Food Chemistry 210 (2016) 212–220

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant-guided isolation and mass spectrometric identification of the major polyphenols in barley (*Hordeum vulgare*) grain

Nirupama Gangopadhyay [a,b,*], Dilip K. Rai [a], Nigel P. Brunton [b], Eimear Gallagher [a], Mohammad B. Hossain [a]

[a] Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland
[b] School of Agriculture and Food Science, University College Dublin, Dublin 4, Ireland

ARTICLE INFO

Article history:
Received 22 February 2016
Received in revised form 19 April 2016
Accepted 20 April 2016
Available online 21 April 2016

Keywords:
Barley
Antioxidant capacity
Flash chromatography
Polyphenols
Flavanols
Mass spectrometry

ABSTRACT

In the present study, the relative contribution of individual/classes of polyphenols in barley, to its antioxidant properties, was evaluated. Flash chromatography was used to fractionate the total polyphenol extract of Irish barley cultivar 'Irina', and fractions with highest antioxidant properties were identified using total phenolic content and three *in vitro* antioxidant assays: DPPH, FRAP, and ORAC. Flavanols (catechin, procyanidin B, prodelphinidin B, procyanidin C) and a novel substituted flavanol (catechin dihexoside, $C_{27}H_{33}O_{16}^-$, *m/z* 613.17), were identified as constituents of the fraction with highest antioxidant capacity. Upon identification of phenolics in the other active fractions, the order of most potent contributors to observed antioxidant capacity of barley extract were, flavanols > flavonols (quercetin) > hydroxycinnamic acids (ferulic, caffeic, coumaric acids). The most abundant polyphenol in the overall extract was ferulic acid (277.7 μg/g dw barley), followed by procyanidin B (73.7 μg/g dw barley).

1. Introduction

The role of dietary polyphenols in human health has been explored to a great extent in the past few decades due to their ability to reduce oxidative stress, induced by the generation of harmful free oxygen radicals in the body. Uncontrolled oxidative stress causes damage to major biomolecules, including the proteins, lipids and DNA, and may be critical to the aetiology of a number of degenerative diseases, such as cancer (Thanan et al., 2014), atherosclerosis (Li, Horke, & Förstermann, 2014) and other inflammatory disorders (Tak, Zvaifler, Green, & Firestein, 2000; Rezaie, Parker, & Abdollahi, 2007). Recently, oxidative stress has also been associated with the neurodegenerative disorder, Alzheimer's disease (Wang et al., 2014). In addition to their possible health benefits, antioxidants can be used to retard oxidative deterioration of lipids in foods which lead to the development of rancid off-flavours. Naturally occurring antioxidants, such as phenolic compounds in food sources, are often preferred to their synthetic counterparts because of consumer concerns associated with health and safety of synthetic antioxidants (Branen, 1975).

The most well-known sources of polyphenols include green tea, fruits, vegetables, beans, and cereals (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Compelling evidence on the *in vitro* and *in vivo* antioxidant activities of dietary polyphenols from a number of sources has been presented (Serafini, Ghiselli, & Ferro-Luzzi, 1996; Nigdikar, Williams, Griffin, & Howard, 1998; Jensen et al., 2008). Whole grains and cereals have also been well explored in the last two decades, and in many cases, their benefits on humans have been linked to their content of phenolic compounds (Slavin, 2004; Dykes & Rooney, 2007). Barley is the most abundantly grown cereal in Ireland that finds maximum applications in the brewing industry or as animal feed, while only about 2% of the grain is used for food directly (Sullivan, Arendt, & Gallagher, 2013). The scope of increasing the use of barley and its products in food applications warrants exploiting the potential of Irish-grown barley, with respect to its associated health benefits.

The purported health benefits of barley are often linked to its antioxidant properties, which are largely derived from its polyphenolic content (Goupy, Hugues, Boivin, & Amiot, 1999; Bonoli,

Abbreviations: FCR, Folin-Ciocalteu reagent; HCl, hydrochloric acid; EA, ethyl acetate; H_2SO_4, sulphuric acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPTZ, 2.4.6-tri (2-pyridyl)-s-triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TPC, total phenolic content; FRAP, ferric ion reducing antioxidant power; ORAC, oxygen radical absorbance capacity; AAPH, 2,2′-Azobis (2-amidinopropane) dihydrochloride; ET, electron transfer; HAT, hydrogen atom transfer; UHPLC-MS/MS, ultra-high performance liquid chromatography coupled with tandem mass spectrometry; MRM, multiple reaction monitoring; Q-TOF, quadrupole Time of Flight; RDA, retro-Diels Alder; CID, collision induced dissociation.

* Corresponding author at: Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland.
E-mail address: nirupama.gangopadhyay@teagasc.ie (N. Gangopadhyay).

http://dx.doi.org/10.1016/j.foodchem.2016.04.098
0308-8146/

Marconi, & Caboni, 2004; Holtekjølen, Kinitz, & Knutsen, 2006). Phenolic compounds in barley exist in so-called free, soluble conjugated, and insoluble bound forms. The insoluble bound forms of phenolic acids are linked by ester or ether linkages to the cell wall material of the grain and require acid, alkaline or enzymatic hydrolysis for their release (Gangopadhyay, Hossain, Rai, & Brunton, 2015). In contrast, free polyphenols can be extracted using solvents, such as methanol, ethanol and acetone. The majority of the free phenolics in barley are flavanols that are usually found in their monomeric form as catechin and epicatechin, or in their polymeric form as proanthocyanidins (Bonoli et al., 2004; Holtekjølen et al., 2006). The bound phenolics in barley include phenolic acids particularly hydroxycinnamic acids, such as ferulic acid, which can also exist in its dimeric or trimeric form. Coumaric acid and caffeic acid are also often reported as part of the bound fraction of barley grains (Bonoli et al., 2004; Holtekjølen et al., 2006; Verardo, Bonoli, Marconi, & Caboni, 2008).

Although previous studies have given meaningful insights into the different polyphenols present in barley extracts, the identities of the individual phenolic compounds which are strongest contributors to the observed antioxidant capacity of barley are still unknown. Antioxidant-guided identification is a method of chromatographically fractionating a sample extract, following which the most antioxidant-active fractions are chosen, and the identity of the predominant contributors to the observed antioxidant capacity of the sample are established. The objective of the current study was to employ a flash chromatography fractionation of the barley grain extract followed by antioxidant-guided identification of polyphenols in the fractions.

