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2 **New insights into antioxidant activity of Brassica crops**

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Abstract

This work was aimed to evaluate the variation of the antioxidant activity of several Brassica vegetables at different plant stages, on their by-products and to study the relationship among the antioxidant activity and phenolic composition. Antioxidant activity of six Brassica crops including broccoli, cabbage, cauliflower, kale, nabicol and tronchuda cabbage was measured at four plant stages with DPPH and FRAP assays, founding that samples taken three months after sowing showed the highest antioxidant activity. Kale crop outstand at this plant stage and also at adult plants stage, while cauliflower showed the highest antioxidant activity in sprouts and in leaves taken two months after sowing. Brassica by-products could be used as sources to obtain derived products with high content of antioxidants. Phenolic content and composition varied depending on the crop under study and on the plant stage, being sprout samples much more reach in hydroxycinnamic acids than the rest of samples. Differences in antioxidant activity of Brassica crops were related to differences in total phenolic content but also to differences in phenolic composition for most samples.

Key words: Brassica vegetables, By-products, Plant stages, ROS, phenolic compounds

1. Introduction

Reactive oxygen species (ROS) are generated during cell aerobic respiration. Under normal physiological conditions, the redox state is tightly controlled by antioxidants. However, increased production of ROS can overwhelm the antioxidant defenses, leading to an imbalance and imposing oxidative stress on the physiological systems. The oxidative damages caused by ROS on lipids, proteins and nucleic acids may trigger various chronic diseases. Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system. Some foods and vegetables are important sources of exogenous antioxidants. In fact, Brassica crops are among the ones having the highest antioxidant activity in the group of vegetable foods, including spinach, carrot, potato, purple onion, green pepper, beet, rhubarb or green bean (Cao, Sofic & Prior, 1996; Kequan & Liangli, 2006).

A high intake of Brassica vegetables reduces the risk of age-related chronic illnesses such as cardiovascular health and other degenerative diseases (Kris-Etherton et al., 2002) and of several types of cancer (Wang, Giovannucci, Hunter, Neuberg, Su & Christiani, 2004). The contribution of Brassica vegetables to health improvement has been partly associated with their antioxidant capacity, and consequently, Brassica crops have been the focus of intense research based on the content of secondary metabolites (Verkerk et al., 2009).

Comparisons of antioxidant activity of the main Brassica crops have been studied *in vitro* by different authors (Jagdish, Upadhyay, Singh & Rai, 2009; Nilsson et al., 2006; Podsedek, Sosnowska, Redzynia & Anders, 2006; Samec, Piljac-Zagarac,

Bogovic, Habjanic & Gruz, 2011; Sikora, Cieslik, Leszczynska, Filipiak-Florkiewicz & Pisulewski, 2008), establishing rankings based on antioxidant potential of hydrophilic and lipophilic extracts. Broccoli, kale, red cabbage and Brussels sprouts show high antioxidant potential, whereas cabbage has a rather low antioxidant activity. These comparisons are normally carried on in the consumed organs (heads of cabbage, broccoli and cauliflower, leaves of kale and tronchuda cabbage) of Brassica crops. However, other organs which are not normally consumed can also show high antioxidant activities. Llorach, Espin, Tomas-Barberan & Ferreres (2003) and Guo, Lee, Chiang, Lin & Chang (2001) found that cauliflower and broccoli by-products extracts showed significant antioxidant activity, so they could be used as sources of antioxidant products. By-products of other Brassica crops may also show different antioxidant activities as well as different growth stages. Samec et al. (2011) found that antioxidant activity of white cabbage and Chinese cabbage leaves reached its maximum in juvenile stages. These aspects of antioxidant activity of Brassica crops, including measurements of antioxidant potential on different stages of the plant and on by-products have not been conveniently studied yet and they may be promising subjects in the field of antioxidant activity of Brassica crops.

Phenolic compounds are known to be the major antioxidants of Brassica crops (Podsedeck, 2007). Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols (Pereira, Valentao, Pereira & Andrade, 2009). The most widespread and diverse group of polyphenols in Brassica species are flavonoids and hydroxycinnamic acids. Flavonoids can act as antioxidants by a number of potential pathways. The most important is likely to be by free radical scavenging, in which the polyphenol can break the free radical chain reaction. Another pathway of apparent antioxidant action of the flavonoids,

1 particularly in oxidation systems using such transition metal ions as copper or iron, is
2 chelation of the metal ions. The hydroxycinnamic acids may also be good antioxidants,
3 particularly those possessing the catechol-type structure such as caffeic acid (Croft,
4 1998). The chemical properties of polyphenols in terms of the availability of the
5 phenolic hydrogens as hydrogen-donating radical scavengers predict their antioxidant
6 activity (Rice-Evans, Miller & Paganga, 1996).

7 Several authors have found significant and high correlations between antioxidant
8 activity measured with electron-transfer based assays and total phenolic content,
9 employing the Folin-Ciocalteu method, in samples of white and red cabbages
10 (Kusznierewicz et al., 2010; Podsedek et al., 2006), cauliflower, savoy cabbage,
11 Brussels sprouts (Podsedek et al., 2006) and broccoli (Kaur, Kumar, Anil & Kapoor,
12 2007; Podsedek et al., 2006). However, the Folin-Ciocalteu method is an indirect
13 measurement of the total phenolic content. Following Huang, Ou & Prior (2005) this
14 method measures a sample's reducing capacity like other electron-transfer based assays
15 such as those based on FRAP (ferric reducing antioxidant power), ABTS (2,2'-azino-bis
16 (3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl),
17 although with differences based for example in their sensitivity to thiols (Blois, 1958).
18 A more reliable quantification of the total phenolic content and its relationship with
19 antioxidant activity of Brassica crops is needed.

