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# Hydroxycinnamic acid composition and in vitro antioxidant activity of selected grain fractions

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**Abstract**

This study was designed to determine the composition in hydroxycinnamic acids and the antioxidant properties of soluble extracts from wheat, rye and buckwheat. Free, soluble and insoluble hydroxycinnamic acids were determined by HPLC-diode array (DAD). Some *C*-glycosyl flavonoid derivatives were also identified by LC/MS/MS. Total antioxidant capacity, inhibition of lipid peroxidation and scavenging of oxygen radicals were used to evaluate the in vitro antioxidant capacity of the cereal soluble extracts. The highest levels of total hydroxycinnamic acids and derivatives were found in the wheat bran and rye bran fractions whereas the buckwheat flours had only trace quantities of these compounds. The most abundant compound present in the wheat and rye fractions was ferulic acid but small quantities of diferulic acids, sinapic acid, *p*-coumaric acid and benzoic acid derivatives were also present. The largest proportions of these phenolic compounds were found covalently bound (esters) in the insoluble pellet but between 10% and 30% of the total compounds were solubilized, mostly in water. Most of the antioxidant capacity was found in the water extracts from all the cereal fractions. Overall, buckwheat and wheat germ products exhibited the highest antioxidant capacity whereas the rye products had the lowest antioxidant values. The antioxidant capacity of these complex cereal extracts cannot be explained by simple correlation with the content of total soluble hydroxycinnamic acids. All other soluble compounds present in the extracts, their possible antioxidant activity and interactions need to be elucidated in order to fully explain the final antioxidant capacity of the extracts.


*Keywords:* Antioxidants; Hydroxycinnamic acids; Ferulic acid; Diferulic acids; Flavonoids; Cereals; Water extracts; Wheat; Rye; Buckwheat

## 1. Introduction

Antioxidants are defined as molecules that, at low concentration and under specific assay conditions, can delay or prevent oxidation of an oxidizable substrate (Halliwell & Whiteman, 2004). Compounds with antioxidant capacity, and which are naturally present in food, are of great interest to the food industry and to consumers because of (i) their potential value to prolong the shelf-life of foodstuffs by protecting them against oxidative deterioration and (ii) their possible beneficial effects on human and animal health (Zhou & Yu, 2004). Epidemiological and animal studies suggest that the regular consumption of plant foods, such as fruits, vegetables and whole grains, reduces the risk of chronic diseases associated with oxidative damage (namely cardiovascular diseases and cancer) (Arts & Hollman, 2005; Kris-Etherton et al., 2002; Slavin, 2003). Significant in vitro antioxidant activity has been determined in a variety of extracts from fruits, vegetables and various cereals (Martínez-Tomé et al., 2004; Miller, Rigelhof, Marquat, Prakash, & Kanter, 2000; Wu et al., 2004) which is mostly due to the combined effects of a range of soluble molecules with antioxidant properties present in the food (Kris-Etherton et al., 2002). It has been postulated that the consumption of these dietary antioxidants may be

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partially responsible for the prevention or delay of oxidative damage in the body, although whether these compounds may actually act as antioxidants in the body, and by which mechanisms, is not yet fully understood.

Amongst other various plant components, phenolic acids are well-recognized powerful antioxidants widely present in plant food. In particular, hydroxycinnamic acids are a group of phenolics highly abundant in cereals (Adom & Liu, 2002; Adom, Sorrells, & Liu, 2003; Andreasen, Christensen, Meyer, & Hansen, 2000; Emmons, Peterson, & Paul, 1999; Garcia-Conesa, Plumb, Waldron, Ralph, & Williamson, 1997; Hatcher & Kruger, 1997; Sun, Sun, & Zhang, 2001; Zhou, Laux, & Yu, 2004a, Zhou, Su, & Yu, 2004b). They exhibit good antioxidant properties (Andreasen, Landbo, Christensen, Hansen, & Meyer, 2001a; Garcia-Conesa, Wilson, Plumb, Ralph, & Williamson, 1999a; Rice-Evans, Miller, & Paganga, 1996) and may be at least partially responsible for the beneficial effects associated with cereal consumption. Ferulic acid and its oxidative products, diferulic acids, are the most abundant hydroxycinnamic acids in cereals (Andreasen et al., 2000; Garcia-Conesa et al., 1997) but small quantities of other hydroxycinnamic acids (sinapic acid, *p*-coumaric acid, caffeic acid) and benzoic acid derivatives have also been described in various grains and derived products (Adom & Liu, 2002; Adom et al., 2003; Emmons et al., 1999; Hatcher & Kruger, 1997; Sun et al., 2001; Zhou & Yu, 2004; Zhou et al., 2004a, 2004b). Most of these compounds are located in the outermost aleuron layers and bran and in the germ of the grains (Garcia-Conesa et al., 1997; Zhou et al., 2004a). They are mostly covalently bound to cell wall polymers and can be released after alkali or acid treatments (Bonoli, Verardo, Marconi, & Caboni, 2004; Garcia-Conesa et al., 1997). A fraction of soluble hydroxycinnamic acids is also present in cereals and can be extracted with a mixture of solvents of various polarities (Adom & Liu, 2002; Adom et al., 2003; Bonoli et al., 2004; Emmons et al., 1999; Hatcher & Kruger, 1997; Zhou et al., 2004a, 2004b; Zieliński & Kozłowska, 2000). It has been shown that several factors, namely grain variety, environmental and growing conditions or milling and refining process of grains, can influence the presence and distribution of hydroxycinnamic acids and other phenolic compounds and the final antioxidant power of cereal products (Adom & Liu, 2002; Adom et al., 2003; Emmons et al., 1999; Zhou & Yu, 2004; Zhou et al., 2004a, 2004b; Zieliński & Kozłowska, 2000). Grain-derived products are a very important component of the human diet. Current recommendations from international health and nutrition organizations include an increase in the consumption of high bran cereals because of the potential benefit to human health. Thus, it is of great importance to understand the association between antioxidant compounds in food, such as those present in cereals, and their possible role in human health. A step in this direction is to fully characterize the antioxidant compounds in cereals destined for human consumption, as well as to determine the effect of processing on the fate of these compounds and on the antioxidant capacity of cereal products.