2. Materials and methods

2.1. Materials

Hulled Irish spring barley cultivar 'Irina' from the 2013 harvest was provided by Seedtech (Waterford, Ireland). Whole barley grains were milled using a Perten Lab mill 3100 (Perten Instruments, AB, Kungens Kurva, Sweden). HPLC-grade ethyl acetate, methanol, hexane and water were purchased from Sigma Aldrich, Wicklow, Ireland. The polyphenols, caffeic acid, *p*-coumaric acid, ferulic acid, catechin and quercetin, were purchased from Sigma-Aldrich, Wicklow, Ireland. The polyphenol standard of procyanidin B1 was purchased from Extrasynthèse, Lyon, France. The purity of the standards and solvents were in the range of 95–99%. Folin-Ciocalteu reagent (FCR), gallic acid, sodium carbonate (Na_2CO_3), sodium acetate anhydrous, α-amylase, cellulase, 98% sulfuric acid (H_2SO_4), ferric chloride hexahydrate, hydrochloric acid (HCl), 2,2-diphenylpicrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, Wicklow, Ireland. The Oxiselect ORAC assay kit was purchased from Cell BioLabs, Inc., San Diego, CA). Fluorescein probe and the radical generator (AAPH) were provided as a part of the ORAC kit.

2.2. Extraction of free and bound phenolics

An illustration of the workflow employed for antioxidant capacity-guided isolation and identification of phenolics in barley extract and its fractions is shown in Fig. 1. Extraction of free (unbound) phenolics from milled barley was carried out using the conditions optimised by Madhujith and Shahidi (2006). This involved sequentially extracting milled barley (100 g) three times in a shaker set at 60.5 °C. The extraction was carried out using 80.2% aqueous methanol for 38.3 min at a solids to solvent ratio of 1:10 (*w/v*) per extraction. The extracts were centrifuged at 5000 rpm for 15 min, and the obtained supernatant (1, 2 and 3), after each extraction, was filtered through a Büchner funnel (pore size ~ 1 μm), while the corresponding residue was used as a substrate for the next round of extraction. The pooled and filtered supernatants were defatted using hexane at a ratio 2:1 (*v/v*). The methanolic phase containing the free phenolics was dried immediately using rotary evaporation (Heidolph, Schwabach Germany). The residue from the third round of extractions of the free phenolics was used for the extraction of bound polyphenols using an acid, α-amylase, and cellulase hydrolysis according to the method of Yu, Vasanthan, and Temelli (2001), with some modifications. The acid hydrolysis step was carried out by mixing the residue with 1 L of 0.1 M H_2SO_4, and heating at 85 °C for 1 h. The sample was cooled for 10 min in an ice-water bath prior to the addition of 200 mL of 2.5 M aqueous sodium acetate solution containing 2% (*w/v*) α-amylase and incubated at 30 °C for 1 h. Following this, 100 mL of a 0.1 M aqueous sodium acetate solution containing 2% cellulase were added, and the sample was further incubated for 10 h at 30 °C. Upon centrifugation at 5000 rpm for 10 min, the obtained supernatant of the aqueous extract was filtered through a Büchner funnel. The filtered extract was defatted using hexane (2:1 v/v), after which the extract was subjected to a liquid–liquid partitioning using an equal volume of ethyl acetate. The ethyl acetate phase containing the bound phenolics from barley was dried using rotary evaporation. The total phenol content of the dried extracts was calculated and the dried extracts were stored at −20 °C until further use.

2.3. Fractionation of the barley polyphenols using reversed-phase flash chromatography

Prior to flash chromatography, the dried extracts of free and bound phenolics were each dissolved in minimal amounts of 80% methanol and mixed with each other to give the total pooled phenol extract from barley. About 1 g of the dry total polyphenol extract was resuspended in minimal amount (approximately 10 mL) of 80% methanol and fractionated on a Varian IntelliFlash flash chromatography system (Model 310). The column used for flash chromatography was a reversed-phase Telos C_{18} column with a sorbent mass of 140 g and an average particle size of 40–60 μm. A binary solvent system consisting of water plus 0.5% formic acid (mobile phase **A**) and acetonitrile plus 0.5% formic acid (mobile phase **B**) was used as the mobile phase. A stepwise gradient (0% **B** for 5 min, 10% **B** from 5 to 10 min, 20% **B** from 10 to 15 min, 30% **B** from 15 to 20 min, 80% **B** from 20 to 25 min, and 100% **B** from 25 to 35 min) at a flow rate of 40 mL/min was employed to separate the polyphenols of the pooled extract (Fig. 2). Fractions were collected at a time interval of 1.0 min over 35 min, resulting in 35 fractions. The eluting fractions were monitored at the wavelengths of 280 and 320 nm. As no visible peaks were detected on the chromatogram in the last 5 fractions, only the first 30 fractions were assayed for antioxidant capacities.

2.4. Determination of TPC and antioxidant capacities of the flash chromatography fractions

The total phenolic content (TPC) of the fractions was determined by Folin-Ciocalteu method and the antioxidant capacities were tested using two types of *in vitro* assays – a) single electron transfer (ET) reaction-based assays such as ferric ion reducing antioxidant power (FRAP), and DPPH b) hydrogen atom transfer (HAT) reaction-based assay, which included the oxygen radical absorbance capacity (ORAC) assay. Prior to the assays, the fractions were dried, and redissolved in 40 mL methanol. The experimental procedures of TPC and the two ET reaction-based assays (DPPH,

