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Study of antioxidant properties of fractionated apple peel phenolics using a multiple-assay approach

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Abstract

The recovery of antioxidant mixtures from fruit waste is an opportunity to find natural alternatives to synthetic compounds for further food applications. Target components are selected based on an understanding of their antioxidant properties in solvent systems of different composition before further food use. Phenolic antioxidants were extracted with an alcoholic solution from the peels of cv Bramley's Seedling apple and then fractionated with solvents of different polarity. The composition of the extracts was analysed through identification of the main classes of phenolics and their components; the antioxidant and metal chelating capacity were assessed using chemical assays in solvent media with different characteristics. The phenolic composition and the type of solvent had an effect on the antioxidant behaviour of the phenolic extracts, especially in the presence of phases with different polarity (e.g. emulsified lipid in water). Apple peel phenolics, in particular hydroxycinnamic acids such as chlorogenic acid, could supply antioxidant protection not only through radical scavenging but also metal chelating capacity depending on the type of medium in which they are added and its environmental conditions. This should be considered when a multiple-assay strategy is adopted for the screening of plant phenolics as novel food antioxidants.

Introduction

There has been a significant interest in the food industry to use plant extracts (also referred to as botanicals) as taste enhancers, colorants, or as natural additives in alternative to synthetic counterparts, especially for those compounds with hazardous effects on consumers' health (Wardhani *et al*., 2013). The research on the physical-chemical stabilisation of food products with botanicals has been boosted by an increased demand for natural antioxidants instead of synthetic additives (Berdahl *et al*., 2010). It is expected that the incorporation of high quality fatty acids for functional/nutritional use will result in novel mixtures of antioxidants to be developed based on their physical-chemical properties and composition of the food (Pokorný, 2007).

Agri-food waste and by-products have been screened as valuable sources of natural antioxidants, particularly phenolics, in order to find valuable alternatives to traditional disposal and recycling routes, thus leveraging the cost of waste disposal through resource efficiency (O'Shea *et al*., 2012). In the case of apple peels, previous work has been carried out for the recovery, isolation and characterisation of dietary fibre and/or phenolics (Rabetafika *et al*., 2014). Little information is available for the peels of cooking apples that are used for canning, sauce or puree making such as cv Bramley's Seedling which is traditionally grown in the British Isles (Massini *et al*., 2010). Further development of the natural food market requires in-depth knowledge of the chemical functionality and stability of the plant antioxidants in foods, especially if the mixtures are applied without further fractionation and the active component(s) are present at low concentrations, which can lead to undesired taste or colour impairment (Pokorný, 2007).

Chemical assays based on different solvent media are commonly used to investigate the role of plant antioxidants before further food application (Koleva *et al*., 2002). Studies of plant phenolics have normally focused on the role of flavonoids as free radical scavengers; however, studies have also reported that various groups of phenolics including flavonoids but mostly phenolic acids can also supply metal chelating capacity (Andjelković *et al*., 2006).

The presence of more than one antioxidant mechanism in the phenolic structure is further complicated by the effect of the solvent medium and

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environmental conditions, especially when more than one phase is present as in heterogeneous systems made of water and lipid phases (Gordon, 2010). The latter would be the ideal choice when screening plant antioxidants in view of food use because of the complex nature of the food matrix; however, such assays are notoriously difficult to be closely reproduced as a result of the interactions between matrix and target components. This is the reason why simple radical scavenging assays based on the colorimetric reactions of chemical probes such as FRAP or $DPPH^{\cdot}$ in polar solvents have been widely used as quick inexpensive methods, and ultimately combined with more complex assays testing the lipid peroxidation of oil-in-water emulsions or emulsified lipid such as in the β-carotene bleaching assay, and thiobarbituric acid assay (TBA) (Moon and Shibamoto, 2009). In particular, DPPH and the β-carotene bleaching assay (BCB) have been used together to compare the radical scavenging capacity of various flavonoids and phenolic acids (Burda and Oleszek, 2001; Koleva *et al*., 2002); nevertheless, it was reported that the antioxidant capacity of phenolics in BCB could be due to mechanisms other than the solely free radical quenching capacity (Pekkarinen *et al*., 1999).

The choice of the antioxidant assays is one of the critical steps towards the feasible application of natural mixtures of plant phenolics as novel food antioxidants; however, it is challenging to decide about the right combination of assays so as to maximise the transfer of results towards food applications while achieving ease of use and reproducibility, thus leading to a comparison of different plant materials.

