Partie 1 : Optimisation du matériel végétal et de l'extraction

1. Introduction

De nombreuses études effectuées partout dans le monde ont révélé que l'alimentation était la cause de nombreux problèmes de santé des populations occidentales. Il faut relever ce défi du "manger autrement", de l'alimentation santé, de la "functional food" pour mieux vivre, plus en forme et plus longtemps, ceci sans bouleverser des habitudes le plus souvent solidement ancrées, ni renoncer aux plaisirs de la table.

Depuis la nuit des temps, les hommes apprécient les vertus des plantes pour vaincre la souffrance et améliorer leur santé. Certaines plantes ou organes comme les baies sont riches en composés phénoliques (Shahidi and Naczk, 2004 ; Zadernowski *et al.,* 2005) tels que des flavonoïdes (Häkkinen *et al.*, 1999). Ces composés peuvent avoir des effets sur la prévention de certaines pathologies (Cao *et al.*, 1998 ; Landbo *et al.*, 2001 ; Mazza *et al.*, 2002), effets dus notamment à leurs propriétés antioxydantes (Wang *et al.*, 1996 ; Skrede and Wrolstad, 2002). Les myrtilles (Prior *et al.,* 1998) et le cassis (Moyer *et al.*, 2002 ; Ehala *et al.*, 2005) sont les baies qui possèdent les plus hauts taux en anthocyanes, ainsi qu'une capacité antioxydante très élevée. La myricétine, la quercétine, et le kaempférol sont les principaux flavonols, un autre groupe de flavonoïdes très important. Les teneurs en ces composés dans les baies sont fonction de différents facteurs comme le degré de maturation (Mikkonen *et al.*, 2001 ; Raffo *et al.*, 2004), les différences génétiques (cultivar), les conditions environnementales avant la récolte, le stockage après la récolte, les traitements pour la conservation (Shahidi and Naczk, 2004 ; Heiberg *et al.*, 1992 ; Wang and Lin, 2000) ainsi que la saison (Howard *et al.*, 2003).

L'industrie agro-alimentaire doit actuellement faire face à une réduction des marges bénéficiaires sur les produits traditionnels. Elle cherche donc à ajouter de la plus-value à certains de ses produits et le développement des produits "santé" est une alternative très intéressante, à une époque où la relation alimentation-santé est reconnue et où nous souffrons de plus en plus de pathologies liées à l'alimentation. Un des principaux défis de l'industrie alimentaire (fruits et légumes) est de valoriser les propriétés de ses produits. Grâce à leurs propriétés de prévention de certaines pathologies, la richesse en composés phénoliques des matières premières ou des produits transformés pourrait constituer un bon argument de vente.
Les baies domestiques et sauvages sont consommées en abondance et de nombreuses études ont été réalisées pour évaluer leur contenu en composés phénoliques et leur pouvoir antioxydant. Actuellement, peu d'études ont été faites sur les propriétés antioxydantes d'autres explants tels que les bourgeons et les feuilles. Declume (1989) et Chrubasik (2000), de leur coté, ont montré que les feuilles de cassis possédaient une activité anti-inflammatoire. Cependant, aucune étude n'a comparé la capacité antioxydante des divers organes du cassis.

Dans cette partie de notre travail, nous avons voulu comparer les capacités antioxydantes de différentes parties de divers cultivars de cassis dans le but de préparer un extrait présentant une haute capacité antioxydante. Pour ce faire, nous avons testé différents paramètres comme le cultivar, le type d'explant prélevé et son stade de développement: « **Antioxidant capacity of black currant varies with organ, season and cultivar.** » (Journal of Agricultural and Food Chemistry **54**, 6271-6276, 2006).

Il est clair que, excepté les baies, d'autres explants ne peuvent être consommés tels quels mais doivent être conditionnés comme complément alimentaire ou être ajoutés à des préparations. Il faut noter que les feuilles et les bourgeons du cassis sont déjà utilisés dans la préparation de compléments alimentaires.

La méthode d'extraction doit permettre l'extraction la plus complète possible des composés d'intérêt et doit éviter leur modification chimique (Zuo *et al.*, 2002). De nombreuses solutions d'extraction sont proposées dans la littérature pour extraire les polyphénols de la matrice végétale (Chavan *et al.*, 2001). Le rendement dépend grandement de la solution extractive mais aussi de la méthode utilisée (Goli *et al.*, 2005) et de la matrice végétale. L'eau, des

mélanges aqueux d'éthanol, de méthanol et d'acétone sont généralement utilisés (Sun and Ho, 2005).

Dans cette partie du travail, nous avons tenté d'optimiser l'extraction des composés phénoliques totaux afin d'obtenir un extrait dont la capacité antioxydante est la plus élevée possible et cela dans des conditions compatibles avec l'alimentation.

Divers explants de cassis ont été testés ainsi que divers facteurs contribuant à l'efficacité de l'extraction : le mélange d'extraction, le pH et l'état du matériel (frais, congelé ou lyophilisé). La stabilité des propriétés antioxydantes de l'extrait a également été étudiée : **« Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage. »** (Food Chemistry **105**, 1268-1275, 2007).

2. Articles

JOURNAL OF
AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Capacity of Black Currant Varies with Organ, Season, and Cultivar

JESSICA TABART,[†] CLAIRE KEVERS,[*,†] JOËL PINCEMAIL,[§] JEAN-OLIVIER DEFRAIGNE,[§] AND JACQUES DOMMES[†]

Plant Molecular Biology and Biotechnology Unit, B22, and CREDEC, Pathology tower B23, University of Liège, Sart Tilman, B-4000 Liege, Belgium

Small berries such as black currant constitute one of the important sources of potential health-promoting phytochemicals because these fruits are rich sources of compounds with high antioxidant properties. In this work, antioxidant capacities of different parts (buds, leaves, fruits) of various black currant cultivars were compared throughout the growing season with the aim to prepare extracts with high antioxidant capacity. Buds (opened, at the end of March) and leaves (in June) had a higher content in phenolics and antioxidants than fully ripened berries (in July) and the best yield (per branch) was obtained with the leaves collected in June due to their higher biomass. The differences observed among the eight cultivars tested were small. Concerning flavonols, quercetin was dominant in all organs and cultivars, myricetin varied widely among the cultivars, and kampferol was very low.

KEYWORDS: Antioxidant; black currant; flavonols; phenolics

INTRODUCTION

Small berries constitute one of the important sources of potential health-promoting phytochemicals. These fruits are rich sources of phenolic compounds (*1*) such as flavonoids (*2*) and other polyphenols (*3*), which display potential health-promoting effects (*4−7*) due to their antioxidant properties (*8−10*). Anthocyanins, which belong to the group of flavonoids, are responsible for the red, violet, and blue colors of most berries and fruits (*7, 11, 12*). Especially high levels of anthocyanins and high antioxidant capacity are found in bilberries (*13*) and black currants (*14, 15*). Myricetin, quercetin, and kaempferol are the principal flavonols, another group of flavonoids. Their amounts change greatly during black currant fruit ripening (*16, 17*).

The content of phenolics in berries is affected by the degree of maturity at harvest, genetic differences (cultivar), preharvest environmental conditions, postharvest storage conditions and processing (*1, 18, 19*), and growing season (*20*) but not by cultivation practices (organic or conventional ways) (*16*). The potential health benefits of these fruits may be greatly increased if berries from cultivars with high contents of antioxidant compounds are used as raw materials (*16*).

Domestic berries, both wild and cultivated species, are consumed in abundance. Although studies have been conducted on these berries in terms of their antioxidant capacity and phenolic profile (*15*), nothing was done on the other parts of these plants.

* Author to whom correspondence should be addressed (e-mail c.kevers@ulg.ac.be; fax +32 43 66 38 72).
[†] Plant Molecular Biology and Biotechnology.
[§] CREDEC.

Declume (*21*) and Chrubasik (*22*) demonstrated that leaf extract of black currant showed significant anti-inflammatory activity. However, no study has compared the antioxidant capacities of various parts of black currant. In this work we wanted to compare the antioxidant capacities of different parts of various black currant cultivars with the aim to prepare an extract with high antioxidant capacity. It is clear that, except for the berries, other parts cannot be consumed just as they are but can be conditioned as food supplements or added to food preparations or juices. Leaves and buds of black currant are already used in the preparation of some food supplements.

MATERIALS AND METHODS

Materials. One-year branches of black currant 'Noir de Bourgogne' were collected on different dates during the year 2004, in the Belgian Ardennes (at Bihain). Approximately 30 branches were randomly selected among more than 100 plants. Branches of eight cultivars of black currant were also collected in the botanical garden of the University of Liège during the year 2005. The cultivars were Black Down (BD), Ben Nevis (BN), Goliath (G), Noir de Bourgogne (NB), Tenah (T), Titania (Ti), Silvergieter (S), and Wellington (W). To obtain comparable samples, buds, leaves, or berries were collected at the same stages of development as described later under Results.

Immediately, the various types of samples (buds, leaves, flowers, berries, or stem pieces) were cut, weighed, frozen in liquid nitrogen, lyophilized, and stored at −20 °C prior to analysis at the end of the sample collection. The stability of the compounds in frozen samples was previously tested.

Sample Preparation. One gram of lyophilized sample was ground with 1 g of quartz and 5 mL of glycine buffer (0.87 M), pH 3 (with HCl). The mixture was shaken during 1 h at 4 °C and centrifuged at 17000*g* for 15 min. The supernatant was removed, and the sample was extracted two more times with 5 mL of the same buffer, incubated for

B

Table 1. Linear Gradient Used for the Separation of Flavonols from Black Currant Extracts

time (min)	solvent A[a] (%)	solvent B[b] (%)
0	85	15
2	80	20
10	80	20
23	70	30
30	60	40
42	0	100
47	0	100
49	85	15

[a] Solvent A: water/acetonitrile (95:5) adjusted to pH 1.8 with perchloric acid. [b] Solvent B: water/acetonitrile (50:50) adjusted to pH 1.8 with perchloric acid.

15 min, and centrifuged using the same procedure. The supernatants were pooled and then diluted as appropriate for the analyses. Each sample was independently extracted in triplicate or more, and analyses were performed the same day.

Extractions concerning the comparison among varieties were performed in acetone mixture (acetone/water/acetic acid, 70:28:2). One gram of lyophilized sample was ground with quartz (1 g) and acetone mixture (10 mL). The mixture was shaken during 1 h at 4 °C and centrifuged at 17000*g* for 15 min. The supernatant was removed and the pellet washed with 5 mL of acetone mixture, shaken for 15 min, and centrifuged using the same procedure. The supernatants were pooled, and 70% of the volume was evaporated at 30 °C. The volume was then adjusted to 15 mL with water. Each sample was independently extracted in triplicate or more, and analyses were performed the same day.

This second type of extraction was used in the second part of the work because it allowed a better extraction of phenolics and antioxidants and the stability of the extracts was better (data not shown).

Total Phenolics. Total phenolics were determined according to the Folin−Ciocalteu method (*23*). If not very precise for phenolics, this protocol gave a good idea of the total phenolic content. Appropriately diluted extracts (3.6 mL) were mixed with 0.2 mL of Folin−Ciocalteu reagent, and 3 min later, 0.8 mL of sodium carbonate (20% w/v) was added. The mixture was heated at 100 °C during 1 min. After cooling, the absorbance at 750 nm was measured. Chlorogenic acid (Sigma) was used as standard, and results were expressed as milligrams of chlorogenic acid equivalents (CAE) per gram of dry weight of plant material. Analyses were performed in duplicate on each sample.

Antioxidant Capacity. Antioxidant capacity was determined by scavenging of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Tadolini et al. (*24*). Stock solution was prepared by stirring 75 mg of DPPH in 1 L of methanol overnight. Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Fluka Chemie GmbH, Buchs, Switzerland] was used as a standard and methanol as a blank.

In the assay, 0.75 mL of extract, standard (0−0.1 mM Trolox), or blank (methanol) and 1.5 mL of DPPH solution were mixed. The absorbance at 517 nm of samples, standards, and blanks was determined after 5 min. For each extract, a blank with 1.5 mL of methanol, instead of DPPH reagent, was included to correct for any sample absorbance at 517 nm. The percentage of the remaining DPPH was proportional to the antioxidant concentration, calculated relative to the antioxidant capacity of Trolox and expressed as milligrams of Trolox equivalent (TE) per gram of dry weight of plant material. Analyses were performed in duplicate.

Flavonol Analysis. Analyses were performed in a liquid Elite Lachrom Merck Hitachi chromatograph equipped with an L2450 photodiode array detector. Separation was carried out using a LiChroCART steel cartridge, 240 mm × 4 mm, filled with 5 μm particles of RP 18 and thermostated at 25 °C.

The mobile phase (*25*) was a linear gradient of water/acetonitrile (50:50) adjusted to pH 1.8 with perchloric acid (solvent B) in water/acetonitrile (95:5) adjusted to pH 1.8 with perchloric acid (solvent A), at a flow rate of 1.2 mL/min, as shown in **Table 1**. Spectra were recorded between 250 and 400 nm (sampling period, 400 ms; spectral bandwidth, 4 nm). Standards of flavonols were purchased from Extrasynthese (Genay, France).

All results presented are the means (± SE) of at least three independent experiments (extraction).

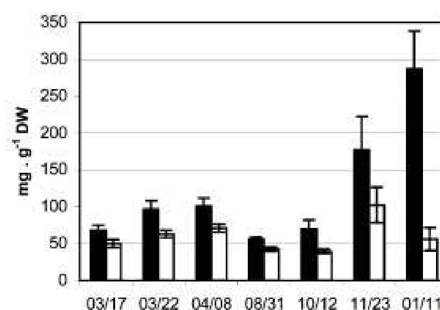


Figure 1. Total phenolics (black bars, mg of CAE g^{-1} of DW) and antioxidant capacities (white bars, mg of TE g^{-1} of DW) of buds of black currant 'Noir de Bourgogne' collected on different dates during the year (n = 4).

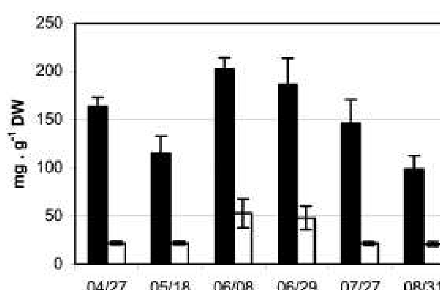


Figure 2. Total phenolics (black bars, mg of CAE g^{-1} of DW) and antioxidant capacities (white bars, mg of TE g^{-1} of DW) of leaves of black currant 'Noir de Bourgogne' collected on different dates during the year (n = 4).

RESULTS

Antioxidants in Various Parts of Black Currant Collected during Growth over the Year. *Buds.* The variations of total phenolics and antioxidant capacity were measured during the year 2004 on buds of black currant 'Noir de Bourgogne' (**Figure 1**). On March 17, the buds were closed (± 6 mm long), on March 22, they were "splinted" (± 8 mm long), and on April 8, the buds were opened (± 15 mm long). At the end of the summer, new buds were present, but they remained very small (2−3 mm long) until the end of the winter.

The level of phenolics did not change significantly from March to October but increased later with a maximum in January. The changes of the antioxidant capacity were similar except in January. Maxima were observed in April and November. Whereas the antioxidant capacity was 1.5−2 times higher in November, the weight of buds by branch was very low (5−10 times less). Thus, the best yield per branch was obtained in the spring, when the buds were opened.

Leaves. The leaves were collected from April to August (**Figure 2**). There was no difference between the measurements done in April (10−25 mm width) and in May (15−35 mm width) on the young leaves, but in June, when the leaves were well developed (± 70 mm width), the level of phenolics and the antioxidant capacity were higher. During the summer, the leaves necrosed except in the apex. The higher antioxidant capacity observed in June corresponded to the best yield because, at this time, the amount of leaves per branch was also the highest.

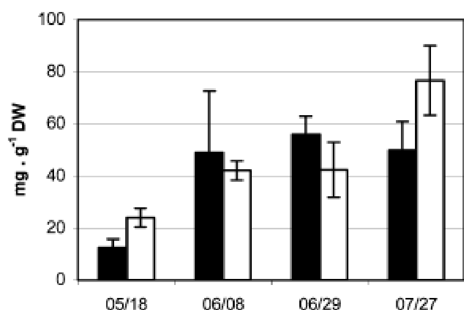


Figure 3. Total phenolics (black bars, mg of CAE g^{-1} of DW) and antioxidant capacities (white bars, mg of TE g^{-1} of DW) of berries of black currant 'Noir de Bourgogne' collected on different dates during the year ($n = 3$).

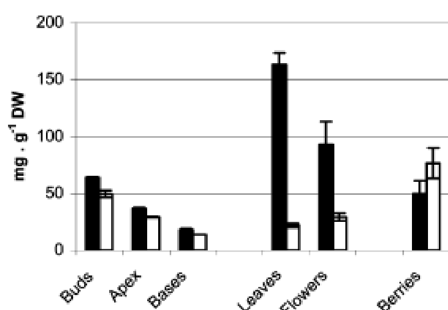


Figure 4. Total phenolics (black bars, mg of CAE g^{-1} of DW) and antioxidant capacities (white bars, mg of TE g^{-1} of DW) of various parts of black currant plants 'Noir de Bourgogne'. Buds, apex (2 cm long), and bases (2 cm long) of stems (without buds) were collected on March 17, leaves and flowers on April 22, and berries on July 27 ($n = 3$).

Berries. The berries were collected from May to July (**Figure 3**). The phenolic content increased during the growth of the berries and then stabilized during ripening. An increase of the antioxidant capacity was observed throughout the sampling period. The antioxidant capacity was maximal when the berries were ripe. At that moment, the fruit weight was highest. Thus, the yield of the antioxidant capacity was also maximal at this time.

Comparison with Other Parts. The phenolic level in leaves and flowers was higher than that in the other parts of the plant (**Figure 4**), whereas antioxidant capacity was superior in buds. The phenolic level and antioxidant capacity decreased from the apex to the basis of the stem.

Antioxidants in Buds, Leaves, and Berries of Various Black Currant Cultivars. Total phenolics and antioxidant capacities were measured in four types of explants (splinted and opened buds, well-developed leaves, and ripe berries) from eight commercial cultivars of black currant. These explants were collected at the time when the yield was previously shown to be maximal (**Figure 5**).

Concerning the buds, there was no significant difference between the different cultivars (**Figure 5**) even though the splinted buds of the Wellington cultivar did show lower phenolic content and antioxidant capacities than all of the other cultivars.

The phenolic contents and the antioxidant capacities of the leaves and the berries were very similar whatever the cultivars except for Black Down and Wellington, in which phenolic contents were lower.

If both phenolic contents and antioxidant capacities, measured per gram of dry weight, were similar for buds and leaves, the results obtained for the berries were lower, 3 times for phenolic contents and 6 times for antioxidant capacities.

The yield by branch was calculated for each explant of each cultivar (**Figure 6**). The results were very different from those obtained by unit of dry weight. There were important differences in the amount of each type of explant between the cultivars.

Yield in total phenolic and antioxidant capacity per branch was >20 times higher for the leaves than for the other explants.

Flavonol Contents in Buds, Leaves, and Berries of Black Currant Cultivars. Flavonols were present as aglycons or as glycosides. The ratios between the three main flavonol aglycon

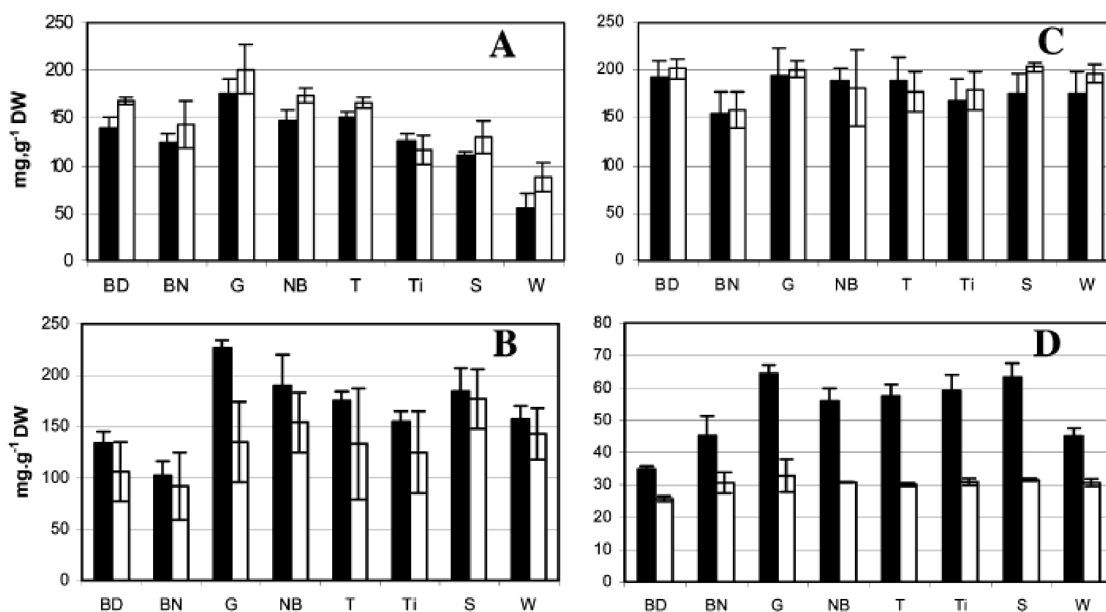


Figure 5. Total phenolics (black bars, mg of CAE g^{-1} of DW) and antioxidant capacities (white bars, mg of TE g^{-1} of DW) of buds (**A**, splinted; **B**, opened), leaves (**C**), and berries (**D**) of various cultivars of black currant: Black Down (BD), Ben Nevis (BN), Goliath (G), Noir de Bourgogne (NB), Tenah (T), Titania (Ti), Silvergieter (S), and Wellington (W) ($n = 3$).

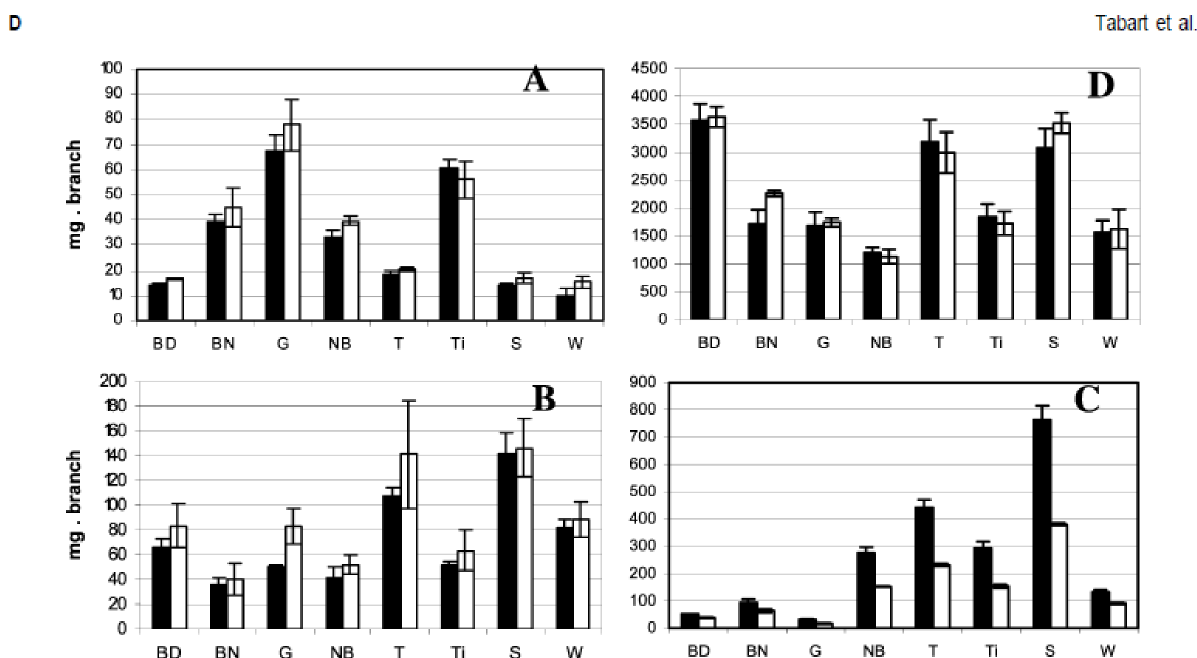


Figure 6. Yield by branch of total phenolics (black bars, mg of CAE branch^{-1}) and of antioxidant capacities (white bars, mg of TE branch^{-1}) of buds (**A**, splinted; **B**, opened), leaves (**C**), and berries (**D**) of various cultivars of black currant: Black Down (BD), Ben Nevis (BN), Goliath (G), Noir de Bourgogne (NB), Tenah (T), and Titania (*n* = 3).

Table 2. Flavonol Levels (Micrograms per Gram of Dry Weight) in Opened Buds of Various Cultivars of Black Currant: Black Down (BD), Ben Nevis (BN), Goliath (G), Noir de Bourgogne (NB), Tenah (T), Titania (Ti), Silvergieter (S), and Wellington (W) (*n* = 3)

flavonol	BD	BN	G	NB	T	Ti	S	W
myricitrin	197 ± 48	97 ± 52	35 ± 34	2 ± 0	169 ± 9	0	153 ± 20	0
rutin	407 ± 94	649 ± 149	433 ± 160	467 ± 80	753 ± 97	279 ± 44	589 ± 142	603 ± 63
isoquercitrin	165 ± 27	178 ± 19	122 ± 41	95 ± 1	178 ± 4	96 ± 5	140 ± 18	170 ± 11
astragalin	105 ± 21	140 ± 21	102 ± 34	70 ± 9	123 ± 9	82 ± 4	109 ± 8	110 ± 14
myricetin	109 ± 15	78 ± 31	26 ± 19	6 ± 1	102 ± 6	5 ± 1	80 ± 16	6 ± 1
quercetin	39 ± 5	27 ± 9	31 ± 4	13 ± 3	27 ± 1	20 ± 3	20 ± 7	16 ± 1
kampferol	4 ± 0	9 ± 1	8 ± 2	5 ± 1	10 ± 1	7 ± 1	9 ± 3	5 ± 1

Table 3. Flavonol Levels (Micrograms per Gram of Dry Weight) in Opened Buds, Leaves and Berries of Black Currant Cultivars Goliath and Noir de Bourgogne (*n* = 3)

	Goliath			Noir de Bourgogne		
flavonol	buds	leaves	berries	buds	leaves	berries
myricitrin	35 ± 5	0	6 ± 1	2 ± 0	0	0
rutin	433 ± 160	2097 ± 427	260 ± 72	467 ± 80	2875 ± 503	277 ± 55
isoquercitrin	122 ± 41	1327 ± 282	75 ± 15	95 ± 1	747 ± 62	61 ± 6
astragalin	102 ± 34	752 ± 162	39 ± 6	70 ± 9	366 ± 18	38 ± 3
myricetin	26 ± 19	82 ± 15	8 ± 3	6 ± 1	41 ± 6	4 ± 1
quercetin	31 ± 4	53 ± 4	3 ± 1	13 ± 3	28 ± 4	2 ± 0
kampferol	8 ± 2	10 ± 2	0	5 ± 1	5 ± 2	0

forms (myricetin, quercetin, and kampferol) were different in the various cultivars tested. In buds, the myricetin level was higher in Black Down, Ben Nevis, Tenah, and Silvergieter, whereas in Wellington, Titania, and Noir de Bourgogne cultivars, quercetin was higher. The level of kampferol was always lower (**Table 2**). Among the glycosides, rutin was the most concentrated form. In all of the varieties, the level of rutin was many times higher than that of the other flavonols. The second form was isoquercitrin, also a glycoside of quercetin. Myricitrin (myricetin glycoside) was not present in some cultivars such as, for instance, Titania and Wellington.

In the leaves, the total level of flavonols, such as that of phenolics or antioxidants, was higher than in buds and berries (**Table 3**). In all of the different plant organs, rutin was always the most abundant among the flavonols and the ratio between the different flavonols measured was the same in the different plant organs of the same cultivar.

DISCUSSION

In all plant material tested, the total phenolic level was correlated with antioxidant activity as already shown in different berries (*15*) and other common foods (*26*, *27*). Due to differences in solubility, different extraction mixtures can affect the yield in total antioxidant capacity (*28*, *29*). This explained the difference between the phenolic amounts and antioxidant

capacities measured on the same type of explant extracted with glycine buffer (used for the variations during the year) or with acetone mixture (used for the cultivar comparison). The results were often higher with this last solvent, never lower.

Antioxidant capacity showed variations throughout the growing season in the different plant organs. Taking into account the biomass available for extraction, we defined an optimal period of harvest to ensure the highest yield in antioxidant activity. For the buds, the best yield was observed when the buds were just opened at the end of March or in the beginning of April. For the leaves, it was in June. For the berries, best yields were obtained in July, when they were fully ripened. Mikkonen et al. (*16*) and Wang and Lin (*19*) have already observed similar phenolic changes during black currant fruit ripening.

If bud extracts had the same content in phenolics and antioxidants as leaf extracts, the best yield was obtained from leaves due to their higher biomass. The antioxidant capacity of black currant leaves is not well studied (*30*), but interest in them as herbal medicinal products for the alleviation of osteoarthritis and rheumatic complaints (*22*) has been demonstrated. The antioxidant activities of the black currant berries have been studied more than for other berries (*2, 3*), but we have shown that their antioxidant capacity was lower than that of the leaves and buds, leading to lower yield of antioxidant capacity. Ehlenfeldt and Prior found similar results in blueberry (*31*).

The differences observed among the eight cultivars tested were not important. Other research groups have observed some larger differences in black currant berries (*14, 16, 19, 32*). Concerning flavonols, our results were in accordance with previous studies showing that black currant berries contained high amounts of quercetin (*2*); this aglycon (alone or as glycoside) was the dominant flavonol in all cultivars. Mikkonen et al. (*16*) have also shown that the amount of myricetin glycosides in black currant berries varied widely among black currant cultivars, whereas kampferol was known as a minor flavonol.

The increasing importance of functional ingredients in food provides new challenges for plant sciences to increase health-promoting phytochemicals in crop plants (*33*). Higher intakes of flavonoïds and other antioxidant compounds from foods are associated with reduced risks of cancer, heart disease, and stroke. Some experimental studies indicate that several plant flavonols, such as quercetin, myricetin, and rutin, are more powerful antioxidants than traditional vitamins (*34, 35*) and have antitumor properties (*36*). The challenge is how to increase the levels of these beneficial phytochemicals in food. Black currant extract of leaves can be used as food supplements or incorporated in foods or beverages. The extract with the highest antioxidant capacity can be obtained with leaves collected in June and extracted with an acetone mixture.

ACKNOWLEDGMENT

Mr. Andrianne from HerbalGem provided the material. The skillful assistance of the APE personnel (provided to CEDEVIT by the government of Wallonia) was greatly appreciated.

LITERATURE CITED

(1) Shahidi, F.; Naczk, M. *Phenolics in Food and Nutraceuticals*; CRC Press: Boca Raton, FL, 2004; pp 131−155.

(2) Häkkinen, S. H.; Heinonen, I. M.; Kärenlampi, S. O.; Mykkänen, H. M.; Ruuskanen, J.; Törrönen, R. Screening o selected flavonoids and phenolic acids in 19 berries. *Food Res. Int.* **1999**, *32*, 345−353.

(3) Zadernowski, R.; Naczk, M.; Nesterowicz, J. Phenolic acid profiles in some small berries. *J. Agric. Food Chem.* **2005**, *53*, 2118−2124.

(4) Cao, G.; Russell, R. M.; Lischner, N.; Prior, R. L. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J. Nutr.* **1998**, *128*, 2383−2390.

(5) Landbo, A. K.; Meyer, A. S. Enzyme-assisted extraction of antioxidative phenols from black currant juice residue (*Ribes nigrum*). *J. Agric. Food Chem.* **2001**, *49*, 3169−3177.

(6) Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **2002**, *50*, 7731−7737.

(7) Kong, J. M.; Chia, I. S.; Goh, N. K.; Chia, T. F.; Brouillard, R. Analyses and biological activities of anthocyanins. *Phytochemistry* **2003**, *64*, 923−933.

(8) Wang, H.; Cao, G.; Prior, R. L. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996**, *44*, 701−705.

(9) Kähkönen, M. J.; Hopia, A. I.; Heinonen, M. Berry phenolics and their antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 4076−4082.

(10) Skrede, G.; Wrolstad, R. E. Flavonoids and other polyphenolics in grapes and other berry fruit. In *Functional Foods—Biochemical Processing Aspects*; Shi, J., Mazza, G., Le Maguer, M., Eds.; CRC Press: Boca Raton, FL, 2002; Vol. II, pp 71−130.

(11) Kähkönen, M. J.; Heinäki, J.; Ollilainen, V.; Heinonen, M. Berry anthocyanins: isolation, identification and antioxidant activities. *J. Sci. Food Agric.* **2003**, *83*, 1403−1411.

(12) Nielsen, I. L.; Haren, G. R.; Magnussen, E. L.; Dragsted, L. O.; Rasmussen, S. E. Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency. *J. Agric. Food Chem.* **2003**, *51*, 5861−5866.

(13) Prior, R. L.; Cao, G.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W.; Krewer, G.; Mainland, C. M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46*, 2686−2693.

(14) Moyer, R. A.; Hummer, K. E.; Finn, C. E.; Frei, B.; Wroldtad, R. E. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. *J. Agric. Food Chem.* **2002**, *50*, 519−525.

(15) Ehala, S.; Vaher, M.; Kaljurand, M. Characterization of phenolic profiles of northern European berries by capillary electrophoresis and determination of their antioxidant activity. *J. Agric. Food Chem.* **2005**, *53*, 6484−6490.

(16) Mikkonen, T. P.; Määttä, K.; Hukkanen, A. T.; Kokko, H. I.; Törrönen, A. R.; Kärenlampi, S. O.; Karjalainen, R. O. Flavonol content varies among black currant cultivars. *J. Agric. Food Chem.* **2001**, *49*, 3274−3277.

(17) Raffo, A.; Paoletti, F.; Antonelli, M. Changes in sugar, organic acid, flavonol and carotenoid composition during ripening of berries of three seabuckthorn (*Hippophae rhamnoides* L.) cultivars. *Eur. Food Res. Technol.* **2004**, *219*, 360−368.

(18) Heiberg, N.; Mage, F.; Haffner, K. Chemical composition of 10 black currant (*Ribes nigrum*) cultivars. *Acta Agric. Scand., Sect. B* **1992**, *42*, 251−254.

(19) Wang, S. Y.; Lin, H. Antioxidant activity in fruits and leaves of blackberry, raspberry and strawberry varies with cultivar and developmental stage. *J. Agric. Food Chem.* **2000**, *48*, 140−146.

(20) Howard, L. R.; Clark, J. R.; Brownmiller, C. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. *J. Sci. Food Agric.* **2003**, *83*, 1238−1247.

(21) Declume, C. Anti-inflammatory evaluation of a hydroalcoholic extract of black currant leaves (*Ribes nigrum*). *J. Ethnopharmacol.* **1989**, *27*, 91−98.

(22) Chrubasik, S. Pain therapy using herbal medicines. *Gynakologe* **2000**, *33*, 59−64.

(23) Caboni, E.; Tonelli, M. G.; Lauri, P.; Iacovacci, P.; Kevers, C.; Damiano, C.; Gaspar, T. Biochemical aspects of almond microcuttings related to in vitro rooting ability. *Biol. Plant.* **1997**, *39*, 91−97.

(24) Tadolini, B.; Juliano, C.; Piu, L.; Franconi, F.; Cabrini, L. Resveratrol inhibition of lipid peroxidation. *Free Radical Res.* **2000**, *33*, 105−114.

(25) Revilla, E.; Ryan, J. M. Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography−photodiode array detection without sample preparation. *J. Chromatogr. A* **2000**, *881*, 461−469.

(26) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States *J. Agric. Food Chem.* **2004**, *52*, 4026−4037.

(27) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(28) Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance acapacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **2003**, *51*, 3273−3279.

(29) Skrede, G.; Bryhn Larsen, V.; Aaby, K.; Skivik Jorgensen, A.; Birkeland, S. E. Antioxidative properties of commercial fruit preparations and stability of bilberry and black currant extracts in milk products. *J. Food Sci.* **2004**, *69*, S351−S356.

(30) YaQin, X.; Yuan, Y. Z.; Fei, W. Studies on the flavonoids and antioxidant activities in leaves of black currant. *J. Northeast Agric. Univ.* **2002**, *9*, 136−140.

(31) Ehlenfeldt, M. K.; Prior, R. L. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *J. Agric. Food Chem.* **2001**, *49*, 2222−2227.

(32) Wu, X.; Liwei, G.; Prior, R. L.; McKay, S. Characterization of anthocyanins and proanthocyanidins in some cultivars of *Ribes*, Aronia and Sambucus, and their antioxidant capacity. *J. Agric. Food Chem.* **2004**, *52*, 7846−7856.

(33) Becker, E.; Nissen, L. R.; Skibsted, L. H. Antioxidant evaluation protocols: food quality or health effects. *Eur. Food Res. Technol.* **2004**, *219*, 561−571.

(34) Vinson, J. A.; Hao, Y.; Xuehui, S.; Zubik, L. Phenol antioxidant quantity and quality in foods: vegetables. *J. Agric. Food Chem.* **1998**, *46*, 3630−3634.

(35) Tsao, R.; Deng, Z. Separation procedures for naturally occurring antioxidant phytochemicals. *J. Chromatogr. B* **2004**, *812*, 85−99.

(36) Elattar, T. M.; Virji, A. S. The inhibitory effects of curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cells *in vitro*. *Anticancer Res.* **2000**, *20*, 1733−1738.

Received for review April 20, 2006. Revised manuscript received June 22, 2006. Accepted June 30, 2006. The Walloon Ministry financed this research for Agriculture.

JF061112Y

ELSEVIER

Available online at www.sciencedirect.com

Food Chemistry 105 (2007) 1268–1275

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage

Jessica Tabart [a], Claire Kevers [a,b,*], Arnaud Sipel [b], Joël Pincemail [c], Jean-Olivier Defraigne [c], Jacques Dommes [a]

[a] *Plant Molecular Biology and Biotechnology Unit, B22, University of Liège, Sart Tilman, B-4000 Liege, Belgium*
[b] *CEDEVIT asbl, B22, University of Liège, Sart Tilman, B-4000 Liege, Belgium*
[c] *CREDEC, University of Liège, Pathology Tower, B23, Sart Tilman, B-4000 Liege, Belgium*

Received 13 November 2006; received in revised form 4 January 2007; accepted 2 March 2007

Abstract

Health benefits of a diet rich in fruits and vegetables are attributed in part to their contents of phenolics and other antioxidant compounds. In this research, the extraction of phenolics and antioxidant compounds from black currant was optimised for different plant organs. The extraction solvent affected yield: aqueous acetone was better than methanol and acetate or glycine buffer. In aqueous buffer, maximum yields of total phenolics and antioxidant activities were obtained at pH 3. Extraction from lyophilised materials yielded extracts with higher phenolic contents and antioxidant activities.

Stability of extracts made with acetate or glycine buffer was limited while the use of a mixture of acetone/acetic acid/water for extraction allowed a high phenolic content and antioxidant capacity in dry extract to be maintained for several months. This type of extract could be incorporated in functional food, beverage or dietary supplement.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Antioxidant; Black currant; Extraction; Phenolics; Stability

1. Introduction

There is a growing body of evidence suggesting that oxidative stress through an increased production of reactive oxygen and nitrogen species (ROS and RNS) plays an important role in the development of tissue damage and pathological events in living organisms (Bhatia, Shukla, Madhu, Gambhir, & Prabhu, 2003; Peuchant et al., 2004). In order to limit the harmful effects of ROS, a high-performance antioxidant system, consisting of enzymes, proteins, vitamins (A, C, E), carotenoids, polyphenols, trace elements and small molecules, such as glutathione, may interact with ROS and regulate their production within a physiological range. Antioxidants may therefore be of major importance in preventing the onset and/or the progression of oxidative pathologies and may provide protection to foods.

Many health-related properties, including anticancer, antiviral, antiinflammatory activities, antioxidant properties, effects on capillary fragility and an ability to inhibit human platelet aggregation, have been ascribed more particularly to phenolics (Spignoli, 2000). The physiological benefits of the plant phenolics have been attributed to their potential role in inhibiting lipid peroxidation, modulating cell signal transduction pathways and inducing apoptosis (Hou, Lambert, Chin, & Yang, 2004). The development and utilisation of more effective antioxidants of natural origin could, therefore, afford potential benefits for the

* Corresponding author. Address: Plant Molecular Biology and Biotechnology Unit, B22, University of Liège, Sart Tilman, B-4000 Liege, Belgium. Tel.: +32 46 66 38 46; fax: +32 46 66 38 72.

E-mail addresses: Jessica.Tabart@student.ulg.ac.be (J. Tabart), c.kevers@ulg.ac.be (C. Kevers), cedevit@guest.ulg.ac.be (A. Sipel), J.Pincemail@chu.ulg.ac.be (J. Pincemail), JO.Defraigne@chu.ulg.ac.be (J.-O. Defraigne), j.dommes@ulg.ac.be (J. Dommes).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.03.005

J. Tabart et al. / Food Chemistry 105 (2007) 1268–1275 1269

optimization of human health (Moure et al., 2001; Panico et al., 2005). Black currants are among the berries that contain very high amounts of phenolic compounds. Fresh black currants are particularly rich in anthocyanins. Other principal phenolics present in black currants include flavonols, procyanidins and various phenolic acids (Benvenuti, Pellati, Melegari, & Bertelli, 2004). Only a very small portion of these berries is consumed fresh; most is processed for juice concentrates. Leaves and buds can also be used (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2006).

The extraction and purification of phytochemicals from natural sources is desired, since these bioactive substances are often used in the preparation of dietary supplements, nutraceuticals, functional food ingredients, food additives, pharmaceuticals and cosmetic products. Different solvent systems have been used for extraction of polyphenols from plant materials (Chavan, Shahidi, & Naczk, 2001). Extraction yield is dependent on the solvent and method of extraction (Goli, Barzegar, & Sahari, 2005). The extraction method must enable complete extraction of the compounds of interest and must avoid their chemical modification (Zuo, Chen, & Deng, 2002). Water, aqueous mixtures of ethanol, methanol and acetone are commonly used (Sun & Ho, 2005).

The objective of this study was to optimise the extraction of total phenolics and antioxidant capacity from black currant material under conditions compatible with food use. The objective in extracting phytochemicals from their plant sources is to liberate these compounds from the vacuolar structures where they are found, either through rupturing plant tissue or through a process of diffusion. The factors that contribute to the efficiency of extraction are, in particular, solvent, pH and material state. The stability of antioxidant properties in extracts was also investigated.

2. Materials and methods

2.1. Materials

Buds and leaves of 2 year-old plants of black currant "Noir de Bourgogne" were collected, respectively, in March and June, in the Belgian Ardennes (at Bihain). Immediately, the various types of samples (buds, leaves) were cut into small pieces, frozen in liquid nitrogen, lyophilized and stored at −20 °C prior to analysis.

2.2. Sample preparation

One gram of lyophilized (exceptionally fresh or frozen) sample was ground with 1 g of quartz before addition of 10 ml of one of the following extraction solvents:

- 80% methanol,
- 50% and 70% acetone with 0–2% acetic acid,
- water,
- acetate buffer [0.05, 0.1 or 0.2 M NaOH (final concentration) adjusted to pH (3–5.4) with acetic acid],
- glycine buffer (0.2 M HCl, pH 2.2–5 with glycine).

