds. The shikimic acid pathway (biosynthesis in plants) and the malonic acid pathway (biosynthesis in fungi and bacteria) are the two basic metabolic processes for aromatic amino acids phenylalanine and tyrosine which are precursors of phenolic compounds (TAIZ and ZEIGER, 2007, SMITH et al., 2010).

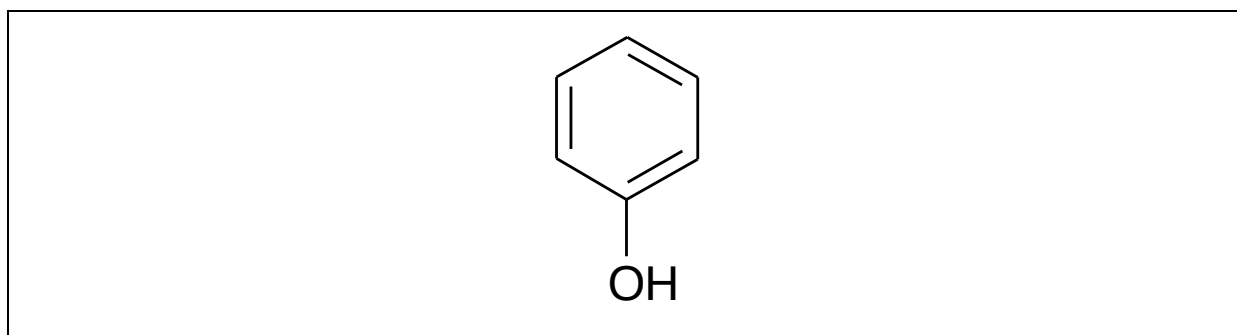


Fig. 3-4: Basic structure of phenolic compounds: phenol

Phenylalanine is an end-product of the shikimic acid pathway and the location of the biosynthesis recently has been proven to be in plastids (YAMADA et al., 2008, RIPPERT et al., 2009). There are about 10,000 different phenolic compounds, mostly bound to sugar as glycosides, which makes them usually water-soluble. Furthermore, there are water-soluble carboxylic acids, organic-soluble phenolic compounds and insoluble polymers (HARBORNE, 1984, TAIZ and ZEIGER, 2007).

In plants phenolic compounds are structural polymers, which can act as antioxidants, attractants, defence responses and UV screens. In humans phenolic compounds function as antioxidants, radical scavengers, inhibitors of radical generation, anticarcinogenic and enzyme inhibitors (PARR and BOLWELL, 2000).

3.2.1 Phenolic acids

Phenolic acids (PA) belong to the group of phytochemicals and own a carboxylic acid functional group. Many functions of phenolic acids are still unknown and have to be analyzed. They are synthesized in the phenylpropanoid pathway in plants where they

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are present everywhere (ROBBINS, 2003). They are found as trans-Isomers rather than as cis-isomers whereas an isomerization takes place under UV-light (PARR and BOLWELL, 2000).

PA existing in two different groups: hydroxycinnamic acids (simple phenylpropanoids, C6-C3) and hydroxybenzoic acids (C6-C1) both hydroxylated derivatives of the corresponding acid (MATTILA and KUMPULAINEN, 2002, WATZL and RECHKEMMER, 2001). Figures 3-5 and 3-6 show the skeletal structure of hydroxybenzoic (C6-C1 compounds) and hydroxycinnamic acids (C6-C3 compounds). Number and position of the hydroxylic group at the aromatic ring lead to various different phenolic acids.

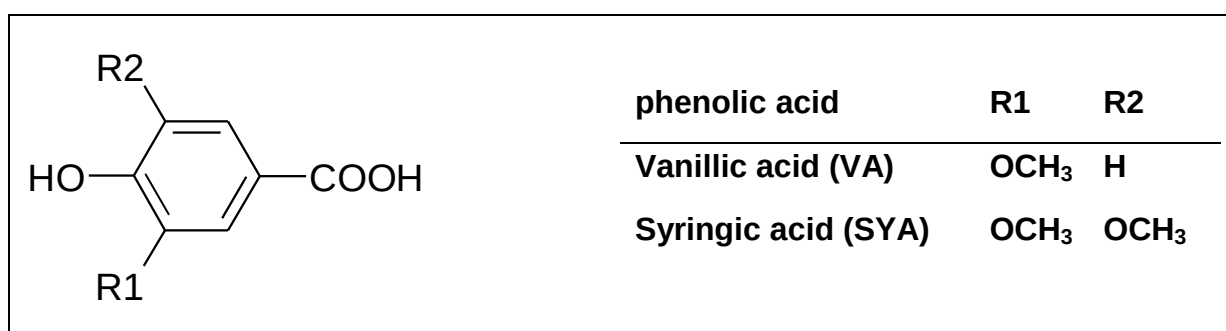


Fig. 3-5: Hydroxybenzoic acids (C6-C1)

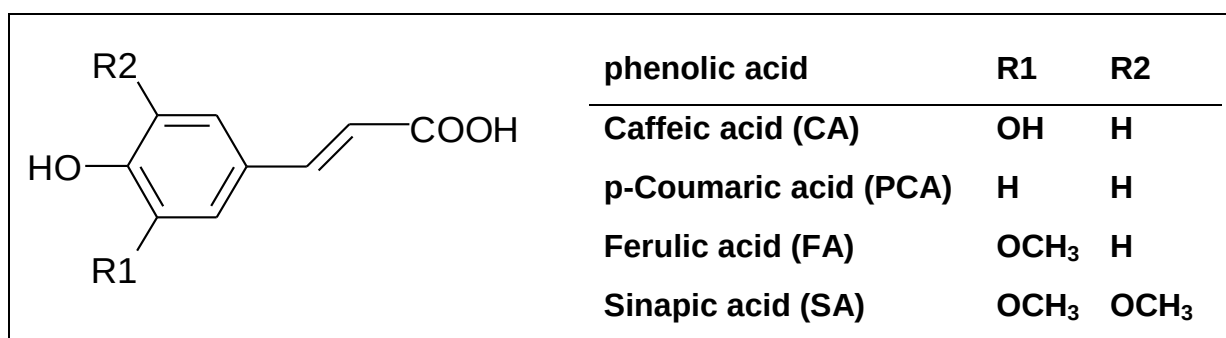


Fig. 3-6: Hydroxycinnamic acids (C6-C3)

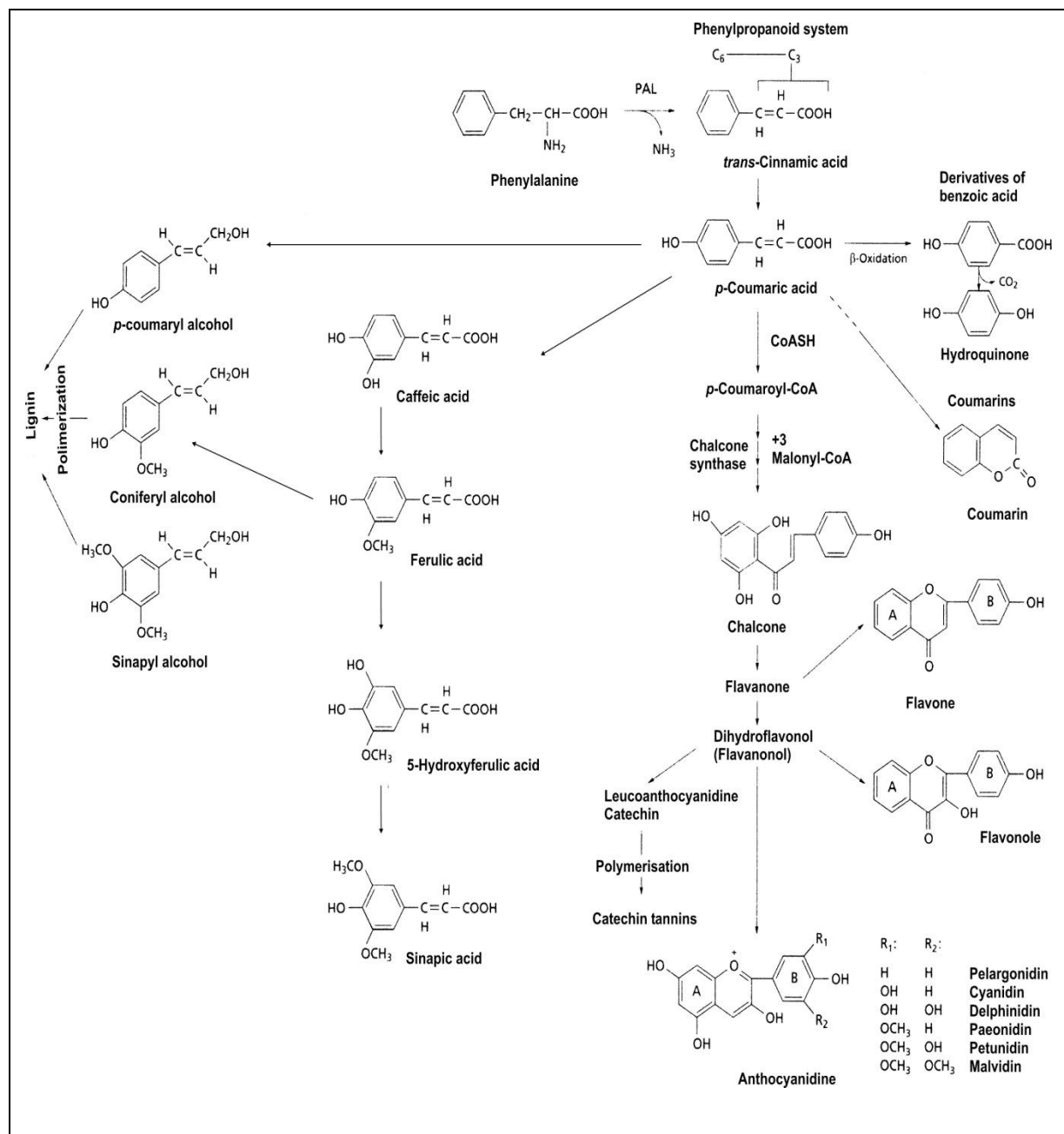


Fig. 3-7: Phenylpropanoid pathway (KULL, 2000)

To explain the phenylpropanoid pathway briefly (Fig. 3-7), one ammonia molecule is eliminated from phenylalanine or tyrosine by a non-oxidative deamination catalyzed through phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL), building *trans*-cinnamic acid (VOGT, 2010). PAL is influenced by low nutrient levels, light and fungal infection. The more PAL is present because of environmental influences the higher the synthesis of phenolic compounds. *Trans*-cinnamic acid is hydroxylated by cinnamic acid hydroxylase to *p*-coumaric acid and from that point on other phenolic compounds such as simple phenolic acids, lignins, flavonoids and coumarins are metabolized by several condensation and modification reactions (LEE

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et al., 1995, KULL, 2000, HELDT, 2005, VOGT, 2010). Cinnamic acid is converted to benzoic acids by different pathways which may co-exist (LEE et al., 1995, VOGT, 2010).

PAs exist rather in bound than in free form. Hydrolysis of plant tissue releases PAs being ester, ether or acetal bound to structural components of plants (cellulose, protein, lignin), to bigger phenolics (flavonoids) or smaller organic molecules (glucose), resulting in an enormous number of derivatives (HARBORNE, 1984, KLINK and HERRMANN, 1988, CLIFFORD, 2000). Freezing or fermentation can release those bound phenolic acids so that processed food might contain free PA (MANACH et al., 2004, EL GHARRAS, 2009).

Hydroxycinnamic acids are the most widespread group of phenolic acids with four major phenolic acids in plants: ferulic acid (FA), sinapic acid (SA), caffeic acid (CA) and p-coumaric acid (PCA). CA is the most abundant PA in fruits with more than 75 % of total phenols and is found in all parts of the fruit (MANACH et al., 2004). Highest amounts of FA are present in cereals with up to 90 % and more of total phenols (RONDINI et al., 2004, MANACH et al., 2004). Those phenolic acids can be detected on paper chromatograms because of fluorescence (blue and green) under UV light (HARBORNE, 1984). Table 3-1 reflects some widespread hydroxycinnamic acids.

Common hydroxybenzoic acids are vanillic (VA) and syringic acid (SYA) which are present e.g. in wheat (Tab. 3-1). Benzoic acid is naturally present in cranberries or lingonberries (VISTI et al., 2003). Generally, hydroxybenzoic acids are found as conjugates. In food and beverages the content of hydroxybenzoic acids are low except for some *Rosaceae* such as blackberry or strawberry and herbs or spices (TOMÁS-BARBERÁN and CLIFFORD, 2000).

Phenolic acids like FA (hydroxycinnamic acid), VA or SYA (hydroxybenzoic acid) are not only bound to structural components, in fact they are an integral part e.g. in lignin. Lignin is an aromatic polymer derived from phenylalanine as a product of the shikimic acid pathway. FA is biosynthesized in the phenylpropanoid pathway leading to the polymer lignin which is abundant in almost every plant cell. It provides strength and rigidity to cell walls and makes them water impermeable (WHETTEN and SEDEROFF,

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1995). Lignin is one important defence compound for plants against abiotic environmental factors and pathogens (BHUIYAN et al., 2009).

Tab. 3-1: IUPAC nomenclature of hydroxycinnamic acids and hydroxybenzoic acids present in wheat (*Triticum* sp.)

hydroxycinnamic acid	IUPAC nomenclature
caffeic acid	(E)-3-(3,4-dihydroxyphenyl)-2-propenoic acid
<i>p</i> -coumaric acid	3-(4-hydroxyphenyl)-2-propenoic acid
ferulic acid	(E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid
sinapic acid	3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid
hydroxybenzoic acid	
vanillic acid	4-Hydroxy-3-methoxybenzoic acid
syringic acid	4-hydroxy-3,5-dimethoxybenzoic acid

3.2.2 Oxidative stress

On the one hand it is a matter of common knowledge that oxygen (dioxygen, O₂) is a gas and essential to human life. Although it is so important for breathing and human survival, oxygen is on the other hand a poison for cells leading to different defense mechanisms. It is a so called strong oxidant and one defending substances against those are antioxidants. A general and widely accepted definition of antioxidant was done by HALLIWELL and GUTTERIDGE (2010) who defined this compound as “any substance that delays, prevents or removes oxidative damage to a target molecule”. This definition includes enzymatic (glutathione peroxidases (GSH), superoxide dismutase (SOD) and catalase) and non-enzymatic compounds (phenolic acids, flavonoids).

Normally, reactive species (RS) are in balance with antioxidant defense systems, regulating the RS level. Oxidative stress means an imbalance in this system because of reduced levels of antioxidants or increased presence of RS, implying oxidative damage to biomolecules (HALLIWELL and WHITEMAN, 2004, HALLIWELL, 2007). There are many different responses on RS (increased proliferation, apoptosis, cell death) to stop oxidation by three different kinds of RS: reactive oxygen (ROS), reactive nitrogen (RNS) or reactive chlorine species (RCS). Important radicals and nonradicals are explained in Table 3-2 (HALLIWELL and WHITEMAN, 2004). The most

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reactive species is the hydroxyl radical ($\text{OH}^\bullet$) because of its ability to abstract hydrogen atoms (PRYOR et al., 2006). Hydrogen peroxide (H_2O_2) which can result of superoxide ($\text{O}_2^{\bullet-}$) is also a very reactive species. Both substances are needed as cell regulators but can be toxic as well (SUZUKI et al., 1997, SUH et al., 1999, HADDAD, 2004, PRYOR et al., 2006).

Tab. 3-2: Nomenclature of reactive species (HALLIWELL and WHITEMAN, 2004)

free radicals		nonradicals	
reactive oxygen species (ROS)			
superoxide	$\text{O}_2^{\bullet-}$	hydrogen peroxide	H_2O_2
hydroxyl	$\text{OH}^\bullet$	hypobromous acid	HOBr
hydroperoxyl	$\text{HO}_2^\bullet$	hypochlorous acid	HOCl
		ozone	O_3
peroxyl	$\text{RO}_2^\bullet$	singlet oxygen	$\text{O}_2^1\Delta\text{g}$
alkoxyl	$\text{RO}^\bullet$	organic peroxides	ROOH
carbonate	$\text{CO}_3^{\bullet-}$	peroxynitrite	ONOO^-
carbon dioxide	$\text{CO}_2^{\bullet-}$	peroxynitrous acid	ONOOH
reactive chlorine species (RCS)			
atomic chlorine	$\text{Cl}^\bullet$	hypochlorous acid	HOCl
		nitryl (nitronium) chloride	NO_2Cl
		chloramines	
		chlorine gas	Cl_2
reactive nitrogen species (RNS)			
nitric oxide	$\text{NO}^\bullet$	nitrous acid	HNO_2
nitrogen dioxide	$\text{NO}_2^\bullet$	nitosyl cation	NO^+
		nitroxyl anion	NO^-
		dinitrogen tetroxide	N_2O_4
		dinitrogen trioxide	N_2O_3
		peroxynitrite	ONOO^-
		peroxynitrous acid	ONOOH
		nitronium (nitryl) cation	NO_2^+
		alkyl peroxynitrites	ROONO
		nitryl (nitronium) chloride	NO_2Cl

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Free radicals such as superoxide ($O_2^{\bullet-}$) or nitric oxide ($NO^{\bullet}$) are activators of subsequent reactions where further reactive metabolites are formed. Figures 3-8 and 3-9 show different pathways of ROS, one is the dismutation reaction of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) by the superoxide oxidoreductase dismutase (SOD). In a further pathway H_2O_2 is converted to water (H_2O) and oxygen (O_2) while another pathway produces hydroxyl radicals ($OH^{\bullet}$) which is iron catalyzed. Both radicals ($O_2^{\bullet-}$ and $OH^{\bullet}$) are capable of damaging cells or initiate a cascade of signal transduction as it is displayed in figure 3-10 (PFEILSCHIFTER et al., 2003, HADDAD, 2004). Hypochlorous acid ($HOCl$) is metabolized out of H_2O_2 and becoming the highly reactive singlet oxygen (O_2^1). Radical-radical coupling between $O_2^{\bullet-}$ and $NO^{\bullet}$ results in peroxynitrite ($ONOO^-$) which is converted into nitric acid ($ONOOH$) where nitric oxide ($NO^{\bullet}$) can be split up resulting in another hydroxyl radical ($OH^{\bullet}$).

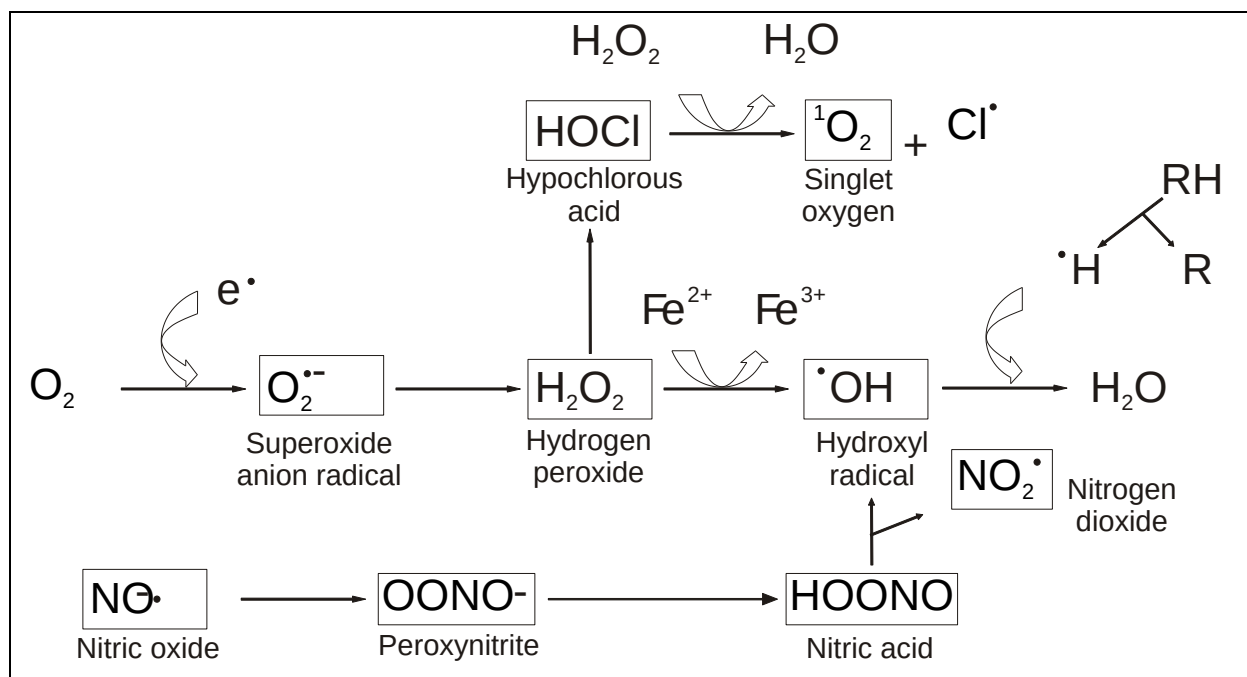


Fig. 3-8: Mutual association between free radicals and their reactive metabolites (DURACKOVA, 2010)

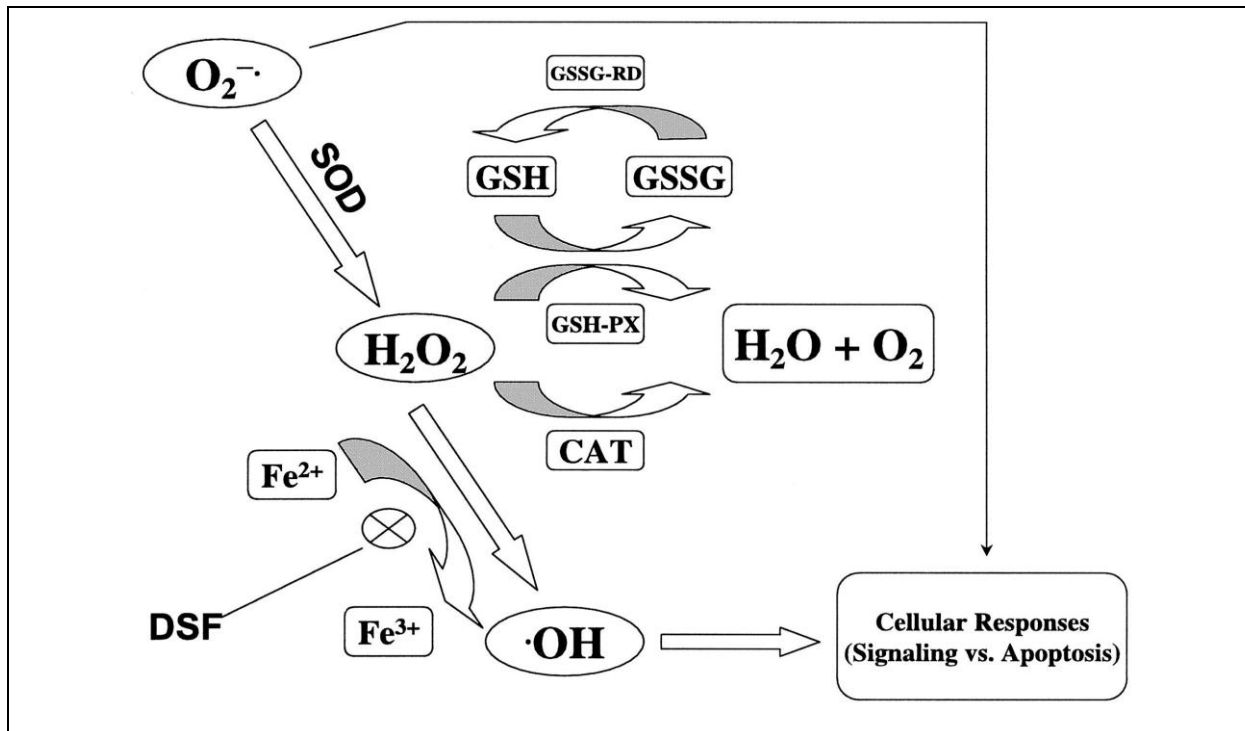


Fig. 3-9: Schematic representation of the pathways leading to the generation of reactive oxygen species (ROS) and their selective dismutation (HADDAD, 2004)

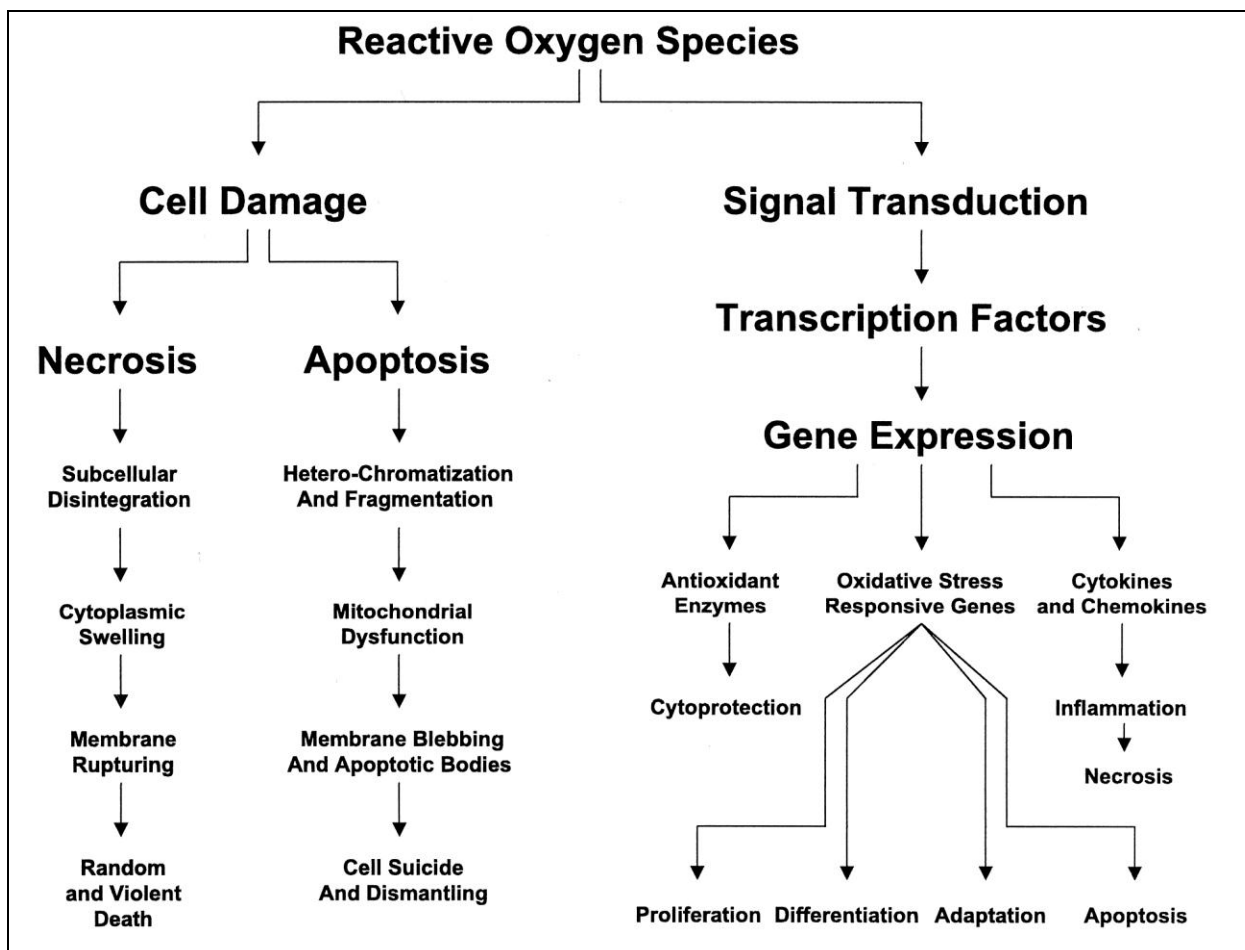


Fig. 3-10: A general schematic showing the regulation of cell death and signal transduction pathways in response to reactive oxygen species (HADDAD, 2004)

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Since oxidative stress is an imbalance between antioxidants and RS it is as well an toxic mechanism (HALLIWELL and WHITEMAN, 2004, PRYOR et al., 2006, HALLIWELL, 2007). Oxidative stress causes damage to proteins, lipids, carbohydrates or nucleic acids when those substances are exposed too much to oxidizing gases or when there is not enough protection against oxidants (PRYOR et al., 2006). Recent studies found out that oxidative stress is the "fuel" for cancer cells and their metabolism (MARTINEZ-OUTSCHOORN et al., 2010). Different conditions lead to the production and accumulation of oxidants which then is called oxidative stress. For example, the oxidative mechanisms described in figures 3-8 and 3-9 or environmental circumstances like air pollution or oxidants in smoke (BOFFETTA and NYBERG, 2003, DURACKOVA, 2010). UV radiation leads as well to oxidants where lipids, DNA and proteins are damaged as it is shown in figure 3-11 (BITO et al., 2010, DURACKOVA, 2010, HALLIWELL and WHITEMAN, 2004).

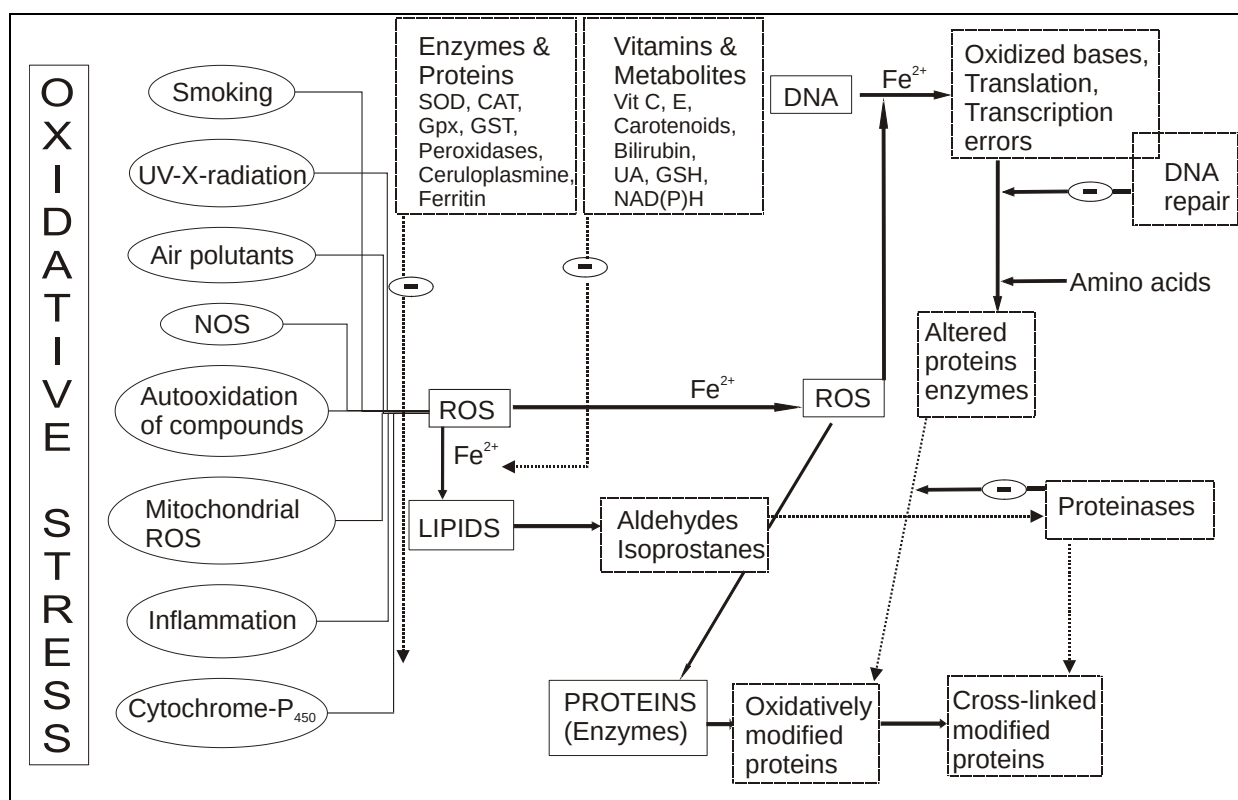


Fig. 3-11: Mutual association between oxidants and antioxidants. NOS- NO-synthase, ROS- reactive oxygen species, CAT- catalase, SOD- superoxide dismutase, GPx- Glutathione peroxidase, GST- glutathione S-transferase, UA- uric acid, GSH- glutathione reduced (modified by DURACKOVA, 2010)

The results of oxidative stress are diseases like atherosclerosis, cardiovascular diseases, skin and tumor diseases. Dietary phenols possess a potent capacity to

fight these ROS with various antioxidant strategies which is shown schematically in figure 3-12 (HAN et al., 2007).