In the present study, apple peels were used as a well established source of natural antioxidants (Schieber *et al*., 2001b; Medina *et al*., 2003; Wolfe *et al*., 2003; Rupasinghe *et al*., 2008). Flavonoids and phenolic acids were first solubilised with aqueous ethanol and then fractionated with partitioning solvents of different polarity. The resulting extracts were tested for their antioxidant capacity using two commonly used chemical assays based on different antioxidant mechanisms and environmental conditions (i.e. type of solvent, pH): a radical scavenging assay ($DPPH^{\cdot}$) and a lipid peroxidation assay in ethyl linoleate (BCB); the metal chelating capacity against Iron(II) was also assessed. This approach aimed at investigating the antioxidant behaviour of mixtures of fractionated apple phenolics under different conditions in view of selecting target component(s) with further application in different food matrices.

Materials and Methods

Plant material and sample preparation

Peel samples were obtained from apples (*Malus domestica* Borkh.) cv Bramley's Seedling that were purchased from a local store (Dublin, Ireland) during the years 2009-2010; the apples were stored at 4°C in polyethylene bags until further processing. In order to carry out the experiment, a minimum of two batches of apples (from 3 to 5 kg each) were purchased; for each batch, three independent samples were prepared by randomly pooling fruits from different trays. After the fruits were washed under tap water, the peels were removed mechanically with a hand peeler and dried using stainless steel trays at 60 ± 2°C in a convection oven with forced air ventilation (BS Oven 250, Weiss Gallenkamp, Loughborough, UK) until a constant weight was achieved. The dried peels were pulverised using a coffee grinder and the powders stored in glass jars at -20°C until further analysis.

Extraction of phenolic compounds

Phenolic compounds were solubilised from dried peels using a liquid extraction method with aqueous ethanol (80%, v/v) (Wolfe and Liu, 2003). Briefly, crude extracts of peel phenolics were obtained through homogenisation of the peel powder with the chilled solvent followed by filtration. The filtrates were pooled and the organic solvent was removed at 40°C in a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland) until a final concentration of 10-20% (v/v) was achieved. The crude extract was washed twice with petroleum ether (1:1); the organic layers were discarded, while the aqueous phase was retained in view of further fractionation.

Fractionation based on acidity

The fractionation of apple peel phenolics into flavonoids and phenolic acids was carried out with a liquid partitioning system of water and ethyl acetate at different pH, according to a previous method (Delage *et al*., 1991). Flavonoids and phenolic acids were selectively partitioned within the organic phase based on their acidic behaviours in water. The pH of the defatted peel extract was first adjusted to pH 7.0 ± 0.2 with sodium hydroxide and then the liquid-liquid extraction (1:1) was carried out at least three times in order to isolate neutral phenolics (i.e. flavonoids). Phenolic compounds that remained in the aqueous layer (i.e. mostly phenolic acids) were extracted with ethyl acetate after adjustment of the pH to 2.0 ± 0.2 with meta-phosphoric acid; the extraction was repeated three times.

The organic layers were collected and dried over

sodium sulphate anhydrous; the organic solvent was then removed using a rotary evaporator at 40°C under vacuum. The remaining solids were resuspended in 5 mL of LC grade methanol (Sigma-Aldrich, Ireland) for further quantification analysis. The phenolic extracts (referred to as APN for neutral; and APA for acidic) were stored at -20°C in capped amber glass bottles until use.

Total phenolic content

Phenolic extracts from apple peels (APN and APA) were quantified for their total phenolic content (TPC) using Folin-Ciocalteu assay (Wolfe and Liu, 2003). The total phenolic content was expressed as mg gallic acid equivalents (GAE) g^{-1} peels (on a dried weight basis, DW). All measurements were carried out in triplicate.

Total proanthocyanidin content

The total proanthocyanidin content (TPAC) was measured as previously described, with minor modifications (Li *et al*., 2010). A volume of 0.25 mL of sample was added with 1.5 mL of acid butanol (1-butanol:HCl, 95:5, v/v) and 0.05 mL of iron reagent (0.041 mol L^{-1} in 2 M HCl) in test tubes that were placed in water at 95°C for 20 min. Once cooled, the absorbance of the resulting anthocyanidin product was recorded at 550 nm using Agilent 8453 spectrophotometer (Agilent Technologies, Ireland) and quantified as mg cyanidin equivalents (CyE) g^{-1} peels (DW); cyanidin was obtained via acid-butanol chemistry from a primary standard of procyanidin B2 (Extrasynthèse, Genay, France). The measurements were carried out in duplicate.