20 The objectives of this work were to compare the antioxidant activity of several
21 Brassica vegetables at different plant stages, to evaluate the antioxidant activity of
22 Brassica crops by-products and to study the relationship among the antioxidant activity
23 and phenolic composition.

2. Material and Methods

2.1. Plant material

Fourteen Brassica varieties were studied: four cabbages (*Brassica oleracea* var. *capitata*), three kales (*B. oleracea* var. *acephala*), two tronchuda cabbages (*B. oleracea* var. *costata*), one broccoli (*B. oleracea* var. *italica*), one cauliflower (*B. oleracea* var. *botrytis*), and three nabicol (*Brassica napus* var. *pabularia*) (Table 1). Broccoli, cauliflower and one cabbage are commercial varieties, while the remaining are landraces kept at the Gene Bank placed at Misión Biológica de Galicia (MBG-CSIC).

Five sample types were collected and analyzed at four different plant stages. Varieties were grown in a controlled environmental chamber to analyze sprout samples seven days after sowing (S1: sprouts, stage 1). The same varieties were grown in the field to analyze several samples: young leaves taken two months after sowing (L2: leaves, stage 2), leaves taken three months after sowing (L3: leaves, stage 3), the consumed organs of each crop (CO4: consumed organs, stage 4) and finally, by-products (BP4: by-products, stage 4). Samples from S1, L2 and L3 were taken at the same time for all varieties, meaning 7, 60 and 90 days after sowing (Table 1). Samples of stage 4 (CO4 and BP4) were taken according the maturation stage of each variety (Table 1). The same plant part was analyzed in all varieties for samples taken at S1, L2 and L3. However, different plant parts were analyzed for samples taken at CO4 and BP4 since the consumed organs and its by-products differ among Brassica crops. For CO4 samples heads were harvested from broccoli, cauliflower and cabbage; inner leaves from tronchuda cabbage, leaves from kales, and tops from nabicol. Alongside the

harvest of consumed organs, a sample of by-products meaning outer leaves from broccoli, cauliflower, cabbage, tronchuda cabbage and nabicol were taken at time 4 (BP4) (Table 1). No by-products samples were taken from kale, since all leaves can be collected for human consumption.

Seeds of each variety were rinsed in Milli-Q water, immersed in 5gl⁻¹ sodium hypochlorite for 2 h and drained. Afterwards, they were soaked overnight in Milli-Q water. Seeds were dried and weighed and 1g of seeds was spread on a tray filled with vermiculite. Trays were transferred to a controlled environment chamber (MLR-351H, Sanyo) with 14h light and 10h dark cycle and temperature of 20 °C. The relative humidity was 60% at light cycle and 80% at dark cycle. Three S1 subsamples from each tray were taken and stored at -80 °C. Antioxidant activity was measured in the three subsamples and phenolic compound analysis in one.

Varieties were planted in multiplot trays, and approximately one month later, seedlings were transplanted into the field at the five- or six-leaf stage. Varieties were transplanted at the experimental station of MBG located at Pontevedra (42°20'N, 8°38'W) in April 29th, 2010 in a randomized design. This location has a humid climate with an annual rainfall of about 1600 mm and an acid sandy loam soil type. Each variety was transplanted in an experimental plot consisted in 4 rows with 25 plants per row. Rows were spaced 0.6 m apart, and plants within rows were spaced 0.5 m apart. Cultural operations, fertilization, and weed control were made according to local practices. Samples L2, L3, CO4 and BP4 were taken on each plot. Variety MBG-BRS0378 was lost in the field trial two months after transplant because of its high susceptibility to different pests and diseases, therefore no samples of consumed organs or by-products were taken. Three subsamples of ten different plants were taken from each plot. Antioxidant activity was measured in the three subsamples and phenolic

compound analysis in one. After its introduction on liquid nitrogen, the material was immediately transferred to the laboratory and frozen at -80 °C. All samples from laboratory and field experiments were lyophilized (BETA 2-8 LD plus, Christ) during 72 h. The dried material was powdered by using and IKA-A10 (IKA-Werke GmbH & Co.KG) mill, and the powder obtained was used for analysis.

2.2. Reagents and standards

DPPH (2,2'-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), chlorogenic acid, sinapic sodium acetate, hydrochloridic acid and trifluoroacetic acid were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), kaempferol 3-rutinoside was obtained from Extrasynthese (Genay, France); acetic acid, ferric chloride and methanol were obtained from Panreac quimica S.A. (Castellar del Valles, Spain).

2.3. Antioxidant activity analysis

For antioxidant activity analysis, extracts were prepared following Ferreres, Sousa, Valentao, Seabra, Pereira & Andrade (2007). 20 mg of powder material was suspended in 2 ml of Milli-Q water then boiled for 1h at 100 °C. Suspension was filtered and supernatant lyophilized. The dry material was weighed and dissolved in Milli-Q water to reach a concentration of 10 mgml⁻¹.