The mixture was shaken during 1 h at 4 °C and centrifuged at 17000*g* for 15 min. The supernatant was removed and the pellet washed with 5 ml of the same solvent, shaken for 15 min and centrifuged using the same procedure. The supernatants were pooled and acetone, methanol or ethanol volume was evaporated at 30 °C. The volume was then adjusted to 15 ml with water. Each sample was independently extracted in triplicate or more and analyses were performed the same day or after storage when the stability was studied.

2.3. Total phenolics

Total phenolics were determined according to the Folin–Ciocalteu method (Caboni et al., 1997). This protocol gave a good idea of the total phenolic content. Appropriately diluted extracts (3.6 ml) were mixed with 0.2 ml of Folin–Ciocalteu reagent (Merck) and 3 min later, 0.8 ml of sodium carbonate (20% w/v) was added. The mixture was heated at 100 °C during 1 min. After cooling, the absorbance at 750 nm was measured. Chlorogenic acid (Sigma) was used as standard, and results were expressed as mg of chlorogenic acid equivalents (CAE) per gramme of dry weight of plant material. Analyses were performed in duplicate on each extract.

2.4. Antioxidant capacity

Antioxidant capacity was determined by scavenging of the radical 2,2-diphenyl-1-picryhydrazyl (DPPH$^{\cdot}$), as described by Tadolini, Juliano, Piu, Franconi, and Cabrini (2000). Stock solution was prepared by stirring 75 mg of DPPH to 1 l of methanol overnight. In the assay, 0.75 ml of extract, standard (0–0.1 mM trolox) or blank (methanol), and 1.5 ml of DPPH solution were mixed. The absorbance at 517 nm of samples, standards and blanks was determined after 5 min. For each extract, a blank with 1.5 ml of methanol, instead of DPPH reagent, was included to correct for any sample absorbance at 517 nm.

Antioxidant capacity was also determined by scavenging of the radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6sulfonic acid (ABTS$^{\cdot}$), as described by Re et al. (1998). Stock solution was prepared by stirring ABTS (7 mM) and potassium persulfate (2.45 M) in water overnight. Before use, this solution was diluted in ethanol to obtain an absorbance of 0.7 at 734 nm. In the assay, 5 µl of extract, standard (0–0.1 mM trolox) or blank (ethanol), and 1 ml of ABTS solution were mixed. The absorbance at 734 nm was determined after 4 min. For each extract, a blank with 1 ml of ethanol, instead of ABTS reagent, was included to correct for any sample absorbance at 734 nm.

Trolox [(±)6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Fluka Chemie GmbH, Switzerland] was used as a standard. The percentage of the remaining DPPH or ABTS was proportional to the antioxidant concentration.

1270 J. Tabart et al. / Food Chemistry 105 (2007) 1268–1275

The antioxidant capacity was expressed as mg of trolox equivalents (TE) per gramme of dry weight of plant material. Each analysis was performed in duplicate.

All results presented are the means (±SE) of at least three independent experiments (extraction).

3. Results

3.1. Effect of extraction solvent on phenolic content and antioxidant capacity

Various types of solvent were used to extract phenolic and antioxidant compounds from leaves of black currant (Fig. 1). The higher phenolic levels were observed with solvent containing acetone. Use of water led to lower yields of phenolics. Antioxidant capacity (measured with DPPH or ABTS) was also higher in the presence of acetone but the differences were not so important than when considering phenolics.

The DPPH and ABTS methods gave consistent results, with similar variations, but antioxidant capacity, measured with ABTS, was always lower than with DPPH.

3.2. Effect of pH on phenolic extraction in acetate buffer

To optimize the extraction of phenolics in aqueous solvent, leaves of black currant were extracted with acetate buffer of various pH values and ionic strengths (Fig. 2). The increase of the ionic strength (NaOH concentration from 0.05 to 0.2 M) led to higher yields of total phenolics at each pH tested. Higher yields of phenolics were observed when the pH was decreased to 3. The best extraction yield with acetate buffer was thus observed at pH 3 and high ionic strength.

3.3. Effect of ethanol on extraction of phenolics and antioxidants in acetate buffer

To improve the phenolic extraction under conditions compatible with food use, leaves of black currant were extracted at different pH values (3, 4 and 4.2) in acetate

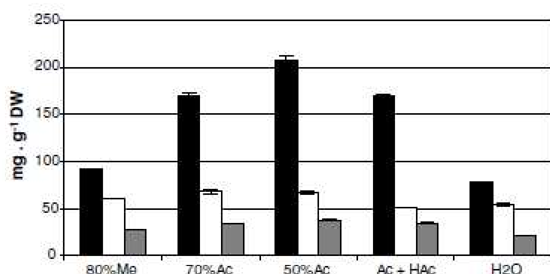


Fig. 1. Total phenolics (■ mg CAE g^{-1} DW) and antioxidant capacities (mg TE g^{-1} DW) measured with DPPH (□) and ABTS (▩) in leaves of black currant extracted with various solvents (80%Me: 80% methanol; Ac: 50 or 70% acetone; Ac + HAc: 70% acetone + 0.5% acetic acid).

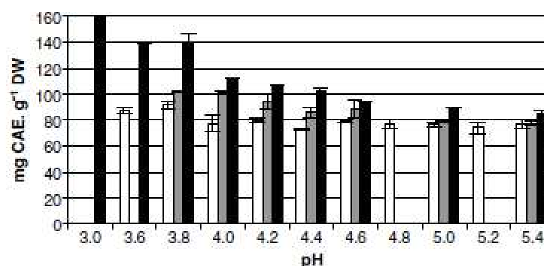


Fig. 2. Total phenolics (mg CAE g^{-1} DW) from leaves of black currant extracted with acetate buffers of various pH (3.0–5.4) and ionic strength: 0.05 M (□), 0.1 M (▩) and 0.2 M (■) of NaOH.

buffers supplemented with ethanol at various concentrations (Fig. 3). At pH 3, the presence of ethanol in the extraction solvent had no effect on yield of total phenolics. At pH 4 and 4.2, the increase of ethanol concentration led to higher yields of total phenolics. The best results were obtained with 38.4% of ethanol. These results were similar to those obtained for the extraction at pH 3 and slightly lower than that obtained for an extraction with 50% acetone 50% (Fig. 4A).

Similar results were observed with buds. In acetate buffer, the best extraction of phenolics was performed in acetate buffer at pH 3 and these results were similar to those obtained with 50% acetone.

For antioxidant capacity in extracts from leaves and buds (Fig. 4B), similar values were obtained after extraction at pH 3 and pH 4, with or without 38.4% ethanol. Use of 50% acetone as extraction solvent allowed higher yields of phenolics from leaves.

3.4. Effect of pH on extraction of phenolics and antioxidant capacity in glycine buffer

Buds of black currant were extracted with glycine buffers of various pH values and comparatively with acetate buffer at pH 3 (Fig. 5). Phenolic level and, to a less extent, antioxidant capacity, increased in glycine buffer extracts from pH 2.2 to pH 3. At pH 5, the phenolic level and the antioxidant capacity were reduced. The values obtained with acetate and glycine buffers at pH 3 were similar.

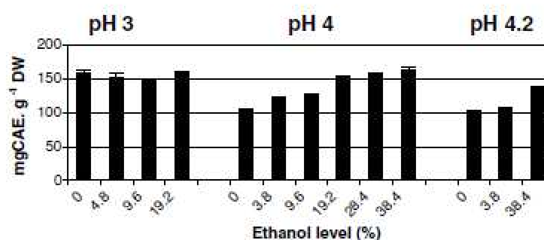
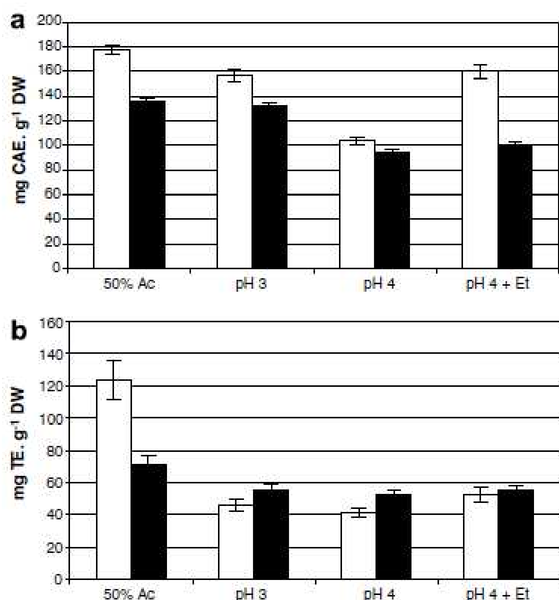


Fig. 3. Total phenolics (mg CAE g^{-1} DW) from leaves of black currant extracted with acetate buffers of pH 3, 4 and 4.2, and supplemented with various levels of ethanol (0–38.4%).

J. Tabart et al. / Food Chemistry 105 (2007) 1268–1275 1271



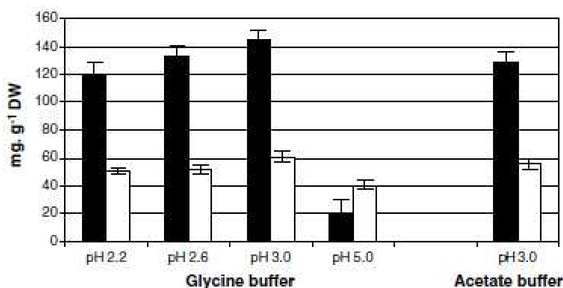


Fig. 4. Total phenolics (a, mg CAE g^{-1} DW) and antioxidant capacities (b, DPPH, mg TE g^{-1} DW) from leaves (□) and buds (■) of black currant extracted with various solvents (50% Ac: 50% acetone, acetate buffers at pH 3 and 4, supplemented or not with 38.4% ethanol (Et)).

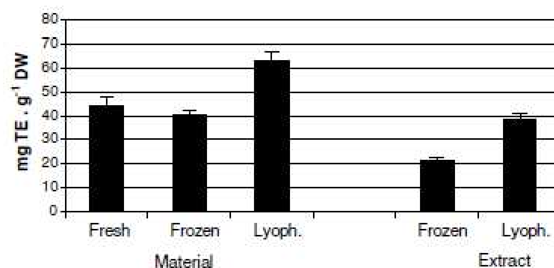


Fig. 5. Total phenolics (■, mg CAE g^{-1} DW) and antioxidant capacities (□, DPPH, mg TE g^{-1} DW) from buds of black currant extracted with various buffers: glycine at pH 2.2 to 5 and acetate, pH 3.

3.5. Effect of acetone and acetic acid concentration

Buds of black currant were extracted with different mixtures of acetone and acetic acid and the results were compared with those obtained after extraction with glycine buffer at pH 3 (Fig. 6). Phenolic level and antioxidant capacity were similar in most of the acetone–acid acetic mixtures and higher than that in extract obtained in glycine buffer at pH 3. The mixture of 70% acetone and 0.5% acetic acid gave lower yields, similar to that obtained with glycine buffer.

3.6. Stability of antioxidant capacity in stored plant material and extracts

Fresh buds of black currant were frozen (−20 °C) during one week or immediately lyophilized and stored for

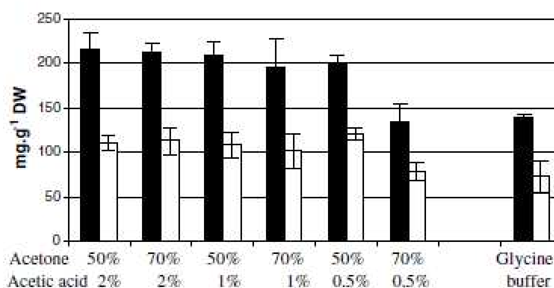


Fig. 6. Total phenolics (■, mg CAE g^{-1} DW) and antioxidant capacities (□, mg TE g^{-1} DW) from buds of black currant extracted with various acetone mixture (50% or 70% acetone with 0.5, 1 or 2% acetic acid) and glycine buffer, pH 3.

one week at 4 °C in darkness. The antioxidant capacities of these three types of buds (fresh, frozen and lyophilized) were measured. The freezing did not modify the antioxidant capacity, whereas the antioxidant capacity of lyophilized material was higher (Fig. 7).

In parallel, extracts of fresh material were kept frozen or lyophilized for one week before a new measurement of antioxidant capacity. This capacity was reduced in the

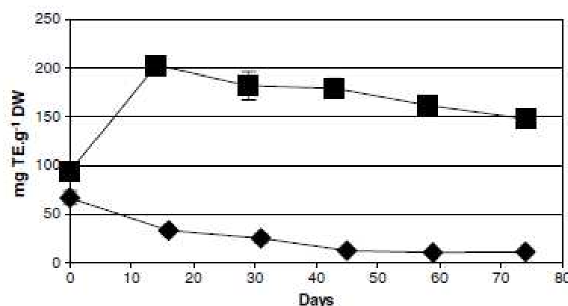


Fig. 7. Antioxidant capacities (DPPH, mg TE g^{-1} DW) of extracts (made with glycine buffer, pH 3) from buds of black currant: fresh material, frozen or lyophilised material stored for one week, extracts from fresh buds frozen or lyophilised for one week.

Fig. 8. Evolution of antioxidant capacities (DPPH, mg TE g^{-1} DW) of lyophilised extracts from buds of black currant made with glycine buffer, pH 3 (♦) or acetone mixture (■, 70% acetone, 2% acetic acid) and stored at 4 °C in darkness.

Table 1
Antioxidant capacity (in percent of the initial value) of extracts of buds made with glycine buffer, pH 3, kept for 75 days at 4 °C in darkness

Conditions	%
Lyophilised	17.0 ± 1.1
Liquid	11.1 ± 2.0
Liquid + nitrogen	6.6 ± 4.1

The extracts were stored dry (lyophilised) or in buffer (liquid) in air or under nitrogen.

frozen extracts whereas, in lyophilized samples, there were no significant differences in comparison to the fresh extract.

Extracts of buds in glycine buffer (pH 3) were immediately lyophilized and stored at 4 °C in darkness. The variations of the antioxidant capacity of the extracts were measured during 75 days (Fig. 8). They decreased regularly during the first 6 weeks of storage and then stabilized at a very reduced value (17% of the initial value). When air of the flask was replaced by nitrogen, no improvements were observed (Table 1).

Some compounds, known for their stabilizing properties, were added to the glycine buffer during the extraction. No improvements were obtained (Table 2).

Extracts made with acetone mixture (70% acetone, 2% HAc) showed a better stability during storage than did those made with glycine buffer (Fig. 8). The antioxidant capacity increased during the first few days and then decreased slowly. After 75 days, this capacity was 1.5 times higher than the initial value (Fig. 8). Various temperatures were used (20 °C, 4 °C and −20 °C) to test the stability of dry and liquid samples. At 20 °C and 4 °C, the stability of the lyophilized extracts was better than that of liquid samples. At −20 °C, the stability of dry extracts decreased, whereas that of liquid extracts increased but remained lower than that of dry samples at 4 or 20 °C. Storage under nitrogen did not lead to any improvement (Table 3).

Table 2
Antioxidant capacity (in percent of the initial value) of lyophilised extracts of buds made with glycine buffer, pH 3, stored for 75 days at 4 °C in darkness

Stabilizing compounds	%
–	17.0 ± 1.01
EDTA	10.8 ± 0.1
$CaCl_2$	12.0 ± 0.4
EDTA + $CaCl_2$	12.4 ± 0.2
Cysteine	18.4 ± 2.5
Sulfite	12.6 ± 0.1

Some stabilizing compounds were added to the buffer during the extraction: 0.2 mM EDTA, 6.8 mM $CaCl_2$, 0.19 mM cysteine, 0.4 mM sodium sulfite.

Table 3
Antioxidant capacity (in percent of the initial value) of extracts of buds made with acetone mixture (acetone 70%, HAc 2%), stored at 20 °C, 4 °C or −20 °C in darkness for 65 days

Conditions	20 °C	4 °C	−20 °C
Lyophylised	169 ± 14.4	157 ± 4.8	130 ± 9.2
Lyophylised + nitrogen	160 ± 9.3	113 ± 8.9	102 ± 10.3
Liquid	66.3 ± 12.3	98.4 ± 6.0	130 ± 9.1

The extracts were preserved dry (lyophilised) in normal atmosphere or under nitrogen or in water (liquid).

4. Discussion

Numerous methods are used to evaluate antioxidant activities of natural compounds in food or biological systems. Two free radicals that are commonly used to assess antioxidant activity *in vitro* are ABTS$^{\cdot}$ and DPPH$^{\cdot}$. The reduction of these two radicals by hydrogen-donating antioxidant is monitored through the decrease of their optical density at long wavelength. These two methods showed good repeatability. Generally, as seen here (Fig. 1), they showed consistent results (Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003). The Folin–Ciocalteu assay has been used for many years as a measure of the total phenolics in natural products, but the basic mechanism is an oxidation/reduction reaction and, as such, can be considered as another method of antioxidant determination (Prior, Wu, & Schaich, 2005). The high correlation (Fig. 4) between total phenolic content and antioxidant activity (DPPH) has already been observed in black currant leaf and bud extracts (Tabart et al., 2006), as in different berries (Ehala, Vaher, & Kaljurand, 2005) and other common foods (Turkmen, Sari, & Velioglu, 2006; Wu et al., 2004). The results obtained by the three methods used here, again showed a good correlation (Fig. 1). Moreover, this study indicated that the extracts obtained from buds or leaves of black currant have remarkable antioxidant activities, the extent of which depends on the extraction conditions.

The principal factors that contribute to the efficiency of extraction are: type of solvent, pH, temperature, number of steps and volume of solvent, and particle size in the sample (Escribano-Bailon & Santos-Buelga, 2003). Some of these parameters were tested in this work on the extraction of phenolics and antioxidants in black currant leaves and buds.

4.1. Nature of the solvent

The most widely used solvents for extracting phenolic substances are methanol, acetone and their water mixtures (Kashiwada, Morita, Nonaka, & Nishioka, 1990). Comparative studies have been carried out to establish extractive efficiency of various solvents. For extraction of the total phenolics from black currant leaves, aqueous acetone was found to be more effective than methanol and water (Fig. 1) as in other plants (Zhou & Yu, 2004). Acetone and methanol seem to have distinct specificities in the extraction of polyphenolic substances. Methanol is the best solvent for catechin extraction, whereas a better yield for procyanidins is obtained with 70% acetone. This fact is in accordance with polarity of the solvent used for the extraction and solubility of phenolic compounds in them (Canadanovic-Brunet et al., 2006; Turkmen et al., 2006).

Aqueous methanol, due to its polarity, is more effective at extracting polyphenols linked to polar fibrous matrices. Acetone/water mixtures are more useful for extracting polyphenols from protein matrices, since they appear to degrade the polyphenol–protein complexes (Hussein, Fattah, & Salem, 1990; Kallithraka, Garcia Viguera, Bridle, & Bakker, 1995). The results obtained with water alone were lower than with organic solvents. However, Khokhar and Magnusdottir (2002) found water to be a better solvent for extracting *Zea* polyphenols than were 80% methanol or 70% ethanol. In some plants, ethanol extraction was the most efficient recovery method for phenolics (Liyana-Pathirana & Shahidi, 2005; Yu, Ahmedna, & Goktepe, 2005). For black currant leaf extraction (Fig. 3); the addition of ethanol to aqueous buffer had an effect only when the aqueous extraction (pH 4 or 4.2, acetate buffer) was not optimum.

Of the different solvents mixed with water and/or acetic acid, the acetone (50% or 80%)/water/acetic acid (1% or 2%) mixtures (Fig. 6) proved to be the best for extracting phenolic compounds and for obtaining extracts with higher antioxidant capacities in blackcurrant. A similar observation was made for dark-chocolate procyanidins extracted in acetone/water/acetic acid (70/28/2) by Counet and Collin (2003). Thus, extraction solvents had significant effects on total extractable phenolics and antioxidant capacities of the extracts.

4.2. pH of the extraction medium

With the aim of using solvent directly compatible with food use, aqueous buffers with various pH values and ionic strengths were tested for the antioxidant extraction from black currant leaves. These two parameters determine the degree of solubility for soluble compounds and also influence the possible solubilisation of the hydrolysable fraction. So, in blueberry extracts, Kalt, McDonald, and Donner (2000) showed that antioxidant capacity was greater in pH 1 extracts than in extracts at pH 4 and 7. In cereals, most efficient antioxidant extraction was achieved by using acidic solvent (pH 2) (Perez-Jimenez & Saura-Calixto, 2005). In this work, acetate buffer and glycine buffer, respectively known for their buffer properties between pH 3 and 5.8, and 1.8 and 3.6 were used to study the extraction of phenolics. The best results were obtained at pH 3.0 with the two buffers (Figs. 2 and 5). The extracts made with higher (acetate buffer) and lower (glycine buffer) pH showed a lower content of phenolics and a reduced antioxidant capacity. pH 3 was also the pH of the various acetone/acetic acid mixtures used that yielded extracts with higher phenolics and antioxidant capacity. The increase of the ionic strength also had a positive effect on the extraction of phenolics at the various pH values tested. A reason of these differences can be the variability in degree of sample hydrolysis occurring during extraction (Baugh & Ignelzi, 2000) because many of the antioxidant compounds are redox-active phenolic molecules, which are commonly found in plants as components of glycosides and starch polymers.

4.3. Particle size and shape

Homogenisation favours the extraction process and can be carried out in contact with the extraction solvent. In this work, the use of lyophilised material allowed a better extraction of antioxidants (Fig. 7). Two reasons can explain this fact: first, a better grinding of the tissues and thus a reduced particle size in the sample and second, degradation of some phenolics and antioxidants in undried plant material. It is known that lyophilisation generally does not affect the phenolics and antioxidant capacity, and allows samples to be kept for longer periods (Arts, Hollman, & Kromhout, 1999) while air-drying methods can induce a decrease of these compounds (Kwok, Hu, Durance, & Kitts, 2004).

Black currant extracts, rich in antioxidants, could be used for the preparation of functional foods or beverages. To this end, the stability of the antioxidant properties during the storage of the extracts has to be addressed. The extracts made in glycine buffer, pH 3, showed a rapid decrease of their properties (Fig. 8) whatever the conditions of storage (liquid, frozen or lyophilised). It has long been known that anthocyanins and ascorbic acid are mutually destructive in the presence of oxygen (Sondheimer & Kertesz, 1953). Starr and Francis (1968) showed that cranberry juice pigments degraded most rapidly when the greatest amounts of ascorbic acid and oxygen were present. Considering the oxygen radical absorbing capacity of anthocyanins, which confers potent antioxidant properties on these compounds, as shown by Wang, Cao, and Prior (1997), this lends support to the theory of oxidation reaction in which the ascorbic acid acts as an activator of molecular oxygen-producing free-radicals (Garcia-Viguera & Bridle, 1999). But, in our extracts, the suppression of the oxygen by replacement of the atmosphere by nitrogen did not improve the conservation of the antioxidant properties (Table 1) nor did the addition of stabilizing compounds (Table 2) generally used in food (Mikova, 2001). The extracts made with acetone mixture showed a better stability (Fig. 8) than did those obtained with glycine buffer, whatever the temperature used (Table 3). This difference can be explained by the difference in the antioxidant compounds extracted by the two types of solvents. Indeed, the protective antioxidative property of some flavonols (e.g., quercetin), which retard the degradation process, has been demonstrated (Shrikhande & Francis, 1974). An analysis of phenolic compounds would elucidate the difference in composition of the two types of extracts.

It is a very big challenge for the food industry to produce a new generation of food products with enriched content of natural antioxidants. Plant extracts, from a wide range of fruits or vegetables, are ideal candidates for product developers to address the health effects of oxidative stress. An extract with high antioxidant capacity can be

obtained from lyophilised leaves or buds of black currant extracted with a mixture of acetone/acetic acid/water (70/2/28). After elimination of acetone, the dry extract can be stored at room temperature for several months.

Acknowledgements

The Walloon Ministry financed this research for Agriculture. Mr P. Andrianne from HerbalGem provided the material. J.T. gratefully acknowledges the Luxemboug Ministry of National Education. The skilful assistance of the APE personnel (provided to CEDEVIT by the government of Wallonia) was greatly appreciated.

References

Arts, I., Hollman, P. C., & Kromhout, D. (1999). Chocolate as a source of tea flavonoids. *The Lancet, 354*, 488.

Awika, J. M., Rooney, L. W., Wu, X., Prior, R. L., & Cisneros-Zevallos, L. (2003). Screening methods to measure antioxidant activity of Sorghum (*Sorghum bicolor*) and Sorghum Products. *Journal of Agricultural and Food Chemistry, 51*, 6657–6662.

Baugh, S., & Ignelzi, S. (2000). Hydrolysis and redox factors affecting analysis of common phenolic marker compounds in botanical extracts and finished products. *Journal of AOAC International, 83*, 1135–1140.

Benvenuti, S., Pellati, F., Melegari, M., & Bertelli, D. (2004). Polyphenols, anthocyanins, ascorbic acid and radical scavenging activity of Rubus, Ribes, and Aronia. *Journal of Food Science, 69*, 164–169.

Bhatia, S., Shukla, R., Madhu, S. V., Gambhir, J. K., & Prabhu, K. M. (2003). Antioxidant status, lipid peroxidation and NO end products in patients of type 2 diabetes mellitus with nephropathy. *Clinical Biochemistry, 36*, 557–562.

Caboni, E., Tonelli, M. G., Lauri, P., Iacovacci, P., Kevers, C., Damiano, C., et al. (1997). Biochemical aspects of almond microcuttings related to in vitro rooting ability. *Biologia Plantarum, 39*, 91–97.

Canadanovic-Brunet, J. M., Djilas, S. M., Cetkovic, G. S., Tumbas, V. T., Mandic, A. I., & Canadanovic, V. M. (2006). Antioxidant activities of different *Teucrium montanum* L. extracts. *International Journal of Food Science and Technology, 41*, 667–673.

Chavan, U. D., Shahidi, F., & Naczk, M. (2001). Extraction of condensed tannins from beach pea (*Luthyrus maritimus* L.) as affected by different solvents. *Food Chemistry, 75*, 509–512.

Counet, C., & Collin, S. (2003). Effect of the number of flavonols units on the antioxidant activity of procyanidin fractions isolated from chocolate. *Journal of Agricultural and Food Chemistry, 51*, 6816–6822.

Ehala, S., Vaher, M., & Kaljurand, M. (2005). Characterization of phenolic profiles of northern European berries by capillary electrophoresis and determination of their antioxidant activity. *Journal of Agricultural and Food Chemistry, 53*, 6484–6490.

Escribano-Bailon, M. T., & Santos-Buelga, C. (2003). Polyphenol extraction from foods. In C. Santos-Buelga & G. Williamson (Eds.), *Methods in Polyphenol Analysis* (pp. 1–16). Cambridge, United Kingdom: Royal Society of Chemistry.

Garcia-Viguera, C., & Bridle, P. (1999). Influence of structure on colour stability of anthocyanins and flavylium salts with ascorbic acid. *Food Chemistry, 64*, 21–26.

Goli, A. H., Barzegar, M., & Sahari, M. A. (2005). Antioxidant activity and total phenolic compounds of pistachio (*Pistachia vera*) hull extracts. *Food Chemistry, 92*, 521–525.

Hou, Z., Lambert, J. D., Chin, K. V., & Yang, C. S. (2004). Effects of tea polyphenols on signal transduction pathways related to cancer chemoprevention. *Mutation Research, 555*, 3–19.

Hussein, L., Fattah, M., & Salem, E. (1990). Characterization of pure anthocyanidins isolated from the hulls of faba beans. *Journal of Agricultural and Food Chemistry, 38*, 95–98.

Kallithraka, S., Garcia Viguera, C., Bridle, P., & Bakker, J. (1995). Survey of solvents for the extraction of grape seed phenolics. *Phytochemical Analysis, 6*, 265–267.

Kalt, W., McDonald, W., & Donner, H. (2000). Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. *Journal Food Science, 65*, 390–393.

Kashiwada, Y., Morita, M., Nonaka, G., & Nishioka, Y. (1990). Tannins and related compounds. XCI. (1) Isolation and characterization of proanthocyanidins with an intramolecularly doubly-linked unit from the fern *Dicranopteris pedata* Houtt. *Chemical and Pharmaceutical Bulletin, 38*, 856–860.

Khokhar, S., & Magnusdottir, S. G. M. (2002). Total phenol, catechin and caffeine contents of teas commonly consumed in the United Kingdom. *Journal of Agricultural and Food Chemistry, 50*, 565–570.

Kwok, B. H. L., Hu, C., Durance, T., & Kitts, D. D. (2004). Dehydration techniques affect phytochemical contents and free radical scavenging activities of saskatoon berries. *Journal of Food Science, 69*, 122–126.

Liyana-Pathirana, C., & Shahidi, F. (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chemistry, 93*, 47–56.

Mikova, K. (2001). The regulation of antioxidants in food. In J. Pokorny, N. Yanishlieva, & M. Gordon (Eds.), *Antioxidants in food* (pp. 267–283). Boca Raton, USA: CRC press LLC.

Moure, A., Cruz, J. M., Franco, D., Dominguez, J. M., Sineiro, J., Dominguez, H., et al. (2001). Natural antioxidants from residual sources. *Food Chemistry, 72*, 145–171.

Panico, A. M., Cardile, V., Garufi, F., Puglia, C., Bonina, F., & Ronsisvalle, G. (2005). Protective effect of *Capparis spinosa* on chondrocytes. *Life Sciences, 77*, 2479–2488.

Perez-Jimenez, J., & Saura-Calixto, F. (2005). Literature data may underestimate the actual antioxidant capacity of cereals. *Journal of Agricultural and Food chemistry, 53*, 5036–5040.

Peuchant, E., Brun, J., Rigalleau, V., Dubourg, L., Thomas, M., Daniel, J., et al. (2004). Oxidative and antioxidative status in pregnant women with either gestational or type 1 diabetes. *Clinical Biochemistry, 37*, 293–298.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*, 4290–4302.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1998). Antioxydant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237.

Shrikhande, A. J., & Francis, F. J. (1974). Effect of flavonols on ascorbic acid and anthocyanin stability in model systems. *Journal of Food Sciences, 39*, 904–906.

Sondheimer, E., & Kertesz, Z. I. (1953). Participation of ascorbic acid in the destruction of anthocyanins in strawberry juice and model systems. *Food Research, 18*, 475–479.

Spignoli, G. (2000). Protective effects of dietary flavonoids on cardiovascular system and circulation. *European Bulletin of Drug Research, 8*, 1–8.

Starr, M., & Francis, F. J. (1968). Oxygen and ascorbic acid effect on the relative stability of four anthocyanin pigments in cranberry juice. *Food Technology, 22*, 1293–1295.

Sun, T., & Ho, C. (2005). Antioxidant activities of buckwheat extracts. *Food Chemistry, 90*, 743–749.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J. O., & Dommes, J. (2006). Antioxidant capacity of black currant varies with organ, season, and cultivar. *Journal of Agricultural Food Chemistry, 54*, 6271–6276.

Tadolini, B., Juliano, C., Piu, L., Franconi, F., & Cabrini, L. (2000). Resveratrol inhibition of lipid peroxidation. *Free Radical Research, 33*, 105–114.

Turkmen, N., Sari, F., & Velioglu, S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea

polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chemistry, 99*, 835–841.
Wang, H., Cao, G., & Prior, R. L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry, 45*, 304–309.
Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *Journal of Agricultural and Food Chemistry, 52*, 4026–4037.
Yu, J., Ahmedna, M., & Goktepe, I. (2005). Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chemistry, 90*, 199–206.
Zhou, K., & Yu, L. (2004). Effects of extraction solvent on wheat bran antioxidant activity estimation. *Lebensmittel-Wissenschaft und Technologie, 37*, 717–721.
Zuo, Y., Chen, H., & Deng, Y. (2002). Simultaneous determination of catechins, caffeine and gallic acids in green, oolong, black and pureh teas using HPLC with a photodiode array detector. *Talanta, 57*, 307–316.

3. *Conclusion*

A l'heure actuelle, un vrai engouement pour les produits enrichis en phytonutriments secoue la planète. Un vrai challenge s'opère au niveau industriel pour produire cette nouvelle génération de produits, enrichis avec ces composés antioxydants ou autres, de base naturelle.

Au niveau des antioxydants, un des premiers points est de trouver un produit naturel riche en composés polyphénoliques et de haute activité antioxydante. En prenant comme modèle le cassis, nous avons pu remarquer que le taux en polyphénols ainsi que la capacité antioxydante variaient en fonction de la saison de prélèvement et ce, quelque soit l'explant analysé (feuilles, fruits, bourgeons ouverts ou fermés). En prenant aussi en compte la biomasse, il s'avère que les feuilles prélevées en juin sont les plus intéressantes. Divers cultivars de cassis ont aussi été analysés, mais aucune différence significative au niveau du contenu en composés phénoliques et de la capacité antioxydante n'a été observée.

Pour tenter d'augmenter le rendement d'extraction des composés phénoliques du cassis, l'étape d'extraction a été optimisée en jouant sur divers paramètres. Les résultats ont été comparés au tampon glycine, tampon aqueux pouvant sans risque être utilisé dans la production de compléments alimentaires. Pour l'extraction des composés phénoliques, les solvants utilisés couramment sont le méthanol, l'acétone ainsi que leurs mélanges aqueux, acidifiés ou non. Pour les feuilles de cassis, le mélange aqueux contenant 70% d'acétone donne le meilleur rendement d'extraction. L'ajout de 2% d'acide acétique permet d'améliorer ce rendement. Nous avons aussi analysé la stabilité de ces extraits au cours du temps et nous avons pu constater que le mélange acétone/eau/acide acétique (70 :28 :2) permettait la conservation des propriétés de nos extraits à plus long terme que le tampon glycine.

Pour analyser ces divers paramètres, nous avons utilisé deux techniques d'analyses courantes : la méthode de décoloration du radical DPPH ainsi que le dosage des phénols totaux. Ces deux techniques spectrophotométriques sont des techniques *in vitro* basées sur un principe chimique propre ne reflétant qu'une infime partie des réactions potentielles des différents composants de l'extrait. Dans la suite de ce travail, la caractérisation de l'activité antioxydante ainsi que le contenu en composés phénoliques sera abordée en utilisant un maximum de techniques tant *in vitro* que sur modèles cellulaires.

Partie 2: Caractérisation des extraits de cassis

1. Introduction

Les molécules antioxydantes des matrices végétales peuvent être caractérisées par une séparation chromatographique (par exemple HPLC, GC,…) suivie éventuellement d'une identification par spectrométrie de masse ou plus globalement par des méthodes spectrophotométriques détectant des groupes spécifiques chimiquement semblables de composés réactifs. Les techniques chromatographiques sont très précises et permettent l'identification d'un grand nombre de composés mais ces techniques sont très longues et coûteuses (Stratil *et al.,* 2006). Il est donc plus simple d'utiliser d'abord des méthodes spectrophotométriques pour quantifier des familles de composés.

Dans un premier temps, nous avons utilisé des standards et tenté au moyen de ceux-ci de valider les méthodes colorimétriques pour différents groupes de composés antioxydants (flavonoïdes, anthocyanines, catéchines, acide ascorbique, et glutathion). Ces méthodes colorimétriques seront aussi appliquées à diverses matrices alimentaires (jus de pomme, jus d'orange, jus de légumes, thé glacé et vin rouge) et les résultats mis en relation avec leur capacité antioxydante totale. Les résultats sont présentés dans l'article: **« Evaluation of spectrophotometric methods for antioxidant compound measurement in relation to total antioxidant capacity in beverages**. **»** (Food Chemistry **120**, 607-614, 2010). Ensuite, ces méthodes colorimétriques ont été appliquées à nos extraits de cassis, parallèlement à des dosages de composés identifiés par chromatographie liquide: « **Ascorbic acid, Phenolic acid, Flavonoid and Carotenoid profiles of selected extracts from *Ribes nigrum.* »** (Journal of Agricultural and Food Chemistry, soumis en novembre 2010).

De nombreuses méthodes rapides et simples ont été développées pour l'évaluation de la capacité antioxydante de matrices végétales. Les méthodes les plus utilisées sont classées en deux groupes: les analyses basées sur un transfert d'électron, visualisées par un changement de couleur lors de la réduction de l'oxydant (TEAC, FRAP, CUPRAC, DPPH), et les analyses basées sur un transfert d'atome d'hydrogène, où l'antioxydant et le substrat (sonde) sont en concurrence face aux radicaux libres (TRAP, ORAC, crossing blancking assay) (Huang *et al.,* 2005). D'autres méthodes non incluses dans ces deux groupes ont aussi été développées :

TOSC, la chemiluminescence, l'électrochemiluminescence (Huang *et al.,* 2005 ; Prior *et al.*, 2005), la RPE (piégeage de l'anion superoxyde par les molécules antioxydantes). Dans un premier temps, nous avons comparé les valeurs de capacité antioxydante obtenues par les différentes méthodes (TEAC, DPPH, ORAC et RPE) pour différents composés antioxydants purs (principalement des composés phénoliques). Ensuite, nous avons proposé une méthode permettant de normaliser la valeur de la capacité antioxydante. L'approche proposée a été testée sur plusieurs boissons (jus de pomme, jus d'orange, jus de légumes, thé glacé et vin rouge): « **Comparative antioxidant capacities of phenolic compounds measured by various tests**. » (Food Chemistry **113**, 1226-1233, 2009).

Toutes les méthodes décrites ci-dessus sont des techniques basées sur l'interaction *in vitro* entre un système de production de radicaux libres, des antioxydants et une sonde. Elles ne reflètent en aucun cas les aspects biologiques. D'autres techniques utilisant une approche plus biologique ont aussi été développées. Nous en avons testé trois sur notre matériel. La première analyse utilisée est le test de résistance des globules rouges soumis à un stress oxydant en présence de composés antioxydants (Girodon *et al.,* 1997). Le deuxième modèle est l'analyse de l'activité antioxydante cellulaire (Wolfe and Liu, 2007) réalisée sur culture cellulaire. Cette analyse permet de mesurer l'inhibition de l'oxydation de colorant par des antioxydants dans des cellules en culture. Cette méthode donne une capacité antioxydante en rapport avec la prise des composés antioxydants par des cellules. Un troisième modèle permet de mettre en relation la production de radicaux libres et les situations inflammatoires. L'activation des neutrophiles avec libération de myéloperoxydase (MPO) est impliquée dans diverses pathologies chroniques inflammatoires chez l'homme et les chevaux. Une stimulation excessive des neutrophiles avec production d'espèces réactives de l'oxygène (ROS) et dégagement de la MPO mène à une activité préjudiciable d'oxydant sur les cellules, les tissus et les molécules voisines. Quelques drogues et composés phénoliques ont été testés pour limiter les effets délétères de l'activité excessive de MPO comme le resvératrol (Kohnen *et al.,* 2007). Ces effets ont été évalués par différentes analyses spécifiques à la MPO.

Les extraits de cassis (feuilles, baies et bourgeons) ont été examinés pour leur potentiel antioxydant par diverses analyses *in vitro* (DPPH, TEAC, ORAC, RPE et phénols totaux) décrites ci-dessus. Nous avons complété notre recherche en étudiant l'effet de ces extraits sur les modèles cellulaires en conditions oxydantes et, sur l'activité spécifique de la MPO équine purifiée, sur la production de ROS et sur la dégranulation des neutrophiles équins : « **Antioxidant and anti-inflammatory activities of *Ribes nigrum* extracts.** » (Journal of Nutritional Biochemistry, soumis en janvier 2011).

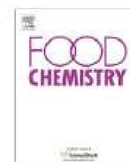
2. Articles

Food Chemistry 113 (2009) 1226–1233

Contents lists available at ScienceDirect

Food Chemistry

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



Analytical Methods

Comparative antioxidant capacities of phenolic compounds measured by various tests

Jessica Tabart [a], Claire Kevers [a,*], Joël Pincemail [b], Jean-Olivier Defraigne [b], Jacques Dommes [a]

[a] University of Liège, Plant Molecular Biology and Biotechnology Unit B22, Sart Tilman, B-4000 Liege, Belgium
[b] CREDEC, Pathology Tower B23, Sart Tilman, B-4000 Liege, Belgium

ARTICLE INFO

Article history:
Received 29 April 2008
Received in revised form 22 July 2008
Accepted 3 August 2008

Keywords:
Antioxidant
Anthocyanins
Flavan-3-ols
Flavonols
Phenolics

ABSTRACT

The purpose of this study was to compare the antioxidant capacities of standard compounds (phenolic compounds, ascorbic acid, and glutathione) as measured by various assays. Five methods were selected so as to span a diversity of technical approaches: TEAC (radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid), DPPH (radical 2,2-diphenyl-1-picryhydrazyl used to measure reducing capacity), ORAC (oxygen radical scavenging capacity), red blood cell haemolysis (protection of biological sample), and ESR (electron spin resonance for direct free radical evaluation). Most compounds showed significant differences in free radical scavenging activity according to the method used. Of the 25 tested compounds, only a few, such as myricetin and gallocatechin, gave comparable activities in the various tests. To standardise reporting on antioxidant capacity, it is proposed to use a weighted mean of the values obtained using the DPPH, ORAC, resistance to haemolysis, and ESR assays.

This strategy was used to test the antioxidant capacity of several beverages. The highest antioxidant capacity was observed for red wine, followed by green tea, orange juice, grape juice, vegetable juice, and apple juice.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

According to numerous epidemiological studies, the antioxidants from foods appear to be closely related to prevention of several pathologies, including different types of cancer, cardiovascular and neurological diseases, and aging-related disorders (Willet, 2001). Besides vitamins A, C, and E, foods of plant origin also provide our diet with other antioxidants in large amounts: carotenoids and phenolic compounds such as flavonoids (anthocyanins, flavonols, catechins, etc.). Some widely consumed beverages like tea, red wine, and cocoa are also rich in phenolic phytochemicals well known for their high antioxidant activities (Fernandez-Pachon, Villano, Troncoso, & Garcia-Parrilla, 2006; Lee, Kim, Lee, & Lee, 2003). Additionally, such compounds display antiviral and antimicrobial activity and can chelate iron, inhibit enzymes (matrix metalloproteinases), regulate gene expression, and significantly improve endothelial function (Lee et al., 2003).

It is therefore of great interest to assay properly the antioxidant capacity of the foods we consume. We thus need convenient methods for quick, simple quantification of the antioxidant capacity. The methods most commonly used to determine the total antioxidant capacity fall into two major groups: assays based on a single electron transfer reaction, monitored through a change in colour as the oxidant is reduced, and assays based on a hydrogen atom transfer reaction, where the antioxidant and the substrate (probe) compete for free radicals (Huang, Ou, & Prior, 2005).

Electron transfer reaction assays include the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric reducing ability of plasma (FRAP) assay, the copper reduction (CUPRAC) assay, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay. Hydrogen atom transfer reaction assays include the crocin bleaching assay, the total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and the oxygen radical absorbance capacity (ORAC) assay. Other methods not included in these two groups have been developed, such as the total oxidant scavenging capacity (TOSC) assay, the chemiluminescence assay, and the electrochemiluminescence assay (Huang et al., 2005; Prior, Wu, & Schaich, 2005).