RP-HPLC-PDA analysis

The identification and quantification of phenolic compounds via RP-HPLC-PDA analysis was carried out with a previous method, with minor modifications (Schieber *et al*., 2001a). A volume of phenolic extract in LC methanol was filtered through 0.45 µm PTFE membrane disc filter (Acrodisc, Pall, Portsmouth, UK) and then 0.02 mL was injected into e2695 Separation Module (Waters Alliance, Dublin, Ireland). The HPLC system consisted of an auto sampler, a column heater, a quaternary solvent system pump, an inline vacuum degasser, a photodiode array detector (PDA 2998), and was equipped with Empower 2 Chromatographic Software (Waters Alliance, Ireland). The components were separated on a Nucleosil C18 column (250 x 4.6 mm I.D., 5 µm packing) (Varian, JVA Analytical, Dublin, Ireland) at a controlled temperature of 25.0°C ± 0.2 and at a flow rate of 0.9 mL min^{-1}. The gradient consisted of a mixture of 0.33 mol L^{-1} acetic acid in water (solvent A) and 0.083 mol L^{-1} acetic acid in water:acetonitrile (50:50) (solvent B); it was increased linearly from 10% to 55% B in 45 min; from 55% to 57% B in 5 min; from 57% to 70% B in 10 min; from 70% to 100% B in 5 min; and from 100% to 10% in 1 min. The system was re-equilibrated with the initial conditions for 10 min, before resuming the analysis.

The elution of target groups of phenolics was simultaneously monitored at 280 nm (flavan-3-ols; dihydrochalcones and derivatives); 320 nm (hydroxicinnamic acids and derivatives); and 370 nm (flavonols and flavonol glycosides). The identification of phenolic components was carried out through comparison of their retention times with commercial standards; the latter were of compatible purity for LC analysis and included: (+)-catechin; phloretin-2'-O-glucoside (phloridzin); quercetin; quercetin-3-O-rutinoside (rutin); gallic acid; caffeic acid; p-coumaric acid; 5'-caffeoylquinic acid (chlorogenic acid) (Sigma-Aldrich, Ireland). Procyanidin B2; (-)-epicatechin; quercetin-3-O-galactoside (hyperoside); and quercetin-3-O-glucoside (isoquercitrin) were from Extrasynthèse (Genay, France).

Provisional identification of unknown compounds for which commercial standards were not available was carried out based on their UV-Vis spectral characteristics and comparison with bibliographical data (Escarpa and González, 1998; Sakakibara *et al*., 2002; Schieber *et al*., 2002; Alonso-Salces *et al*., 2004). The quantification study was carried out with calibration curves of standard phenolic compounds (20-200 mg L^{-1}): (+)-catechin for flavan-3-ols (280 nm); quercetin for flavonols and flavonol glycosides (370 nm); phloridzin for dihydrochalcones and derivatives (280 nm); chlorogenic acid for hydroxycinnamic acids and derivatives (320 nm).

Antioxidant capacity

The antioxidant and metal chelating properties of the phenolic extracts were tested using the following chemical assays: 1) DPPH; 2) β-carotene bleaching assay (BCB); 3) Iron(II) chelation with Ferrozine reagent. Depending on the assay, neutral and acidic extracts were dissolved in water or ethanol and their concentration was expressed as mmol GAE L^{-1}.

DPPH radical scavenging capacity

The radical scavenging capacity of the aqueous phenolic extracts was measured as previously described (Massini *et al*., 2013). The absorbance of 0.08 mmol L^{-1} $DPPH^{\cdot}$(2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich, Ireland) solution

in ethanol with added phenolic extracts (0.06-2.94 mmol L^{-1} as GAE, in distilled water) was measured in a spectrophotometer at 515 nm and compared with a control $DPPH^{\cdot}$ solution with distilled water instead of sample. The antioxidant capacity was calculated as:

$$\% \text{ Reduced } DPPH^{\cdot} = \left[\left(Abs_{control} - Abs_{sample}\right)/Abs_{control}\right] * 100 \quad \text{Equation 1}$$

Where Abs = absorbance at 515 nm.

The IC_{50} or sample concentration giving a 50% reduction of the initial concentration of $DPPH^{\cdot}$ was calculated using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). L-ascorbic acid (0.06-0.6 mmol L^{-1}) was used as the reference standard. All measurements were carried out in triplicate.

β-carotene bleaching assay

The oxidative bleaching of ß-carotene in a model ethyl linoleate emulsion in water (BCB assay) was used to measure the percentage of inhibition against lipid oxidation of the phenolic extracts (Barros *et al.*, 2008). A volume (0.4 mL) of ß-carotene solution that was prepared with 10 mg of ß-carotene in 10 mL chloroform was aliquoted into a 100 mL round-bottom flask together with 1.6 mL of 0.089 mol L^{-1} linoleic acid (Sigma-Aldrich, Dublin, Ireland) in ethanol and 400 mg of Tween 80 as emulsifier. After removal of the organic solvent at 40°C in a rotary evaporator, 25 mL of aerated water that was prepared by bubbling air into distilled water through an air compressor was added to the flask. The emulsion was vigorously shaken and sonicated in an ultrasound bath for 10 minutes. Aliquots of the emulsions (0.96 mL) were added to tubes containing 0.04 mL of aqueous phenolic extracts at different concentrations (0.30-2.94 mmol L^{-1} as GAE); an emulsion with distilled water instead of samples was used as the assay control. The tubes were incubated in a water bath at 50°C. The oxidation of ß-carotene was monitored in a spectrophotometer at 470 nm every 20 minutes, until discolouration occurred in the assay control (120 min). The amount of residual ß-carotene in the emulsions with and without added extracts at each i-th interval of the incubation time was calculated with Equation 2:

$$\% \text{ Residual } \beta\text{-carotene}_{ti} = \left[1 - \left(\frac{Abs_{t0} - Abs_{ti}}{Abs_{t0}}\right)\right] * 100 \quad \text{Equation 2}$$