Antioxidant activity of the samples was determined by monitoring the disappearance of radical DPPH spectrophotometrically according to Brand-Williams, Cuveleir & Berset (1995). The working DPPH reagent was prepared by solving DPPH in methanol to a final concentration 75 μM . For each extract, a dilution series of 0.00, 0.06, 0.12, 0.18, 0.24 and 0.30 mgml^{-1} was prepared in a 96-well plate. 50 μl of extract were added to 250 μl of freshly prepared DPPH reagent and mixed thoroughly. Readings were taken at 517 nm after 30 min of incubation at room temperature in a plate spectrum (Spectra MR, DYNEX Technologies). Three replications were analyzed for each concentration. After extracting the value of the blank to each sample, percentage of inhibition was plotted against concentration of samples. Standard prepared with different concentration of Trolox® (0, 0.008, 0.016, 0.024, 0.032, 0.04 mM) was also measured. EC50 values (concentrations which produced 50% inhibition) were computed for each extract and normalized to Trolox® equivalents per gram of dry weight

Antioxidant activity of the samples was also measured by ferric reducing antioxidant power (FRAP) assay of Benzie & Strain (1996). The working FRAP reagent was prepared by mixing 10 vol of 300 mM acetate buffer, pH 3.6, 1 vol of 10 mM TPTZ in 40 mM hydrochloric acid and 1 vol of 20 mM ferric chloride and then heated at 37 °C . For each extract, a dilution series of 0.00, 0.06, 0.12 and 0.18 mgml^{-1} was prepared in a 96-well plate. 50 μl of extract were added to 250 μl of freshly prepared FRAP reagent and mixed thoroughly. Readings were taken at 593 nm after 20 min in a plate spectrum (Spectra MR, DYNEX Technologies). Three replications were analyzed for each concentration. After extracting the value of the blank to each sample, absorbance was plotted against concentration. Standard prepared with different concentration of Trolox® (0, 0.008, 0.016, 0.024, 0.032, 0.04 mM) was also measured.

Concentrations where absorbance reached a value of 1.00 were computed for each extract and normalized to Trolox[®] equivalents per gram of dry weight

2.4. Phenolic compounds analysis

Samples were prepared and phenolic compounds extracted as it was described by Velasco, Francisco, Moreno, Ferreres, Garcia-Viguera & Cartea (2011). Chromatographic analyses were carried out on a Luna C18 column (250 mm × 4.6 mm, 5 µm particle size; Phenomenex, Macclesfield, UK). The mobile phase was a mixture of (A) ultrapure water/trifluoroacetic acid (TFA) (99.9:0.1) and (B) methanol/TFA (99.9:0.1). The flow rate was 1 ml min⁻¹ in a linear gradient starting with 0% B after 55-65 min. The injection volume was 20 µl, and chromatograms were recorded at 330 nm in a model 600 HPLC instrument (Waters) equipped with a model 486 UV turnable absorbance detector (Waters). Phenolic compounds were identified by comparing our HPLC-DAD chromatograms with the ones obtained by Velasco et al. (2011) in *B. oleracea* and *B. napus* cros. In this publication a detailed explanation on the methods employed to identify phenolic compounds is given, including the use of mass and UV chromatograms. Caffeoylquinic and *p*-coumaroylquinic acid derivatives were quantified as chlorogenic acid (5-caffeoylquinic acid), flavonoids as kaempferol 3-rutinoside and sinapic acid derivatives as sinapic acid.

2.5. Statistical analysis

1 Analysis of variance were performed for DPPH and FRAP by PROC GLM of SAS v
2 9.2 (SAS Institute, 2008). Crops, varieties and sample types were considered fixed
3 effects, whereas replications were considered as a random factor. Comparisons of
4 means were performed for each trait by using Fisher's protected least significant
5 difference at the 0.05 level of probability. Simple correlations coefficients were
6 computed among DDPH, FRAP and total phenolic content with PROC CORR of SAS v
7 9.2 (SAS Institute, 2008). To determine the relationship among antioxidant activity of
8 Brassica crops and individual phenolic compounds, multiple regression analysis was
9 carried out with PROC REG of SAS v 9.2 (SAS Institute, 2008) employing a stepwise
10 procedure, allowing a variable to stay in the model for $p \leq 0.10$.

3. Results and Discussion

3.1. Antioxidant activity analysis

3.1.1. Comparisons among crops

For this analysis, samples S1, L2, L3 and CO4 (Table 1) were taken into account. The source of variation due to crops, stages and their interaction was significant for DPPH and FRAP assays ($P < 0.01$, Table 2). Individual analysis by stage was carried out for each type of assay and significant differences were found among crops at each plant stage (Table 2). Different crops stand out over the rest, depending on the plant stage, (Table 3). Cauliflower sprouts had significantly higher antioxidant activity than the other crops for DPPH ($3.23 \mu\text{mol Trolox g}^{-1} \text{ dw}$) and FRAP assays ($6.75 \mu\text{mol Trolox g}^{-1} \text{ dw}$), followed by broccoli sprouts, which significantly differ from the rest of crops for FRAP assay but not for DPPH (Table 3). Cauliflower still performed significantly better than the rest of the crops for DPPH assay ($4.25 \mu\text{mol Trolox g}^{-1} \text{ dw}$) when L2 were measured, and it did not differ significantly from broccoli and naborcol for FRAP assay ($5.74 \mu\text{mol Trolox g}^{-1} \text{ dw}$). Thus, cauliflower could be recommended for sprouts and young plants production because of its high antioxidant activity at these stages. However, results should be taken cautiously because only one variety of cauliflower was included in the experiments.

Kale showed the highest antioxidant activity when L3 samples were measured (6.40 and $8.44 \mu\text{mol Trolox g}^{-1} \text{ dw}$ for DPPH and FRAP assays, respectively), although

it did not differ significantly from tronchuda cabbage and cabbage for DPPH assay and it did not differ significantly from broccoli, cauliflower, cabbage and tronchuda cabbage for FRAP assay. Antioxidant activity of CO4 samples (meaning heads of broccoli, cabbage and cauliflower, inner leaves of tronchuda cabbage and tender leaves of kale) was significantly higher for kale ($4.71 \mu\text{mol Trolox g}^{-1} \text{ dw}$) compared to the rest of crops for DPPH assay, followed by broccoli and tronchuda cabbage. For FRAP assay, kale also showed the highest antioxidant activity ($8.91 \mu\text{mol Trolox g}^{-1} \text{ dw}$) and it did not differ significantly from broccoli (Table 3).