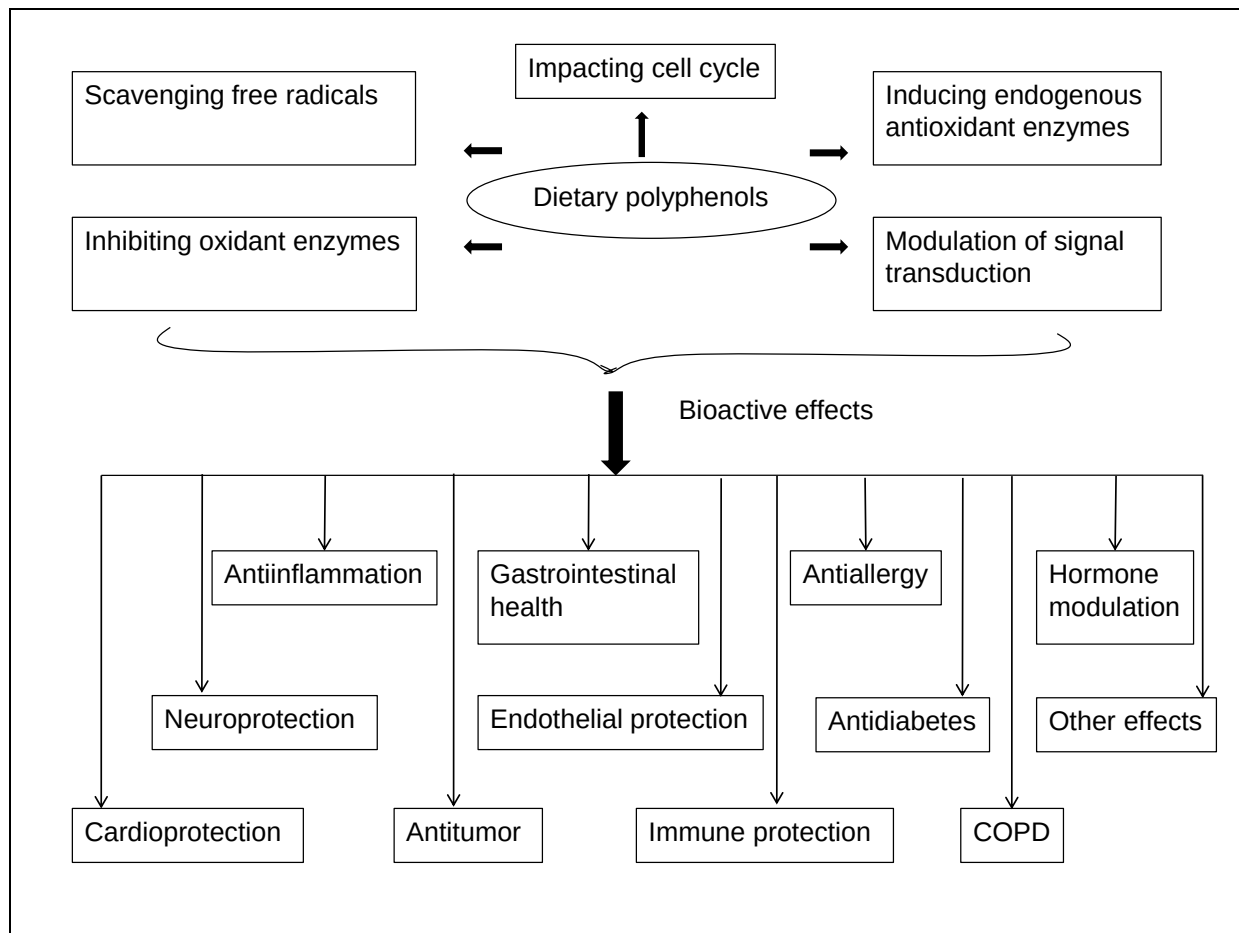


Fig. 3-12: Bioactivities of dietary polyphenols (modified by HAN et al., 2007)

3.2.3 Health effecting potentials and nutrition of phenolic compounds

Most phenolic compounds can be found in fruits such as apples or strawberries, cereals such as wheat and beverages such as red wine or coffee (EL GHARRAS, 2009). The extent of intake of phenolic compounds is highly variable depending on the food source and dietary habits. Intake of polyphenolic compounds can reach up to 1 g/d with phenolic acids ranging between 9 and 989 mg/d in Germany (RADTKE, 1998, CLIFFORD, 2000). The individual food choice like the consumption of coffee (hydroxycinnamic acids) or citrus fruits (flavanones) plays an important role in the determination of the extent of phenolic intake. Coffee, fruit and fruit juices might be the main source of phenolic acids (MANACH et al., 2004, CLIFFORD, 2000). Intake and bioavailability define the health beneficial effect of phenolic compounds. Because of

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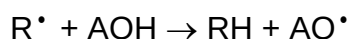
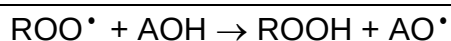
their ability of scavenging free radicals phenolic compounds are interesting for possible therapies preventing diseases associated with oxidative stress (EL GHARRAS, 2009).

Several studies document diverse positive health beneficial effects of phenolic compounds on human health after a dietary intake of fruits, vegetables and whole grain. They are associated with effects such as:

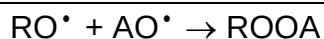
- *protection against free radicals* (antioxidativ, radical scavenging)

Free radicals produced by oxidative stress have to be scavenged in order to make them effectless and to hinder damages described above. When the primary protective system fails, antioxidants like PAs are of importance to turn the already metabolized radicals into nonradicals and not toxic substances by trapping and scavenging (DURACKOVA, 2010).

The antioxidative ability of PAs is to inhibit lipid oxidation by trapping peroxy radicals. One way is that the antioxidant like a phenolic compound containing an active hydrogen atom (AOH) spends the hydrogen proton to the radical ($R^\bullet$ or $ROO^\bullet$) and the generation of new radicals by chain reaction is stopped in a termination reaction. The antioxidant itself becomes a radical ($AO^\bullet$) (Eq. 3-1) which is chemically a lot stabler than the initial radical. The resulting free radical doesn't participate in the chain propagation and diffuses away. It can be reduced by glutathione or ascorbate to its starting substance (PARR and BOLWELL, 2000, KOLTOVER, 2010). Another way is to "spend" an electron to the radical ($RO^\bullet$) when the antioxidant itself is an radical ($AO^\bullet$) by radical-radical coupling to form a unreactive addition product (ROOA) (Eq. 3-2) (CUPETT et al., 1997, PARR and BOLWELL, 2000).



Eq. 3-1: Spending an hydrogen atom (KOLTOVER, 2010)



Eq. 3-2: Radical-radical coupling (modified by CHIMI et al., 1991)

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In order to measure the antioxidative ability of antioxidants such as PAs the term antioxidative capacity is used for different kinds of assays. There are diverse in vitro antioxidative capacity assays to describe the ability and dimension of antioxidants to scavenge radicals (Tab. 3-3).

Tab. 3-3: In vitro Antioxidant capacity Assays (modified by Huang et al., 2005)

Assay type	Assay
<i>Assays involving hydrogen atom transfer reactions (HAT)</i> (radical scavenging activity assays)	ORAC (oxygen radical absorbance capacity)
	TRAP (total radical trapping antioxidant parameter)
	Crocin bleaching assay
	IOU (inhibited oxygen uptake)
	Inhibition of linoleic acid oxidation
	Inhibition of LDL oxidation
<i>Assays by electron-transfer reaction (ET)</i> (redox assay)	TEAC (Trolox equivalent antioxidant capacity)
	FRAP (ferric ion reducing antioxidant parameter)
	DPPH (diphenyl-1-picrylhydrazyl)
	Copper(II) reduction capacity
	Total phenols assay by Folin-Ciocalteu reagent

Hydroxycinnamic acids such as ferulic acid, caffeic acid and sinapic acid provide such antioxidative effects. For example, sinapic acid is a potent antioxidant and provides higher antioxidative effects than ferulic acid. Even higher but sometimes comparable to sinapic acid is the antioxidative capacity of caffeic acid. Sinapic acid as well as other PAs is a effective cell protectant and it is important in oxidative related disease for instance because of its peroxynitrite (ONOO^-) scavenging ability (ZOU et al., 2002, KIKUZAKI et al., 2002, THIYAM et al., 2006, NENADIS et al., 2007).

In a study about the antioxidative activity of sinapic acid and its alkyl esters by GASPARI et al. (2010) free sinapic acid and synthesized derivatives were analyzed to get more knowledge about the free radical scavenging potential. The measurement

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of the electrochemical behavior of sinapic acid and its synthesized esters was conducted by differential pulse and cyclic voltammetry. It was assumed by the results that the electrochemical oxidation of free sinapic acid and its derivatives happens through electron transfer. Because of these results the oxidation mechanism for sinapic acid is thought to involve an electron transfer from the phenolic acid ion and is followed by an irreversible radical-radical coupling of two phenyl radicals. Thermoxidative stability was tested by differential scanning calorimetry (DSC). The oxidation induction temperature (OIT) was measured of linoleic acid (LA) as a lipid model system to find out about the efficiency of antioxidants such as sinapic acid. The transfer of oxygen to an unsaturated fatty acid requires energy that can be measured by thermal analysis to determine the oxidative stability. The OIT of LA without sinapic acid (SA) was at 108.1 °C while after adding the OIT increased to 150.3 °C revealing the evidence that SA is an antioxidant and significantly slows down LA oxidation (GASPAR et al., 2010).

The same results according to the electron transfer from the phenolic acid ion were found for ferulic acid. Antioxidative capacity tests with DPPH and ABTS confirmed the scavenging potential of pure hydroxycinnamic acids and their derivatives. The results in the study by GASPAR et al. (2009) showed that caffeates (CA) and ferulates (FA) scavenged about 80 and 60 % of the DPPH radical. Both, caffeic acid and ferulic acid have radical scavenging capacities while CA was stronger than FA. Furthermore, the tested phenolic acids showed activities against ABTS even more than trolox which is a vitamin E analogue (GASPAR et al., 2009). FA and CA have been found to be active radical scavengers by several other studies (SILVA et al., 2000, KARMAK et al., 2005, ROLEIRA et al., 2010). In an additional study the electrochemical oxidation of ferulic acid lead to phenoxy radicals of four mesomeric forms by electron transfer which can even be quantitatively oxidized to malic acid, oxalic acid and formic acid (KALLEL TRABELSI et al., 2005).

In a research by NENADIS et al. (2007) different assays such as FRAP, Folin-Ciocalteu or ORAC, DPPH, and ABTS were chosen to assess the antioxidative capacity of different antioxidative relevant compounds. FRAP assay illustrated that caffeic acid (CA) and sinapic acid (SA) were able to reduce Fe^{3+} at acidic pH (3.6) although SA is less active than CA. The reason can be explained by the fact that SA exhibits only one hydroxyl group. FRAP expresses the electron-donating ability of

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compounds as well as Folin-Ciocalteu. In the Folin-Ciocalteu assay the availability of hydroxyl groups is of importance and again CA is more active than SA even though it has methoxy substituents. These tests illustrated the potential ability of phenolic acids to participate in redox reactions with transition metals (Fe, Mo, W). The ORAC assay and other radical scavenging assays showed the ability of CA and SA to act as efficient antioxidants (NENADIS et al., 2007).

- *reducing risk of cancer* (anticarcinogenic)

Cancer is mainly due to lifestyle factors such as the diet, smoking, infections, alcohol, physical activity or sun exposure and environmental factors like pollutants (Fig. 3-13). These lifestyle factors can strongly influence the incidence of cancer because only 5-10 % is due to a gene defect (ANAND et al., 2008, GONZALEZ and RIBOLI, 2010). Figure 3-13 displays the interaction of genes and environment in the development of cancer. A is the percentage of environmental and genetic factors to cancer. B shows the genetic or family risk for some cancers with familial risk ratios while C reveals the percentage of the environmental factors (ANAND et al., 2008).

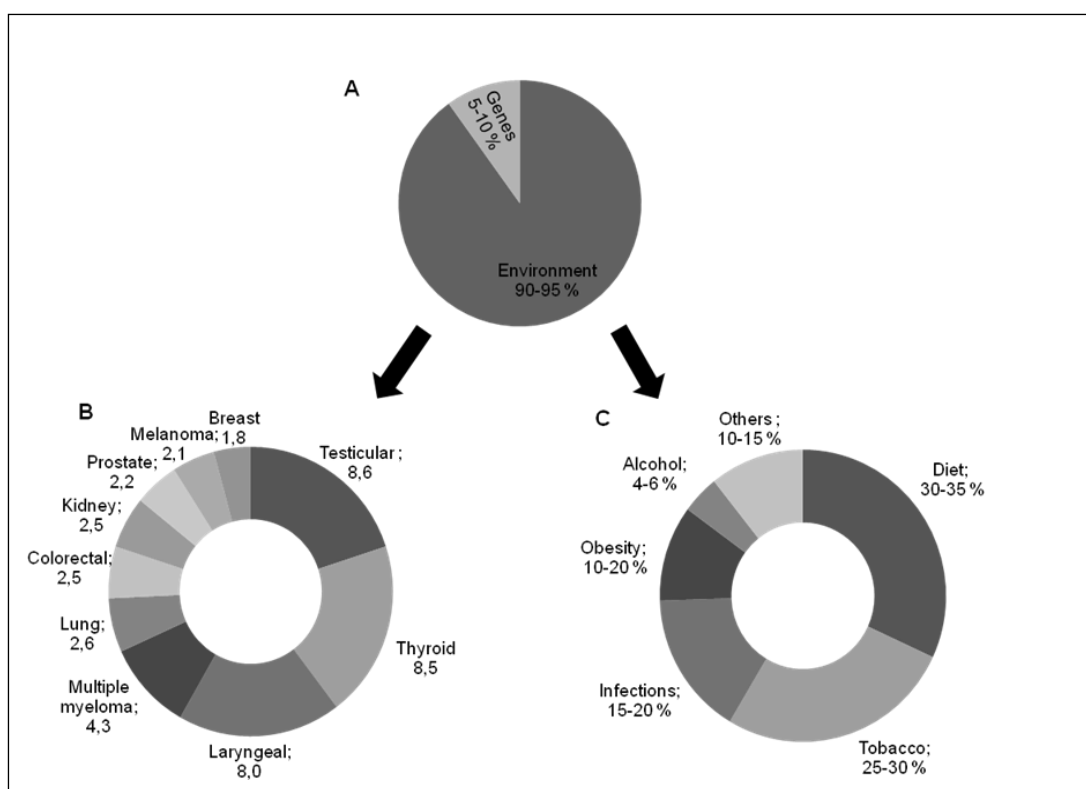


Fig. 3-13: Cancer risk factors (modified by ANAND et al., 2008)

A large prospective study on the influence of lifestyle and environmental factors and cancer was conducted in 10 European countries with 519,978 subjects. The intake of

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fruits, vegetables and cereals containing phenolic compounds was associated with the reduction of gastric cancer while the intake of red and processed meat increased the risk. They also investigated an inverse association between the intake of dietary fiber and colorectal cancer and an influence of fruits and vegetables on the risk of colon cancer (GONZALEZ and RIBOLI, 2010).

Phenolic acids and flavonoids activate naturally provided functions of the human body against carcinogenic substances like nitrosamines. During the initiation phase of cancer free radicals are destroyed by phenolic acids or carotenoids. In the second phase (promotion phase) the velocity of cleavage of the tumor cells is reduced (LEITZMANN and GROENEVELD, 1997). That means that blocking agents are active before or during the initiation of carcinogenesis (Fig. 3-14). So called suppressing agents interfere after the initiation during promotion and progression (JOHNSON, 2007). WATTENBERG defined 1985 anticarcinogenes derived from food as blocking agents like phenolic acids, coumarins and flavones or suppressing agents such as carotenoids depending on their site of action (WATTENBERG, 1985).

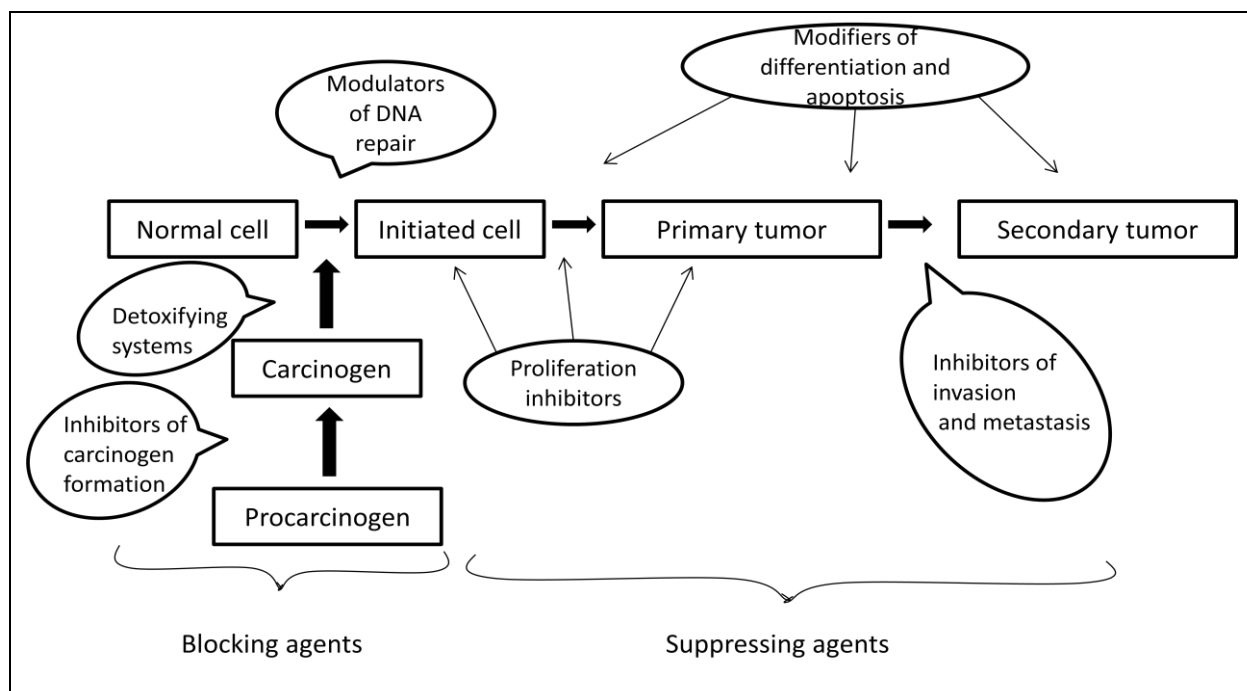


Fig. 3-14: Intervention of antioxidants as blocking agents and suppressing agents during carcinogenesis (neoplasia) (JOHNSON, 2007)

LEMARCHAND et al. (2000) conducted a study with lung cancer patients to reveal the association between the risk of lung cancer and the intake of phenolic compounds derived from food. Smoking was connected with lung cancer as the background

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characteristics of the case patients and the control subjects shows. Case patients consumed more saturated fat, less phenolic compounds due to a lower consumption of vegetables as the control subjects did. In this study it was confirmed that lung cancer can be reduced by the intake of flavonols and flavanones derived from onions, apples (quercetin) or citrus fruits (naringin) (LE MARCHAND et al., 2000). The same findings revealed an earlier Finnish longtime study of 10,000 men and women for a time period of at least 20 years. KNEKT et al. (1997) found an inverse connection between flavonoid intake and lung cancer incidence after consumption of fruits and vegetables.

Further studies indicate that carotenoids are cancer protective and they suggest a high variety of fruits and vegetables with an overall carotenoid intake and not a high intake of a distinct carotenoid (DONALDSON, 2004). OZASA et al. (2005) analyzed the urothelial cancer risk in a nested case-control study in Japanese men and showed that serum carotenoids may have a protective effect. They name smoking as the key risk factor for urothelial cancer and confirmed the relation of serum carotenoids to the intake of carotenoids. The protective effect of carotenoids was strengthened after the adjustment of risk factors like alcohol, body mass index (BMI) and total cholesterol (OZASA et al., 2005).

A population-based case-control study of incident colorectal cancer (CRC) with 2,186 subjects was conducted between 1998 and 2004. Corresponding control subjects consumed significantly more carotenoids like β -carotenes or lutein than the CRC patients resulting in the inverse association between carotenoid intake and CRC. Again, smoking is found as a strong risk in developing cancer (CHAITER et al., 2009).

- *reducing microbial infection (antimicrobial)*

Antibacterial activities against antibiotic resistant bacterial strains have been proven for some flavonoids like quercetin. Flavones or quercetin for example inhibit methicillin-resistant *Staphylococcus aureus* (GROSS, 2004). The flavonol myricetin can even inhibit other antibiotic resistant organisms. In a study conducted by XU and LEE (2001) 38 flavonoids have been analyzed whether they are effective against multidrug-resistant bacteria like MRSA (methicillin-resistant *Staphylococcus aureus*), VRE (vancomycin resistant enterococci), *Burkholderia cepacia* and *Klebsiella pneumoniae* which are nosocomial pathogens. The flavonols datiscetin, kaempferol,

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myritecin and quercetin and the flavones luteolin and flavone showed antibacterial impacts against MRSA. Other flavonoids such as flavanones, flavanols, isoflavones, chalcones and biflavones didn't show any activity. The tested VRE were resistant against the flavonoids and only myritecin showed activity against *Burkholderia cepacia* and *Klebsiella pneumonia* (XU and LEE, 2001).

Several flavonols (morin, quercetin, galangin, kaempferol, fisetin, quercitrin, rutin) and flavanones (naringenin, eriodictiol) have been tested against *Salmonella enteritidis* and *Bacillus cereus* by ARIMA et al. (2002) to reveal the antibacterial potential of those flavonoids. Antibacterial activities were shown for some flavonoids used alone but in combination they enhanced antibacterial activity (ARIMA et al., 2002).

Since *Escherichia coli* is a bacteria of the gastrointestinal (GI) tract of humans but a pathogenic one on meat products a study was conducted to analyze phenolic compounds as natural protectants against food-borne illnesses. RODRÍGUEZ VAQUERO et al. (2010) studied synergetic effects of phenolic compounds with their antibacterial activity. Phenolic acids (vanillic acid, caffeic acid), flavonoids (quercetin, rutin) and tannins were tested. Nonflavonoid mixtures such as gallic-caffeic acid, gallic-vanillic acid or gallic-protocatechuic acid as well as flavonoid mixtures like rutin- quercetin or rutin-catechin reduced the microorganisms in nutrient mixtures. Gallic-caffeic, gallic-protocatechuic acid and rutin-quercetin reduced the bacterial activities even in meat between 30 and 100 % (RODRÍGUEZ VAQUERO et al., 2010).

These results display that some flavonoids and phenolic acids might reduce bacterial infections and therefore inhibit inflammatory responses in humans. Inflammation is associated with a high risk of coronary heart disease (WILLERSON and RIDKER, 2004).

- *reducing cardiovascular diseases (CVD)*

CVD are associated with the inverse correlation of phenolic compounds. Several studies report about these findings rather for flavonoids than for phenolic acids. Research results of a 12-year prospective study of middle-aged men expose a negative correlation between plasma enterolactones and the risk of CVD. Enterolactones result from a bacterial metabolization of plant lignans derived from whole grain cereals, seeds fruits and vegetables in the colon (HEINONEN et al., 2001,

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JACOBS JR. et al., 2002). Low plasma enterolactone levels were associated with higher risks of CVD and CHD (coronary heart disease) mortality. A high level of serum enterolactones reduced the mortality (VANHARANTA et al., 2003).

KNEKT et al. (2002) analyzed the total dietary intake of 10,054 men and women to estimate the flavonoid intake in relation to chronic disease like CVD. They report an inverse association between flavonoid intake and CVD which is probably due to quercetin of apples. They support the suggestion that a flavonoid rich lifestyle might decrease the risk of chronic diseases (KNEKT et al., 2002).

The Zutphen Elderly Study was a longitudinal within population-cohort study of the risk for chronic diseases in elderly men with a 5-year follow up (HERTOG et al., 1993). They found out that a flavonoid rich diet might reduce the death from coronary heart disease (CHD). The seven country cross-cultural long-term study was conducted to determine the association between flavonoid intake and coronary heart disease. The mortality from 16 cohorts was observed after a 25-year follow up and the results showed that flavonoid intake was inversely correlated with coronary heart disease. These results were consistent with the above mentioned Zutphen Elderly Study and another study which was strengthened by the 10-year follow up by the same working group (HERTOG et al., 1995, HERTOG et al., 1997).

The Iowa Women's Health study with 34,489 participants was conducted to get to know the association between dietary flavonoid intake and CVD, CHD, stroke and total mortality. There was a follow-up of 16 years and total flavonoids along with seven subclasses of flavonoids were studied. In this longtime study flavanones and anthocyanidins decreased the risk of CHD and CVD mortality but there was no association with stroke mortality. In conclusion, the study suggests that the intake of some flavonoids might be associated with reduced CVD and CHD mortality in postmenopausal women although the results are not conclusive and comprehensive information on the sources of flavonoids were needed (MINK et al., 2007).

The Kuopio Ischaemic Heart Disease (KIHD) Risk Factor Study was conducted in Finland with 2,682 subjects. It is an ongoing population-based study to explore the risk factors for CVD and atherosclerosis. Different flavonoids were tested such as flavonols, flavones, flavonones or anthocyanidins and a follow-up time of about 15

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years ensued. The study revealed that flavonols and flavan-3-ols had the highest potential of decreasing the risk of ischaemic strokes. Flavonones and flavones might reduce the risk of CVD mortality which wasn't significant for this study. Since atherosclerosis results in CVD and ischaemic strokes a feasible explanation could be that atherosclerosis results earlier in a stroke because of smaller arterioles leading to stronger results than for CVD (WILLERSON and RIDKER, 2004, MURSU et al., 2008). As well as for cancer the results of this study are associated with a healthier lifestyle. Men who took in more flavonoids were less likely to be smokers and had a lower intake of fat, saturated fatty acids (SFA) or alcohol and higher intakes of vitamins and fibers (MURSU et al., 2008).

Those contradictory results show that many factors can influence the risk and incidence of chronic disease. Therefore the reducing capacity for cardiovascular and related disease has still to be analyzed further in well designed analyses to be able to tell if the decreased risk of CVD or stroke is actually due to phenolic compounds or a healthier lifestyle. In this context only little is known about the role of phenolic acids and needs to be included in such longtime studies (HOOPER et al., 2008).

An *in vitro* study on vascular smooth muscle cells (VSMC) showed that ferulic acid (FA) is able to inhibit the proliferation induced by angiotensin II. Dose effects of FA in the proliferation of VSMC were significant. These findings indicate that FA is a potential agent for cardiovascular disease (HOU et al., 2004).

3.2.4 Bioavailability

Phenolic compounds can be released from the food matrix by digestive processes making them bioaccessible for the human organism. Bioaccessibility is influenced by chemical characters of the phenolic component and by the food matrix where it is derived from (GONI et al., 2006). Phenolic compounds are potentially bioavailable and absorbable for the gut barrier not until they are bioaccessible which might change with the alteration of the colonic microflora due to nutritional shifts or progressive change (aging). Some of the phenolic compounds are more bioaccessible and absorbed by the small intestine epithelium, some by the colon epithelium and the rest is excreted by the feces (GONI et al., 2006).

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KERN et al. (2003b) showed in an in vitro study with Caco-2 cells of the human small intestinal epithelium that hydroxycinnamates such as ferulic acid (FA), sinapic acid (SA), p-coumaric acid (PCA) or caffeic acid (CA) can be absorbed in the human small intestine either conjugated or free. A metabolism by sulfation might occur as the preferred metabolic pathway for hydroxycinnamates (KERN et al., 2003). Bioaccessibility of beverages is very high while they are merging directly with intestinal fluids. Polyphenols in solid food matrixes have to be released first in order to be available in the small and large intestine. Bioavailability of phenolic compounds in the small intestine through the mucosa is very low with values reported between 5-10 % (CLIFFORD, 2004, SAURA-CALIXTO et al., 2007). The other 90-95 % reaches the colon where they have to undergo a fermentation and microbial metabolism in order to be bioavailable. The absorption follows as intact molecules or after breaking down of the polyphenols into metabolites (WILLIAMSON and MANACH, 2005, SAURA-CALIXTO et al., 2007).

The intestinal absorption as well as the bioaccessibility of phenolic compounds is determined by the chemical structure of the phenolics. The absorption is possibly influenced by the number of attached glucose molecules (OLTHOF et al., 2000). For a passive absorption through the small intestine brush border glycosylated phenolics have to be hydrolyzed by enzymes (glycosidases) first. Endogenous glycosidase like β -glycosidase is expressed by human cells in order to detach glucose, arabinose or xylose. Esterified phenolics such as hydroxycinnamates are hydrolyzed through the colonic microflora and the aglycone is released (TAPIERO et al., 2002, HOLLMAN, 2001). The caffeic acid derivatives such as ferulic acid or isoferulic acid are absorbed and metabolized in the GI tract (RECHNER et al., 2001).

Absorption of caffeic acid is reduced by esterification resulting in chlorogenic acid which has to undergo a microbial hydrolysis by esterases in order of being absorbed in the colon. Not more than 33 % chlorogenic acid and 95 % caffeic acid were absorbed in the small intestine of humans (OLTHOF et al., 2001). In a study on healthy volunteers RENOUEF et al. (2010) verified the absorption of phenolic acids like chlorogenic acid, CA and FA after the consumption of coffee in the small intestine. They also point out that the colon with its containing microflora is an important site for the absorption and metabolism of chlorogenic acid derived from coffee (RENOUEF et al., 2010). The release of caffeic acid and ferulic acid from caffeoylquinic acid and

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feruloylquinic acid present in coffee happens presumably in the small intestine whereas the metabolization of these compounds to their sulfated forms occurs in the liver and in the small intestine. The colon is probably the site where ferulic acid and caffeic acid are metabolized further to their dehydrogenated forms (STALMACH et al., 2010).

Free ferulic acid is present e.g. in tomatoes and beer and is absorbed easily whereas in cereals they are esterified to arabinoxylans of grain cell walls and the absorption is constrained (BOURNE et al., 2000). An earlier study by BOURNE and RICE-EVANS (1998) proved the bioavailability and absorption of free ferulic acid derived from tomatoes with a recovery rate of 11-25 % of free ferulic acid and feruloyl glucuronide in the urine. The maximum of the excretion was reached after 7 h after ingestion. It is suggested that phenolic compounds are absorbed intact and have a long enough lifetime in the human organism in order to function physiologically. This lifetime is a pharmacokinetic property which suits for ferulic acid as proved in this study (BOURNE and RICE-EVANS, 1998).