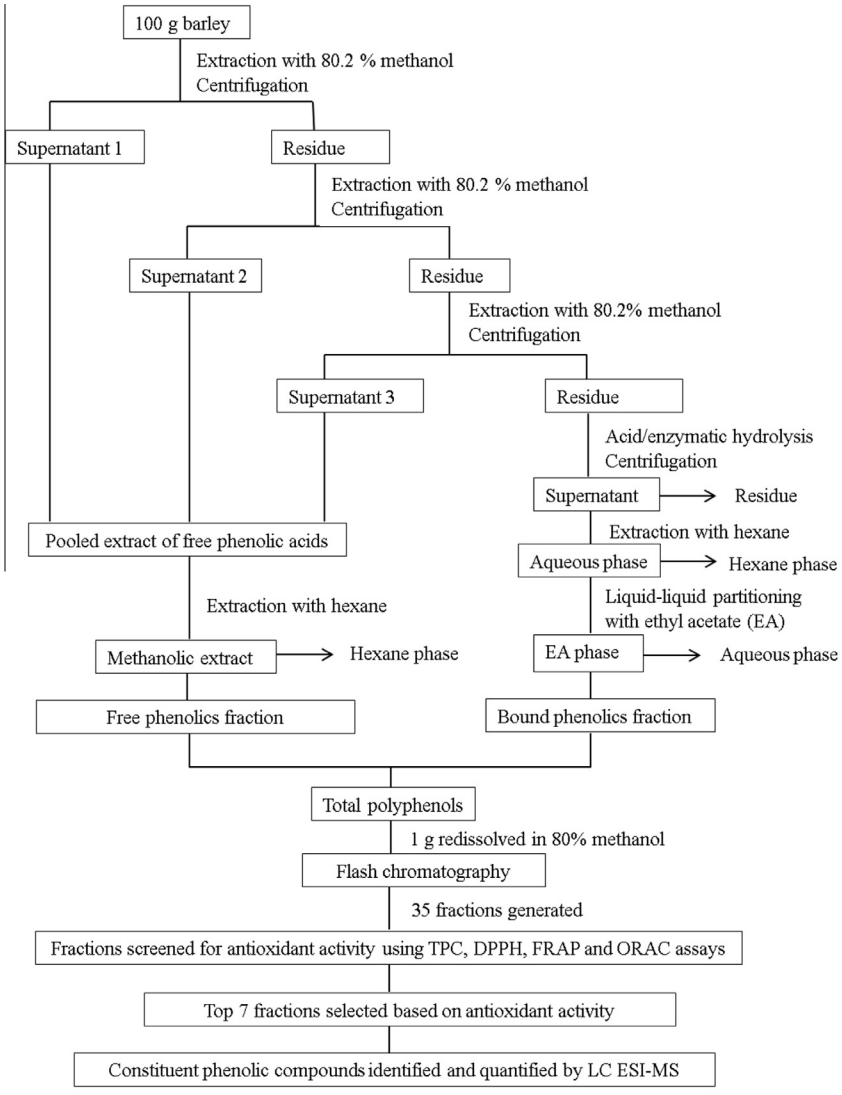

Fig. 1. Schematic representation of the extraction, fractionation, and antioxidant activity-guided identification of the major phenolics in barley extract.

FRAP) were adapted from our previous work (Hossain, Camphuis, Aguiló-Aguayo, Gangopadhyay, & Rai, 2014).

2.4.1. Determination of TPC

For the assay, 100 μL methanol, 100 μL Folin-Ciocalteu reagent (FCR) and 700 μL 20% Na_2CO_3 were added to 100 μL sample extract. The contents of the tube were mixed by vortexing. After 20 min of reaction in the dark at room temperature, the mixture was centrifuged at 13,000 rpm for 3 min. The absorbance of the supernatant was measured against the blank (methanol) at 735 nm by UV-Vis spectrophotometry (Hitachi U-2900; Hitachi High-Technologies, Tokyo, Japan).

A standard curve of gallic acid dissolved in methanol in the range of 10–400 mg/ L was plotted, and TPC was expressed as μg gallic acid (GA) equivalents/ mL of each fraction. The assay was performed in triplicate for all the 30 fractions and the standards.

2.4.2. DPPH assay

DPPH assay, with Trolox as a standard, was used to measure *in vitro* antioxidant capacity of the fractions. Briefly, 100 μL of a methanolic solution of DPPH (0.0476 mg/ mL) were added to 100 μL of the sample or standard in a 96-well plate. The 96-well plate was then incubated in the dark for 30 min at room temperature. The absorbance of the mixtures was measured at 515 nm using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany).

A standard curve of Trolox in methanol in the range of 0.005–0.05 mM was plotted, and the antioxidant capacity was expressed as mM Trolox equivalents. The assay was performed in triplicates for the standards and appropriately diluted samples.

2.4.3. FRAP assay

A working FRAP reagent was prepared fresh by mixing 300 mM acetate buffer, pH 3.6, with 20 mM ferric chloride in distilled water and 10 mM TPTZ (2,4,6-tri(2-pyridyl)-*s*-triazine) in 40 mM hydrochloric acid in a proportion of 10:1:1. Briefly, 180 μL FRAP reagent were added to 20 μL of the sample or standard in a 96-well plate. The reaction mixture was incubated at 37 °C for 40 min. Then, the absorbance of the samples was measured at 592 nm using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany).

The antioxidant capacity of the samples was tested against the reference standard Trolox. A standard curve of Trolox in methanol in the concentration range of 0.1–1.0 mM was plotted, and the

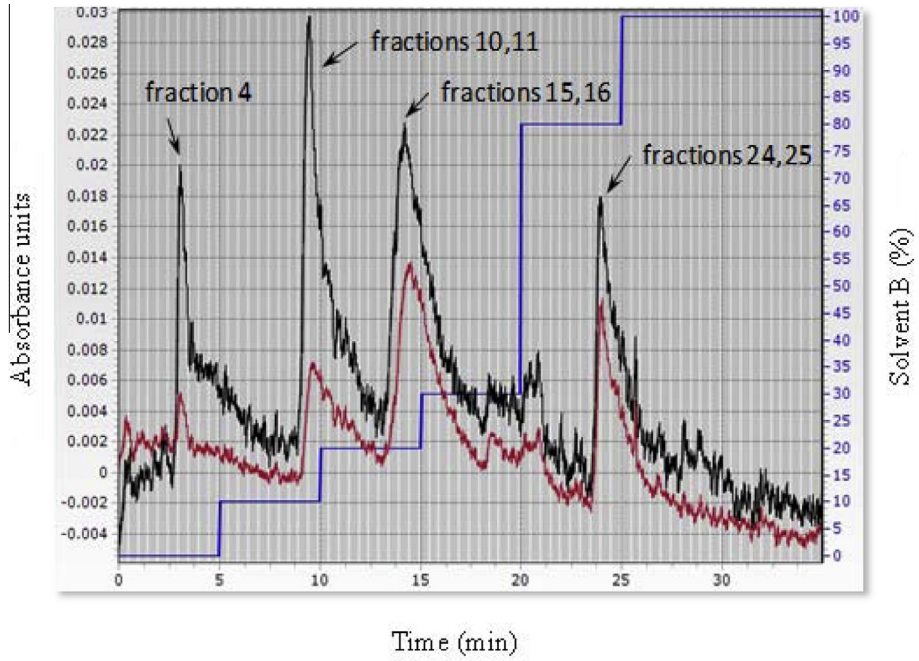

Fig. 2. Chromatogram resulting from the flash chromatographic separation of pooled bound and free polyphenol extract from barley grain at 280 nm (black line) and 320 nm (red line). The stepwise gradient used is indicated by the blue line.

antioxidant capacity of the samples was expressed as mM Trolox equivalents. All the samples and standards were assayed in triplicates.

2.4.4. ORAC assay

ORAC assay kit was used as per manufacturer's instructions for carrying out the assay. Each standard and sample was tested in duplicates. Samples were diluted 80 times prior to analysis. Briefly, 25 μL of diluted sample or standard were incubated with 150 μL of fluorescein probe (substrate) at 37 °C for 30 min. Following this, 25 μL of the peroxyl radical generator, 2,2′- azobis(2-amidinopropane) dihydrochloride (AAPH), were added to each well and the resulting fluorescence was recorded for 60 min using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively.

Trolox in the concentration range of 0–50 μM was used for plotting the standard curve, using area under the curve (AUC) method. The antioxidant capacities of the fractions were expressed as mM Trolox equivalents.