Comparisons among the antioxidant activity of Brassica crops are normally being carried on only in the edible parts used for human consumption, i.e., in the consumed organs of each crop. In this work, for CO4 samples, kale varieties showed a higher antioxidant activity than the rest of crops for both antioxidant assay systems (Table 3). Different works have demonstrated the higher antioxidant activity of kale compared to the most commonly consumed vegetables including garlic, beets, pepper, onion, lettuce, celery, cucumber, spinach, carrot, potato, tomato, rhubarb or green bean and other Brassica vegetables as Brussels sprouts, cauliflower, broccoli or cabbage (Cao et al., 1996; Kequan et al., 2006). Antioxidant activity of curly kale was at least 10-fold higher than that of cauliflower and white cabbage in the study carried out by Nilsson et al. (2006) and kale showed by large the highest antioxidant activity compared to broccoli, Brussels sprouts, and green and white cauliflower. Therefore, kale is a very interesting crop to be grown due to its high antioxidant activity, higher than broccoli, which is the Brassica crop which has been the most deeply studied for its antioxidant activity and its phenolic composition (Podsedeck et al., 2006). In our work, antioxidant activity of broccoli was also high, followed by that of tronchuda cabbage and nabicol, while antioxidant activity of cabbage and cauliflower were the lowest. This result is in

agreement to other authors who found that antioxidant activity of heads of white cabbage and cauliflower was low (Jagdish et al., 2009; Podsedek et al., 2006; Sikora et al., 2008).

As a conclusion, different crops can be recommended for their higher antioxidant activity depending on plant stage. Cauliflower showed high antioxidant activities when sprouts and two-month leaves samples were considered, even though these plant stages are not usually used for human consumption, afterwards it decreases gradually and it is overcome by other crops, including kale varieties, whose leaves showed the highest antioxidant activity compared to other brassica crops evaluated three months after sowing and when consumed organs samples are measured (Table 3). Kale varieties evaluated in this work are landraces grown in the NW Spain in a traditional way. They have shown a large genetic variance for nutritional quality (Cartea, Velasco, Obregon, Padilla & de Haro, 2008), which could allow us to even increase the antioxidant activity by selecting those outstanding genotypes.

3.1.2. Variation of antioxidant activity with plant stage

Antioxidant activity of young leaves, taken three months after sowing was significantly higher than the rest of plant stages for DPPH and FRAP assays (Table 3). Generally speaking, there is an increase of antioxidant activity of Brassica crops on time, starting when samples are sprouts, until it reaches its maximum three months after sowing, after that, antioxidant activity decreases again, which means that crops reach their maximum antioxidant activity when leaves are young. This result is in agreement to Samec et al. (2011) who measured the antioxidant activity of white cabbage and Chinese cabbage

leaves periodically between transplanting until full maturation (head harvesting) and observed that there was an increase in the antioxidant activity in the first 8 to 12 weeks and afterwards there was a gradual decreasing trend, probably as a consequence of a more active plant metabolism which accompanies active/rapid growth in the first few months. Also, Kim, Padilla-Zakour & Griffiths (2004) found that three-weeks cabbages possessed relatively higher amounts of flavonols compared with mature cabbage, suggesting that maturity may affect the phenolic composition in cabbage and therefore their antioxidant activity. In this study we have demonstrated that there is a period between germination and maturation, in which the antioxidant activity of Brassica crops is maximum, independently of the crop under study. This period should be taken into account if Brassica crops with high antioxidant properties are meant to be produced.

3.1.3. Antioxidant activity of Brassica by-products

In this analysis, CO4 samples and BP4 samples (meaning outer leaves for broccoli, cabbage, cauliflower and tronchuda cabbage nabicol) were compared for each crop (Figure 1). Non by-products samples were taken from kale, since all the leaves are normally consumed. For all crops tested, unless cauliflower, consumed organs showed higher DPPH values than by-products although differences were only significant for broccoli (Figure 1). Differences between consumed organs and by-products were more evident when antioxidant activity was measured by a FRAP assay (Figure 2). In this case, significant differences between consumed organs and by-products were found for broccoli, nabicol and tronchuda cabbage. In the case of cauliflower, by-products had higher antioxidant activity than consumed organs, probably due to the lack of pigments

of cauliflower heads. Similarly Llorach et al. (2003) found that cauliflower by-products extracts showed significant antioxidant activity. They also found that the flavonoid concentration in cauliflower by-products was much higher than that found in the edible parts where only trace amounts were detected.

In the last years, some authors have studied the antioxidant activity of several Brassica crops by-products. Ferreres et al. (2006) measured the antioxidant activity of internal and external leaves of tronchuda cabbage and they found that both have high antioxidant activity, but in general terms, internal leaves exhibited lower antioxidant activity than external leaves, which is in disagreement with our results. Discrepancies can be explained by different varieties under study in both works. Guo et al. (2001) studied antioxidant activity of three different parts of broccoli: stems, leaves and heads . They found that antioxidant activity of leaves and stems was of the same magnitude of that showed by heads, so they could also be used to develop a new type of product and therefore to enhance the utilization of broccoli.