The present study was conducted to determine and compare the profile and distribution of hydroxycinnamic acids, as well as the in vitro antioxidant capacity of several cereal products from wheat, rye and buckwheat. This research aims to contribute to the understanding of the relationship between phenolics and antioxidant capacity in foods, as well as to look into the influence of processing on the antioxidant capacity.

## 2. Materials and methods

### 2.1. Reagents

*trans*-Ferulic acid (*trans*-FA), *trans*-*p*-coumaric acid (*p*-CA), vanillic acid, *p*-OH-benzoic acid, *trans*-sinapic acid (SA), vanillin, rutin and quercetin were obtained from Sigma Chemical Co. US. *cis*-Ferulic acid (*cis*-FA) was prepared by radiation with UV light (Lewis, Dubelsten, Eberhardt, Yamamoto, & Towers, 1987). The 5-5-diferulic acid (5-5-diFA), 8-*O*-4-diferulic acid (8-*O*-4-diFA), 8-5-benzofuran-diferulic acid (8-5-benzofurandiFA) and 8-5-diferulic acid (open ring) (8-5-diFA) were obtained by alkaline hydrolysis of the corresponding diethyl diferulates, followed by purification on reverse preparative chromatography (García-Conesa et al., 1999b).

### 2.2. Cereal samples

The cereal products used in this study were provided by Danone Vitapole (France) and are described in Table 1. On arrival, the samples were stored and kept at −20 °C prior to analysis. Moisture content of the cereal samples was determined by drying at 130 °C for 90 min (Reference Method, International Organization for Standardization, 2001). Analyses were done in duplicate.

### 2.3. Preparation of extracts

The cereal samples were extracted sequentially with water at 50 °C, followed by 80% methanol and ethyl acetate. Extractions with each solvent were carried out twice for 30 min under $N_2$, in the dark and constant agitation (200 rpm). Ethyl acetate extracts were evaporated under vacuum and re-dissolved in 80% methanol. The final insoluble pellet was dried to constant weight (37 °C). The soluble fractions and the insoluble pellet were stored at 4 °C (under $N_2$ and in the dark) prior to the analysis of phenolic compounds and determination of the antioxidant activity.

### 2.4. Analysis of hydroxycinnamic acids and derivatives

To determine free soluble hydroxycinnamic acids and benzoic acid derivatives, internal standard (IS, *o*-coumaric acid) was added to each of the soluble extracts prior to concentration, filtering (0.2 μm) and analysis by HPLC-diode

Table 1
Description of the cereal fractions used in this study

| Fraction | Name | Humidity (%)[a] | Description |
|---|---|---|---|
| 1 | Wheat middling | 13.0 | Wheat by-product of milling industry. Mix of fine particles of flour and bran which can not be sieved one from each other and fine particles of husk bonded to albumen particles |
| 2 | Air separated wheat middling | 11.6 | Wheat fine middlings obtained by air classifier separation which operates by a combination of a centrifugal force and an opposing force (air velocity) according to the weight and the size of the particles |
| 3 | Reduction wheat flour | 13.4 | Wheat flour obtained during grinding on smooth reduction rolls. Fine particles of albumen (30–200 μm) |
| 4 | Fresh wheat germ | 13.3 | Fresh germs are a by-product of the wheat flour milling industry |
| 5 | Wheat bran | 8.3 | By product of wheat milling industry. Outer cell layers of the wheat kernel. Bits of husk (0.5–5 mm main dimension: 0.5–10 $mm^2$) free of any albumen |
| 6 | Fermented wheat bran | 9.6 | Wheat bran mixed with water and fermented by *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Saccharomyces cerevisiae*. The mixture is freeze-dried after fermentation |
| 7 | Rye flour | 12.9 | Fine particles of albumen (30–200 μm) |
| 8 | Rye middling | 10.6 | By-product of rye milling industry. Mix of fine particles of flour and fine particles of bran which can not be sieved one from each other. |
| 9 | Rye bran | 12.1 | By product of rye milling industry. Outer cell layers of the rye kernel. Bits of husk (0.5–5 mm main dimension: 0.5–10 $mm^2$) free of any albumen |
| 10 | Cold pressed wheat germ | 5.6 | Stabilized germs from wheat (obtained during milling). Stabilization is obtained by pressing the germs in order to remove the oil and avoid oxidation |
| 11 | Buckwheat flour | 13.5 | Pseudo-cereal, Polygonaceae – *Fagopyrum esculentum*. Flour is obtained by grinding the small edible triangular seeds into flour after removal of the husks |
| 12 | Buckwheat husks | 6.8 | Pseudo-cereal, Polygonaceae – *Fagopyrum esculentum*. Husks are the outer cell layers of the seed. It is a by-product of the buckwheat milling industry |