2.5. Mass spectrometric determination of polyphenols in the antioxidant-active fractions

The polyphenols in the selected bioactive fractions were analysed using Waters Acquity (Waters Corporation, Milford, MA) ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Separation of the compounds was achieved on a Waters Acquity HSS T3 column (particle size 1.8 μm, 2.1 × 100 mm) using a binary solvent system consisting of water plus 0.5% formic acid (mobile phase **A**) and acetonitrile/methanol (50/50) plus 0.5% formic acid (mobile phase **B**). The following gradient program was carried out: 0–2.5 min 2% **B**, 2.5–3 min 10% **B**, 3–7.5 min 15% B, 7.5–8.5 min 35% **B**, 8.5–9.5 min 98% **B** and 9.5–10.0 min 2% **B** at a flow rate of 0.5 mL/ min. The injection volume for all the selected fractions was 5 μL. Mass spectrometry detection and quantification of the polyphenols were performed in the negative ion mode using multiple reaction monitoring (MRM) experiments in which the first quadrupole was set to scan a specific precursor ion mass-to-charge (*m/z*) ratio, and the third quadrupole was set to scan structurally distinctive product ions (*m/z*). A pre-existing database of MRM transitions of about 55 polyphenols, which included the most commonly found phenolics in barley, was used for screening the phenolic compounds in the bioactive fractions. The ionization source conditions were as described by Hossain, Rai, and Brunton (2015): capillary voltage 3 kV, cone voltage 40 V, extractor voltage 3 V, source temperature 120 °C, desolvation temperature 350 °C, desolvation gas flow 800 L/h, cone gas flow 50 L/h, and collision gas flow 0.1 mL/min. The desolvation gas used was nitrogen. For quantification purposes, standards of polyphenols were prepared in 80% methanol in the concentration range of 0.1–1.0 μg/ mL for caffeic and coumaric acids, 0.5–10.0 μg/mL for catechin and quercetin, 1.0–10.0 μg/mL for procyanidin B1 and ferulic acid. The standard curve of procyanidin B1 was used for quantification of prodelphinidin B and procyanidin C. Waters TargetLynx™ software was used for data evaluation.

Since MRM only scans for target analytes with known mass transitions, there exists a possibility for novel analytes or analytes with unknown mass transitions to go undetected. Hence, the fractions were also analysed using an Alliance 2695 HPLC system (Waters Corporation, Milford, MA) coupled with a quadrupole time of flight (Q-TOF) mass analyser, to identify any such analyte that might not have been detected by MRM. The Q-TOF premier mass spectrometer was also used for accurate mass measurement of the identified polyphenols, based on the method previously described by Hossain, Rai, Brunton, Martin-Diana, and Barry-Ryan (2010), using leucine enkephalin as lock mass reference compound.

3. Results and discussion

3.1. Extraction of polyphenols

The goal of this work was to identify the polyphenols associated with the antioxidant capacity of barley extracts. The selection of a barley cultivar for this study was based on the observed total

phenol content (TPC) of five barley cultivars. Only the cultivar Irina, with the highest TPC, was chosen for the study, to avoid the solvent consumption involved in extraction and fractionation of all the cultivars. The approach was to not discriminate between bound and free polyphenols, and thus the extracts obtained of the same were pooled prior to further fractionation by flash chromatography. The 80% methanol used for the extraction of free phenolics has been used extensively as an extraction solvent for phenolic compounds from cereals and other plant matrices (Velioglu, Mazza, Gao, & Oomah, 1998; Zielinski, & Kozlowska, 2000). This mixture of water and methanol is deemed capable of solubilising a wide range of polyphenolic compounds including the very polar compounds like flavanols, intermediately polar compounds like phenolic acids and less polar compounds like flavonols. The residual sample matrix from the exhaustive extraction of free phenolics was subjected to extraction of the bound phenolics using acid hydrolysis followed by enzymatic hydrolysis using α-amylase and cellulase. This combination of sequential acid hydrolysis and enzymatic treatment is efficient in releasing the potent hydroxycinnamic acids that are ester-linked to starch and other polysaccharides of the barley cell wall (Yu et al., 2001). The aqueous extract containing the bound phenolic acids was liquid/liquid partitioned using ethyl acetate, as it was necessary to eliminate any interference from the acid and enzymes in the aqueous extract.

The TPC of the free polyphenols extract from wholegrain barley was recorded as 0.98 ± 0.1 and that of the bound polyphenols extract was 0.51 ± 0.02, both of which were measured as mg gallic acid/g of flour. Following extraction, the free and bound fractions of barley polyphenols were combined and the total extract was fractionated using flash chromatography.

3.2. Antioxidant capacities of the fractions

Considering the limitations of antioxidant assays, the use of at least two or more assays with different mechanisms of oxidation is strongly recommended. Three *in vitro* assays (DPPH, FRAP, ORAC) to analyse the antioxidant capacities, together with TPC determination, were performed on the 30 fractions, results of which are shown in Fig. 3. Although all the above mentioned assays claim to measure the total antioxidant capacity of samples, our data indicated low correlations between ORAC and the other assays ($r = 0.84$, $r = 0.83$ and $r = 0.82$ with TPC, DPPH and FRAP respectively). Nevertheless, results from the TPC, DPPH and FRAP assay correlated well with each other, wherein a highest correlation of $r = 0.98$ was obtained between TPC and DPPH, and also between DPPH and FRAP (Table 1). The most active fractions could be ranked in the following order based on ORAC assay: fraction 10 > fraction 11 > fraction 25 > fraction 24 > fraction 15 > fraction 16 > fraction 4, whereas the rank order based on TPC, DPPH and FRAP assays was: fraction 10 > fraction 11 > fraction 4 > fraction 15 > fraction 16 > fraction 25 > fraction 24 (Fig. 3).

Such discrepancy in the values obtained by ORAC and the other assays was reported before, and has been assigned to the differences in the mechanisms involved (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002; Dudonne, Vitrac, Coutiere, Woillez, & Mérillon, 2009). The TPC and electron transfer (ET) based antioxidant assays: DPPH and FRAP, involve a single redox reaction and measure the electron-donating capacity of the antioxidant to reduce the oxidant (probe). However the hydrogen atom transfer (HAT) based assays, such as ORAC, employ biologically relevant reactive oxygen species, and apply competitive reaction kinetics, in which the antioxidant and the substrate compete to scavenge the generated oxygen radical species (Huang, Ou, & Prior, 2005). It has been speculated that some phenolic species, owing to their structure, might not be a good electron-donating radical scavenger; however, they might be able to entrap the peroxyl radicals and form stable compounds with them, which is indicated in differences in their activities by the ET and HAT based assays (Oettl et al., 2001). However, on account of the biological relevance of the ORAC assay, antioxidant capacities of samples have often been based on the results obtained by ORAC as compared to the ET-based assays (Ou et al., 2002; Dudonne et al., 2009). The antioxidant rank order of the fractions was based on ORAC results in the current study.