In general, by-products from handling and commercialization of vegetables have been traditionally valorized as animal feedstuff, fiber production and fuel production, however in the last years, a number of studies have proposed some vegetable by-products as new sources of natural antioxidants in order to valorize these wastes (reviewed by Llorach et al., 2003). High antioxidant activity was found in the by-products analyzed. These results supported by other previous works show that Brassica by-products could be used as sources to obtain derived products with high content of antioxidants, interesting from both the industrial point of view and as ingredients to functionalize foodstuffs.

3.2. Phenolic compounds analysis

3.2.1. Phenolic compounds composition

Thirty one peaks corresponding to different phenolic compounds were detected in the Brassica crops analyzed. Seventeen phenolic compounds were identified by comparing HPLC-DAD chromatograms of Brassica vegetables extracts with the ones obtained by Velasco et al. (2011) in extracts of two *B. oleracea* crops (kale and cabbage) and in one *B. napus* crop (nabicol). Among flavonoids, derivatives of kaempferol, quercetin and isorhamnetin were identified. Seven kaempferol derivatives were identified: kaempferol-3,7-di-*O*-glucoside, kaempferol-3-*O*-sophoroside-7-*O*-glucoside, kaempferol-3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside, kaempferol-3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol-3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside, kaempferol-3-*O*-(feruloyl)-sophoroside-7-*O*-glucoside and kaempferol-3-*O*-(*p*-coumaroyl)-sophoroside-7-*O*-glucoside. Two quercetin derivatives (quercetin-3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside and quercetin-3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside) and one isorhamnetin derivative (isorhamnetin-3,7-di-*O*-glucoside) were identified. Among hydroxycinnamic acids, sinapic acid and several derivatives (1,2-disinapoylgentiobioside, 1-sinapoyl-2-feruloylgentiobioside, 1,2,2'-trisinapoylgentiobioside and 1,2'-disinapoyl-2-feruloylgentiobioside), 3-caffeoyl quinic acid and 3-*p*-coumaroyl quinic acid were detected. The seventeen compounds were present in all crops analyzed. Contrarily, Velasco et al. (2011) found that isorhamnetin was only present in the *B. napus* crop. Three peaks, corresponding to hydroxycinnamic

acids, nine peaks corresponding to acylated derivatives of kaempferol and two peaks corresponding to derivatives of kaempferol and quercetin could not be identified.

Respecting the phenolic profile of each sample type and crop, sprouts samples (S1) were composed mostly by sinapic acid derivatives and a small portion of the total amount of phenolic compounds was due to other hydroxycinnamic acids and kaempferol derivatives (Figure 2). Broccoli showed the highest amount of total phenolic compounds at this stage ($43.7 \mu\text{mol g}^{-1} \text{ dw}$). Two-month leaves (L2), three-month leaves (L3), consumed organs (CO4) and by-products (BP4) samples were composed mostly by kaempferol and sinapic acid derivatives, also quercetin derivatives supposed a significant portion of the total amount of phenolic compounds. Tronchuda cabbage outstand over the rest of crops for its high content in phenolic compounds in L2, L3 and CO4 samples (36.3 , 43.9 and $43.5 \mu\text{mol g}^{-1} \text{ dw}$). Kale also showed a high content of phenolic compounds in CO4 ($38.7 \mu\text{mol g}^{-1} \text{ dw}$) and cauliflower showed a very low content at this plant stage ($15.0 \mu\text{mol g}^{-1} \text{ dw}$).

The total phenolic content computed as an average of Brassica varieties studied was quite constant over plant stages (Figure 3). However, the content in hydroxycinnamic acids and flavonoids varied significantly with plant stage. In S1 stage, plants show a maximum peak of hydroxycinnamic acids content, afterwards it decreases severely in L2 and experiments a gradual increase in L3 and CO4 samples. The evolution of the concentration of flavonoids on time is a mirror image of the graphic of hydroxycinnamic acids. At CO4 stage, the content of hydroxycinnamic acids and flavonoids is almost equal and their ratio is close to 1 (Figure 3). Profile and content of phenolic compounds between different stages have been compared before. Hydroxycinnamic acids were the main phenolics in seeds of tronchuda cabbage,

contrarily to what happens in the leaves where the main phenolics are flavonoids (Ferrerres et al., 2007; Ferreres et al., 2006).

The biosynthesis of phenolic compounds involves a complex network of routes based principally on the shikimate, phenylpropanoid and flavonoid pathways. Different branches of the network generate various classes of phenolic compounds, with essential functions in plant development and environmental interactions, such as lignin for structural support, flavones and flavonols for UV protection, anthocyanins, chalcones and aurones as pigments for the attraction of pollinators and seed distributors and isoflavonoids and furanocoumarins as phytoalexins for pathogen defense (Hamberger & Hahlbrock, 2004). How these metabolite networks are organized and differentially controlled is not yet fully understood (Costa, Collins, Anterola, Cochrane, Davin & Lewis, 2003), although it is known that key enzymes in the network are regulated by different stimuli, such as light, pathogen attack, several abiotic stresses, metabolites and plant growth regulators. Hydroxycinnamic acids are precursors of lignin biosynthesis, important in the first plant stages to rigidify cell walls and rendering them impermeable to water. This could explain why sprouts have the higher concentration of hydroxycinnamic acids. When plants are two-months old, the phenolic compounds network is switched to produce flavonoids, probably because plants are more active photosynthetically and they need protection from the UV light.