A study by MATEO ANSON et al. (2009) was conducted to determine the bioaccessibility of FA of wheat and bread samples in order to get more information about its bioavailability. The term bioaccessibility refers to the release of bound FA from the analyzed matrix in the GI tract. Aleurone layer, bran, flour and freeze-dried bread were used to determine their free FA and total FA (free+bound) content. Total FA contents ranged between 33 and 6,780 µg/g with only low free FA contents of about 1-7 %. Therefore the bioaccessibility of FA was very low ranging between 0.5 and 0.6 % for the different fractions as well as for bread. These findings indicate that the bioavailability of FA is determined by its food matrix and the release of free FA. FA acid firstly had to be released from its bond to arabinoxylan and cell wall polysaccharides in order to be bioavailable. Free FA added to a flour (60 %) was highly bioaccessible (MATEO ANSON et al., 2009).

Free hydroxycinnamic acids are absorbed in the small and the large intestine whereas ester bound PA can only be absorbed in the large intestine. The small intestine has a lack of enzymes to break the ester bonds other than the large intestine where microbial enzymes are present to metabolize phenolic acid esters. 95 % of the hydrolysis of FA by fermentation processes is located in the large

intestine. Free FA is metabolized by microorganisms or transformed into other phenolic compounds (WATZL and RECHKEMMER, 2001, Zhao et al., 2003). Antioxidative mechanisms in the human body still have to be analyzed and resolved further (GALLARDO et al., 2006).

3.3 Phenolic acids in wheat

Different studies proved that phenolic acids as strong antioxidants are present in wheat. Hydroxycinnamic acids provide the major part of phenolic acids in cereals with FA as the abundant PA, followed by SA, PCA, CA and VA as a hydroxybenzoic acid (ADOM and LIU, 2002, ADOM et al., 2003, ANDREASEN et al., 2000a, ANDREASEN et al., 2000b, EMMONS et al., 1999, GARCIA-CONESA et al., 1997, HATCHER and KRUGER, 1997, SLAVIN, 2003, SUN et al., 2001, ZHOU and YU, 2004, ZHOU et al., 2004a, ZHOU et al., 2004b).

The biggest part with about 98 % of phenolic compounds is situated in the aleurone layer, bran and germ of the caryopsis which makes whole wheat flours a good source of phenolic acids (GARCIA-CONESA et al., 1997, ZHOU et al., 2004a). Different cereals adhere to a variable amount of FA which is mainly covalently bound to cell wall polysaccharides through ester bonds (VITAGLIONE et al., 2008). In the case of FA it is arabinoxylan. The antioxidative capacity of dietary fiber with bound phenolic compounds (PC) has been underestimated for some time. The extraction of dietary fiber to release the PC requires several steps in order to release the water insoluble compounds like ferulic acid. Dietary fiber survive in the GI tract for some time and are released slowly. Ferulates cross-link polysaccharides to lignin and influence the physical characters of dietary fibers which is important for the bioavailability of FA (HATFIELD et al., 1999). A degradation of cell wall polymers of wheat bran fibers is done by xylanase (hydrolytic enzyme) to release FA (BARTOLOMÉ et al., 1995). About 95 % of the covalently bound feruloyl groups are freed during fermentation in the colon of humans whereas only small quantities are hydrolyzed in the small intestine. Feruloyl groups esterified to arabinoxylans of insoluble wheat bran fibers are released by xylanase resulting in soluble feruloylated oligosaccharides. Afterwards this molecule is hydrolyzed by ferulic acid esterase (FAE) to liberate the free FA (KROON et al., 1997, ZHAO et al., 2003).

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In organic wheat bran analyzed by RONDINI et al. (2004) FA was present mainly in bound form with 95.8 %. Free and conjugated FA was absorbed quickly after application to rats and it was metabolized to sulfated ferulic acid as the main metabolite found in plasma and urine. The linkage of FA strongly influenced the bioavailability of FA, prolonging the time of absorption. The antioxidative capacity of plasma was better after bran consumption than after supplementation of pure FA indicating that bran provides further antioxidants like tocopherols or some enzymatic cofactors like Se or Cu (RONDINI et al., 2004).

In a high-bran cereal meal study by KERN et al. (2003a) the main phenolic acids were ferulic acid and sinapic acid with highest absorption rates between 1 and 3 h. Since the amount of excreted phenolic acids was greater than the ingested amount of total free phenolic acids from the test meal metabolism in form of de-esterification must have occurred (KERN et al., 2003a). Bioavailability of FA is higher for the endosperm fraction than for the bran fraction where the absorption is depressed by complex matrix (ADAM et al., 2002).

A recent study by FUNK et al. (2007) showed that a low or moderate level of ferulates or diferulates did not hinder the degradation of cell walls which were not lignified (FUNK et al., 2007). The more soluble dietary fibers were present the higher was the bioaccessibility of FA by bacteria β -glucosidases and esterases in the gut (ZHAO et al., 2003).

The major site of the absorption of conjugated ferulic acid is the colonic epithelium. The main transportation form is the free form of FA, previously degraded by bacterial enzymes (POQUET et al., 2008). Transportation mainly takes place by a transcellular passive diffusion (SILBERBERG et al., 2006). This transport mechanism probably occurs in other tissues, too, e.g. the gastric mucosa. FA, CA, PCA, gallic acid and chlorogenic acid are absorbed through the gastric mucosa (KONISHI et al., 2006, LAFAY et al., 2006).

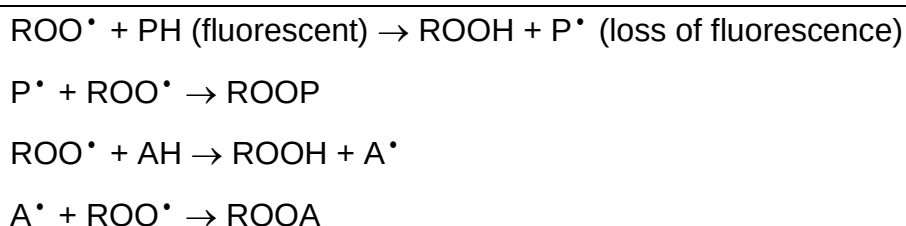
3.4 Antioxidative capacity tests

As described above there are diverse in vitro tests to analyze the antioxidative capacity. Two of them are of importance for the current work, one HAT (hydrogen

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atom transfer) based reaction assay namely ORAC (oxygen radical absorbance capacity) assay and one ET (electron transfer) based reaction assay namely Folin-Ciocalteu assay. The end product is the same, both are scavenging radicals but the kinetic is different.

HAT based assays measure the quenching ability of free radicals by hydrogen donation of antioxidants and examine competitive reaction kinetics using an oxidizable molecular probe like fluorescein, a free radical generator such as AAPH in case of the ORAC assay and an antioxidant (PRIOR et al., 2005). AAPH is an azo radical initiator while fluorescein is fluorescent to monitor the reaction progress. If the antioxidants present in the carried out analysis are working efficiently and scavenge radicals, the fluorescence of the molecular probe will last. The antioxidant (AH) competes with the molecular probe (PH) for radicals like peroxy radicals ($\text{ROO}^\bullet$) derived from the decomposition of the azo compound. The basic kinetic of this reaction is displayed in equation 3-3 (HUANG et al., 2005).



Eq. 3-3: HAT kinetics (HUANG et al., 2005, PRIOR et al., 2005)

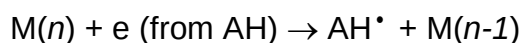
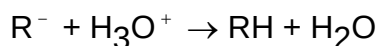
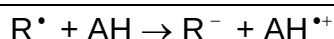
The antioxidant is metabolized by the peroxy radicals and when it is run down the reaction as well as the fluorescence will stop. In case of the ORAC assay the area under curve (AUC) is calculated to measure the antioxidative capacity (HUANG et al., 2005). At the beginning, when this test first was applied, B-phycoerythrin a fluorescent protein was the probe. Since it interacted with polyphenols and was photobleached under plate-reader conditions it was replaced by the synthetic nonprotein fluorescein by Ou et al. (2001). Trolox is used as the standard antioxidant to obtain a standard curve and Trolox equivalents (TE) are calculated for the tested samples (HUANG et al., 2005). The net integrated areas under curve (AUC_{net}) [$\text{AUC}_{\text{AH}} - \text{AUC}_{\text{no AH}}$] are determined to obtain the antioxidative capacity of the measured sample. With the help of a linear or quadratic function ($y=a+bx$ or $y=a+bx+cx^2$) the TE of the samples are estimated between the trolox concentration

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(y [μM]) and the netto AUC (AUC_{net}). The dynamic range of the assay is widened a little bit by the quadratic regression and results are expressed as μmol TE/g (PRIOR et al., 2005).

The advantage of HAT based assays is their solvent and pH independence while they are fast. In case of the ORAC assay only hydrophilic chain breaking antioxidant capacity against peroxy radicals is calculated and does not take into account lipophilic antioxidants unless it is adapted by using a 50 % acetone/50 % water (v/v) with 7 % randomly methylated β-cyclodextrin (RMCD). By adjusting the method the hydrophilic and lipophilic chain-breaking antioxidant capacity can be measured without any undesirable interactions of the probe. In the contrary ET based assays are usually slow and pH dependent. It is a simple and accurate method although it can be influenced by many substances (sugars, aromatic amines sulfur dioxide, ascorbic acid or Fe(II)). Normally both types of reactions (HAT and ET) occur together in samples (PRIOR et al., 2005).

ET based assays occupy one redox reaction with the oxidant and as described above one proton-coupled electron is transferred from the antioxidant (AH) to reduce radicals, carbonyls or metals (M) (PRIOR et al., 2005). Folin-Ciocalteu assay also known as total phenolic content (TPC) assay belongs to this type and the general reaction shows equation 3-4. This time the probe such as the Folin-Ciocalteu reagent is the oxidant that abstracts the electron from the antioxidant leading to a color change of the probe and a blue complex is build. The probe is reduced and the antioxidant oxidized. As long as there are antioxidants present the reactions proceeds and the color changes. The higher the antioxidant concentration the stronger are color changes to dark blue (HUANG et al., 2005). The optical density is measured at $\lambda_{max}=765$ nm (PRIOR et al., 2005).



Eq. 3-4: ET reaction (HUANG et al., 2005, PRIOR et al., 2005)

To determine the antioxidative capacity a linear function is calculated where the slope reflects the reducing capacity of the antioxidant. Since the used standard is gallic acid the results are expressed as gallic acid equivalents (GAE) and it is assumed that the antioxidant capacity is equal to the reducing capacity which is actually measured by this type of assay (HUANG et al., 2005).

The Folin-Ciocalteu assay belongs to the type of ET based reactions but the name total phenolic content (TPC) assay is misleading because the Folin-Ciocalteu reagent (FCR) is not only specific to phenolic compounds. FCR contains sodium tungstate ($Na_2WO_4 \cdot 2H_2O$, 100 g), sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$, 25 g) concentrated hydrochloric acid (100 ml), 85 % phosphoric acid (50 ml), water (700 ml) and lithium sulfate ($Li_2SO_4 \cdot 4H_2O$, 150 g) leading to a dark yellow color. A reversible one or two electron reduction results in blue complexes $(PMoW_{11}O_{40})^{4-}$. Sodium carbonate is needed to get basic conditions so that phenolic compounds can react with this reagent (HUANG et al., 2005).

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4 Material and methods

4.1 Characterization of the experiments

Experiment 1 “Evaluation of ancient wheat species” (chapter 5.1)

In 2006 different ancient wheat species were tested in a field experiment at the research station “Weilburger Grenze” Gießen of the Institute of Agronomy and Plant Breeding (degree of longitude: 8° 39' 16" E degree of latitude: 50° 36' 12" N, altitude: 158 m). Climate conditions were characterized by the annual total precipitation of 600 mm and mean air temperature of 8.5 °C. The field experiment was carried out on an alluvial soil which is characterized by a clay content ($< 2 \mu\text{m}$) of 33 % and a silt content ($2 - 63 \mu\text{m}$) of 58 %. The pH value (0 – 30 cm soil depth) was 6.3, and the total carbon content (humus) of the soil (soil depth 0 – 30 cm) was 1.42 %. In total 20 accessions (species and/or cultivars) of the genus *Triticum* (T.) were evaluated under these field conditions. The accessions include three of the section *monococcon* (diploid), seven of the section *dicoccoidea* (tetraploid), and 10 of the section *triticum* (hexaploid) (chapter 5.1 Tab. 1). Standard procedures of soil tillage, sowing and fertilization (nitrogen: 80 kg N/ha applied in April 2006) were carried out. No pesticides and no plant growth regulators (PGRs) were applied which supposedly led to lodging during ripening. After reaching the full ripening stage all accessions were harvested by harvest combine. After harvest hulled wheat was dehulled automatically by a Saatmeister-Allesdrescher K35 (Kurt Pelz Maschinenbau, Germany). The dehulled and free threshing samples were ground on a Cyclotec 1073 Sample Mill (Foss, Germany) with a 500 μm sieve to get a whole wheat flour. The flour was mixed to ensure homogeneity and was extracted subsequently.

Experiment 2 “Sprouting experiment with bread wheat” (chapter 5.2)

In 2008 a field experiment with three winter wheat cultivars was carried out in the research station “Rauischholzhausen” of the Institute of Agronomy and Plant Breeding (degree of longitude: 8° 52' 05" E, degree of latitude: 50° 46' 41" N, altitude: 222 m). Three bread wheat cultivars (Tommi, Privileg, Estevan) of *T. aestivum* ssp. *aestivum* L. with four randomized replications were obtained from the executed field experiment. The cultivars which are officially licensed for

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cultivation in Germany or Austria by the Federal Plant Variety Office belong to the following groups of wheat quality: Tommi – A quality, Privileg – E quality and Estevan – E quality. Two nitrogen levels were analyzed: N₁ without N fertilization and N₂ with 150 kg N/ha in total (applied at three times: 40, 40, 70 kg N/ha). To avoid lodging of wheat plants all plots were treated with the plant growth regulator chlorocholin chloride (CCC, 720 g/l) at two times. To protect the wheat against diseases fungicides were applied at two times (first: 0.8 l/ha Proline + 1.5 l/ha Pugil 75 WG and second: 0.7 l/ha Diamant + 0.7 l/ha Champion). After harvesting with plot harvest combine the samples were stored in darkness at 20 °C until they were used for the sprouting experiment.

To induce sprouting 30 g of wheat grain were steeped with 15 ml aqua dest. in petri dishes which were covered with ashless circular filters (MN 640 m, 90 mm, Macherey-Nagel). Three different sprouting times were tested at room temperature, without direct solar radiation: 0 h (no sprouting), 24 h (presprouted wheat samples) and 48 h (fully sprouted wheat samples) (chapter 5.2 Fig. 1). To stop the sprouting process, all samples were dried for 24 h at 36 °C.

In total 72 wheat samples (3 cultivars x 3 sprouting levels x 2 N fertilization doses x 4 replications) were included and ground with a 500 µm sieve on a Cyclotec 1073 Sample Mill (Foss, Germany) to get a whole wheat flour. The samples were mixed thoroughly to ensure homogeneity. Extraction followed immediately.

Experiment 3 “Evaluation of spelt wheat cultivars” (chapter 5.3)

In 2008 field trials with spelt wheat (*Triticum aestivum* ssp *spelta* L.) were conducted in Baden-Württemberg (southern Germany) at four different sites namely Kirchberg-Dörrmenz, Bremelau, Döggingen and Eiselau. At the location Kirchberg-Dörrmenz organic farming was induced while the trials of the other locations (Bremelau, Döggingen, Eiselau) were carried out under conventionally farming methods. Badengold, Franckenkorn, Oberkulmer Rotkorn, Schwabenkorn and Zollernspelz were the five spelt wheat cultivars which were tested in field trials and analyzed in the laboratory. In total 19 wheat grain samples were obtained from the different sites while Schwabenkorn was the only cultivar which wasn't available from the organic farming location Kirchberg-Dörrmenz. Until beginning the lab analysis the wheat grain samples were stored at 20 °C under dry conditions.

4.2 Grain quality parameters

4.3 Sample preparation for HPLC and antioxidative capacity

The extraction (Fig. 4-1) of the grain samples was done in three separate fractions: (1) soluble free, (2) soluble conjugated, and (3) bound phenolic acids as previously described by using the methods of ADOM and LIU (2002) and KRYGIER et al. (1982) with modifications. Briefly: 1 g whole wheat flour was extracted for one hour with (7:7:6, v/v/v) methanol/acetone/water and centrifuged. The supernatant provided fraction 1 and 2, the residue fraction 3. Before extracting all three fractions with ethyl acetate, the bound and the soluble conjugated phenolic acids had to undergo an alkaline pulping with 5 M NaOH. After evaporating the extracts they were resumed in 5 % acetonitrile or 10 % methanol depending on the HPLC method. Until analysis extracts were stored at -18 °C. The extracts were also used for the antioxidative capacity assays namely Folin-Ciocalteu and ORAC assay.

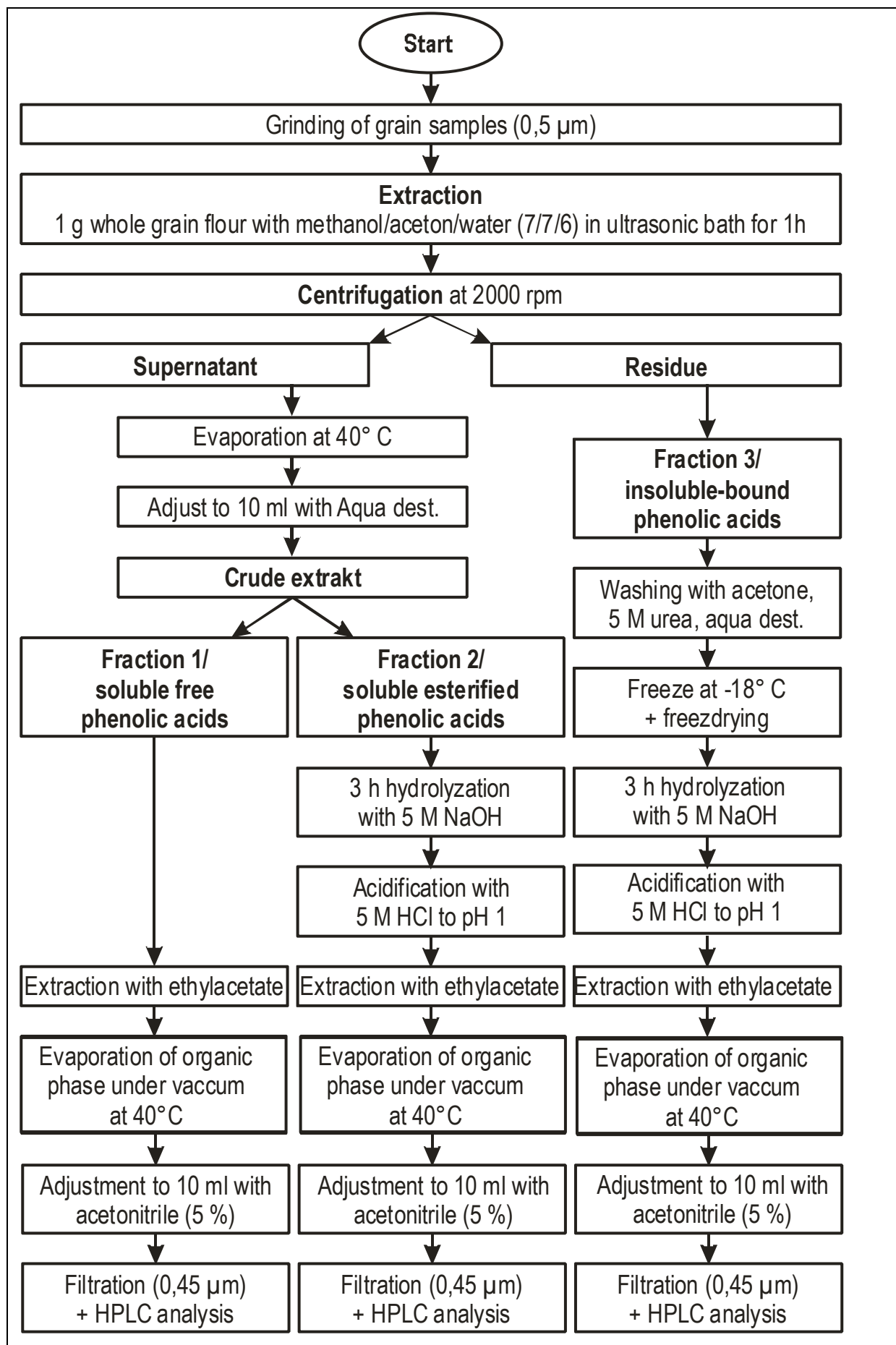


Fig. 4-1: Procedure of the preparation of the used wheat grain samples

4.4 HPLC analysis

4.4.1 HPLC method 1

Six phenolic acids were identified by HPLC-DAD analysis on a C18 column (EC 250x4 Nucleodur Sphinx RP, 5 μm (MN)). For quantification of the hydroxybenzoic acids the diode array detection recorded at the wavelength of 250 nm and for the hydroxycinnamic acids at 290 nm. The method was modified according to ZIELINSKI et al. (2001) and WEIDNER et al. (2000). The temperature for the column was set to $T = 25\text{ }^{\circ}\text{C}$. The injection volume was 100 μl and elution took place with a gradient mobile system: (A) acetonitrile, (B) acetic acid (0.5 %, pH 4.5) at 1 ml min^{-1} following the program: 0-14.5 min 8 % A, 15-30 min 10 % A, 31-40 min 80 % A and 40.5-45 min 8 % A. The identification was made by retention times and UV/VIS spectra compared with commercially available reference compounds. The quantification of all six phenolic acids was done by a five point calibration curve, where the standards were added to a reference flour before extraction to exclude matrix effects. Results were expressed as $\mu\text{g/g}$ and converted in $\mu\text{g GAE/g}$. This HPLC method was used for the analysis of experiment 1 (chapter 5.1).

4.4.2 HPLC method 2

The HPLC method according to WEIDNER et al. (2000) and ZIELINSKI et al. (2001) was used with some modifications. The analysis was performed on a HPLC-DAD system by Knauer on a C18 column (EC 250 x 4 Nucleodur Sphinx RP, 5 μm (MN)) at a temperature of $25\text{ }^{\circ}\text{C}$, a flow rate of 1 ml min^{-1} and an injection volume of 100 μl . The detection was carried out at 250 nm (hydroxybenzoic acids) and 290 nm (hydroxycinnamic acids) following the program: 0-3 min 20 % A, 3.5-7.5 min 0 % A, 8-14 min 8 % A, 14.5-32 min 30 % A, 33-39 min 95 % and 40-45 min 20 %. The gradient system consisted of (A) 95 % methanol with acetic acid (about 200 μl) adjusted to pH 4.5 and (B) water adjusted to pH 4.5 with about 5 μl acetic acid. This HPLC method was used for the analysis of the spelt wheat samples in experiment 2 (chapter 5.2) and 3 (chapter 5.3).

4.5 Analysis of antioxidative capacity

4.5.1 Folin-Ciocalteu assay

The total phenolic content was determined by using the Folin-Ciocalteu micro method (WATERHOUSE, 2001) using gallic acid as a standard. This assay is electron transfer reaction based which measures the sample's reducing capacity (HUANG et al., 2005). Folin-Ciocalteu reagent consists of phosphotungstic ($\text{H}_3\text{PW}_{12}\text{O}_{40}$) and phosphomolybdic ($\text{H}_3\text{PMo}_{12}\text{O}_{40}$) acids. 40 μL of the sample extract was mixed with 3.16 ml aqua dest. and 200 μL Folin-Ciocalteu reagent was added. After 5 min 600 μL saturated sodium carbonate solution was added. The reaction blend was mixed and kept at 40 °C for 30 min in a water bath in darkness. Folin-Ciocalteu solution was reduced to blue oxides of tungsten and molybdenum and the absorbance was measured spectrophotometrically at 765 nm. The total phenolic content was expressed as gallic acid equivalent (GAE).

4.5.2 ORAC assay

The determination of the antioxidative capacity with the ORAC assay, described previously by HUANG et al. (2002), was operated on the 96-well plate fluorescence reader Fluoroskan (Fisher Scientific). The assay is hydrogen atom transfer reaction based and measures antioxidant capacity towards peroxy radicals (HUANG et al., 2005). In general, 150 μL fluorescein (6-hydroxy-9-(2-carboxyphenyl)-(3*H*)-xanthen-3-on) was mixed with 25 μL of the sample extract and incubated at 37 °C for 30 min. To initiate the reaction the ROS-generator AAPH (2,2'-azobis(2-methylpropion-amidine)dihydrochloride) had to be added and the fluorescence was measured at every 60 s for 90 min (excitation wavelength: 485 nm, emission wavelength: 538 nm). As a standard Trolox[®] (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a vitamin E analogue, was used. The antioxidative capacity was expressed as Trolox equivalents (TE).

4.6 Statistical analysis

Experiment 1

Results are reported as mean values and the statistical analysis was performed with PASW Statistics 18 (SPSS Inc., Chicago, IL). The following characteristics were statistically tested by the analysis of variance (ANOVA) in dependency on species with a general linear model: total phenolic content (Folin-Ciocalteu assay), antioxidative capacity (ORAC assay) and phenolic acid concentration (HPLC). Correlation analysis was performed by the Pearson test, bivariate.

Experiment 2

One-way analysis of variance (ANOVA) was performed with PASW Statistics 18 (SPSS Inc., Chicago, IL) for all wheat cultivars (Tommi, Privileg, Estevan) and nitrogen levels (N_1 and N_2) within all sprouting times (no sprouting, presprouted, fully sprouted) to determine the effects on the measured parameters. When significant differences occurred ($p \leq 0.05$) multiple mean comparisons were performed using the Tukey t-test. To determine the influence of sprouting time on the analyzed parameters a paired samples test (t-test) was performed. Spearman's correlation (r_s) factors (bivariate) were calculated between total phenolic acids (TPA), total phenolic content (TPC) and antioxidative capacity (ORAC). Results are reported as mean values and the significance level was at a 95 % confidence interval ($\alpha = 0.05$).

Experiment 3

ANOVA (analysis of variance) was executed by the statistical program PASW Statistics 18 (SPSS Inc., Chicago, IL) for analyzing the effect of the used spelt cultivars regarding phenolic acid contents and the antioxidative capacity (Folin-Ciocalteu and ORAC assay). Tukey's HSD post-hoc test was used for multiple mean comparisons when significant differences were calculated ($p \leq 0.05$). Pearson's correlation (r) factors (bivariate) were computed between total phenolic acids (TPA), total phenolic content (TPC) and antioxidative capacity (ORAC). To evaluate significant ($p \leq 0.05$) differences of the three analyzed fractions of each parameter a

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ANOVA with repeated measures was used. Results were reported as mean values and the significance level was at a 95 % confidence interval ($\alpha=0.05$).

5 Publications

5.1 Experiment 1 “Evaluation of ancient wheat species”

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Characterization of grain quality and phenolic acids in ancient wheat species (*Triticum* sp.)

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Summary:

The matter of this study was to determine the phenolic acid profile of different ancient wheat, furthermore, to analyze the total phenolic content (TPC) with the Folin-Ciocalteu assay and the antioxidative capacity with the ORAC (oxygen radical absorbance capacity) assay. The concentration and composition of free, conjugated, and insoluble bound phenolic acids were analyzed in 20 accessions of wheat (*Triticum* sp.), including 16 ancient wheat and 4 bread wheat samples grown in Germany. Six phenolic acids were analyzed by HPLC, and ferulic acid (FA) was identified to be the abundant phenolic acid. The content of phenolic acids in total was comparable to bread wheat, ranging between 141.0 and 542.0 µg GAE/g (gallic acid equivalent/g) whole wheat flour. In the current study no significant distinction between the analyzed species could be observed. There was no significant impact by the *Triticum* species, neither on the total phenolic content by Folin-Ciocalteu nor on the antioxidative capacity by ORAC. Correlation analysis between ORAC values and total phenolic acids demonstrated a positive correlation ($r=0.469$, $p=0.05$). Phenolic acids, TPC and ORAC values of the analyzed ancient wheat samples were comparable to bread wheat.

Keywords: ancient wheat, old wheat, emmer, einkorn, antioxidants, phenolics, phenolic acids, antioxidative capacity, Folin-Ciocalteu, ORAC

Introduction

Wheat (*Triticum aestivum* L. ssp. *aestivum*) is one of the most important agricultural commodities worldwide with 670 million tons in 2009, and it is constantly rising (USDA, 2010). The domestication of wheat goes back to 10,000 BC, where wild species were taken into cultivation (FELDMAN, 2001; NESBITT and SAMUEL, 1995). Wheat is one of the most important vegetable foods which provides human with basic nutrients (carbohydrates, proteins, vitamins, minerals). Besides basic nutrients, wheat caryopses contain secondary plant metabolites, such as carotenoids, flavonoids and phenolic acids. These compounds can have an influence on the utility value and can influence the quality characteristics of wheat products. Additionally, secondary plant metabolites are bioactive compounds because of their antioxidative capacity (WATZL and RECHKEMMER, 2001; SLAVIN, 2003).

Einkorn (*T. monococcum* L.), emmer (*T. dicoccum* L.) and spelt (*T. aestivum* L. ssp. *spelt*) are ancient wheat species with low grain yields and non-threshable grain. Wild einkorn, an one-grained wheat, is known as the progenitor of cultivated diploid wheat, which later results in tetraploid wheat (emmer) and hexaploid wheat (spelt) (FELDMAN, 2001). In the last time interest in ancient wheat and a higher variety in nutrition grew in some countries, e. g. Germany. For instance, the harvested area of spelt rose from 2003 to 2009 more than 300 % in Germany (STATISTISCHES BUNDESAMT, 2010). Einkorn, emmer and the hexaploid spelt are very robust and modest cereals which can be harvested under moderate environmentally conditions like poor soils and water deficiency (STAGNARI et al., 2008). Thus, ancient wheat is recommended for organic production, where no synthetic fertilizers or pesticides are used to strengthen the plant.

One reason for increasing interest in ancient wheat is the consumer, who claims more biologically grown products because of their environmentally friendlier production (ZHAO et al., 2007; DANGOUR et al., 2009). Another reason is the health beneficial-aspect of wheat products, especially of whole grain products. Investigations report that whole grain wheat products are reducing high blood pressure (BEHALL et al., 2006). Ancient wheat products often can be found as whole grain products, where the health aspect is higher than in white flour products.

Phenolic acids (PA), such as ferulic, *p*-coumaric, vanillic, sinapic, syringic, and caffeic acid are valuable antioxidativ ingredients in wheat (MPOFU, 2006). It is reported that phenolic acids inhibit lipid oxidation by scavenging free radicals such as hydroxyl radicals (CUPPETT et al., 1997). The phenolic acid profile in ancient wheat subjects a

high range of variation (MPOFU, 2006). In wheat are three different forms of phenolic compounds existing, free phenolic acids, soluble conjugated (e. g. esterified to sugars) and bound phenolic acids (e. g. esterified to cell walls). The predominant phenolic acid is ferulic acid (HATCHER and KRUGER, 1997; SOSULSKI et al., 1982; WEIDNER et al., 1999).

The aim of this study is to quantitatively investigate phenolic acids, their composition, and their range of variation in ancient wheat. Furthermore, the determination of the antioxidative capacity as a nutritionally positive and health affecting potential in emmer, einkorn and spelt growing in Germany is carried out in this study.