[a] Drying at 130 °C for 90 min (25)

array (DAD) (Andreasen, Kroon, Williamson, & García-Conesa, 2001b). For analysis of total hydroxycinnamates, diferulates and benzoate compounds, soluble fractions and pellets were hydrolyzed in 1.0 M NaOH for 16 h under $N_2$ and in the dark to ensure the release of ester-linked compounds (Garcia-Conesa et al., 1997). IS was added to the hydrolyzed sample prior to acidification (pH < 2) and extraction with ethyl acetate (3× volume). The combined organic extracts were evaporated under vacuum and re-dissolved in 50% (v/v) aqueous methanol, centrifuged and filtered (0.2 μm) prior to analysis by HPLC-DAD (Andreasen et al., 2001). Hydroxycinnamic acids, diferulic acids and benzoic acid derivatives were detected and quantified by comparison with authentic compounds at 280 and 325 nm. The response factors for the free acids were calculated from appropriate solutions (50% (v/v) aqueous methanol) of the synthetic compounds and external calibration curves were prepared in the linear range. All samples were analyzed in duplicate. Results are expressed as mg/100 g of dry matter (d.m.).

### 2.5. LC/MS/MS detection of flavonoids

Samples were separated using a Jasco PU-1585 triple pump HPLC equipped with an UV-1575 UV detector. Spectra were acquired using a Micromass Quatro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray™ electrospray ionization ion source. Mass spectra were acquired continuously at unit mass resolution over a mass range $m/z$ 50–500 when measuring protonated product ions $[M + H]^+$ at $m/z$ 433 and 449; range $m/z$ 50–600 for product ions at $m/z$ 565; range $m/z$ 50–800 for product ions at $m/z$ 741 and range $m/z$ 50–850 for product ions at $m/z$ 771, at a scan rate of 2 s with an interscan delay of 0.1 s. The collision gas (argon) was maintained at a pressure of $1.1 \times 10^{-3}$ mbar and the collision energy was 18 eV. MS1 mass resolution was set to the same values as for conventional scanning, with MS3 LM and HM resolution parameters each set to 12.5.

### 2.6. Analysis of antioxidant activity

The antioxidant in vitro activity of the water and methanol extracts obtained from selected cereal products (wheat fractions **2**, **4**, **5**, **6** and **10**, rye fractions **8** and **9** and buckwheat fraction **11**) was measured using three standard assays: (i) trolox equivalent antioxidant capacity (TEAC) (Salah et al., 1995). The extent of quenching of the $ABTS^+$ radical by a test sample was compared with standard amounts of trolox C. TEAC values are expressed as μmol of trolox equivalents (TE) per 100 g of cereal product (d.m.); (ii) thiobarbituric acid-reactive substances (TBARS) which determines the antioxidant activity in lipid phase and measures the inhibition of lipid peroxidation by a test sample (Plumb et al., 1996). Results are expressed as mg of starting cereal fraction (d.m.) needed to obtain 50% inhibition of lipid peroxidation ($IC_{50}$) where 100% inhibition is baseline peroxidation of liposome without added iron/ascorbate and 0% inhibition is peroxidation of liposomes with added iron/ascorbate. Calculation of $IC_{50}$ value was performed by fitting a third order polynomial curve to

the data, (iii) oxygen radical absorbance capacity (ORAC) assay determines the capacity of a test sample to scavenge peroxyl radicals in an aqueous phase system (Cao, Alessio, & Culter, 1993). Values are expressed as μmol of trolox equivalents (TE) per 100 g of cereal product (d.m.). We found that methanol interfered with some of the antioxidant activity assays; thus the methanol extracts were evaporated under vacuum and redissolved with an equal volume of milliQ water prior to the assay. The antioxidant tests were conducted in triplicate.