Determination of the antioxidant capacity of food should take into account the overall concentrations and compositions of diverse antioxidants, because the total antioxidant capacity is due to the combined activities of diverse antioxidants, including phenolics.

The purpose of this study was to compare the antioxidant capacity values obtained by different methods (TEAC, DPPH, ORAC, red blood cell haemolysis, and ESR) for various standard

Abbreviations: AAPH, 2,2′-azobis-2-methyl-propanimidamide; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity; ESR, electron spin resonance; ORAC, oxygen radical absorbance capacity; SOD, superoxide dismutase; TEAC, Trolox equivalent antioxidant capacity.

* Corresponding author. Tel.: +32 4 366 38 46; fax: +32 4 366 38 72.
E-mail address: c.kevers@ulg.ac.be (C. Kevers).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.08.013

J. Tabart et al. / Food Chemistry 113 (2009) 1226–1233 1227

compounds (mainly phenolics) known as antioxidants. A second goal was to propose a way to standardise reporting on antioxidant capacity. The proposed approach was tested on several beverages (apple, orange, grape, and vegetable juices, ice tea, and red wine).

2. Materials and methods

2.1. Materials

Chemicals: Standards of flavonols and anthocyanins were obtained from Extrasynthese (Genay, France), flavanons, flavan-3-ols, phenolic acids, ascorbic acid, and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). Trolox [(±) 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] was obtained from Fluka Chemie GmbH, Switzerland.

Beverages: Apple and orange juices (Minute Maid®, Coca-Cola Enterprises, Belgium), grape juice (N°1, Carrefour), vegetable juice V8 (Cambells Foods Belgium), ice tea green® (Lipton), red wine (Merlot 2004, Vallé des Rappel, Chile).

2.2. Sample preparation

Stock standard solutions (1 mmol/l) in methanol were prepared for each product and appropriate dilutions were done for each type of measurement with the specific solvent of each method.

The various beverages were centrifuged at 17,000*g* for 15 min. The resulting supernatants were used directly as final samples except in the case of wine. From the wine sample, alcohol was removed by evaporation at 38 °C to avoid ethanol interference and the volume was adjusted with distilled water as proposed by Fernandez-Pachon et al. (2006).

2.3. Evaluation of antioxidant capacity

One assay used to determine the antioxidant capacity was the TEAC assay (scavenging of the radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid, ABTS) described by Re et al. (1999). The stock solution was prepared by stirring ABTS (7 mmol/l) and potassium persulfate (2.45 mol/l) in water overnight. Before use, this solution was diluted in ethanol to obtain an absorbance of 0.7 at 734 nm. In the assay, 5 μl extract, standard (0–0.1 mmol Trolox), or blank (ethanol) and 1 ml ABTS solution were mixed. The absorbance at 734 nm was determined after 4 min. For each extract, a blank with 1 ml ethanol; instead of the ABTS reagent, was included to correct for any sample absorbance at 734 nm.

Antioxidant capacity was also determined by scavenging of the radical 2,2-diphenyl-1-picryhydrazyl (DPPH) as described by Tadolini, Juliano, Piu, Franconi, and Cabrini (2000). The stock solution was prepared by stirring 75 mg DPPH in 1 l methanol overnight. In the assay, 0.75 ml extract, standard (0–0.1 mmol Trolox), or blank (methanol) and 1.5 ml DPPH solution were mixed. The absorbance of samples, standards, and blanks at 517 nm was determined after 5 min. For each extract, a blank with 1.5 ml methanol, instead of the DPPH reagent, was included to correct for any sample absorbance at 517 nm.

ORAC assays were carried out on a Fluoroskan Ascent FL Thermolabsystems (Finland) plate reader. The temperature of the incubator was set at 37 °C. Procedures were based on the method of Wu et al. (2004). Briefly, AAPH was used as a peroxyl radical generator, Trolox as a standard, and fluorescein as a fluorescent probe. Filters were used to select an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Twenty-five microlitres of diluted sample, blank, or Trolox calibration solution (0–100 μmol) were mixed with 150 μl of 4 μmol fluorescein and incubated for 15 min at 37 °C before injection of 25 μl AAPH solution (173 mmol/l). The fluorescence was measured every 2 min for 4 h. All samples were analysed in duplicate at three different dilutions. The final ORAC values were calculated using the net area under the decay curves.

Red blood cell resistance to oxidative stress (haemolysis) was measured on a reader plate (Labsystems IEMS reader MF). Blood was centrifuged at 3000*g* and 4 °C for 5 min to separate the red cells from the plasma. The cells were washed twice with PBS (pH 7.4) and centrifuged at 3000*g* for 2 min. A 5% suspension of red blood cells was used for the test. In each well of a 96-well plate, 100 μl cell suspension, 160 μl AAPH, and 30 μl sample or PBS (blank) were incubated for 3 h at 37 °C. Various concentrations of Trolox (0.01–1 mmol) were used to obtain a calibration curve. Over the 3-h incubation period, the absorbance at 450 nm was determined every 2 min. Then the time corresponding to 50% haemolysis was determined and compared with the calibration curve (Girodon et al., 1997).

For these four methods, Trolox was used as a standard and the antioxidant capacity was expressed in μmol Trolox equivalent (TE) per mmol standard or per 100 ml beverage.

Superoxide anion (O_2^{-})-scavenging capacities were measured by electron spin resonance (ESR) spectroscopy. Measurements were performed at room temperature on a JEOL – Jes-FR30 spectrophotometer. A standard superoxide-generating mixture of xanthine and xanthine oxidase (XOD) was prepared in PBS (pH 7.4). The reaction was initiated by addition of XOD and recorded after 2 min. In this system, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) dissolved in NaCl (9 g/l) was used at 44 mmol concentration as a spin-trap. Various final concentrations of SOD (0.25–1.5 U/ml) were added to the system to generate a calibration curve. The mixture finally contained 0.4 mmol xanthine, 2.5 U/ml XOD, 2.2 mmol DMPO, and 50 μl sample, PBS, or SOD in a final volume of 1 ml (Koide, Noda, Liu, Agata, & Kamada, 2000). The results were expressed in units of SOD equivalent (U SODE) per mmol standard or per 100 ml beverage.

The running conditions were as follows: 9.5 GHz frequency, 100 kHz modulation frequency, 4 mW microwave power, 335.6 mT centre field, 2500G Gauss modulation amplitude, 1 s time constant, and 4 min sweep time.

2.4. Total phenolics

Total phenolics were determined by the Folin–Ciocalteu method (Caboni et al., 1997). Although not very specific for phenolics, this protocol gives a good idea of the total phenolic content. Appropriately diluted extract (3.6 ml) was mixed with 0.2 ml Folin–Ciocalteu reagent and 3 min later, 0.8 ml sodium carbonate (20% w/v) was added. The mixture was heated at 100 °C for 1 min. After cooling, the absorbance at 750 nm was measured. Chlorogenic acid (CA) was used as standard, and the results were expressed in mg of CA equivalent (CAE) per mmol standard or per 100 ml beverage.

All samples were prepared in triplicate. Each sample analysis was performed in triplicate. All results presented are means (±SEM) of at least three independent experiments. Statistical analysis (ANOVA with a statistical significance level set at $P < 0.05$ and linear regression) was carried out with Microsoft Excel (Microsoft, Roselle, IL).

3. Results

3.1. Antioxidant capacity of standard compounds

The antioxidant capacities of flavonols, anthocyanins, flavanons, flavan-3-ols, several phenolic acids, ascorbic acid, Trolox, and reduced glutathione were evaluated by the TEAC, DPPH, ORAC, haemolysis, and ESR assays. The results clearly varied according to the assay used.

With the TEAC assay (Fig. 1), kaempferol and rutin showed the same activity as Trolox, whereas quercetin, myricetin, and especially myricetin-3-rhamnoside showed higher activities; kaempferol-3-O-glucoside showed lower activity. The tested anthocyanins showed rather similar antioxidant activities, at least twice as high as the Trolox activity. In the flavanon group, the activity of hesperidin was low and that of naringenin was almost zero. Amongst the flavan-3-ols, the activities of gallocatechin and epigallocatechin gallate were higher than that of Trolox (five and two times, respectively). Of the phenolic acids, only gallic acid differed significantly from Trolox, its activity being twice as high. The activity of ascorbic acid was lower than that of Trolox.

In the DPPH assay (Fig. 1), the antioxidant activity of flavonols was similar to that of Trolox used as the reference antioxidant, except for myricetin and myricetin-3-rhamnoside (twice as high) and kaempferol-3-O-glucide (no significant activity). The anthocyanins

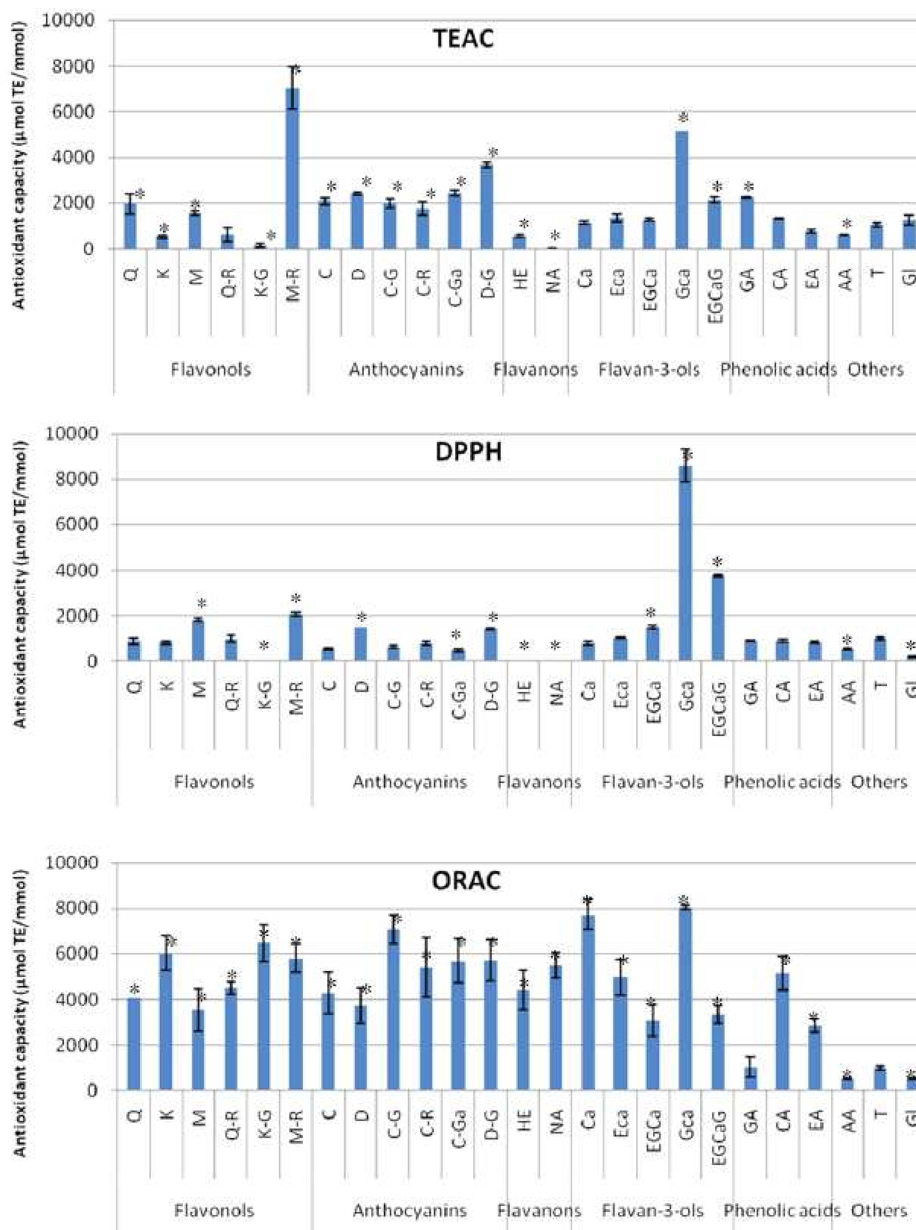


Fig. 1. Antioxidant activities (µmol TE/mmol) of standard compounds (1 mmol) determined by the TEAC, DPPH, and ORAC assays. *indicates a significant difference with respect to Trolox, determined by AVONA ($p < 0.05$). AA, ascorbic acid; C, cyanidin; C-G, cyanidin-3-O-glucoside; C-Ga, cyanidin-3-O-galactoside; C-R, cyanidin-3-O-rutinoside; Ca, catechin; CA, chlorogenic acid; D, delphinidin; D-G, delphinidin-3-O-glucoside; EA, ellagic acid; ECa, epicatechin; EGCa, epigallocatechin; EGCaG, epigallocatechin gallate; GA, gallic acid; GCa, gallocatechin; Gl, reduced glutathione; HE, hesperidin; K, kaempferol; K-G, kaempferol-3-O-glucoside; M, myricetin; M-R, myricetin-3-rhamnoside; NA, naringenin; Q, quercetin; Q-R, rutin; T, Trolox.

J. Tabart et al./Food Chemistry 113 (2009) 1226–1233 1229

showed a similar range of activities, with delphinidin and delphinidin-3-O-glucoside exhibiting higher activity and cyanidin and cyanidin-3-O-galactoside lower activity than Trolox. With this assay the variability amongst anthocyanins was greater than with the TEAC assay. The antioxidant activity of the flavanons was near zero. As with the TEAC assay, the activities of some flavan-3-ols (gallocatechin and epigallocatechin gallate) were very high (respectively, four and eight times as high as Trolox). The phenolic acids tested had the same activity as Trolox, whereas ascorbic acid and reduced glutathione displayed lower activity.

In general, the antioxidant activities measured by the ORAC assay (Fig. 1) were considerably higher than that of Trolox (4–6 times for all flavonols, anthocyanins, and flavanons, 3–8 times for the flavan-3-ols, 3–5 times for the phenolic acids). The only exceptions were gallic acid, with similar activity, and ascorbic acid and reduced glutathione, with lower activity. The antioxidant capacities of most tested compounds ranged between 3000 and 8000 µmol TE/mmol, far higher than most of the TEAC and DPPH values. It is noteworthy that kaempferol-O-glucoside (flavonol), hesperidin, and naringenin (flavanons), displaying low to no antioxidant activity in the TEAC and DPPH assays, behaved as strong antioxidants in the ORAC assay.

Ten of the compounds (four flavonols, three anthocyanins, one flavanon, and two flavan-3-ols) delayed AAPH-induced haemolysis of red blood cells significantly longer than Trolox (Fig. 2). Of the remaining compounds, kaempferol, ellagic acid, ascorbic acid,

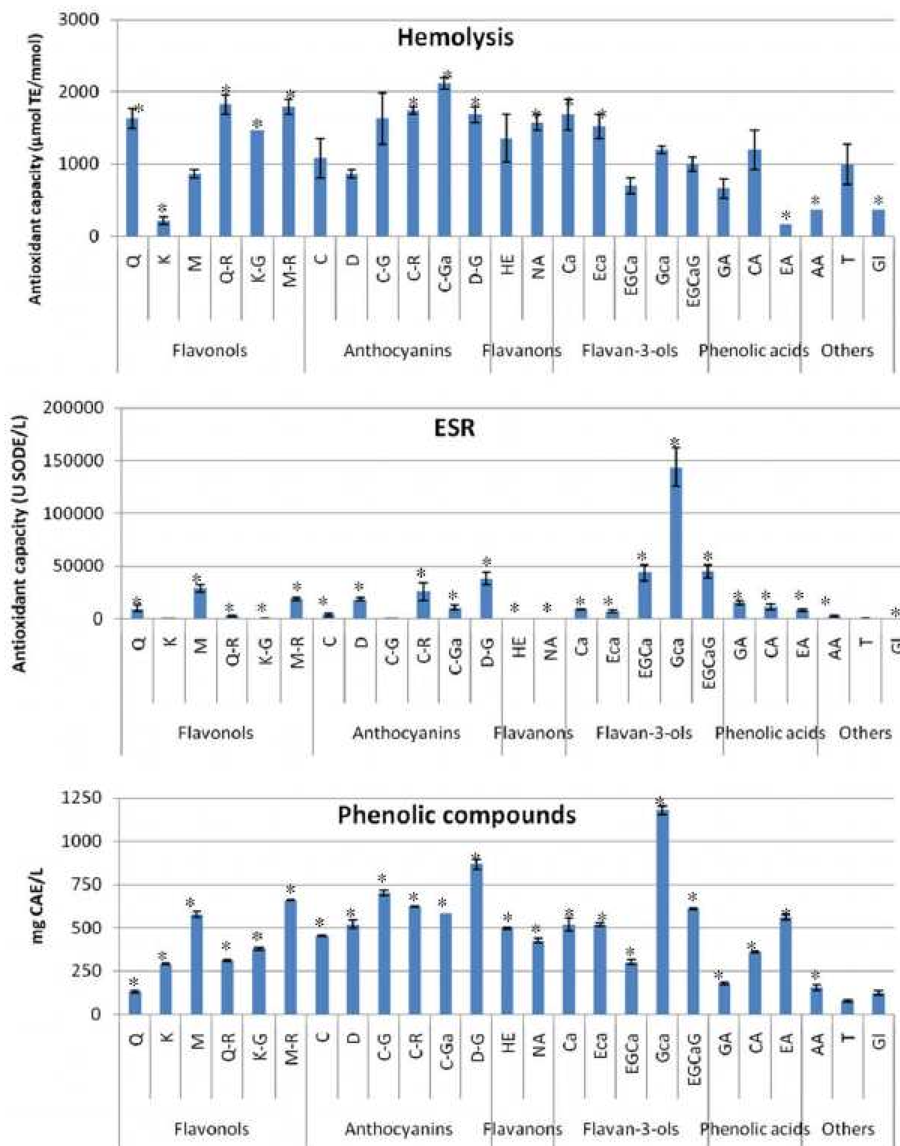


Fig. 2. Antioxidant activities of standard compounds (1 mmol) determined by the haemolysis (µmol TE/mmol) and ESR (U SODE/l) assays and Folin–Ciocalteu measurement of the phenolic content (mg CAE/l). *indicates a significant difference with respect to Trolox, as determined by AVONA ($p < 0.05$). *Abbreviations:* see legend of Fig. 1.

1230 J. Tabart et al. / Food Chemistry 113 (2009) 1226–1233

and reduced glutathione showed significantly lower antioxidant activity than Trolox, with myricetin, cyanidin, delphinidin, cyanidin-3-O-glucoside, hesperidin, epigallocatechin, gallocatechin, epigallocatechin gallate, and gallic acid, and chlorogenic acid displaying no significant difference with respect to the reference compound.

With the ESR method, Trolox exhibited very low antioxidant activity (Fig. 2), so the data were expressed using SOD (superoxide anion scavenger) as the reference antioxidant. Compounds showing a significantly higher antioxidant capacity than Trolox included the flavanols quercetin, myricetin, and myricetin-3-rhamnoside, and (just barely) rutin, the anthocyanins, the flavan-3-ols, the phenolic acids, and (just barely) ascorbic acid. The highest activities were recorded for the flavan-3-ols gallocatechin, epigallocatechin, and epigallocatechin gallate. Although this assay is quite different from the DPPH assay, it is noteworthy that these two assays yielded quite similar profiles.

All the phenolic compounds tested reacted with Folin–Ciocalteu reagent (Fig. 2). Amongst the flavonols, the intensity of the reaction varied by a factor of 5 from quercetin (which gave the lowest result of all phenolics tested) to myricetin-3-rhamnoside (myricetin also gave a strong reaction). The various anthocyanins also reacted differently but the differences were less pronounced. The flavanons reacted similarly; the results being near the average for all tested compounds. Amongst the flavan-3-ols, gallocatechin reacted about twice as strongly as the other tested compounds. The various phenolic acids also reacted quite differently. Although the non-phenolic compounds also reacted with the Folin–Ciocalteu reagent, the results were lower than those obtained with most phenolics.

3.2. Relative antioxidant capacities of various beverages

Apple, orange, grape, and vegetable juice, ice green tea, and red wine were tested for their antioxidant capacity by the five different methods (Fig. 3). Of all the assays, DPPH clearly emerged as the least sensitive. The TEAC, ORAC, and haemolysis assays generally yielded quite similar results for each beverage, with the exception of grape juice, in which case the ORAC assay gave a much higher result than the TEAC and haemolysis assays. The ESR method compared variably with the other methods according to the beverage studied: with red wine, grape juice, and ice tea the antioxidant activity determined by ESR was very high, but with vegetable, apple, and orange juice it was low.

The measured phenolic compound content was highest in wine and orange juice and lowest in apple and vegetable juice (Fig. 4).

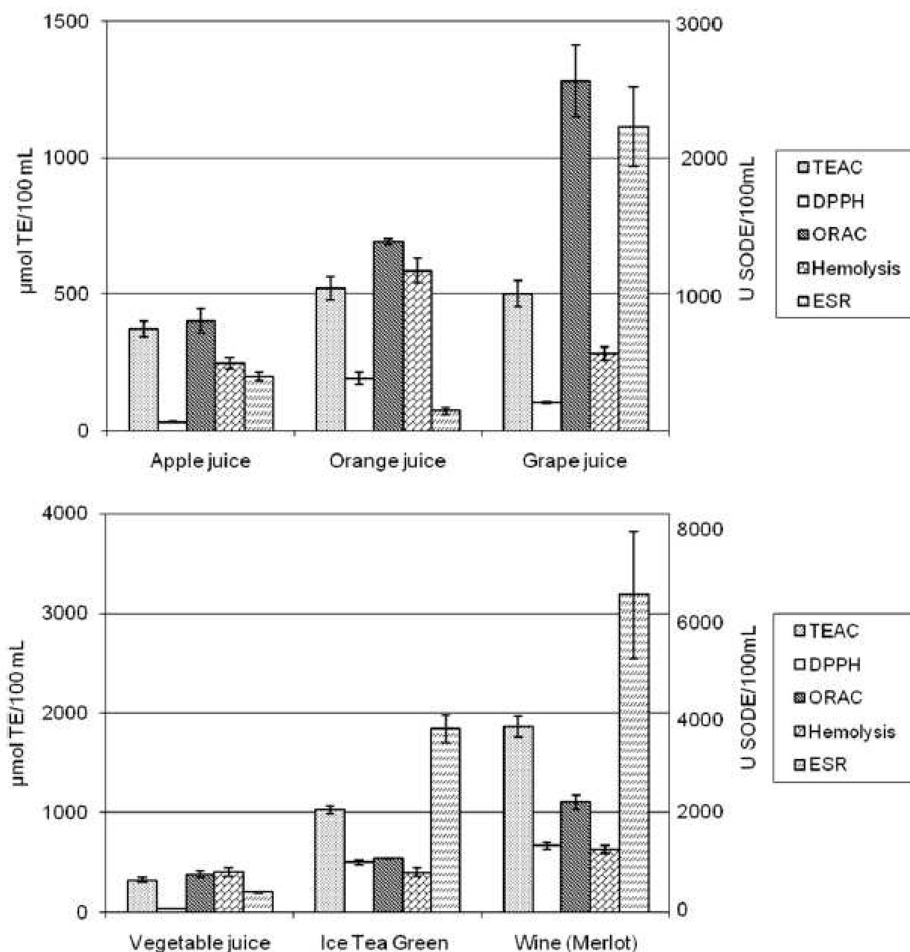


Fig. 3. Evaluation of the antioxidant capacities of various beverages by five methods: TEAC, DPPH, ORAC, haemolysis (µmol TE/100 ml), and ESR (U SODE/100 ml).

J. Tabart et al. / Food Chemistry 113 (2009) 1226–1233 1231

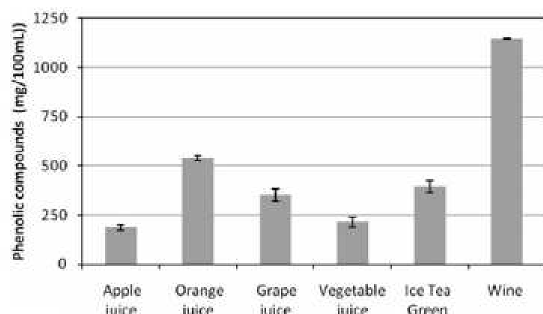


Fig. 4. Phenolic contents of various beverages (mg CAE/100 ml).

4. Discussion

4.1. Antioxidant capacities of standard compounds with the different methods

The antioxidant activities of some phenolic compounds have been determined previously by the TEAC assay under various experimental conditions, with results varying greatly from author to author. Our results additionally highlight the variability of results obtained, whatever the flavonoid family considered, according to the assay method used (see Table 1 for values normalised with respect to Trolox). As generally observed (Kevers et al., 2007), we noted a slight correlation ($r^2 = 0.356$) between the TEAC and DPPH values obtained for the various phenolic compounds, but the DPPH assay generally yielded a value closer to the Trolox activity (lower average, Table 1). TEAC and DPPH are commonly used to assess antioxidant activity *in vitro*. Reduction of the radicals used by a hydrogen-donating antioxidant is monitored through a decrease in optical density. The fact that both reactions have same mechanism explains the correlation between their results (Prior et al., 2005).

Table 1
Antioxidant activities normalised with respect to the Trolox measurement

		ABTS	DPPH	ORAC	Haemolysis	ESR	Weighted average
Flavonols	Q	1.8	0.9	4.2	1.6	16.0	0.9
	K	0.5	0.8	6.2	0.2	2.7	0.5
	M	1.5	1.8	3.6	0.9	47.1	1.1
	Q-R	0.6	1.0	4.6	1.8	5.1	0.9
	K-G	0.2	0.0	6.6	1.5	0.6	0.7
	M-R	6.6	2.0	6.0	1.8	30.9	1.3
Anthocyanins	C	2.0	0.5	4.4	1.1	6.4	0.6
	D	2.3	1.5	3.8	0.9	30.5	0.9
	C-G	1.9	0.6	7.3	1.6	2.6	0.9
	C-R	1.7	0.8	5.5	1.7	42.0	1.1
	C-Ga	2.3	0.5	5.8	2.1	17.4	1.0
	D-G	3.5	1.4	5.9	1.7	61.4	1.4
Flavanons	HE	0.5	0.0	4.5	1.4	0.4	0.5
	NA	0.0	0.0	5.6	1.6	0.2	0.6
Flavan-3-ols	Ca	1.1	0.8	7.9	1.7	15.3	1.1
	ECa	1.3	1.0	5.1	1.5	11.5	0.9
	EGCa	1.2	1.5	3.1	0.7	70.7	1.2
	GCa	4.9	8.5	8.3	1.2	232.7	4.2
	EGCaG	2.0	3.7	3.4	1.0	72.5	1.7
Phenolic acids	GA	2.1	0.9	1.0	0.7	24.9	0.6
	CA	1.3	0.9	5.3	1.2	18.4	0.9
	EA	0.7	0.8	2.9	0.2	13.5	0.5
Others	AA	0.6	0.5	0.5	0.4	4.6	0.2
	Gl	1.2	0.2	0.5	0.4	0.3	0.2
Average		1.7	1.3	4.7	1.2	30.3	

The right-hand column shows the weighted average (mmol TE/mmol) obtained by (1) dividing the antioxidant capacity of each compound, as determined by the specified method, by the average capacity determined for the whole set of compounds by the same method (bottom row), (2) summing the four (DPPH, ORAC, haemolysis, and ESR) results of this calculation, and (3) dividing the sum by four. *Abbreviations:* see legend of Fig. 1.

There was no correlation between either the TEAC or the DPPH data and the ORAC data, whatever the class of phenolics considered ($r^2 < 0.1$, Table 1). ORAC data have a different meaning, the results reflecting more than just radical scavenging. In this assay, free radicals from the thermal decomposition of AAPH are generated at a constant rate throughout the test. The ORAC assay is the only method combining both an inhibition time and a degree of inhibition, and it also has the particularity of resulting in a complete reaction. With this test, all the phenolic compounds tested displayed an antioxidant activity more than three times as high as the Trolox activity, whilst ascorbic acid and reduced glutathione showed lower values (as with the TEAC and DPPH assays). For each of these methods, differences between phenolic compounds can be explained largely by the specific structure of each compound (number of OH groups, side chain on benzoic acid, etc.) (Cao, Sofic, & Prior, 1997; Rice-Evans, Miller, & Paganga, 1996; Wang, Cao, & Prior, 1997), rather than by the phenolic family to which it belongs (Paquay, Haenen, Korthouwer, & Bast, 1997).

Electron spin resonance (ESR) spectroscopy determines the presence of unpaired electrons of oxygen, and is commonly used for free radical evaluation. It has been applied to some foods to measure free radical production and to establish their antioxidant capacities (Noda et al., 1997). Phenolic compounds are the most prevalent antioxidant phytochemicals in the plant kingdom and reportedly possess both singlet oxygen quenching activity and radical scavenging activity (Guo, Zhao, Shen, Hou, & Xin, 1999). Their reactivity in this assay was high, far higher than that of Trolox (on the average, 30 times as high), with the exception of flavanons. The ESR and DPPH profiles of this set of compounds were quite similar, a very high correlation ($r^2 = 0.917$) being found between the results obtained by these two methods.

In vitro oxidative haemolysis of human red blood cells is used as a model to study free radical-induced damage of biological membranes and the protective effect of phenolic compounds and known antioxidants. Here, most of the phenolic compounds inhibited oxidative damage as efficiently as, or more efficiently than Trolox (Table 1). Dai, Miao, Zhou, Yang, and Liu (2006) have previously reported that flavonols and their glycosides are effective antioxidants protecting human red blood cells from free radical-induced oxidative haemolysis. In our study, kaempferol was not effective, but as mentioned by these authors, this may be due to a dose-dependency effect (Dai et al., 2006). The protective effect of flavonoids can be linked to their binding to the plasma membrane (Blasa, Candiracci, Accorsi, Piacentini, & Piatti, 2007) and their ability to penetrate lipid bilayers (Lopez-Revuelta, Sanchez-Gallego, Hernandez-Hernandez, Sanchez-Yague, & Llanillo, 2006). The results obtained by this method were different from those obtained with the TEAC, DPPH, and ESR assays ($r^2 < 0.08$). A slight correlation ($r^2 = 0.396$) was found with the ORAC assay. The antioxidant effects of ascorbic acid and reduced glutathione were always very low.

The Folin–Ciocalteu method, generally used to assay the total phenolic compound content also measures the total reducing capacity of a sample. Total phenolics generally correlate with redox and antioxidant capacities measured by the TEAC or DPPH method (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2006; Tabart et al., 2007) or with ORAC values (Andre et al., 2007). For the standard compounds used here, the correlation with the other methods used was never very good ($r^2 < 0.5$). Dissociation of the phenolic proton leads to a phenolate anion, which is capable of reducing the Folin–Ciocalteu reagent. Yet this method is not specific to phenolic com-

pounds. Many non-phenolic compounds, such as ascorbic acid (Fig. 2) and saccharides present in fruits and vegetables, can reduce the Folin–Ciocalteu reagent (Stratil, Klejdus, & Kuban, 2007). Ethanol, furthermore, can interfere with antioxidant capacity measurement by the ORAC assay (Fernandez-Pachon et al., 2006).

4.2. A standardised antioxidant capacity: weighted average based on four methods

As stressed by Frankel and Meyer (2000) and Huang et al. (2005), no single method is adequate for evaluating the antioxidant capacity of foods, since different methods can yield widely diverging results. Various methods, based on different mechanisms, must be used. Here we have used assays based on four different principles: DPPH or TEAC (reducing capacity), ORAC (peroxyl radical scavenging capacity), haemolysis (protection of a biological sample), and ESR (free radical evaluation). To standardise reports on antioxidant capacity, we propose using an average of the results obtained by the DPPH, ORAC, haemolysis, and ESR methods with Trolox as reference. A simple mathematical mean is not adequate, because two of the four methods (ORAC and ESR) gave much higher values because of the poor performance of Trolox in these assays. This would give those assays undue preponderance in the mean. We therefore propose calculating a global antioxidant capacity as a weighted mean of the results obtained by the DPPH, ORAC, resistance to haemolysis, and ESR assays.

As the antioxidants analysed in this paper are the major antioxidants found in plants, we propose calculating, for each compound, a weighted average where the weighting factor applied to the antioxidant capacity determined by a given method is equal to 1 divided by the average capacity determined for this whole set of compounds by the same method. Results of this calculation are shown in Table 1 (last column). On this basis, gallocatechin appears as the best antioxidant, with a weighted average of 4.2. Some other flavan-3-ols (epigallocatechin and epigallocatechin gallate), anthocyanins (delphinidin-3-*O*-glucoside), and flavonols (myricetin and myricetin-3-rhamnoside) also show a high antioxidant capacity when it is calculated in this way.

4.3. Antioxidant capacity of beverages

As discussed above, assessment of the antioxidant capacity of food matrices requires the parallel use of several methods. Here, we have applied the TEAC, DPPH, ORAC, resistance to haemolysis, and ESP assays to various beverages. Although the DPPH results were almost always lower than the others, the results of the TEAC, ORAC, and haemolysis assays were quite similar in the case of apple, orange, and vegetable juice (Fig. 3). ESR gave very high results (grape juice, ice tea green, and especially wine). Of all the beverages tested, wine showed the best antioxidant capacity regardless of the method used. Phenolic compounds can explain this high antioxidant capacity (Fernandez-Pachon, Villano, Garcia-Parrilla, & Troncoso, 2004; Fernandez-Pachon et al., 2006; Kevers et al., 2007; Mullen, Marks, & Crozier, 2007). By calculating a weighted average of the results obtained in the four assays as described above it was possible to rank the beverages as follows: apple (84 µmol TE/100 ml) < vegetable juice (117 µmol TE/100 ml) < grape juice (176 µmol TE/(100 ml) < orange juice (198 µmol TE/100 ml) < ice green tea (256 µmol TE/100 ml) < wine (402 µmol TE/100 ml). Similar results have been reported for apple, orange, and grape juice on the basis of TRAP and TEAC assays (Pellegrini et al., 2003), but not when the FRAP assay was used. It would be interesting to know the type of compounds responsible for the antioxidant capacity. After specific analysis of different compounds present in red wine, Fernandez-Pachon et al. (2004) showed that 50% of the total radical scavenging activity (TEAC, DPPH assays) was attributable to polymeric phenolic compounds. The remaining activity was mainly attributed to anthocyanins and flavan-3-ols, followed by phenolic acids and flavonols.

Given the complex composition of foods, separating each antioxidant compound and studying it individually is costly and inefficient, notwithstanding the possible synergistic or antagonistic interactions amongst the antioxidant compounds in a food mixture. For instance, gallic acid and epigallocatechin gallate, the major antioxidants in tea, show strong antioxidant activity but may also act as pro-oxidants (Johnson & Loo, 2000).

5. Conclusion

Various methods, based on different mechanisms, must be used in parallel to evaluate the antioxidant capacity of compounds or beverages, since different methods can give very different results. We confirm here that different methods can give widely divergent results. We propose a means of standardising reporting on plant antioxidant capacities by using a weighted mean of the results of four methods based on different principles: DPPH (reducing capacity), ORAC (peroxyl radical scavenging capacity), haemolysis (protection of a biological sample), and ESR (estimation of free radicals). This approach highlighted considerable differences amongst the phenolic compounds, which all showed a greater antioxidant capacity than ascorbic acid. The same approach enabled us to rank various beverages according to their antioxidant activity.

Acknowledgments

We thank HEPL – Rennequin Sualem and J. Bleret for technical assistance. J.T. gratefully acknowledges the support of the Luxemburg Ministry of National Education. The skillful assistance of the CEDEVIT staff (supported by the Regional Government of Wallonia through the APE program) was greatly appreciated.

References

Andre, C. M., Ghislain, M., Bertin, P., Oufir, M., Herrera, M., Hoffmann, L., et al. (2007). Andean potato cultivars as a source of antioxidant and mineral micronutrients. *Journal of Agricultural and Food Chemistry, 55*, 366–378.

Blasa, M., Candiracci, M., Accorsi, A., Piacentini, M. P., & Piatti, E. (2007). Honey flavonoids as protection agents against oxidative damage to human red blood cells. *Food Chemistry, 104*, 1635–1640.

Caboni, E., Tonelli, M. G., Lauri, P., Iacovacci, P., Kevers, C., Damiano, C., et al. (1997). Biochemical aspects of almond microcuttings related to in vitro rooting ability. *Biologia Plantarum, 39*, 91–97.

Cao, G., Sofic, E., & Prior, R. L. (1997). Free radic. Antioxidant and prooxidant behavior of flavonoids: Structure–activity relationships. *Biologia Medica, 22*, 749–760.

Dai, F., Miao, Q., Zhou, B., Yang, L., & Liu, Z. L. (2006). Protective effects of flavonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells. *Life Sciences, 78*, 2488–2493.

Fernandez-Pachon, M. S., Villano, D., Garcia-Parrilla, M. C., & Troncoso, A. M. (2004). Antioxidant activity of wines and relation with their polyphenolic composition. *Analytica Chimica Acta, 513*, 113–118.

Fernandez-Pachon, M. S., Villano, D., Troncoso, A. M., & Garcia-Parrilla, M. C. (2006). Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with antioxidant activity. *Analytica Chemica Acta, 563*, 101–108.

Frankel, E., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture, 80*, 1925–1941.

Girodon, F., Blache, D., Monget, A. L., Lombart, M., Brunet-Lecompte, P., Arnaud, J., et al. (1997). Effect of two-year supplementation with low doses of antioxidant vitamins and/or minerals in elderly subjects on levels of nutrients and antioxidant defence parameters. *Journal of the American College of Nutrition, 4*, 357–365.

Guo, Q., Zhao, B., Shen, S., Hou, J., & Xin, W. (1999). ESR study on the structure–antioxidant activity relationship of tea flavan-3-ols and their epimers. *Biochimica et Biophysica Acta, 1427*, 13–23.

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

Johnson, M. K., & Loo, G. (2000). Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutation Research, 459*, 211–218.

Kevers, C., Falkowski, M., Tabart, J., Defraigne, J. O., Dommes, J., & Pincemail, J. (2007). Evolution of antioxidant capacity during storage of selected fruits and vegetables. *Journal of Agricultural and Food Chemistry, 55*, 8596–8603.

Koide, T., Noda, H., Liu, W., Agata, T., & Kamada, H. (2000). Determination of superoxide scavenging activity of a sample containing xanthine oxidase inhibitor by ESR spin trapping. *Analytical Sciences, 16*, 1029–1032.

Lee, K. W., Kim, Y. J., Lee, H. J., & Lee, C. Y. (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *Journal of Agricultural and Food Chemistry, 51*, 7292–7295.

Lopez-Revuelta, A., Sanchez-Gallego, J. I., Hernandez-Hernandez, A., Sanchez-Yague, J., & Llanillo, M. (2006). Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress. *Chemico-Biological Interactions, 161*, 79–91.

Mullen, W., Marks, S. C., & Crozier, A. (2007). Evaluation of phenolic compounds in commercial fruit juices and fruit drinks. *Journal of Agricultural and Food Chemistry, 55*, 3148–3157.

Noda, Y., Anzai, K., Mori, A., Kohno, M., Shinmei, M., & Packer, L. (1997). Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized jes-fr30 ESR spectrometer system. *Biochemistry and Molecular Biology International, 42*, 35–44.

Paquay, J. B., Haenen, G. R., Korthouwer, R. E., & Bast, A. (1997). Peroxynitrite scavenging by wines. *Journal of Agricultural and Food Chemistry, 45*, 3357–3358.

Pellegrini, N., Serafini, M., Colombi, B., Del Rio, D., Salvatore, S., Bianchi, M., et al. (2003). Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *Journal of Nutrition, 133*, 2812–2819.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*, 4290–4302.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*, 933–956.

Stratil, P., Klejdus, B., & Kuban, V. (2007). Determination of phenolic compounds and their antioxidant activity in fruits and cereals. *Talanta, 71*, 1741–1751.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J. O., & Dommes, J. (2006). Antioxidant capacity of black currant varies with organ, season, and cultivar. *Journal of Agricultural and Food Chemistry, 54*, 6271–6276.

Tabart, J., Kevers, C., Sipel, A., Pincemail, J., Defraigne, J. O., & Dommes, J. (2007). Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage. *Food Chemistry, 105*, 1268–1275.

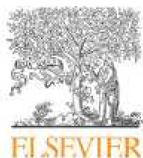
Tadolini, B., Juliano, C., Piu, L., Franconi, F., & Cabrini, L. (2000). Resveratrol inhibition of lipid peroxidation. *Free Radical Research, 33*, 105–114.

Wang, H., Cao, G., & Prior, R. L. (1997). The oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry, 45*, 304–309.

Willet, W. C. (2001). *Eat, drink, and be healthy – The Harvard Medical School guide to healthy eating*. New York: Simon and Schuster.

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *Journal of Agricultural and Food Chemistry, 52*, 4026–4037.

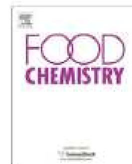
Food Chemistry 120 (2010) 607–614



Contents lists available at ScienceDirect

Food Chemistry

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



Analytical Methods

Evaluation of spectrophotometric methods for antioxidant compound measurement in relation to total antioxidant capacity in beverages

Jessica Tabart [a], Claire Kevers [a,*], Joël Pincemail [b], Jean-Olivier Defraigne [b], Jacques Dommes [a]

[a] University of Liège, Plant Molecular Biology and Biotechnology Unit, B22, Sart Tilman, B-4000 Liege, Belgium
[b] University of Liège, CREDEC, Pathology Tower, B23, Sart Tilman, B-4000 Liege, Belgium

A R T I C L E I N F O

Article history:
Received 22 October 2008
Received in revised form 27 July 2009
Accepted 14 October 2009

Keywords:
Antioxidant
Flavonols
Flavonoids
Anthocyanins
Flavanols
Ascorbic acid

A B S T R A C T

The validity of different colorimetric methods used to quantify various families of antioxidant compounds was evaluated with standard compounds. The colorimetric tests for global evaluation of flavonoids, anthocyans, and flavanols were found generally unreliable, as reactions could be different for individual compounds within a family (anthocyanins or flavonols or flavan-3-ols) and not specific to one family. In the flavonoid test, for example, flavonols reacted very well, anthocyanins did not react, and flavanons reacted only slightly. The same methods were applied also to beverages known for their antioxidant content (apple, orange, grape, and vegetable juices, ice tea, and red wine) and the data were compared with the results of HPLC analysis of specific compounds. The values obtained in a colorimetric test were generally higher than the sum of the values obtained for the corresponding individual compounds by HPLC analysis, mainly because other compounds can interfere with the colorimetric tests. For example, in wine, anthocyanin concentrations obtained by colorimetric test were 9068 ± 1407 μmol/100 ml (mean ± SEM), higher than the sum of the six main anthocyanidins detected by HPLC, only 41 μmol/100 ml. The relative antioxidant capacity values determined for beverages on the basis of colorimetric tests could exceed by far the values previously measured in radical-scavenging tests (for instance, the antioxidant capacity attributable to anthocyans in wine on the basis of the colorimetric test was 50 times higher than the total antioxidant capacity measured by the ORAC assay). In conclusion, colorimetric tests for flavonoids, anthocyans, and flavanols appeared generally unreliable for estimating their content and thus the antioxidant capacity reliable to these compounds.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

The study of phytochemicals as bioactive food components is of major interest. Ecological and epidemiological studies indicate that high consumption of fruits and vegetables has protective effects against degenerative diseases (e.g. cancer, cardiovascular disease) in which development oxidative stress – resulting in enhanced free radical activity – plays a key role (Arts & Hollman, 2005). In addition to supplying nutrients such as mineral salts, trace elements, fibers, folates, etc., diets rich in fruits and vegetables supply antioxidants that can limit oxidative damage to lipids, DNA, and proteins and which may be largely responsible for the benefits ascribed to such diets. Although much of the antioxidant capacity of a fruit or vegetable can be attributed to compounds such as vitamins C and E, carotenoids, and glutathione, high antioxidant activities have also been recorded for members of the large polyphenol family (which includes flavonoids such as flavones, isoflavones, flavanones, anthocyanins, catechin, and isocatechin), frequent components of the human diet (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009; Villano, Fernandez-Pachon, Troncoso, & Garcia-Parrilla, 2005).