Materials and Methods

Wheat Materials

In 2006 ancient wheat species were grown in field plots of the experimental station “Weilburger Grenze” of the Institute of Agronomy and Plant Breeding, (degree of longitude: 8° 39' 16" E degree of latitude: 50° 36' 12" N, altitude: 158 m). Climate conditions were characterized by the annual total precipitation of 600 mm and mean air temperature of 8.5 °C. The experiment was carried out on an alluvial soil which is characterized by a clay content (< 2 µm) of 33 %, and a silt content (2 – 63 µm) of 58 %. The pH value was 6.3, and the total carbon content (humus) of the soil (soil depth 0 – 30 cm) was 1.42 %. In total 20 accessions (species and/or cultivars) of the genus *Triticum* (*T.*) were obtained from the field experiment, including three of the section *monococcon* (diploid), seven of the section *dicoccoidea* (tetraploid), and 10 of the section *triticum* (hexaploid) (Tab. 1). No plant growth regulators (PGRs) were applied which supposedly led to lodging during ripening.

Hulled wheat was dehulled automatically by a Saatmeister-Allesdrescher K35 (Kurt Pelz Maschinenbau, Germany). The dehulled and freethreshing samples were ground on a Cyclotec 1073 Sample Mill (Foss, Germany) with a 500 µm sieve to get an whole wheat flour. The flour was mixed to ensure homogeneity and was extracted subsequently.

Tab. 1: Wheat material of the eight species with corresponding accessions

species [No.]	section	species	accession	Cv. [No.]
1	<i>monococcon</i>	<i>T. boeiticum</i> L. subsp. <i>boeiticum</i>	<i>hausknechtii</i>	1
			<i>pseudoreuteri</i>	2
2	<i>monococcon</i>	<i>T. monococcum</i> L. subsp. <i>monococcum</i>	<i>hohensteinii</i>	1
3	<i>dicoccoidea</i>	<i>T. turgidum</i> L. subsp. <i>dicoccoides</i>	<i>spontaneovillosum</i>	1
			<i>artratum</i>	2
			<i>semicanum</i>	3
4	<i>dicoccoidea</i>	<i>T. turgidum</i> L. subsp. <i>turgidum</i>	<i>griseo buccale</i>	1
			<i>centigranum</i>	2
			<i>mirabile</i>	3
5	<i>dicoccoidea</i>	<i>T. timopheevii</i> Zhuk. subsp. <i>timopheevii</i>	<i>timopheevii</i>	1
6	<i>triticum</i>	<i>T. aestivum</i> L. subsp. <i>spelta</i>	<i>albispicatum</i>	1
			<i>durhamelianum</i>	2
			<i>arduini</i>	3
7	<i>triticum</i>	<i>T. aestivum</i> L. subsp. <i>aestivum</i>	<i>erythrospermum</i>	1
			<i>ferrugineum</i>	2
			<i>lutescens</i>	3
			<i>miturum</i>	4
8	<i>triticum</i>	<i>T. aestivum</i> L. subsp. <i>compactum</i>	<i>humboldtii</i>	1
			<i>erinaceum</i>	2
			<i>pseudo-rubiceps</i>	3

Chemicals

Vanillic acid (VA), syringic acid (SYA), caffeic acid (CA), p-coumaric acid (PCA), ferulic acid (FA), as well as sinapic acid (SA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Gallic acid, acetonitrile, acetic acid, acetone, ethyl acetate, and methanol were obtained from Roth (Karlsruhe, Germany). Water (gradient grade) was acquired by AppliChem (Darmstadt, Germany) and ethanol by Schmidt (Dillenburg, Germany). Sodium carbonate was purchased from Acros organics (New Jersey, USA).

Analytics

Analysis of Quality Parameters

Firstly, thousand grain weight ([g], ISTA 1999) and the proportion of germinated caryopses [%] were analyzed. According to ICC standard methods the following analyses were carried out: total nitrogen (Dumas method, ICC 167), falling number (Hagbert-Perten, ICC 107/1) and sedimentation test (ICC 116/1).

Extraction of Bioactive Compounds

The extraction of the grain samples was done in three separate fractions ((1) soluble free, (2) soluble conjugated, and (3) bound phenolic acids) as previously described by using the methods of ADOM and LIU (2002) and KRYGIER et al. (1982) with modifications. Briefly: 1 g whole wheat flour was extracted for one hour with (7:7:6, v/v/v) methanol/acetone/water and centrifuged. The supernatant provided fraction 1 and 2, the residue fraction 3. Before extracting all three fractions with ethyl acetate, bound and soluble conjugated phenolic acids had to undergo an alkaline pulping with 5 M NaOH. After evaporating the extracts they were resumed in 10 % acetonitrile and stored at -18 °C until analysis. The extracts were used for all further analyses (HPLC, Folin, ORAC).

Analysis of Phenolic Acids by HPLC

Six phenolic acids were identified by HPLC-DAD analysis on a C18 column (EC 250x4 Nucleodur Sphinx RP, 5 µm (MN)) (Fig. 1). For quantification of the hydroxybenzoic acids the diode array detection recorded at the wavelength of 250 nm and for the hydroxycinnamic acids at 290 nm. The method was modified according to ZIELINSKI et al. (2001) and WEIDNER et al. (2000). The temperature for the column was set to 25 °C. The injection volume was 100 µl and elution took place with a gradient mobile system: (A) acetonitrile, (B) acetic acid (0.5 %, pH 4.5) at 1 ml min⁻¹ following the program: 0-14.5 min 8 % A, 15-30 min 10 % A, 31-40 min 80 % A and 40.5-45 min 8 % A. The identification was made by retention times and UV/VIS spectra compared with commercially available reference compounds. The quantification of all six phenolic acids was done by a five point calibration curve, where the standards were added to a reference flour before extraction to exclude matrix effects. Results were expressed as µg/g and converted in µg GAE/g.

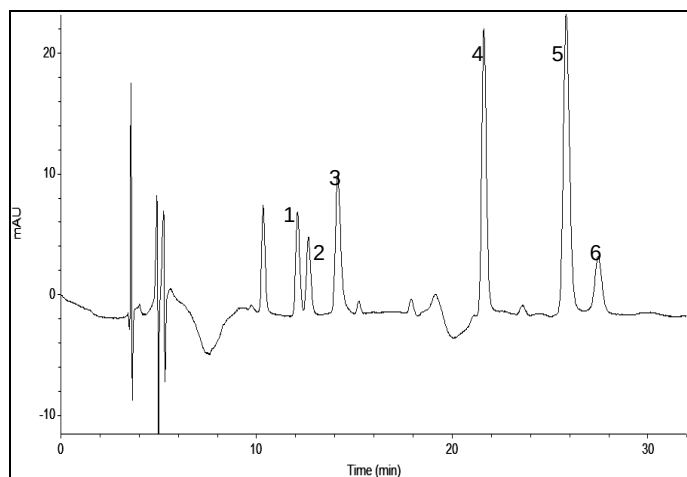


Fig. 1: HPLC chromatogram of a wheat sample with standards. Peak 1 vanillic acid (VA), 2 syringic acid (SYA), 3 caffeic acid (CA), 4 p-coumaric acid (PCA), 5 ferulic acid (FA), 6 sinapic acid (SIA)

Total Phenolic Assay by Folin-Ciocalteu

The total phenolic content was determined by using the Folin-Ciocalteu micro method (WATERHOUSE, 2001) using gallic acid as a standard. This assay is electron transfer reaction based, which measures the sample's reducing capacity (HUANG et al., 2005). Folin-Ciocalteu reagent consists of phosphotungstic ($\text{H}_3\text{PW}_{12}\text{O}_{40}$) and phosphomolybdic ($\text{H}_3\text{PMo}_{12}\text{O}_{40}$) acids. 40 μL of the sample extract was mixed with 3.16 ml aqua dest. and 200 μL Folin-Ciocalteu reagent was added. After 5 min 600 μL saturated sodium carbonate solution was added. The reaction blend was mixed and kept at 40 °C for 30 min in a water bath in darkness. Folin-Ciocalteu solution was reduced to blue oxides of tungsten and molybdenum and the absorbance was measured spectrophotometrically at 765 nm. The total phenolic content was expressed as gallic acid equivalent (GAE).

ORAC (oxygen radical absorbance capacity) Assay

The determination of the antioxidative capacity with the ORAC assay, described previously by HUANG et al. (2002), was operated on the 96-well plate fluorescence reader Fluoroskan (Fisher Scientific). The assay is hydrogen atom transfer reaction based and measures antioxidant capacity towards peroxy radicals (HUANG et al., 2005). In general, 150 μL fluorescein (6-hydroxy-9-(2-carboxyphenyl)-(3*H*)-xanthen-3-on) was mixed with 25 μL of the sample extract and incubated at 37 °C for 30 min. To initiate the reaction the ROS-generator AAPH (2,2'-azobis(2-methylpropionamidine)dihydrochloride) had to be added and the fluorescence was

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measured at every 60 s for 90 min (excitation wavelength: 485 nm, emission wavelength: 538 nm). As a standard Trolox[®] (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a vitamin E analogue, was used. The antioxidative capacity was expressed as Trolox equivalents (TE).

Statistical Analysis

Results are reported as mean values and the statistical analysis was performed with PASW Statistics 18 (SPSS Inc., Chicago, IL). The following characteristics were statistically tested by the analysis of variance (ANOVA) in dependency on species with a general linear model: total phenolic content (Folin-Ciocalteu assay), antioxidative capacity (ORAC assay) and phenolic acid concentration (HPLC). Correlation analysis was performed by the Pearson test, bivariate.

Results

In this study grain quality parameters, concentrations of phenolic acids (TPA), the antioxidant capacity expressed as total phenolic contents (TPC) and ORAC values were analyzed. Despite this, the correlation between the concentration of phenolic acids, total phenolic content and ORAC values was calculated.

Grain Quality Parameters

Thousand grain weight (TGW) of the ancient wheat species ranged from 24.3 to 49.4 g. The tetraploid species *T. timopheevii* Zhuk. ssp. *timopheevii* showed highest TGW, therefore the tetraploid species showed in total the highest TGW followed by the hexaploid species. No significant differences were observed in this set of data (Fig. 2). The experiment was characterized by a very high germination level of the grain samples, which partly had visible symptoms of germination. *T. turgidum* L. ssp. *turgidum* showed in total the highest germination with more than 40 % of the analyzed caryopses. The species seem to have a significant influence on germination rate because the tetraploid species showed the highest germination followed by the hexaploid species (Fig. 2). In the case of the parameter falling number (FN) the observed values were very low (62 s) because of a high amylase activity by germination (Fig. 3). These low falling numbers indicate a very high level of degradation of starch in caryopses. For the quality parameter protein there was no

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significant difference identifiable. The relative proportion ranged between 12.4 and 20.4 % crude protein of the whole grain (Fig. 3). As opposed to crude protein significant differences were observed for the sedimentation test. The results ranged from 8 to 34 with the highest mean value for the tetraploid species, whereas one cultivar of *T. aestivum* L. ssp. *compactum* showed the highest value overall (Fig. 3).

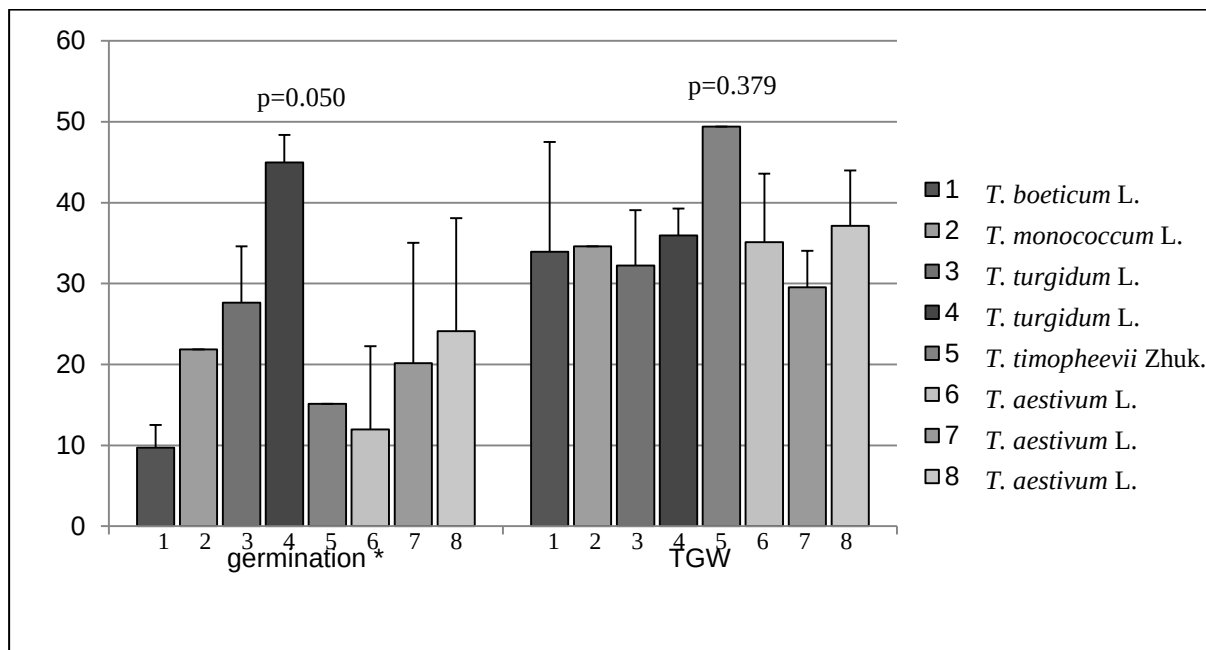


Fig. 2: Germination rate [%], and thousand grain weight (TGW) [g] of different wheat species (mean $\pm$ SD, $\alpha=0.05$)

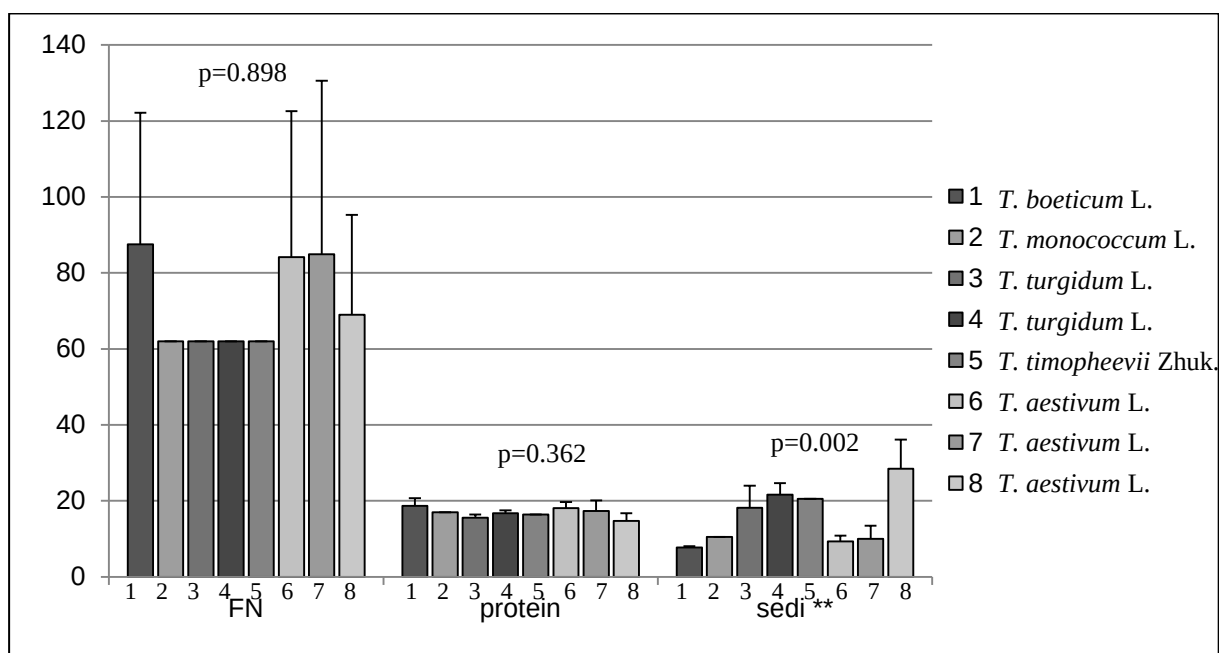


Fig. 3: Falling number (FN) [s], crude protein content [%], and sedimentation test of different wheat species (mean $\pm$ SD, $\alpha=0.05$)

Phenolic Acids by HPLC

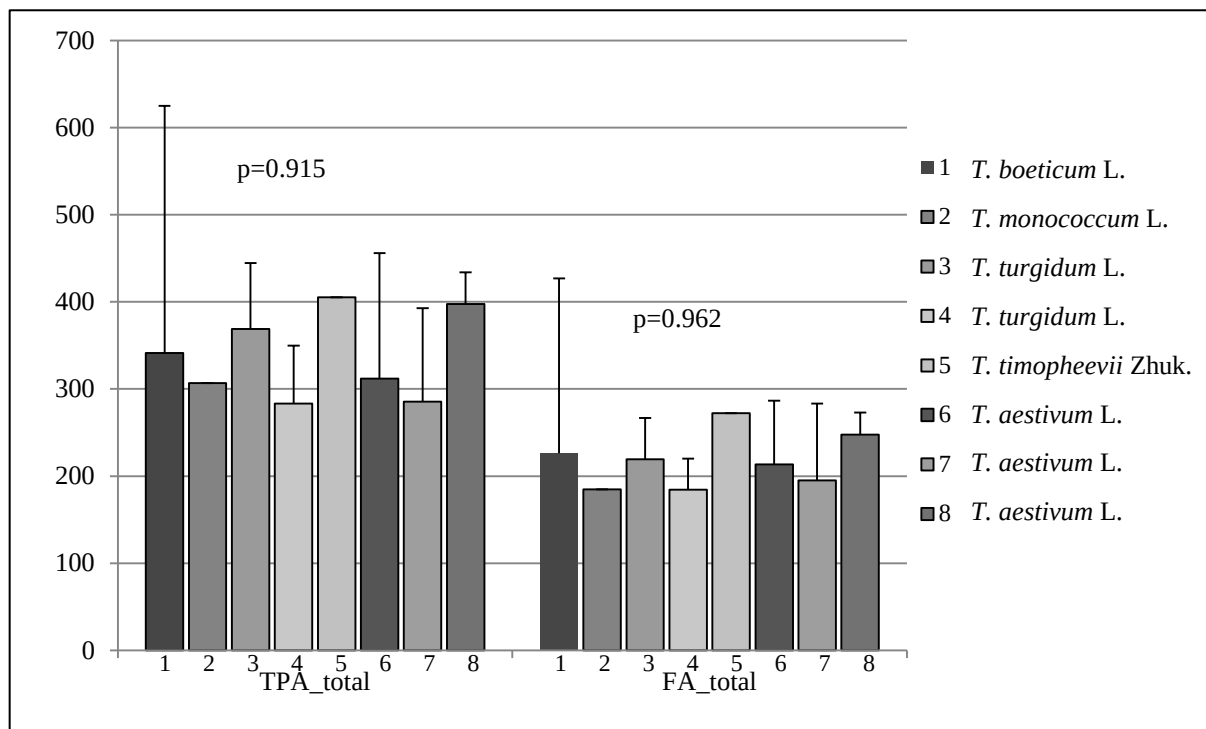
The mean value of TPA_{total} was 334.9 µg GAE/g (Tab. 2) and the individual concentration of the TPA_{total} in all accessions varied from minimum 141.0 µg GAE/g to maximum 542.0 µg GAE/g (not displayed). There was a large species variation with the highest concentration for *T. boeiticum* L. ssp. *boeiticum*. Neither between the species nor between the sections significant differences could be found for TPA_{total} or FA (Fig. 4). The value of the TPA consisted of six detected phenolic acids: vanillic acid, syringic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid. 70 % of phenolic acids were found as cell wall esterified compounds (TPA 3) followed by 23 % of free esters (TPA 2) and 7 % of free compounds (TPA 1) (Tab. 2). The main phenolic acid was ferulic acid with about 65 % (217.9 µg GAE/g) (Tab. 3) of the phenolic acids (Fig. 4).

Tab. 2: Concentration of phenolic acids (TPA) [µg GAE/g] in caryopses of different wheat species

[No.]	species	TPA 1	SD	TPA 2	SD	TPA 3	SD	TPA _{total}	SD
1	<i>T. boeiticum</i> L.	27.31 ±	15.66	81.75 ±	76.33	232.43 ±	191.55	341.49 ±	283.54
2	<i>T. monococcum</i> L.	15.38 ±	.	98.50 ±	.	192.89 ±	.	306.76 ±	.
3	<i>T. turgidum</i> L.	31.98 ±	13.39	88.75 ±	28.11	247.99 ±	46.86	368.71 ±	75.99
4	<i>T. turgidum</i> L.	14.92 ±	3.95	63.14 ±	10.82	205.20 ±	73.38	283.26 ±	66.34
5	<i>T. timopheevii</i> Zhuk.	21.67 ±	.	68.11 ±	.	315.51 ±	.	405.29 ±	.
6	<i>T. aestivum</i> L.	18.92 ±	15.06	68.61 ±	22.91	202.98 ±	84.76	312.02 ±	143.90
7	<i>T. aestivum</i> L.	22.76 ±	18.70	61.10 ±	7.69	201.45 ±	95.37	285.31 ±	107.59
8	<i>T. aestivum</i> L.	21.71 ±	5.71	87.58 ±	31.45	288.36 ±	27.47	397.65 ±	36.20
mean		21.83		77.19		235.85		334.87	
relative concentration [%]		6.52		23.05		70.43		100.00	
p (α=0.05)		0.823		0.859		0.818		0.915	

Tab. 3: Concentration of ferulic acid (FA) [μg GAE/g] in caryopses of different wheat species

[No.]	species	FA 1	SD	FA 2	SD	FA 3	SD	FA_total	SD
1	<i>T. boeiticum</i> L.	5.09 $\pm$ 1.33		25.88 $\pm$ 25.05		195.48 $\pm$ 173.94		226.44 $\pm$ 200.32	
2	<i>T. monococcum</i> L.	5.16 $\pm$.		25.73 $\pm$.		153.84 $\pm$.		184.73 $\pm$.	
3	<i>T. turgidum</i> L.	5.86 $\pm$ 0.03		26.30 $\pm$ 6.08		187.20 $\pm$ 42.49		219.36 $\pm$ 47.41	
4	<i>T. turgidum</i> L.	4.54 $\pm$ 0.93		26.99 $\pm$ 4.45		152.70 $\pm$ 39.14		184.23 $\pm$ 35.91	
5	<i>T. timopheevii</i> Zhuk.	5.32 $\pm$.		23.39 $\pm$.		243.54 $\pm$.		272.26 $\pm$.	
6	<i>T. aestivum</i> L.	4.86 $\pm$ 0.68		28.71 $\pm$ 4.68		213.86 $\pm$ 24.38		247.43 $\pm$ 25.42	
7	<i>T. aestivum</i> L.	4.55 $\pm$ 1.91		30.02 $\pm$ 3.43		178.86 $\pm$ 71.98		213.44 $\pm$ 73.06	
8	<i>T. aestivum</i> L.	2.77 $\pm$ 2.52		28.27 $\pm$ 2.95		163.89 $\pm$ 83.88		194.92 $\pm$ 88.21	
mean		4.77		26.91		186.17		217.85	
relative concentration [%]		2.19		12.35		85.46		100.00	
p ($\alpha=0.05$)		0.478		0.807		0.943		0.962	

**Fig. 4:** Total phenolic acids (TPA_total) and total ferulic acid (FA_total) values [μg GAE/g] in different wheat species (mean $\pm$ SD, $\alpha=0.05$)

Total Phenolic Assay by Folin-Ciocalteu

The TPC_total of the whole wheat flour (sum of all three fractions) measured by the Folin-Ciocalteu assay ranged from 2.3 to 2.6 mg GAE/g. Fraction 3 (TPC 3) was characterized by the highest total phenolic concentration (40.1 %) with a mean value of 1.0 mg GAE/g followed by fraction 1 (36.2 %) with a mean value of 0.9 mg GAE/g. The highest concentration of all samples was observed in fraction 1 (TPC 1) for *T. turgidum* L. ssp. *turgidum* with a value of 1.1 mg GAE/g. No significant difference

between the species could be found (Fig. 5). A strong correlation between TPA_total and TPC_total values was calculated ($r=0.847$, $p=0.00$). There was also a strong relationship between TPA_total and FA_total ($r=0.816$, $p=0.00$) (Tab. 4).

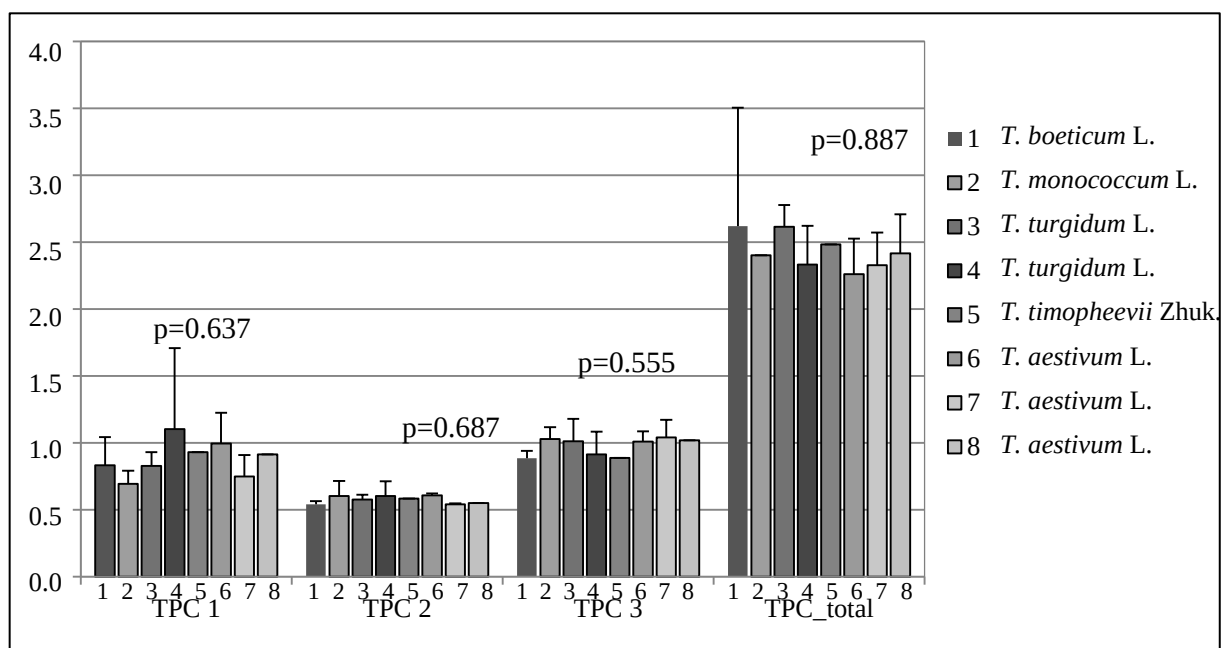


Fig. 5: Total phenolic content (TPC) [mg GAE/g] in different wheat species (mean $\pm$ SD, $\alpha=0.05$)

ORAC (oxygen radical absorbance capacity) Assay

The antioxidant capacity by the ORAC assay ranged from 18.2 to 23.8 $\mu\text{mol TE/g}$ whole wheat flour for the sum of all three fractions (ORAC_total). Highest ORAC values were measured in fraction 1 (ORAC 1) for the hexaploid wheat species *T. aestivum* L. ssp. *aestivum* with 11.1 $\mu\text{mol TE/g}$ followed by the diploid *T. boeiticum* L. ssp. *boeiticum* and the tetraploid *T. turgidum* L. ssp. *dicoccoides*. The lowest value was observed in *T. timopheevii* Zhuk. ssp. *timopheevii* in fraction 2 (ORAC 2) with 3.0 $\mu\text{mol TE/g}$ (Fig. 6). It was noticeable, that fraction 1 (ORAC 1) had the highest values holding 46.3 % of the ORAC values in total followed by fraction 3 (ORAC 3: 33.8 %). ORAC values were positively correlating with TPC_total on a moderate level (Tab. 4). A strong positive relationship between ORAC_total and fraction 1 of the TPC was observed ($r=0.618$, $p=0.01$) and no correlation between ORAC_total and fraction 2 and 3 of the TPC was found (Tab. 4).

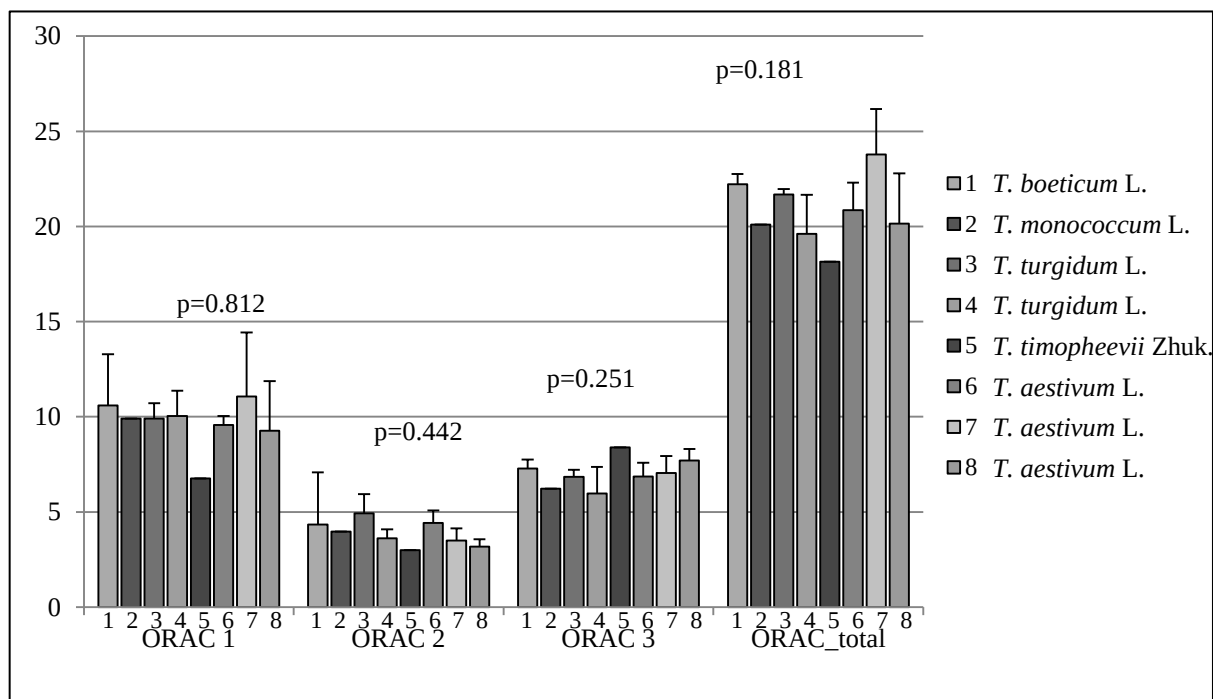


Fig. 6: Antioxidant capacity (ORAC) [$\mu\text{mol TE/g}$] in different wheat species for fraction 1, 2 and 3 (mean $\pm$ SD, $\alpha=0.05$)

Tab. 4: Pearson's correlation coefficients (r) between phenolic acids (TPA) and antioxidant capacity (TPC, ORAC) of different wheat species

	Phenolic acids				
fraction	TPA 1	TPA 2	TPA 3	TPA_total	FA_total
TPC_total	0.576 **	0.700 **	0.814 **	0.847 **	0.816 **
ORAC_total	0.618 **	0.182	0.116	0.469 *	0.371

* significant at $p < 0.05$

** significant at $p < 0.01$

Discussion

To our knowledge there are only a few studies published on the comparison of phenolic acid concentrations and the antioxidative capacity of ancient wheat (Li et al., 2008; ABDEL-AAL and RABALSKI, 2008; SERPEN et al., 2009). For that reason the aim of this study is to evaluate the phenolic acid concentrations of different ancient wheat varieties (accessions) to learn more about those varieties. It is necessary to find out more about phenolic compounds and the antioxidative capacity in ancient wheat, as they are an alternative to bread wheat. Secondary plant metabolites, like phenolic acids, contribute to health promoting effects of wheat and consumers are increasingly interested in health promoting substances. Since the highest proportion

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of phenolic acids is located in bran and aleurone layer, the analysis of phenolic acids, total phenolic content and antioxidative capacity was carried out in whole wheat flour (PUSSAYANAWIN et al., 1988; ADOM et al., 2005; ZHOU et al., 2004; ANSON et al., 2008).