Where Abs = absorbance of reaction mixture at 470 nm; t_0 = beginning of reaction; t_i = *i-th* interval of time.

The antioxidant capacity of the samples was expressed as percentage of Lipid Oxidation (LOX) Inhibition after 120 min of incubation (Liu *et al.*, 2009):

$$\% \text{ LOX Inhibition} = 100^{*}(DR_c - DR_s)/DR_c \quad \text{Equation 3}$$

Where: DR_c = degradation rate of the control = [ln(a/b)/120]; DR_s = degradation rate in the presence of the sample = [ln*(a/b)*/120], where a = Abs at λ = 470 nm at t_0 and *b* = Abs at λ = 470 nm at 120 min.

The percent inhibition values with Equation 3 were plotted versus sample concentrations and the IC_{50} value was calculated using GraphPad Prism 5.0. The antioxidant tert-butyl hydroquinone (TBHQ) (Sigma-Aldrich, Dublin, Ireland) (0.06-3 mmol L^{-1}) was used as the reference standard.

Metal chelating capacity

The metal chelating capacity of the phenolic extracts (APN and APA) was measured according to a previous method (Andjelković *et al.*, 2006). Phenolic extracts were dissolved in ethanol (0.5 mL) and diluted with 1.5 mL of 50 mmol L^{-1} HEPES buffer (pH 7.4) until final concentrations of 6-1180 µmol L^{-1} as GAE; the water was ultrapure and de-aerated before use. A volume of 0.05 mL of 2 mmol L^{-1} ferrous chloride was added to the reaction mixtures by gently mixing; after 2 min, 0.15 mL of 5 mmol L^{-1} Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate) (Sigma Aldrich, Ireland) was added. The reaction was incubated for 10 min, and then the absorbance was recorded in a spectrophotometer at 562 nm. The measurements were carried out in triplicate. An assay control with HEPES buffer instead of sample was prepared. The iron chelating capacity of the samples was calculated as:

$$\% \text{ Chelated Iron} = \left[\left(Abs_{control} - Abs_{sample}\right)/Abs_{control}\right] * 100 \quad \text{Equation 4}$$

Where Abs: absorbance at 562 nm.

The sample concentration (IC_{50}) chelating 50% of the initial amount of Iron(II) was calculated with GraphPad Prism 5.0. The chelating capacity of EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich, Ireland) (3-684 µmol L^{-1}) was used for comparison.

Statistical analysis

In order to assess the effect of applied phenolic extracts, a statistical analysis was performed with GraphPad Prism 5.0 for Windows which included ANOVA and Tukey and Bonferroni tests for comparing means at a significance level at $p<0.05$. Linear and non-linear regression models were used to fit the experimental data in order to obtain concentration-response curves for the antioxidant

Table 1. Phenolic composition (mg g^{-1} peels, DW) of neutral and acidic extracts from apple peels assessed with HPLC and spectrophotometric methods

Component(s)	Phenolic extracts APN (Neutral)	Phenolic extracts APA (Acidic)	Apple Peels (Total)
(+)-Catechin	0.14 ± 0.01	-	0.14 ± 0.01
(-)-Epicatechin	1.40 ± 0.23	-	1.40 ± 0.23
Procyanidin B2	1.27 ± 0.06	-	1.27 ± 0.06
Other PAs[i]	1.19 ± 0.11	0.97 ± 0.31	2.16 ± 0.36
PAs	*2.46 ± 0.16*	*0.97 ± 0.31*	*3.43 ±0.37*
Flavan-3-ols	*4.01 ± 0.06*	*0.97 ± 0.31*	*4.98 ± 0.32*
Quercetin-3-O-galactoside	0.38 ± 0.09	-	0.38 ± 0.09
Quercetin-3-O-glucoside	0.21 ± 0.02	-	0.21 ± 0.02
Other FLOs[ii]	1.04 ± 0.15	-	1.04 ± 0.15
FLOs	*1.63 ± 0.26*	-	*1.63 ± 0.26*
Phloridzin	0.51 ± 0.02	-	0.51 ± 0.02
Phloretin derivative[iii]	0.11 ± 0.02	0.20 ± 0.02	0.33 ± 0.01
DCHAs[iv]	*0.62 ± 0.03*	*0.20 ± 0.02*	*0.83 ± 0.03*
Chlorogenic acid	-	0.61 ± 0.10	0.61 ± 0.10
Other HCAs[v]	-	0.27 ± 0.05	0.27 ± 0.05
HCAs	-	*0.88 ± 0.15*	*0.88 ± 0.15*
Total phenolics (with HPLC method)	6.23 ± 0.17	2.04 ± 0.45	8.39 ± 0.24
TPC [vi] (with Folin-Ciocalteu assay)	6.32 ± 0.52	2.64 ± 0.26	8.96 ± 0.57
TPAC [vii] (with Acid-Butanol assay)	2.02 ± 0.22	0.90 ± 0.11	2.92 ± 0.32