3.2.2. Relationship between antioxidant activity and phenolic composition

Correlations between antioxidant activity measured with DPPH and FRAP assays were highly significant ($p \leq 0.01$) for all the sample types measured. Correlation coefficients

in CO4 and S1 (0.884) were the highest, followed by BP4 (0.850), L3 (0.689) and L2 (0.650). Correlation coefficients between antioxidant activity measured with DPPH assay with total phenolic content were significant for L2 (0.617), CO4 (0.761) and BP4 (0.837), while correlations between antioxidant activity measured with FRAP assay with total phenolic content were significant for L2 (0.480) and CO4 (0.771) (Table 4), meaning that for S1 and L3 there is a no clear relationship among total phenolic content and antioxidant activity among different Brassica crops and that the tightest relationship was found in CO4 samples.

Several authors have found significant and high correlations (ranging from 0.7 to 1) between antioxidant activity measured with DPPH and FRAP assays and total phenolic content measured with Folin-Ciocalteu method in hydrophilic samples of heads of white and red cabbages (Kusznierewicz et al., 2010; Podsdek et al., 2006), cauliflower, savoy cabbage and Brussels sprouts (Podsdek et al., 2006) and broccoli (Kaur et al., 2007; Podsdek et al., 2006). However, other authors have found no correlation between antioxidant activity of Brassica hydrophilic extracts and total phenolic content of broccoli (Cogo et al., 2011; Gawlik-Dziki, 2008).. In our work, total phenolic content was assessed by HPLC analysis and therefore, correlations with antioxidant activity should be more reliable.

Flavonoid content was further divided into two different classes: flavonoids and hydroxycinnamic acids. Antioxidant activity of S1 was significantly correlated to hydroxycinnamic acids content for FRAP assay but no for DPPH assay (Table 4). Antioxidant activity of L2 was significantly correlated to flavonoid content for both assays. Antioxidant activity of L3 leaves showed no significant correlation with any class of phenolic compounds, while antioxidant activity of CO4 was significantly correlated to content on both classes for DPPH and FRAP assays. BP4 antioxidant

activity was significantly correlated to flavonoids and hydroxycinnamic acid content for DPPH assay but not for FRAP assay (Table 4). CO4 samples showed the strongest relationship with total phenolic content but also with flavonoids and hydroxycinnamic acid content, meaning that at this plant stage, antioxidant activity is mainly determined by phenolic compounds.

To determine which individual phenolic components are responsible for antioxidant properties of Brassica extracts, a multiple regression analysis was employed, on which single phenolic compound contents were independent variables and antioxidant activity measured as DPPH and FRAP assays were dependent variables. The coefficient of determination (R^2) is the proportion of variability in a data set that is accounted by the statistical model and provides a measure of how well future outcomes are likely to be predicted by the model. Model R^2 showed different values depending on the sample type under consideration, being close to 1 in L2, CO4 and BP4 for DPPH assay. For the same antioxidant assay, model R^2 was 0.88 for L3 and 0.28 for S1 (Table 5). For FRAP assay, model R^2 reached a value of 1.00 in BP4, 0.76 in L2, 0.88 in CO4 and 0.56 in S1 (Table 5). No one of the independent variables reached the significance threshold to be considered in the model in L3 for FRAP assay. Clearly, most part of variation among crops for antioxidant activity in L2, CO4 and BP4 can be explained by using a model where individual phenolic compounds are taken into account. Mrkic et al. (2006) found that the total antioxidant activity of broccoli extracts was better correlated with the polyphenol content than with the concentration of a single compound or a group of compounds. Discrepancy may be explained if we take into account that Mrkic et al. (2006) studied antioxidant activity of different broccoli cultivars while in this work, six different crops, one of them a *B. napus* crop, with different phenolic profile

were studied and therefore, our model shows much more variability in the content of individual phenolic compounds.

In several cases, a particular phenolic compound explains a considerable amount of variability, giving a R^2 close to 0.50. These are the cases of kaempferol-3-*O*-(feruloyl)-sophoroside-7-*O*-glucoside in two-month leaves samples for DPPH assay, a conjugated form of kaempferol with an undetermined hydroxycinnamic acid in three-month leaves samples for DPPH assay, derivatives of sinapic acid named 1-sinapoyl-2-feruloylgentiobioside in consumed organs samples for DPPH and FRAP assays, 1,2'-disinapoyl-2-feruloylgentiobioside in by-products for FRAP assay and 3-caffeoyl quinic acid in by-products for DPPH assay (Table 5). Therefore, a high part of the variability for antioxidant activity among the six Brassica crops studied may be explained by the variability of several individual phenolic compounds, including flavonoids and hydroxycinnamic acids (Table 5). In this work, differences in antioxidant activity among consumed organs of Brassica crops are mostly due to a derivative from sinapic acid. The crops which showed the higher antioxidant activity for both types of assays also had a high content on 1-sinapoyl-2-feruloylgentiobioside ($R^2=0.472$ and 0.62 for DPPH and FRAP assays, respectively).

As conclusion, differences in antioxidant activity of Brassica crops are related to differences in total phenolic content and composition. These relationships are not so clear in sprouts and three-month leaves samples, therefore other compounds different from phenolics are also important in determining their variability for antioxidant activity. Although this model explains a great part of the variability for antioxidant activity, it is a linear model which does not include the interactions among phenolic compounds, meaning synergistic and antagonistic effects. The study of the interactions among phenolic compounds should clarify the relationship with antioxidant activity.

1 Novel aspects of antioxidant activity in Brassica crops and its relationship to
2 individual phenolic compounds have been presented. Antioxidant activity varies with
3 plant stage, and it reaches a peak when plants are young, meaning three months after
4 sowing. Kale crops showed the highest antioxidant activity at this developmental stage
5 and when the plants are adult. Brassica by-products were found to be an important
6 source of antioxidant compounds. Phenolic compounds composition varied depending
7 on the crop and plant stage and antioxidant activity is related to total phenolic content
8 and to phenolic composition.