The germination rate was relatively high, ranging between 0 and 48 % which resulted in low falling numbers with an average of 62 s. Low falling numbers are induced by the high degradation of starch during the process of germination. It can be supposed that humid conditions during grain ripening and a tendency of lodging led to germination before harvesting.

The analyzed phenolic acids (TPA_{total}), which result by addition of the concentrations of the three fractions, differed between 283.3 µg GAE/g for *T. turgidum* L. subsp. *turgidum* and 405.3 µg GAE/g for *T. aestivum* L. subsp. *compactum*. Einkorn (*T. monococcum* L. subsp. *monococcum*) showed the average concentration of 306.8 µg GAE/g and emmer (*T. turgidum* L. subsp. *dicoccoides*) 368.7 µg GAE/g. The phenolic acid concentrations ranged widely and they were comparable to those reported for bread wheat in the literature. For instance, STRACKE et al. (2009) reported concentrations between 282 µg/g and 1262 µg/g in wheat samples, ZHOU et al. (2005) analyzed the phenolic acid composition of hard red winter wheat bran extracts with low concentrations between 202.6 µg/g and 244.1 µg/g and LI et al. (2008) reported average contents of 615 µg/g (einkorn), 779 µg/g (emmer), and 579 µg/g (spelt).

Highest concentration of phenolic acids in the used ancient wheat samples showed fraction 3 (TPA 3, bound phenolic acids), which was also in line with literature (STRACKE et al., 2009; ABDEL-AAL et al., 2001). Phenolic acids are bound to hydrolysable tannins, lignins, cellulose and proteins which are mainly structural components of bran, building a protective layer to the seed. Phenolics, among phenolic acids, play a role in defending mechanisms against pathogens, parasites and predators (LIU, 2004). Also, they have antioxidant properties in plants (PARR and BOLWELL, 2000; GRAF, 1992). During cell elongation ferulic acid is proposed to increase wall extensibility (GRAF, 1992). In this study about 70 % were bound to cell wall components, which was lower than in other studies, where up to 98 % were bound phenolic components (STRACKE et al., 2009; LI et al., 2008). Free phenolic acids showed the smallest contribution to total phenolic acids ranging between 1 % and 2 % (LI et al., 2008; ABDEL-AAL et al., 2001). In the present study fraction 1 (TPA 1) contributed 5 to 8 % to TPA_{total}, which was higher than in the other

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studies. Reasons for the ranging results could be that einkorn and emmer produce more free phenolic acids than bound forms to protect the plant material against antioxidative stress. If it is the case, that ancient wheat have a higher concentration of soluble free phenolic acids, the bioavailability and bioaccessibility could be higher than in bread wheat. E. g. free ferulic acid can be resorbed in the small intestine, whereas bound forms have to be released by bacterial hydrolytic enzymes during fermentation in the large intestine (ANSON et al., 2009; KROON et al., 1997; ZHAO et al. 2003). Furthermore, different extraction methods are the reason for ranging amounts of phenolic compounds of the analyzed fractions, which leads to the suggestion, that a standardized method should be implemented.

Ferulic acid was the predominant phenolic acid with about 66 % in wheat, averaging between 185 µg GAE/g and 247 µg GAE/g. Highest concentration of ferulic acid was found in the hexaploid species (*T. aestivum*). The diploid species (*T. boeoticum* and *T. monococcum*) and the tetraploid species (*T. turgidum* and *T. timopheevii*) could keep up with the hexaploid ones. Bound ferulic acid (FA 3) contributed the highest concentration of the total ferulic acid in the analyzed ancient wheat samples. Ferulic acid is mostly bound to arabinoxylans and other indigestible polysaccharides restricting its release in the small intestine (ANSON et al., 2009). In the present study the concentrations of ferulic acids were lower compared to those reported previously. LI et al. (2008) stated 298 µg/g for einkorn, 476 µg/g for emmer, 365 µg/g for spelt, and 395 µg/g for winter wheat. STRACKE et al. (2009) found about 85 % (239-1072 µg/g) ferulic acid of the analyzed phenolic acids in total.

Total phenolic contents of the present ancient wheat samples were reported as gallic acid equivalents in mg/g whole wheat flour. The highest TPC was found in fraction 3 of the whole grain flour, where phenolic compounds exist in bound forms (STRACKE et al., 2009; ABDEL-AAL et al., 2001; Adom and Liu, 2002). In addition, there were high values of fraction 1. The Folin-Ciocalteu assay does not only display phenolics, it also reacts with nonphenolic substances such as vitamin C or other organic acids (HUANG et al., 2005; GEORGÉ et al., 2005). That could be an explanation for the high values of fraction 1, where further free phenolic and nonphenolic substances could be present. Other studies did not analyze those three fractions, as they divided into flour and bran. The values of the present study for the total phenolic contents in whole wheat flour (TPC_{total}: 2.4 mg GAE/g) were higher than in the literature reported for wheat samples. ADOM et al. (2005) reported 176-195 µmol GAE/100 g (0.3-0.4 mg GAE/g) for the endosperm fraction of hexaploid wheat. SERPEN et al.

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(2008) found total phenolic content values for einkorn with the average of 3.37 $\mu\text{mol/g}$ (0.6 mg GAE/g), for emmer with 6.33 $\mu\text{mol/g}$ (1.2 mg GAE/g) and for bread wheat with 4.36 $\mu\text{mol/g}$ (0.8 mg GAE/g). The widely ranging total phenolic contents may be due to different extracting methods, such as inclusion of soluble free, soluble conjugated and bound phenolic acids. DEWANTO et al. (2002) and LIYANA-PATHIRANA and SHAHIDI (2006) drew a similar conclusion. In the current study there were no significant differences in total phenolic contents between the analyzed *Triticum* L. species. Einkorn and emmer were at about the same level of total phenolic contents as bread wheat. TPA_{total} could be attributed to TPC_{total} because of a strong correlation between the total phenolic acids and total phenolics ($r=0.847$, $p=0.00$).

Comparing the antioxidative capacity of the ORAC test in whole wheat flour with the literature was difficult because different ORAC assays and extraction methods had been applied (MOORE et al., 2005; ZHOU et al., 2007; LIYANA-PATHIRANA and SHAHIDI, 2006; OKARTER et al., 2010). In the present study ORAC values (ORAC_{total}) ranged from 15.1 to 25.2 $\mu\text{mol TE/g}$, which was lower than in most of other investigations of wheat reported in the literature. For instance, ORAC values of soft wheat cultivars investigated by MOORE et al. (2005) ranged from 32.9 to 47.7 $\mu\text{mol TE/g}$. Slightly higher ORAC values of 51 to 96 $\mu\text{mol TE/g}$ for whole wheat flours of different cultivars could be found by OKARTER et al. (2010). In contrary much higher values of 3406 $\mu\text{mol TE/g}$ defatted material were measured by LIYANA-PATHIRANA and SHAHIDI (2006). The analyzed soft wheat cultivars of ZHOU et al. (2007) varied between 15.5 $\mu\text{mol TE/g}$ and 24.5 $\mu\text{mol TE/g}$, which is in line with the current study.

In the current study three separate fractions of the grain samples (soluble free, soluble conjugated, and bound phenolic acids) were used for HPLC and ORAC analysis. It could be observed that ORAC values in fraction 1 (ORAC 1, free phenolic acids) contribute 47.7 % to the total ORAC values and they are even higher than in fraction 3 (ORAC 3, bound phenolic acids). The bound fraction was reported to contribute about 86 % to the total ORAC (LIYANA-PATHIRANA and SHAHIDI, 2006). However, in the present study fraction 1 of the total phenolic acids was correlating with the ORAC values ($r=0.618$, $p=0.05$), therefore the sum of all three TPA fractions were correlating with ORAC_{total} ($r=0.469$, $p=0.04$).

In conclusion, the current study showed, that TPA and TPC values of the applied ancient wheat samples were comparable to the values in the literature reported for bread wheat or spelt wheat. It could be supposed that phenolic acid contents have

not been considerably changed during the evolutionary development of wheat although there is a genetic diversity between einkorn, emmer, spelt and bread wheat. Therefore, health-beneficial effects reported for bread wheat seem to be present in ancient wheat, too. The antioxidative capacity of ancient wheat, e.g. einkorn and emmer, is comparable to bread wheat which leads to the suggestion of using those wheat varieties as an alternative to bread wheat. Einkorn, emmer and spelt, especially used as whole grain flours, could be taken into consideration for human diets, with health benefits. But still, the low bioaccessibility of phenolic acids, especially of ferulic acid from cereals, has to be improved. Reasons for the wide variation of the phenolic acid concentrations within and between species have to be analyzed further. The effect of the germination rate on phenolic acids, total phenolic content and antioxidative capacity needs to be conducted in further studies.

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5.2 Experiment 2 “Sprouting experiment with bread wheat”

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Germany

EFFECT OF SPROUTING ON THE CONCENTRATION OF PHENOLIC ACIDS AND ANTIOXIDATIVE CAPACITY IN WHEAT CULTIVARS (*TRITICUM AESTIVUM* SSP. *AESTIVUM* L.)

IN DEPENDENCY OF NITROGEN FERTILIZATION

N. Engert, A. John, W. Henning, B. Honermeier

Summary

The study was conducted to analyze the effects of sprouting on content and composition of phenolic acids (PA) and antioxidative capacity by the Folin-Ciocalteu (total phenolic content (TPC)) and ORAC (Oxygen Radical Absorbance Capacity) assay depending on nitrogen application and cultivar. Three wheat cultivars (cv. Tommi, cv. Privileg and cv. Estevan) were treated with two different nitrogen (N) levels (N₁ without N and N₂ with 150 kg N/ha). Three fractions of the grinded wheat caryopses (free, free-insoluble and bound phenolic acids) were extracted. Mean values for total phenolic acids (TPA) ranged between 498.1 and 1726.2 µg GAE/g with significant differences for the cultivars in not sprouted samples. Nitrogen fertilization showed significant differences with lower TPA contents for not fertilized grain. Even the interaction cultivar x nitrogen was significant for not sprouted grain. The statistical analysis of sprouting time did not show effects on TPA values after 24 h and 48 h of sprouting. In the present study no significant influence of cultivar or nitrogen application was identified for TPC and ORAC values. In contrary to that, the effects of sprouting time on the antioxidative capacity by Folin-Ciocalteu and ORAC were significant. After 48 h of sprouting the values of TPC (2575.6 µg GAE/g) and ORAC (32.6 µmol TE/g) were significantly higher than for not sprouted wheat samples (TPC: 2054.8 µg GAE/g, ORAC: 28.2 µmol TE/g). The longer the sprouting time was, the higher the antioxidative capacity of free phenolic acids, indicating that more free phenolic acids were released and other phytochemicals might have been present after sprouting.

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Since there are only little information about the effect of sprouting time on phenolic acids and antioxidative capacity in dependence on nitrogen application further studies need to be conducted.

Keywords: wheat, sprouting, phenolic compounds, phenolic acids, antioxidative capacity, Folin-Ciocalteu, ORAC

Introduction

Cereals, especially wheat (*Triticum aestivum* L. ssp. *aestivum*), provide an important contribution to the human diet with their basic nutrients carbohydrates, protein, dietary fiber, vitamins and minerals. Besides, they contain secondary plant metabolites such as carotenoids, flavonoids and phenolic acids that complement a balanced diet by means of their antioxidative potential. As a result of enhanced health awareness during the last years, natural antioxidants gained considerable interest due to their health beneficial functions. E. g. phenolic acids show health affecting potentials because of their antioxidativity (SERPEN, 2008). Potential effects of phenolic compounds are to reduce the risk of cardiovascular diseases and cancer (KNEKT et al. 2002, PETERSON et al., 2003, SESSO et al. 2003, ARTS and HOLLMAN, 2005, GRAF et al. 2005). Wheat phenolic acids (PA), such as ferulic (FA), *p*-coumaric (PCA), vanillic (VA), sinapic (SIA), syringic (SYA) and caffeic acid (CA) are valuable antioxidativ ingredients (MPOFU, 2006). It is reported that phenolic acids inhibit lipid oxidation by scavenging free radicals such as hydroxyl radicals (CUPPETT et al., 1997). In addition, sprouts are considered to be health beneficial, even more than the cereal grain itself (KAHLIL and MANSOUR, 1995, PRODANOV et al., 1997, YANG et al., 2001, ARORA et al., 2010, TIAN et al., 2010). However, for some processes, like the production of bread, it is extremely necessary that wheat caryopses are of a very high quality with no degradation of their compounds. Sprouting is a result of humid conditions during and after grain development or storage. By reason of sprouting the starchy endosperm can be reduced because of enzymatic degradation processes, e.g. by α -amylase.

Little information is available about the effect of nitrogen fertilization on the content of phenolic acids. Germination of the caryopses induces a higher amylase activity in caryopses resulting in lower starch contents. During this process other components like phenolic acids might be changed, too. Thus, the aim of this study was to

investigate phenolic acids, their composition and their variation in three different wheat cultivars in dependency on sprouting time and nitrogen fertilization.

Materials and Methods

In 2008 winter wheat cultivars were grown in field plots of the experimental station “Rauischholzhausen” of the Institute of Agronomy and Plant Breeding (degree of longitude: 8° 52' 05" E, degree of latitude: 50° 46' 41" N, altitude: 222 m). Three cultivars (cv. Tommi, cv. Privileg, cv. Estevan) of *T. aestivum* ssp. *aestivum* L. with four randomized replications were obtained from the field experiment. Two nitrogen levels were analyzed: N₁ without N fertilization and N₂ with 150 kg N/ha (40, 40, 70 kg N/ha). To avoid lodging of wheat plants all plots were treated with the plant growth regulator chlorcholin chloride (CCC, 720 g/l) at two times. To protect the wheat against diseases fungicides were applied at two times (first: 0.8 l/ha Proline + 1.5 l/ha Pugil and second: 0.7 l/ha Diamant + 0.7 l/ha Champion). After harvesting the samples were stored in darkness at 20 °C until they were used for the sprouting experiment.

To induce sprouting 30 g wheat were steeped with 15 ml aqua dest. in petri dishes which were covered with ashless circular filters (MN 640 m, 90 mm, Macherey-Nagel). Three different sprouting times were tested at room temperature, without direct solar radiation: 0 h (no sprouting), 24 h (presprouted wheat samples) and 48 h (fully sprouted wheat samples) (Fig. 1). To stop the sprouting process, all samples were dried for 24 h at 36 °C.

In total 72 wheat samples (3 cultivars x 3 sprouting levels x 2 N fertilization doses x 4 replications) were included and ground with a 500 µm sieve on a Cyclotec 1073 Sample Mill (Foss, Germany) to get a whole wheat flour. The samples were mixed thoroughly to ensure homogeneity. Extraction followed immediately.

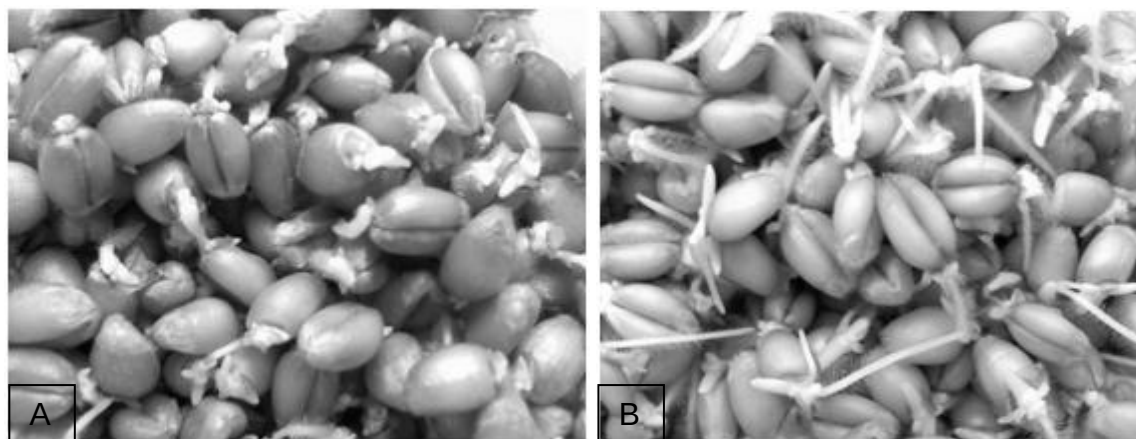


Fig. 1: Different sprouting levels of wheat: A- presprouted grain after 24 h; B- fully sprouted grain after 48 h of sprouting

Extraction: For the analysis of phenolic acids, samples were extracted using a modified method by KRYGIER et al. (1982) and ADOM and LIU (2002) as follows. Whole wheat flour (1 g) was extracted with 10 ml methanol/acetone/water (7:7:6, v/v/v) and divided into three fractions: (1) soluble free, (2) soluble conjugated, and (3) bound phenolic acids. The supernatant provided fraction 1 and 2, the residue fraction 3. Before all fractions were extracted with ethyl acetate, bound and soluble conjugated phenolic acids had to be hydrolyzed with 5 M NaOH for three hours. The reaction was stopped with 5 M HCL and the extracts were shaken out with ethyl acetate. The organic extracts were transferred into flasks and ethyl acetate was evaporated. Referring to that, extracts were resumed in 10 ml of 10 % methanol and stored at -18 °C for all further analysis (HPLC, Folin, ORAC).

Quality Parameters: Generally, parameters concerning grain quality were measured to characterize the used wheat cultivars before starting the sprouting experiment. According to ICC and ISTA standard methods thousand grain weight (TGW), crude protein (CP), falling number (FN), gluten index and sedimentation test by Zeleny (Sedi) were analyzed. The parameter falling number was additionally used to determine the state of sprouting. That means, grain with no sprouting showed highest mean falling numbers (419 s), grain which was presprouted showed lower (104 s) and fully sprouted grain had lowest falling numbers (62 s).

HPLC: The HPLC method according to WEIDNER et al. (2000) and ZIELINSKI et al. (2001) was used with some modifications for determination of phenolic acids (PA) and total phenolic acids (TPA) as the sum of all analyzed phenolic acids. The analysis was performed on a HPLC-DAD system by Knauer on a C18 column (EC 250 x 4 Nucleodur Sphinx RP, 5 µm (MN)) at a temperature of 25 °C, a flow rate of

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1 ml min⁻¹ and an injection volume of 100 µl. The detection was carried out at 250 nm (hydroxybenzoic acids) and 290 nm (hydroxycinnamic acids) following the program: 0-3 min 20 % A, 3.5-7.5 min 0 % A, 8-14 min 8 % A, 14.5-32 min 30 % A, 33-39 min 95 % and 40-45 min 20 %. The gradient system consisted of (A) 95 % methanol with acetic acid (about 200 µl) adjusted to pH 4.5 and (B) water adjusted to pH 4.5 with about 5 µl acetic acid.

Folin-Ciocalteu Assay: The Folin-Ciocalteu Assay is an electron transfer based reaction assay, and measures the reducing capacity of the sample (HUANG et al., 2005). In the present analysis it is called total phenolic content (TPC) and the Folin-Ciocalteu micro method by WATERHOUSE (2001) was used. Folin-Ciocalteu reagent consisted of phosphotungstic (H₃PW₁₂O₄₀) and phosphomolybdic (H₃PMo₁₂O₄₀) acids, additionally gallic acid was used as standard. To measure the absorbance spectrophotometrically at 765 nm with a Specord 205 – 222A430 (Analytik Jena AG, Germany), the Folin-Ciocalteu reagent had to be reduced to blue oxides of tungsten and molybdenum. The total phenolic content was expressed as gallic acid equivalent (GAE).

ORAC Assay: ORAC (Oxygen Radical Absorbance Capacity) is a hydrogen atom transfer based reaction assay to measure the antioxidative capacity towards peroxy radicals (HUANG et al., 2005). A previously described method by HUANG et al. (2002) was used and the analysis was operated on a 96-well plate fluorescence reader Fluoroskan (Fisher Scientific GmbH, Germany). Very briefly, fluorescein (6-hydroxy-9-(2-carboxyphenyl)-(3h)-xanthen-3-on) and the sample were mixed and incubated at 37 °C. To initiate the reaction the ROS-generator AAPH (2,2'-azobis(2-methylpropionamidine)dihydrochloride) was added to measure fluorescence at excitation wavelength 485 nm and emission wavelength 538 nm every 60 s for 90 min. As a standard Trolox[®] (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a vitamin E analogue, was used and the antioxidative capacity was expressed as Trolox equivalent (TE).

Statistical Analysis: One-way analysis of variance (ANOVA) was performed with PASW Statistics 18 (SPSS Inc., Chicago, IL) for all wheat cultivars (cv. Tommi, cv. Privileg, cv. Estevan) and nitrogen levels (N₁ and N₂) within all sprouting times (no sprouting, presprouted, fully sprouted) to determine the effects on the measured parameters. When significant differences occurred (p≤0.05) multiple mean comparisons were performed using the Tukey t-test. To determine the influence of sprouting time on the analyzed parameters a paired samples test (t-test) was

performed. Spearman's correlation (r_s) factors (bivariate) were calculated between total phenolic acids (TPA), total phenolic content (TPC) and antioxidative capacity (ORAC). Results are reported as mean values and the significance level was at a 95 % confidence interval ($\alpha=0.05$).

Results

Quality Parameters: Thousand grain weight (TGW) ranged from 40.1 to 45.0 g with significant differences (≤ 0.001) between all three cultivars (table 1). Cv. Privileg could be characterized as the cultivar with largest grain (45.0 g) followed by cv. Tommi (42.8 g) and cv. Estevan (40.1 g). Analyzed falling numbers (FN) ranged from 323 to 388 s significantly affected by the cultivars ($p\leq 0.001$) and N fertilization ($p\leq 0.001$). Cv. Tommi with a mean value of 388 s showed the highest falling number which was significantly higher than cv. Estevan with 323 s. Cv. Tommi and cv. Privileg (377 s) were at the same level, which indicates a very low amylase activity in the caryopses. Nitrogen fertilized samples had higher FN (381 s) than not fertilized (345 s) ones. Sedimentation volumes (Fig. 2) differed between 33 ml and 58 ml with significantly ($p=0.003$) interaction effects for cultivar and nitrogen treatment. Nitrogen application to cv. Estevan (58 ml) and cv. Tommi (55 ml) induced significantly higher sedimentation volumes than nitrogen application to cv. Privileg (44 ml). Gluten Indices (GI) ranged between 81 and 92 indicating a high gluten quality. N fertilization ($p\leq 0.001$) and cultivar ($p\leq 0.001$) were significant with regard to GI. Fertilized wheat samples had significantly lower GI than not fertilized wheat samples. The cultivar Estevan (92) reached significantly higher GI than cv. Tommi (81) and cv. Privileg (84) which were at the same level. Crude protein (CP) ranged between 12.5 and 12.9 %. As to expect, nitrogen treatment (13.6 %) led to higher contents of crude protein ($p\leq 0.001$) in wheat caryopses than in unfertilized (11.6 %) ones, whereas between cultivars no significant CP differences were observed.

Tab. 1: Effect of N fertilization and cultivar on thousand grain weight (TGW, [g]), falling number (FN, [s]), gluten index (GI) and crude protein (CP, [% DM]) in wheat samples

Treatment (cultivar/nitrogen)	TGW g	FN s	GI	CP % DM
Main effect cv.				
Estevan	40.1 c	323 b	92 a	12.9 n.s.
Privileg	45.0 a	377 a	84 b	12.5 n.s.
Tommi	42.8 b	388 a	81 b	12.5 n.s.
Main effect N				
N ₁	42.7 n.s.	345 a	93 a	11.6 a
N ₂	42.6 n.s.	381 b	78 b	13.6 b
p cultivar (cv.)	0.000	0.000	0.000	n.s.
p nitrogen (N)	n.s.	0.001	0.000	0.000
p cv. x N	n.s.	n.s.	n.s.	n.s.

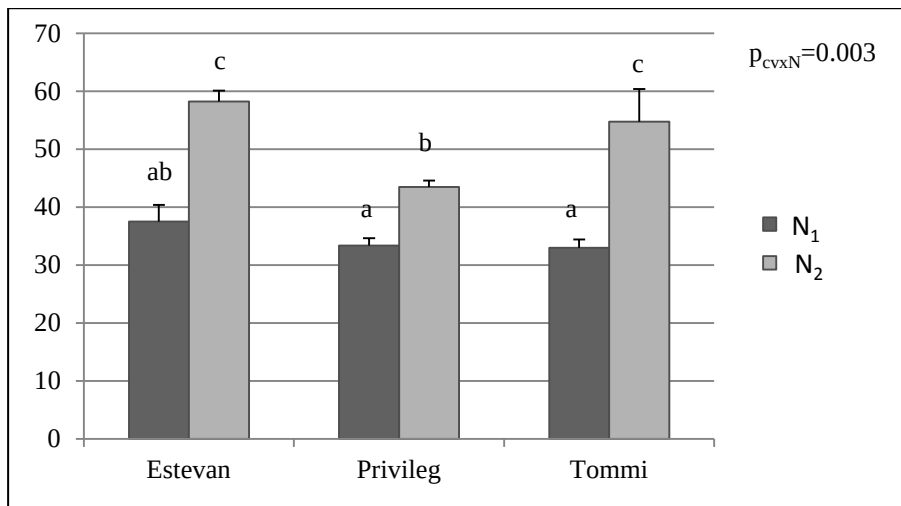


Fig. 2: Interaction effect of N fertilization and cultivar on sedimentation by Zeleny in wheat samples (mean ± SD, $\alpha=0.05$)

Phenolic Acids (PA): The analyzed total phenolic acids (TPA) consisted of vanillic acid (VA), syringic acid (SYA), caffeic acid (CA), p-coumaric acid (PCA) and ferulic acid (FA), measured by the HPLC method. Results were expressed as mean values in sum of the measured phenolic acids, ranging between 498.1 and 1726.2 $\mu\text{g GAE/g}$. Cultivars which were not sprouted showed significant differences ($p \leq 0.001$) in their TPA contents. However, cv. Privileg (1066.1 $\mu\text{g GAE/g}$) and cv. Estevan (1154.3 $\mu\text{g GAE/g}$) had significantly lower values than cv. Tommi (1726.2 $\mu\text{g GAE/g}$). TPA values of non-fertilized wheat ($N_1=1085.7 \mu\text{g GAE/g}$) were significantly lower than of fertilized ones ($N_2=1545.3 \mu\text{g GAE/g}$). Moreover, significant ($p \leq 0.001$) interaction effects between cultivar and nitrogen occurred for not sprouted wheat samples mainly resulting from cv. Tommi x N_2 which was about

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twice as high than the other cultivar x nitrogen interactions. After a sprouting time of 24 h an interaction effect between cultivar and nitrogen fertilization ($p=0.044$) was observed. The combination of cv. Tommi x N₂ showed once more the highest TPA value (1669.0 µg GAE/g) which significantly differed only from cv. Tommi x N₁ (760.5 µg GAE/g). After a sprouting time of 48 h a significant cultivar effect on TPA was noticeable (table 2). The cultivars cv. Tommi (1642.2 µg GAE/g) and cv. Estevan (1554.0 µg GAE/g) had the same level of TPA contents which was significantly higher than the TPA of cv. Privileg (498.1 µg GAE/g). Nitrogen application had no significant effects on TPA contents of the analyzed wheat samples but a tendency of higher TPA contents of N₂ fertilized samples in comparison with the N₁ fertilization level was observed.

Looking at the means of TPA values of the three sprouting treatments, which varied from 1315.5 µg (0 h) to 1099.2 µg (24 h) and 1231.4 µg GAE/g (48 h), no significant differences were detectable.

Tab. 2: Effect of sprouting time on the total phenolic acids (TPA) [µg GAE/g] in wheat samples depending on N fertilization and cultivar

Treatment (cultivar/nitrogen)	Sprouting time [h]		
	0	24	48
Main effect cv.			
Estevan	1154.3 a	1148.6 n.s.	1554.0 a
Privileg	1066.1 a	934.4 n.s.	498.1 b
Tommi	1726.2 b	1214.8 n.s.	1642.2 a
Main effect N			
N ₁	1085.7 a	1048.5 n.s.	1151.7 n.s.
N ₂	1545.3 b	1149.9 n.s.	1311.2 n.s.
Interaction effect			
Estevan x N ₁	1136.9 a	1500.5 ab	1542.7 n.s.
Estevan x N ₂	1171.8 a	796.7 ab	1565.3 n.s.
Privileg x N ₁	1057.3 a	884.5 ab	359.1 n.s.
Privileg x N ₂	1074.9 a	984.2 ab	637.1 n.s.
Tommi x N ₁	1063.0 a	760.5 a	1553.3 n.s.
Tommi x N ₂	2389.3 b	1669.0 b	1731.1 n.s.
Mean	1315.5 n.s.	1099.2 n.s.	1231.4 n.s.
p cultivar (cv.)	0.000	n.s.	0.002
p nitrogen (N)	0.000	n.s.	n.s.
p cv. x N	0.000	0.044	n.s.

Total phenolic acids (TPA) were most concentrated in fraction 3 (TPA3) for all three sprouting times (fig. 3). Unsprouted wheat samples contained 86 % (1128.4 µg GAE/g) in fraction 3 followed by fraction 1 (TPA1, 77.9 µg GAE/g) and

fraction 2 (TPA2, 109.3 $\mu\text{g GAE/g}$) with less than 10 %. While sprouting for 24 h the relative and absolute values did not change much. After 48 h TPA1 increased to 28 % (346.3 $\mu\text{g GAE/g}$) and TPA3 provided 59 % (722.3 $\mu\text{g GAE/g}$). TPA2 rose up to 13 % (162.8 $\mu\text{g GAE/g}$).

Looking at the composition of the five analyzed phenolic acids, the predominant phenolic acid was ferulic acid. During sprouting the proportion of each analyzed phenolic acid shifted. Nevertheless, ferulic acid (FA) was the main phenolic acid with 67 % (881.8 $\mu\text{g GAE/g}$) for not sprouted, 74 % (809.3 $\mu\text{g GAE/g}$) for presprouted and 54 % (669.9 $\mu\text{g GAE/g}$) for fully sprouted grain (fig. 4).