Values are expressed as mean values ± standard deviation (SD) (n = 6). DW: dry weight basis.
[i] PAs: proanthocyanidins (oligomers), quantified as (+)-catechin.
[ii] FLOs: flavonol glycosides, quantified as quercetin.
[iii] Phloretin derivative, quantified as phloridzin.
[iv] DCHAs: dihydrochalcones.
[v] HCAs: Hydroxycinnamic acids, quantified as chlorogenic acid.
[vi] TPC: total phenolic content, expressed as mg GAE g^{-1} (DW).
[vii] TPAC: total proanthocyanidin content, expressed as mg CyE g^{-1} peels (DW).

and metal chelating capacity of phenolic extracts and reference standard compounds and to calculate the IC_{50} values; the goodness of fit (R^2) and standard deviation of the residuals (Std. error) were also reported.

Results and Discussion

Phenolic composition of apple peels

The phenolic composition of apple peels has been widely studied (Neveu *et al*., 2010), therefore a HPLC-RP-PDA analysis is routinely used for the identification of main components in plant mixtures and their quantification using commercially available standards. Peel phenolics from Bramley apple comprised mostly of flavonoids (> 50%), especially flavan-3-ols consisting of monomers such as catechins, and dimers (namely procyanidin B2) (Table 2). These findings were in agreement with previous studies with the same cultivar (Price *et al*., 1999; Khanizadeh *et al*., 2008). Flavonoids in APN mostly comprised of monomeric catechins and oligomeric proanthocyanidins (PAs) such as trimers and tetramers; the latter were also detected in APA but were less abundant (Delage *et al*., 1991). Other flavonoids detected in APN included flavonol glycosides, in particular quercetin-3-O-galactoside and quercetin-3-O-glucoside; other flavonols were tentatively identified as quercetin-3-O-xyloside (λ_{max} = 254.7, 353.5 nm), quercetin-3-O-arabinoside (λ_{max} = 254.7; 352.3 nm), and quercetin-3-O-rhamnoside (λ_{max} = 254.7, 349.9 nm) (Sakakibara *et al*., 2002; Schieber *et al*., 2002). The most abundant dihydrochalcone in apple peels was phloridzin, which was isolated in APN. Another dihydrochalcone that was isolated in both phenolic extracts, mostly in APA, was identified as a phloretin derivative (λ_{max} = 227.5, 284.3 nm) (Escarpa and González, 1998). Finally, hydroxycinnamic acids consisting of derivatives of caffeic (λ_{max} = 229.9, 322.4 nm) and coumaric acids (λ_{max} = 221.6, 315.3 nm; λ_{max} = 231.1, 311.7 nm) were detected in APA (Sakakibara *et al*., 2002; Alonso-Salces *et al*., 2004). Chlorogenic acid (or 5'-caffeoyilquininic acid) was the most abundant phenolic acid that was identified in apple peels. The quantification carried out with the RP-HPLC-PDA method was consistent with the total phenolic and

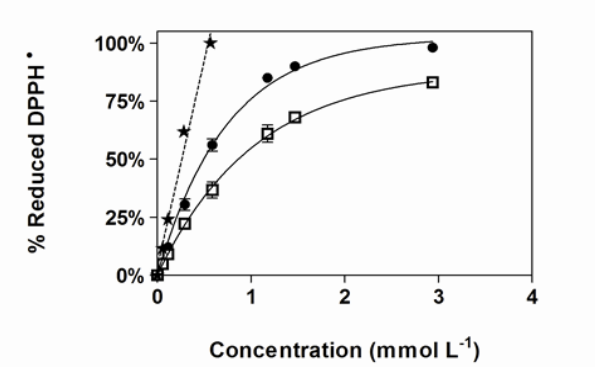

Figure 1. Radical scavenging capacity of phenolic extracts from apple peels (APN; APA) and L-ascorbic acid (AA) ( ) using DPPH assay. The values are given as mean ± SD of three replicates. For phenolic extracts, concentration is expressed as gallic acid equivalents (GAE). The fitted equations to the experimental data are: (APN) $Y = 102.6^{*}[1-e^{(-1.35X)}]$ ($R^2 = 0.99$; Std. error = 3.64); (APA) $Y = 88.68^{*}[1-e^{(-0.96X)}]$ ($R^2 = 0.99$; Std. error = 3.49); (AA) $Y = 177.7^{*}X + 3.166$ ($R^2 = 0.98$; Std. error = 5.68).