9

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2

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8

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1 **Figure captions**

2

3 **Figure 1.** Comparison of antioxidant activity between consumed organs and by-
4 products for six Brassica crops analyzed by two different assays

5 **Figure 2.** Phenolic compound profile of six Brassica crops quantified in five different
6 sample types

7 **Figure 34.** Variation of total phenolic, flavonoid and hydroxycinnamic acids content,
8 computed as the average of six Brassica crops, with plant stage

9

Table 1. Crop, origin and description of varieties studied, type of samples analyzed and number of days from sowing to collecting

Crop	Specie	Variety name	Description and origin ^a	Samples analyzed				
				Sprouts (S1)	Two-months leaves (L2)	Three-months leaves (L3)	Consumed organs (CO4)	By-products (BP4)
Broccoli	<i>B. oleracea</i>	Brocoletto	commercial (Rainbow hortícolas)	7 d	60 d	90 d	105 d	105 d
Cabbage	<i>B. oleracea</i>	Corazón de buey	commercial (Rainbow hortícolas)	7 d	60 d	90 d	105 d	105 d
		MBG-BRS0072	landrace (MBG)	7 d	60 d	90 d	114 d	114 d
		MBG-BRS0425	landrace (MBG)	7 d	60 d	90 d	114 d	114 d
		MBG-BRS0452	landrace (MBG)	7 d	60 d	90 d	114 d	114 d
Cauliflower	<i>B. oleracea</i>	Bola de nieve	commercial	7 d	60 d	90 d	105 d	105 d
Kale	<i>B. oleracea</i>	MBG-BRS0062	landrace (MBG)	7 d	60 d	90 d	135 d	^c
		MBG-BRS0106	landrace (MBG)	7 d	60 d	90 d	135 d	^c

		MBG-BRS0281	landrace (MBG)	7 d	60 d	90 d	135 d	^c
Tronchuda cabbage	<i>B. oleracea</i>	MBG-BRS0121	landrace (MBG)	7 d	60 d	90 d	114 d	114 d
		MBG-BRS0226	landrace (MBG)	7 d	60 d	90 d	114 d	114 d
		MBG-BRS0063	landrace (MBG)	7 d	60 d	90 d	105 d	105 d
Nabicol	<i>B. napus</i>	MBG-BRS0113	landrace (MBG)	7 d	60 d	90 d	105 d	105 d
		MBG-BRS0378	landrace (MBG)	7 d	60 d	90 d	^b	^b

^a MBG: Germplasm Bank at the Misión Biológica de Galicia, Pontevedra, Spain.

^b Variety MBG-BRS0378 was lost three months after sowing, thus no samples of consumed organs and by-products could be collected ^c No by-products samples were taken for kale varieties

Table 2. Combined and individual analysis of variance for the antioxidant activity of six Brassica crops across four plant stages measured with two different antioxidant assay systems (DPPH and FRAP)

Combined analysis of variance							
DPPH assay					FRAP assay		
Sources of variation	DF	Mean square	F value	Pr > F	Mean square	F value	Pr > F
Crop	5	6.92	6.94	<0.01	12.16	7.74	<0.01
Replication	2	0.34	0.34	0.72	0.06	0.04	0.96
Plant stage	3	31.89	31.98	<0.01	37.67	23.97	<0.01
Crop*plant stage	15	5.22	5.24	<0.01	10.81	6.88	<0.01
Error	136	1.00			1.57		
Individual analysis of variance							
DPPH assay					FRAP assay		
Sources of variation	DF	Mean square	F value	Pr > F	Mean square	F value	Pr > F
<i>Sprouts (S1)</i>							

Crop	5	1.09	8.64	<0.01	4.10	9.90	<0.01
Replication	2	0.04	0.35	0.71	0.48	1.16	0.33
Error	34	0.123			0.41		
<i>Two-months leaves (L2)</i>							
Crop	5	1.08	3.50	0.01	3.03	3.48	0.01
Replication	2	0.33	1.08	0.35	0.01	0.01	0.99
Error	33	0.31			0.87		
<i>Three months-leaves (L3)</i>							
Crop	5	10.28	3.47	0.01	9.59	2.95	0.03
Replication	2	0.59	0.20	0.82	0.56	0.17	0.84
Error	33	2.99			3.26		
<i>Consumed organs (CO4)</i>							
Crop	5	9.94	14.24	<0.01	27.96	13.50	<0.01
Replication	2	0.07	0.10	0.91	0.55	0.26	0.77
Error	30	0.70			2.09		

Table 3. Antioxidant activity of six Brassica crops measured in four plant stages with two different antioxidant assay systems (DPPH and FRAP)

Crop	DPPH assay							
	N	Sprouts (S1)	N	Two-month leaves (L2)	N	Three-months leaves (L3)	N	Consumed organs (CO4) ^b
Broccoli	3	2.23b	3	3.27b	3	3.72bc	3	3.43b
Cabbage	12	1.89bc	12	3.12b	12	4.54abc	11	1.82d
Cauliflower	3	3.23a	3	4.25a	3	3.16c	3	1.81d
Kale	9	1.91bc	9	2.86b	9	6.40a	9	4.71a
Tronchuda cabbage	6	1.70c	6	2.76b	6	5.87ab	6	3.07bc
Nabicol	9	2.03bc	8	2.95b	8	3.70bc	6	2.24cd
LSD ^c		0.44		0.7		2.18		1.15
Total ^d	42	2.17c	41	3.20b	41	4.57a	38	2.85b
Crop	FRAP assay							
	N	Sprouts (S1)	N	Two-month leaves (L2)	N	Three-months leaves (L3)	N	Consumed organs (CO4) ^b
Broccoli	3	5.48b	3	5.16ab	3	7.53a	3	7.40ab
Cabbage	12	4.31c	12	4.30bc	12	6.77ab	11	4.11c
Cauliflower	3	6.75a	3	5.74a	3	7.03ab	3	3.58c
Kale	9	4.57c	9	3.59c	9	8.44a	9	8.91a
Tronchuda cabbage	6	4.08c	6	3.81c	6	6.46ab	6	6.46b
Nabicol	9	4.16c	8	4.61abc	8	5.19b	6	6.71b
LSD ^c		0.81		1.18		2.28		2
Total ^d	42	4.89c	41	4.54c	41	6.90a	38	6.20b