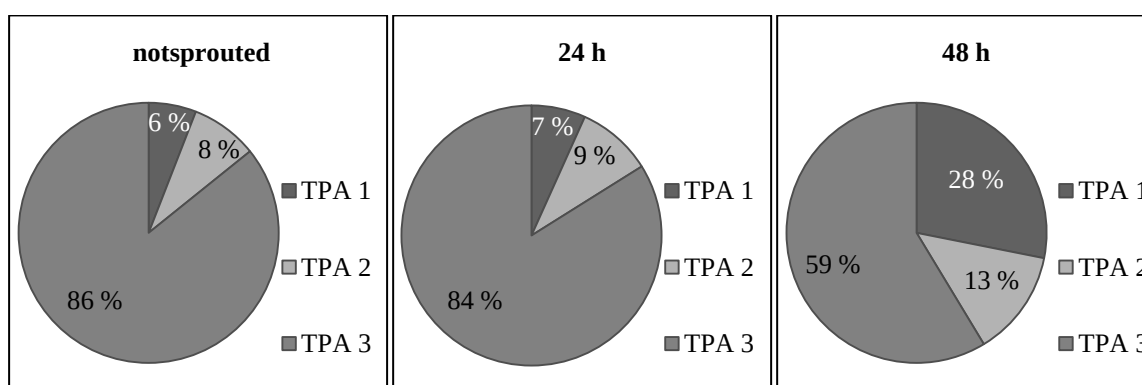


Fig. 3: Effect of sprouting time on the composition of the extracted fractions (TPA1, TPA2, TPA3) of total phenolic acids (TPA) in wheat samples

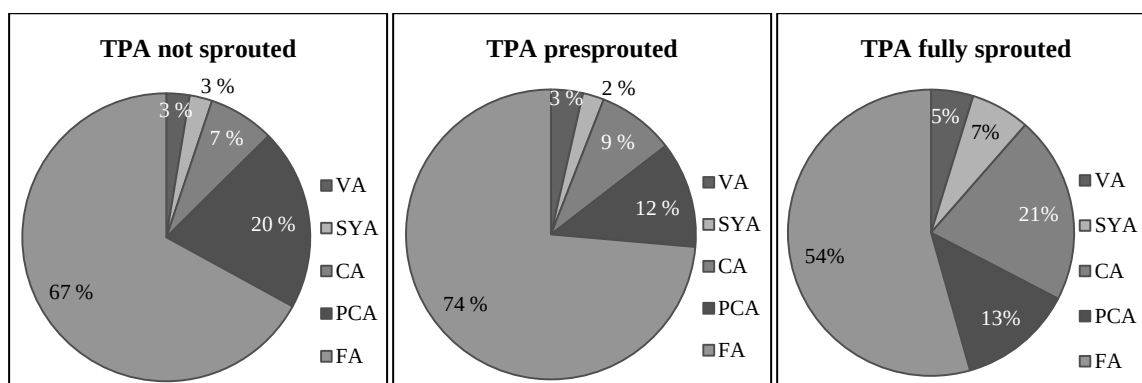


Fig. 4: Effect of sprouting time on the composition of phenolic acids (TPA) in wheat grain

Total Phenolic content (TPC): The total phenolic content (TPC) analyzed by the Folin-Ciocalteu assay ranged between 1523.8 and 2836.7 $\mu\text{g GAE/g}$ (tab. 3). At the beginning of the experiment not sprouted wheat samples were characterized by a small variability of TPC values. Neither main effects (cultivars, nitrogen) nor interaction effects (cv. x N) could be observed. Contrary to that after 24 h of sprouting

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there was a significant nitrogen effect of TPC expressed by lower TPC for N₁ (1464.6 µg GAE/g) in comparison to N₂ (2038.6 µg GAE/g). After 48 h of sprouting the treatments cultivar ($p=0.008$) and fertilization ($p=0.011$) showed significantly effects, however, the interaction between both parameters is not significant. Cv. Estevan (2836.7 µg GAE/g) and cv. Tommi (2684.9 µg GAE/g) had significantly higher TPC values than cv. Privileg (2205.2 µg GAE/g). TPC values for fertilized wheat (N₂=2790.1 µg GAE/g) were significantly higher than for non-fertilized samples (N₁=2361.0 µg GAE/g).

A sprouting time of 48 h significantly ($p\leq 0.001$) influenced the TPC. After 48 h (2575.6 µg GAE/g) of sprouting the TPC were higher than after 24 h (1751.6 µg GAE/g) and for unsprouted wheat samples (2054.8 µg GAE/g) (tab. 3). The highest TPC provided fraction 1 (TPC1) for all three sprouting times. Unsprouted wheat samples showed a total phenolic antioxidative capacity of 46 % (940.2 µg GAE/g for TPC1, followed by fraction 3 (TPC3) with 28 % (574.7 µg GAE/g) and fraction 2 (TPC2) 26 % (539.7 µg GAE/g). During 24 h of sprouting, TPC1 (38 %, 678.1 µg GAE/g) lost some of its antioxidative capacity while TPC3 (35 %, 574.2 µg GAE/g) gained. After 48 h TPC1 (46 %, 1194.3 µg GAE/g) and TPC2 (31 %, 799.8 µg GAE/g) values increased whereas TPC3 (23 %, 581.5 µg GAE/g) decreased.

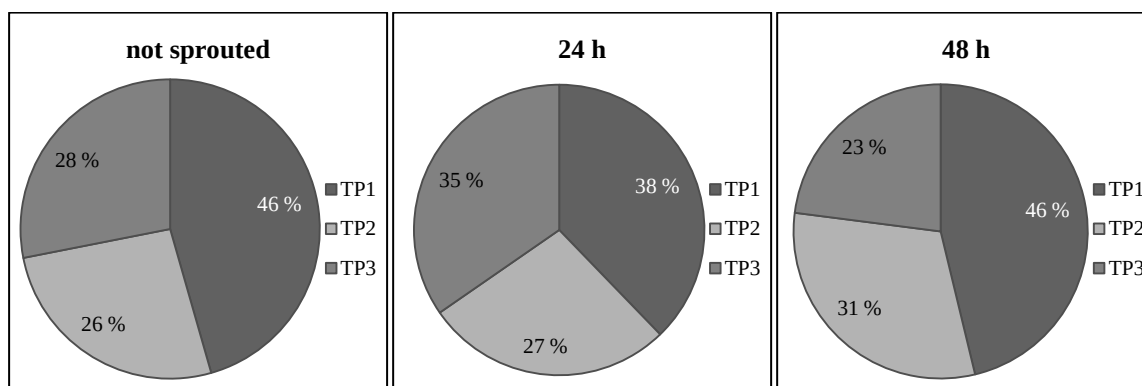


Fig. 1: Effect of sprouting time on the composition of the extracted fractions (TPC1, TPC2, TPC3) of total phenolic contents (TPC) in wheat samples

Tab. 3: Effect of sprouting time on the total phenolic content (TPC) [$\mu\text{g GAE/g}$] in wheat samples depending on N fertilization and cultivar

Treatment (cultivar/nitrogen)	Sprouting time [h]		
	0	24	48
Main effect cv.			
Estevan	2012.0n.s.	1523.8n.s.	2836.7a
Privileg	2170.5n.s.	1646.7n.s.	2205.2b
Tommi	1981.9n.s.	2084.4n.s.	2684.9a
Main effect N			
N ₁	1970.6n.s.	1464.6a	2361.0a
N ₂	2139.0n.s.	2038.6b	2790.1b
Interaction effect			
Estevan x N ₁	2090.9n.s.	1031.6n.s.	2634.9n.s.
Estevan x N ₂	1933.1n.s.	2015.9n.s.	3038.5n.s.
Privileg x N ₁	2005.9n.s.	1656.9n.s.	2151.2n.s.
Privileg x N ₂	2335.2n.s.	1636.6n.s.	2259.1n.s.
Tommi x N ₁	1815.1n.s.	1705.4n.s.	2297.0n.s.
Tommi x N ₂	2148.7n.s.	2463.5n.s.	3072.7n.s.
Mean	2054.8a	1751.6a	2575.6b
p cultivar (cv.)	n.s.	n.s.	0.008
p nitrogen (N)	n.s.	0.029	0.011
p cv. x N	n.s.	n.s.	n.s.

ORAC: The antioxidative capacity by the ORAC assay ranged from 24.4 to 35.1 $\mu\text{mol TE/g}$ whole wheat flour (Tab.4). In unsprouted wheat samples no significant effects could be observed, neither for main effects nor for the interaction of both treatments (cv x N). After 24 h of sprouting the factor cultivar ($p \leq 0.001$) and the interaction cv. x N had significant influence ($p = 0.006$) on the antioxidative capacity. Cv. Privileg x N₁ (28.3 $\mu\text{mol TE/g}$), cv. Privileg x N₂ (22.6 $\mu\text{mol TE/g}$) and cv. Tommi x N₂ (36.5 $\mu\text{mol TE/g}$) showed significant differences whereas cv. Estevan x N₁ (24.1 $\mu\text{mol TE/g}$), cv. Estevan x N₂ (24.6 $\mu\text{mol TE/g}$) and cv. Tommi x N₁ (28.2 $\mu\text{mol TE/g}$) were at the same level. After 48 h of sprouting no significant differences neither for main effects nor for the interaction effect could be observed. The results showed a tendency that wheat samples fertilized with 150 kg N/ha (N₂) had higher ORAC values than N₁ ones. Furthermore, table 4 reveals a significant influence of sprouting time on the antioxidative capacity. The values were significantly higher after 48 h (32.6 $\mu\text{mol TE/g}$) of sprouting than after 24 h (27.4 $\mu\text{mol TE/g}$) or with no sprouting (28.2 $\mu\text{mol TE/g}$).

The highest antioxidative capacity contributed fraction 3 (ORAC3) for not sprouted wheat samples (39 %, 10.9 $\mu\text{mol TE/g}$) and presprouted cultivars (40 %, 10.9 $\mu\text{mol TE/g}$).

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10.8 $\mu\text{mol TE/g}$). After a sprouting time of 48 h the values of ORAC1 increased to 48 % (15.7 $\mu\text{mol TE/g}$) whereas ORAC3 (26 %, 8.6 $\mu\text{mol TE/g}$) decreased (fig. 6).

Tab. 4: Effect of sprouting time on the antioxidative capacity by the ORAC assay [$\mu\text{mol TE/g}$] in wheat samples depending on N fertilization and cultivar

Treatment (cultivar/nitrogen)	Sprouting time [h]		
	0	24	48
Main effect cv.			
Estevan	27.9 n.s.	24.4 a	35.1 n.s.
Privileg	26.4 n.s.	25.4 a	29.5 n.s.
Tommi	30.2 n.s.	32.3 b	33.3 n.s.
Main effect N			
N ₁	28.0 n.s.	26.9 n.s.	30.8 n.s.
N ₂	28.3 n.s.	27.9 n.s.	34.5 n.s.
Interaction effect			
Estevan x N ₁	28.4 n.s.	24.1 ab	35.1 n.s.
Estevan x N ₂	27.3 n.s.	24.6 ab	35.1 n.s.
Privileg x N ₁	27.6 n.s.	28.3 a	29.3 n.s.
Privileg x N ₂	25.2 n.s.	22.6 b	29.8 n.s.
Tommi x N ₁	28.1 n.s.	28.2 ab	27.9 n.s.
Tommi x N ₂	32.4 n.s.	36.5 c	38.7 n.s.
Mean	28.2 a	27.4 a	32.6 b
p cultivar (cv.)	n.s.	0.001	n.s.
p nitrogen (N)	n.s.	n.s.	n.s.
p cv. x N	n.s.	0.006	n.s.

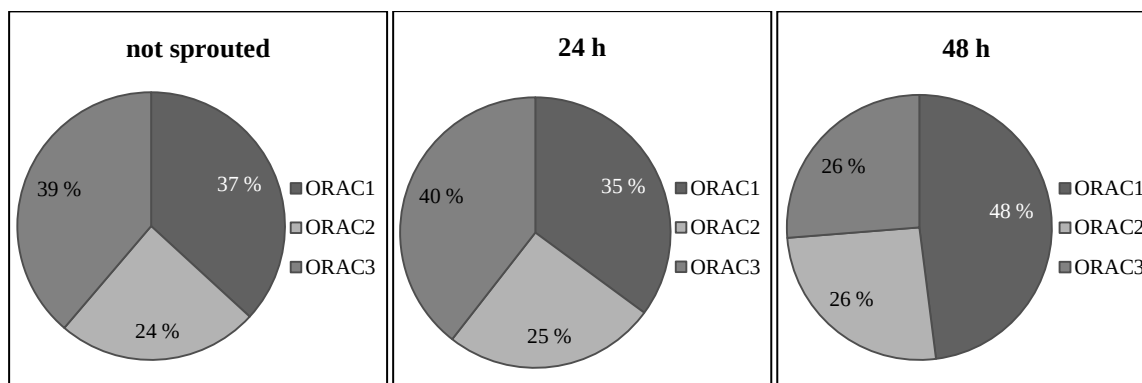


Fig. 6: Effect of sprouting time on the composition of the extracted fractions (ORAC1, ORAC2, ORAC3) of the antioxidative capacity by the ORAC assay in wheat samples

Discussion

The present study focused on the effects of sprouting time on phenolic acids and antioxidative capacity of whole wheat samples depending on cultivar and nitrogen fertilization. Sprouting is the first important stage during biogenesis of wheat. Enzymatic processes, like starch degradation by α -amylase, take place to provide the

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embryo with nutrients. Normally sprouting occurs after a certain time of dormancy when the caryopsis has come to full ripeness (HAUMANN and DIETZSCH, 2000). If it starts too early, because of humid conditions during ripening, it is a negative parameter for grain quality, e.g. for flour processing. Nevertheless it is necessary to know, if deliberately induced sprouting has an influence on phenolic acids and their antioxidative capacity with regard to their health beneficial effects (KAHLIL and MANSOUR, 1995, PRODANOV et al., 1997, YANG et al., 2001, TIAN et al., 2004, ARORA et al., 2010, TIAN et al., 2010). In the conducted study analysis of phenolic acids (TPA) and antioxidative capacity by the Folin-Ciocalteu and ORAC assay was carried out in whole wheat flour because of highest concentrations of phenolic acids in the bran and aleurone layer of caryopses (PUSSAYANAWIN et al., 1988, ZHOU et al., 2004, ADOM et al., 2005, ANSON et al., 2008).

The analyzed phenolic acids (TPA) didn't show a significantly influence for the different sprouting times, indicating that enzymatic processes during sprouting did not affect phenolic acids while differences for the analyzed cultivars were visible. After 48 h of sprouting in the current study cultivar Estevan (1554.0 µg GAE/g) showed higher TPA values than after 0 h of sprouting (1154.3 µg GAE/g), whereas cv. Privileg and cv. Tommi showed lower values (tab. 2). The cultivar seems to have an influence whether the TPA concentration is higher in sprouted wheat samples than in not sprouted ones or not. Until now it is known that genotype and environment influence the content of phenolic acids (ABDEL-AAL et al., 2001, MPOFU et al., 2006, MOORE, 2006). The current study presents higher results than in literature which could be due to different extraction methods. STRACKE et al. (2009) reported concentrations between 282 µg/g and 1262 µg/g in soft wheat samples whereas ZHOU et al. (2007) only reported concentrations between 219.7 µg/g and 389.1 µg/g in eleven soft red winter wheat grain cultivars. MOORE et al. (2005) analyzed eight soft red winter wheat genotypes with TPA contents of 486.6 µg/g - 656.0 µg/g and MPOFU et al. (2006) conducted a study with six western Canadian wheat genotypes where TPA values ranged between 580.2 µg/g and 724.7 µg/g. Different cultivars were used in these studies which displayed a variation in TPA values between the used cultivars. Even though only three cultivars were analyzed in the current study and a genetic determination was found for not sprouted cultivars, as well. N fertilization was significantly influencing TPA contents for not sprouted grain resulting in higher values after application. MARGNA (1977) traced this effect back to the sufficiently available amino acid phenylalanine which is primarily used for protein

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biosynthesis and which can be released by protein molecules. Since there is only little information about the correlation between N application and phenolic acid content, especially for wheat caryopses, further studies need to be conducted, to get a greater knowledge about the influence of nitrogen on phenolic acids in wheat caryopses.

Fraction 3 (TPA 3) represented cell wall bound phenolic acids and provided the biggest proportion of all three fractions, being well in line with further studies (STRACKE et al., 2009, ABDEL-AAL et al., 2001). According to other authors free phenolic acids (fraction 1) contributed the smallest part to total phenolic acids ranging between 1 % and 2 %. This is in agreement with the detected results for not sprouted grain but not for fully sprouted samples (LI et al., 2008, ABDEL-AAL et al., 2001). Generally, phenolic acids are bound to hydrolysable tannins, lignins, cellulose and proteins which are mainly structural components of bran, building a protective layer to inner parts of the caryopsis. Furthermore, important functions of phenolics, including phenolic acids, are defending mechanisms against pathogens, parasites and predators in plants (GRAF, 1992, LIU, 2004, PARR and BOLWELL, 2000). The highest ratio in all sprouting times was observed for TPA 3 (fig. 3). Nevertheless it is noticeable that after 48 h of sprouting TPA 3 decreases from 86 % (not sprouted) to 59 % for fully sprouted grain and TPA 1 increases from 6 % (not sprouted) to 28 %. It can be assumed that conjugated phenolic acids are released from the breakdown of cell walls, maybe to protect the inner parts of the caryopsis which is still needed to support the developing germ. A study by Cheng et al. (2006) suggests, that the conjugation of polyphenolics, e.g. tannins, are broken and simple phenolics are released.

It is reported that the main phenolic acid in wheat is ferulic acid (FA) which is proposed to enhance wall extensibility during cell elongation (GRAF, 1992). Figure 4 shows that the composition of the phenolic acids shifted from 67 % FA and 7 % caffeic acid (CA) for not sprouted wheat samples to 54 % FA and 21 % CA for fully sprouted samples. Since CA is a precursor of FA it suggests itself that breakdown processes of cellular constituents are in progress during sprouting, e.g. when the coleoptile breaks through. Another assumption is that FA and CA, as intermediates in the biosynthesis of lignin, are released to provide substrates for the lignification. For human nutrition the use of fully sprouted grain could provide positive effects because of the higher amounts of free phenolic acids. In most findings the bioavailability and bioaccessibility of free phenolic acids is higher than of bound forms, which leads to a

better resorption, e.g. of ferulic acid, in the small intestine. Bound phenolic acids firstly have to be released by bacterial hydrolytic enzymes during fermentation in the large intestine (KROON et al., 1997, ZHAO et al. 2003, ANSON et al., 2009).

In contrary to TPA values the antioxidative capacity by the Folin-Ciocalteu (TPC) and by the ORAC assay showed significant differences for sprouting times. After 48 h of sprouting the TPC was significantly higher (2575.6 $\mu\text{g GAE/g}$) than for not sprouted wheat samples. The same was visible for ORAC values, which were higher for fully sprouted samples (32.6 $\mu\text{mol TE/g}$) than for not sprouted samples (28.2 $\mu\text{mol TE/g}$).

In the present study significant influences of the cultivar on TPC or on ORAC in not sprouted samples were not observed. According to other authors, there should be a significant variation in ORAC and TPC values of different cultivars due to genetic variability (MOORE et al., 2005, MOORE et al., 2006, MPOFU et al., 2006, ZHOU et al., 2007). One reason for the differing results might be the small number of treatments (three cultivars) analyzed in the present study. The effect of N fertilization on the antioxidative capacity by Folin-Ciocalteu and ORAC assay was not significant for not sprouted grain. However, there was the tendency of higher TPC and ORAC values after nitrogen application which needs to be analyzed further to get more information about the influence of nitrogen application on the antioxidative capacity.

These two tests of the antioxidative capacity are working differently with the Folin-Ciocalteu assay being an electron transfer based reaction assay and ORAC a hydrogen atom transfer based reaction assay (HUANG et al., 2005). Nevertheless, they both are testing the antioxidative activity which is the reason for the same tendency. MOORE et al. (2006) and OKARTER et al. (2010) reported a correlation between TPC and ORAC values. In the current study TPC were correlating strongly with ORAC values after 48 h of sprouting ($r_s=0.714$, $p\leq 0.001$). For not sprouted samples it is at least a positively tendency ($r_s=0.327$). Highest TPC were present for free phenolic acids with 46 % in not sprouted grain and fully sprouted grain, even though fraction 3 supplied the highest amount of phenolic acids. The same was visible for the ORAC values where ORAC 1 and ORAC 3 were about at the same level in unsprouted grain. After 48 h of sprouting ORAC 1 was the predominant fraction in terms of antioxidative capacity by the ORAC assay. These results indicate that sprouting alters the phenolic acids and other free phytochemicals might be present, leading to an improved antioxidative capacity in fully sprouted grain. The presence of tocopherols in wheat grain including the vitamers α -tocopherol, γ -tocopherol and δ -tocopherol and carotenoids including β -carotene, zeaxanthin and

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lutein was reported by several studies (ZHOU et al., 2004, MOORE et al., 2005, OKARTER et al., 2010). During the biosynthetic pathway the different tocopherol isomers can be transformed into α -tocopherol which has the highest vitamin E activity (BRAMLEY et al., 2000). The antioxidative capacity of the tocopherol isomers seems to differ, too. It is possible that tocopherols and carotenoids are released to a greater extent from the aleurone fraction and improved the antioxidative capacity after 48 h of sprouting. ZHOU et al. (2004) reported a correlation between δ -tocopherol and TPC, with the strongest antioxidative capacity among the tocopherols. Those phytochemicals, including phenolic acids could be the reason for a health benefit of whole wheat products or sprouted products, which is in line with the assumption of OKARTER et al. (2010).

In conclusion, the current study showed that the TPA composition was comparable with the literature while TPA values were higher than reported. Phenolic acids contribute together with other phytochemicals in wheat, especially in sprouted wheat, a high potential health benefit by scavenging free radicals in caryopses as well as in the human organism. The composition of free, free-insoluble and bound phenolic acids changed during the process of sprouting for the benefit of free phenolic acids after 48 h. Furthermore, the antioxidative capacity by the Folin-Ciocalteu (TPC) and ORAC assay was increased after the caryopses were fully sprouted. The composition of the three extracted fractions changed for the benefit of the antioxidative capacity of free phenolic acids. This indicated that the more free phenolic acids and other free phytochemicals were present, the higher was the antioxidative capacity. Sprouted grain is a negative quality parameter with regard to standard backing processes. Nevertheless, it should be taken in consideration for human diets, e.g. in bread with whole cereals or in special breads named “Essener bread” with up to 100 % spouted grain flour (BECK et al., 2010). It is assumed that bioavailability along with bioaccessibility is upgraded because of free phytochemicals. Further studies need to be conducted with more than three cultivars to show whether the interaction between cultivar and fertilization has an effect on phenolic acids and antioxidative capacity of sprouted grain.

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5.3 Experiment 3 “Evaluation of spelt wheat cultivars”

Manuscript under review in Cereal technology.

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Grain quality, phenolic acid contents and antioxidative capacity of different spelt wheat (*Triticum aestivum* ssp. *spelta*) cultivars

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Summary

The aim of the current study was to characterize five different spelt wheat cultivars (*T. aestivum* L. ssp. *spelta*) by their quality parameters of grain morphology, protein characteristics and phenolic compounds. The following parameters were detected: thousand kernel weight (TKW), hectoliter weight (HLW), SDSS (sodium dodecyl sulphate sedimentation) test, falling number (FN), crude protein (CP), gluten and Gluten-Index (GI) as well as total phenolic acid (TPA) contents in total and in three different extracted fractions. Additionally, the antioxidative capacity was analysed by Folin-Ciocalteu (TPC) and ORAC assay. It was observed that most of the quality parameters of spelt wheat grain were determined by the cultivars. TPA contents ranged from 287.2 to 408.7 µg GAE/g. Ferulic acid (FA) was the abundant phenolic acid in grain of the analyzed spelt wheat samples. FA contributed 85 % in total and 98 % for the cell wall bound fraction. TPC values ranged from 1175.0 µg GAE/g to 1375.0 µg GAE/g in total with no significant differences between cultivars. Cell wall bound phenolic acids (fraction 3) contributed the highest antioxidative capacity for the Folin-Ciocalteu assay as well as for the ORAC assay. ORAC values were ranging between 21.5 µmol TE/g and 24.6 µmol TE/g. The current results indicate that spelt wheat as ancient wheat contributes health beneficial potentials regarding the presence of phenolic acids and the resulting antioxidative capacity. Especially cell wall bound phenolic acids showed highest values which lead to the suggestion of consuming whole wheat products of spelt in order to benefit of their antioxidative effects.

Keywords: wheat, spelt, phenolic compounds, phenolic acids, antioxidative capacity, Folin-Ciocalteu, ORAC

1. Introduction

Spelt wheat (*T. aestivum* L. ssp. *spelta*) is a hulled cereal with low grain yield. Because of lower performance it lost attention during the time while bread wheat (*T. aestivum* L. ssp. *aestivum*) was improved by cultivation and breeding efforts. Spelt wheat varieties gained importance in cultivation and in human diet over the last years (BICKERT, 2006). In the period between 2003-2009 spelt wheat cultivation area more than trippled in Germany from 2003 with about 11,293 ha until 2009 with 37,474 ha (STATISTISCHES BUNDESAMT, 2010). Since spelt wheat is a robust cereal which grows under restrained environmentally conditions it is an interesting cereal for organic farming where no synthetic fertilizers or pesticides are used. The products of spelt wheat derived from organic farming are demanded strongly by the consumer because of an assumed enhanced health beneficial value and environmentally sustainability of organically produced goods (ZHAO et al., 2007, DANGOUR et al., 2009).

Basic nutrients of spelt wheat such as carbohydrates, proteins, dietary fibers, vitamins and minerals contribute to a well-balanced human diet containing higher amounts of soluble fibers and higher protein contents than bread wheat (BONAFACCIA et al., 2000, SKRABANJA et al., 2001). Additionally secondary plant metabolites such as phenolic acids (PA), ferulic acid (FA), *p*-coumaric acid (PCA), vanillic acid (VA), sinapic acid (SIA), syringic acid (SYA) and caffeic acid (CA) are present in spelt wheat, too. These phenolic compounds can supply health beneficial effects to human consumers by their antioxidative, carcinogenic and cardiovascular disease lowering attributes (ARTS et al., 2001, GRAF et al., 2005, MPOFU et al., 2006, GONZALEZ and RIBOLI, 2010, RODRÍGUEZ VAQUERO et al., 2010).

Only little is known about the content, composition and variation of secondary plant metabolites of ancient wheat species like spelt wheat. While spelt wheat and its products are used more and more by the consumer it is valuable to know complementary information about ingredients and quality characteristics of this species. Thus, the aim of the present study was to identify phenolic acids, their composition and antioxidative capacity, analysed by Folin-Ciocalteu and ORAC assay, of spelt wheat in dependence on different cultivars.

2. Materials and Methods

In 2008 field trials were conducted as randomized experiments with four replications at four different sites of the federal state Baden-Württemberg (southern Germany). At one location (Kirchberg-Dörrmenz) the method of organic farming was realized while at the other three locations (Bremelau, Döggingen, Eiselau) conventionally farming methods were carried out. Five different cultivars namely Badengold, Franckenkorn, Oberkulmer Rotkorn (RK), Schwabenkorn and Zollernspelz were cultivated and analyzed from which only representative samples per cultivar were used. Schwabenkorn was the only cultivar which wasn't available from the organic farming location Kirchberg-Dörrmenz. Until analysis samples were stored at 20 °C.

At the beginning of lab analysis grain quality parameters such as thousand kernel weight (TKW), hektoliter weight (HLW), SDSS (sodium dodecyl sulphate sedimentation) test, falling number (FN), crude protein (CP), gluten and Gluten-Index (GI) were evaluated using ICC-standard methods. In total 19 grain samples (3 sites x 5 cultivars and 1 site x 4 cultivars) were ground with a 500 µm sieve on a Cyclotec 1073 Sample Mill (Foss, Germany) to produce a whole grain flour for the following extraction. After mixing the samples carefully the extraction of PAs was pursued immediately and extensively. Until analyzing the extracts they were stored at -18 °C. An HPLC method was used to determine vanillic acid (VA), syringic acid (SYA), caffeic acid (CA), *p*-coumaric acid (PCA), ferulic acid (FA) and sinapic acid (SIA). The phenolic acid extracts were used to estimate the antioxidative capacity by Folin-Ciocalteu assay and ORAC assay.

The extraction was done by the method described previously (ENGERT and HONERMEIER, 2011). In short, the ground samples were extracted with methanol/acetone/water (7:7:6, v/v/v) and centrifuged. The supernatant and the residue were used for further extraction to produce three different fractions depending on the type of bond of the present phenolic acids: fraction 1 soluble free, fraction 2 soluble conjugated, and fraction 3 bound phenolic acids. Fraction 2 and 3 had to undergo an alkaline hydrolysis to release bound phenolic acids. Furthermore, all three fractions were extracted with ethyl acetate and the organic phase was evaporated. After resumption in 5 % acetonitrile the extracts were stored at -18°C until analysis by HPLC, Folin-Ciocalteu and ORAC assay was conducted.

HPLC analysis was performed on a HPLC-DAD system by Knauer on a C18 column (EC 250 x 4 Nucleodur Sphinx RP, 5 µm (MN)) at a temperature of 25 °C, a flow rate of 1 ml min⁻¹ and an injection volume of 100 µl. Two hydroxybenzoic acids namely

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vanillic acid (VA) and syringic acid (SYA) were measured at 250 nm as well as four hydroxycinnamic acids namely caffeic acid (CA), p-coumaric acid (PCA), ferulic acid (FA) and sinapic acid (SIA) were measured at 290 nm. The method was modified according to WEIDNER et al. (2000) and ZIELINSKI et al. (2001) using acetonitrile and acetic acid as the mobile phase. Results were measured as $\mu\text{g/g}$ and transformed into $\mu\text{g GAE/g}$.

The Folin-Ciocalteu assay belongs to the type of electron transfer based assays where an electron of the antioxidant reduces the radical in a redox reaction (PRIOR et al., 2005). The Folin-Ciocalteu micro method by WATERHOUSE (2001) was modified according to the method described previously to measure the samples of the present study (ENGERT and HONERMEIER, 2011). The oxidant is the Folin-Ciocalteu-reagent consisting of phosphotungstic ($\text{H}_3\text{PW}_{12}\text{O}_{40}$) and phosphomolybdic ($\text{H}_3\text{PMo}_{12}\text{O}_{40}$) which is reduced to blue oxides of tungsten and molybdenum by the standard gallic acid or by the phenolic acids of the samples. The induced change of colors was measured spectrophotometrically at $\lambda_{\text{max}} = 765 \text{ nm}$ on a Specord 205 – 222A430 (Analytik Jena AG, Germany) and results were expressed as gallic acid equivalents (GAE).

The ORAC (oxygen radical absorbance capacity) assay was applied to measure the ability of antioxidants to scavenge free radicals by transferring a hydrogen atom (HUANG et al., 2005, PRIOR et al., 2005). To operate the assay the 96-well plate fluorescence reader Fluoroskan (Fisher Scientific GmbH, Germany) was run with the method described by HUANG et al. (2002). The molecular probe fluorescein (6-hydroxy-9-(2-carboxyphenyl)-(3h)-xanthen-3-on), the azo radical initiator AAPH (2,2'-azobis(2-methylpropionamidine)dihydrochloride) and the standard Trolox[®] (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were used to determine the antioxidative capacity of the samples. The fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 538 nm every 60 s for 90 min. The results were expressed as Trolox equivalents (TE).

Statistical analysis was executed by the statistical program PASW Statistics 18 (SPSS Inc., Chicago, IL) using an ANOVA (analysis of variance) to test significances between the analyzed spelt cultivars regarding phenolic acid contents and antioxidative capacity (Folin-Ciocalteu and ORAC assay). Tukey's HSD post-hoc test was used for multiple mean comparisons when significant differences were calculated ($p \leq 0.05$). Pearson's correlation (r) factors (bivariate) were computed between total phenolic acids (TPA), total phenolic content (TPC) and antioxidative

capacity (ORAC). To evaluate significant ($p \leq 0.05$) differences of the three analyzed fractions of each parameter an ANOVA with repeated measures was used. Results were reported as mean values and the significance level was at a 95 % confidence interval ($\alpha = 0.05$).