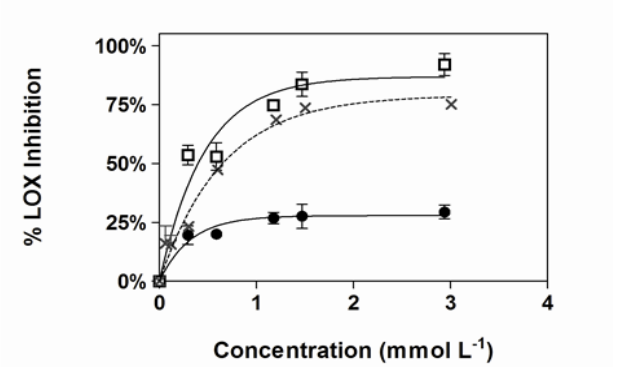

Figure 2. Lipid oxidation inhibition of phenolic extracts from apple peels (APN; APA) and TBHQ ( ) using BCB assay. The values are given as mean ± SD of three replicates. For phenolic extracts, concentration is expressed as GAE. The fitted equations to the experimental data are: (APN) $Y = 27.86^{*}[1-e^{(-3.13X)}]$ ($R^2 = 0.81$; Std. error = 3.58); (APA) $Y = 86.86^{*}[1-e^{(-2.16X)}]$ ($R^2 = 0.83$; Std. error = 10.56); (TBHQ) $Y = 78.93^{*}[1-e^{(-1.54X)}]$ ($R^2 = 0.96$; Std. error = 5.58).

proanthocyanidin contents measured with Folin-Ciocalteu and Acid-Butanol reagents, respectively (Table 1).

Antioxidant and metal chelating capacities

Phenolic extracts from apple peels were capable of scavenging the free radical $DPPH^{\cdot}$ and lipid radicals in a model emulsified system (BCB assay) (Figures 1 and 2, respectively); they were also capable of chelating ferrous ion at physiological pH (Figure 3). The combined use of the three antioxidant assays led to different results depending on the phenolic composition of the extract and the type of assay.

Radical scavenging capacity (DPPH)

The neutral extract (APN) that was enriched with flavan-3-ols (catechins and oligomers) and flavonol glycosides had significantly higher DPPH radical scavenging capacity ($p<0.05$) than the acidic extract; phenolic extracts also had lower antioxidant capacity than ascorbic acid ($p<0.05$) (Table 2). It has been widely reported that the scavenging capacity of peel phenolics, particularly flavonoids, is mostly attributed to their H-atom donor and electron transfer capacities, which lead to chain breaking activity against the progression of lipid oxidation (Apak *et al*., 2007). Their chemical structure, including the number of hydroxyl substituents on the flavonoid molecule, their positions, presence of glycosides or aglycones, degree of conjugation and/or unsaturation play a crucial role in determining such activity (Burda and Oleszek, 2001). Flavan-3-ols and flavonols contribute highly to the radical scavenging capacity of apple peels (Tsao *et al*., 2005; Sekhon-Loodu *et al*., 2013); particularly, monomeric and oligomeric flavonoids with orthodiphenolic structure in the B ring and those with the unsaturation of the hydrocarbon ring C (Katalinić *et al.*, 2004). Phenolic acids and dihydrochalcones have lower radical scavenging capacity than other apple phenolics (Wolfe *et al*., 2003).

Lipid oxidation inhibition (BCB)

Unlike what observed with DPPH assay, the antioxidant capacity of the phenolic extracts with the β-carotene bleaching assay (BCB) led to a significantly lower IC_{50} value of the acidic extract (APA) ($p<0.05$) than that of the neutral extract (APN); the inhibition against lipid oxidation of APA was also significantly higher than TBHQ (Table 2). It was previously reported that in heterophasic emulsified systems such as in BCB, the antioxidant activity is affected by several parameters including partitioning behaviours and interactions with other compounds; on the contrary, the DPPH assay is independent of sample polarity (Koleva *et al.*, 2002). Due to the chemical structure of plant phenolics, these compounds can be located at the interface between lipid and water where they show different partitioning behaviours depending on their level of substitution (Burda and Oleszek, 2001). Less polar antioxidants such as the most abundant flavan-3-ols in apple peels are normally expected to be stronger radical scavengers than more polar antioxidants such as flavonol glycosides and hydroxycinnamic acids in emulsified systems due to their lower polarity i.e. favourable partitioning towards the lipid phase (Sekhon-Loodu *et al.*, 2013). However, the so-called