Mullen, Marks, and Crozier (2007) suggest that protective effects against a number of degenerative diseases could be enhanced by regular consumption of a combination of juices rich in phenolics, so as to provide an abundance and diversity of such compounds. In addition to juices derived from purple grapes, grapefruit, cranberries, and apples, red wine and green tea are also viewed as exceptional dietary sources of various classes of phenols, including benzoic and cinnamic acid derivatives, flavan-3-ols, flavonols, and anthocyanins (Bravo, 1998).

The phenolic compound content of plant materials can be determined either by separation methods (e.g. high-performance liquid chromatography, gas chromatography, capillary zone electrophoresis) as a set of individual substances or by spectrophotometric assays detecting specific groups of chemically similar reactive compounds. Chromatographic determination is very precise, accurate, and informative, but identifying all phenolic compounds

* Corresponding author. Tel.: +32 43 66 38 46; fax: +32 43 66 38 72.
E-mail addresses: Jessica.Tabart@student.ulg.ac.be (J. Tabart), c.kevers@ulg.ac.be (C. Kevers), J.Pincemail@chu.ulg.ac.be (J. Pincemail), JO.Defraigne@chu.ulg.ac.be (J.-O. Defraigne), j.dommes@ulg.ac.be (J. Dommes).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.10.031

in a specific plant material can be daunting in terms of time consumed and analysis costs (Stratil, Klejdus, & Kuban, 2006).

One aim of the present study was to test the validity of existing colorimetric methods for evaluating various families of antioxidant compounds, mainly phenolics, using standard antioxidant compounds (flavonoids, anthocyanins, catechins, ascorbic acid, and glutathione). Another was to apply the same methods to several beverages (apple, orange, grape, and vegetable juices, ice tea, and red wine) and to compare the results with those obtained by HPLC analyses. We have further sought to relate the measured antioxidant levels in beverages to previously established antioxidant capacity values (Tabart et al., 2009), with a view to determining how different polyphenol families contribute to the total antioxidant capacity.

2. Materials and methods

2.1. Materials

Chemicals. Standards of flavonols and anthocyanins were obtained from Extrasynthese (Genay, France). Flavanons, flavan-3-ols, phenolic acids, ascorbic acid, glutathione, and Trolox [(±)6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] were purchased from Sigma Chemical Co. (St. Louis, MO).

Beverages. Apple and orange juices (Minute Maid®, Coca-Cola Enterprises, Brussels, Belgium), purple grape juice (No. 1, Carrefour, Brussels, Belgium), vegetable juice V8 (Cambells Foods Belgium, Puurs, Belgium), ice green tea® (Lipton, Unilever Belgium, Brussels, Belgium), red wine (Merlot 2004, Vallé des Rappel, Chile) were tested.

2.2. Sample preparation

Stock standard solutions (1 mmol) in methanol were prepared for each standard and appropriate dilutions were done for each type of measurement, with the specific solvent used for polyphenol quantification (colorimetric tests, HPLC).

The various beverages were centrifuged (Beckman Coulter Avanti JE) at 17,000*g* for 15 min and at 20 °C. The resulting supernatants were used directly as final samples except for wine, in which case alcohol was removed by evaporation under reduced pressure at 38 °C and the volume adjusted with distilled water as proposed by Fernandez-Pachon, Villano, Troncoso, and Garcia-Parrilla (2006) to avoid ethanol interference.

The extracts were hydrolyzed before measurement of flavonol aglycons and anthocyanins, as proposed by Häkkinen, Kärenlampi, Jeinonen, Mykkänen, and Torronen (1998). In short, extracts were incubated for one hour in the presence of 1.2 M HCl, MeOH (50%), and 300 mg/L ascorbic acid.

2.3. Colorimetric tests

Total flavonoid content was measured as in Lamaison and Carnat (1990). Appropriately diluted extracts (1 mL) were mixed with 1 mL reagent ($AlCl_3 \cdot 6H_2O$, 2% in methanol). The absorbance at 430 nm (spectrophotometer Kontron Unikon 931) was measured 10 min later. Quercetin was used as standard, and results are expressed in μmol quercetin equivalents (QE) per mmol standard or per 100 mL beverage.

Total anthocyan quantification was performed by the pH-differential method (Nielsen, Haren, Magnussen, Dragsted, & Rasmussen, 2003). The extract was diluted in a pH 1.0 solution (0.1 M HCl, 25 mM KCl) and in a pH 4.5 solution (0.4 M CH_3COONa). The absorbance of each mixture was then measured at 535 and 700 nm against distilled water. The value $(Abs_{535} - Abs_{700})_{pH1.0} - (Abs_{535} - Abs_{700})_{pH4.5}$ corresponds to the absorbance due to anthocyans. Calculation of anthocyan concentrations was based on a cyanidin 3-glucoside (molar extinction coefficient 25.965 cm^{-1}, molecular weight 449.2 g mol^{-1}). Results are expressed in μmol cyanidin 3-glucoside equivalents per mmol standard or per 100 mL beverage.

Total flavanol content was evaluated by the vanillin assay (Nakamura, Tsuji, & Tonogai, 2003), in which one molecule of vanillin reacts with one molecule of flavanol to produce a red chromophore. The conversion was monitored as an increase in the absorbance at 500 nm. One volume of sample diluted in methanol was mixed with 2.5 volumes of vanillin (1% in methanol) and 2.5 volumes of HCl (9 M in methanol). The mixture was incubated for 20 min at 35° before analysis. For each sample, a blank was used in which the vanillin solution was replaced with methanol alone. A standard curve was constructed with catechin.

Reduced ascorbic acid (AA) was measured by the 2,6-dichloroindophenol (DCIP) method of the Association of Vitamin Chemists (1961). In this assay, briefly, each molecule of vitamin C converts a molecule of DCIP into a molecule of $DCIPH_2$, and this conversion is monitored as a decrease in the absorbance at 520 nm. A standard curve was prepared with a series of known AA concentrations. One milliliter of diluted sample (in 5% metaphosphoric acid) or ascorbic acid calibration solution was mixed with 500 μL 10% metaphosphoric acid. With 600 μL of this mixture were mixed 300 μL citric acid, citrate buffer (pH 4.15) and 300 μL DCIP (0.1 mg mL^{-1}). Optical density blanching was used; for each sample, the blank value was determined after addition of 60 μL AA (1 mg mL^{-1}) so as to measure the inference due to the sample color. Results are expressed in μmol AA per mmol standard or per 100 mL beverage.

Reduced glutathione (GSH) was oxidized with 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to yield oxidized glutathione (GSSG) and 2-nitro-5-thiobenzoic acid (TNB). GSSG was reduced to GSH by action of the highly specific glutathione reductase and NADPH. TNB formation was monitored as the rate change in absorbance at 412 nm and was proportional to total glutathione (GSH + GSSG). Oxidized glutathione was determined after removal of reduced glutathione with N-ethylmaleimide. GSH was determined by subtracting GSSG from total glutathione (Franck et al., 1998).

2.4. Separation and measurement of compounds by HPLC

Analyses of extracts were performed in a liquid Elite Lachrom Merck Hitachi chromatograph equipped with an L2450 photodiode array detector (sampling period: 400 ms, spectral bandwidth: 4 nm). Separation was carried out with a LiChroCART steel cartridge (250 mm × 4.6 mm) (Merck) filled with 5-μm RP 18 particles and maintained thermostatically at 30 °C (for flavonols and flavan-3-ols) or 40° (for anthocyanidins).

For *flavonol aglycone* analysis of hydrolyzed extracts, the mobile phase was a linear gradient of water–acetonitrile (50:50, adjusted to pH 1.8 with perchloric acid) and water–acetonitrile (95:5, adjusted to pH 1.8 with perchloric acid), at a flow rate of 1.2 mL/min as previously described by Tabart, Kevers, Pincemail, Defraigne, and Dommes (2006). Spectra were recorded at 365 nm.

For *anthocyanidin analysis* of hydrolyzed extracts, the mobile phase was a gradient of water–acetonitrile–formic acid (87:3:10 to 40:50:10) at a flow rate of 0.8 mL/min as previously described by Dutruc-Rosset (2003). Spectra were recorded at 518 nm.

For *flavan-3-ol analysis*, the mobile phase described by Sharma, Gulate, Ravindranath, and Kumar (2005) was modified. It was composed of acetonitrile (90%), orthophosphoric acid (0.1%) and water. A flow gradient was used: 0.4 mL min^{-1} for 3 min, a linear decrease to 0.3 mL min^{-1} at 10 min and to 0.2 mL min^{-1} at 13 min, a steady state to 25 min, followed by a linear increase to 0.4 mL min^{-1} at 35 min. Spectra were recorded at 230 nm.

J. Tabart et al. / Food Chemistry 120 (2010) 607–614 609

All samples were prepared in triplicate and each sample analysis was performed in triplicate. All results presented are the means (±SEM). One-way analysis of variance (ANOVA-1) was used to determine significant differences between each standard compound or each beverage in specific spectrophotometric tests. ANOVA-1 was also applied to determine significant difference between the flavonoid content in various beverages tested by HPLC. Differences at $P < 0.05$ were considered significant. Statistical analysis was performed using Microsoft Excel (Microsoft, Roselle, IL).

3. Results

3.1. Measurement of standard compounds by colorimetric tests

Antioxidant compounds (flavonols, anthocyanins, flavanons, flavan-3-ols, several phenolic acids, ascorbic acid, and reduced glutathione) were evaluated by colorimetric assays purporting to measure specifically the total flavonoid, anthocyan, flavanol, ascorbic acid, or glutathione content. As shown in Fig. 1, assay performances varied according to the compound studied.

Flavonoids. The reactions of the tested standards are illustrated in Fig. 1A. Flavonols reacted well in this test described by Lamaison et al. (1990). The reaction was quite similar for three aglycons (quercetin, kampferol and myricetin) and the quercetin glycoside (rutin). Kaempferol-3-O-glucoside was less reactive. Slight reactivity (less than 10% of that observed with quercetin) was seen with the flavanons, two phenolic acids (chlorogenic acid and ellagic acid), and glutathione. Other groups measured did not cross-react with this test.

Anthocyans. The various anthocyanins tested reacted differently with this method (Fig. 1B). Among the anthocyanidins, the strongest reaction was observed with delphinidin and malvidin. This

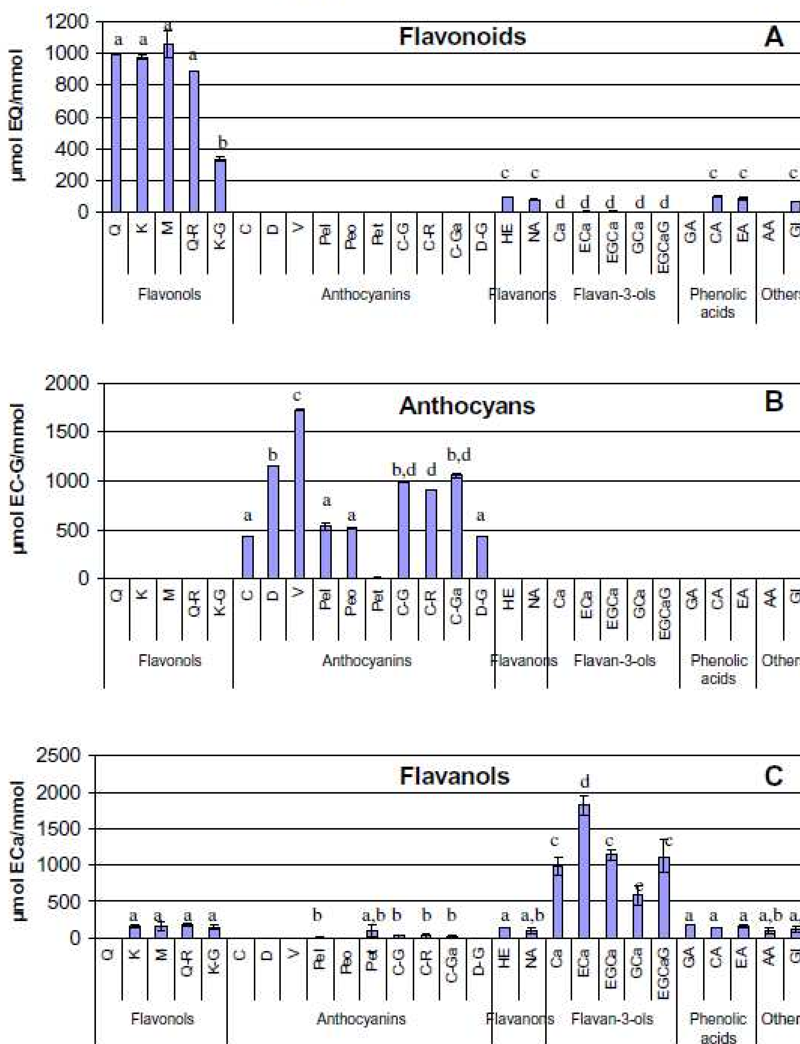


Fig. 1. Detection of standard compounds (1 mmol) determined by the flavonoid (A, µmol EQ/mmol), anthocyan (B, µmol EC-G/mmol), and flavanol (C, µmol ECa/mmol) colorimetric tests (error bars represent +SEM). AA: ascorbic acid; C: cyanidin; C-G: cyanidin-3-O-glucoside; C-Ga: cyanidin-3-O-galactoside; C-R: cyanidin-3-O-rutinoside; Ca: catechin; CA: chlorogenic acid; D: delphinidin; D-G: delphinidin-3-O-glucoside; EA: ellagic acid; ECa: epicatechin; EGCa: epigallocatechin; EGCaG: epigallocatechin gallate; GA: gallic acid; GCa: gallocatechin; Gl: reduced glutathione; HE: hesperidin; K: kaempferol; K-G: kaempferol-3-O-glucoside; M: myricetin; NA: naringenin; Pel: pelargonidin; Peo: peonidin; Pet: petunidin; Q: quercetin; Q-R: rutin; V: malvidin. Letters (a, b, c, d and e) indicates a significant difference determined by ($P < 0.05$) between compounds illustrated within graph.

610 J. Tabart et al. / Food Chemistry 120 (2010) 607–614

response was more than twice as high as the difference of coloration observed with cyanidin, pelargonidin, and peonidin. Petunidin was not detected. The glycosides of cyanidin reacted more strongly than cyanidin, whereas the glycoside of delphinidin reacted less strongly than delphinidin. The other standards tested did not react, apart from a very slight reaction of the flavanon standards (amounting to less than 0.5% of the reactivity of cyanidin-3-O-glucoside).

Flavanols. With this assay (Fig. 1C), flavan-3-ols showed the strongest reaction. Epicatechin showed higher reactivity than catechin, epigallocatechin, and epigallocatechin gallate. Among the other compounds, the intensity of the reaction varied from 0 (quercetin and some anthocyanins) to 20% of the intensity of the catechin reaction. For the other flavonols, the flavanons, the phenolic acids, ascorbic acid, and glutathione, the reaction intensity was between 10% and 20% of that of catechin.

Ascorbic acid. Only ascorbic acid was significantly detected with the specific method used. A very weak reaction was also observed with myricetin (less than 0.5% of the intensity of the ascorbic acid reaction (data not shown)).

Glutathione. The method used was very specific to reduced glutathione. No reaction was recorded with any other standard tested (data not shown).

3.2. Measurement of antioxidant compounds in beverages by colorimetric tests

Apple, orange, grape, and vegetable juices, ice green tea, and red wine were tested for their content in various antioxidant compounds by the different colorimetric methods described above (Fig. 2).

Flavonoids. A high flavonoid content was observed in red wine (Fig. 2A), three times higher than for ice tea and orange juice. Practically no flavonoids were detected in apple, grape, or vegetable juice.

Anthocyans. A high anthocyan content was measured in red wine, at least 10 times higher than those recorded in the other beverages (Fig. 2B). Grape juice also showed a significant anthocyan content, exceeding the level recorded for apple juice and ice green tea. No anthocyans were found in orange or vegetable juice.

Flavanols. As observed for anthocyans, red wine showed a high flavanol content, 10 times higher than those of grape juice and ice green tea (Fig. 2C). No flavanols were detected in apple, orange, or vegetable juice.

Ascorbic acid. All the beverages tested contained ascorbic acid, but orange juice showed the highest level, followed by ice green tea and red wine (Fig. 2D). The ascorbic acid content of the apple, grape, and vegetable juices was lower.

Glutathione. This compound was not found in any beverage (data non shown).

3.3. Separation and measurement of antioxidant compounds

The aglycons of flavonols and anthocyanins were analyzed by HPLC after hydrolysis of the beverage. Flavan-3-ols were also separated by HPLC and measured in each beverage.

Flavonols. Quercetin was detected only in vegetable juice and red wine, in similar amount (Fig. 3). Red wine also showed a similar concentration of kaempferol. Myricetin was not detected in any beverage.

Anthocyanidins. Some anthocyanidins were detected in all tested beverages, at various levels (Fig. 3). Red wine showed the

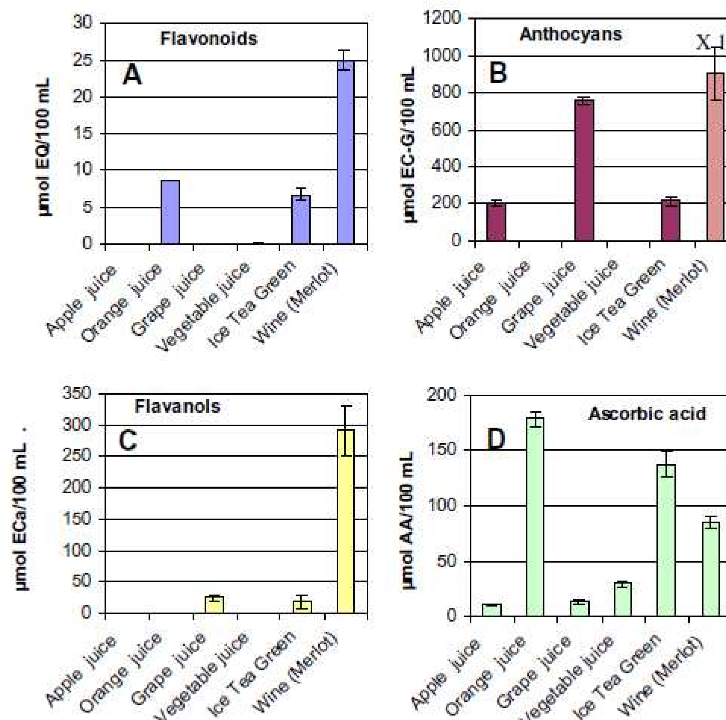


Fig. 2. Evaluation of total flavonoid (A, µmol EQ/100 mL), anthocyan (B, µmol EC-G/100 mL), flavanol (C, µmol ECa/100 mL) and ascorbic acid (D, µmol AA/100 mL) content in various beverages by colorimetric tests (error bars represent +SEM). X10 on (B) indicates that anthocyan content of wine must be multiply by 10.

J. Tabart et al. / Food Chemistry 120 (2010) 607–614 611

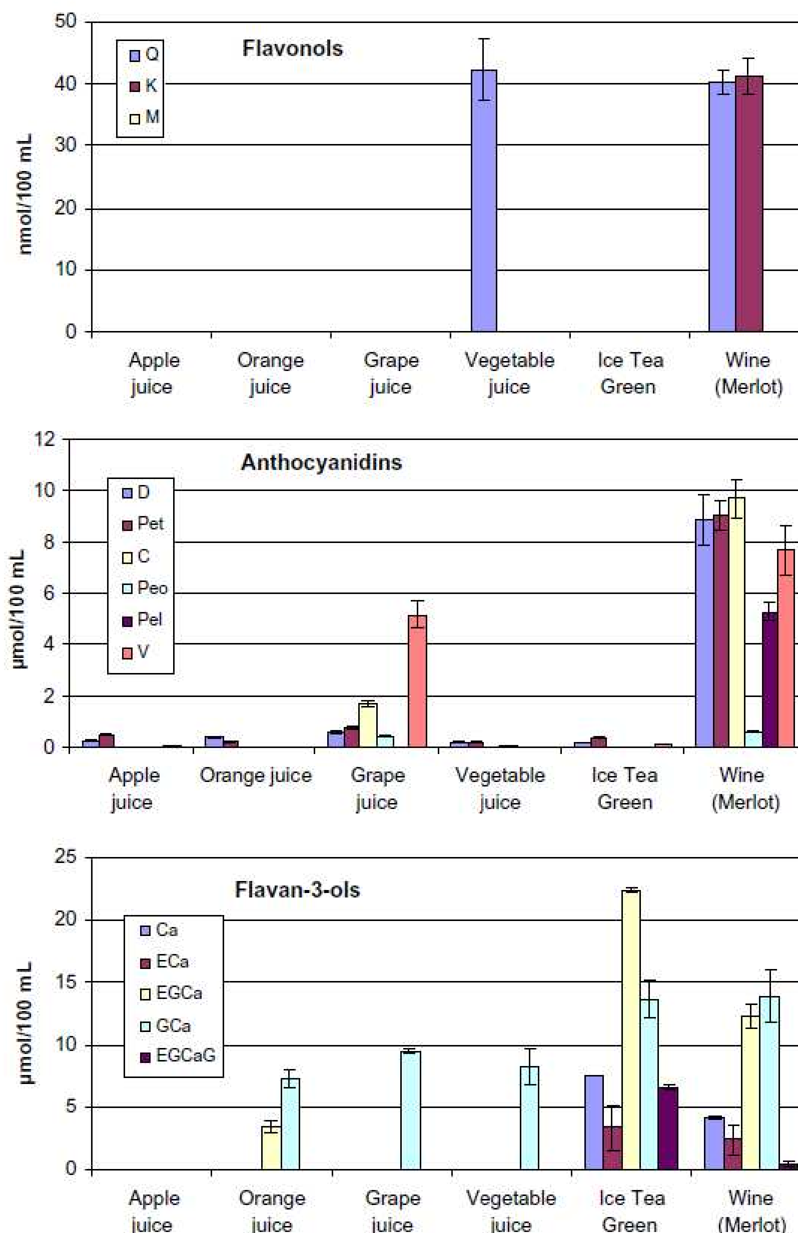


Fig. 3. HPLC analyses of flavonol aglycons (A, nmol/100 mL), anthocyanidins (B, µmol/100 mL) and flavan-3-ols (C, µmol/100 mL) in some beverages (error bars represent +SEM).

highest concentrations of all anthocyanidins except peonidin. All the anthocyanidins (except pelargonidin) were found in grape juice, at levels considerably lower than in red wine. In the other beverages, all anthocyanidin contents were very low, but in each case delphinidin and petunidin were the most abundant.

Flavan-3-ols. The five species measured were present in ice green tea and red wine, most levels being higher in the former than in the latter (except the epicatechin and gallocatechin levels, which were the same in both beverages) (Fig. 3). Only gallocatechin was found in grape, vegetable, and orange juice. In orange juice, epigallocatechin was also present. None of these flavan-3-ols was detected in apple juice.

4. Discussion

The colorimetric methods here used to quantify ascorbic acid (DCIP method) and glutathione proved to be very specific. Anthocyan quantification by the pH-differential method also appears very specific to anthocyans and has been used to detect them

(Gonzales-Rodriguez, Perez-Juan, & Luque de castro, 2002), but different anthocyans respond very differently. In contrast, the method of Lamaison et al. (1990) for the measurement of flavonoids appears adequate only for flavonols. Some other flavonoids, such as flavanons, could also be detected but with lower sensitivity.

Anthocyans and flavan-3-ols were not detected. It is worth mentioning that the color intensifies with a higher degree of hydroxylation as a result of Al^{3+} complexation (Adelmann et al., 2007). Among the standards tested, the flavan-3-ols reacted very well with vanillin (total flavanol colorimetric test), but a lot of other compounds, such as flavanons, also reacted to a lesser extent (Sarkar & Howarth, 1976). Other polyphenolics reacting in the vanillin–HCl assay include procyanidins and dihydrochalcone because of their structure (a single bond at the 2.3 position and free meta–ortho-hydroxy groups on the ring, Nakamura et al., 2003; Sarkar & Howarth, 1976). Other structural factors influencing the reaction sensitivity include the degree of polymerization of proanthocyanidin (sensitivity increases with increasing polymerization) and the degree of esterification of flavan-3-ols (sensitivity decreases with increasing esterification) (Nakamura et al., 2003; Sun, Ricardo-da-Silva, & Spronger, 1998).

Separation by HPLC followed by absorbance measurement is needed to evaluate concentrations of the various compounds correctly. A comparison of Figs. 2 and 3 reveals great differences between the results obtained by colorimetric tests and HPLC analysis. Although the flavonoid test used here detects mainly flavonols, the levels of flavonols separated and measured by HPLC were too low to account for the results of this test, which must thus have detected additional compounds (unknown or not tested). In wine (Table 1), for instance, the total flavonoid content measured was 25 µmol/100 ml, as opposed to only 0.08 µmol/100 ml flavonols (sum of relevant flavonol aglycons: quercetin and kampferol) detected by HPLC analysis. For anthocyans likewise, the colorimetric test yielded higher values than HPLC analysis. For wine (Table 1), the total anthocyan content was 9069 µmol/100 ml according to the colorimetric test, but only 41 µmol/100 ml anthocyanidins (sum of the six main anthocyanidins detected by HPLC) were found. For flavanols a major difference was again observed, but by HPLC we separated only the free flavan-3-ols. The colorimetric test is not specific to these free compounds; tannins can also be detected (Nakamura et al., 2003).

In a previous paper (Tabart et al., 2009), the antioxidant capacity of the different standards and beverages used in the present study was determined by two different assays: one measuring scavenging of the 2,2-diphenyl-1-picryhydrazil (DPPH) radical and one measuring the oxygen radical-scavenging capacity (ORAC). With these values of the antioxidant capacity of standard compounds, we have converted the present measurements of each compound found in beverages (by colorimetric tests or by HPLC) into antioxidant capacity. Thus, by integrating the antioxidant capacities of polyphenolic standard compounds with the present measurements of specific compounds by colorimetric tests and HPLC analyses, we have tried to determine how each type of compound contributes to the total antioxidant capacity of beverages. The results of this conversion (amount to antioxidant capacity) were indicated in Tables 1 and 2 for wine, orange juice, and tea, respectively.

The results based on the colorimetric tests are quite surprising. In wine (Table 1), ascorbic acid (46 and 43 µmol ET/100 ml measured, respectively, by DPPH and ORAC assays) and flavonoids (22 and 101 µmol ET/100 ml measured, respectively, by DPPH and ORAC assays) can together account for 10 (DPPH assay) or 13% (ORAC assay) of the antioxidant capacity, depending on the method used to determine this capacity. Anthocyans alone (in C-G equivalents) seem to account for an antioxidant activity several times higher than the capacity determined by either method. This is also the case when the flavanol results are converted to an antioxidant capacity on the basis of the ORAC assay. Addition of the calculated values gives abnormally high results as compared to those previously measured by the DPPH or ORAC assay. Specific HPLC analysis of different compounds (flavonols, anthocyanidins, flavan-3-ols) present in wine, on the other hand, shows that 35% or 41% of the total wine antioxidant capacity (as determined, respectively, by the DPPH or ORAC assay) can be attributed to flavan-3-ols, anthocyanins, and ascorbic acid (the most active compounds), followed by flavonols. In red wine, Fernandez-Pachon, Villano, Garcia-Parrilla, and Troncoso (2004) have shown that 50% of the total radical-scavenging activity of red wines (ABTS, DPPH methods) can be attributed to polymeric phenolic compounds. Phenolic acids and some other compounds (Fernandez-Pachon et al., 2006) such as proanthocyanidins (Cimino, Sulfaro, Trombetta, Saija, & Tomaino, 2007) can explain the difference.

In orange juice (Table 2), the ascorbic acid content can explain 50% of the antioxidant capacity measured by the DPPH assay, but only 13% of that measured by the ORAC assay. The two methods, based on different mechanisms (DPPH: reducing capacity and ORAC: peroxyl radical-scavenging capacity) can be used to evaluate the antioxidant capacity of compounds or beverages, but it was known that the different methods could give very different results, sometime widely divergent (Tabart et al., 2009). By colorimetric assays, only flavonoids were detected while by HPLC analysis, anthocyanidins and flavan-3-ols were detected also, thus accounting for part of the antioxidant capacity. In all, ascorbic acid, anthocyanidins, and flavan-3-ols can explain 85% of the

Table 1
The present measurements realized (content, columns 2 and 3) of each compound found in wine by colorimetric tests (a) or by HPLC (b) were converted into their relative antioxidant capacity (column 4–7) by using the antioxidant value previously obtained (Tabart et al., 2009) for each standard of phenolic compounds by two methods: DPPH and ORAC (µmol ETrolox/100 mL) assays. This calculation permits an evaluation of the contribution of the different compounds measured to the total antioxidant capacity of wine (per 100 ml). Nd, not detectable, –, not determined.

Compounds	Content (µmol/100 mL)	Calculated values of relative antioxidant capacity			
		(DPPH µmol ET/100 mL)		(ORAC µmol ET/100 mL)	
		(a)	(a) + (b)	(a)	(a) + (b)
Glutathione	nd (a)	nd	nd	nd	nd
Ascorbic acid	84.86 (a)	46	46.49	43	42.67
Flavonoids (Q)	24.86 (a)	22	–	101	–
Anthocyans (C-G)	9068.56 (a)	5831	–	64160	–
Flavanols (Ca)	290.71 (a)	235	–	2245	–
Flavonols (3)					
Quercetin	0.04 (b)	–	0.04	–	0.16
Myricetin	nd (b)	–	nd	–	nd
Kampferol	0.04 (b)	–	0.03	–	0.25
Anthocyanidins (6)					
Delphinidin	8.86 (b)	–	13.30	–	32.86
Petunidin	9.03 (b)	–	5.81	–	63.91
Cyanidin	9.71 (b)	–	5.29	–	41.52
Peonidin	0.62 (b)	–	0.50	–	3.35
Pelargonidin	5.29 (b)	–	2.60	–	30.08
Malvidin	7.71 (b)	–	11.10	–	43.96
Flavan-3-ols (5)					
Catechin	4.17 (b)	–	3.37	–	32.17
Epicatechin	2.44 (b)	–	2.52	–	12.13
Epigallocatechin	12.34 (b)	–	18.72	–	37.77
Gallocatechin	13.95 (b)	–	119.72	–	112.40
Epigallocatechin gallate	0.47 (b)	–	1.75	–	1.56
Total calculated		6134	231.24	66,999	454.79
Total antioxidant capacity measured (Tabart et al., 2009)		663		1100	

Table 2
Conversion of the present measurements into their relative antioxidant capacity (column 4–7) as explained in Table 1 and evaluation of the contribution of the different compounds measured to the total antioxidant capacity of orange juice and ice green tea. The relative antioxidant capacity corresponding to the concentration obtained for each flavonol, anthocyanidin and flavan-3-ol was calculated and the results were summed per family. Nd, not detectable, –, not determined.

Compounds	Content µmol/100 mL	Calculated values of relative antioxidant capacity			
		(DPPH µmol ET/100 mL)		(ORAC µmol ET/100 mL)	
		(a)	(a) + (b)	(a)	(a) + (b)
Orange juice					
Ascorbic acid	178.14	97	97.37	89	89.36
Flavonoids (Q)	8.64	8	–	35	–
Anthocyans (C-G)	nd	nd	–	nd	–
Flavanols (Ca)	nd	nd	–	nd	–
Flavonols (3)	nd	–	nd	–	nd
Anthocyanidins (6)	0.62	–	0.74	–	3.01
Flavan-3-ols (5)	10.76	–	67.74	–	69.29
Total calculated		105	165.85	124	161.66
Total antioxidant capacity measured (Tabart et al., 2009)		192		690	
Ice green tea					
Ascorbic acid	137.40	75	74.94	69	68.77
Flavonoids (Q)	6.69	6	–	27	–
Anthocyans (C-G)	214.97	138	–	1521	–
Flavanols (Ca)	20.30	16	–	157	–
Flavonols (3)	nd	–	nd	–	nd
Anthocyanidins (6)	0.68	–	0.70	–	3.98
Flavan-3-ols (5)	53.53	–	185.58	–	275.56
Total calculated		235	261.22	1774	348.31
Total antioxidant capacity measured (Tabart et al., 2009)		497		537	

antioxidant capacity measured by the DPPH assay but only 23% of that measured by the ORAC assay. Flavanones (another type of flavonoid) and hydrocinnamic acids, not measured, are present in orange juice (Klimczak, Malecka, Szlachta, & Gliszczynka-Swiglo, 2007) and may explain part of the difference.

For tea (Table 2) as for wine, there is a discrepancy between the high level of anthocyans measured by the colorimetric test and the low anthocyanidin levels measured by HPLC analysis. The different specificity of the anthocyanins (anthocyanidins and glycolysed forms) for the colorimetric test (Fig. 1) could partially explained this discrepancy while in HPLC analysis, only the aglycon forms (after hydrolysis) were measured. This discrepancy leads to a much higher calculated ORAC-based antioxidant capacity value than previously measured in ice green tea. In contrast, the colorimetric test yielded a flavanol level lower than the summed flavan-3-ol levels determined by HPLC analysis. Tea is known for its high content in flavan-3-ols and for their high antioxidant capacity (Sultana et al., 2008).

In conclusion, colorimetric tests for flavonoids, anthocyans, and flavanols appear generally unreliable for estimating the antioxidant capacity. Compounds of the same type (anthocyanins, flavonols, or flavan-3-ols) react differently in some tests. In the flavonoid test, for example, flavonols reacted very well, anthocyanins failed to react, and flavanons reacted only slightly. Other colorimetric tests (for anthocyans and flavanols) show a lack of specificity for a single type.

The Folin–Ciocalteu method can be used to assay the total phenolic compound content but it also measures the total reducing capacity of a sample. Moreover, this method is not specific to phenolic compounds. Many non-phenolic compounds, such as ascorbic acid (Tabart et al., 2009) and saccharides present in fruits and vegetables, can reduce the Folin–Ciocalteu reagent (Stratil, Klejdus, & Kuban, 2007). When HPLC analysis was applied to beverages, the levels obtained by summing the levels of individual compounds were very different from those determined by colorimetry, generally much lower. The values given by the colorimetric tests were generally too high, suggesting that other compounds can react in the tests. Likewise, the relative antioxidant capacity values determined for beverages on the basis of colorimetric tests could exceed by far the values actually measured in radical-scavenging tests (for instance, the antioxidant capacity attributable to anthocyans in wine on the basis of the colorimetric test was 50 times higher than the total antioxidant capacity measured by the ORAC assay).

Investigations into the antioxidant capacity of foods should take into account the chemical natures and concentrations of the various antioxidants present in each food matrix, since the total antioxidant capacity of a food may reflect the combined activities of diverse antioxidants rather than being attributable to any particular phenolics. Yet the complexity of the food composition makes it costly and inefficient to separate each antioxidant compound and to study it individually. It is also necessary to consider the possibility of synergistic or antagonistic interactions among antioxidant compounds in a food mixture. For instance gallic acid and epigallocatechin gallate, major antioxidants in tea, show strong antioxidant activity but can also act as pro-oxidants (Johnson & Loo, 2000).

It is important to have the best analytical tools for determining the antioxidant content of any food matrix, as the agro-food industry will soon have to provide strong scientific evidence in support of allegations regarding the health benefits conferred by foods containing various antioxidants. The present study shows that results may be significantly different according to the method of investigation. Scientists must therefore focus on developing standardized methods for antioxidant determination in food.

Acknowledgments

We thank HEPL-Rennequin Sualem and J. Bleret for technical assistance. J.T. gratefully acknowledges the support of the Luxembourg Ministry of National Education. The skilful assistance of the APE personnel (provided to CEDEVIT by the Regional Government of Wallonia) was greatly appreciated.

References

Adelmann, J., Passos, M., Breyer, D. H., Rocha dos Santos, M. H., Lenz, C., Leite, N. F., et al. (2007). Exotic flora dependence of an unusual Brazilian propolis: The pinocembrin biomarker by capillary techniques. *Journal of Pharmaceutical and Biomedical Analysis, 43*, 174–176.

Association of Vitamin Chemists. (1961). *Methods of vitamin assay* (3rd ed.). New York: Interscience Publishers.

Arts, I. C., & Hollman, P. C. (2005). Polyphenols and disease risk in epidemiologic studies. *American Journal of Clinical Nutrition, 81*, 317s–325s.

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews, 56*, 317–333.

Cimino, F., Sulfaro, V., Trombetta, D., Saija, A., & Tomaino, A. (2007). Radical-scavenging capacity of several Italian red wines. *Food Chemistry, 103*, 75–81.

Dutruc-Rosset, G. (2003). Détermination par CLHP de neuf anthocyanes principales dans le vin rouge et rosé. *Résolution OENO*, 22.

Fernandez-Pachon, M. S., Villano, D., Garcia-Parrilla, M. C., & Troncoso, A. M. (2004). Antioxidant activity of wines and relation with their polyphenolic composition. *Analytica Chimica Acta, 513*, 113–118.

Fernandez-Pachon, M. S., Villano, D., Troncoso, A. M., & Garcia-Parrilla, M. C. (2006). Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with antioxidant activity. *Analytica Chimica Acta, 563*, 101–108.

Franck, T., Kevers, C., Penel, C., Greppin, H., Hausman, J. F., & Gaspar, T. (1998). Reducing properties and markers of lipid peroxidation in normal and hyperhydrating shoots of *Prunus avium* L. *Journal of Plant Physiology, 153*, 339–346.

Gonzales-Rodriguez, J., Perez-Juan, P., & Luque de castro, M. D. (2002). Method for the simultaneous determination of total polyphenol and anthocyan indexes in red wines using a flow injection approach. *Talanta, 56*, 53–59.

Häkkinen, S., Kärenlampi, S., Jeinonen, I. M., Mykkänen, H., & Torronen, R. (1998). HPLC method for screening of flavonoids and phenolics acids in berries. *Journal of the Science of Food and Agriculture, 77*, 543–551.

Johnson, M. K., & Loo, G. (2000). Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutation Research, 459*, 211–218.

Klimczak, I., Malecka, M., Szlachta, M., & Gliszczynka-Swiglo, A. (2007). Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. *Journal of Food Composition and Analysis, 20*, 313–322.

Lamaison, J. L., & Carnat, J. (1990). Teneur en principaux flavono des fleurs et des feuilles de *Crataegus monogyna* Jacq. et de *Crataegus laevigata* (Poiret) DC. (Rosaceae) A.. *Pharmaceutica Acta Helvetiae, 65*, 315–320.

Mullen, W., Marks, S. C., & Crozier, A. (2007). Evaluation of phenolic compounds in commercial fruit juices and fruit drinks. *Journal of Agricultural and Food Chemistry, 55*, 3148–3157.

Nakamura, Y., Tsuji, S., & Tonogai, Y. (2003). Analysis of proanthocyanidins in grape seed extracts, health foods and grape seed oils. *Journal of Health Science, 49*, 45–54.

Nielsen, I. L., Haren, G. R., Magnussen, E. L., Dragsted, L. O., & Rasmussen, S. E. (2003). Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency. *Journal of Agricultural and Food Chemistry, 51*, 5861–5866.

Sarkar, S. K., & Howarth, R. E. (1976). Specificity of the vanillin test for flavonols. *Journal of Agricultural and Food Chemistry, 24*, 317–320.

Sharma, V., Gulate, A., Ravindranath, S. D., & Kumar, V. (2005). A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. *Journal of Food Composition and Analysis, 18*, 583–594.

Stratil, P., Klejdus, B., & Kuban, V. (2006). Determination of total content of phenolic compounds and their antioxidant activity in vegetables – Evaluation of spectrophotometric methods. *Journal of Agricultural and Food Chemistry, 54*, 607–616.

Stratil, P., Klejdus, B., & Kuban, V. (2007). Determination of phenolic compounds and their antioxidant activity in fruits and cereals. *Talanta, 71*, 1741–1751.

Sultana, T., Stecher, G., Mayer, R., Trojer, L., Qureshi, M. N., Abel, G., et al. (2008). Quality assessment and quantitative analysis of flavonoids from tea samples of different origins by HPLC-DAD-ESI-MS. *Journal of Agricultural and Food Chemistry, 56*, 3444–3453.

Sun, B., Ricardo-da-Silva, J. M., & Spronger, I. (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. *Journal of Agricultural and Food Chemistry, 46*, 4267–4274.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J. O., & Dommes, J. (2006). Antioxidant capacity of black currant varies with organ, season, and cultivar. *Journal of Agricultural and Food Chemistry, 54*, 6271–6276.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J. O., & Dommes, J. (2009). Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chemistry, 113*, 1226–1233.

Villano, D., Fernandez-Pachon, M. S., Troncoso, A. M., & Garcia-Parrilla, M. C. (2005). Comparison of antioxidant activity of wine phenolic compounds and metabolites in vitro. *Analytica Chimica Acta, 538*, 391–398.

Ascorbic acid, Phenolic acid, Flavonoid and Carotenoid profiles of selected extracts from *Ribes nigrum.*

Jessica TABART[a], Claire KEVERS[a], Danièle EVERS[b], Jacques DOMMES[a]

[a]Plant Molecular Biology and Biotechnology Unit, University of Liège, B22, Sart –Tilman, B-4000 Liège (Belgium)

[b]Public Research Center - Gabriel Lippmann, Rue du Brill 41, L-4422 Belvaux (Luxembourg)

Corresponding author: Jessica TABART: Jessica.Tabart@doct.ulg.ac.be
Other authors: C.Kevers@ulg.ac.be, Evers@lippmann.lu, J.Dommes@ulg.ac.be;

TITLE RUNNING HEAD: Phenolics from *Ribes nigrum*

ABSTRACT

Small fruits such as berries have low energy contents, but high content of vitamins, micronutrients, and dietary fibres and constitute a good source of natural antioxidant compounds that are important constituents of the human diet. This study allowed to identify a large number of compounds in an extract of blackcurrant showing high antioxidant activity and to compare their profile in various parts of the plants (leaves, buds and berries). If it was known that berries contained very high levels of natural phenolic compounds, here we showed that leaves and buds could also be considered as a good sources of natural antioxidants. Indeed, they contained high amount of ascorbic acid, phenolic acids, flavonoids and carotenoids. An acetone mixture can extract several classes of phenolic compounds with a good yield of ascorbic acid, flavonols, flavan-3-ols and anthocyanins. For phenolic acids, it extracted fewer compounds than a specific extraction solution.

KEYWORDS: Antioxidant, flavonoids, flavonols, anthocyanins, flavanols, ascorbic acid, phenolic acids, carotenoids.