Tyl and Bunzel (2012) tested all the fractions generated by HPLC, in an extract from blue wheat, at the same concentration, to find the most active polyphenols and not the most predominant ones. However, the observed antioxidant capacity of a sample matrix can be accrued from two factors - 1) the inherent activity of the polyphenols/group of polyphenols that are present in the sample and 2) the abundance of the polyphenols. In many cases,

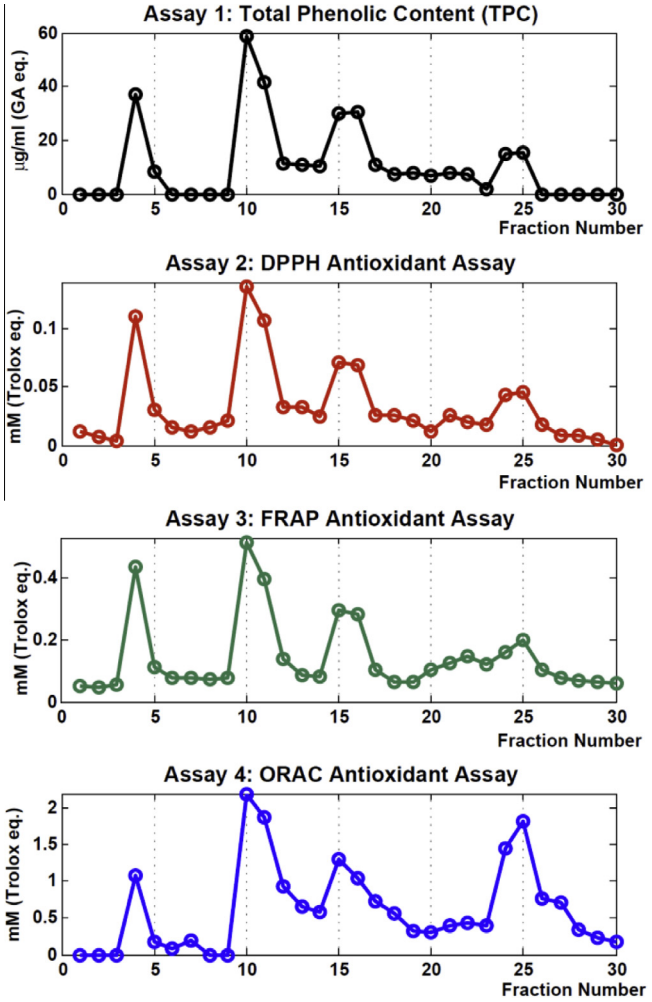

Fig. 3. TPC determination and antioxidant capacities of the 30 fractions obtained from flash chromatography, as measured by DPPH, FRAP and ORAC antioxidant assays.

Table 1
Pearson's correlation coefficients between TPC and three *in vitro* antioxidant capacity assays: DPPH, FRAP and ORAC.

	TPC	DPPH	FRAP	ORAC
TPC	1	0.98	0.96	0.84
DPPH	0.98	1	0.98	0.83
FRAP	0.96	0.98	1	0.82
ORAC	0.84	0.83	0.82	1

a polyphenol might inherently be very bioactive, however it might be present in amounts too low to be a potent contributor of the sample's observed antioxidant capacity. The same also applies for very abundant polyphenols that perhaps possess minimal or no activity. Thus, the intrinsic bioactivity as well as abundance of the reported polyphenols must be taken into account to determine the most influential contributor to the observed antioxidant capacity of a particular sample. In the present study, to account for both the activity and abundance of polyphenols, same volume of all the 30 fractions obtained from flash chromatography, irrespective of the concentration of polyphenols/ group of polyphenols present in them, was used for determining their antioxidant capacities.

3.3. Identification and quantification of polyphenols in the bioactive fractions by mass spectrometry

A total of nine phenolic and two non-phenolic compounds were identified in the seven selected fractions with high antioxidant activities. Identification of six of the phenolic compounds (catechin, procyanidin B, quercetin, ferulic acid, *p*-coumaric acid, caffeic acid) was carried out by comparing their retention times and MRM mass transitions with those of authentic standards, and determination of their accurate masses. Three other phenolic compounds (procyanidin C, prodelphinidin B, and catechin dihexoside), for which standards were not available, were identified on the basis of accurate mass measurement (Observed mass error < 5 ppm to theoretical exact mass) of their $[M - H]^-$ ions. The product ions (MS/MS) of these two compounds were compared with previous reports, and used for their quantification by MRM. The MRM chromatograms of all the identified polyphenols have been provided as Supplementary Data. The detection of the two other non-phenolic compounds was based on accurate mass measurement and the generated product ions. The MRM parameters used for identification and quantification of the polyphenols in the antioxidant-active fractions are given in Table 2. Data obtained on accurate masses of detected analytes are given in Table 3. All the antioxidant-active fractions detected in our study existed in pairs (10/11, 24/25, 15/16), and identical polyphenolic compounds were detected in them. This is most likely due to an inherent limitation in the resolving power of flash chromatography. For the sake of quantitation, these pairs of fractions have been treated as one, and a total account of the amount of polyphenols in them has been provided in Table 3.

Amongst the 30 tested fractions, fraction 10 followed by fraction 11 had the highest antioxidant activities, representing about a quarter (24%) of the total antioxidant activities of all the fractions based on ORAC assay. Both the fractions largely consisted of a number of flavan-3-ols/flavanols, which were present in higher amounts in fraction 10. The monomer catechin, dimers procyanidin B and prodelphinidin B, and trimer procyanidin C were the flavanols detected in these fractions, all of which have been previously reported in barley (Holtekjølen et al., 2006).

Identification of the monomer catechin was based on the MRM transition wherein the precursor ion mass of *m/z* 289.1 produces a fragment ion mass of *m/z* 245.0. This particular transition of catechin (289 → 245) is very well reported and arises from an interflavan bond breakage (Callemien, & Collin, 2008). A similar mechanism is responsible for production of the fragments ions from the dimers and the trimer. The dimers procyanidin B ($[M - H]^-$ *m/z* 577.1) and prodelphinidin B ($[M - H]^-$ *m/z* 593.1) involve the monomeric units of catechin/epicatechin and catechin/gallocatechin respectively. Albeit having different molecular masses, both these dimers produce the same dominant product ions at *m/z* 407.0 and *m/z* 289.0, upon fragmentation using collision-induced dissociation (CID). The fragment ion *m/z* 407.0 arises from the dimers due to a retro-Diels-Alder (RDA) fission of ring C and further elimination of a molecule of water, while the ion *m/z* 289.0 arises after a quinone-methide (QM) cleavage of the interflavan bond (Gu et al., 2003; Friedrich, Eberhardt, & Galensa, 2000). While the identification of B type procyanidin dimer could be established based on mass transitions of the standard procyanidin B1, no conclusion could be drawn on the precise type of this dimer (B1, B2, B3, B4), due to lack of availability of all the dimeric standards. Similarly, type identity of the dimer prodelphinidin B and the trimer procyanidin C was inconclusive due to unavailability of standards, though their molecular mass, elemental composition and fragment ion masses were validated from previous reports (Friedrich et al., 2000). The two major product ions with *m/z* 577.0 and *m/z* 124.9 were detected arising from the fragmentation of the parent ion of procyanidin C with $[M - H]^-$ *m/z* 865.1. These two fragments are produced from the trimer by a similar sequence of RDA fissions as the dimers (Bittner, Rzeppa, & Humpf, 2013).