## 3. Results

### 3.1. Total hydroxycinnamic acids, diferulic acids and benzoic derivatives

The composition, in total hydroxycinnamic acids, diferulic acids and benzoic derivatives, of the 12 cereal fractions investigated is shown in Fig. 1. The highest levels of these compounds are present in fractions **5**, **6** and **9** which are predominantly bran products, whereas flours **3** and **7**, constituted mostly of albumen, have the lowest contents of these phenolic compounds. The most abundant compound present in the wheat fractions **1**–**6** and **10** and the rye fractions **7**–**9** is ferulic acid (*trans* + *cis*; 50–86% of total hydroxycinnamic acids). Diferulic acids, sinapic acid, *p*-coumaric acid and some benzoic acid derivatives (*p*-OH-benzoic acid, vanillic acid and vanillin) are also present in small quantities. The buckwheat flours, **11** and **12**, contain only trace quantities of *p*-coumaric acid and benzoic acid derivatives. On average, the largest proportions of ferulic acid (85%), diferulic acids (100%) and other hydroxycinnamic and benzoic acid derivatives (50%) are found covalently bound (esters) in the insoluble pellet of the wheat and rye fractions. Wheat bran fractions **5** and **6** and rye bran fraction **9** had the highest levels of insoluble ferulate (≈400 mg of ferulate/100 g (d.m.) for the wheat bran samples and 230 mg of ferulate/100 g (d.m.) for the rye bran product). Wheat bran samples **5** and **6** also had the maximum levels of insoluble diferulates (28.7 and 39.9 mg of total diferulic acids/100 g (d.m.), respectively) followed by rye products **9** and **8** (22.5 and 20.1 mg of total diFAs/100 g (d.m.)) (Fig. 2). The most abundant diferulate in all the cereal samples analyzed was 8-*O*-4-diFA, followed by the 8-5-diferulate (benzofuran + open ring forms), whereas the 5-5-diFA accounted for a much smaller fraction. No diferulates were detected in the buckwheat flours.

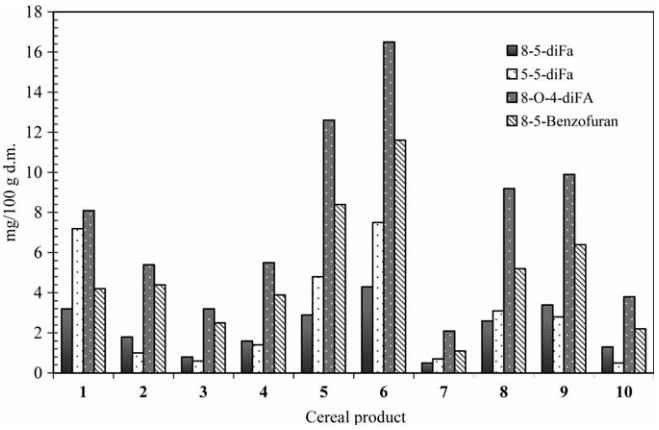


Fig. 2. Composition in insoluble diferulates (mg/100 g d.m.) of the wheat and rye products.

### 3.2. Soluble phenolic compounds

Between 10% and 30% of the total hydroxycinnamic acids and benzoic acid derivatives can be sequentially extracted from the different cereal fractions. Total soluble compounds (free + ester-linked) ranged from 8.7 mg/100 g (d.m.) for the rye flour **7** to 60.0 mg/100 g (d.m.) for the stabilized wheat germ **10** (Fig. 3). The largest part of the soluble detected compounds was as hydroxycinnamic

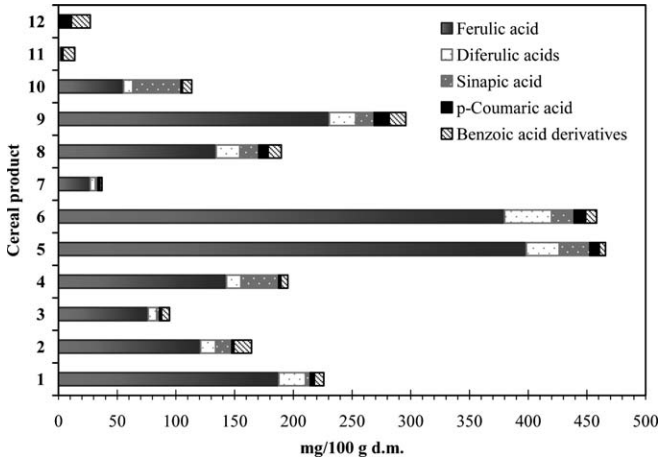


Fig. 1. Total hydroxycinnamic acids, diferulic acids and benzoic acid derivatives (mg/100 g d.m.) of the 12 cereal products investigated (see Table 1 for description of cereal products).

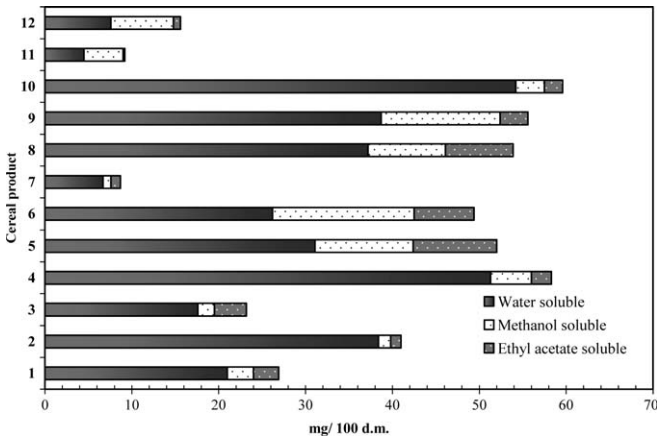


Fig. 3. Total hydroxycinnamic acids and benzoic acid derivatives (mg/100 g d.m.) as sequentially extracted in water, methanol and ethyl acetate from the 12 cereal products investigated.