INTRODUCTION

Phenolic compounds are one of the most widely occurring groups of phytochemicals. In the plant kingdom, these compounds can range from simple molecules such as phenolic acids, to highly polymerized compounds such as tannins (1). Different classes of phenolic compounds can be distinguished. Flavonoids and phenolic acids are the most abundant. Phenolic acids are synthesised from hydroxybenzoic acid and hydroxycinnamic acid. The flavonoids are subdivided in different classes: flavonols, anthocyanins, flavones, flavan-3-ols, flavanones, isoflavones (2). In the plant, they play an important role in growth and reproduction, providing protection against pathogens and predators as well as against abiotic stresses (1, 3). In the human diet, fruits and vegetables have low energy content, but high content of vitamins, essential micronutrients, and dietary fibres. They are also the predominant source of flavonoids and phenolic acids. Many health-related properties, including anti-viral, anti-inflammatory activities, antioxidant properties and ability to inhibit human platelet aggregation have been described (4-6).

Carotenoids belong to another important group of natural pigments because of their wide distribution, structural diversity and numerous functions for photosynthesis and for life in an oxygen-containing atmosphere. The carotenoids are subdivided in two groups: the carotenes and the xanthophylls (or oxycarotenoids) (7). Recently, these pigments have been described to be implicated in the prevention of human health disorders such as heart disease and photosensitivity disease but also of certain forms of cancer (8, 9).

Small fruits constitute a good source of natural antioxidant substances. Extracts of fruits from various blackberry, raspberry and gooseberry cultivars act effectively as free radical inhibitors (10, 11). In addition, the flavonoid content of small fruits has been investigated (12). Blackcurrant berries contain very high amounts of phenolic compounds. Fresh blackcurrants are particularly rich in anthocyanins. But other phenolic compounds like flavonols are also present (13). Only a very small proportion of these berries are consumed fresh, most is processed for juice concentrates. Leaves and buds can also be used (14); leaf micronisates and glycerinate extracts of buds are especially commercialized as food supplements. Declume (15) and Chrubasik (16) also demonstrate that leaf extracts of blackcurrant show significant anti-inflammatory activity.

The first objective of this study was to identify most of the compounds present in an extract of blackcurrant with high antioxidant activity. We also compared the profile in ascorbic acid, phenolic acids, flavonoïds and carotenoids of extracts from various parts of the plants (leaves, buds and berries). The second objective was to check whether an acetone extraction method optimised for high antioxidant capacity (17) did provide good yields of various classes of antioxidant compounds.

MATERIAL AND METHODS

Materials

The buds, berries and leaves of two years old blackcurrant plants (Noir de Bourgogne) were harvested respectively in March, July and August in the Belgian Ardennes (Bihain). The various explants were directly cut out in pieces, frozen in liquid nitrogen, lyophilised and stored at -20°C.

Sample preparation

Acetone Extraction:

One gram of fresh sample (berries, buds or leaves) was ground with 1 gram of quartz and 10 mL of extraction solution: acetone/water/acetic acid (70:28:2) (14). The mixture was shaken during 1 h at 4°C and centrifuged at 17 000g for 15 min. The supernatant was removed, and the sample was extracted again with the same procedure but incubated only 15 min. The supernatants were pooled and then diluted as appropriate for the analyses.

Specific Extractions:

For ascorbic acid: 4 gram of fresh material (berries, buds, leaves) were ground with 1 gram of quartz and 80 mL of extraction solution (20 g/L metaphosphoric acid). The mixture was shaken during 1 h at 4°C and centrifuged at 15 000 g for 15 min. For HPLC analysis, 10 mL of a L-cystein solution (40g/L) were added to 20 mL of the supernatant and the pH was adjusted between 7.0 and 7.2 with a solution of trisodium phosphate (200 g/L). After 5 minutes, the pH was adjusted between 2.5 and 2.8 with a solution of metaphosphoric acid (20 g/L) (18).

For phenolic acids (19): 3.4 gram of fresh material (berries, buds, leaves) were ground with 1 gram of quartz and 49 mL of extraction solution (methanol/water/acetic acid; 90:1.5:8.5; v/v/v) containing 2 g/L of BHA (butylated hydroxyanisol). The mixture was sonicated during 1 h at room temperature. 50 mL of 20 g/L ascorbic acid and 25 mL of 10 M NaOH were added for the hydrolysis of esterified phenolic acids. Then 12.5 mL of 12 N HCl were added for the extraction of glycoside forms of phenolic acids and the mixture was incubated for 3 h at 85°C before cooling on ice. The mixture was extracted 5 times with a solution of diethyl ether/ethyl acetate (50:50; v/v) and centrifuged at 1 200 g for 2 min. The organic phases were pooled and evaporated. All the residues were dissolved in 2 mL of 2% acetic acid and filtered for HPLC analysis.

For anthocyanins: One gram of fresh sample (berries, buds or leaves) was ground with 1 gram of quartz and 15 mL of 1% HCl in methanol (20). The mixture was shaken during 2 h at room temperature and incubated one night in the dark at -20°C before centrifugation at 4 000g for 15 min. The supernatant was collected and the sample was extracted again with 15 mL of 1% HCl in methanol. The supernatants were pooled and then diluted as appropriate for the analyses.

For flavanols (based on Parva-Uzunalic et al. (21))**:** One gram of fresh sample (berries, buds or leaves) was ground with 1 gram of quartz and 10 mL of acetone 100%. The mixture was shaken during 1 h at 70°C and centrifuged at 17 000 g for 15 min. The supernatant was removed and the sample was extracted again with the same procedure but incubated only 15 min. The supernatants were pooled and then diluted as appropriate for the analyses.

For carotenoids: One gram of fresh sample (berries, buds or leaves) was ground with 1 gram of quartz and 15 mL of 1% BHT (2,6-O-tert-butyl-4-methylphenol) in acetone (22). The mixture was shaken during 30 min at 4°C in the dark and centrifuged at 17 000 g for 10 min. The supernatant was removed, and the sample was re-extracted until the sample was colorless. The supernatants were pooled.

Colorimetric assays

Reduced ascorbic acid was measured with the 2,6-dichloroindophenol (DCIP) method of the Association of Vitamin Chemists (23). Briefly, each molecule of vitamin C converted a molecule of DCIP into a molecule of $DCIPH_2$, and that conversion was monitored as a decrease in the absorbance at 520 nm. A standard curve was prepared using a series of

known ascorbic acid concentrations. 1 mL of diluted samples (in 5% metaphosphoric acid) or ascorbic acid calibration solutions was mixed with 500 µL 10% metaphosphoric acid. 300 µL citrate buffer (pH 4.15) and 300 µL DCIP (0.1 mg/mL) were added to 600 µL of this mixture. The optical density blanching was used; for each sample, the blank value was determined after addition of 60 µL ascorbic acid (1 mg/mL) to take into account interference due to the sample colour. The results were expressed as mg of ascorbic acid (AA) per gram of fresh weight.

Total phenolic content was determined according to the Folin-Ciocalteu method (24). 3.6 mL of appropriate dilution of extracts were mixed with 0.2 mL of Folin-Ciocalteu reagent and after 3 minutes of incubation, 0.8 mL of sodium carbonate solution (20% w/v) was added. The mixture was heated at 100°C during one minute. The absorbance at 750 nm was measured after cooling. A standard curve was done with chlorogenic acid. The results were expressed in mg equivalent chlorogenic acid (CAE) per gram of fresh weight.

Total flavonol content was measured following the method of total flavonoids described by Lamaison and Carmat (25). In a previous paper, we had demonstrated that this technique appears adequate only for flavonols (26). Appropriately diluted extracts (1 mL) were mixed with 1 mL of 2% $AlCl_3.6H_2O$ in methanol. The absorbance at 430 nm was measured 10 minutes later. Quercetin was used as standard, and results were expressed as mg of quercetin equivalents (QE) per gram of fresh weight.

Total anthocyanin quantification was performed by the pH-differential method (27). The extract was diluted in a pH 1.0 solution (0.1 M HCl, 25 mM KCl) and in a pH 4.5 solution (0.4 M CH_3COONa). The absorbance of the mixtures was then measured at 535 and 700 nm against distilled water. The value $(Abs_{535}\text{-}Abs_{700})_{pH1.0}$ - $(Abs_{535}\text{-}Abs_{700})_{pH4.5}$ corresponds to the absorbance due to the anthocyanins. Calculation of the anthocyanin concentrations was based on a cyanidin 3-glucoside molar extinction coefficient of 25.965 cm^{-1}. Results were expressed as µg of Kuromanin equivalents (KuE) per gram of fresh weight.

Total flavanol content was evaluated by the vanillin assay (28). Each molecule of vanillin reacted with a molecule of flavanol to produce a red chromophore. The conversion was monitored as an increase in the absorbance at 500 nm. One volume of sample diluted in methanol was mixed with 2.5 volumes of 1% vanillin in methanol and 2.5 volumes of 9 M HCl in methanol. The mixture was incubated 20 minutes at 35° C before analysis. For each sample, a blank value was measured where vanillin solution was replaced by

methanol alone. Catechin (0 to 1 mg/mL) was used as standard and results were expressed as mg of catechin equivalents (CaE) per gram of fresh weight.

Total carotenoid content was evaluated by a spectrophotometric method described by Rodriguez-Amaya (29) using the measurement of the absorbance at 450 nm. From this value we subtracted the turbidity of the sample (assayed through absorbance at 700 nm). We used an extinction coefficient recommended for the mixture of carotenoids ($\varepsilon = 2500$). The results were expressed as µg of carotenoids per gram of fresh weight.

Separation and measurement of compounds by HPLC

Analyses were performed in a liquid Elite Lachrom Merck Hitachi chromatograph equipped with a L2450 photodiode array detector (sampling period: 400 ms, spectral bandwidth: 4 nm). Separation was carried out using a LiChroCART steel cartridge (Merck), 250 mm x 4.6 mm, filled with 5 µm particles RP 18 at 30°C for flavonols and 40°C for anthocyanidins and flavan-3-ols. Other separations were carried out using a Grace Smart RP 18 (Grace Davison Discovery Sciences), 250 mm x 4.6 mm filled with 5 µm particles RP 18 at 30°C for ascorbic acid and phenolic acids.

For **ascorbic acid analysis**, the mobile phase was a gradient of water- metaphosphoric acid (199:1; v/v) and 100% acetonitrile, at a flow rate of 1 mL/min. Optical density was recorded at 254 nm.

For **phenolic acid analysis**, the mobile phase was a gradient of 2% ascorbic acid and 100% acetonitrile, at a flow rate of 0.5 mL/min. Optical densities were recorded at 260, 275 and 325 nm.

For **flavonol aglycone analysis** of hydrolyzed extracts, the mobile phase (14) was a linear gradient of water-acetonitrile (50:50) adjusted to pH 1.8 with perchloric acid and water-acetonitrile (95:5) adjusted to pH 1.8 with perchloric acid, at a flow rate of 1.2 mL/min. Optical density was recorded at 365 nm

For **anthocyanidin analysis** of hydrolyzed extracts, the mobile phase (30) was a gradient of water-acetonitrile-formic acid (87:3:10) and (40:50:10), at a flow-rate of 0.8 mL/min. Optical density was recorded at 518 nm.

For **flavan-3-ol analysis** the mobile phase (31) was composed of 90% acetonitrile, 0.1% orthophosphoric acid and 9.9% water. A gradient of flow was used: 0.4 mL/min to 3 min, a linear decrease to 0.3 mL/min at 10 min and to 0.2 mL/min at 13 min, a

steady state to 25 min followed by a linear increase to 0.4 mL/min at 35 min. Optical density was recorded at 230 nm.

All the samples were prepared in triplicates. Each sample analysis was performed in duplicate or triplicate. All the results presented were the mean (± SE) of at least three independent experiments.

Statistical analysis

The data were subjected to the statistical analysis of the variance (ANOVA-1) to evaluate significant differences between various explants of blackcurrant. The difference was regarded as significant when $p < 0.05$.

RESULTS AND DISCUSSION

The first aim of this study was to identify antioxidant compounds in the blackcurrant acetone extracts (acetone/water/acetic acid, 70/28/2) from leaves, buds and berries. This method of extraction of the antioxidant compounds was previously optimised (14) for the high antioxidant capacity of the extracts. We first determined the contents of total phenolics, phenolic acids, ascorbic acid, flavonols, anthocyanins, flavan-3-ols and carotenoids. The second objective of this study was to check whether the acetone mixture was an adequate solvent for high yield extraction of different antioxidant compounds (by comparing the content of various antioxidant compounds in acetone extract and extracts obtained through compound-specific methods).

Ascorbic acid content

Extraction of ascorbic acid (AA) in the acetone mixture was compared to the specific extraction method. First AA was assayed by the colorimetric method (**Figure 1**). Both extraction methods gave similar results for leaves and berries with approximately 1.5 mg AA/ g FW. Results were very different for buds: about 1.5 mg AA/ g FW were found when using the acetone mixture, while AA was barely detectable in the specific extract. HPLC determination confirmed the absence of AA in the bud specific extract, but also showed contrasting results for leaves and berries (**Figure 1**). Compared to specific extraction, acetone extraction gave higher yields for berries (2347 vs 748 µg/g FW) and lower yields for leaves (384 vs 28 µg/g FW). The results obtained for berries by the colorimetric assay after acetone extraction were similar to those obtained by Benvenuti et al. (13) on the blackcurrant "Noir de

Bourgogne" by HPLC. For the determination of the ascorbic acid, we could show great differences between the two assays used. These observations were due to the characteristics of the quantification methods: In the DCIP assay, only reduced L(+)-ascorbic acid was measured but DCIP could also react with other reducing substances contained in the extracts like myricetin (26). In HPLC assay, vitamin C was quantified in its two forms: reduced L(+)-ascorbic acid and D(-)-deshydroascorbic acid. HPLC method had greater sensibility and specificity than colorimetric assays. We could show also that bud extracts (by the two extracting methods) did not contain ascorbic acid. Information about AA content in buds is scarce. In soybean, the buds contain ascorbic acid and its concentration increases at flower induction (32).

Total phenolic compounds' content

Total phenolic content was assayed in acetone extracts of leaves, buds and berries (**Figure 2**). Leaves and buds showed significantly higher content (46 ± 8.4 and 45 ± 7.5 mg CAE/ g FW respectively) than berries (21 ± 8.0 mg CAE/g FW). Benvenuti et al. (13) obtained contents in berries of 5.5 ± 0.1 mg gallic acid equivalent / g of FW (extracted in methanol/HCl 2% (95:5, v/v), while Cacace and Mazza (33) obtained 88.9 ± 2.4 mg CAE/g FW in an aqueous sulphured dioxide extraction solution at pH 3.8 on milled berries.

Phenolic acid content

Concerning **phenolic acids** (**Figures 3** and **4**), the extraction by specific techniques yielded higher results compared to extraction in acetone mixture. In acetone extraction, only the free forms were detected. Phenolic acids are rarely free but generally linked or esterified. The acetone mixture was able to liberate a lot of gallic acid (from the three explants) and gentisic acid (only from leaves), and small amounts of cafeic and ferulic acids (only from buds). With the specific extraction and hydrolysis (**Figure 4**), others compounds were found. Gallic acid was present in the extracts from the three explants with higher content in leaves. Leaf specific extracts contained about twice the amount of gallic acid than acetone extracts (1883 ± 90 vs 1015 ± 54 µg/g FW). Two other phenolic acids were also found in high quantity: p-hydroxybenzoic acid in leaves (1572 ± 32 µg/g FW) and vanillic acid in buds (2677 ± 206 µg/g FW). Zadernowski et al. (34) used a similar extraction method to the specific extraction used here on berries, and generally found the same phenolic acids but in lower amounts. They also found that m-coumaric acid was predominant (1872 ± 145 µg/g FW); this phenolic acid

was not found in our specific berry extract. These contrasting results could be due to varietal differences and/or to difference in ripening stage of the berries.

Flavonoid content

Flavonoids are important plant secondary metabolites accumulating in stressing conditions (35). They are largely studied for their benefit on human health.

First, we evaluated the content in total **flavonols** in the three extracts (leaves, buds and berries) (**Table 1**). The colorimetric technique used was described in the literature as able to quantify the content in total flavonoids. But it is based on the formation of a complex between the flavonoids and $AlCl_3$. We showed previously that only the flavonols were measured (26) by this technique. The contents in total flavonols of the berries (0.5 ± 0.1 mg QE/ g FW) were significantly lower than in leaves and buds. The leaves and the buds had similar contents (2.05 ± 0.34 and 2.15 ± 0.09 QE/ g FW respectively). Cacace and Mazza (33) assayed flavonols in extracts from frozen berries by HPLC-MS analysis and reported slightly higher contents (1.9 ± 0.14 mg QE / g of FW). After separation and HPLC analysis, we showed that quercetin was more abundant than the two other aglycons (myricetin and kampferol) in the three explants with a higher amount in leaf extracts (1.6 and 10 times more than in buds and berries, respectively). Similar results were obtained by Borges et al. (36). This group determined eight flavonols by HPLC-MS analysis with a better yield than in the present study. Häkkinen et al. (37) also reported similar contents in berries for the three aglycons (quercetin: 3.3 to 6.8; K: <0.01 to 1, myricetin: 55 µg/g FW). The same conclusions were obtained by the group of Jakobek (38): 44 µg myricetin / g FW, 21 µg quercetin / g FW and 8 µg kampferol / g FW. For the majority of cultivars, quercetin is prevalent, followed by myricetin and finally, kaempferol. But, the content in quercetin and myricetin considerably varied with the cultivars (37). For the variety “Silvergieter”, no myricetin was present while for the “Rosenthals langtraubige Schwarze” variety, myricetin was prevalent, followed by quercetin.

Anthocyanins are another group of pigments in plants showing health benefits. As shown in table 3, total anthocyanins were significantly different among the explants studied and the extraction methods used. For acetone extraction, the berry extract had a very high content (1468 ± 222 µg KuE/ g FW) compared with the two other explants (381 ± 72 and 260 ± 72 µg KuE/ g FW, for leaf and bud extracts respectively). The specific extraction for anthocyanins described by Awika et al. (20) using 1% HCl in methanol gave similar results for leaves (429

± 87 µg KuE/ g FW). On the contrary, for berries and buds, the acetone mixture extracted less anthocyanins than specific extraction (3180 ± 996 µg KuE/ g FW). The berries had an anthocyanin content 4-5 times higher than leaves, and than buds. The extracts obtained by the two extraction types were hydrolyzed before being analyzed by HPLC. With this technique, we were able to quantify the total content of the major anthocyanidins (aglycons of the anthocyanins) (**Table 2**): delphinidin, petunidin, cyanidin, peonidin, pelargonidin and malvidin. Petunidin was the prevalent anthocyanidin found in the three types of explants with both extraction methods. Malvidin was also found in great quantity in the berries extracted with the acetone mixture. Extraction with 1% HCl led to higher or equivalent yields, except for petunidin in buds, cyanidin in berries and malvidin in buds and berries. Anthocyanin content in berries was reported to be 2189 ± 20 mg KuE/kg FW by the pH-differential method by the group of Jakobek (38), 15.3 ± 0.4 mg KuE/g of frozen berries by Cacace and Mazza (33) and 2.287 mg KuE/g FW by HPLC analysis by Benvenuti et al. (13). In this study we observed a great difference between the content determined by colorimetric and HPLC assays. The colorimetric method gave lower contents than HPLC analysis. This was due to the specific reactivity of each anthocyanins in the assay (26) and the use of a glycosylated standard.

Concerning the total content in **flavan-3-ols** (**Table 3**) in acetone extracts, significantly higher contents were found in the buds (15.3 ± 0.9 mg CaE / g FW) compared to the two other explants. The same trend was observed in extract prepared by the specific method, although this extract always contained more flavan-3-ols than acetone extract. But the analyses by HPLC of the monomers present in the different extracts showed that acetone mixture extracted a higher amount of monomers than the specific extraction. To determine the contents in total monomers of flavan-3-ols, various techniques of acid hydrolysis and depolymerisation described in the literature (39) were tested without success.

Carotenoid content

For **carotenoids,** Morris et al. (40) and André et al. (22) used as extractive solvent 1% BHT in acetone. Compared to our acetone mixture, this specific extraction gave higher yields from leaves (549 ± 8 vs 10 ± 20 µg of carotenoids / g FW) and to a lesser extent from buds (**Figure 5**). For berries, the yields for the two types of extraction were similar. An extractive mixture containing a high percentage of acetone is advised for the extraction of xanthophylls. But

those were probably degraded in the acetone method because no precaution was taken here to allow their correct extraction and their conservation.

In general, the black currant berries contained higher levels of natural phenolic compounds than other fruits like *Rubus fruticosus*, *Rubus Idaeus* L., *Ribes Rubum* and *Sambucus nigra* (13, 37-38). The blackcurrant variety "Noir de Bourgogne" has a lower level in phenolic compounds and ascorbic acid than other varieties of blackcurrant but a higher level in anthocyanins (13-14). In this work, we have shown that leaves and buds could be considered as a good source of natural antioxidant compounds.

CONCLUSION

To summarize, the acetone extracts of leaves and buds had total phenolic contents largely higher than the berry extracts, which was also confirmed after analyzing the content in total flavonoids. The most abundant flavonol aglycon in these extracts was quercetin. The content in ascorbic acid was similar in the three types of extracts if measured by spectrophotometric method while by HPLC analysis, berries showed a higher content in ascorbic acid than the two other plant organs. The extracts also contained phenolic acids: gallic acid in extracts from the three explants, gentisic acid in leaves and ferulic acid in buds and berries. The most abundant anthocyanidin, whatever the explants, was petunidin. Concerning the flavan-3-ols, the bud extracts presented a higher content compared to other explants. The predominant monomers of flavan-3-ols were gallocatechin followed by epigallocatechin.

In conclusion, the acetone mixture can extract several classes of phenolic compounds with a good yield for ascorbic acid, flavonols, flavan-3-ols and anthocyanins. This method is not adequate for carotenoids. For phenolic acids, it extracts fewer compounds than the specific method.

ACKNOWLEDGMENT:

J.T. gratefully acknowledges the AFR Luxembourg and Mr Andrianne from HerbalGem for providing the material. The skilful assistance of the APE staff (provided to CEDEVIT by the regional government of Wallonia) was greatly appreciated.

REFERENCES:

(1) Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317-333.

(2) Williamson, G.; Santos-Buelga, C. In *Methods in polyphenol analysis*; The Royal Society of Chemistry: United Kingdom, **2003**.

(3) Teutter, D. Significance of flavonoïds in plant resistance: a review. *Environ. Chem. Lett.* **2006**, *4*, 147-157.

(4) Guardia, T.; Rotelli, A. E.; Juarez, A. O.; Pelzer, L. E. Anti-inflammatory properties of plant flavonoids; Effects of rutin, quercetin and hesperetin on adjuvant arthiris in rat. *Il Farm.* **2001**, *56*, 683-687.

(5) Ghedria, K. Les Flavonoïdes: structure, propriétés biologiques, rôle prophylactique et emplois en thérapeutique. *Phytothér.* **2005**, *4*, 162-169.

(6) Scalbert, A.; Claudine, C.; Morand, C.; Rémésy, C. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287-306.

(7) Olivier, J.; Palou, A. Chromatographic determination of carotenoids in foods. *J. Chromatogr. A* **2000**, *881*, 543-555.

(8) Mayne, S. T. Beta-carotene, carotenoids, and disease prevention in humans. *FASEB J* **1996**, *10,* 691-701.

(9) Kanofsky, J. R.; Sima, P. D. Activity of a cationic carotenoid derivative in a mouse model of protoporphyria. *J. Photochem. Photobiol. B Biol.* **2007**, *87*, 124-129.

(10) Heinonen, I. M.; Meyer, A. S.; Frankel, E. N. Antioxidant activity of berry phenolics on human low density lipoprotein and liposome oxidation. *J. Agric. Food Chem.* **1998**, *46*, 4107–4112.

(11) Su, M. S.; Silva, J. L. Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (*Vaccinim ashei*) by-products as affected by fermentation. *Food Chem.* **2006**, *97*, 447–451.

(12) Pantelidis, G. E; Vasilakakis, M.; Manganaris, G. A.; Diamantidis, G. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. *Food Chem.* **2007**, *102*, 777–783.

(13) Benvenuti, S.; Pellati, F.;Melegari, M.; Bertelli, D. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes*, and *Aronia*. *Food Chem. Toxicol.* **2004**, *69*, 164-169.

(14) Tabart, J.; Kevers, C.; Pincemail, J.; Defraigne, J. O.; Dommes, J. Antioxidant capacity of black currant varies with organ, season, and cultivar. *J. Agric. Food Chem.* **2006,** *54*, 6271-6276.

(15) Declume, C. Anti-inflammatory evaluation of hydroalcoholic extract of black currant leaves (*Ribes* nigrum). *J. Ethnopharmacol.* **1989**, *27*, 91-98.

(16) Chrubasik, S. Pain therapy using herbal medicines. *Gynakol.* **2000**, *33*, 59-64.

(17) Tabart, J.; Kevers, C.; Sipel, A.; Pincemail, J.; Defraigne, J. O.; Dommes, J. Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and stability during storage. *Food Chem.* **2007**, *105*, 1268-1275.

(18) AFNOR. Foodstuffs – Determination of vitamin C by high performance liquid chromatography. *NF EN 1430* **2003**.

(19) Barberousse, H.; Roiseux, O.; Robert, C.; Paquot, M.; Deroanne, C.; Blecker, C. Analytical methodologies for quantification of ferulic acid and its oligomers. *J. Sci. Food Agric.* **2008**, *88*, 1494–1511.

(20) Awika, J. M.; Rooney, L. W.; Waniska, R. D. Anthocyanins from black sorghum and their antioxidant properties. *Food Chem.* **2004**, *90*, 293-301.

(21) Parva-Uzunalic, A.; Skerget, M.; Knez, Z.; Einreich, B.; Otto, F.; Grüner, S. Extraction of active ingredients from green tea (*Camellia sinensis*): Extraction efficiency of major catechins and caffeine. *Food Chem.* **2006**, *96*, 597-605.

(22) Andre, C. M.; Ghislain, M.; Bertin, P.; Oufir, M.; Del Rosario Herrera, M.; Hoffman, L.; Hausman, J. F.; Larondelle, Y.; Evers, D. Andean potato cultivars (*Solanum tuberosum* L.) as a source of antioxidant and mineral micronutrients. *J. Agric. Food Chem.* **2006**, *55*, 366-378.

(23) Association of vitamin chemists. *Methods of vitamin assay*, 3rd ed.; Interscience Publishers: New York, 1961.

(24) Caboni, E.; Tonelli, M. G.; Lauri, P.; Iacovacci, P.; Kevers, C.; Damiano, C.; Gaspar, T. Biochemical aspects of almond microcuttings related to *in vitro* rooting ability. *Biol. Plantar.* **1997**, *39*, 91-97.

(25) Lamaison, J. L.; Carmat, A. Teneur en principaux flavonoïdes des fleurs et des feuilles de *Crataegus monogyna Jacq.* et de *Crataegus laevigata* (Poiret) DC en fonction de la végétation. *Plant. Méd. Phytothér.* **19990**, *XXV*, 12-16.

(26) Tabart, J.; Kevers, C.; Pincemail, J.; Defraigne, J. O.; Dommes, J. Evaluation of spectrophotometric methods for antioxidant compound measurement in relation to total antioxidant capacity in beverages. *Food Chem.* **2010**, *120*, 607-614.

(27) Nielsen, I. L.; Haren, G. R.; Magnussen, E. L.; Dragsted, L. O.; Rasmussen, S. E. Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography, Investigation of their pH stability and antioxidative potency. *J. Agric. Food Chem.* **2003**, *51*, 5861-5866.

(28) Nakamura, Y.; Tsuji, S.; Tonogai, Y. Analysis of proanthocyanidins in grape seed extracts, health foods and grape seed oils. *J. Health Sci.* **2003**, *49*, 45-54.

(29) Rodriguez-Amaya, D. B. *A guide to carotenoid analysis in foods*, Omni Research: ILSI Press, 2001.

(30) Dutruc-Rosset, G. Détermination par CLHP de neuf anthocyanes principales dans le vin rouge et rosé. *Résolution OENO 22/2003*.

(31) Sharma, V.; Gulati, A.; Ravindranath, S. D.; Kumar, V. A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. *J. Food Comp. Anal.* **2005**, *18*, 583-594.

(32) Bharti, S.; Garg, P. P. Changes in the ascorbic acid content of the lateral buds of soybean in relation to flower induction. *Plant Cell Physiol.* **1970**, *11*, 723-727.

(33) Cacace, J. E.; Mazza, G. Extraction of anthocyanins and other phenolics from black currants with sulfured water. *J. Agric. Food Chem.* **2002**, *50*, 5939-5946.

(34) Zadernowski, R.; Naczk, M.; Nesterowicz, J. Phenolic acid profiles in some small berries. *J. Agric. Food Chem.* **2005**, *53*, 2118-2124.

(35) Hardorne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochem.* **2000**, *55*, 481-504.

(36) Borges, G.; Degeneve, A.; Mullen, W.; Crozier, A. Identification of flavonoid and phenolic antioxidants in black Currants, blueberries, red currants, and cranberries. *J. Agric. Food Chem.* **2010**, *58*, 3901-3909.

(37) Häkkinen, S.; Heinonen, M.; Kärenlampi, S.; Mykkänen, H.; Ruuskanen, J.; Törrönen, R. Screening of selected flavonoids and phenolic acids in 19 berries. *Food Res. Internat.* **1999**, *32*, 345-353.

(38) Jakobek, L.; Seruga, M.; Novak, I.; Medvidovic-Kosanovic, M. Flavonols, phenolic acids and antioxidant activity of some red fruits. *Deutsche Lebensmit.Rund.* **2007**, *103*, 369-378.

(39) Kelm, M. A., Hammerstone, J. F.; Schmitz, H. H. Identification and quantitation of flavanols and proanthocyanidins in foods: How good are the datas? *Clin. Developm. Immunol.* **2005**, *12*, 35-41.

(40) Morris, W. L; Ducreux, L.; Griffiths, D.; Steward, D.; Davies, H. V.; Taylor, M. Carotenogenesis during tuber development and storage in potatoes. *J. Exp.l Bot.* **2004**, *399*, 975-982.

FIG. AND TABLE CAPTIONS:

Fig. 1: Determination of the contents in ascorbic acid of three blackcurrants organs (berries, buds and leaves) obtained by acetone and specific extractions: Spectrophotometric (A) and HPLC (B) determinations. The concentration is expressed in milligram of ascorbic acid per gram of fresh weight. No significant difference ($p>0.05$) was observed for spectrophotometric assay between the three explants tested and between the two types of extraction used, except for specific extraction of buds (n=3). For the three explants, analysed by HPLC, a significant difference ($p>0.05$) was observed (n=3).

Fig. 2: Determination of the total phenolic contents of three blackcurrant plant organs (berries, buds and leaves). The concentration is expressed in milligram equivalent chlorogenic acid per gram of fresh weight. For three analysed explants, a significant difference ($p<0.05$) was observed for the fruits compared with the leaves and the buds (n=3).

Fig. 3: HPLC determination of the content in phenolic acids of three blackcurrant extracts (leaves, buds and berries) obtained by acetone extraction. The concentration is expressed in microgram of phenolic acids per gram of fresh weight (n=3).

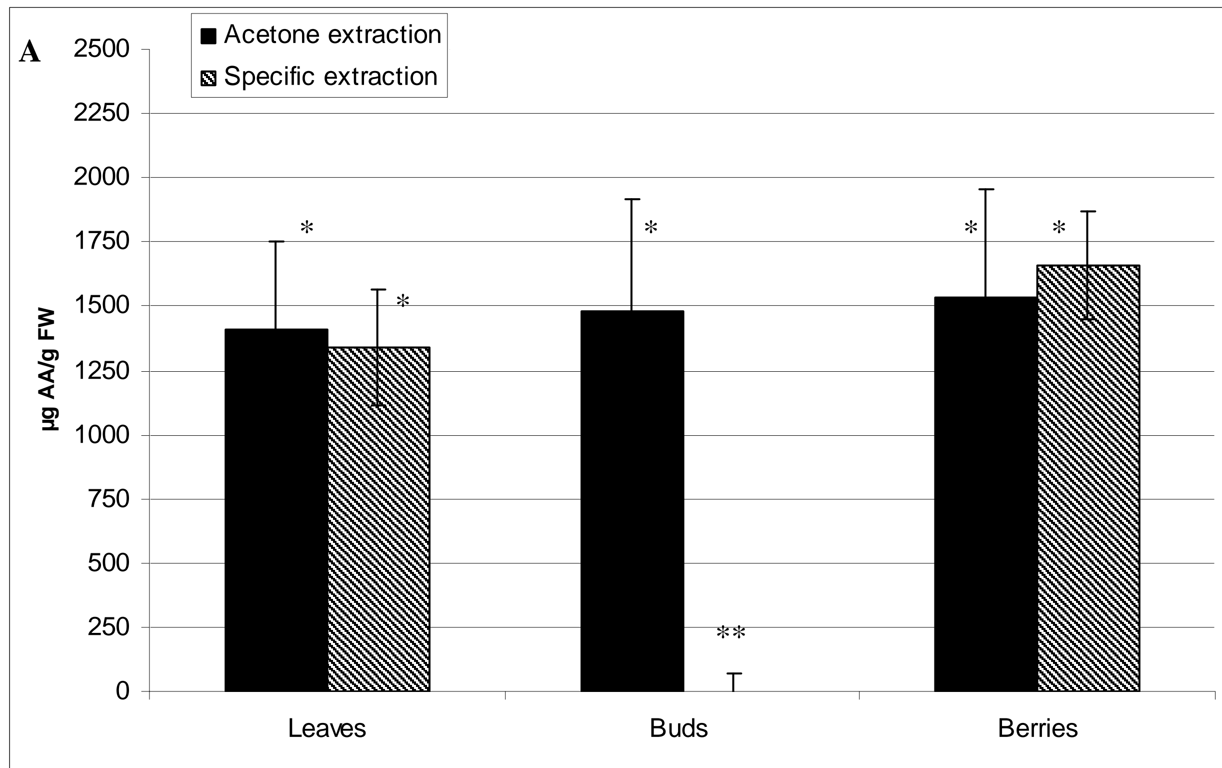
Fig. 4: HPLC determination of the content in phenolic acids of three blackcurrant extracts (leaves, buds and berries) obtained by specific extraction. The concentration is expressed in microgram of phenolic acids per gram of fresh weight (n=3).

Fig. 5: Total carotenoid content in extracts of blackcurrant plant organs (leaves, buds and berries) after acetone and specific extractions. The results were expressed in microgram of carotenoids per gram of fresh weight (n=3). A significant difference on the total carotenoid content ($p< 0.05$) was observed between the three explants analysed (n=3).

Table 1: Contents in various flavonol aglycons in the three extracts (leaves, buds and berries), expressed in microgram of aglycon per gram of fresh weight and quantification of the total flavonol content expressed in milligram quercetin equivalent per gram of fresh weight. Among the three explants, a significant difference in the total flavonol content ($p< 0.05$) was observed for the berries compared to the buds and leaves (n=3).

Table 2: Contents of various anthocyanidins in the three extracts (leaves, buds and berries) obtained by acetone extraction (A) and specific extraction (B), expressed in microgram of aglycon per gram of fresh weight and quantification of the total anthocyanin content expressed in microgram kuromanin equivalent per gram of fresh weight. Among the three explants, a significant difference in the total anthocyanin content ($p<0.05$) was observed for the berries compared to the buds and leaves (n=3).

Table 3 : Identification and quantification of monomers of flavan-3-ols in blackcurrant extracts (leaves, buds and berries) obtained by acetone (A) and specific (B) extraction in microgram of monomer per gram of fresh weight and quantification of the total content in flavanols expressed in milligram catechin equivalent per gram of fresh weight. Among the three explants, a significant difference on the total flavanol content ($p<0.05$) was observed for buds compared to leaves and berries (n=3).



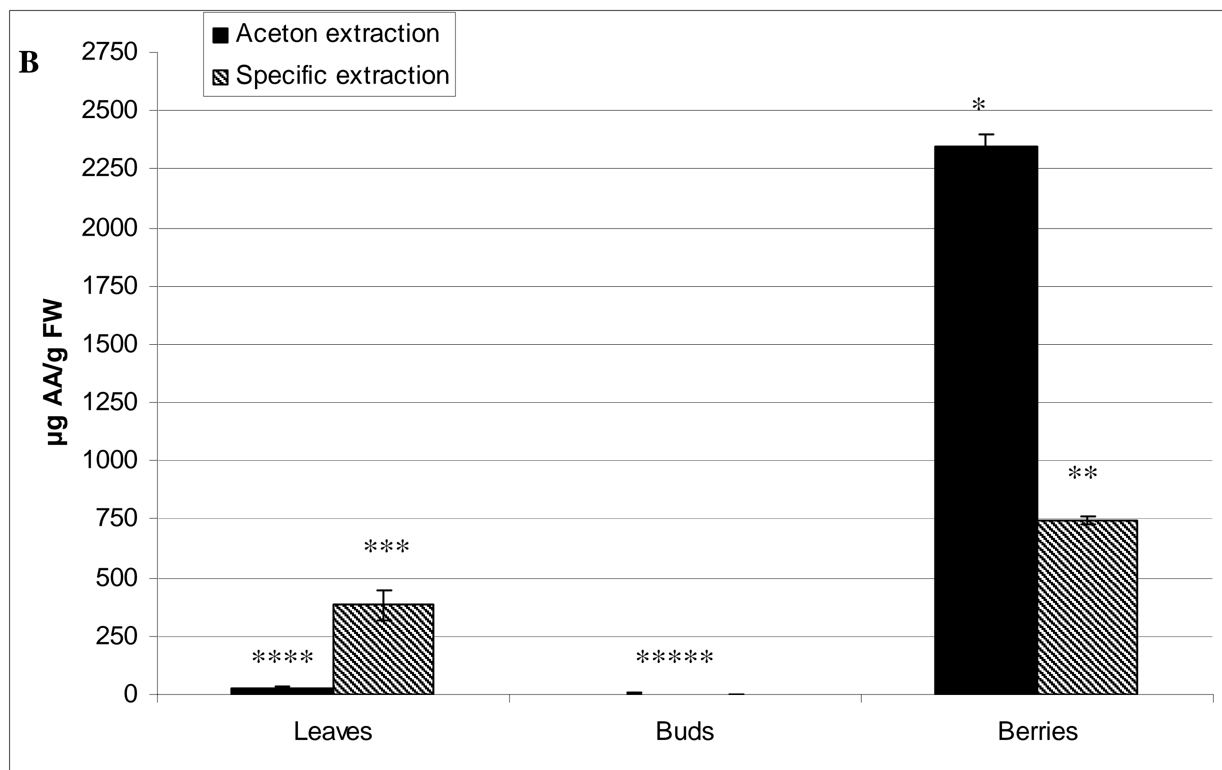


Figure 1:

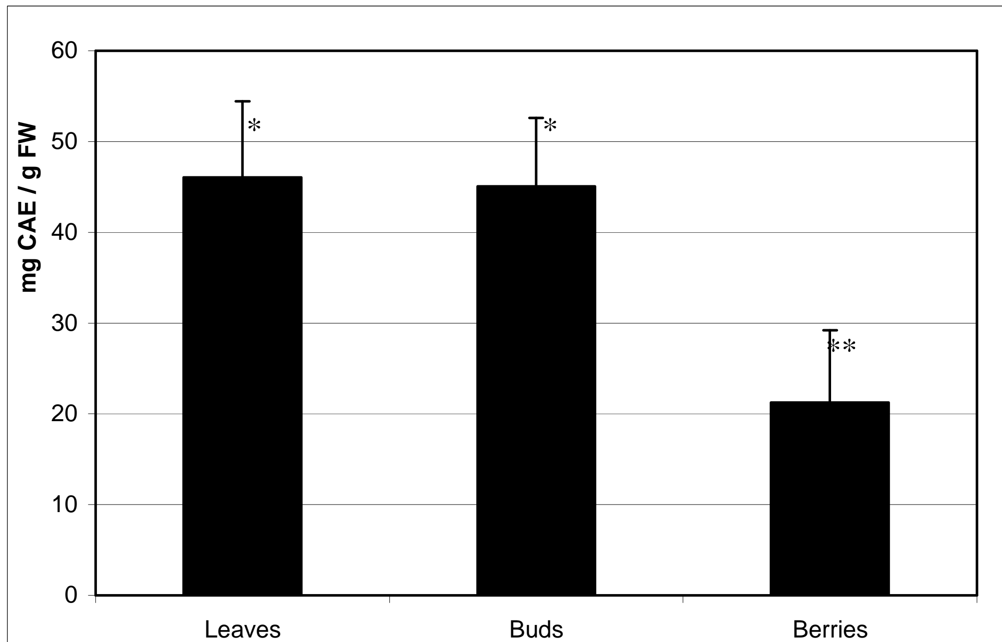


Figure 2:

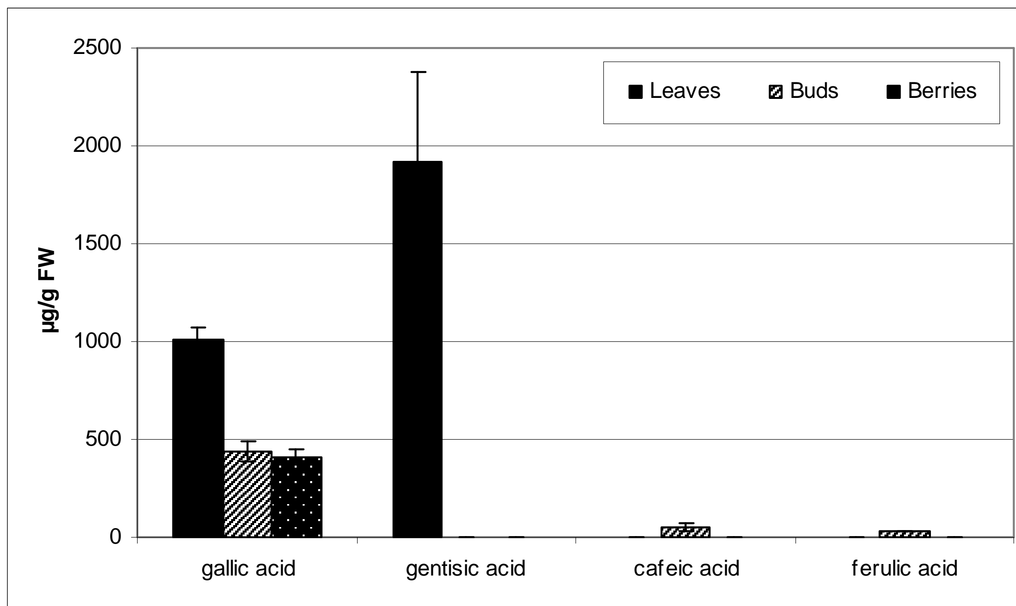


Figure 3:

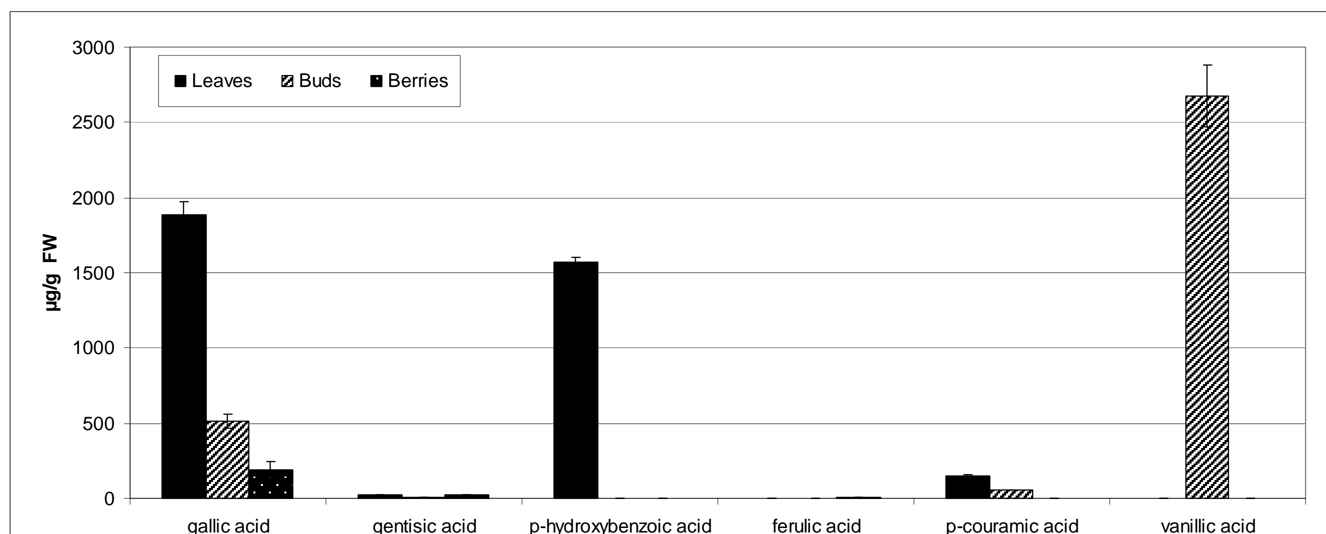


Figure 4:

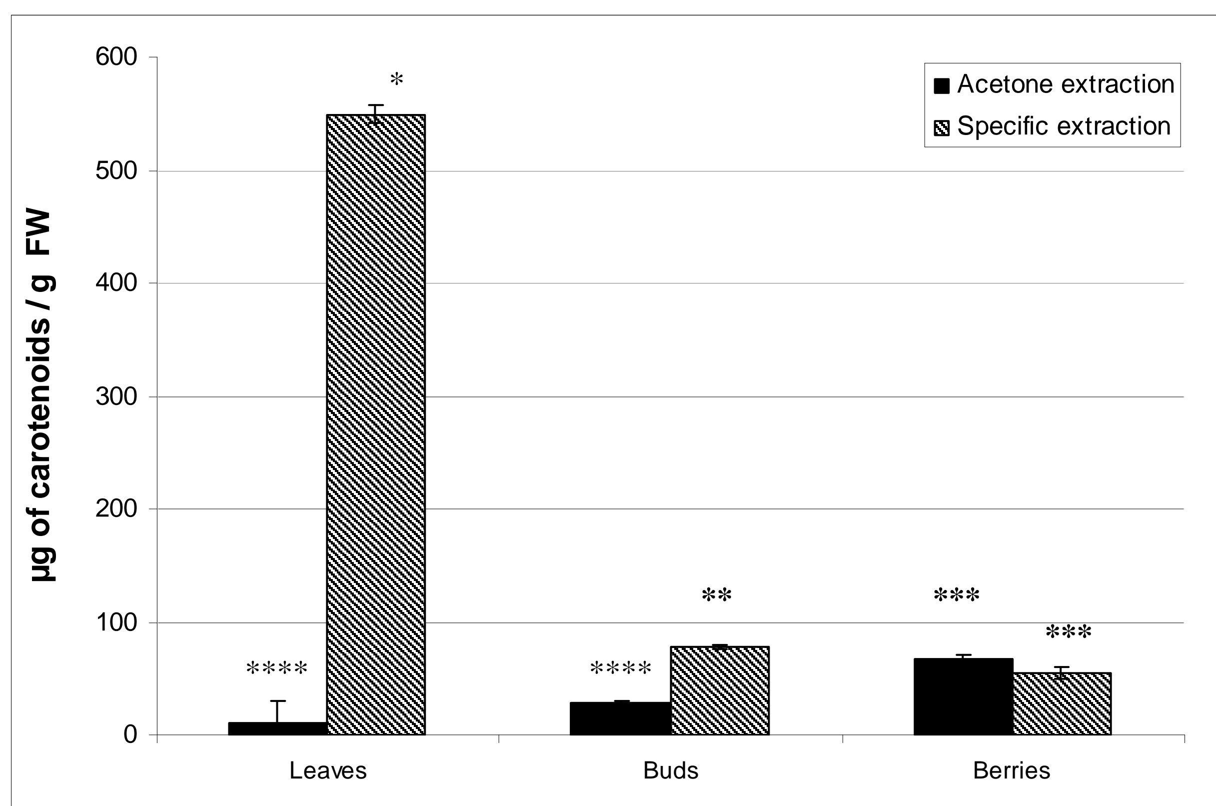


Figure 5:

	Flavonols (µg/ g FW)			Total Flavonols
	Myricetin	Quercetin	Kaempferol	(mg QE/ g FW)
Leaves	139 ± 47	778 ± 203	322 ± 151	2.05 ± 0.34 [a]
Buds	241 ± 57	480 ± 74	87 ± 22	2.15 ± 0.09 [a]
Berries	35 ± 3	77 ± 19	10 ± 5	0.50 ± 0.09 [b]

Table 1:

A

	Anthocyanidins (µg/ g FW)						Total Anthocyanins
	Delphinidin	Petunidin	Cyanidin	Peonidin	Pelargonidin	Malvidin	(µg KuE/ g FW)
Leaves	85 ± 14	514 ± 152	65 ± 19	35 ± 7	0	0	381 ± 72 [a]
Buds	70 ± 5	493 ± 140	14 ± 7	0	0	64 ± 18	260 ± 72 [a]
Berries	155 ± 56	641 ± 61	123 ± 41	21 ± 13	0	503 ± 74	1468 ± 222 [b]

B

	Anthocyanidins (µg/ g FW)						Total Anthocyanins
	Delphinidin	Petunidin	Cyanidin	Peonidin	Pelargonidin	Malvidin	(µg KuE/ g FW)
Leaves	88 ± 18	1181 ± 115	363 ± 138	133 ± 75	258 ± 196	178 ± 119	429 ± 87 [a]
Buds	66 ± 7	114 ± 13	0	0	0	0	665 ± 92 [b]
Berries	92 ± 14	941 ± 167	3 ± 2	19 ± 10	0	10 ± 8	3180 ± 996 [c]

Table 2:

A

	Falvan3-ols (µg/ g FW)					Total Flavan-3-ols
	Epigallocatechin	Gallocatechin	Catechin	Epicatechin	Epigallocatechin Gallate	(mg CaE/ g FW)
Leaves	150 ± 86	382 ± 132	19 ± 9	2,5 ±1,6	0	1.47 ± 0.87 [a]
Buds	599 ± 113	667 ± 45	91 ± 9	48 ± 21	37 ± 35	15.30 ± 0.93 [b]
Berries	114 ± 20	226 ± 4.5	29 ± 10	8 ± 0.5	0	3.42 ± 0.15 [c]

B

	Falvan3-ols (µg/ g FW)					Total Flavan-3-ols
	Epigallocatechin	Gallocatechin	Catechin	Epicatechin	Epigallocatechin Gallate	(mg CaE/ g FW)
Leaves	0	0	0	0	0	9.0 ± 4.4 [a]
Buds	110 ± 4	241 ± 21	36 ± 3	69 ± 22	44 ± 7	32.0 ± 3.4 [b]
Berries	79 ± 33	108 ± 35	78 ± 1	35 ± 4	25 ± 16	9.7 ± 1.4 [a]

Table 3:

Antioxidant and anti-inflammatory activities of *Ribes nigrum* extracts.

Jessica TABART [1], Thierry FRANCK [2], Claire KEVERS [1], Joël PINCEMAIL [3], Didier SERTEYN [2], Jean-Olivier DEFRAIGNE [3], Jacques DOMMES [1]

University of Liège,

[1]Plant Molecular Biology and Biotechnology Unit, B22

[2]CORD, B6

[3]CREDEC, B35

Sart Tilman,

B-4000 LIEGE

Belgium

Corresponding author: Jessica TABART: Jessica.Tabart@doct.ulg.ac.be
Other authors: T.Franck@ulg.ac.be, C.Kevers@ulg.ac.be, J.Pincemail@chu.ulg.ac.be, Didier.Serteyn@ulg.ac.be, JO. Defraigne@ulg.ac.be, J.Dommes@ulg.ac.be.

TITLE RUNNING HEAD: Antioxidant activity of blackcurrant extracts

ABSTRACT
Blackcurrant berries contain very high amounts of flavonoids with a number of health benefit attributed to the antioxidant potential. Recent studies indicate that blackcurrant flavonoids also exhibit anti-inflammatory properties. Leaves and buds actually used to produce food complement could also exhibit such interesting properties.

In the literature, many methods are used to evaluate the antioxidant capacity of food compounds or biological systems, the main ones being the DPPH, TEAC, ORAC and ESR assays. These methods are valid indicators of the antioxidant potential of dietary substances. However this type of assay does not provide evidence that a substance or a mixture of antioxidants has an *in viv*o antioxidant activity when consumed. To obtain more biologically relevant information, the antioxidant activities of the extracts were evaluated on cellular models implicating the measurement of blood haemolysis, of the Cellular Antioxidant Activity on endothelial cells and of the anti-inflammatory activities on isolated equine stimulated neutrophils and purified myeloperoxidase.

The classical in vitro tests, as well the cellular models generally showed that the blackcurrant leaf extract have the highest antioxidant capacity followed by buds and berries. These antioxidant activities are correlated to the total phenolics content of the extracts.

KEYWORDS: Antioxidant, Cellular Antioxidant Activity, Myeloperoxidase, EAHy926, Neutrophils, SIEFED

INTRODUCTION

Oxidative stress results from a decrease of endogenous antioxidant capacities or an increase of reactive oxygen species (ROS) concentration in organisms. It can cause deleterious damages on cell constituents like DNA, proteins, … and finally, could induce several pathologies [1, 2]. Phytochemicals of fruits and vegetables such as polyphenols have been considered of crucial nutritional importance in the prevention of chronic diseases such as cancer, cardiovascular and neurodegenerative diseases. This may be related to their antioxidant activity as well their ability to regulate cellular activities of inflammation-related cells (mast cells, macrophages, lymphocytes and neutrophils).

Blackcurrant berries contain very high amounts of phenolic compounds [3, 4]. They are sometime called “super fruit” because they show a number of health benefits [5]. These benefits are attributed to the antioxidant potential of berries but recent studies indicate that blackcurrant flavonoids exhibit also anti-inflammatory properties. A supplementation with blackcurrant fruit concentrate in monocytic THP-1 cell culture modulates inflammatory response with a decrease of NFκB, TNFα and IL-6 production by an unknown mechanism [5].

Several studies demonstrated that berries have an effect on the prevention of the eye fatigue [6]. There are only few studies on involved mechanisms and the majority of these studies focused on berries [6, 7, 8, 9]. Leaves and buds can also used to produce food supplement. Leaves are used in European traditional medicine for treatment of inflammatory disorders such as rheumatic disease [10]. The proanthocyanidins contained in blackcurrant leaves interfere with the accumulation of circulatory leukocytes, associated with a decrease of pro-inflammatory factors such as TNFα, IL-1β, NO and CINC-1 [11]. To our knowledge, bud health properties were never studied but they are used for the production of food complements.

The objective of this work was to evaluate the antioxidant capacities of the blackcurrant extracts from leave, buds and berries. To perform this study, we used several in vitro classical techniques applied for the measurement of antioxidant capacity and efficacy of food antioxidants. The most popular assay is the ORAC assay (Oxygen Radical Antioxidant Capacity) initially developed by Cao and Prior [12] and using 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) as free radical generator. Other tests include the reaction of antioxidant with stable free radicals (2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2.2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid cation radical ($ABTS^{\cdot+}$)) or the ability of antioxidants to reduce ferric (FRAP assay) and cupric ions (CUPRAC assay). Less unknown is the use of the Electron Spin Resonance (ESR) spectroscopy, the only one method allowing to directly evidence the free radical formation in a biological sample. In presence of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as spin trapping agent, the system xanthine/xanthine oxidase is used to generate and detect superoxide anion radical ($O_2^{\cdot-}$) which has the great advantage to be a physiological species.

In order to get more biologically relevant information, *in vitro* oxidative stress models on cells have also been developed. The first assay used is the haemolysis assay where blood cells were placed in oxidative conditions with or without antioxidant compounds [13]. The second model is the Cellular Antioxidant Activity (CAA) assay on hepatic cells (HepG2). This assay can measure the inhibition of dye oxidation by antioxidants in cell cultures. This method is more biologically relevant because it gives an antioxidant capacity taking into account the uptake of compounds by cells [14]. A third model is linked to ROS production in connection to inflammatory situations. The activation of neutrophils leads to the formation of superoxide

anion through NADPH oxidase cascade. At the same time, activated neutrophils also release myeloperoxidase (MPO) [15] which catalyses the conversion of hydrogen peroxide (H_20_2) to the more potent oxidant hypochlorous acid (HOCl).

For different reasons linked to instruments limitations, mechanisms, endpoint, quantification method and biological relevance, there is often a lack of correlation between results obtained through these differentassays. Actually, it is well admitted that there is no simple universal assay by which antioxidant capacity of food can be assessed accurately and quantitatively. It is the reason why we used in this study several in vitro test including cellular models to better evidence and understand the antioxidant and anti-inflammatory potency of blackcurrant extracts (leaves, berries and buds).

MATERIALS AND METHODS

Sample preparation

The buds, berries and leaves of blackcurrant (noir de Bourgogne), two years old, were harvested respectively in March, July and August in the Belgian Ardennes (Bihain). The various explants were directly cut out into pieces, frozen in liquid nitrogen, lyophilized and stored at -20°C for some months.

For extraction, one gram of fresh sample (berries, buds or leaves) was ground with 1 gram of quartz and 10 ml of extraction solution: acetone/water/acetic acid (70:28:2) [16]. The mixture was shaken during 1 h at 4°C and centrifuged at 17 000 g for 15 min. The supernatant was removed, and the sample was extracted again with the same procedure but incubated only 15 min. The supernatants were pooled and then diluted as appropriate for the immediate analysis.

For experiments on EAHy926 cells and neutrophils, the acetone contained in the supernatant was evaporated; the remainder of supernatant was freeze-dried and stored at – 20°C. For different assays, the extracts were suspended in the adequate medium and filtered.

Determination of total phenolic contents

Total phenolics were determined according to the Folin-Ciocalteu method described in a previous study [17] The results were expressed in mg equivalent chlorogenic acid (CAE) per gram of fresh weight.

In vitro evaluation of antioxidant capacity

To determine the antioxidant capacity, the first method used was the TEAC assay (scavenging of the radical 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid, ABTS). Antioxidant capacity was also determined by scavenging of the radical 2,2-diphenyl-1-picryhydrazyl (DPPH). ORAC assays were carried out on a Fluoroskan Ascent FL Plate Reader (ThermoLabsystems, Finland) at 37°C. For these three methods described in a previous study [17], Trolox was used as standard and the antioxidant capacity was expressed in mg Trolox equivalent (TE) per gram of fresh weight. All samples were analysed in duplicate.

Superoxide anion (O_2^-)-scavenging capacities were measured by electron spin resonance (ESR) spectroscopy described in a previous study [17]. Measurements were performed at room temperature on a JEOL–Jes-FR30 spectrophotometer. The results were expressed in units of SOD equivalent (U SODE) per gram of fresh weight. The running conditions were as follows: 9.5 GHz frequency, 100 kHz modulation frequency, 4mW microwave power, 335.6 mT centre field, 2500G Gauss modulation amplitude, 1 s time constant, and 4 min sweep time.

Evaluation of antioxidant capacity on cells

a) Red blood cell resistance to oxidative stress (haemolysis)

The resistance of red blood cell was measured on a microliter plate reader (Labsystems IEMS reader MF) as described by Tabart et al. [17]. The results were expressed in mmole Ascorbic Acid (AA) equivalent per gram of fresh weight.

b) Cellular Antioxidant Activity (CAA) on EAHy926 cells

Cell Culture: The human endothelial-like EAhy926 cell line [18] derived from the fusion of human umbilical vein endothelial cells with the A549 carcinoma cell line. EAHy926 were grown in DMEM (Biowithaker, Lonza) supplemented with 10% of heat-inactived FBS (Fetal serum bovine), 10 µM HEPES, 5000 unit of penicillin and streptomycin, 1 mM sodium pyruvate, 2 mM glutamine and 2% HAT (hypoxanthin, aminoprotein and thymidin) and were maintained at 37°C and 5% CO_2.

Cellular Antioxidant Activity (CAA) was measured using the method of Wolfe and Liu [14] with various modifications. EAHy926 cells were seeded at a density of 5000 cells/ well on a 96 well microplate in 100 µl of supplemented DMEM. 100 µL of blackcurrant extract were added in each well, together with 100 µl of DCFH-DA (dichlorofluorescein diacetate, 25 µM, Sigma) dissolved in PBS at 37°C. After 1 h at 37°C, wells were washed with 100 µl PBS (3 times). Then, 100 µl of APPH (600 µM) were added in each well. The plate was placed into a Fluoroskan Ascent FL Plate Reader at 37°C. Emission of fluorescence was recorded every five minutes during 1h (emission at 538 nm and excitation at 485 nm). We used the method of quantification described by Wolfe and Liu [14]. Results were expressed in microgram quercetin equivalent (QE) per gram of freeze-dried extract. Each experiment was repeated with different cell batches.

Evaluation of anti-inflammatory activity of ribes extracts on the oxidant response of neutrophils and on MPO activity

Isolation of equine neutrophils: They were isolated from EDTA (1.6 mg/ml) treated blood drawn from jugular vein of horses not under medical treatment (Faculty of veterinary Medicine, University of Liège, Belgium). They were isolated at room temperature by centrifugation on a percoll density gradient according to the method of Pycock et al. [19]. After washing in physiological saline solution, the cell pellets were suspended in 20 mM phosphate buffer saline (PBS) at pH 7.4. The cells were used within four hours after isolation, and each assay was performed in triplicate. Each experiment was repeated at least twice with different cell batches.

Effect of the extract on the ROS production by stimulated neutrophils

Neutrophil suspensions (10^6 neutrophils/200 µL PBS) were distributed in the wells of a 96-well microtiter plate (White Combiplate 8, Fischer Scientific, Tournai, Belgium) and incubated for 10 min at 37°C with the extracts at final concentration of 50, 25, 10, 7.5, 5, 2.5, 1, 0.5 µg mL^{-1} After incubation, neutrophils were stimulated with phorbol myristate acetate (PMA) according to the method of Kohnen et al. (2007). Immediately after the addition of PMA, the CL response of the neutrophils was monitored for 30 min (Multiscan Ascent, Fischer Scientific, Tournai, Belgium) and expressed as the integral value of the total CL emission. Control assays made with the vehicle solution of extracts (PBS) were taken as

100% of chemiluminescence response. Each experiment was repeated with different cell batches.

Effect of the extracts on the release of MPO by stimulated neutrophils:

The neutrophil suspensions (10^6 cells/ml) were incubated for 10 min at 37 °C with the extracts at final concentration of 50, 25, 10, 7.5, 5, 2.5, 1, 0.5 µg mL^{-1} and then activated for 30 min at 37°C with PMA as described in Kohnen et al. (2007). After activation, the neutrophil suspensions were centrifuged (450 x g, 10 min), and the supernatants were collected. MPO released by the neutrophils was measured in the supernatants by an ELISA assay raised against equine MPO (Franck et al., 2005) and distributed by BIOPTIS (Liege, Belgium). The control assay taken as 100% of MPO release was made with the supernatant obtained from PMA stimulated neutrophils where the extracts were replaced by PBS. Each experiment was repeated with different cell batches.

Effect of the extracts on the specific activity of MPO measured by SIEFED

The Specific Immunological Extraction Followed by Enzymatic Detection (SIEFED) is an original method developed for the specific detection of active equine neutrophil MPO (Franck et al., 2006) The first step is an immunoextraction of MPO from a solution or a biological sample by specific immobilized antibodies; the next step consist of washings to eliminate unspecifically bound compounds or interfering substances and the third step is the detection of MPO enzymatic activity. The extracts at final concentrations of 50, 25, 10, 7.5, 5, 2.5, 1, 0.5 µg mL^{-1} were incubated for 10 min with pure equine MPO (50 ng/ml) in the dilution buffer (PBS 20 mM with 5 g/L BSA and 0.1 % Tween 20) before the immunoextraction step. The control taken as 100 % MPO activity was performed with purified MPO in PBS.

Statistical analysis

For antioxidant capacity assays, the data were subjected to the statistical analysis of the variance (ANOVA-1) to evaluate the significant differences between various explants of blackcurrant. The difference was regarded as significant when $p < 0.05$ or 0.01.

For anti-inflammatory assays, a one-way ANOVA followed by Dunnett's post-test was performed to compare the inhibitory percentage of the extract with the corresponding 100 % controls. A p value < 0.05 was considered as significant.

RESULTS

Evaluation of antioxidant capacity of blackcurrant extracts by *in vitro* assays

Blackcurrant extracts were prepared from leaves, berries and buds. Their antioxidant capacity was evaluated by several commonly used assays (Table 1). With all these methods, we can show a similar profile for the three explants analyzed. Leaf and bud extracts showed a significant ($p<0.01$) higher antioxidant capacity than berry extracts. In the ESR assay, leaves and buds also showed a higher activity than berries.

For all assays, the observations were correlated with the total phenol content: leaves and buds contained two times more phenolic compounds (46.1 ± 8.4 and 45.1 ± 7.5 mg ACE/ g of FW, respectively) than berries (21.25 ± 8.0 mg ACE/ g of FW).

Evaluation of antioxidant capacity of blackcurrant extracts on cellular models

The cellular antioxidant activity of blackcurrant extracts (leaves, berries and buds) was measured using the CAA assay on EAHy926 cells (Table 2). Leaf extracts had the highest CAA value (12.89 ± 0.77 µmole QE/ g of LE) followed by buds (8.8 ± 1.92 µmole QE/ g of LE) and finally, berries (0.40 ± 0.10 µmole QE/ g of LE). For each extract, we also determined the concentration to obtain 50% of fluorescence inhibition (EC_{50}): 113 ± 7 µg /ml for leaf extract, 166 ± 36 µg /ml for bud extract and 3688 ± 910 µg /ml for berry extract. For the haemolysis assay, also called "Resistance Test of blood cells in oxidative stress conditions", the antioxidant capacities were the same for leaves and buds (around 1.2 mmole AA/ g of FW) and lower for berries (0.394 ± 0.053 mmole AA/ g of FW).

To determine if this inhibition was really due to a decreased of ROS production in the presence of the extracts and not to a cytotoxic effect on endothelial cells, a viability assay using MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltatrazolium bromide; Cell Growth Determination Kit MTT, Sigma-Aldrich) was made in the range of concentrations from 0 to 1 mg extract/ml. The extracts (data not shown) were not cytotoxic.

Effects of blackcurrant extracts on total ROS production by neutrophils

The influence of blackcurrant extracts (leaves, berries and buds) on the ROS production of PMA-activated neutrophils was determined through a lucigenin dependent chemiluminescence assay (Figure 1). A significant dose-dependent inhibition was observed for all extracts. We reached 50% of inhibition with a concentration of 5 ± 0.5 µg of leaf extract/ml, 13 ± 4.3 µg of bud extract/ml and 44 ± 20 µg of berry extract/ml. To determine if this inhibition was really due to a decrease of ROS production in the presence of the extracts and not by a toxic effect on equine neutrophils, a tryptan blue exclusion test was made in the range of concentrations used and above. The extracts (data not shown) were not cytotoxic. Only some cell aggregates were formed with high extract concentrations (1 mg/ml) versus PBS controls.

Effects of blackcurrant extracts on the total MPO released by stimulated neutrophils

The effects of blackcurrant extracts (leaves, berries and buds) on the total MPO released by activated neutrophils were measured by an ELISA assay. Extract addition did not induce significant decrease of MPO release versus control (data not presented). The higher concentration of blackcurrant extracts (1 mg extract/ ml) gave only 15 to 20 % of inhibition. Thus, only slight effects of blackcurrant extracts were observed on MPO release by neutrophils.

Effects of blackcurrant extracts on MPO activity (*in vitro*)

To determine the effects of blackcurrant extracts (leaves, berries and buds) on MPO activity, we used the SIEFFED method developed by Franck et al. [21]. We observed a dose-dependent inhibition of the MPO activity with all the explants tested (Figure 2). For leaves, the percentage of inhibition was superior to 50% with the smallest concentration tested (around 60 % inhibition at 0.5 µg/ml for leaf extract). For buds, we reached 50% inhibition at 0.9 ± 0.2 µg/ml; and for berries, 14 ± 4 µg/ml. At the smallest concentrations of berry extract (1 µg/ml), a significantly increased MPO activity was observed.

DISCUSSION

Many methods are used in different studies to evaluate the antioxidant capacity of food compounds or biological systems. The two oldest and most usually used *in vitro* methods are the assays based on DPPH and ABTS radicals, two methods based on the transfer of hydrogen between the free radicals and the antioxidants. These two methods give similar results for the

blackcurrant extracts as well as for the majority of plant extracts [22, 23]. But, the technique of discolouration of $ABTS^{\cdot+}$ radical presents nevertheless a disadvantage compared to the DPPH method. Indeed, radical $ABTS^{\cdot+}$ is very unstable, a loss of absorbance of 30% is already observed over a 1 hour incubation while radical DPPH is very stable [23]. The ORAC method is more widely used in the agro-food sector. At the present time, it is considered as the method of reference to evaluate the total antioxidant capacity of a food sample. This assay largely differs from the two other methods: a fluorescent dye is destroyed by AAPH radical and the antioxidants present in the sample will protect this molecule against oxidation. This technique gives higher values of antioxidant capacity than the two first methods. With regard to another technique, the ESR assay, the results are not easily comparable with the methods described above owing to the fact that the results are not expressed in the same units. ESR gives us information as for the action of antioxidants on a free radical, the anion superoxide, found abundantly in the human body. $O_2^{\cdot-}$ is mainly produced in cellular respiratory processes in mitochondria and by the NADPH oxidase on endothelial cells and phagocytes.

In this first part of the study, we can observe that the choice of the method has a notable influence on the determination of the antioxidant potential of a sample. This is due to the various antioxidant compounds found in the food matrices and the sources of free radicals used in the assays. But we observed a similar profile on the antioxidant capacity: leaves ≥ buds > berries, even if the intensity of the answer is variable from one technique to another due to the type of reaction implemented but also to the choice of standard (Trolox, chlorogenic acid or SOD). These methods are valid indicators of the antioxidant potential of dietary substance but this type of assays did not provide evidence that a substance or a mixture of antioxidant compounds has an *in vivo* antioxidant activity when consumed.

Complementary to the classical *in vitro* tests to measure the antioxidant potential, we also studied the effect of blackcurrant extracts on the CAA and anti-inflammatory activities of cellular models. The CAA assay, developed by Wolfe and Liu [14], and the haemolysis assays are complementary but are more biologically relevant than *in vitro* assays since they used cellular models to evaluate the antioxidant capacity of fruits, vegetables, and foods. The haemolysis assay is on *in vivo* situation since this method proposes the influence of an antioxidant contribution on red blood cells near to an oxidizing stress state. The rate of cell lysis can be regarded as an *in vitro* marker of the oxidative damage. The susceptibility of the

red blood cells could be determined by their submission to an oxidative system that produces ROS and by measuring at regular laps time the rate of haemolysis. The latency time before the beginning of haemolysis is proportional to the quantity of antioxidant content present in the reaction mixture [24]. For the CAA, the dye is taken up by cells and it is deacetylated by intracellular enzymes (making it unable to exit the cell). The CAA assay is based on the ability of hydroxyl radicals to react with a fluorescent probe in the cell and on the prevention of this reaction in the presence of antioxidant compounds inside the cell. The DCFH-DA is a commonly used dye in biological systems [25, 26]. DCFH-DA is apolar and it diffuses through the biological membranes. In the cell, the dye is deacetylated by cellular esterases to form DCFH that is more polar and is trapped in the cell. In presence of ROS, DCFH is oxidized into DCF that is fluorescent. We have worked in the conditions described by Wolfe and Liu [14] but we have chosen endothelial cells for our investigations. Endothelial cells are involved in the development of several vascular pathologies through endothelial dysfunction [27] by various mechanisms like increasing endothelial oxidase activity like NADPH oxidase enzyme [28], decreased the endothelial nitric oxide (vasodilator agent) bioavailability by using it as a substrate [28, 29].

DCFH-DA was already used on EAHy926 cells [26] to measure the protection afforded by honey on oxidative stress. The optimization of the CAA assay on EAHy926 cells did need some modifications: cell density was 5000 cells/ well and PBS was used for dye solubilisation instead of William's E medium. This assay was used to determine the antioxidant potential of some berries by Wolfe and Liu [30]: pomegranate, blackberry, blueberry, strawberry, raspberry and cranberry. The extracts had an antioxidant action in the CAA model at two levels. First, they can act at the cell membrane, breaking peroxyl radical chain reactions at the cell surface. Second, they can be taken up by the cells and then react with ROS intracellulary [14]. In our study, we found with these two tests the same profile than *in vitro* assays.

A second cellular model is used and consists of polymorphonuclear neutrophils which are the primary effector cells in host responses to injury and infection [15]. The antimicrobial function of stimulated PMNs is based on their phagocytic capacity and ability to release oxidant and proteolytic enzymes and ROS involved in the destruction of microorganisms. ROS play important roles in the pathogenesis of various diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation [31,

32]. Myeloperoxidase (MPO) is an haemic enzyme stored in the azurophilic granules of neutrophil and has an essential role in the destruction of bacteria inside PMNs. By using hydrogen peroxide (H_2O_2) and chloride anion (Cl^-), it produces hypochlorous acid (HOCl), an important microbial killer [15]. MPO also catalyzes tyrosine nitration in proteins from nitrite and H_2O_2 [20]. In some conditions of acute and chronic inflammations, MPO is released from cells and can cause severe damage to macromolecules in cell membranes and in extracellular space [33]. Thus, the decreasing oxidant activity of neutrophils and MPO may prove beneficial for the treatment of inflammation-related diseases.

When neutrophils were stimulated in the presence of blackcurrant extracts, a dose dependent inhibitory effect on ROS production was observed for each extract. These results showed that some molecules contained into the extract have the abilities to scavenge ROS produced by neutrophils or to inhibit the activation of NADPH oxidase by interfering with the intracellular pathway or membrane elements involved in the activation of the enzymatic complex. We suspected that the effect of ribes extract on the oxidative burst of PMNs is mainly extracellular. Indeed, ribes extracts had poor effect on the degranulation pathway of MPO probably because the molecules which contain do not interfere with membrane receptors, cytoskeleton, intracellular Ca^{2+} and PKC activation pathway [34, 35]. Such hypothesis should be confirmed by testing longer incubation time between the cell and the extract or by adapting on PMNs, the DCFH technique used with endothelial cells. Nevertheless, our results showed that blackcurrant extract had important extracellular scavenging activity on ROS produced by stimulated neutrophils.

Although blackcurrant extract had a poor effect on the release of total MPO, we were interested about their effect on the activity of this oxidant enzyme. Evidence has emerged that MPO-derived oxidants such HOCl and nitrogen dioxide ($^{\bullet}NO_2$) contribute to tissue damage and the initiation and propagation of acute and chronic vascular inflammatory disease that attracts considerable interest in the development of therapeutically useful MPO inhibitors including natural compounds [36]. All the tested extracts, mainly leaves and buds, exerted a strong dose-dependent inhibitory effect on the specific activity of equine MPO as measured by the SIEFED assay. In SIEFED assay, the interferences of the extract with the revelation system were not possible since the tested compound or the extract was washed before the measurement of the activity. Therefore, this technique constitute a useful pharmacological

tool to study specific inhibitors of MPO because if an inhibition of the enzyme is measured after the washing of the tested compound, it means that this compound can interact directly with the enzyme by inducing conformational changes in the protein moiety or by blocking or hindering the active site of the enzyme.

Interestingly, all the antioxidant activities of the blackcurrant extracts are correlated with their content in total polyphenolic compounds. In a previous study [37], we showed that leaf and bud extracts had a higher flavonol content (2.05 ± 0.34 and 2.15 ± 0.09 mg quercetin equivalent per gram of fresh weight, respectively) than berry extract (0.5 ± 0.1 mg quercetin equivalent per gram of fresh weight), with a prevalence of quercetin. Some flavonoids, like quercetin, rutin, resveratrol, are especially known for their scavenging properties [38]. In addition, several studies showed that some polyphenolic compounds could have a potential inhibitory effect on inflammation by decreasing the ROS production and degranulation [39] of stimulated neutrophils, but also by decreasing the activities of NADPH oxidase [40, 41] and MPO [42]. Other studies showed that flavonoids could prevent cardiovascular diseases [43] by inhibiting the activity of MPO, which could be responsible of the oxidation of low-density lipoproteins in arteries [43].

In conclusion, blackcurrant extracts possess strong antioxidant activities as demonstrated by *in vitro* classical assays, but also by cellular models such as endothelial cells and polymorphonuclear neutrophils. Our study emphasized the intracellular and extracellular scavenging properties of blackcurrant extracts showing not any cell toxicity. Although blackcurrant extracts do not interfere with the degranulation of MPO by stimulated neutrophils, they strongly inhibited the activity of MPO. By inhibiting the ROS production by endothelial cells and polymorphonuclear neutrophils, blackcurrant extract present an interesting potential against inflammatory and cardiovascular diseases. For all assays, a better effect was observed for leaves and buds in comparison to berries, and these effects seemed to be correlated to the total polyphenolic contents of the extracts. Very few studies have characterized other explants than berries. The fruits are a good source of anthocyanins and flavonols [44, 45]. Leaves contain small quantity of essential oil but also numerous flavonoid glycosides, especially derivates of quercetin and kampferol, and prodelphinidins [10]. Further investigations will focus on the characterization of the compounds present in the extracts to identify the active compounds or to show possible synergic interactions between several

compounds which could have benefit effect against inflammatory diseases in animals and inhuman.

ACKNOWLEDGMENT:

J.T. gratefully acknowledges the AFR Luxembourg and the memberships of CEDEVIT A.S.B.L (Liège).

REFERENCES

[1] Halliwell B, Aruoma OI. DNA and free radicals. FEBS Lett. 1993; 335: 140-1.

[2] Halliwell B. Lipid peroxidation, antioxidants and cardiovascular disease: How should we move forward? Cardiovasc Res. 2000; 47: 410-8.

[3] Häkkinen S, Heinonen M, Kärenlampi S, Mykkänen H, Ruuskanen J, Törrönen R. Screening of selected flavonoids and phenolics acids in 19 berries. Food Res Intern. 1999; 32: 345-53.

[4] Benvenuti S, Pellati F, Melegari M, Bertelli D. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes*, and *Aronia*. Food Chem Toxicol. 2004; 69: 164-9.

[5] Lyall KA, Hurst SM, Cooney J, Jensen D, Lo K, Hurst RD, Stevenson LM. Short-term blackcurrant extract consumption modulates exercise-induce oxidative stress and lipopolysaccharide-stimulated inflammatory responses. Am J Physiol Regul Integr Comp Physiol. 2009; 297: R70-R81.

[6] Nakamura Y, Matsumoto H, Todoki K. Endothelium-dependent vasorelaxation induced by black currant concentrate in rat thoracic aorta. Jpn J Pharmacol. 2002; 89: 29-35.

[7] Moller P, Loft S, Alfthan G, Freese R. Oxidative DNA damage in circulatoring mononuclear blood cells after ingestion of blackcurrant juice or anthocyanin-rich drink. Mut Res. 2004; 551:119-26.

[8] McGhie TK, Walton MC, Barnett LE, Vather R, Martin H, Au J, Alspach PA, Booth CL, Kruger MC. Boysenberry and blackcurrant drinks increased the plasma antioxidant capacity in an elderly population bud had little effect on other markers of oxidative stress. J Sci Food Agric. 2007; 87: 2519-27.

[9] Dalgard C, Nielsen F, Morrow JD, Enghusen-Poulsen H, Jonung T, Horder M, and de Maat MPM. Supplementation with orange and blackcurrant juice, but not vitamin E, improves inflammatory markers in patients with peripheral arterial disease. Br J Nutr. 2009; 101: 263-9.

[10] Wichtl M, Anton R. Plantes thérapeutiques : Tradition, pratique officinale, science et thérapeutique. Ed. Tec. & Doc. Paris; 1999.

[11] Garbacki N, Kinet M, Nusgens B, Desmecht D, Damas J. Proanthocyanidins, from *Ribes nigrum* leaves, reduce endothelial adhesion molecules ICAM-1 and VCAM-1. J Inflamm. 2005; 2: 9-21.

[12] Cao G, Prior RL. Measurment of oxygen radical absorbance capacity in biological samples. Methods Enzymol. 1999; 299: 50-62.

[13] Girodon F, Blache D, Monget AL, Lombart M, Brunet-Lecompte P, Arnaud J, Richard MJ, Galan P. Effect of two-year supplementation with low doses of antioxidant vitamins and/ or minerals in elderly subjects on levels of nutrients and antioxidant defense parameters. J Am Col. Nutr. 1997; 4: 357-65.

[14] Wolfe KL, Liu RH. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. J Agric Food Chem. 2007; 55: 8896-07.

[15] Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005; 77: 598-625.

[16] Tabart J, Kevers C, Sipel A, Pincemail J, Defraigne JO, Dommes J. Antioxidant capacity of black currant varies with organ, season, and cultivar. J Agric Food Chem. 2006; 54: 6271-6.

[17] Tabart J, Kevers C, Pincemail J, Defraigne JO, Dommes J. Comparative antioxidant capacities of phenolic compounds measured by various tests. Food Chem. 2009; 113: 1226-33.

[18] Edgell CIS, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA. 1983; 80: 3734-7.

[19] Pycock JF, Allen WE, Morris TH. Rapid, single-step isolation of aquine neutrophils on a discontinuous Percoll density gradient. Res Vet Sci. 1987; 42: 411-2.

[20] Kohnen S, Franck T, Van Antwerpen P, Zouaoui Boudjeltia K, Mouithys-Mickalad A, Deby C, Moguilevsky N, Deby-Dupont G, Lamy M, Serteyn D. Resveratrol inhibts the activity of equine neutrophil myeloperoxidase by a direct interaction with the enzyme. J Agric Food Chem. 2007; 55: 8080-7.

[21] Franck T, Kohnen S, Deby-Dupont G, Grulke S, Deby C., Serteyn D. A specific method for measurement of equine active myeloperoxidase in biological samples and in *in vitro* tests. J Vet Diagn Invest. 2006; 18: 326-34.

[22] Awika JM, Rooney LW, Waniska RD. Anthocyanins from black sorghum and their antioxidant properties. Food Chem. 2004; 90: 293-301.

[23] Stratil P, Klejdus B, Kuban V. Determination of total Content of Phenolic Compounds and Their Antioxidant Activity in Vegetables – Evaluation of Spectrophotometric Methods. J Agric Food Chem. 2006; 54: 607-16.

[24] Pincemail J, Meurisse M, Limet R, Defraigne JO. L'évaluation du stress oxydatif d'un individu : une réalité pour le médecin. Vaisseaux, Cœur, Poumons. 1999 ; 4 (5).

[25] Wang H, Joseph JA. Structure-activity relationships of quercetin in antagonizing hydrogen peroxide-induced calcium dysregulation in PC12 cells. Free Radical Biol Med. 1999; 27: 683-94.

[26] Beretta G, Orioli M, Facino RM. Antioxidant and radical scavenging activity of honey in endothelial cell cultures (EAHy926). Planta Med. 2007; 73: 1182-9.

[27]Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae K, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in pathophisiology of vascular disorders. Blood. 1998; 91: 3527-61.

[28] Ray R, Shah AM. NADPH oxidase and endothelial cell function. Clin Sci.2005; 109: 217-26.

[29] Baldus S, Rudolph V, Roiss M, Ito WD, Rudolph TK, Eiserich JP, Sydow K, Lau D, Szöcs K, Linke A, Kubala L, Berglund L, Schrepfer S, Deuse T, Haddad M, Risius T, Klemm H, Reichenspurner HC, Meinertz T and Heitzer T. Heparins increase endothelial nitric oxide bioavailability by liberating vessel-immobilized myeloperoxidase. Circ. 2006; 113: 1871-8.

[30] Wolfe KL, Liu RH. Cellular antioxidant activity of common fruits. J Agric Food Chem. 2008; 56: 8418-26.

[31] Parke A, Parke DV. The pathogenesis of inflammatory disease: Surgical shock and multiple system organ failure. Inflammopharmacol. 1995; 3: 168-78.

[32] Conforti F, Sosa S, Marrelli M, Menichini F, Statti GA, Uzunov D, Tubaro A, Menichini F, Loggia RD. *In vivo* anti-inflammatory and *in vitro* antioxidant activities of Mediterranean dietary plants. J Ethnopharmacol. 2008; 116: 144-51.

[33] Deby-Dupont G, Deby C, Lamy M. Neutrophil myeloperoxidase revisited: its role in health and disease. Intensivmed. 1999; 36: 500-13.

[34] Sheppard FR, Kelher MR, Moore EE, Mclaughlin NJD, Banerjee A., Silliman C.C. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. J Leukoc Biol. 2005; 78: 1025-42.

[35] Jog NR, Rane MJ, Lominadze G, Luerman GC, Ward RA, McLeish KR. The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets. Am J Physiol Cell Physiol. 2007; 292: 1690- 00.

[36] Malle E, Furtmuller PG, Sattler W, Obinger C. Myeloperoxidase: a target for new drug development? Br J Pharmacol. 2007; 152: 838-54.

[37] Tabart J, Kevers C, Evers D, Dommes J. Ascorbic acid, Phenolic acid, Flavonoid and Carotenoid profiles of selected extracts from Ribes nigrum. J Agric Food Chem.; article submit in November.

[38] Iacopini P, Baldi M, Storchi P, Sebastiani L. Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, in vitro antioxidant activity and interactions. J Food Comp Anal. 2008; 21: 589-598.

[39] Blackburn WD, Heck LW, Wallance RW. The bioflavonid quercetin inhibits neutrophil degranulation, superoxide production, and the phosphorylation of specific neutrophil proteins. Biochem Biophys Acta. 1987; 144: 1229-36.

[40] Schneider C, Berton G, Spisani S, Traniello S, Romeo D. Quercetin, a regulator of polymorphonuclear leukocyte (PMNL) functions. Adv Exp Biol Med. 1978; 221: 371-9.

[41] Tauber AI. Fay JR, Marletta MM. Flavonoid inhibition of the human neutrophil NADPH-oxidase. Biochem Pharmacol. 1984; 33: 1367-9.

[42] Pincemail J, Deby C, Thirion A, de Bruyn-Dister M, Goutier R. Human myeloperoxidase activity is inhibited in vitro by quercetin, comparison with three related compounds. Experient. 1988 ; 44 : 450–3.