^a Values followed by the same character within the same column do not differ significantly at $P \leq 0.05$

^b Consumed organs: heads of broccoli, cabbage and cauliflower, inner leaves of tronchuda cabbage and leaves from kales

^c Least significant difference

^d LSD for comparisons among plant stages= 0.44 and 0.56 for DPPH and FRAP assays, respectively at $P \leq 0.05$

Table 4. Correlation coefficients between antioxidant activity measured with DPPH and FRAP assays and phenolic, flavonoid and hydroxycinnamic acid content

Antioxidant activity	N	Phenolics	Flavonoids	Hydroxycinnamic acids
<i>DPPH assay</i>				
Sprouts (S1)	14	0.27	-0.17	0.31
Two-month leaves (L2)	14	0.62*	0.67**	0.47
Three-month leaves (L3)	14	0.41	0.48	0.30
Consumed organs (CO4)	13	0.76**	0.77**	0.63*
By-products (BP4)	10	0.84**	0.76**	0.82**
<i>FRAP assay</i>				
Sprouts (S1)	14	0.51	-0.05	0.54*
Two-month leaves (L2)	14	0.48	0.56*	0.31
Three-month leaves (L3)	14	0.24	0.23	0.22
Consumed organs (CO4)	13	0.77**	0.74**	0.67**
By-products (BP4)	10	0.47	0.37	0.58

** , *: significant at $P \leq 0.01$ and $P \leq 0.05$, respectively

Table 5. Partial and model coefficient of determination (R^2), and partial regression coefficients (b) for the multiple regression analysis carried on with antioxidant activity assays (DPPH and FRAP) and individual phenolic compounds content for each sample type

DPPH assay				FRAP assay			
Phenolic ^a compounds	Partial R^2	Model R^2	Partial b	Phenolic ^a compounds	Partial R^2	Model R^2	Partial b
<i>Sprouts (S1)</i>							
AC-1	0.28	0.28	0.41	AC-1	0.43	0.43	0.91
				SA-1	0.13	0.56	0.19
<i>Two-month leaves (L2)</i>							
K-1	0.55	0.55	0.48**	K-1	0.30	0.30	0.85*
Q-1	0.17	0.73	1.13**	SA-1	0.20	0.51	-4.51*
KAc-1	0.15	0.87	-0.37*	Q-1	0.17	0.67	1.16*
K-2	0.06	0.94	0.29**	K-2	0.09	0.76	0.59
AC-2	0.02	0.96	1.67**				

K-3	0.03	0.98	-0.40**
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SA-1	0.01	0.99	0.84*
------	------	------	-------

Three-months leaves (L3)

KAc-2	0.46	0.46	1.53**
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AC-3	0.23	0.69	-37.59**
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KAc-3	0.12	0.81	25.71*
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KAc-4	0.07	0.88	-2.88*
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Consumed organs (CO4)

SA-2	0.47	0.47	0.44**	SA-2	0.62	0.62	1.06**
------	------	------	--------	------	------	------	--------

KAc-5	0.25	0.72	4.00**	K-3	0.21	0.83	1.05**
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KAc-6	0.16	0.88	4.83**	K-5	0.05	0.88	1
-------	------	------	--------	-----	------	------	---

AC-4	0.07	0.95	5.01**
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AC-3	0.04	0.99	2.88**
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SA	0.01	1	-0.19**
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By-products (BP4)

3CQA	0.52	0.52	0.75**	SA-3	0.58	0.58	2.66**
SA-4	0.26	0.78	0.28**	K-1	0.23	0.81	0.99**
SA-3	0.16	0.93	0.91**	K-3	0.13	0.94	-0.44**
				I-1	0.05	0.99	-7.93**
				KAc-6	0.01	1	-1.95**

^a**Hydroxycinnamic acids**- AC-1, AC-2, AC-3, AC-4: undetermined hydroxycinnamic acids; 3CQA: 3-Caffeoyl quinic acid; SA: sinapic acid; SA-1: 1,2,2'-trisinapoylgentiobioside; SA-2: 1-sinapoyl-2-feruloylgentiobioside; SA-3: 1,2'-disinapoyl-2-feruloylgentiobioside; SA-4: undetermined derivative of sinapic acid

Flavonoids- I-1: Isohamnetin-3,7-di-*O*-glc; K-1: Kaempf-3-*O*-(feruloyl)-soph-7-*O*-glc; K-2- Kaempf-3-*O*-(caffeoyl)-soph-7-*O*-glc; K-3: Kaempf-3-*O*-(*p*-coumaroyl)-soph-7-*O*-glc; K-4: undetermined derivative of kaempferol; KAc-1, KAc-2, KAc-3, KAc-4, KAc-5, KAc-6: kaempferol conjugated with undetermined hydroxycinnamic acids; Q-1: undetermined derivative of quercetin