Along with the abovementioned flavanols, a new flavanol was detected in fractions 10 and 11 following untargeted analyses by LC-TOF-MS. This flavanol exhibited a peak for its $[M - H]^-$ molecule at *m/z* 613.2, and produced two prominent fragment ions at *m/z* 451.0 and 289.0. The fragment ions can be accounted for by

Table 2
MRM parameters for UHPLC-MS/MS data acquisition of polyphenols in the most active fractions of barley extract obtained by flash chromatography.

Compound	Retention time in min (fr[a])	MRM transitions	Cone voltage (V)	Collision energy (eV)	Quantity[b] (μg/g dry wt barley flour)
Procyanidin dimer B	3.55 (10/11)	*m/z* 577.1 → *m/z* 406.8 *m/z* 577.1 → *m/z* 288.8	40	26	73.7
Procyanidin trimer C	3.58 (10/11)	*m/z* 865.1 → *m/z* 577.1 *m/z* 865.1 → *m/z* 124.9	30	16	24.6 [c]
Catechin	3.67 (10/11)	*m/z* 289.1 → *m/z* 245.0	40	16	7.6
Prodelphinidin dimer B	3.22 (10/11)	*m/z* 593.1 → *m/z* 407.0 *m/z* 593.1 → *m/z* 289.0	40	26	23.1[c]
Catechin dihexoside	3.20 (10/11)	*m/z* 613.0 → *m/z* 451.0 *m/z* 613.0 → *m/z* 289.0	40	16	12.4[d]
Quercetin	8.47 (25/24)	*m/z* 301.0 → *m/z* 150.9	48	22	15.1
Ferulic acid	8.03 (25/24)	*m/z* 192.9 → *m/z* 177.8 *m/z* 192.9 → *m/z* 133.9	66	12	277.7
Caffeic acid	4.57 (15/16)	*m/z* 179.0 → *m/z* 134.9	34	16	4.8
Coumaric acid	5.91 (15/16)	*m/z* 163.0 → *m/z* 118.9	40	20	2.1

[a] Fraction number (fr).
[b] Quantities of the detected phenolic compounds (except prodelphinidin B3 and catechin diglucoside) in the whole barley grain were determined using commercial standards. The dwell time for acquiring the MRM transitions was 7 ms.
[c] Procyanidin B equivalent.
[d] Catechin equivalent.

Table 3
LC-MS/MS profile of the phenolic compounds in the most active fractions of barley extract obtained by flash chromatography.

Fraction (fr) number	Detected phenolic compounds	Empirical formula	Calculated $[M-H]^-$ (*m/z*)	Observed $[M-H]^-$ (*m/z*)	Quantity[a] (μg)
10,11	Procyanidin dimer B	$C_{30}H_{25}O_{12}^-$	577.1346	577.1362	536.9
	Procyanidin trimer C	$C_{45}H_{37}O_{18}^-$	865.1980	865.2018	169.2 [b]
	Catechin	$C_{15}H_{13}O_6^-$	289.0712	289.0701	61.9
	Prodelphinidin B	$C_{30}H_{25}O_{13}^-$	593.1295	593.1273	166.4[b]
	Catechin dihexoside	$C_{27}H_{33}O_{16}^-$	613.1710	613.1739	102.0[c]
15,16	Caffeic acid	$C_9H_7O_4^-$	179.0344	ND[d]	28.0
	Coumaric acid	$C_9H_7O_3^-$	163.0395	ND	20.3
24,25	Quercetin	$C_{15}H_9O_7^-$	301.0334	ND	122.7
	Ferulic acid	$C_{10}H_9O_4^-$	193.0501	193.0510	2248.0

[a] Quantities of the detected phenolic compounds (except prodelphinidin B3 and catechin diglucoside) in the flash chromatography fractions were determined using commercial standards. The quantities of the compounds in this column are a summation of the quantities in the two fractions mentioned in the first column.
[b] Procyanidin B equivalent.
[c] Catechin equivalent.
[d] Not detected.

the loss of two successive anhydro-hexose groups (*m/z* 162), which yielded the aglycone at *m/z* 289.0. Since the *m/z* 289 corresponds to the $[M-H]^-$ molecule of catechin, this novel flavanol could most likely be a molecule of catechin dihexoside. Accurate mass measurement of the flavanol has further revealed its elemental composition to be $C_{27}H_{33}O_{16}^-$ Catechin-di-glucopyranoside which has the exact same elemental composition, molar mass (*m/z* 613) and fragment ions (*m/z* 451, 289), has been previously identified from a rhubarb extract (Kashiwada, Nonaka, & Nishioka, 1986). Although catechin glucoside has been previously reported in barley (Friedrich & Galensa, 2002), this appears to be the first report on the presence of catechin dihexoside in barley.

Amongst the five flavanols (total 1036 μg) identified in fraction 10 and 11, the B type procyanidin dimer (537 μg) was the most dominant (Table 3). Previous reports have assigned the antioxidant activities of procyanidins to their degree of polymerisation (Lotito et al., 2000). Thus, the dimeric structure of procyanidin B coupled with its abundance in fractions 10/11, could account for most of the antioxidant capacity of these two fractions; however, no certain conclusions could be drawn from this effect.

Based on ORAC results, fraction 25 and fraction 24 were the next most active fractions, representing about 19% of the total antioxidant capacity of the fractions. Quercetin and ferulic acid were the polyphenols detected in these fractions, quercetin being more abundant in fraction 25, whereas ferulic acid was more abundant in fraction 24. Although barley has not been widely reported as a source of flavonols, some cultivars have shown presence of the flavonol quercetin (Etoh et al., 2004; Maillard, Soum, Boivin, & Berset, 1996). Like the flavanols, RDA fission also plays a role in fragmenting flavonols like quercetin (Tsimogiannis, Samiotaki, Panayotou, & Oreopoulou, 2007), which displays a characteristic MRM transition from the $[M-H]^-$ *m/z* 301.0 to *m/z* 150.9 upon CID. Ferulic acid, on the other hand, belongs to the group of hydroxycinnamic acids, and follows a distinct pattern of fragmentation. It has a precursor ion mass of $[M-H]^-$ *m/z* 193.0, which on dissociation produced ions with *m/z* 177.8 and *m/z* 133.9. These fragment ions arise from ferulic acid due to the subsequent loss of methyl (CH_3, −15 Da) and carboxyl (CO_2, −44 Da) groups (Kuhnert, Jaiswal, Matei, Sovdat, & Deshpande, 2010). Ferulic acid has been associated with the cell wall constituents of the grain and has been reported as the dominant phenolic acid in barley (Holtekjølen et al., 2006). This result can be confirmed in our study, as ferulic acid was the most abundant (278 μg/g dry weight of barley) polyphenol amongst the identified ones.