3. Results

Thousand kernel weights (TKW) of the five spelt wheat cultivars ranged not significantly between 48 g (Badengold) and 56 g (Oberkulmer) (Tab. 1). Only a small variation of hectoliter weights (HLW) which were not significant were observed. SDSS values showed high significant ($p = 0.010$) differences between cultivars. The cultivar Oberkulmer RK had the lowest HLW as well as the lowest SDSS value. Falling numbers (FN) of the analyzed grain samples were at a high level (313 and 423 s) showing no enzymatic activity as well as no significance ($p = 0.133$). High significant ($p = 0.000$) variation was also observed in protein contents which ranged from minimal 12.8 (Badengold) to maximal 15.7 % (Schwabenkorn). Gluten diverged significantly ($p = 0.003$) between 29.2 and 38.3 % and Gluten Indices significantly ($p = 0.000$) between 14.8 and 48.7.

Tab. 1: Morphological and quality parameters of spelt wheat grain (*Triticum aestivum* ssp. *spelta*) dependent on cultivar: TKW (thousand kernel weight, [g]), HLW (hektoliter weight ([kg/hl]), SDSS (sodium dodecyl sulphate sedimentation) test, FN (falling number, [s]), CP (crude protein, [%]), gluten index (GI, [%]) and gluten [%] (mean, $\alpha = 0.05$)

Cultivar	TKW [g]	HLW [kg/hl]	SDSS	FN [s]	CP [%]	Gluten [%]	GI [%]
Badengold	48 a	78 a	31 a	313 a	12.8 a	29.2 a	48.3 b
Franckenkorn	50 a	78 a	45 b	373 a	13.8 ab	30.0 a	48.7 b
Oberkulmer	56 a	77 a	32 a	330 a	15.5 c	38.3 b	24.1 a
Schwabenkorn	50 a	79 a	39 ab	349 a	15.7 c	32.8 ab	14.8 a
Zollernspelz	53 a	78 a	43 ab	423 a	14.1 b	35.5 ab	39.1 b
p	0.116	0.381	0.010	0.133	0.000	0.003	0.000

All six phenolic acids (PA) cumulated reveal the TPA content of each fraction or in total of all three fractions. In total of all measured spelt wheat samples 76 % (245.0 μg GAE/g) were originated from fraction 3 (TPA3), 21 % (67.4 μg GAE/g) from fraction 2 (TPA2) and the rest (11.5 μg GAE/g) from fraction 1. The differences between the analyzed fractions were significant implying that TPA1 was significantly

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different from TPA2 ($p=0.000$) and TPA3 ($p=0.000$) while TPA2 was also significantly different from TPA3 ($p=0.000$) (Tab. 2).

Tab. 2: Relative parts [%] of total phenolic acids (TPA), antioxidative capacity by TPC and ORAC assay of three fractions in sum of all analyzed cultivars: fraction 1 (soluble free phenolic acids), fraction 2 (soluble conjugated phenolic acids) and fraction 3 (bound phenolic acids)

	fraction 1	fraction 2	fraction 3
TPA	3	21	76
TPC	27	16	57
ORAC	36	20	44

Tab. 4: Concentration of total phenolic acids (TPA) [$\mu\text{g GAE/g}$] of five spelt wheat cultivars in three extracted fractions (mean $\pm$ SD)

Cultivar	TPA1	SD	TPA2	SD	TPA3	SD
Badengold	7.7 $\pm$ 5.4		93.1 $\pm$ 43.9		218.5 $\pm$ 114.7	
Franckenkorn	13.5 $\pm$ 1.6		73.4 $\pm$ 13.5		279.0 $\pm$ 54.7	
Oberkulmer RK	14.3 $\pm$ 6.0		51.5 $\pm$ 16.3		287.3 $\pm$ 41.6	
Schwabenkorn	8.5 $\pm$ 0.3		66.5 $\pm$ 29.8		218.8 $\pm$ 32.3	
Zollernspelz	13.6 $\pm$ 3.5		52.4 $\pm$ 18.0		221.1 $\pm$ 118.3	

TPA values of fraction 1 in dependence on the cultivar varied from 7.7 $\mu\text{g GAE/g}$ to 69.8 $\mu\text{g GAE/g}$ while the values for Franckenkorn and Oberkulmer RK were the highest which was due to outliers since the standard deviation was also very high. TPA2 showed higher values than TPA1 which fluctuated within 51.5 and 93.1 $\mu\text{g GAE/g}$. TPA3 ranged between 218.5 and 287.3 $\mu\text{g GAE/g}$. In total TPA values varied from 287.2 for Zollernspelz to 408.7 $\mu\text{g GAE/g}$ for Oberkulmer RK with no significant ($p=0.513$) differences between the cultivars. Oberkulmer RK had the highest contents in fraction 3 which lead to highest total phenolic acid contents (Tab. 3).

FA was the abundant phenolic acid for all analyzed spelt wheat cultivars although no significant distinction of cultivars could be found ($p=0.692$) (Fig. 1). The phenolic compound FA contributed 91 % of all phenolic acids detected in TPA3 and in total 74 % of all analyzed spelt wheat samples (not displayed).

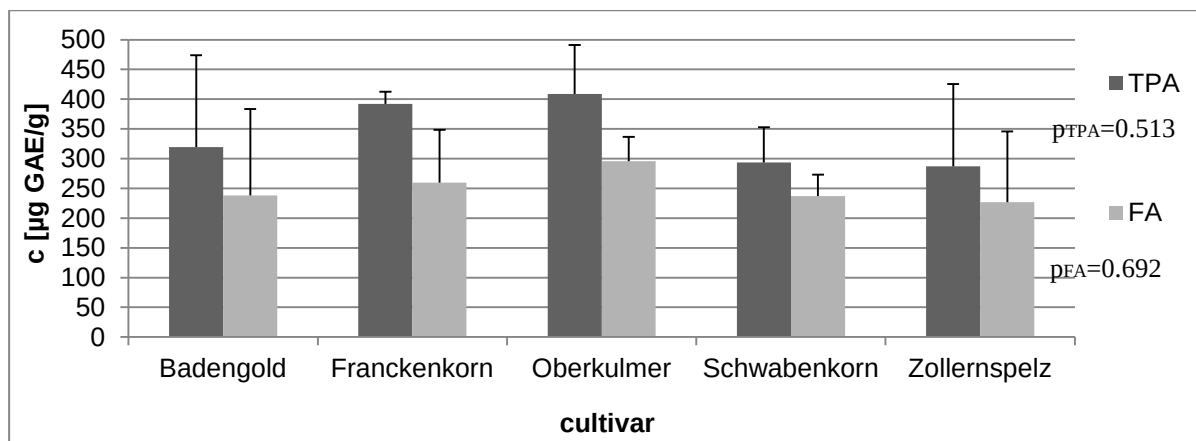


Fig. 2: Concentration of total phenolic acids (TPA) [$\mu\text{g GAE/g}$] and ferulic acid (FA) [$\mu\text{g GAE/g}$] of five spelt wheat cultivars in sum of all three extracted fractions (mean $\pm$ SD, $\alpha=0.05$)

The antioxidative capacity measured by Folin-Ciocalteu assay is called total phenolic content (TPC) in this continuative assay. TPC values in total varied between 1175.0 $\mu\text{g GAE/g}$ for Oberkulmer RK and 1375.0 $\mu\text{g GAE/g}$ for Schwabenkorn without significance ($p=0.382$) for the analyzed spelt wheat cultivars (Tab. 4).

All three fractions were significantly different from each other with $p=0.002$ for the comparison of TPA1 with TPA2, $p=0.000$ for TPA1 and TPA3 and $p=0.000$ for the comparison of TPA2 with TPA3. Table 2 displays the ratio of the three fractions where TPC1 contributed 27 %, TPC2 16 % and TPC3 57 % of all measured spelt wheat samples. Fraction 1 (TPC1) showed a higher antioxidative capacity than TPC2 with mean values between 225.0 $\mu\text{g GAE/g}$ for Badengold and 400.0 $\mu\text{g GAE/g}$ for Zollernspelz. TPC2 ranged between 162.5 $\mu\text{g GAE/g}$ for Oberkulmer RK as well as for Zollernspelz and 266.7 $\mu\text{g GAE/g}$ for Schwabenkorn. Highest values were observed for fraction 3 where they ranged from 600.0 $\mu\text{g GAE/g}$ for Schwabenkorn to 800.0 $\mu\text{g GAE/g}$ for Franckenkorn (Tab. 4).

Tab. 4: Total phenolic content (TPC) [$\mu\text{g GAE/g}$] of five spelt wheat cultivars in three extracted fractions and in sum of all three fractions (mean $\pm$ SD)

Cultivar	TPC1	SD	TPC2	SD	TPC3	SD	TPC	SD
Badengold	225.0 $\pm$ 64.5		262.5 $\pm$ 249.6		700.0 $\pm$ 227.3		1187.5 $\pm$ 460.8	
Franckenkorn	387.5 $\pm$ 165.2		187.5 $\pm$ 103.1		800.0 $\pm$ 158.1		1375.0 $\pm$ 263.0	
Oberkulmer RK	312.5 $\pm$ 160.1		162.5 $\pm$ 160.1		700.0 $\pm$ 108.0		1175.0 $\pm$ 425.2	
Schwabenkorn	383.3 $\pm$ 202.1		266.7 $\pm$ 246.6		600.0 $\pm$ 173.2		1250.0 $\pm$ 614.4	
Zollernspelz	400.0 $\pm$ 187.1		162.5 $\pm$ 131.5		712.5 $\pm$ 165.2		1275.0 $\pm$ 417.3	

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ORAC values in total ranged from 21.5 $\mu\text{mol TE/g}$ for Zollernspelz to 24.6 $\mu\text{mol TE/g}$ for Oberkulmer RK with no significant differences between the cultivars (Tab. 4). Comparing all three different fractions in total the differences were significant. ORAC1 accounted 36 % for the antioxidative capacity and was significantly ($p=0.000$) higher than ORAC2 with 20 %. Furthermore, ORAC1 ($p=0.006$) and ORAC2 ($p=0.000$) were significantly lower than ORAC3 which contributed 44 % (Tab. 2).

Once more, fraction 3 (ORAC3) contributed highest antioxidative capacity values fluctuating between 8.4 $\mu\text{mol TE/g}$ for Schwabenkorn and 10.5 $\mu\text{mol TE/g}$ for Franckenkorn followed by ORAC1 which was almost at the same level ranging from 6.2 $\mu\text{mol TE/g}$ for Badengold to 8.4 $\mu\text{mol TE/g}$ for Zollernspelz. ORAC2 supplied lowest ORAC values between 3.4 $\mu\text{mol TE/g}$ for Zollernspelz and 6.0 $\mu\text{mol TE/g}$ for Badengold (Tab. 5).

Tab. 5: Oxygen radical absorbance capacity (ORAC) values [$\mu\text{mol TE/g}$] of five spelt wheat cultivars in three extracted fractions and in sum of all three fractions (mean $\pm$ SD)

Cultivar	ORAC1	SD	ORAC2	SD	ORAC3	SD	ORAC	SD
Badengold	6.2 $\pm$ 1.3		6.0 $\pm$ 3.0		9.3 $\pm$ 0.9		22.8 $\pm$ 4.4	
Franckenkorn	8.3 $\pm$ 2.8		3.6 $\pm$ 1.4		10.5 $\pm$ 0.2		23.5 $\pm$ 1.9	
Oberkulmer RK	8.2 $\pm$ 0.6		4.9 $\pm$ 1.2		10.4 $\pm$ 0.6		24.6 $\pm$ 1.5	
Schwabenkorn	8.1 $\pm$ 2.6		4.0 $\pm$ 2.2		8.4 $\pm$ 2.2		22.1 $\pm$ 5.6	
Zollernspelz	8.4 $\pm$ 1.5		3.4 $\pm$ 1.3		8.5 $\pm$ 2.2		21.5 $\pm$ 4.5	

Looking at Pearson's correlation coefficient between TPA and TPC a positive relationship was observed, explicitly $r=0.504$ ($p=0.033$). As well as for TPC and ORAC which were the two assays for the antioxidative capacity ($r=0.522$, $r=0.022$) a positive correlation was surveyed. In addition a strong positive correlation was computed between TPA and ORAC values ($r=0.666$, $p=0.003$) (Tab. 6).

Tab. 6: Pearson correlation coefficient r between total phenolic acids (TPA). total phenolic content (TPC) and oxygen radical absorbance capacity (ORAC)

	TPA	TPC	ORAC
TPA	-	0.504*	0.666**
TPC	0.504*	-	0.522*
ORAC	0.666**	0.522*	-

4. Discussion

Phenolic acids are secondary plant metabolites occurring in cereal grain and are able to promote health because of its antioxidativ effects (ZHOU et al., 2007, ANSON et al., 2008, GASPAR et al., 2010). Spelt wheat, an ancient subspecies of bread wheat, has come to new importance in foods like bread, pasta or breakfast cereals because of health promoting compounds and other nutritionally valuable qualities such as higher protein, dietary fiber, magnesium, phosphorus, iron, copper and zinc contents (ABDEL-AAL et al., 1995, RANHOTRA et al., 1995, BONAFACCIA et al., 2000, MARCONI et al. 2002, ZIELINSKI et al., 2008, ABDEL-AAL and RABALSKI, 2008b, ESCARNOT et al., 2010). Until now only little research was done on phenolic acids and their antioxidative capacity in ancient wheat like spelt wheat (LI et al., 2008, ABDEL-AAL and RABALSKI, 2008a). Consequently the aim of the present study was to characterize selective spelt wheat cultivars according to their quality paramters, total phenolic acid contents and the resulting antioxidative capacity which was measured by two different kinds of assays to be precise Folin-Cioclatau assay and ORAC assay.

Morphological grain properties of the analyzed spelt wheat cultivars were characterized by thousand kernel weight (TKW) ranging between 48 and 56 g and hectoliter weight (HLW) ranging from 77 to 79 kg/hl. TKW values of the spelt wheat samples were comparable to those of other studies. KLING et al. (2006) reported TKW values of 45,6-59,1 g for spelt wheat and ZANETTI et al. (2001) found values of 40.0 to 55.2 g. HLWs average around 74 kg/hl for common wheat species which is lower than the results of the analyzed spelt wheat cultivars. Usually HLWs are used to determine wheat prices and flour yields (KLEIJER et al., 2007). Both grain properties can be affected by cultivars (genetically determined seed formation) as well as by growing conditions.

High crude protein (CP) contents for spelt wheat samples of the current study ranging between 12.8 and 15.7 % with significantly highest values for Schwabenkorn (15.7 %) and Oberkulmer RK (15.5 %) were well in line with those reported in literature (RANHOTRA et al., 1996, ABDEL-AAL et al., 1997, ZANETTI et al., 2001, KLING et al., 2006) while those reported by BONFACCIA et al. (2000) were even higher with values between 15.9 and 17.1 %. Crude protein is an important quality parameter for wheat which is influenced especially by two factors cultivar as well as nitrogen application.

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For good baking purposes of bread wheat gluten content of total protein should be at least 25 %. In the present study the gluten content ranged between 29.2 and 38.3 % which was lower than the findings of BOJNANSKA and FRANCAKOVA (2002) who detected values between 30.6 and 51.8 %. They conducted a study over three years where one year was extremely dry and revealed those high gluten values averaging 47.02 %. The other two years with an average of 32.6 and 31.78 % are at the level of the own results. Gluten contents of a study conducted by ZIELINSKI et al. (2008) showed as well values between 30.1 and 37.2 % which are at the same level as the present results. A common parameter for baking quality of bread wheat flour is the Gluten Index (GI) which gives a lead for baking properties such as gas retention during fermentation or baking volumes. Gluten indices of the current analysis were between 14.8 and 48.7 % which is widely ranging. Lowest values were analyzed for the cultivars Schwabenkorn (14.8 %) and Oberkulmer RK (24.1 %). Weak Gluten Indices below 35 % result in low baking qualities and reduced gas retention.

The swelling capacity of proteins which is measured by the sedimentation test is a parameter to be able to draw conclusions about the volume yield of dough. SDS sedimentation test values of the analyzed spelt wheat cultivars were ranging between 31 and 45 which were lower than for bread wheat but still predicting reasonable protein contents and gluten strength. The same was found by BOJNANSKA and FRANCAKOVA (2002) who tested five spelt wheat cultivars with volumes of 31 to 46 ml and by KLING (2006) where SDS sedimentation volumes ranged from 28 to 43 ml (n=4).

The examined high falling numbers of more than 300 s and even more than 400 s for Zollernspelz indicate a low enzymatic activity of the five different spelt wheat samples. A falling number with a level of more than 300 s might be negative with regard to backing quality characteristics although it was found in literature as well (ABDEL-AAL et al., 1997, BOJNANSKA and FRANCAKOVA, 2002, ZIELINSKI et al., 2008). Falling numbers are inversely proportional to α -amylase activity leading to low volume yields of dough for high falling numbers. In order to reduce dough viscosity some α -amylase enzymatic activity is needed to get reasonable dough volumes.

Total phenolic acids were analyzed in three fractions with ferulic acid (FA) as the abundant phenolic acid in total as well as in fraction 3 (TPA3) which were cell wall esterified phenolic acids. Compared to the findings of LI et al. (2008) who examined five spelt wheat samples with TPA values ranging between 382 and 726 $\mu\text{g/g}$ the present TPA contents were lower ranging from 287.2 to 408.7 $\mu\text{g GAE/g}$ with a mean

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value of 340.2 µg GAE/g. According to TPA values of spelt wheat conducted in a previous study (312.0 µg GAE/g) TPA values of the current analysis were a little higher but not completely different.

The analyzed spelt wheat samples indicate lower concentrations of phenolic acids compared to bread wheat investigated by MPOFU et al. (2006) where six phenolic acids including FA as the main compound were detected in six bread wheat cultivars ranging between 580.2 and 694.6 µg/g. However, in comparison with eleven red winter wheat samples analyzed by ZHOU et al. (2007) the own results were even higher. ZHOU et al. (2007) reported that values for insoluble phenolic acids ranged between 189.0 and 336.8 µg/g (mean 253.0 µg/g) whereas soluble phenolic acids were at a low level of 30.7 to 58.4 µg/g (mean 48.3 µg/g). FA acid was as well the main phenolic acid in their study with more than 98 % in the insoluble fraction and more than 90 % in the total phenolic acid fraction. This relation is higher than the findings of the own study for TPA3 with 91 % and for the TPA content with 74 % of all three fractions.

Generally it can be stated that TPA and FA values of spelt wheat and bread wheat are fairly comparable and not significantly different. It can be supposed that within the hexaploid species *Triticum aestivum* (ssp. *aestivum* and ssp. *spelta*) there is only a small diversity regarding concentration and composition of phenolic acids

The Folin-Ciocalteu or total phenolic content (TPC) assay was conducted to measure the antioxidative capacity expressed by gallic acid equivalents (GAE). Some studies express the results of their analyzed wheat samples as ferulic acid equivalents (FAE) which reveals the fact that methods are different (LI et al., 2005; LIYANA-PATHIRANA and SHAHIDI, 2006; SIEBENHANDL et al., 2007; ABDEL-AAL and RABALSKI, 2008a). Resulting TPC values in total of the current analysis between 1175.0 and 1375.0 µg GAE/g were difficult to compare to other ancient wheat species because of absent literature. A previous study conducted revealed TPC values ranging between 2.3 and 2.6 mg GAE/g for different ancient wheat species such as einkorn, emmer and spelt wheat. Einkorn values ranged between 2.4 and 2.6 mg GAE/g, emmer between 2.3 and 2.6 mg GAE/g and spelt wheat was at 2.3 mg GAE/g which shows that spelt wheat was lower than einkorn and emmer (ENGERT and HONERMEIER, 2011). Moreover, the present results were about 1 mg GAE/g lower than the previous results which might be due to cultivar effects. The present study confirmed previous findings that TPC3 (present: 57 %, previous: 40 %) had the highest antioxidative

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capacity and was followed by TPC1 (present: 27 %, previous: 36 %) although found ratios were differing to the benefit of TPC3.

In the current study there was a correlation between TPA and TPC values ($r=0.504$, $p=0.033$) which confirmed the finding of another own study ($r=0.847$, $p=0.000$) carried out with ancient wheat (ENGERT and HONERMEIER, 2011). From these results can be concluded that phenolic acids of the analyzed spelt wheat cultivars are acting as antioxidants and could contribute to health benefits when whole wheat products of spelt wheat are consumed. Since the three fractions were significantly different from each other it is advisable to consume whole wheat products which contain fraction 3 with the highest content of TPA and TPC in order to benefit from these findings. MOORE et al. (2005) analyzed soft winter wheat genotypes with TPC values between 0.4 and 0.8 mg GAE/g which was lower than the own results of the analyzed spelt wheat cultivars. ABDEL-AAL and RABALSKI (2008a) analyzed the TPC contents of two einkorn, one emmer, six spelt wheat as well as six soft (bread) wheat samples expressed in μg ferulic acid equivalents (FAE)/g. Einkorn ranged between 2319 and 2355 μg FAE/g (2658-2699 μg GAE/g), emmer was at 2323 μg FAE/g (2662 μg GAE/g), spelt varied from 1121 to 2382 μg FAE/g (1285-2730 μg GAE/g) and soft wheat varied between 1575 and 2190 μg FAE/g (1805-2510 μg GAE/g). These results were at a higher level than the present ones, nevertheless, they confirmed previous findings that spelt seems to be lower in its TP contents than einkorn and emmer but higher than bread wheat samples. Since results were widely ranging standardized extraction and analysis methods are needed in order to compare the results properly. However, the findings of the present study and literature confirm the presence of phenolic acids and their antioxidative capacity in spelt wheat and other ancient wheat cultivars.

The antioxidative capacity measured by the ORAC assay ranged between 21.5 μmol TE/g and 24.6 μmol TE/g whole wheat sample. As well as for TPC values ORAC3 (44 %) contributed the highest antioxidative capacity followed by ORAC1 (36 %). Results of the study previously conducted were ranging about at the same level between 18.2 and 23.8 μmol TE/g. ORAC values for einkorn were ranging between 20.1 and 22.2 μmol TE/g, those for emmer between 18.2 and 21.7 μmol TE/g and spelt was at 23.8 μmol TE/g which shows that spelt might provide the highest antioxidative capacity of the ancient wheat species when hydrogen transfer based reaction takes place (ENGERT and HONERMEIER, 2011). On the other hand the antioxidative capacity for spelt wheat measured by the ORAC

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assay seems to be lower than for bread wheat. MOORE et al. (2005) analyzed soft winter wheat samples and found values ranging from 32.9 to 47.7 $\mu\text{mol TE/g}$. Additionally OKARTER et al. (2010) even found ORAC values of 51.5 to 96.2 $\mu\text{mol TE/g}$ when they analyzed six bread wheat varieties.

Generally it can be concluded that the antioxidative capacity of spelt grain depends on tissue fractions which are analyzed. Phenolic acids of the cell wall bound fraction seem to be more important for the antioxidative capacity in comparison with other fractions no matter if it is an electron or a hydrogen transfer based reaction to dispose oxidants. The importance of phenolic acids for antioxidative reactions can be confirmed by a correlation between total phenolic acids and the antioxidative capacity measured by the ORAC assay ($r=0.666$, $p=0.003$). The result that cell wall bound phenolic compounds significantly contribute to ORAC values was confirmed by the study of LIYANA-PATHIRANA and SHAHIDI (2006). In agreement with LIYANA-PATHIRANA and SHAHIDI (2006) it can be concluded that a preventive activity by phenolic acids is mainly derived from whole wheat products than from refined flour which is also relevant in spelt wheat.

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6 Discussion

The present work was focused on the following questions:

- Is there an influence of species on the concentration, composition and antioxidative capacity of phenolic acids in ancient whole wheat flours (einkorn, emmer, spelt) and on the difference of the three types of bonds (free phenolic acids, soluble conjugated and bound phenolic acids) of phenolic acids in ancient wheat?
- Does sprouting or nitrogen fertilization make a difference in the concentration, composition and antioxidative capacity of phenolic acids in total and in the three types of bonds of common whole wheat species?
- Are there differences in the concentration, composition and antioxidative capacity of phenolic acids in spelt wheat cultivars and their different types of bonds?

Those questions above were highlighted in chapters 5.1, 5.2 and 5.3 where the three conducted experiments are described and discussed. The following discussion is about all three experiments together to answer the question whether ancient wheat cultivars and bread wheat cultivars are comparable in their phenolic acid contents and their antioxidative capacity.

Experiment 1 (chapter 5.1) concentrates on the evaluation of several ancient wheat species tested under the same growing conditions in Gießen. In total seed samples from 19 diploid, tetraploid and hexaploid wheat cultivars and accessions were extracted into three different fractions depending on the type of bond of the phenolic acids and analyzed for their concentration and composition of phenolic acids in an HPLC system. Moreover, the antioxidative capacity of the different extracts was analyzed in two different ways (Folin-Ciocalteu and ORAC method). Chapter 5.2 reveals experiment 2 where three different common wheat cultivars were fertilized with nitrogen and controlled sprouting was executed to analyze partly sprouted and fully sprouted grain in comparison with not sprouted grain samples. The question of nitrogen fertilization was examined, as well in experiment 2. To get further information about the meanwhile regained importance of spelt wheat products experiment 3 (chapter 5.3) was conducted. In total grain samples of 19 spelt wheat

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cultivars were extracted into three fractions and the concentration and composition of phenolic acids was determined as well as their antioxidative capacity.

The influence of the species didn't seem to be present when looking at the composition of phenolic acids (PA). Ferulic acid (FA) was the predominant PA for all conducted experiments in this work (Tab. 6-1). Experiment 1 illustrated FA to be the major PA for all analyzed species and accessions (chapter 5.1 Fig. 4) no matter if it was a diploid, tetraploid or hexaploid wheat. Concentrations of FAs were ranging between 184.2 and 272.3 $\mu\text{g GAE/g}$ whole wheat flour which were about 65 % of all analyzed PAs (chapter 5.1 Tab. 3). Experiment 2 where three bread wheat cultivars with different nitrogen levels were fertilized revealed the same tendency. FA was the predominant PA with 67 % (881.8 $\mu\text{g GAE/g}$) for not sprouted, 74 % (809.3 $\mu\text{g GAE/g}$) for presprouted and 54 % (669.9 $\mu\text{g GAE/g}$) for fully sprouted grain (Tab. 6-1 and chapter 5.2 Fig. 4). As it can be seen FA in bread wheat samples was at a much higher absolute level than in the ancient wheat samples of experiment 1 and sprouting seemed to have an effect on FA for the benefit of caffeic acid (CA). Another point is that the concentration of FA declined for the total concentration (sum of all three fractions) during sprouting while the concentration of free PAs rose (chapter 5.2 Fig. 3). Bound PAs such as FA or CA were probably released from some cell walls for biosynthetic processes like lignification of newly build cells. Normally, they are part of structural components such as cellulose, hemicelluloses or lignin and function as supporting and strengthening compounds (Liu, 2004). Experiment 3 where various spelt wheat cultivars were analyzed showed as well the predominance of FA (chapter 5.3 Fig. 1) ranging between 226.7 and 295.8 $\mu\text{g GAE/g}$ (mean 251.5 $\mu\text{g GAE/g}$) which was 74 % of the total phenolic acids (Tab. 6-1). This was about at the same level as for the analyzed spelt wheat cultivars of experiment 1 where the analyzed FA contents with a mean of 247.4 $\mu\text{g GAE/g}$ only accounted for 79 % of the total phenolic acids (Tab. 6-1, chapter 5.1 Tab. 2 and chapter 5.1 Tab. 3). The proportion of FA as part of TPA was higher for the used spelt wheat cultivars than for bread wheat or einkorn and emmer following the order: spelt > bread wheat > einkorn = emmer (chapter 5.1 Tab. 2 and chapter 5.1 Tab. 3).

Tab. 6-1: TPA and FA concentrations in µg GAE/g whole wheat flour and relation (%) of FA to TPA for all three experiments (exp.) (experiment 1 chapter 5.1, experiment 2 chapter 5.2, experiment 3 chapter 5.3) and different sprouting times (mean)

Exp.	species	Sprouting [h]	TPA [µg GAE/g]	FA [µg GAE/g]	Relation FA/TPA [%]
1	<i>T. aestivum</i> L. ssp. <i>spelta</i>	0	312.0	247.4	79
	<i>T. monococcum</i> L. ssp. <i>monococcum</i>	0	306.8	184.7	60
	<i>T. turgidum</i> L. ssp. <i>dicoccoides</i>	0	368.7	219.4	59
2	<i>T. aestivum</i> L. ssp. <i>aestivum</i>	0	1315.5	881.8	67
	<i>T. aestivum</i> L. ssp. <i>aestivum</i>	24	1099.2	809.3	74
	<i>T. aestivum</i> L. ssp. <i>aestivum</i>	48	1231.4	669.9	54
3	<i>T. aestivum</i> L. ssp. <i>spelta</i>	0	340.2	251.5	74

The working group of Li et al. (2008) analyzed 175 samples of different wheat varieties for their phenolic acid content and composition using three fractions (soluble free, soluble conjugated, and bound). They found that FA concentrations (in total) were higher for ancient wheat samples like einkorn (298 µg/g, 48 % of TPA), emmer (476 µg/g, 61 % of TPA) or spelt wheat (365 µg/g, 63 % of TPA) than in the present work. The results for emmer were much higher than those for einkorn or spelt which was not the same for the present results. Furthermore, the relative proportion of FA for einkorn was much lower than for emmer in the analysis of Li et al. (2008) which was not the case for the present work with around 60 % for einkorn and emmer and 74-79 % for spelt wheat. ABDEL-AAL and RABALSKI (2008a) conducted a study on various primitive and modern wheat species with mean FA concentrations for einkorn 390 µg/g, emmer 323 µg/g and spelt 424 µg/g which was as well much higher than the own results but this time no fractionation into soluble free, soluble conjugated and bound PA was done by the working group.

FA concentrations of bread wheat samples of the present work were much higher than those of ancient wheat samples (Tab. 6-1). In the literature results were widely ranging making it hard to compare and value the results. Li et al. (2008) found FA concentrations of 395 µg/g (59 % of TPA) in winter wheat and 401 µg/g (66 % of

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TPA) in spring wheat. STRACKE et al. (2009) analyzed two winter wheat varieties of three years under different farming systems which were also extracted into three fractions namely soluble free, soluble conjugated and bound phenolic acids. Results of FA contents in total were ranging approximately between 239-1072 $\mu\text{g/g}$ (85 % of total phenolic acids). MOORE et al. (2005) conducted a study on eight soft red winter wheat samples which were extracted into soluble free, soluble conjugated and insoluble bound phenolic acids to analyze the phenolic acid composition. In total (soluble free, soluble conjugated and insoluble bound) FA concentrations were ranging between 455.9 and 621.5 $\mu\text{g/g}$ expressing about 94 % of the total phenolic acids which was much higher than for the present results. ABDEL-AAL and RABALSKI (2008a) analyzed common wheat with FA contents ranging between 441-556 $\mu\text{g/g}$ and a mean of 488 $\mu\text{g/g}$.

In general, the own results of FA contents of ancient wheat were lower and those of common wheat were higher than in literature reported. The fact that different extraction methods were used might be one reason for these differences. Other reasons for deviated findings might be the wheat material (genetically background) which was used and the specific growing conditions of the wheat crops which were tested. Several studies reported that climate factors and genotype had an impact on phenolic acids (Abdel-Aal et al., 2001c, ABDEL-AAL and RABALSKI, 2008a, STRACKE et al., 2009).