acids, extracted mostly with water (between 50% and 90% of total soluble compounds) although some quantities were further extracted with methanol (3–50%), followed by ethyl acetate (2–18%). The major soluble compound detected was ferulic acid (on average about 44% of the total soluble hydroxycinnamic acids) and it was found predominantly as an ester (≈70%) and the remaining compound as free ferulic acid (Fig. 4). Other soluble hydroxycinnamic acids and benzoic aid derivatives were mainly present as ester-linked derivatives with sinapate and vanillate being the most abundant ones (Fig. 5). Several *C*-glycosyl flavonoid-type compounds were also solubilized from some of the wheat and rye fractions with methanol (**1**, **2**, **5**, **6**, **8** and **9**) or water (**4** and **10**) and tentatively identified on the basis of their spectral properties and molecular masses. The main flavonoid derivatives detected are presented in Table 2. The mass spectrometry analysis shows the presence of several separate peaks with protonated molecular ions $[M+H]^+$ at *m*/*z* 565, 741 or 771 in the wheat extracts. For example, various peaks with the molecular mass of 741 were found in the water extract from wheat germ **10** and could correspond to feruloyl derivatives of the *C*-glycosyl flavonoids vicenin, schaftoside or isoschaftoside. Only the compounds with ions at *m*/*z* 565 and 771 were detected in the rye products. The buckwheat flours **11** and **12** contained rutin (0.7 and 11.2 mg/100 g (d.m.), respectively) and quercetin (4.0 and 2.8 mg/100 g (d.m.), respectively), as well as other flavonoids identified on the basis of their molecular masses: ions at *m*/*z* 449 (orientin or isoorientin) and 433 (vitexin or isovitexin).

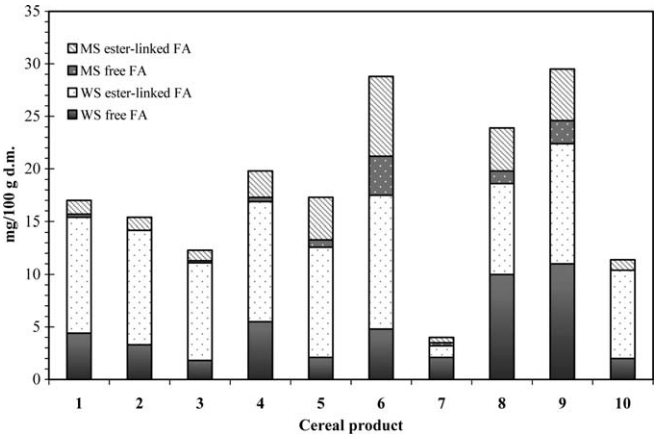


Fig. 4. Distribution of soluble ferulic acid (FA) (free + ester-linked; mg/100 g d.m.) in the wheat and rye products.

### 3.3. Antioxidant properties

Total soluble hydroxycinnamic and benzoic acid derivatives and antioxidant properties (TEAC, TBARS and ORAC assays) of the water and methanol extracts from selected cereal products are shown in Table 3. The greatest antioxidant capacity, as measured by the TEAC and

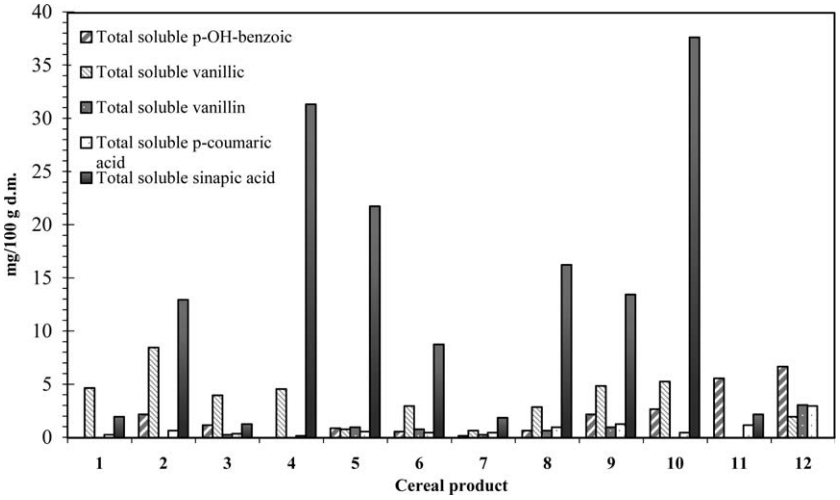


Fig. 5. Total soluble hydroxycinnamic acids and benzoic acid derivatives (mg/100 g d.m.) of the 12 cereal products investigated.