Flavonols, owing to their planar structure and presence of a 3-hydroxyl group, have been reported to exhibit higher antioxidant activity as compared to hydroxycinnamic acids (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). Therefore, the higher antioxidant capacity of fraction 25 possibly arises from the abundance of quercetin in this fraction as compared to fraction 24, which is more abundant in ferulic acid. Also, flavan-3-ols have been reported to exhibit higher antioxidant activity, compared to hydroxycinnamic acids. This has been attributed to the presence of conjugated double bonds and multiple hydroxyl groups in the structure of flavan-3-ols (Soobrattee et al., 2005). This accounts for the fact that although ferulic acid was present in high amount (2248 μg) in fractions 25/24 (Table 3), these fractions were not as high in their antioxidant capacities as the fractions 10/11, which contained flavan-3-ols, albeit in lower amounts (1036 μg). These results corroborate the point made earlier that the structure-dependent intrinsic activity of a polyphenol is crucial along with its abundance, in determining its contribution to the overall antioxidant potential of a given substrate.

Other fractions that demonstrated high antioxidant capacities based on ORAC were fractions 15 and 16, which represented about 12% of the total antioxidant capacity of the fractions. Two hydroxycinnamic acids, caffeic and coumaric acids were detected in these fractions, with the greater proportion being present in fraction 15. These hydroxycinnamic acids and their glycosyl derivatives have been previously reported in barley (Verardo et al., 2008). Caffeic acid is the hydroxylated form of coumaric acid, and both these phenolic acids show an identical pattern of fragmentation. Caffeic acid has a precursor ion mass $[M-H]^-$ of *m/z* 179.0, which on fragmentation loses a carboxyl group to produce a fragment ion at *m/z* 134.9, whereas *p*-coumaric acid has a mass of $[M-H]^-$ *m/z* 163.0 and produces the fragment ion *m/z* 118.9 on loss of the carboxyl group.

Fraction 4 which demonstrated the least antioxidant capacity amongst the seven selected fractions was initially analysed using UHPLC-MS/MS. Interestingly, no polyphenols based on the existing database of MRM transitions were detected in this fraction. The fraction was further analysed on a Q-TOF premier mass spectrometer, whereby two abundant peaks with masses $[M-H]^-$ *m/z* 377.03 and *m/z* 341.06 were identified. Accurate mass measurement revealed the elemental composition of $[M-H]^-$ *m/z* 377.03 to be $C_{12}H_{22}O_{11}Cl$ and that of $[M-H]^-$ *m/z* 341.06 to be $C_{12}H_{21}O_{11}$, which corresponds to the molecules of chlorinated adduct of a hexose dimer and deprotonated hexose dimer respectively. On MS/MS analysis, the precursor ion of *m/z* 377.03, lost the group of chlorine (Cl, 35 Da), to produce a major fragment ion at *m/z* 341.18, while the precursor ion of the hexose dimer (*m/z* 341.06) lost an anhydro–hexose (162 Da), and a molecule of water to produce two dominant fragment ions at *m/z* 179 and *m/z* 161 respectively. Although glucosides by themselves might not possess antioxidant capacities, in a recent study, chlorinated iridoid glucosides from speedwell (*Veronica longifolia*), exhibited enhanced radical-scavenging activities, as compared to the unsubstituted iridoid glucosides, against DPPH, nitric oxide and superoxide

radicals (Jensen, Gotfredsen, Harput, & Saracoglu, 2010). The antioxidant capacity of fraction 4 in the current study would qualify as false-positive, and presumably arises from the abundant presence of the chlorinated adduct of hexose dimer, since no major polyphenols were detected in this fraction.

4. Conclusions

The antioxidant-guided separation was effective in identifying the important polyphenolic contributors to the antioxidant capacity of barley cultivar Irina, as assessed by TPC and *in vitro* antioxidant assays. Some flash chromatography fractions were more active in the ORAC (HAT-based) assay, as compared to the ET-based assays, suggesting the need for performing both types of these assays. Flavanols, were identified as the most influential contributors to the observed *in vitro* antioxidant capacity of barley extracts, of which procyanidin B was the most abundant flavanol. Although ferulic acid was the most abundant phenolic acid in the barley crude extract, contribution of this phenolic acid to the antioxidant capacity of barley was not as significant as the flavanols. The flavanol catechin dihexoside ($C_{27}H_{33}O_{16}^-$, *m/z* 613.1739), novel to barley, was identified in the flash fractions 10 and 11.

Studies have ascribed the antioxidant potential of barley to a number of its components including polyphenols and tocols (Vitamin E). Although the likelihood of a concerted action of the different categories of antioxidants cannot be denied, it can be proposed from our results that the flavanols in barley could play a protective role against oxidative stress. Further confirmation of these results by *in vivo* studies, will allow breeders to select and crossbreed varieties that are rich in their levels of flavanols, for food uses and human consumption, so as to ensure a higher intake of these phytochemicals.

The authors declare a no conflict of interest.

Acknowledgements

The authors acknowledge financial support from the NutriCerealIreland project funded under the Food Institutional Research Measure (FIRM 11/SF/317) of the Irish Department of Agriculture, Food and Marine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.098.

References

Bittner, K., Rzeppa, S., & Humpf, H. U. (2013). Distribution and quantification of flavan-3-ols and procyanidins with low degree of polymerization in nuts, cereals, and legumes. *Journal of Agricultural and Food Chemistry, 61*, 9148–9154.

Bonoli, M., Marconi, E., & Caboni, M. F. (2004). Free and bound phenolic compounds in barley (*Hordeum vulgare* L.) flours: Evaluation of the extraction capability of different solvent mixtures and pressurized liquid methods by micellar electrokinetic chromatography and spectrophotometry. *Journal of Chromatography A, 1057*, 1–12.

Branen, A. (1975). Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *Journal of the American Oil Chemists' Society, 52*, 59–63.

Callemien, D., & Collin, S. (2008). Use of RP-HPLC-ESI (-)-MS/MS to differentiate various proanthocyanidin isomers in lager beer extracts. *Journal of the American Society of Brewing Chemists, 66*, 109–115.

Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., & Mérillon, J.-M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry, 57*, 1768–1774.

Dykes, L., & Rooney, L. (2007). Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World, 52*, 105–111.

Etoh, H., Murakami, K., Yogoh, T., Ishikawa, H., Fukuyama, Y., & Tanaka, H. (2004). Anti-oxidative compounds in barley tea. *Bioscience, Biotechnology, and Biochemistry, 68*, 2616–2618.

Friedrich, W., Eberhardt, A., & Galensa, R. (2000). Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *European Food Research and Technology, 211*, 56–64.