Since FA mainly is bound to arabinoxylan polysaccharides in outer layers of wheat caryopses it has got worthful functions for the grain such as protection from wheat midge or other pathogens, parasites or predators, it is a structural component of bran bound to lignin, tannin, cellulose or protein and part of biosynthetic processes like lignification (GRAF, 1992, LIU, 2004, PARR and BOLWELL, 2000, Abdel-Aal et al., 2001c, VITAGLIONE et al., 2008). For human health the function as antioxidant is highly interesting because of radical scavenging processes in the human organism and therefore protective against diseases like cancer or CVD reported in chapter 3.2.3 of the literature review. FA is the major phenolic acid in whole wheat samples mainly derived from fraction 3 the bound phenolic acids no matter if it is an ancient wheat cultivar or a modern one (chapter 5.1 Tab. 2 and chapter 5.1 Tab. 3, chapter 5.2 Fig. 4 and chapter 5.3 Fig. 1). In order to profit of this phenolic acid it is necessary to use whole wheat products in human nutrition. Spelt wheat might be a

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very good alternative to common wheat since it has got a very good ratio of FA:TPA (Tab. 6-1). Looking at the sprouting experiment (chapter 5.2) with common wheat it is noticeable that common wheat has got much higher FA and TPA values but with a worse ratio of FA:TPA (Tab. 6-1). Sprouting reveals changes in the FA concentrations which are not significant for FA (Fig. 6-1). These findings have to be analyzed further with more samples to understand the results much better.

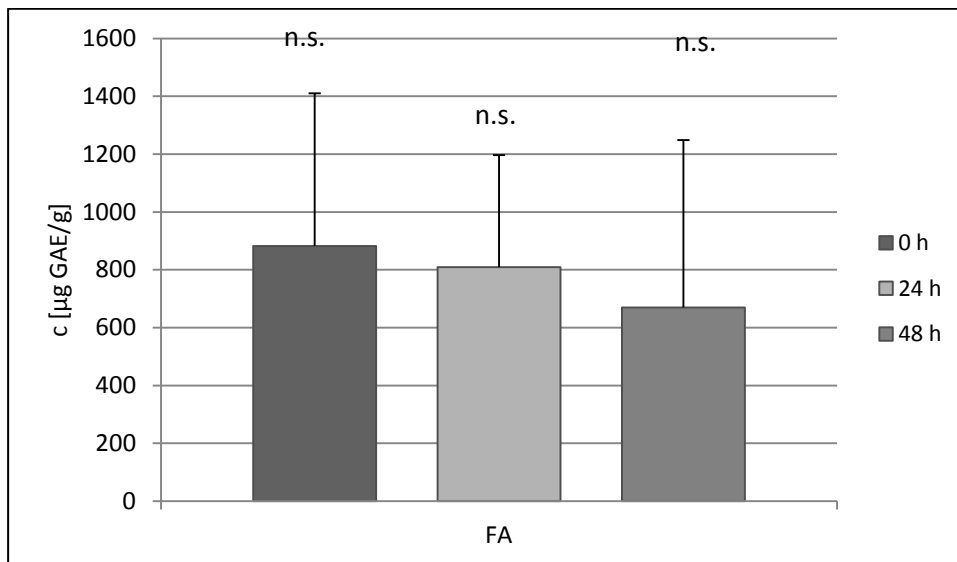


Fig. 6-1: Effects of sprouting time on FA concentrations [µg GAE/g] in wheat samples of experiment 2 (chapter 5.2) (mean, $\alpha=0.05$)

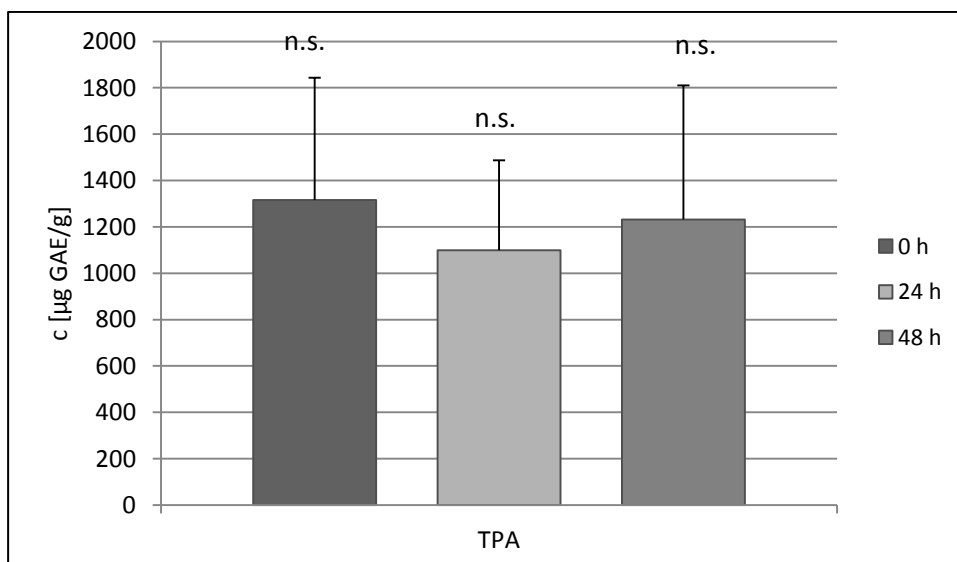


Fig. 6-2: Effects of sprouting time on TPA concentrations [µg GAE/g] in wheat samples of experiment 2 (chapter 5.2) (mean, $\alpha=0.05$)

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When looking at all phenolic acids (TPA) common wheat samples were at a much higher concentration level than ancient wheat samples (Tab. 6-1). TPA values of ancient wheat were around 300-370 µg GAE/g to be exact: experiment 1: einkorn 306.8 µg GAE/g, emmer 368.7 µg GAE/g and spelt 312.0 µg GAE/g (chapter 5.2 Tab. 2) along with spelt of experiment 3 340.2 µg GAE/g (chapter 5.3 Fig. 1). The analyzed common wheat samples of experiment 2 which were not sprouted had a concentration of 1315.5 µg GAE/g (chapter 5.2 Tab. 2). The highest total phenolic acid concentrations are derived from fraction 3. The TPA concentration of fraction 3 of Einkorn in experiment 1 is 192.0 µg GAE/g, of emmer 248.0 µg GAE/g and of spelt 203.0 µg GAE/g (chapter 5.1 Tab. 2). Spelt of experiment 3 had a TPA content of 245.0 µg GAE/g (chapter 5.3 Tab. 2 and chapter 5.3 Tab. 3) whereas not sprouted common wheat of experiment 2 contained 1128.4 µg GAE/g being the highest concentration of all three fractions (chapter 5.2 Fig. 3).

Only little information is available about the effect of sprouting on common wheat or ancient wheat seeds. Although concentration levels were varying between the sprouting times of experiment 2 sprouting didn't result in significant differences for TPA concentrations (Fig. 6-2). While sprouting TPA concentrations were declining and then rising again. This was also observed in a study of YANG et al. (2001) where wheat grain were steeped for 24 and 48 h and germination followed for 1-8 days at 16.5°C. The working group assumed washing out effects of water-soluble phenolic acids during the first four days which resulted in a decline of PAs. Later on PA concentrations increased for the sprouts. In the present work steeping and sprouting was one step and sprouting states were defined by their falling number which was at 100-120 s for presprouted grain after 24 h and 62 s for fully sprouted grain after 48 h. In both cases the coleoptile and roots were visible but not yet green parts of the plant. The own results indicated the same tendency like the results by YANG et al. (2001) but much faster. The endosperm was still present, however, decreased by the process of sprouting. These findings lead to the assumption that TPA concentrations of the ancient wheat of experiment 1 would probably be higher if they weren't partially sprouted. Since the partially sprouted ancient wheat showed falling numbers of 62 s which made them comparable to the fully sprouted samples of experiment 2 the effect of lower PA contents should be marginal given that ancient wheat behaves similar like common wheat.

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Significant differences of TPAs between the analyzed bread wheat cultivars (not sprouted) of experiment 2 were observed indicating a genetically determination (chapter 5.2 Tab. 2). This was affirmed by the findings of ABDEL-AAL et al., 2001c, MPOFU et al., 2006 and MOORE et al., 2006 who analyzed several bread wheat cultivars with genetically determined differences of their TPA concentration. Moreover, it was conspicuous that cultivar Tommi which is characterized as “A” quality wheat showed the significantly highest TPA value. Cultivars Privileg and Estevan both characterized as “E” quality bread wheat had lower values although belonging to a better quality group. The quality group E is determined by higher crude protein (CP) contents than group A. In the present work CP values were at about the same level (chapter 5.2 Tab. 1) and not significantly different for the analyzed cultivars. To answer the question whether TPA contents of different wheat cultivars could be determined by their belonging to a quality group further examinations should be conducted.

In the literature the presence of FA and other phenolic acids was reported variously (ADOM and LIU, 2002, ADOM et al., 2003, ANDREASEN et al., 2000a, ANDREASEN et al., 2000b, EMMONS et al., 1999, GARCIA-CONESA et al., 1997, HATCHER and KRUGER, 1997, SLAVIN, 2003, SUN et al., 2001, ZHOU and YU, 2004, ZHOU et al., 2004a, ZHOU et al., 2004b). Since most phenolic acids are bound to cell wall polymers they have to be released in order to be bioavailable and bioaccessible for the human organism. This process mainly takes part in the colon during fermentation by xylanases (BARTOLOMÉ et al., 1995, KROON et al., 1997, ZHAO et al., 2003). Bioavailability and bioaccessibility of FA and other phenolic acids was analyzed and proven in several studies reported in chapter 3.2.4 and 3.3 of the literature review. When phenolic acids are present in the human organism they can perform their functions such as acting as antioxidants in order to fight oxidative stress and scavenge free radicals. To measure their potential antioxidative capacity two different tests were conducted. The antioxidative capacity was measured by the Folin-Ciocalteu on the one hand and by the ORAC assay on the other hand (chapter 4.5).

Tab. 6-2: Total phenolic compounds (TPC) [$\mu\text{g GAE/g}$] and ORAC values [$\mu\text{mol TE/g}$] of wheat species in the three executed investigations (exp.) (experiment 1 chapter 5.1, experiment 2 chapter 5.2, experiment 3 chapter 5.3) (mean)

Exp	Species	Sprouting [h]	TPC [$\mu\text{g GAE/g}$]	ORAC [$\mu\text{mol TE/g}$]
1	<i>T. aestivum</i> L. ssp. <i>spelta</i>	0	2259.9	20.9
	<i>T. monococcum</i> L. ssp. <i>monococcum</i>	0	2402.9	20.1
	<i>T. turgidum</i> L. ssp. <i>dicoccoides</i>	0	2613.7	21.7
2	<i>T. aestivum</i> L. ssp. <i>aestivum</i>	0	2054.8	28.2
	<i>T. aestivum</i> L. ssp. <i>aestivum</i>	24	1751.6	27.4
	<i>T. aestivum</i> L. ssp. <i>aestivum</i>	48	2575.6	32.6
3	<i>T. aestivum</i> L. ssp. <i>spelta</i>	0	1252.5	22.9

The antioxidative capacity measured by Folin-Ciocalteu was expressed as total phenolic content (TPC). TPC values of experiment 1 are displayed in Fig. 5 of chapter 5.1 which are mean values. Spelt wheat reached TPC concentrations of 2259.9 $\mu\text{g GAE/g}$ which was at a much higher level than for the analyzed spelt wheat cultivars of experiment 3 (Tab. 6-2 and chapter 5.3 Tab. 4) where mean TPC of 1252.5 $\mu\text{g GAE/g}$ had been found. Reasons for those diverging results can only be assumed. It might be the case that cultivar effects were the reason. But still it is hard to explain since TPC and TPA were correlating with each other for both experiments indicating that higher TPA values result in a higher antioxidative capacity. Spelt wheat of experiment 1 had a little lower TPA (312.0 $\mu\text{g GAE/g}$) concentration than spelt of experiment 3 (340.2 $\mu\text{g GAE/g}$) (Tab. 6-1). Another vague supposition could be that spelt and the other analyzed ancient wheat cultivars of experiment 1 had a higher level of further antioxidant constituents which may contribute to the antioxidative capacity especially in fraction 1 of the total phenolic content (TPC1). In experiment 3 TPA values of fraction 1 with a mean of 21.8 $\mu\text{g GAE/g}$ were higher compared to TPA1 of experiment 3 with 11.5 $\mu\text{g GAE/g}$ (chapter 5.1 Tab. 2 and chapter 5.3 Tab. 3). Besides phenolic acids in wheat additional antioxidative active substances were found by ABDEL-AAL and RABALSKI (2008a). This working group analyzed einkorn, emmer, spelt and common wheat samples where they could find following antioxidative active compounds: carotenoids (all-trans lutein), tocopherols (α -tocopherol, β -tocopherol) and tocotrienols (α -tocotrienol, β -tocotrienol - predominant). Carotenoids such as β -carotene, lutein and zeaxanthin also tocopherols such as α -tocopherol were found in soft red winter wheat genotypes analyzed by MOORE et al. (2005). OKARTER et al. (2010) analyzed six different wheat

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varieties where they could confirm the findings of carotenoids such as lutein, zeaxanthin, β -carotene and tocopherols and tocotrienols as well. The question why TPC values of experiment 3 were much lower than the others has to be left open. It can be assumed if other phytochemicals were present in experiment 1 and 2 they contribute to the antioxidative capacity by Folin-Ciocalteu. Those phytochemicals must be substances which rather act in electron transfer reactions (Folin-Ciocalteu assay) than in hydrogen donating ones which is the case for ORAC assays. Moreover, these antioxidative active substances must be present as free compounds because TPC1 was higher than the concentration of phenolic acids in TPA1 (chapter 5.1 Tab. 2 and chapter 5.1 Fig. 5, chapter 5.2 Fig. 3 and chapter 5.2 Fig. 5, chapter 5.3 Tab. 2).

Looking at mean values of the antioxidative capacity measured by the ORAC assay the difference between spelt wheat cultivars of experiment 1 and 3 was not as big as for the TPC values (Tab. 6-2). The ORAC value of spelt wheat in experiment 1 was 20.9 $\mu\text{mol TE/g}$ and in experiment 3 22.9 $\mu\text{mol TE/g}$ (chapter 5.1 Fig. 6 and chapter 5.3 Tab. 5). Einkorn and emmer were at about the same level with 20.1 and 21.7 $\mu\text{mol TE/g}$. The ORAC value of experiment 2 for not sprouted bread wheat was at a higher level with 28.2 $\mu\text{mol TE/g}$ as well as for the sprouted samples (Tab. 6-2 and chapter 5.2 Tab. 4). In the literature ORAC values were ranging probably due to different extraction methods. ZHOU et al. (2007) conducted a study with 11 soft red winter wheat samples which varied between 15.5 $\mu\text{mol TE/g}$ and 24.5 $\mu\text{mol TE/g}$, in the study by MOORE et al. (2005) the analyzed soft winter wheat samples ranged from 32.9 to 47.7 $\mu\text{mol TE/g}$ and the six wheat cultivars analyzed by OKRATER et al. (2010) ranged between 51 to 96 $\mu\text{mol TE/g}$. Results of the antioxidative capacity of three different extracted PA fractions like it was the case in the present work could not be found in literature.

Evaluating the results of the three fractions of TPA and ORAC the tendency was similar to the comparison of TPA and TPC results. ORAC1 showed a higher antioxidative capacity than one lead to assume when looking at the TPA1 concentration. Although ORAC1 was higher than ORAC2 the PA concentration of TPA1 was lower than TPA2 (chapter 5.1 Tab. 2 and chapter 5.1 Fig. 6, chapter 5.2 Fig. 3 and chapter 5.2 Fig. 6, chapter 5.3 Tab. 2). Further antioxidative free substances must be present like it was already discussed for the TPC values. There

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must be substances like vitamin C or other organic substances, assumed by HUANG et al. (2005) and GEORGÉ et al. (2005) which contribute to the Folin-Ciocalteu test values as well as to the ORAC values. ORAC and TPC values were correlating in the own results (chapter 5.3 Tab. 6) which is also confirmed by the findings reported by MOORE et al. (2006) and OKARTER et al. (2010).

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7 Summary

Wheat (*Triticum aestivum* L.) is one of the world's staple foods and important agricultural commodities. During evolutionary development the today used bread wheat (*T. aestivum* ssp. *aestivum*) which is free threshing and provides high grain yields evolved from ancient wheat species which were non-threshable and had low grain yields. In context with a balanced diet those ancient wheat species such as einkorn (*T. monococcum* L.), emmer (*T. dicoccum* L.) and spelt wheat (*T. aestivum* L. ssp. *spelta*) became interesting again in the recent years. The focus of this work was on phenolic acids as natural antioxidants measured by HPLC and their potential antioxidative capacity measured with the Folin-Ciocalteu and ORAC assay.

Experiment 1 (chapter 5.1) was an evaluation of ancient wheat species including einkorn, emmer and spelt wheat. Ferulic acid (FA) as one analyzed phenolic acid was the abundant one with about 65 % of all analyzed phenolic acids. Total phenolic acid values were ranging from 283.3 to 405.3 µg GAE/g whole wheat flour and were comparable to the literature. For the analyses three fractions (soluble free, soluble conjugated and bound phenolic acids) were extracted and most phenolic acids were found as bound forms which was supported by the literature as well. Experiment 2 (chapter 5.2) was a sprouting experiment with bread wheat cultivars. The experiment was conducted to see whether sprouting influenced the phenolic acid contents and the antioxidative capacity. FA was again the predominant (881.8 µg GAE/g) phenolic acid in all three sprouting steps ((1) not sprouted, (2) presprouted and (3) fully sprouted) with 67 % for not sprouted and 54 % for fully sprouted samples. Bound phenolic acids contributed the main part of the total phenolic acids although sprouting had an influence on that. The third experiment (chapter 5.3) evaluated spelt wheat cultivars as the most important ancient wheat species. Similar results to the first experiment were found with FA as the abundant phenolic acid ranging between 226.7 and 295.8 µg GAE/g. Fraction 3 (bound phenolic acids) was the predominant fraction for that experiment as well. In general, TPA contents and composition along with FA contents were comparable to the literature. No evolutionary changes seem to be present in the composition of phenolic acids with FA as the abundant phenolic acid in ancient wheat plus in modern wheat species. Looking at the results of all three experiments there were some differences in TPA and FA contents. Whether

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those differences were due to species or environmental effects can only be assumed nevertheless it was reported in the literature. Many diverse extraction methods had been used by other working groups which makes a reliable comparison difficult. Therefore, a standardized method should be implemented in order to get more comparable evidences.

The antioxidative capacity by Folin-Ciocalteu expressed as total phenolic contents (TPC) measures not only phenolic compounds also other organic compounds are reacting with this test. Since the most phenolic acids were found in the bound fraction and TPA along with TPC were correlating with each other this fraction contributed the highest TPC values in experiment 1. In total 2.4 mg GAE/g were found for the analyzed ancient wheat species which was higher than in the literature reported. Experiment 3 was at a much lower level with 1.3 mg GAE/g in spelt wheat flour and the bound fraction supplied the highest TPC values as well. The TPC values of the bread wheat samples of experiment 2 were at about the same level with 2.1 mg GAE/g as the ancient wheat samples of the first experiment which leads to the assumption that ancient wheat are comparable to bread wheat samples used today. In the literature TPC values of the total extracts were analyzed and ranged widely which made it hard to judge present results. The same obstacle was found for the antioxidative capacity measured by the ORAC assay.

ORAC values of experiment 1 in total ranged from 18.2 to 23.8 $\mu\text{mol TE/g}$ whole wheat flour of ancient wheat which was at about the same level as for spelt wheat flour of experiment 3 ranging between 21.5 and 24.6 $\mu\text{mol TE/g}$. A higher antioxidative capacity was found for the bread wheat samples of experiment 2 with a mean of 28.2 $\mu\text{mol TE/g}$ for not sprouted samples. Sprouting time had a significant influence on the antioxidative capacity and after 48 h of sprouting the mean ORAC value was at 32.6 $\mu\text{mol TE/g}$. The differences between the ORAC values of the conducted three experiments were not as big as for TPC values. Both tests showed the same tendency for the antioxidative capacity which implies a direct proportionality of TPC and ORAC values.

Thus, ancient wheat, sprouted wheat and bread wheat do not only consist of basic nutrients such as carbohydrates, protein, dietary fiber, vitamins or minerals they also contain secondary plant metabolites like phenolic acids. Their ability to scavenge free

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radicals and therefore their antioxidative potential makes them interesting in order to reduce the risk of chronic diseases. Health beneficial effects of phenolic acids are rather derived from whole wheat products than from refined flours because of their predominant occurrence as bound forms to cell wall components. Thus, whole wheat products of ancient wheat species, e.g. derived from spelt wheat, could be a real alternative in a balanced human diet.

8 Zusammenfassung

Weizen (*Triticum aestivum* L.) ist eines der wichtigsten Grundnahrungsmittel und landwirtschaftlichen Erzeugnisse weltweit. Der heute angebaute Brotweizen (*T. aestivum* ssp. *aestivum*) hat sich aus alten Weizenarten entwickelt, ist unbespelzt (freidreschend) und liefert hohe Kornerträge, wohingegen alte Weizenarten bespelzt (nicht freidreschend) sind und geringe Kornerträge hervorbringen. Im Zusammenhang mit einer ausgewogenen Ernährung sind diese alten Weizenarten, wie beispielsweise Einkorn (*T. monococcum* L.), Emmer (*T. dicoccum* L.) und Dinkel (*T. aestivum* L. ssp. *spelta*), in den letzten Jahren wieder interessant geworden. Der Schwerpunkt der vorliegenden Arbeit ist auf Phenolsäuren als natürliche Antioxidantien und ihre mögliche antioxidative Kapazität gesetzt worden. Die Phenolsäuren sind durch HPLC Analysen und die antioxidative Kapazität durch den Folin-Ciocalteu und den ORAC Assay bestimmt worden.

Experiment 1 (Kapitel 5.1) ist eine Auswertung alter Weizenarten einschließlich Einkorn, Emmer und Dinkel. Die Phenolsäure Ferulasäure (FA) ist die Hauptphenolsäure mit 65 % der gesamten untersuchten Phenolsäuren. Die Gesamtphenolsäurewerte, welche mit der Literatur vergleichbar sind, bewegen sich zwischen 283,3 und 405,3 µg GAE/g Vollkornweizenmehl. Für die Analysen sind drei Fraktionen ((1) löslich freie, (2) löslich konjugierte und (3) gebundene Phenolsäuren) extrahiert worden. Die meisten Phenolsäuren sind als gebundene Form vorgekommen, was auch durch die Literatur bekräftigt worden ist. Im Experiment 2 (Kapitel 5.2) sind Keimungsversuche mit Brotweizensorten durchgeführt worden, um festzustellen, ob die Keimung einen Einfluss auf die Phenolsäuregehalte und antioxidative Kapazität hat. Die FA ist hier ebenso die vorherrschende Phenolsäure (881,8 µg GAE/g) in allen drei Keimungsstufen (nicht gekeimt (0 h), angekeimt (24 h) und durchgekeimt (48 h)) mit 67 % bei den nicht gekeimten und 54 % bei den durchgekeimten Weizenproben. Den größten Anteil der gesamten Phenolsäure liefern die gebundenen Phenolsäuren, obwohl die Keimung einen Einfluss darauf hatte. Im dritten Experiment (Kapitel 5.3) ist Dinkel als die wichtigste der alten Weizenarten untersucht worden und es sind ähnliche Ergebnisse wie im ersten Experiment festgestellt worden. Die FA ist ebenfalls in diesem Experiment als die Hauptphenolsäure mit Werten zwischen 226.7 und 295.8 µg GAE/g identifiziert

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worden. Die meisten Phenolsäuren sind als gebundene Bestandteile aus Fraktion 3 zu finden. Allgemein kann die Aussage getroffen werden, dass die Konzentrationen der Gesamtphenolsäuren und der FA mit den Werten der Literatur vergleichbar sind. Die Zusammensetzung der Phenolsäuren, mit FA als Hauptphenolsäure in alten als auch in heutigen Weizenarten, scheint im Laufe der Evolution keine große Veränderung erfahren zu haben. Allerdings sind einige Unterschiede der Gesamtphenolsäuren und FA im Vergleich mit allen drei Experimenten festzustellen. Ob diese Unterschiede in der eigenen Untersuchung aufgrund von Sorten- oder Umwelteinflüssen zustande kommen, kann nur vermutet werden. Die Literatur bestätigt hingegen diesen Zusammenhang. Des Weiteren sind verschiedene Extraktionsmethoden durch andere Arbeitsgruppen angewendet worden, was einen verlässlichen Vergleich schwierig gestaltet. Deshalb sollte eine standardisierte Extraktionsmethode angewendet werden, um besser vergleichbare Aussagen treffen zu können.

Die antioxidative Kapazität nach Folin-Ciocalteu wird als Gesamtphenolgehalt (TPC) ausgedrückt und erfasst nicht nur phenolische, sondern auch andere organische Verbindungen, welche mit diesem Test reagieren. Da der größte Anteil der Phenolsäuren in gebundener Form vorkommt (Fraktion 3) und die Gesamtphenolsäuren mit den Gesamtphenolen korrelieren, liefert diese Fraktion auch die höchsten TPC Werte im ersten Experiment (Kapitel 5.1). Insgesamt sind 2,4 mg GAE/g Vollkornmehl in den untersuchten alten Weizenarten gefunden worden. Dieses Ergebnis ist höher als in der Literatur. Allerdings sind die Untersuchungsergebnisse des Dinkels in Experiment 3 (Kapitel 5.3) mit 1,3 mg GAE/g auf einem viel geringeren Niveau. Zudem können wiederholt die höchsten TPC Werte in der gebundenen Fraktion gefunden werden. Die TPC Werte der Brotweizenproben aus Experiment 2 (Kapitel 5.2) sind mit 2,1 mg GAE/g ungefähr auf demselben Niveau wie die alten Weizenarten des ersten Experiments. Dies führt zur Annahme, dass alte Weizenarten mit modernen Weizenarten vergleichbar sind. In der Literatur sind TPC Werte von Gesamtextrakten untersucht worden, welche nicht in drei Fraktionen unterteilt worden sind. Die Ergebnisse schwanken sehr stark, was die Schwierigkeit der Vergleichbarkeit mit den vorliegenden Ergebnissen mit sich bringt. Ebenso ist diese Schwierigkeit für die Vergleichbarkeit der antioxidativen Kapazität mit Hilfe der ORAC Methode festgestellt worden.

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Die Gesamt-ORAC Werte des ersten Experiments (Kapitel 5.1) bewegen sich zwischen 18,2 und 23,8 $\mu\text{mol TE/g}$ Vollkornmehl der alten Weizenarten. Dieses Ergebnis kann ungefähr mit den Ergebnissen des Dinkels aus Experiment 3 (Kapitel 5.3), welche sich zwischen 21,5 und 24,6 $\mu\text{mol TE/g}$ bewegen, verglichen werden. Eine höhere antioxidative Kapazität weisen die ungekeimten Brotweizenproben aus Experiment 2 (Kapitel 5.2) mit 28,2 $\mu\text{mol TE/g}$ auf. Einen signifikanten Einfluss auf die antioxidative Kapazität hat die Keimungszeit, so dass der mittlere ORAC Wert nach 48 h Keimung bei 32,6 $\mu\text{mol TE/g}$ gelegen hat. Insgesamt sind die Unterschiede der antioxidativen Kapazität der ORAC-Methode nicht so groß, wie die der Folin-Ciocalteu-Methode, bezogen auf alle drei Experimente. Beide Messmethoden der antioxidativen Kapazität zeigen dieselbe Tendenz, so dass eine direkte Proportionalität zwischen TPC und ORAC angenommen wird.

Alte Weizen, gekeimter Weizen und Brotweizen bestehen also nicht nur aus Hauptnährstoffen wie Kohlenhydrate, Proteine, Ballaststoffe, Vitamine oder Mineralstoffe, sondern beinhalten auch sekundäre Pflanzeninhaltsstoffe, wie z.B. Phenolsäuren. Ihre Fähigkeit freie Radikale abzufangen und damit ihr antioxidatives Potential macht Phenolsäuren interessant, um das Risiko chronischer Erkrankungen zu senken. Eine gesundheitsfördernde Wirkung der Phenolsäuren wird eher durch Weizenvollkornprodukte erreicht, als durch Weißmehl. Der Grund liegt darin, dass Phenolsäuren hauptsächlich als zellwandgebundene Bestandteile vorkommen. Aus diesem Grund können Vollkornprodukte der alten Weizenarten, bspw. von Dinkel, eine echte Alternative in einer ausgewogenen Ernährung sein.

9 Declaration

“I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation.

I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me.

At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the ‘Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice’.”

Gießen, June 24, 2011

Nadine Engert

10 Publication list

1. **ENGERT N., PÄTZOLD R., HONERMEIER B., 2009:** Phenolsäuren in alten Weizenarten (*Triticum* sp.) – Vorstellung der Methode und erster Ergebnisse. In: Deutsche Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) DGQ e.V. (Hrsg.) (2009): Pflanzenphenole für die Gesundheit von Pflanze, Mensch und Tier. 44. Vortragstagung am 16./17.03.09 in Freising, 58-60.
2. **ENGERT N., HONERMEIER B., 2009:** Vorkommen von Phenolsäuren in alten Weizenarten. In: Gesellschaft für Pflanzenbauwissenschaften e.V. (Hrsg.) (2009): Pflanzenbauwissenschaften – Systembezug und Modellierung. 52. Jahrestagung am 01.-03.09.09 in Halle/Saale, 59-60.
3. **ENGERT N., PÄTZOLD R., HONERMEIER B., 2010:** Lebensmittelchemische Gesellschaft- Regionalverband Südwest – Tagung am 02./03.03.09 in Koblenz: Variation der Phenolsäuregehalte in alten Weizenarten. Lebensmittelchemie 64, 12-13.
4. **ENGERT N., STRANZ A., HONERMEIER B., 2011:** Lebensmittelchemische Gesellschaft- Regionalverband Südwest – Tagung am 08./09.03.10 in Erlangen: Phenolsäuregehalte und antioxidative Kapazität von Dinkelweizen (*Triticum aestivum* ssp. *spelta*). Lebensmittelchemie 64, 167-168.
5. **ENGERT N., STRANZ A., HONERMEIER B., 2011:** Backqualität und Phenolsäuregehalte von Dinkelweizen (*Triticum aestivum* ssp. *spelta*) unter dem Einfluss von Sorte und Standort. Deutsch-Türkische Agrarforschung. 9. Symposium des VDTAN am 22.-26.03.2010 in Antakya-Hatay (Türkei).
6. **ENGERT N., HONERMEIER B., 2011:** Characterization of grain quality and phenolic acids in ancient wheat species (*Triticum* sp.). Journal of Applied Botany and Food Quality 84 - in print.

10 Publication list

7. **ENGERT N., JOHN A., HENNING, W., HONERMEIER B.:** Effect of sprouting on the concentration of phenolic acids and antioxidative capacity in wheat cultivars (*Triticum aestivum* ssp. *aestivum* L.) in dependency of nitrogen fertilization. Journal of Applied Botany and Food Quality – in print.
8. **ENGERT N., STRANZ A., HENNING, W., HONERMEIER B.:** Grain quality, phenolic acid contents and antioxidative capacity of different spelt wheat (*Triticum aestivum* ssp. *spelta*) cultivars. Cereal Technology - under review.

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