Table 2
Flavonoid-type compounds detected in soluble extracts from wheat and rye fractions

| Spectral properties[a] | $[M+H]^+$ and main fragments | Putative identification |
|---|---|---|
| $\lambda_{max1}=271$, $\lambda_{max2}=335$ | *565*, 512, 427, 525 | Vicenin, schaftoside, isoschaftoside |
| $\lambda_{max1}=272$, $\lambda_{max2}=330$ | *741*, 651, 339, 177[b] | Feruloyl-linked to flavonoid (i.e., vicenin, schaftoside, isoschaftoside) |
| $\lambda_{max1}=273$, $\lambda_{max2}=331$ | *771*, 681, 369, 207[c] | Sinapoyl-linked to flavonoid (i.e., vicenin, schaftoside, isoschaftoside) |

[a] Typical of a flavonoid compound.
[b] Fragment 177 corresponds to a feruloyl residue.
[c] Fragment 207 corresponds to a sinapoyl residue.

Table 3
Total soluble hydroxycinnamic and benzoic acid derivatives and antioxidant capacity in selected wheat, rye and buckwheat products

| | Total soluble phenolic acids[a] (mg/100 g dm) | TEAC[b] (μmol TE/100 g dm) | $IC_{50}$[b] (mg dm) | ORAC[b] (μmol TE/100 g dm) |
|---|---|---|---|---|
| *Product **2**: air separated wheat middling* | | | | |
| Water extract | 38.4 ± 1.9 | 1910 ± 129 | 2.2 ± 0.3 | 3602 ± 582 |
| Methanol extract | 1.4 ± 0.5 | 1528 ± 61 | 22.3 ± 1.2 | 867 ± 190 |
| *Product **4**: fresh wheat germ* | | | | |
| Water extract | 51.3 ± 7.5 | 1308 ± 98 | 3.7 ± 0.4 | 4315 ± 487 |
| Methanol extract | 4.7 ± 0.3 | 387 ± 19 | 17.6 ± 1.0 | 930 ± 309 |
| *Product **5**: wheat bran* | | | | |
| Water extract | 31.1 ± 1.2 | 1641 ± 173 | 3.8 ± 0.4 | 2937 ± 879 |
| Methanol extract | 11.3 ± 0.7 | 546 ± 33 | 11.6 ± 0.9 | 568 ± 138 |
| *Product **6**: fermented wheat bran* | | | | |
| Water extract | 26.2 ± 1.6 | 1641 ± 70 | 4.7 ± 0.4 | 3194 ± 577 |
| Methanol extract | 16.3 ± 0.4 | 329 ± 12 | 13.8 ± 0.9 | 804 ± 187 |
| *Product **8**: rye middling* | | | | |
| Water extract | 37.2 ± 2.5 | 275 ± 36 | 10.5 ± 0.5 | 1180 ± 647 |
| Methanol extract | 8.9 ± 0.3 | 226 ± 8 | 12.3 ± 0.7 | 498 ± 165 |
| *Product **9**: rye bran* | | | | |
| Water extract | 38.7 ± 0.3 | 380 ± 11 | 9.7 ± 0.5 | 1555 ± 792 |
| Methanol extract | 13.7 ± 0.7 | 291 ± 9 | 4.7 ± 0.4 | 798 ± 33 |
| *Product **10**: cold pressed wheat germ* | | | | |
| Water extract | 54.3 ± 3.1 | 4220 ± 316 | 2.3 ± 0.3 | 3870 ± 617 |
| Methanol extract | 3.3 ± 0.2 | 528 ± 20 | 1.9 ± 0.3 | 694 ± 45 |
| *Product **11**: buckwheat flour* | | | | |
| Water extract | 4.5 ± 0.2 | 5897 ± 100 | 0.5 ± 0.1 | 3351 ± 285 |
| Methanol extract | 4.4 ± 0.2 | 1435 ± 135 | 0.5 ± 0.2 | 705 ± 79 |

[a] Analyses done in duplicate.
[b] Analyses done in triplicate.

ORAC assays, was found in the water extracts for all the cereals examined. The quantities of total soluble hydroxycinnamic and benzoic acid derivatives from the wheat and rye products were also higher in the water extracts than in the subsequent methanol extracts. However, no significant correlation was found between the antioxidant capacity (TEAC or ORAC) and the total soluble measured phenolic acids of the water extracts. The capacity for inhibition of lipid peroxidation was also higher in the water extracts from wheat products **2**, **4**, **5** and **6** than in the corresponding methanol extracts. Rye products **8** and **9**, pressed wheat germ **10** and buckwheat flour **11** exhibited similar capacities for inhibition of lipid oxidation, in both the water and the methanol extracts. Overall, of the cereals examined, water-soluble compounds from cold-pressed wheat germ **10** and buckwheat flour **11** exhibited the best antioxidant capacity but, while the wheat germ water extract had the highest levels of soluble hydroxycinnamic and benzoic acid derivatives, these compounds were only present in trace quantities in the buckwheat extract. To estimate the contribution of soluble hydroxycinnamic and benzoic acid derivatives to the antioxidant capacity of water extracts from cereal products, we prepared two mixtures, **A** and **B**, containing the same quantities of these compounds as those solubilized from 100 g of cereal products **2** and **10**, respectively. We then determined the antioxidant capacity using the TEAC and the ORAC tests. The results presented in Table 4 show that both mixtures exhibited very good antioxidant capacity by both assays. The TEAC values determined for the synthetic mixtures were comparable to those measured in the corresponding original extracts whereas the ORAC values were much higher in the extracts than in the standard mixtures. The major difference between mixtures **A** and **B** was the increased level of sinapic acid (≈3-fold) in mixture **B**. This was reflected by an increase in the in vitro TEAC and ORAC antioxidant capacities of mixture **B** (by 3.8- and 3.0-fold, respectively).