Table 2. Antioxidant and metal chelating capacity (as IC_{50}) of phenolic extracts and reference compounds

Parameter	Phenolic extracts		Reference compounds[ii]
	Neutral (APN)	Acidic (APA)	
Radical scavenging capacity (DPPH)	0.51 ± 0.09[b]	0.90 ± 0.05[a]	0.26 ± 0.02[c]
LOX Inhibition (BCB)	> 2.94[c]	0.41 ± 0.12[b]	1.94 ± 0.11[a]
Metal chelating capacity	141 ± 4[c]	43 ± 3[b]	29 ± 1[a]

[i] IC_{50} values are given as mean ± SD (n = 3) and expressed as mmol L^{-1} (as GAE for phenolic extracts), except for metal chelating capacity for which IC_{50} is expressed as µmol L^{-1}. Different superscript letters denote significant difference ($p<0.05$) within rows.
[ii] L-ascorbic acid for DPPH assay; TBHQ for BCB assay; EDTA for metal chelating capacity assay.

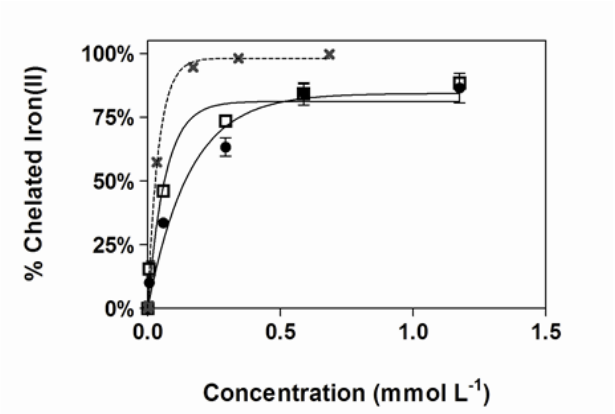

Figure 3. Metal chelating capacity of phenolic extracts from apple peels (APN; APA) and EDTA ( ). The values are given as mean ± standard deviation (SD) of three replicates. For phenolic extracts, concentration is expressed as GAE. The fitted equations to the experimental data are:
(APN) $Y = 84.28^{*}[1-e^{(-6.37X)}]$ ($R^2 = 0.97$; Std. error = 6.88);
(APA) $Y = 81.17^{*}[1-e^{(-15.21X)}]$ ($R^2 = 0.97$; Std. error = 6.82);
(EDTA) $Y = 98.05^{*}[1-e^{(-24.46X)}]$ ($R^2 = 0.97$; Std. error = 3.33).

polar paradox was not able to explain why the APN extract enriched with oligomeric flavan-3-ols had lower antioxidant capacity than APA. Factors other than the sole polarity of the compounds have been suggested in order to explain similar findings with the BCB assay (Burda and Oleszek, 2001). In order to obtain further information regarding this, the study of the metal chelating capacity of the phenolic extracts at physiological pH was also assessed as it was reported that plant polyphenols could prevent or reduce the rate of lipid oxidation through chelation of pro-oxidant metals in emulsions (Gordon, 2010).

Metal chelating capacity

Phenolic extracts from apple peels were capable of chelating ferrous ion (Figure 3). However, the metal chelating capacity of the acidic extract (APA) was significantly higher ($p<0.05$) than that of the neutral extract (APN); both extracts had lower chelating capacity than EDTA (Table 2). Hydroxycinnamic acids such as chlorogenic acid that were exclusively detected in APA could contribute highly to the metal chelating capacity of this mixture as a result of the ortho-dihydroxy substitution in the aromatic ring (catechol) which is effective at chelating transition metals such as Iron(II) (Hider *et al*., 2001). Flavonoids present in APN were also capable of metal chelation but were less effective than phenolics in APA. This difference could be explained as due to the different ionization patterns of neutral and acidic phenolics at physiological pH which ultimately affects their chelating sites.

Phenolic composition and antioxidant capacity

Findings obtained in the present study suggested that the combination of the three chosen chemical assays was useful to highlight different antioxidant behaviours of mixtures of phenolic acids and flavonoids extracted from apple peels in different solvent systems. Under the investigated conditions, the rates of lipid oxidation of BCB emulsions with added acidic phenolics (APA) were significantly lower than with the added flavonoids, thus leading to increased antioxidant capacity (Figure 4). These findings were in agreement with the results of the metal chelating capacity assay, but not with the radical scavenging capacity measured with DPPH assay (Table 2). A similar lack of correlation between results of DPPH and BCB assays was previously observed by Pekkarinen *et al.* (1999); these authors concluded that the antioxidant capacity of phenolic acids should not be solely measured in terms of free radical scavenging capacity in this emulsified system. In another study by Sekhon-Loodu *et al.* (2013), chlorogenic acid was reported as being the most effective apple phenolic against lipid oxidation in a fish oil emulsion, followed in the order by phloridzin > catechins and flavonols; these results did not reflect