[43] Shiba Y, Kinoshita T, Chuman H, Taketani Y, Takeda E, Kato Y, Naito M, Kawabata K, Ishisaka A, Terao J, Kawai Y. Flavonoids as sibstrates and inhibitors of myeloperoxidase : molecular actions of aglycone and metabolites. Chem Res Toxicol. 2008; 21: 1600-9.

[44] Wu X, Gu L, Prior RL, McKay S. Characterization of anthocyanins and proanthocyanidins in some cultivars of *Ribes*, *Aronia*, and *Sambucus* and their antioxidant capacity. J Agric Food Chem. 2004; 52: 7846-56.

[45] Borges G, Degeneve A, Mullen W, Crozier A. Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. J Agric Food Chem. 2010; 58: 3901-9.

FIG. AND CAPTIONS

Table 1: Evaluation of the antioxidant capacity measured by various assays in the extracts of three blackcurrant explants (leaves, berries and buds): TEAC, DPPH and ORAC in mg TE/ g FW, Total phenolics in mg ACE/ g FW, and ESR in u ESOD/g FW. Significant differences were observed ($p < 0.01$) between leaves and buds, and berries (n=3).

Table 2: Evaluation of the antioxidant capacity by the Cellular Antioxidant Activity assay (µmole Quercetin equivalent/ g freeze-dried extract) and the Haemolysis assay (mmole of Ascorbic Acid equivalent/ g fresh weight) of the extracts of the three blackcurrant explants (leaves, berries and buds). A significant difference was observed between explants ($p < 0.05$) (n = 6) in the two assays on cells.

Figure 1: Effects of different blackcurrant extracts (leaves, berries and buds) on the chemiluminescence response produced by $1x10^6$ equine neutrophils stimulated with PMA. The relative chemiluminescence was compared to the cell stimulated with PMA without extract (Ctrl PBS) taken as 100%. A *p-value <0.05 (one-way ANOVA followed by Dunnett's post-test) was considered to indicate significance vs. the control solution [Ctrl PBS which was set as 100% (mean ±SD), n=3]. NA, no activated neutrophils.

Figure 2: Effects of different blackcurrant extracts (leaves, berries and buds) on the specific activity of MPO (50 ng/ml). The relative activity was compared to the MPO activity obtained in PBS without extract (Ctrl PBS) and taken as 100%. . A *p-value <0.05 (one-way ANOVA followed by Dunnett's post-test) was considered to indicate significance vs. the control solution [Ctrl PBS which was set as 100% (mean ±SD), n=3].

Methods	Units	Leaves	Berries	Buds
TEAC	Mg TE / g FW	53,7 ± 6,2 ***	21,9 ± 1,6 *	36,1 ± 3,8 **
DPPH		52,7 ± 10,6 **	25,0 ± 4,1 *	42,1 ± 4,5 **
ORAC		222,0 ± 66,8 **	34,5 ± 1,9 *	155,0 ± 32,3 **
Total Phenolics	ACE / gFW	46,1 ± 8,4 **	21,25 ± 8,0 *	45,1 ± 7,5 **
ESR	E SOD/ g/FW	1232,7 ± 50,4 **	604,2 ± 51,9 *	1277,4 ± 144,4 **

Table 3:

	CAA	Haemolysis
Unit	µmole QE/ g freeze-dried extract	mmole AA/g FW
Leaves	12,89 ± 0,77 *	1,247 ± 0,179 **
Berries	0,40 ± 0,10 **	0,394 ± 0,053 *
Buds	8,82 ± 1,92 ***	1,235 ± 0,053 **

Table 4:

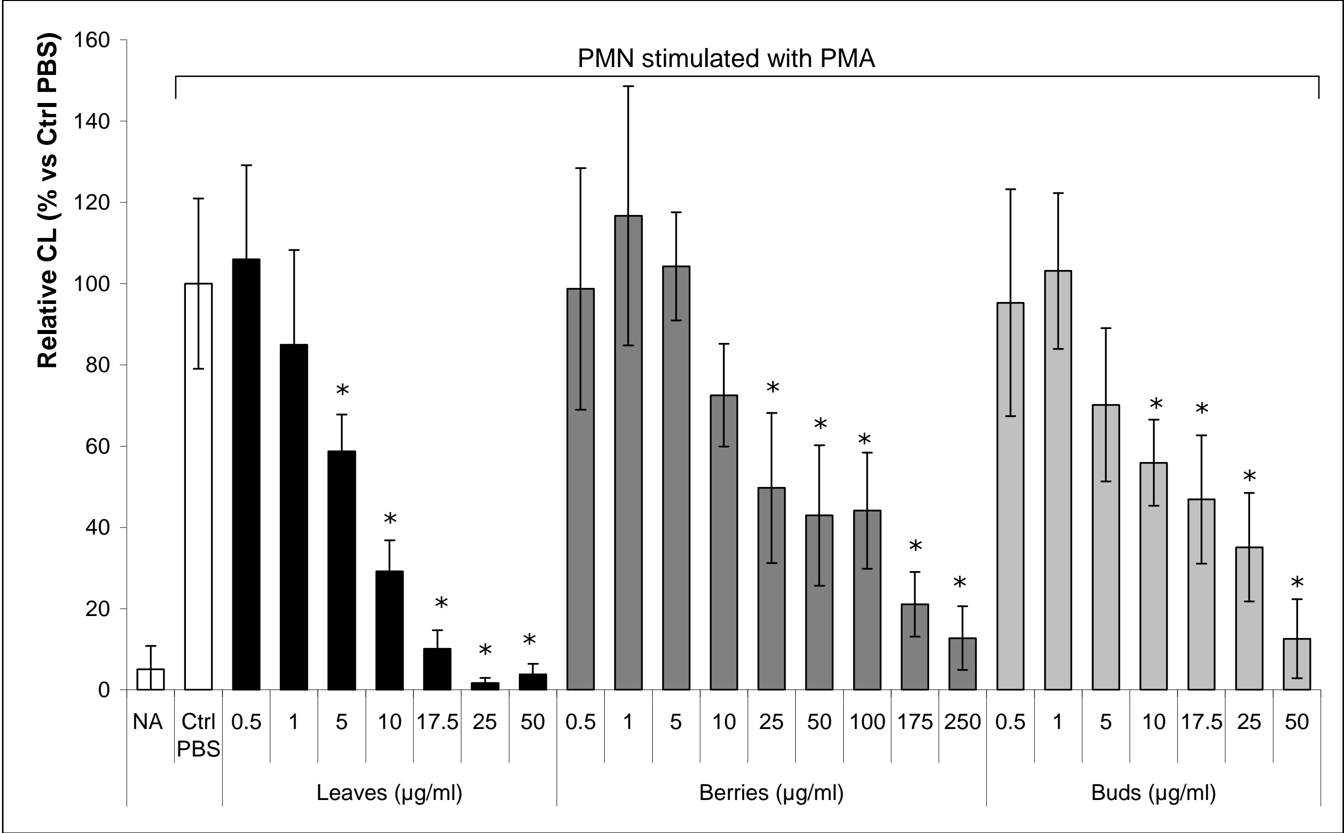


Figure 1:

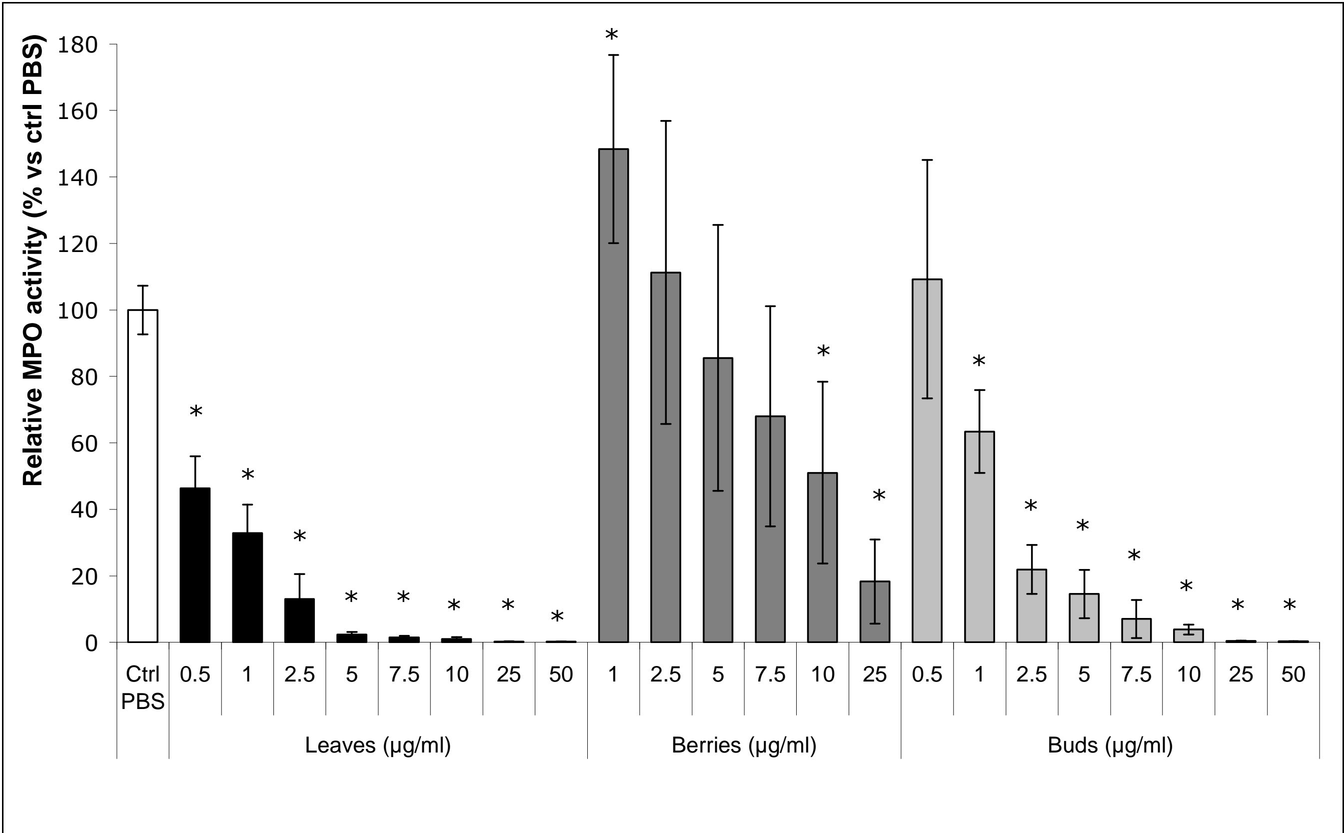


Figure 2:

3. *Conclusion*

La caractérisation des molécules antioxydantes des matrices végétales peut être réalisée par de nombreuses approches différentes. Avant d'utiliser cette large gamme de techniques en vue de caractériser au mieux nos extraits de cassis, nous avons mené un certain nombre d'investigations sur la validation de ces techniques au moyen d'une large gamme de standards commerciaux ainsi que de divers produits alimentaires. Etant donné la diversité des résultats obtenus pour le dosage de la capacité antioxydante, nous avons proposé une méthode permettant de normaliser la valeur de la capacité antioxydante en employant une moyenne pondérée basée sur les résultats de quatre méthodes aux principes différents : DPPH (oxydoréduction), ORAC (cinétique de piégeage du radical peroxyle), Hémolyse (protection d'un échantillon biologique) et l'EPR (évaluation d'un radical libre particulier).

Au niveau des extraits de cassis obtenus par le mélange acétone-eau- acide acétique (70 :28 :2), nous avons caractérisé le contenu en composés antioxydants par des analyses chromatographiques et/ou spectrophotométriques. Les feuilles et les bourgeons présentent un contenu plus élevé en composés phénoliques et en flavonols, avec une prédominance de la quercétine. Par contre, les fruits possèdent un contenu en acide ascorbique très élevé. Au niveau des flavan-3-ols, les bourgeons sont les plus riches, avec une prédominance pour la gallocatéchine et l'épigallocatéchine. Au niveau de la caractérisation des propriétés antioxydante et anti-inflammatoire de nos extraits, notre étude a mis en évidence des propriétés intracellulaires et extracellulaires en tant que piégeurs de radicaux libres et une activité inhibitrice de la myéloperoxydase chez les neutrophiles. Nous avons observé un meilleur effet pour les feuilles, suivit par les bourgeons et finalement, loin derrière, les baies. Cette différence semble être corrélée avec le taux en phénols totaux.

En empêchant la production de radicaux libres dans les cellules comme les neutrophiles ou les cellules endothéliales, l'extrait de feuilles de cassis possèderait un potentiel élevé pour le traitement de pathologies tant inflammatoires que cardio-vasculaires. Dans la suite de ce travail, les effets de l'extrait de feuilles de cassis seront étudiés sur le processus de vasorelaxation en utilisant des modèles cellulaires et animaux.

Partie 3 : Biodisponibilité et Processus de vasorelaxation

1. Introduction

En plus des effets antioxydants et anti-inflammatoires, les polyphénols agissent aussi au niveau de la vasomotricité. Ces polyphénols peuvent agir directement au niveau de l'endothélium, en interagissant avec des facteurs endothéliaux (monoxyde d'azote et endothéline-1). Ils peuvent également contribuer à préserver l'intégrité de l'endothélium par divers mécanismes : la régulation de la prolifération, de la migration et de l'apoptose des cellules endothéliales. Les polyphénols, de par leurs multiples effets vasculaires (anti-inflammatoires, vasorelaxants, ...) présentent un intérêt certain pour la prévention de nombreuses pathologies.

Des études utilisant un concentré de jus de cassis comme modèle ont déjà permis de démonter les effets protecteurs du cassis sur le processus de relaxation vasculaire (Nakamura *et al*., 2002). Comme vu dans les précédents résultats, les feuilles ont des propriétés beaucoup plus importantes que les baies. Dans cette dernière partie des résultats, nous avons tenté de montrer les effets de cet extrait de feuilles de cassis sur la relaxation vasculaire dépendante de l'endothélium en utilisant des modèles cellulaires mais aussi un modèle d'organes isolés. Un deuxième point abordé dans cette partie consiste à voir si notre extrait peut être consommé sans danger et si oui, quelle est la partie ingérée potentiellement active au niveau de l'organisme. Ce dernier aspect est abordé en utilisant un modèle animal.

2. *Article*

Bioavailability and vasorelaxant effect of *Ribes nigrum* leaf extract on human endothelial cells and rat aorta.

Jessica TABART [1], Claire KEVERS [1], Joël PINCEMAIL [2], Jean-Olivier DEFRAIGNE [2], Sophie VINCENT [3], Arlette DELAMARCHE [3], Jacques DOMMES [1]

University of Liège,

[1]Plant Molecular Biology and Biotechnology Unit, B22

[2]CREDEC, B35

Sart Tilman,

B-4000 LIEGE

Belgium

University of Rennes
[3]Laboratory « Mouvement Sport Santé » (EA1274), and ENS Cachan, UFR-APS,
F-Rennes Cedex
France

Corresponding author: Jessica TABART: Jessica.Tabart@doct.ulg.ac.be
Other authors: C.Kevers@ulg.ac.be, J.Pincemail@chu.ulg.ac.be, JO.Defraigne@ulg.ac.be, sophie_vincent_1999@yahoo.fr, arlette.delamarche@univ-rennes2.fr, J.Dommes@ulg.ac.be;

TITLE RUNNING HEAD: Vasorelaxant effect of blackcurrant leaf extract

ABSTRACT

The dysfunction of endothelium is involved in an important cause of mortality in developed countries, atherosclerosis. Many plants contain compounds that cause endothelium-dependent vasorelaxation in vivo. In our study, we used a leaf extract of blackcurrant to investigate the endothelium-dependent relaxing properties in cell model and on isolated organ model, and complementary, the toxicity and the bioavailability in an animal model. Our results indicated the regulation of *eNOS* mRNA with an increase of NO production. In organ bath chambers, we could also show a relaxant effect of the extract on vessel. With the animal model, we did not observe any toxic effect after 28-days of consumption. But we showed an increase of some flavonoids in plasma and a regulating effect on body weight.

KEYWORDS: Endothelium-dependent relaxation, antioxidant, endothelium, NO synthase, bioavailability, toxicity, blackcurrant.

INTRODUCTION

Atherosclerosis is the leading overall direct or indirect cause of mortality in developed countries (Beaudeux et al., 2006). The physiopathological mechanisms for formation and evolution of vascular atheromogenic damages are correlated with various risk factors like oxidative stress. The abnormal increase of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in endothelial cells and vascular smooth muscle cells induces an endothelial activation, an inflammatory response and the proliferation of vascular muscular smooth cells which cause atherosclerotic damages (Beaudeux et al., 2006).

Endothelium has many functions like control of vascular permeability, inflammatory cell adhesion, coagulation and platelet aggregation. Therefore, the functional integrity of the endothelium is essential to prevent vascular leakage and formation of the atherosclerosis. The vascular endothelium plays an important role in homeostasis by modulating vascular smooth muscle tone and acts as a main target site in hypertension and atherosclerosis (Moncada et al., 1991). Endothelial cells produce various vasodilating substances such as endothelium-derived relaxing factors or nitric oxide (NO), endothelium-derived hyperpolarizing factors (EDHF) (Fletou and Vanhoutte, 1988) and vasodilator prostaglandins.

NO produced by endothelial-type nitric oxide synthase is a key factor in vascular protection as vasodilator agent. NO can act also as antithrombic agent by inhibiting platelet aggregation and adhesion. Besides these properties, NO possesses also many antiartherosclerotic properties which include: the prevention of leukocyte adhesion to vascular endothelium and their migration into the vascular wall, the decrease of endothelium permeability, the inhibition of LDL oxidation, and the inhibition of proliferation of vascular smooth cells (Li and Föstermann, 2000). One of the mechanisms involved in the endothelium dysfunction is a reduced NO synthesis and/or an increase of vascular NO degradation (Barton et al., 1997; Li and Föstermann, 2000).

Many plants including grapes contain extractable compounds that cause endothelium-dependent vasorelaxation *in vitro* (Fitzpatrick et al., 1993). Plant flavonoids exhibit various biological properties that have been demonstrated in a variety of experimental systems. The benefit of flavonoid-rich food has been attributed to the antioxidant activity of these compounds (Di Carlo et al., 1999) but also to the modulation of various factors involved in the development of vascular diseases. In rats, polyphenols could decrease arterial pressure, prevent low density lipoprotein (LDL) oxidation, inhibit vascular smooth muscle cells, and prevent platelet aggregation (Martin and Andriantsihaina, 2002).

Blackcurrants are among the berries with the higher amount of phenolic compounds, especially anthocyanins. Other major phenolics present in blackcurrants include flavonols, procyanidins and various phenolic acids (Benvenuti et al., 2004). Only a very small portion of these berries is consumed fresh; most is processed for industrial use in making juice and liquors. Nakaishi et al. (2000) found that oral intake of blackcurrant concentrate prepared from blackcurrant juice had significant and beneficial effects on visual functions. Nakamura et al. (2002) suggested that the prevention of eye fatigue resulted probably from increased blood supply to these areas caused by vasorelaxation induced by blackcurrant concentrate. Their work demonstrated that blackcurrant concentrate causes endothelium-dependent vasorelaxation *in vitro* in rat aorta rings by increasing NO production, via the histamine H_1-receptors on the endothelium. Leaves and buds can also be used (Tabart et al., 2006). In a previous study (Tabart et al., 2011), we have demonstrated that leaf and bud extracts have higher antioxidant and anti-inflammatory properties than berry extract.

This study aimed at investigating the effect of blackcurrant leaf extract on the endothelium relaxation by examining *in vitro* the expression modulation of eNOS and the NO production by human endothelial cells. Its effect was also observed on the relaxation of isolated vessels. The second aim of this study was to examine, *in vivo*, the toxicity and the bioavailability of leaf extract in a chronic experiment on an animal model.

MATERIAL AND METHODS

Sample preparation

The leaves of blackcurrant (Noir de Bourgogne), two years old, were harvested in August in the Belgian Ardennes (Bihain). The various explants were directly cut out into pieces and were frozen in liquid nitrogen, lyophilized and preserved at -20°C for a few months.
For preparing extract, 50 grams of frozen sample (berries, buds or leaves) were ground with a few grams of quartz and 500 ml of extraction solution: acetone/water/acetic acid (70:28:2) (Tabart et al., 2006). The mixture was shaken during 1 h at 4°C and centrifuged at 17 000 g for 15 min. The supernatant was removed, and the sample was extracted again with the same procedure but incubated only 15 min. The acetone was evaporated and the residue was filtered and lyophilised before storage at -20°C. The residue was resuspended in appropriate medium for analysis (DMEM supplemented for cell analysis, Water for chronic administration of extract in rats and DMSO for analysis on isolated vessels).

Cell culture

The human endothelial-like EAhy926 cell line (Edgell et al., 1983), is derived from the fusion of human umbilical vein endothelial cells with the A549 carcinoma cell line. EAHy926 cells were grown in DMEM (Biowithaker, Lonza) supplemented with 10% of heat-inactivated fetal bovine serum, 10 µM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5000 units/l of penicillin and streptomycin, 1 mM pyruvate sodium, 2 mM glutamin and 2% HAT medium supplement (hypoxanthin, aminoprotein and thimidin), and were maintained at 37°C and 5% CO_2.

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide viability assay

Potential toxic effects of blackcurrant leaf extracts were tested using the Cell Growth Determination kit MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide, Sigma-Aldrich, St Louis, MO). The EAhy926 cell line was plated in a 96-well plate at 5000 cells/well and samples treated in sestuplicate with different concentrations of blackcurrant leaf extract (0-1 mg/ml) or complete DMEM as a control. The cell density was assessed at 24 hour time intervals over 5 consecutive days. Cell viability was evaluated as recommended by the manufacturer. The absorbance was measured on a microliter plate reader (Labsystems IEMS reader MF) at 570 nm. The background absorbance at 690 nm was subtracted.

Determination of nitrite content

Nitrite accumulation in EAhy926 cell culture supernatant was measured using the Griess method for the determination of the total nitrite content (nitrite and nitrate), adapted for 96-wells plates (Bani et al., 1998). 500 000 cells were incubated in 6-well plates with leaf extract at different concentration (0 to 1 mg/ml) or quercetin solution (50 µM) during 5 min, 6 h, 12 h, 24 h and 48 h). At the end of incubation, 100 µl culture supernatant were mixed with 25 µl of NADPH (93 µg/ml final concentration) and 10 µl of nitrate reductase (0.2 U) in a 96-well plates. After incubation at 25°C for 1.5 h, 100 µl of Griess reagent (1 part 1% sulfanilamide and 1 part 0.1% *N*-naphtylethyl-ethylenediamine (NED) in 5% ortho-phosphoric acid) were added. The absorbance at 540 nm was measured after 10 minutes of incubation. Various concentrations of sodium nitrate (0–500 µM) were used to obtain a calibration curve. The results were expressed as µM of $NaNO_3$.

Semi-quantitative RT-PCR for *eNOS* mRNA analyses

eNOS mRNA expression was analyzed with semi-quantitative RT-PCR. In the first part, EAhy926 cell cultures were incubated with leaf extract at various concentrations (0 to 1 mg/ ml) during 24 h. In the second part, cell cultures were incubated with leaf extract at 250 µg/ml for different times (5 min, 6 h, 12 h, 24 h and 48 h). After incubation, total RNA was isolated with the RNeasy mini kit (Qiagen) as recommended by the supplier. RNA (1µg) was transcribed into cDNA by reverse transcription by M-MLV RT RNase H(-) Point Mutant enzyme (Promega). For the PCR, 2.5 µl of cDNA product of each sample was amplified on Gene Amp® PCR System 9700 (PE Applied BioSystems) with Taq DNA polymerase using a primer pair specific to eNOS (forward primer: 5'-GCT GCG CCA GGC TCT CAC CTT C-3',

reverse primer: 5'-GGC TGC AGC CCT TTG CTC TCA A-3', 35 cycles, 554 bp, annealing temperature: 55°C), a primer pair specific to iNOS (forward primer: 5'-TCT TGG TCA AAG CTG TGC TC-3', reverse primer: 5'-CAT TGC CAA ACG TAC TGG TC-3', 35 cycles, 237 bp, annealing temperature: 60°C) and two primer pairs specific to β-actin ([forward primer: 5'-GGC ATC GTG ATG GAC TCC G-3', reverse primer: 5'-GCT GGA AGG TGG ACA GCG A-3', 23 cycles, 603 bp, annealing temperature: 68 °C] [forward primer: 5'- ATG GAT GAT GAT ATC GCC GCG -3', reverse primer: 5' - TCT CCA TGT CGT CCC AGT TG - 3', 30 cycles, 248 bp, annealing temperature: 58°C]). At last, the PCR amplified samples were visualized by electrophoresis through 1.5% agarose gel in the presence of ethidium bromide. The quantification was carried out using the G:Box Imaging System (Syngene, Wesburg) and the GeneSnap and GeneTools softwares.

Animals and ethical approval

Male Wistar rats purchased in the central animalery of University of Liege (Belgium) (12-14 weeks old) were housed in cages under standard experimental conditions (23 ± 1°C, 12 h light/dark cycle). All experimental protocols were approved by the Animal Care and Use Committee of University of Liège (Belgium), and conduced humanely.

Characterization of the relaxant effect of leaf extract on vascular reactivity

Male Wistar rats were killed by an injection of pentobarbital (80 mg/Kg bodyweight) and then exsanguinated. The aorta was removed and carefully cleaned of adhering fat and connective tissue, and cut into rings (3-4 mm). The rings were mounted in standard organ baths (IOX, EMKA Technologies, France) filled with physiological salt solution (Krebs buffer: 118 mM NaCl, 25 mM $NaHCO_3$, 5 mM Glucose, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 2.5mM $CaCl_2$), maintained at 37°C and continuously bubbled with 95% O_2 - 5% CO_2 mixture. Resting tension was adjusted to 3 g. Tension was measured with an isometric force transducer.

After an equilibration period of 90 min, the vessels were contracted three times with KCl (0.08 M) in order to test their contractile capacity. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (Ach, 10 µM) to induce more than 50% relaxation of rings precontracted with noradrenalin (0.1 µM). Vessels

were considered to be devoid of functional endothelium when there was no relaxation response to Ach.

After washing and returning to baseline tension, aortic rings with functional endothelium were pre-contracted to 80% of KCl maximal tension with phenylephrine (0.5 µM). When the contraction reached a steady state, increasing concentrations of Ach, quercetin or leaf extract were added cumulatively.

Oral toxicity and bioavailability of leaf extract flavonoids

The rats were fed with a standard diet. The animals were divided into three groups of 5 animals (n=5). The diet of two groups was supplemented with blackcurrant leaf extract (dissolved in water) amounting to 896 ± 30 and 1528 ± 137 mg/kg bodyweight every day during 28 days. Weight of animals was monitored for 28 consecutive days. The general behaviour of each animal was observed every day throughout the study period, such as food consumption.

At the end of the treatment, animals were sacrificed by an injection of pentobarbital (80 mg/Kg of bodyweight) and then exsanguinated. The blood and the liver were collected for analysis.

Blood was analysed for toxicity markers and bioavailability of phenolic compounds. For the toxicity analysis, various markers were quantified versus control by the Department of Clinical Biology of CHU-Liège (Belgium): oral orientation, hepatic and cardiac markers. The plasma was used to analyse the content of total phenolics (by a spectrophotometric method), flavonol aglycons and favan-3-ols monomers (by HPLC following methods described by Tabart et al., 2009; 2010b).

Statistical analysis

The data were subjected to the statistical analysis of the variance (ANOVA-1) to evaluate the significant differences between various explants of blackcurrant. The difference was regarded as significant when $p < 0.05$.

RESULTS

1. Toxicity of blackcurrant leaf extract

The toxicity of blackcurrant leaf extract was determined at two levels: on cell model and on animal model.

a. Cytotoxicity on human endothelial cells

Before testing the cytotoxic effect of the extract on animal, a viability assay was used to determine the effect of leaf extract on the viability of cells during 120 hours (Figure 1).

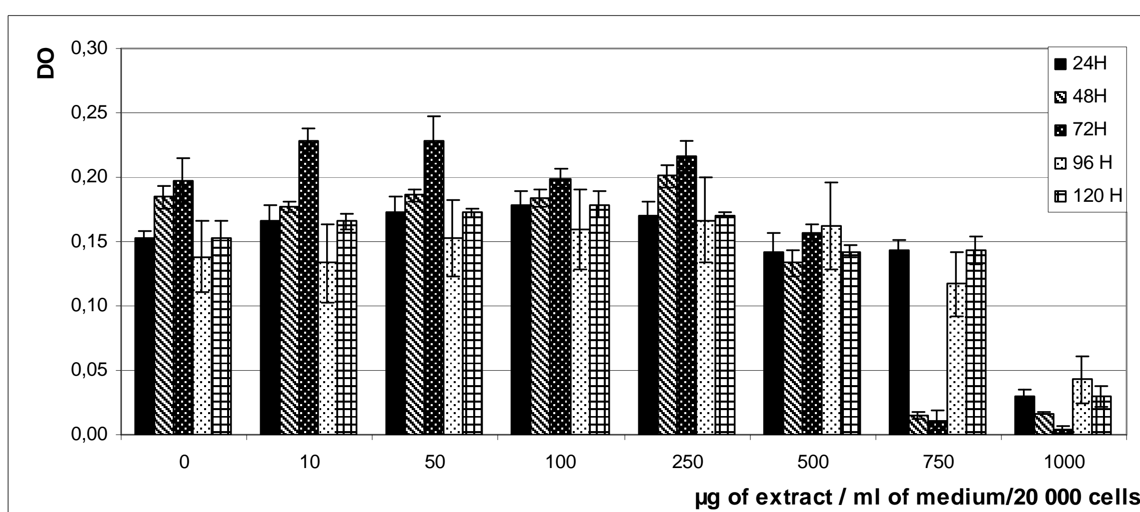


Figure 1: Evolution of cell viability during 120 h of incubation with leaf extract at different concentrations (0 to 1 mg/ml). Data represented the means ± SD (n = 6).

In control conditions, the viability increased to 72h. The addition of leaf extract at a concentration lower than 500 µg/ ml did not modify the cell viability during the first 72 hours. With higher concentrations of leaf extract (1 mg/ ml), the viability decreased and a cytotoxicity was observed.

b. Toxicity on rat models after 28-days chronic ingestion

In the 28-d chronic oral toxicological tests in rats, leaf extract was administrated orally to rats at doses of 0 (control), 896 ± 30 and 1528 ± 137 mg/Kg of body weight per day (Figure 2). All animals survived in all groups.

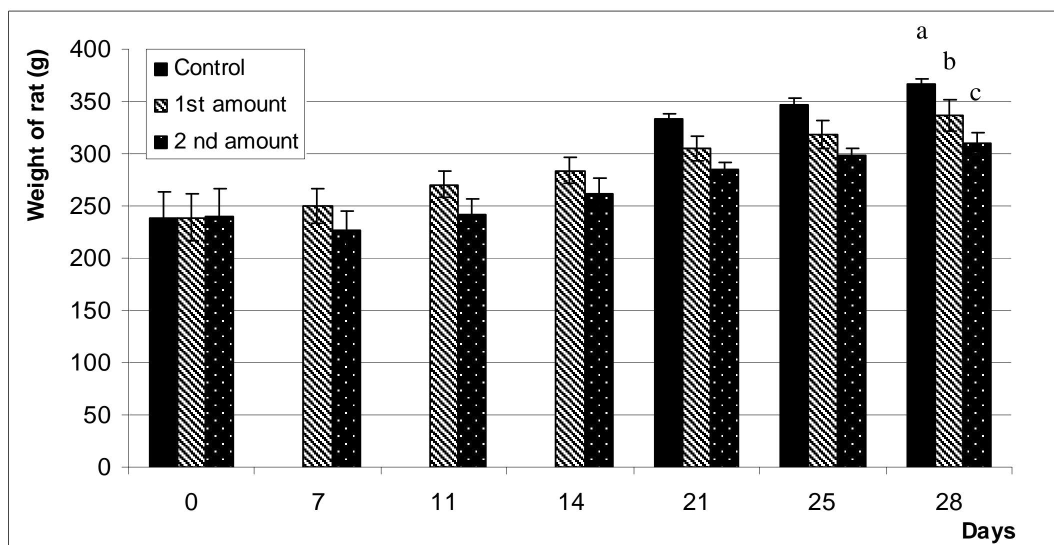


Figure 2: Evolution of body weight during 28-d chronic toxicological test with leaf extract (0 (control), 896 ± 30 (1st amount) and 1528 ± 137 (2nd amount) mg/Kg of body weight). Data represented the means ± SD. A significantly difference was observed at p>0.05 between the three groups (a, b, c) (n = 5).

At the end of the period, the body weight of treated animals was significantly lower than that of control rats, without any abnormal clinical-related parameter. Liver weight was lower in animal fed with the highest amount leaf extract as compared to control (Figure 3).

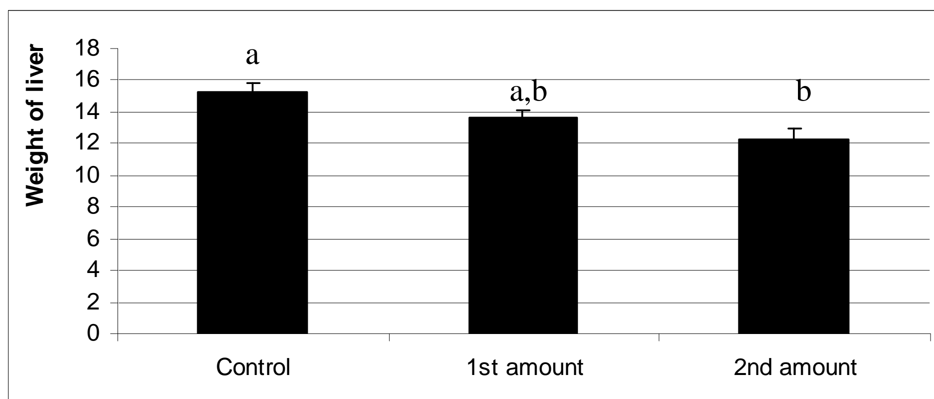


Figure 3: Weight of liver after 28-d chronic toxicological test with leaf extract (0 (control), 896 ± 30 (1st amount) and 1528 ± 137 (2nd amount) mg/Kg of body weight). Data represented the means ± SD. A significantly difference was observed at p>0.05 between control and the 2nd group (a, b) (n = 5).

Concerning toxicological markers, no difference was found between the three groups (Table 1).

	General parameters			Hepatic exploration				Cardiac Markers
	Urea (g/L)	Creatin (mg/L)	Alkalin Phosphatase	GGT	GOT	GPT	LDH	Creatin kinase (UI/L)
Control	0.662 ± 0.008	4.20 ± 0.15	136.4 ± 13.7	<5	174.2 ± 28.4	60 ± 8.1	661 ± 138	6551 ± 1981
1st amount	0.632 ± 0.020	4.52 ± 0.47	130.6 ± 9.8	<5	148.0 ± 47.2	53.7 ± 2.3	570 ± 217	6093 ± 1403
2nd amount	0,618 ± 0.127	5.50 ± 0.23	115.0 ± 37.8	<5	137.8 ± 23.4	49 ± 1.3	573 ± 252	4464 ± 951

Table 1: Analysis of different markers of toxicity after 28-days chronic toxicological ingestion of leaf extract (0 (control), 896 ± 30 (1st amount) and 1528 ± 137 (2nd amount) mg/Kg of body weight) (n = 5). (GGT: gamma glutamyl transpeptidase, GOT: glutamic oxalo-acetic transaminase, GPT: glutamic pyruvic transaminase, LDL: low-density protein).

2. Bioavailability of blackcurrant leaf extract after 28-days chronic ingestion

After the 28-days chronic oral test in rats, we determined the concentration of various compounds of blackcurrant leaf extract in the plasma (Table 2).

	Myricetin (µg/ml)	Quercetin (µg/ml)	Kampferol (µg/ml)	EGC (µg/ml)	Total Phenols (mg/ml)
Control	0*	0.03 ± 0.02*	0*	23.16 ± 5.46*	4.65 ± 0.22*
1st amount	1.29 ± 1.15*	9.28 ± 2.75**	4.93 ± 2.01**	54.04 ± 5.22**	5.14 ± 0.15**
2nd amount	9.29 ± 3.01**	6.40 ± 3.18**	3.34 ± 2.05**	16.59 ± 9.14*	5.88 ± 0.42***

Table 2: Analysis of the flavanols, flavonols and total phenol content of plasma after 28-d chronic ingestion of two amount (896 ± 30 (1st amount) and 1528 ± 137 (2nd amount) mg/Kg of body weight) of leaf extract versus control. Data represent the means ± SD. A significantly difference was observed between the three ingested amounts at p<0.05 (n = 5 for total phenolics analysis and n = 3 for the others).

Total phenols content significantly increased in correlation with the administrated amount. Some flavonoids were analysed by HPLC: flavonols, anthocyanins and flavan-3-ols. For flavonols, the quantity present in the plasma of rats that have ingested the extract was significantly higher versus control, without correlation with the amount administrated except for myricetin. No anthocyanin could be detected. For flavan-3-ols, only epigallocatechin (EGC) monomer was detected. The concentration of this compound was not correlated with

the quantity ingested. The highest quantity present in the plasma was found in rats that had ingested 826 ± 30 mg of leaf extract / Kg of body weight.

3. Effect of blackcurrant leaf extract on endothelium-dependent relaxation process

To analyse the potential effect of blackcurrant leaf extract on vasorelaxation, an endothelial cell model and an isolated organ model were used.

a. Effect on NO production and modulation of eNOS mRNA expression

Leaf extract concentrations between 300 and 750 µg/ml induced a significant increase of NO production after 12 hours of incubation (Table 3). At 1000 µg/ml, the increase was observed after 48 hours of incubation.

	Concentration		**Production of NO (µM $NaNO_3$)**				
			5 min	6 h	12 h	24 h	48 h
Control	Medium		0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Leaf extract	100	µg/ml	1.55 ± 0.58	0.96 ± 0.45	2.20 ± 0.68	1.40 ± 0.90	4.05 ± 0.79
	200	µg/ml	5.57 ± 0.70	9.73 ± 0.41	8.35 ± 2.54	6.35 ± 1.21	7.31 ± 0.88
	300	µg/ml	2.12 ± 1.84*	12.84 ± 0.81*	11.38 ± 0.87*	4.17 ± 1.16*	6.79 ± 1.52*
	400	µg/ml	9.79 ± 1.32*	11.52 ± 1.08*	15.96 ± 1.64**	7.64 ± 2.06*	6.50 ± 1.79*
	500	µg/ml	9.38 ± 3.72*	9.99 ± 1.72*	23.50 ± 2.16**	7.65 ± 1.71*	6.27 ± 1.44*
	750	µg/ml	0 ± 0	1.87 ± 4.03*	22.09 ± 2.83**	4.97 ± 1.37*	12.17 ± 2.94***
	1000	µg/ml	0 ± 0	9.60 ± 7.12*	3,00 ±7.87*	7.22 ± 1.37*	31.90 ± 6.53**
Quercetin	50	µM	0.0070 ± 0.0003[a]	0.005 ± 0.0006 [b]	0.007 ± 0.001[c]	0.008 ± 0.002 [c]	0.007 ± 0.001 [d]

Table 3: Effect of leaf extract on NO production in EAHy926 cells. 5 x 10^5 cells were treated with DMEM, Quercetin 50 µM and various concentrations of leaf extract. After 5 min, 6 h, 12 h, 24 h and 48 h, the content of NO was assessed by Griess method. Data represented the means ± SD of six independent experiments. A significantly difference was observed at p<0.05 during the kinetic (n = 6).

Semi quantitative RT-PCR was used to examine whether *eNOS* gene expression was increased in endothelial cells after incubation with leaf extract for different times and various concentrations. *eNOS* mRNA accumulation was enhanced after 24 hours of incubation with leaf extract at 250 µg/ml (Figure 4).

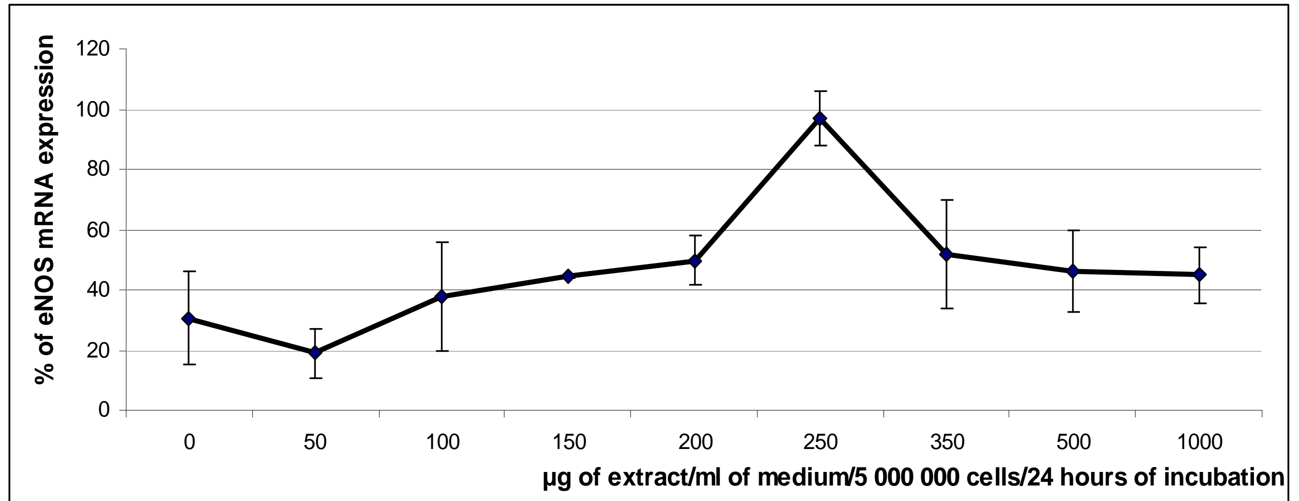


Figure 4: Effect of leaf extract on *eNOS* mRNA expression in human endothelial EAHy926 cells (5 10^6 cells) after 24 hours of incubation with leaf extract at various concentrations (0 to 1 mg/ml). The results were expressed in % of expression and were compared with *β-actin* mRNA expression taken as 0%. Data represented means ± SD (n = 3).

The *eNOS* mRNA level was monitored over a 48 h time period in cells incubated with 250 µg of leaf extract / ml (Figure 5).