## 4. Discussion

In the past two decades increasing interest has been focussed on the study of dietary phenolic antioxidants and their possible association with prevention or delay of oxidative damage in the body. As part of the efforts to unravel the means by which these compounds may provide a beneficial effect in vivo, it is of major importance to determine which actual compounds are present in the food and in which molecular forms. As a result, there has been a substantial amount of research to characterize the phenolic composition and antioxidant capacity of many plant-derived foods (Martínez-Tomé et al., 2004; Miller et al., 2000; Wu et al., 2004) and in particular of grains and cereal products (Adom & Liu, 2002; Adom et al., 2003; Andreasen et al., 2001a; Bonoli et al., 2004; Emmons et al., 1999; Martínez-Tomé

Table 4
Antioxidant activity of mixtures **A** and **B**, containing the major soluble hydroxycinnamic and benzoic acid derivatives present in the water extracts from wheat middling **2** and wheat germ **10**

| Product | Compound | Quantity[a] (mg/l) | TEAC (μmol TE/100 g d.m.) | ORAC (μmol TE/100 g d.m.) |
|---|---|---|---|---|
| Mixture **A** (product **2**) | Vanillic acid | 5.3 | 1493 ± 496 (1919 ± 129) | 579 ± 171 (3602 ± 582) |
| | *p*-Coumaric acid | 0.4 | | |
| | Sinapic acid | 8.2 | | |
| | Ferulic acid | 8.9 | | |
| Mixture **B** (product **10**) | Vanillic acid | 3.3 | 5622 ± 958 (4220 ± 316) | 1674 ± 54 (3870 ± 617) |
| | *p*-Coumaric acid | 0.3 | | |
| | Sinapic acid | 22.3 | | |
| | Ferulic acid | 6.5 | | |

[a] Final concentration of water solubilized phenolics from 100 g of cereal product (d.m.).

et al., 2004; Zhou & Yu, 2004; Zhou et al., 2004a, 2004b; Zieliński & Kozłowska, 2000). Complete characterization of the profile and distribution of the antioxidant components in cereals, and in food in general, is a complex task. In an attempt to understand the link between the antioxidant capacity of cereals and the responsible components, most researchers have partially solubilized and described some of the known antioxidant compounds in cereals and have tried to correlate them to the in vitro antioxidant capacity of soluble extracts prepared from these cereals. However, the methods used for extraction, identification of phenolic compounds and evaluation of antioxidant activity vary considerably, making comparisons between studies and extrapolating general conclusions a rather difficult job. For example, published extraction protocols differ in the solvent or mixture of solvents used and the extraction time applied. Fine-powdered flour is commonly extracted with ethanol (Zhou & Yu, 2004), methanol (Emmons et al., 1999), 50% acetone (Zhou et al., 2004a, 2004b), water (Martínez-Tomé et al., 2004; Zieliński & Kozłowska, 2000) or mixtures of water–alcohol (Adom & Liu, 2002; Adom et al., 2003; Bonoli et al., 2004; Zieliński & Kozłowska, 2000) and extraction times range from a few minutes to several hours. Most methods avoid light, $O_2$ and high temperature to diminish decomposition of the extracted compounds and maximize extraction yields. From a chemical point of view, and based on the polarity of phenolic compounds, extraction with 70–95% alcohol or acetone (in water) is generally recommended to ensure maximum solubilization of most groups of phenolics. However, from an in vivo perspective, water extracts are probably of greatest interest as they contain the antioxidant compounds most easily available from the food matrix. We have applied a sequential extraction procedure to our cereal samples and have shown that the largest parts of soluble hydroxycinnamic acids, benzoic acid derivatives and flavonoids are already extracted in water and that only small quantities of these compounds are further extracted using less polar solvents. These residual compounds may have been entrapped in the food matrix and only freed after repeated extraction with methanol or ethyl acetate but the presence of some more lipophilic compounds, such as sterol ferulates, cannot be disregarded (Iwatsuki et al., 2003).