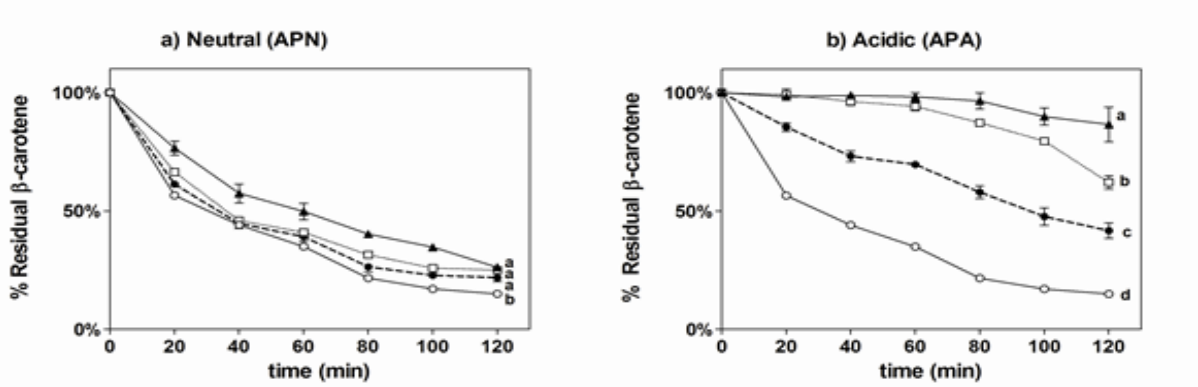

Figure 4. Degradation rates of β-carotene in ethyl linoleate emulsions (BCB assay) in the presence of added phenolic extracts from apple peels (APN and APA) at difference concentrations (▲ 2.94; □ 1.18; ◆ 0.29 mmol GAE L^{-1}) and control (○). Values are expressed as mean ± SD (n = 3). Different superscript letters indicate significant difference ($p<0.05$) at the end of the reaction time (120 min).

the radical-quenching ability of apple phenolics: procyanidins > quercetin glycosides > chlorogenic acid > phloridzin (Tsao *et al.*, 2005).

One of the possible explanations for this could be the role that metal chelation plays in the modulation of lipid peroxidation inhibition in the emulsified lipid model system, especially in the presence of phenolic acids, particularly esters of hydroxycinnamic acids such as chlorogenic acid. It was reported that these compounds could prevent the formation of lipid radicals by chelating pro-oxidant metals in the water phase and/or could scavenge them in the lipid phase as a result of their surface activity (Sasaki *et al.,* 2010). Such possibility could explain the different oxidation rates of the BCB emulsions in the present study (Figure 4). Under the same accelerated oxidative conditions (light, oxygen and heat), the emulsions with the added phenolic acids maintained their antioxidant protection over time, above all at higher concentrations, while the initial protection supplied by the flavonoid extract was lost towards the end of the reaction. Unlike phenolic acids that could supply their antioxidant capacity by retarding the onset of lipid oxidation, flavonoids would preferably supply a chain-breaking activity as a result of their lower metal chelating capacity and reduced surface activity i.e. oligomeric flavan-3-ols have lower polarity than hydroxycinnamic acids and are preferably partitioned towards the oil rather than the water phase.

The possibility for certain apple phenolics, in particular hydroxycinnamic acids, to act with more than one antioxidant mechanism makes the measurement of their antioxidant behaviours in the emulsified model system more challenging than in assays based on polar solvents or mixtures thereof such as in the DPPH assay. Nevertheless, the combination of both assays can ultimately reflect the complex behaviour of those compounds in the food matrix.

The measurement of the metal chelating capacity under environmental conditions such as solvent type, polarity and pH should be considered when screening plant sources rich in phenolics, especially hydroxycinnamic acids, as this could significantly contribute to their antioxidant protection in food applications.

Conclusions

Different classes of apple peel phenolics, in particular flavonoids and phenolic acids, are capable of supplying different antioxidant mechanisms (radical scavenging and/or metal chelating capacity) with regard to the type of selected medium and its environmental conditions.

A multiple-assay approach based on the combined measurement of shear radical scavenging capacity and metal chelating capacity is then useful to interpret such complexity when studying their food applications as natural antioxidants, especially in heterophasic emulsified systems.

The development of tailor-made mixtures of peel phenolics with different antioxidant properties and mechanisms could supply enhanced antioxidant properties in foods of particular structure. Their application at different stages of food processing or storage is worth of further investigation.

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