Friedrich, W., & Galensa, R. (2002). Identification of a new flavanol glucoside from barley (Hordeum vulgare L.) and malt. *European Food Research and Technology, 214*, 388–393.

Gangopadhyay, N., Hossain, M. B., Rai, D. K., & Brunton, N. P. (2015). A review of extraction and analysis of bioactives in oat and barley and scope for use of novel food processing technologies. *Molecules, 20*, 10884–10909.

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

Gu, L., Kelm, M. A., Hammerstone, J. F., Zhang, Z., Beecher, G., Holden, J., ... Prior, R. L. (2003). Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *Journal of Mass Spectrometry, 38*, 1272–1280.

Holtekjølen, A. K., Kinitz, C., & Knutsen, S. H. (2006). Flavanol and bound phenolic acid contents in different barley varieties. *Journal of Agricultural and Food Chemistry, 54*, 2253–2260.

Hossain, M. B., Rai, D. K., Brunton, N. P., Martin-Diana, A. B., & Barry-Ryan, C. (2010). Characterization of phenolic composition in Lamiaceae spices by LC-ESI-MS/MS. *Journal of Agricultural and Food Chemistry, 58*, 10576–10581.

Hossain, M. B., Camphuis, G., Aguiló-Aguayo, I., Gangopadhyay, N., & Rai, D. K. (2014). Antioxidant activity guided separation of major polyphenols of marjoram (Origanum majorana L.) using flash chromatography and their identification by liquid chromatography coupled with electrospray ionization tandem mass spectrometry†. *Journal of Separation Science, 37*, 3205–3213.

Hossain, M. B., Rai, D. K., & Brunton, N. P. (2015). Optimisation and validation of ultra-high performance liquid chromatographic-tandem mass spectrometry method for qualitative and quantitative analysis of potato steroidal alkaloids. *Journal of Chromatography B, 997*, 110–115.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841–1856.

Jensen, G. S., Wu, X., Patterson, K. M., Barnes, J., Carter, S. G., Scherwitz, L., ... Schauss, A. G. (2008). In vitro and in vivo antioxidant and anti-inflammatory capacities of an antioxidant-rich fruit and berry juice blend. Results of a pilot and randomized, double-blinded, placebo-controlled, crossover study. *Journal of Agricultural and Food Chemistry, 56*, 8326–8333.

Jensen, S. R., Gotfredsen, C. H., Harput, U. S., & Saracoglu, I. (2010). Chlorinated iridoid glucosides from Veronica longifolia and their antioxidant activity. *Journal of Natural Products, 73*, 1593–1596.

Kashiwada, Y., Nonaka, G. I., & Nishioka, I. (1986). Tannins and related compounds. XLV Rhubarb. 5 Isolation and characterization of flavan-3-ol and procyanidin glucosides. *Chemical and Pharmaceutical Bulletin, 34*, 3208–3222.

Kuhnert, N., Jaiswal, R., Matei, M. F., Sovdat, T., & Deshpande, S. (2010). How to distinguish between feruloyl quinic acids and isoferuloyl quinic acids by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 24*, 1575–1582.

Li, H., Horke, S., & Förstermann, U. (2014). Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis, 237*, 208–219.

Lotito, S. B., Actis-Goretta, L., Renart, M. L., Caligiuri, M., Rein, D., Schmitz, H. H., ... Fraga, C. G. (2000). Influence of oligomer chain length on the antioxidant activity of procyanidins. *Biochemical and Biophysical Research Communications, 276*, 945–951.

Madhujith, T., & Shahidi, F. (2006). Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *Journal of Agricultural and Food Chemistry, 54*, 8048–8057.

Maillard, M.-N., Soum, M.-H., Boivin, P., & Berset, C. (1996). Antioxidant activity of barley and malt: relationship with phenolic content. *LWT-Food Science and Technology, 29*, 238–244.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition, 79*, 727–747.

Nigdikar, S. V., Williams, N. R., Griffin, B. A., & Howard, A. N. (1998). Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation in vivo. *The American Journal of Clinical Nutrition, 68*, 258–265.

Oettl, K., Greilberger, J., Zangger, K., Haslinger, E., Reibnegger, G., & Jürgens, G. (2001). Radical-scavenging and iron-chelating properties of carvedilol, an antihypertensive drug with antioxidative activity. *Biochemical Pharmacology, 62*, 241–248.

Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *Journal of Agricultural and Food Chemistry, 50*, 3122–3128.

Rezaie, A., Parker, R. D., & Abdollahi, M. (2007). Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Digestive Diseases and Sciences, 52*, 2015–2021.

Serafini, M., Ghiselli, A., & Ferro-Luzzi, A. (1996). In vivo antioxidant effect of green and black tea in man. *European Journal of Clinical Nutrition, 50*, 28–32.

Slavin, J. (2004). Whole grains and human health. *Nutrition Research Reviews, 17*, 99–110.

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (2005). Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 579*, 200–213.

Sullivan, P., Arendt, E., & Gallagher, E. (2013). The increasing use of barley and barley by-products in the production of healthier baked goods. *Trends in Food Science & Technology, 29*, 124–134.

Tak, P. P., Zvaifler, N. J., Green, D. R., & Firestein, G. S. (2000). Rheumatoid arthritis and p53: how oxidative stress might alter the course of inflammatory diseases. *Immunology Today, 21*, 78–82.

Thanan, R., Oikawa, S., Hiraku, Y., Ohnishi, S., Ma, N., Pinlaor, S., ... Murata, M. (2014). Oxidative stress and its significant roles in neurodegenerative diseases and cancer. *International Journal of Molecular Sciences, 16*, 193–217.

Tsimogiannis, D., Samiotaki, M., Panayotou, G., & Oreopoulou, V. (2007). Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS. *Molecules, 12*, 593–606.

Tyl, C. E., & Bunzel, M. (2012). Antioxidant activity-guided fractionation of blue wheat (UC66049 Triticum aestivum L.). *Journal of Agricultural and Food Chemistry, 60*, 731–739.

Velioglu, Y., Mazza, G., Gao, L., & Oomah, B. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry, 46*, 4113–4117.

Verardo, V., Bonoli, M., Marconi, E., & Caboni, M. F. (2008). Distribution of bound hydroxycinnamic acids and their glycosyl esters in barley (Hordeum vulgare L.) air-classified flour: comparative study between reversed phase-high performance chromatography– mass spectrometry (RP-HPLC/MS) and spectrophotometric analysis. *Journal of Agricultural and Food Chemistry, 56*, 11900–11905.

Wang, X., Wang, W., Li, L., Perry, G., Lee, H.-G., & Zhu, X. (2014). Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1842*, 1240–1247.

Yu, J., Vasanthan, T., & Temelli, F. (2001). Analysis of phenolic acids in barley by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry, 49*, 4352–4358.

Zielinski, H., & Kozlowska, H. (2000). Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *Journal of Agricultural and Food Chemistry, 48*, 2008–2016.