Processing (and fractionation of grains) has a significant effect on the composition and properties of the final cereal product. Our results show that, for example, wheat products containing germ have better antioxidant properties than other wheat products and that, in general, the bran-enriched products from rye and wheat provide the worst antioxidant soluble extracts. Although the bran products do have the highest levels of total hydroxycinnamates, most of these compounds are not solubilized and do not make any input into the antioxidant capacity of the extracts. The beneficial effects derived from the consumption of high-bran cereals must be associated with other properties or effects in vivo of the bran not related to in vitro antioxidant capacity. It is the solubilized compounds that are responsible for the in vitro antioxidant capacity of the extracts and the wheat germ products do contain the highest quantities of soluble hydroxycinnamic and benzoic acid derivatives, as well as other flavonoid-type compounds, mostly extracted in water. The water-solubilized hydroxycinnamic and benzoic acid derivatives are present mainly as soluble esters and a small proportion of free acids. There are some reports of the antioxidant capacity of ferulic acid sugar esters (Katapodis et al., 2003; Ohta, Semboku, Kuchii, Egashira, & Sanada, 1997) but the majority of studies on antioxidant activities of phenolic compounds refer to these compounds as free soluble acids (Andreasen et al., 2001a; Garcia-Conesa et al., 1999a; Rice-Evans et al., 1996). It has been shown that esterases in the human gut can cleave esterified hydroxycinnamates and form free acids in the small intestine (Andreasen, Kroon, Williamson, & García-Conesa, 2001c). It is possible that both the ester-linked and the free soluble hydroxycinnamic acids may exert some antioxidant effect in the luminal side of the intestinal tract. The remaining insoluble covalently bound hydroxycinnamates and diferulates arrive almost intact in the large intestine, where a small proportion of these bound compounds can be released in the gut by human and microbial esterases (Andreasen et al., 2001b).The free compounds may exert some antioxidant effect in the colon or may suffer further modifications by the action of bacterial enzymes which may modify the antioxidant power of the parent compounds. The determination of antioxidant

activity in food extracts after alkaline hydrolysis of the ester-linked hydroxycinnamates (Adom et al., 2003) has little biological relevance as not all the esterified compounds will be released and remain intact in vivo.

The antioxidant capacity measured in the water cereal extracts could be partially explained by the combined effect of the soluble hydroxycinnamic and benzoic acid derivatives. We have shown that mixtures prepared with synthetic compounds at the concentrations found in the extracts exhibit a high antioxidant capacity and, with some specific tests (TEAC), this activity is comparable to the capacity of the cereal extract itself. However, we were not able to establish a significant correlation between antioxidant values and levels of total measured soluble phenolic compounds. For example, rye water extracts with a very high content of soluble phenolic acids had the lowest antioxidant activity values whereas buckwheat flour which showed one of the highest antioxidant powers contained only trace quantities of these compounds. Attempts to correlate the antioxidant activities of a variety of cereals and derived products with total or specific groups of phenolics have generated contradictory results. For example, total soluble ferulic acid in various fractions obtained from wheat grain was well-correlated with scavenging activities against $ABTS^{+}$ and superoxide anion (Zhou et al., 2004a). However, the same authors found no correlation between total soluble ferulic acid and antioxidant activity in different wheat bran samples (Zhou et al., 2004b). Correlations between total phenolics (measured by the Folin–Ciocalteu method) and antioxidant activity by various scavenging tests are also inconsistent (Zhou et al., 2004a, 2004b; Zieliński & Kozłowska, 2000). Differences in the type of starting material, extraction procedures and measuring methods may explain, to some extent, the discrepancy in the results. For example, other soluble compounds, such as simple carbohydrates or amino acids, may be present in the extracts and may interfere with the antioxidant test or with the determination of total phenolics, as is the case for the non-specific Folin–Ciocalteu method (Zieliński & Kozłowska, 2000). Cereals also contain other compounds, such as carotenoids or tocopherols (Adom et al., 2003; Zhou et al., 2004b), or other non-characterized phenolics with a significant input to the final antioxidant capacity of the cereal extracts. For example, buckwheat or wheat germ contain various flavonoids which are also well-known powerful antioxidants (Adom et al., 2003; Quettier-Delu et al., 2000). Our results and previous published results clearly show that a complete determination of all the soluble components present in the food extracts and of their antioxidant activity and interactions is needed to fully explain the final antioxidant capacity of the extracts.

In conclusion, there is a wide variation in the composition of hydroxycinnamic acids and derivatives in the various cereals examined. The highest levels of these compounds were found in the bran fractions, whereas the buckwheat flours had only trace quantities. The most abundant compound in the wheat and rye fractions was ferulic acid but small quantities of diferulic acids, sinapic acid, *p*-coumaric acid and benzoic acid derivatives were also present. The largest proportions of these hydroxycinnamic acids were found covalently bound (esters) in the insoluble pellet but up to 30% of the total compounds were water solubilized. Most of the antioxidant capacity was found in the water extracts from all the cereal fractions. Buckwheat and wheat germ products exhibited the highest antioxidant capacity whereas the rye products had the lowest antioxidant values. The antioxidant capacity in cereal extracts cannot be totally explained by the content of soluble antioxidant hydroxycinnamic and benzoic acid derivatives and is most likely due to the combined effect of all the solubilized compounds. For the complete characterization and distribution of all the soluble phenolics in cereals and for inter-laboratory comparisons, a universal and exhaustive extraction protocol that ensures maximal solubilization needs to be established. From a biological point of view though, solubilization in water appears to be more relevant. Our results suggest that sequential extraction starting with water and followed by solvents of decreasing polarity, in combination with sensitive and specific detection methods such as HPLC-DAD and LC–MS, is a convenient approach for determining both total and most easily available soluble antioxidant phenolic compounds in cereals. A better and full characterization of the phenolics present in complex mixtures will lead to better understanding of the antioxidant activity of food extracts, which in turn will help us to improve our knowledge of their possible in vivo antioxidant role.

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