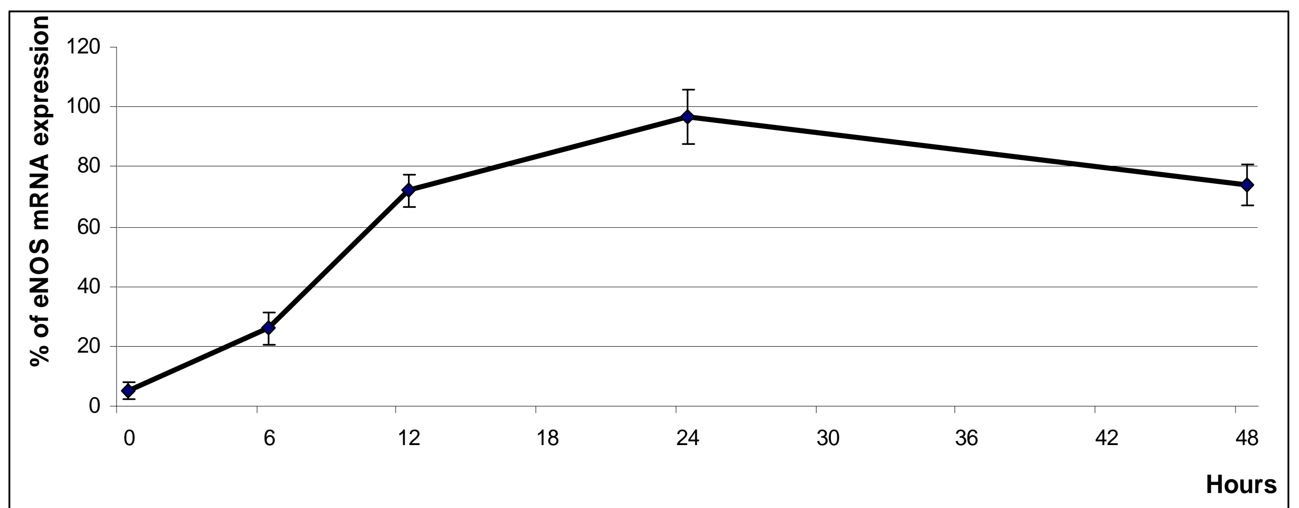


Figure 5: Kinetic of *eNOS* mRNA expression after incubation of 5 10^6 cells with 250 µg/ml of leaf extract in the medium during 48 hours. The results were expressed in % of expression and were compared with *β-actin* mRNA expression taken as 0%. Data represented means ± SD (n = 3).

The increase of *eNOS* mRNA expression was first observed after 6 hours and this expression increased until a steady state at 12 hours.

b. In vitro determination of vascular reactivity

To investigate the functional consequences of the enhanced *eNOS* mRNA expression, we studied the effects of quercetin and blackcurrant leaf extract on vasorelaxation in comparison to acetylcholine (Figure 6).

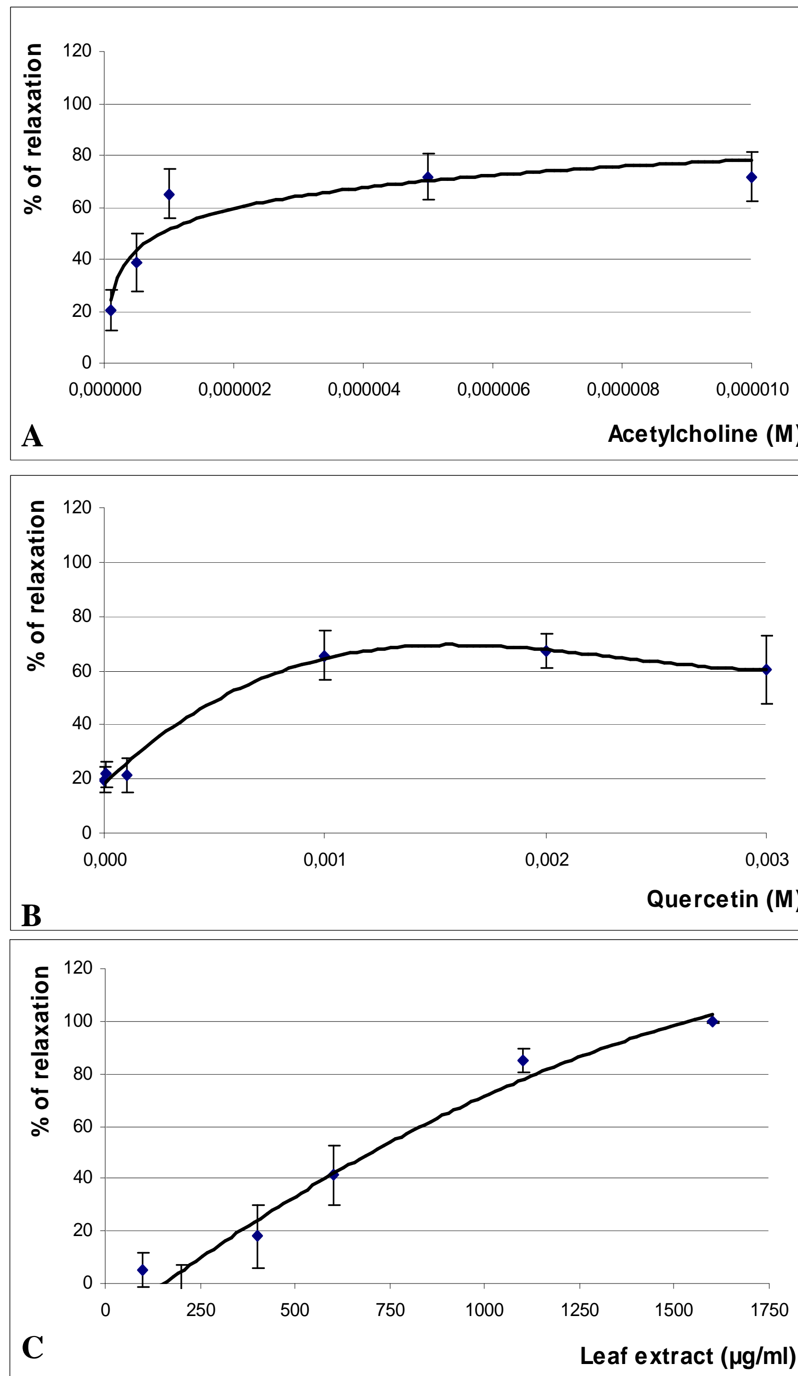


Figure 6: Cumulative concentration-relaxation curves for Acetylcholine (0 to 10^{-5} M, A), Quercetin (0 to 3 µM, B) and leaf extract of blackcurrant (0 to 1.60 mg/ ml, C) in endothelium-intact Rat aortic rings precontracted with phenylephrine (µM). The results were the means ± SD (n = 6).

In organ bath experiments, the extract induced a significant dose-dependent relaxation of aortic rings precontracted with phenylephrine. With Ach, ± 70% of relaxation took place with a concentration of ± 5µM; the same was observed for quercetin at 1µM. For leaf extract, we reached 70% of relaxation with a concentration around 1 mg/ ml.

DISCUSSION

In a previous study (Tabart et al., 2011), various antioxidant compounds were identified and measured in leaf extract of blackcurrant. This extract possessed a high content of many flavonoids except those of the anthocyanin sub-class. The most predominant flavonoids present into the extract were quercetin (778 ± 203 µg/ g of fresh weight), kampferol (322 ± 151 µg/ g of fresh weight), petunidin (514 ± 152 µg/ g of fresh weight) and gallocatechin (382 ± 132 µg/ g of fresh weight). The present study investigated the effect of blackcurrant leaf extract on the endothelium relaxation. The toxicity of this extract was previously studied on endothelial cells and animal model.

Toxicity and bioavailability

Despite the apparently beneficial health effects of flavonoids, several studies indicated their mutagenicity and their genotoxicity in several bacterial and mammalian experiments. This may be due to their pro-oxidant activity. High intake of these compounds may have potential deleterious effects. But it was also evidenced that a flavonoid-rich diet may promote good health and provides protection to age-related diseases (Skiloba and Smith, 2000). In our study, the blackcurrant leaf extract was not cytotoxic for endothelial cells except at very high concentrations (more than 500 µg of extract/ml) (Figure 1). During the 28-days chronic administration of our extract, the body weight of rats increased but this weight gain was inversely correlated with the quantity of extract ingested (Figure 2), without abnormal clinical related sign (Table 1, Figure 3). The decrease in the weight gain is probably due to the action of polyphenols contained in our extract. Actually, numerous studies demonstrated the usefulness of plant extracts in the treatment of obesity through inhibition of the pancreatic lipase, stimulation of thermogenesis, induction of pre-adipocytes apoptosis, appetite suppressant and other effects (Yun, 2010). For instance, leaf extract of *Nelumbo nucifera*, with a high antioxidant capacity, had a beneficial effect on obesity because it increased thermogenesis, up-regulated lipolysis in adipocytes and inhibited the absorption in female ICR (Imprinting Control Region) mice and male Wistar rats. Obesity results from a

disequilibrium between energy intake and expenditure and is known to be a strong risk factor for type 2-diabeted associated with insulin resistance (Ono et al., 2006). Lipid disorders are also induced in abnormal vasomotor process through the high concentration of oxidized LDL, the release of endothelin by endothelial cells, the increase of vessel permeability and so on (Lebranchu, 2000).

The metabolism of polyphenols is now well understood. Actually, numerous studies show that the quantities of polyphenols found intact in urine vary from one phenolic compound to another. Inter-individual variations have also been observed; probably due to the different composition of the intestinal flora which can differently affect their metabolism (D'Archivio et al., 2007). The concentration of polyphenols reached after their consumption varies highly according to the nature of polyphenols and the food sources. The plasma concentrations of intact flavonoids rarely exceeded 1 µM and the maintenance of high concentration in plasma requests repeated ingestions over time; in fact the maximum concentrations are most often reached 1-2 hours after ingestion, except for polyphenols which require to be modified prior to absorption (D'Archivio et al., 2007). Quercetin is the most predominant flavonoid in the human diet and is one of the most extensively studied polyphenols. It serves as a good example because its metabolism in humans is well understood. Estimated human consumption is in the range of 4 to 68 mg per day based on epidemiological studies (Knekt et al., 1997; Hertog et al., 1993, 1995; Rimm et al., 1996). Due to absorption differences of different conjugated forms of quercetin, its bioavailability varies greatly in different foods. Generally, the absorption of glycosylated flavonol was far greater than the absorption of the aglycon (Skiloba and Smith, 2000). In our study (Table 2), the total phenolics in plasma increased with the quantity of extract ingested. However when we analyzed different sub-classes of flavonoids, only myricetin increased in a dose-dependent manner. Quercetin and kampferol also increased in plasma but with no significant difference between the two amounts administrated. The healthy effects of polyphenols depend both on their intake and bioavailability. The concept of bioavailability integrates several variables such as intestinal absorption, metabolism by the microflora, binding to albumin, cellular uptake, accumulation in tissues, and biliary and urinary excretion (D'Archivio et al., 2007).

Nitric oxide and vasorelaxation process

Numerous *in vitro* studies showed that polyphenols induced at low concentration (<10 g/L) an endothelium-dependent vasorelaxation and at high concentration (> 10 g/L) a vascular smooth muscle-dependent vasorelaxation (Andriambeloson et al., 1997, 1998; Flesch et al., 1998). The released nitric oxide contributes to the relaxation of vascular smooth muscle. On another hand, many studies focused on the beneficial vascular effect of phenolic compounds in humans. The current study provides evidence that blackcurrant leaf extract can up-regulate the *eNOS* gene in human endothelial cells (Figure 5). The enhanced mRNA expression results in an increased production of bioactive nitric oxide, as measured by the Griess reagent (Table 3). Similar results were often found in studies on other food products such as wine (Wallerath et al., 2003) or strawberry aqueous extract (Soares et al., 2006). The increase of NO production was observed after 6 hours of incubation with a maximum at 12 hours. Such results were also obtained by Xu et al. (2007) focusing on icariin effect. Increased production of *eNOS* mRNA (Figure 4) most probably contributes to increased NO production. Further investigations must be done to analyze the activity of eNOS protein and his expression.

An increased production of NO may contribute to the vascular protection and antiatherosclerotic properties through a number of mechanisms. Therefore, we hypothesized that the generation of NO induced by blackcurrant leaf extract may be associated with its protective capacity on the vascular system. In this study, we have investigated the effect of blackcurrant leaf extract on aortic rings of rats with intact endothelium. 70% of relaxation was reached with leaf extract at ± 1 mg/ ml (Figure 6C). Nishida and Satoh (2004) showed that *Gingko biloba* extract at 0.03 to 3 mg/ ml had a potent concentration-dependent relaxation: 70 ± 4.5 % at 3 mg/ml. For strawberry extract (Edirisinghe et al., 2008), 70% of relaxation was reached with a concentration of 1 mg/ml. In a recent study (Tabart et al., 2011), we have shown that the predominant flavonoid constituent of our extract was quercetin. The potent relaxation of this compound was assessed. Effectively, we quercetin showed an endothelium-dependent vasorelaxation potential at concentrations between 1 µM and 1 mM. In 1 mg /ml leaf extract, the concentration of quercetin was around 15 µM (Figure 6B). At 1 mM, we had approximatively 60% of relaxation. Therefore the vasorelaxation effect of the blackcurrant leaf extract should depend on other compounds in addition to quercetin. Various effects of quercetin were reported in the literature. The group of Benito (2002) showed that quercetin increased eNOS protein activity and induced endothelium-dependent vasorelaxation in

hypersensitive rats. The same result on eNOS protein activity was obtained by Xu et al. (2007) with quercetin at 50 µM. But Wallerath et al. (2005) found that quercetin (1 to 33 µM) reduced *eNOS* mRNA expression in a concentration dependent manner in EAHy926 cells for 24 hours. To explain this surprising result, they suggested mRNA destabilization, because eNOS promoter activity was not modified by quercetin.

The relaxation of blood vessels is subjected to a complex control mechanism. Increased relaxation of vascular smooth muscle can result from increased eNOS expression and/or NOS activity or from activation of guanylate cyclase, all leading to the accumulation of cyclic GMP (Moncada et al., 1991; Sennequier and Vadon-Le Goff, 1998).

In conclusion, our results indicate that blackcurrant extract are not cytotoxic and that its oral uptake leads to an increase of some flavonoids in plasma with, perhaps, a regulation of body weight. We show also that blackcurrant leaf extract can moderately up-regulate the production of NO and the expression of *eNOS* mRNA. These effects could contribute to the cardiovascular protection from atherosclerosis. Further investigations must be done for a good comprehension of the mechanism by which our extract acts on endothelium-dependent vasorelaxation.

ACKNOWLEDGMENT:

J.T. gratefully acknowledges the AFR Luxembourg and the memberships of CEDEVIT and A.S.B.L (Liège).

REFERENCES

1. Andriambeloson E., Kleschoyv A., Muller N., Beretz A., Stoclet J.C., and Andrianrsitohaina R., 1997. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. British Journal of Pharmacology, 120, 1053-1058.
2. Andriambeloson E., Magnier C., Haan-Archipoff G., Lobstein A., Anton R., Beretz A., Stoclet J.C., and Andriantsitohaina R., 1998. Natural dieatry polyphenolic compounds cause endothelium-dependant vasorelaxation in rat thoracic aorta. The Journal of Nutrition, 128, 2324-2333.
3. Bani D., Failli P., Bello M.G., Thiemermann C., Bani Sacchi T., bigazzi M., and Masini E., 1998. Relaxin activates the L-Arginine-nitric oxide pathway vascular smooth muscle cells in culture. Hypertension, 31, 1240-1247.
4. Barton M., Cosentino F., Brandes R.P., Moreau P., Shaw S., and Lüsher T.F., 1997. Anatomic heterogeneity of vascular aging: Role of nitric oxide and endothelin. Hypertension, 30, 817-824.
5. Beaudeux J.L., Delattre J., Therond P., Bonnefont-Rousselot D., Legrand A., and Peynet J., 2006. Oxidative stress in the atherosclerotic process. Immuno-analyse & Biologie spécialisée, 21, 144-150.
6. Benito S., Lopez D., Saiz M.P., Buxaderas S., Sanchez J., Puig-Parellada P., and Mitjavila M.T., 2002. A flavonoid-rich diet increases nitric oxide production in rat aorta. British Journal of Pharmacology, 135, 910-916.
7. Benvenuti S., Pellati F., Melegari M., and Bertelli D., 2004. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes*, and *Aronia*. Food and Chemical Toxicology., 69, 164-169.
8. D'Archivio M., Filesi C., Di Benetto R., Gargiulo R., Giovannini C., and Masella R., 2007. Polyphenols, dietary sources and bioavailability. Annali Dell'Istituto Superiore di Sanità, 43, 348-361.
9. Di Carlo G., Mascolo N., and Izzo A.A., 1999. Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sciences, 65, 337-353.
10. Edgell C.-I. S., McDonald C.C., and Graham J.B., 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proceedings of the National Academy of Sciences USA, 80, 3734-3737.
11. Edirisinghe I., Burton-freeman B., Varelis P., and Kappagoda T., 2008. Strawberry extract caused endothelium-dependent relaxation through the activation of PI3 kinase/Akt. Journal of Agricultural and Food Chemistry, 56, 9383-9390.
12. Fitzpatrick D.F., Hirscjfield S.L., and Coffey R.G., 1993. Endothelium-dependent vasorelaxing activity of wine and other grape products. American Journal of Physiology Heart and Circulatory Physiology, 265, 774-778.
13. Flesch M., Schwarz A., and Bohm M., 1998. Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. American Journal of Physiology Heart and Circulatory Physiology, 275, 1183-1190.
14. Fletou M., and Vanhoutte P.M., 1988. Endothelium dependent hyperpolarization of canince coronary smooth muscle. British Journal of Pharmacology, 93, 515-524.
15. Hertog M.G.L., Freskens E.J.M., Hollman P.C.H., Katan M.B., and Kromhout D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet, 342, 1007-1011.

16. Hertog M.G.L., Kromhout D., Aravanis C., Blackburn H., Buzina F., Fidanza R., Giampaoli S., Jansen A., Menotti A., Nedeljkovic S., Pekkarinen M., Simic B.S., Toshima H., Freskens J.M., Hollman P.S.H., and Katan M.B., 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Archives of Internal Medicine, 155, 381–386.
17. Knekt P., Järvinen R., Seppänen R., Heliövaara M., Teppo L., Pukkula E., and Aromaa A., 1997. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. American Journal of Epidemiology, 146, 223-230.
18. Lebranchu Y., 2000. Lipides et dysfonctionnement de l'endothélium. Néphrologie, 21, 373-376.
19. Li H., and Föstermann U., 2000. Nitric oxide in the pathogenesis of vascular disease. Journal of Pathology, 190, 244-254.
20. Martin S., and Andriantsitohaina R., 2002. Cellular mechanism of vasculo-protection induced by polyphenols on the endothelium. Annales de Cancérologie et d'Angéiologie, 51, 304-315.
21. Moncada M., Palmer R.M., and Higgs E.A., 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacological Reviews, 43, 109-141.
22. Nakaishi H., Matsumoto H., Tominage S., and Hirayama M., 2000. Effects of blackcurrant anthocyanoside intake on dark adaptation and VDT work-induced transient refractive alternation in healthy humans. Alternative Medicine Review, 5, 553-562.
23. Nakamura Y., Matsumoto H., and Todoki K., 2002. Endothelium-dependent vasorelaxation induced by blackcurrant concentrate in rat thoracic aorta. Japanese Journal of Pharmacology, 89, 29-35.
24. Nishida S., and Satoh H., 2004. Comparative vasodilating actions among terpenoids and flavonoids contained in Ginkgo biloba extract. Clinica Chimica Acta, 339, 129-133.
25. Ono Y., Hattori E., Fukaya Y., Imai S., and Ohizumi Y., 2006. Anti-obesity effect of *Nelumbo nucifera* leaves extract in mica and rats. Journal of Ethnopharmacology, 106, 238-244.
26. Rimm E.B., Katan M.B., Ascherio A., Stampfer M.J., and Willett W.C., 1996. Relation between intake of flavonoids and risk for coronary heart disease in male health professionals. Annals of Internal Medicine, 125, 384-389.
27. Sennequier N., and Vadon-Le Goff S., 1998. Biosynthèse du monoxyde d'azote (NO) : mécanisme, régulation et contrôle. Médecine/Sciences, 14, 1185-1195.
28. Skiloba C.F., and Smith M.T., 2000. Potential health impact of excessive flavonoid intake. Free Radical Biology and Medicine, 29, 375-383.
29. Soares V.C., Varanda E.A., and Raddi M.S., 2006. In vitro basal and metabolism-mediated cytotoxicity of flavonoids. Food and Chemical Toxicology, 44, 835-838.
30. Tabart J., Kevers C., Sipel A., Pincemail J., Defraigne J.O., and Dommes J. 2006. Antioxidant capacity of black currant varies with organ, season, and cultivar. Journal of Agricultural and Food Chemistry, 54, 6271-6276.
31. Tabart J., Kevers C., Pincemail J., Defraigne J.-O., and Dommes J., 2009. Comparative antioxidant capacities of phenolic compounds measured by various tests. Food Chemistry, 113, 1226-1233.
32. Tabart J., Franck T., Kevers C., Pincemail J., Serteyn D., Defraigne J.-O., and Dommes J., 2010a. Antioxidant and anti-inflammatory activities of *Ribes nigrum* extracts. Journal of Nutritional Biochemistry, submitted.

33. Tabart J., Kevers C., Pincemail J., Defraigne J.-O., and Dommes J., 2010b. Evaluation of spectrophotometric methods for antioxidant compound measurement in relation to total antioxidant capacity in beverages. Food Chemistry 120, 607-614.
34. Tabart J., Kevers C., Evers D., and Dommes J., 2011. Ascorbic acid, phenolic acid, flavonoid and carotenoid profiles of selected extracts from *Ribes nigrum.* Journal of Agricultural and Food Chemistry, submitted.
35. Wallerath T., Poleo D., Li H., and Föstermann U., 2003. Red wine increases the expression of human endothelial nitric oxide synthase. Journal of the American College of Cardiology, 41, 471-478.
36. Wallerath T., Li H., Gödtel-Ambrust U., Schwarz P.M., and Föstermann U., 2005. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide, 12, 97-104.
37. Xu H.B., and Huang Z.Q., 2007. Icariin enhances endothelial nitric-oxide synthase expression on human endothelial cells in vitro. Vascular Pharmacology, 47, 18-24.
38. Yun J.W., 2010. Possible anti-obesity therapeutics from nature-A review. Phytochemistry, 71, 1625-1641.

3. Conclusion

L'extrait de feuilles de cassis a une potentialité non négligeable au niveau du processus de relaxation vasculaire dépendante de l'endothélium : augmentation de l'expression de l'eNOS, ce qui se traduit par une augmentation du taux de NO. D'autres investigations devraient également être réalisées sur le modèle des aortes isolées pour une meilleure compréhension du mécanisme par lequel les composants de l'extrait agissent sur la motricité vasculaire dépendante de l'endothélium, par exemple des inhibiteurs enzymatiques comme le L-NAME (N^G-nitro-L-arginine methyl ester, inhibiteur de l'eNOS) et l'ODQ (1*H*-[1,2,4] oxadiazolo-[4,3-α] quinoxalin-1-one, inhibiteur de la Guanylate Cyclase).

La toxicité de l'extrait de feuilles de cassis a été analysée par une expérience d'administration de l'extrait quotidiennement et ce, durant 28 jours. A la fin de cette expérience, aucun signe clinique n'a laissé apparaître d'effet toxique de l'extrait sur les animaux. Il semblerait seulement que l'administration de cet extrait régule la prise de poids puisque l'on peut voir une corrélation inverse entre la dose de l'extrait administré et la prise de poids. Ce qui pourrait être expliqué par le fait que de nombreuses études montrent un effet positif des polyphénols dans le traitement de l'obésité (Ono et al., 2006 ; Yun, 2010). Au niveau de la biodisponibilité, il semblerait que le taux plasmatique de certains composés ne soit pas directement corrélé avec les quantités ingérées.

Une des revues pouvant convenir pour la soumission de l'article serait la revue Nitric Oxide, avec un facteur d'impact de 2,506.

Discussion et Conclusions Générales

Les petits fruits (mûres, framboises et groseilles) constituent une bonne source de substances antioxydantes, plus importante que les autres fruits, et cela principalement parce qu'ils ont un contenu assez élevé en anthocyanes. Ces fruits agissent effectivement comme des inhibiteurs de radicaux libres (Heinonen *et al.*, 1998 ; Su and Silva, 2006) du fait notamment de leur teneur élevée en flavonoïdes (Pantelidis *et al.*, 2007). Dans le cas du cassis, les baies largement utilisées dans la production industrielle de sirops et de concentrés, contiennent des quantités très élevées de composés phénoliques, particulièrement des anthocyanes mais aussi des flavonols (Benvenuti *et al.*, 2004). Les bourgeons et les feuilles de cassis sont également utilisés en tant que complément alimentaire conseillés comme adaptogène tonique, diurétique ainsi que dans le traitement d'affections rhumatismales.

L'objectif de ce travail était de mettre au point, à l'échelle du laboratoire, un extrait de cassis riche en flavonoïdes et stable dans le temps, de le caractériser au niveau de sa composition en composés phénoliques et, enfin, de démontrer ses activités biologiques : capacité antioxydante, capacité anti-inflammatoire et effets sur le processus de relaxation des vaisseaux sanguins. Pour ce faire, différents points ont été traités :

1. **L'optimisation du matériel végétal et l'optimisation de l'extraction des flavonoïdes**
   Il est communément admis que le contenu en composés phénoliques des baies est affecté par divers facteurs environnementaux, le degré de maturité, les différences génétiques, la saison (Heiberg *et al.*, 1992 ; Wang and Lin, 2000 ; Howard *et al.*, 2003). Les conditions de stockage et les procédés de fabrication industriels influencent également ce contenu. Au niveau des autres explants de ces plantes (feuilles et bourgeons), très peu de données existent (Swiderski *et al.*, 2004). Nous avons dans un premier temps examiné divers explants de cassis (feuilles, fruits et bourgeons) en fonction du cultivar ainsi qu'à différents stades du développement. Nous avons pu remarquer que le taux en polyphénols ainsi que la capacité antioxydante (DPPH) variaient en fonction de la saison de prélèvement et ce, quelque soit l'explant analysé. Par contre, aucune différence significative n'a été observée entre ces explants prélevés sur divers cultivars. En prenant en compte la biomasse, il s'avère que les feuilles prélevées en juin sont les plus intéressantes.

Pour tenter d'augmenter le rendement d'extraction des composés phénoliques du cassis, l'étape d'extraction a été optimisée en jouant sur divers paramètres comme le pH, la température, le nombre d'étapes durant l'extraction, le solvant d'extraction, la fragmentation du matériel végétal. Les résultats ont été comparés à ceux obtenus au moyen de tampon glycine, tampon aqueux pouvant être utilisé dans la production de compléments alimentaires. Dans notre étude, le mélange aqueux contenant 70% d'acétone et 2% d'acide acétique donne le meilleur rendement d'extraction, en utilisant du matériel lyophilisé. Des observations similaires ont été faites lors d'étude portant sur le chocolat (Counet and Collin, 2002). Au niveau de la stabilité, nous avons pu constater que le mélange acétone permettait l'obtention d'extraits plus stables que ceux obtenus avec le tampon glycine, et stabilité qui se maintenait durant plusieurs mois.

2. La caractérisation des extraits et l'évaluation de propriétés-santé (capacités antioxydante et anti-inflammatoire)

Avant d'utiliser la large gamme de techniques couramment utilisées en vue d'évaluer la capacité antioxydante, nous avons mené un certain nombre d'investigations sur leur validation. Le groupe de Prior (2005) a émis certaines recommandations pour standardiser les méthodes d'évaluation de la capacité antioxydante. Le premier facteur à prendre en considération est de mettre en relation le mécanisme de réaction avec l'application cible à analyser. Exemple, pour étudier l'activité classique des antioxydants, il est préférable d'utiliser les méthodes basées sur un transfert de proton et d'électron (HAT) plutôt que des méthodes basées uniquement sur un transfert d'électron (SET) car le radical peroxyl est le radical le plus abondamment trouvé dans les systèmes biologiques. Un autre facteur à prendre en considération est la pertinence biologique. De nouvelles techniques utilisant d'autres radicaux libres actifs au sein des systèmes biologiques (anion superoxyde, peroxynitrite, radical hydroxyl, …) devraient être développées et utilisées afin d'avoir un bon aperçu des potentiels antioxydants (Prior *et al.*, 2005). Il est clair qu'il n'y a pas de technique simple et unique permettant de mettre en évidence la capacité antioxydante globale d'un échantillon, permettant ainsi des comparaisons entre extraits de différentes origines et diverses matrices alimentaires. Dans le cadre d'une standardisation, nous avons proposé une méthode permettant de normaliser la valeur de la capacité antioxydante en employant une moyenne pondérée basée sur les résultats de quatre méthodes choisies pour leurs principes différents : DPPH (oxydoréduction), ORAC (cinétique de piégeage du radical peroxyl), résistance à l'hémolyse (protection d'un échantillon biologique) et l'EPR

(évaluation d'un radical libre particulier). Sur cette base, la gallocatéchine apparaît comme le meilleur antioxydant et pourrait dès lors être utilisée comme standard de référence dans les diverses analyses en remplacement du traditionnel trolox. Nous avons également analysé les méthodes spectrophotométriques de quantification des groupes de flavonoïdes où nous avons pu constater quelques anomalies. Par exemple, pour le dosage spectrophotométrique des flavonoïdes totaux, seuls les flavonols réagissent très bien. Les autres classes de flavonoïdes n'interagissent pas ou très peu. Au contraire, dans le test de la vanilline pour le dosage des flavan-3-ols et le test des anthocyanes, seules quelques interférences sont observées. Cependant pour évaluer correctement le contenu en composés phénoliques, le recourt à l'utilisation des techniques de chromatographie est indispensable mais ces méthodes sont assez coûteuses et lourdes à mettre en œuvre.

Après cette analyse critique des diverses méthodes utilisées dans la littérature, nous avons dans un premier temps caractérisé le contenu des extraits de cassis obtenus par l'extraction au mélange acétone-eau-acide acétique (70 /28/2) en utilisant des techniques spectrophotométriques et chromatographiques (HPLC). Les feuilles et les bourgeons présentent un contenu plus élevé, par rapport aux baies, en composés phénoliques et en flavonols, avec une prédominance de la quercétine. Par contre, nous avons observé un contenu en acide ascorbique plus élevé dans les fruits que dans les autres explants, avec une concentration de 2,35 µg/ g de poids frais. Au niveau des flavan-3-ols, les bourgeons sont les plus riches, avec une prédominance pour la gallocatéchine et l'épigallocatéchine. Les trois extraits ont aussi un contenu non négligeable en acides phénoliques avec des prédominances propre à chaque explant : acide gentisique pour les feuilles, l'acide férulique dans les bourgeons et les baies. Au niveau des anthocyanines, on retrouve abondamment la pétunidine, avec, comme attendu, un contenu plus élevé dans les baies.

Ces rendements d'extraction ont été comparés à des techniques d'extraction spécifiques à chaque classe de composés phénoliques. Les résultats permettent de démontrer que le mélange acétone-eau-acide acétique (70/28/2) permet d'extraire efficacement plusieurs classes de flavonoïdes et d'autres composés phénoliques : acide ascorbique, flavonols, flavan-3-ols et anthocyanes. Par contre, cette technique n'est pas adéquate pour l'extraction des caroténoïdes, probablement suite au caractère plus hydrophobe de ceux-ci.

Nous avons ensuite évalué deux propriétés santé connues des mélanges de polyphénols : propriétés antioxydante et anti-inflammatoire. Au niveau de la capacité antioxydante, nous avons utilisé les méthodes traditionnelles *in vitro* (DPPH, TEAC, phénols totaux, RPE et ORAC) ainsi que deux techniques sur modèles cellulaires. La première technique cellulaire utilisée est le test de résistance des globules rouges face à un stress oxydant (APPH). Par cette technique, l'effet protecteur des flavonoïdes peut être lié à leur interaction avec la membrane plasmique des globules rouges (Blasa *et al.*, 2007) et leur capacité à pénétrer dans la couche lipidique (Lopez-Revuelta *et al.*, 2006). Le deuxième test cellulaire utilisé est l'évaluation de l'activité antioxydante cellulaire (AAC), développée par Wolfe et Liu (2007). Cette technique est un dérivé de la technique ORAC mais la protection de la sonde se fait à l'intérieur même de la cellule. Une corrélation significative des résultats de ce test avec le contenu en phénols totaux (R^2= 0.793) et les résultats ORAC (R^2= 0.522) est observée en analysant les résultats obtenus pour 25 fruits (Wolfe and Liu, 2008a). Par contre, en utilisant des standards des composés antioxydants, Wolfe et Liu (2008b) n'ont observé aucune corrélation entre les résultats AAC (Activité antioxydante Cellulaire) et ORAC (R^2= 0.080). La plupart des antioxydantes ayant une haute activité dans le test ORAC n'ont pas (rutine, apigénine, naringénine, genistein) ou ont une faible activité (taxifolin, catéchine, épicatéchine, kampférol) dans le test AAC (Wolfe and Liu, 2008b). La lipophilicité des flavonoïdes joue également un rôle dans le test AAC puisqu'il tient compte de leur accessibilité aux radicaux libres intracellulaires. Les flavonoïdes ayant une forte lipophilicité ont, comme attendu, une faible activité antioxydante cellulaire. Cependant, les flavonoïdes présentant une très faible lipophilicité présentent aussi cette faible activité (Wolfe and Liu, 2008b).

D'une manière générale, nous avons pu observer que le choix de la méthode a une influence non négligeable sur les capacités antioxydantes mesurées. Cela est dû entre autre à la grande variabilité des composés présents dans les matrices végétales mais aussi aux différentes sources de radicaux libres utilisés dans les méthodes d'analyses. De telles différences ont déjà été observées, notamment par le groupe de Bors (Bors *et al.,* 1990). L'activité antioxydante ou la propriété de piégeur de radicaux libres des flavonoïdes est influencée par trois de leurs propriétés structurales : (1) le groupement *o*-hydroxyl sur le cycle B, (2) la liaison double entre les carbones en position 2 et 3 combinée à un groupe 4-oxo sur le cycle C, et (3) les groupements hydroxyls en position C_3 et C_5 (Bors *et al.*,

1990 ; Wolfe and Liu, 2008b). Les flavonoïdes cumulant ces caractéristiques possèdent généralement une haute capacité antioxydante, mais le choix de la technique d'évaluation a aussi son importance (Bors *et al.*, 1990). Pour les trois extraits de cassis et quelque soit la technique utilisée, nous avons observé un profil semblable même si l'intensité de la réponse est variable d'une technique à l'autre: feuilles > bourgeons > baies. Cette corrélation presque parfaite entre les différentes méthodes n'est pas couramment obtenue pour d'autres matrices végétales. Généralement, on trouve une assez bonne corrélation uniquement entre la technique des phénols totaux et les techniques TEAC et DPPH (Tabart *et al.*, 2006, 2007) ou avec les valeurs ORAC (André *et al.*, 2007).

Un autre modèle cellulaire utilisé met en relation la production de radicaux libres et les processus inflammatoires. Ce modèle a été développé sur des neutrophiles équins activés au Centre de l'Oxygène, Recherche et Développement (CORD) de l'Université de Liège. Ces modèles expérimentaux ont déjà été utilisés pour mettre en avant les propriétés anti-inflammatoires de composés antioxydants comme les curcuminoïdes, les tétrahydrocurcuminoïdes (Franck *et al.*, 2008), le resvératrol (Kohnen *et al.*, 2007) ainsi que sur certains flavonols (Pincemail *et al.*, 1988). Pour les extraits de cassis, notre étude a mis en évidence à la fois au niveau intracellulaire qu'extracellulaire des propriétés de piégeurs de radicaux libres et une activité inhibitrice de la myéloperoxydase chez les neutrophiles. Nous avons observé un meilleur effet des extraits de feuilles, suivis des extraits de bourgeons et finalement et loin derrière ceux des baies. Cette différence est corrélée avec la teneur en phénols totaux. Tout comme le resvératrol (Kohnen *et al.*, 2007), nos extraits ne montrent aucun effet au niveau du processus de dégranulation. D'autres flavonoïdes ont également montré une activité anti-inflammatoire comme la quercétine, ses analogues et ses métabolites (Shiba *et al.*, 2008). Ces composés induisent une inhibition de l'activité de la myéloperoxydase dans les modèles de lésions athéroscléreuses chez l'homme mais aussi une activité de piégeurs de l'acide hypochloreux (Shiba *et al.*, 2008). Ce groupe met également en évidence le fait que l'activité des flavonoïdes ne dépend pas uniquement de leur activité de piégeurs de radicaux libres mais également de leur hydrophobicité.

En empêchant ainsi la production de radicaux libres dans les cellules comme les neutrophiles ou les cellules endothéliales, l'extrait de feuilles de cassis possèderait un potentiel élevé pour le traitement de pathologies tant inflammatoires que cardio-vasculaires.

3. Effet des extraits de cassis sur la motricité vasculaire

De nombreuses études ont montré l'effet bénéfique des composés phénoliques au niveau de la motricité vasculaire. Un intérêt croissant leurs est donc porté en vue de développer des traitements alternatifs de pathologies vasculaires comme l'athérosclérose, les maladies neurodégénératives ainsi que les maladies cardiovasculaires. Des travaux récents ont montré que des extraits de plantes riches en polyphénols pouvaient induire une vasorelaxation dépendante de l'endothélium, probablement via le relargage de monoxyde d'azote (Nakamura *et al.*, 2002). Nos investigations se sont portées sur l'effet vasorelaxant dépendant de l'endothélium. Pour ce faire, nous avons utilisé dans un premier temps des modèles cellulaires (production de NO et modulation de l'expression de l'ARNm eNOS dans les cellules endothéliales) et finalement, un modèle d'organe isolé (aortes isolées placées dans un bain à organes). Nos résultats, sur cellules, ont montré une augmentation de la production de monoxyde d'azote simultanément avec une augmentation du taux d'ARNm *eNOS* lorsque les cellules sont incubées en présence d'extrait de feuilles de cassis. Des observations similaires ont été faites pour l'icariine, flavonoïde présent dans *Epimedii herba*, plante médicinale chinoise (Xu *et al.*, 2007). Ces observations ont été renforcées en utilisant les aortes isolées pré-contractées, où l'extrait de cassis permet d'obtenir 70% de relaxation avec une concentration aux alentours de 1 mg/ ml. Des résultats similaires ont été obtenus avec un extrait de fraise (Edirisinghe *et al.*, 2008). Une autre plante médicinale, le *Gingko biloba*, utilisée dans le traitement des désordres circulatoires, les insuffisances cérébrovasculaires et autres pathologies, présente une activité de relaxation vasculaire dépendante de l'endothélium. Une concentration de 3 mg/ml sur aorte isolée donne une relaxation de 70 ± 0.45%. Cette propriété est principalement due à deux fractions : la fraction des flavonoïdes (22 à 27%) et la fraction des terpénoïdes (6%) (Nishida and Satoh, 2004). Nous avons également testé sur ce modèle la quercétine, flavonol le plus abondamment trouvé dans notre alimentation et aussi le plus étudié. Ce flavonol permet une augmentation de l'expression de l'ARNm *eNOS* et a également un potentiel vasorelaxant dépendant de l'endothélium à une

concentration allant de 1 µM à 1 mM (± 60% de relaxation). Les résultats obtenus en utilisant l'extrait de feuille de cassis ont donc mis en évidence ses potentialités vasorelaxantes. Il pourrait donc contribuer à la protection des vaisseaux. Le mécanisme par lequel les polyphénols induisent une relaxation vasculaire n'est pas encore connu. Mendes *et al.* (2003) ont montré que la vasorelaxation induite par les polyphénols était induite par un relarguage d'ATP et probablement d'autres nucléotides comme l'ADP et l'UTP des cellules vasculaires via les récepteurs $P2Y_1$ et/ou $P2Y_2$. Nakamura *et al.* (2002) ont montré que la relaxation des vaisseaux par un concentré de jus de cassis était causée par une augmentation de la production de monoxyde d'azote, via les récepteurs histamines H_1 de l'endothélium. D'autres extraits de plantes n'agissent pas via la voie du NO. C'est le cas d'extraits d'ail dont l'effet relaxant se fait par une voie dépendante de l'endothélium (inhibition des cyclooxygénases et production de facteurs relaxants autres que le NO) et par une voie indépendante de l'endothélium (hyperpolarisation des muscles lisses) (Ashraf *et al.*, 2004).

Des investigations plus poussées devraient être réalisées afin de mieux comprendre les processus impliqués. Ainsi, au niveau cellulaire, il serait intéressant de compléter nos travaux avec des études sur l'expression et l'activité de la protéine eNOS. Au niveau des fragments d'aortes isolées, des investigations devraient être menées en vue d'une meilleure compréhension des mécanismes par lesquels l'extrait de cassis agit sur la motricité vasculaire. Diverses drogues pourraient être utilisées, dans un premier temps, pour démontrer à quel niveau agissent les composants de l'extrait pour la production du NO : le L-NAME (inhibiteur des NO synthases) et l'ODQ (inhibiteur de la guanylate cyclase). Dans un deuxième temps, nous pourrions également chercher à comprendre comment les composants phénoliques modulent l'activité de certaines enzymes impliquées dans la motricité vasculaire. Après une meilleure compréhension de l'action de cet extrait sur la motricité vasculaire, nous pourrions le tester sur des modèles expérimentaux de développement de pathologies comme l'athérosclérose.

4. Toxicité et biodisponibilité des composés phénoliques de l'extrait de feuilles de cassis

Les polyphénols peuvent agir comme antioxydants mais aussi comme pro-oxydants, ce qui suggère que ces composés peuvent être potentiellement dommageables comme bénéfiques. En effet, les cycles phénols contenus dans les flavonoïdes, sous oxydation par les peroxydases, peuvent conduire à la formation de radicaux phénoxyls, extrêmement cytotoxiques. Ces radicaux peuvent oxyder les lipides insaturés, le GSH, le NADH et les acides nucléiques et ainsi, conduire à la production d'autres radicaux (Galati *et al.*, 1999, 2000 ; Chan *et al.*, 1999 ; Galati and O'Brien, 2004). Dans notre étude, la toxicité des extraits de feuilles de cassis a été analysée au niveau cellulaire (cellules endothéliales et neutrophiles) et dans une étude sur modèle animal. Au niveau cellulaire, les extraits n'ont pas montré d'effets cytotoxiques. Au niveau du modèle animal, une administration quotidienne de l'extrait sur une période de 28 jours n'a montré aucun effet toxique. Mais nous avons pu observer une réduction de la prise de poids par rapport aux témoins. De nombreuses études démontrent l'effet des composés phénoliques dans le traitement de l'obésité : inhibition de la lipase pancréatique, stimulation de la thermogenèse, diminution de l'appétit, … (Ono *et al.*, 2006 ; Yun, 2010).

Comme mentionné plus haut, les concentrations nécessaires à une cytotoxicité ne devraient pas être atteintes dans le cadre d'une alimentation normale. Par contre, l'utilisation de compléments alimentaires dits « antioxydants » pourrait conduire à une exposition à des taux potentiellement toxiques. Certains producteurs de ce type de complément recommandent des doses de 500 à 1000 mg de quercétine par jour, ce qui est 10 à 20 fois supérieur aux doses ingérées dans une alimentation typiquement végétarienne (Skiloba and Smith, 2000). Donc, des investigations devront être menées en vue de déterminer les potentiels toxiques associés aux flavonoïdes et autres composés phénoliques avant l'utilisation de ces produits dérivés des plantes en tant que traitement (Galati and O'Brien, 2004).

Les effets positifs ou négatifs pour la santé d'extraits végétaux dépendent non seulement des quantités ingérées mais également de la biodisponibilité des différents composés phénoliques. Cette biodisponibilité varie grandement en fonction de la source alimentaire mais aussi au niveau même des molécules (classe, type de sucre, …). Ce sont les flavanones ainsi que les isoflavones, retrouvées principalement dans le citron et le soja,

qui montrent les meilleurs profils de biodisponibilité avec des concentrations plasmatiques pouvant atteindre 5 µM. Pour les flavonols, les flavanols et les flavones, la concentration plasmatique atteint rarement 1 µM (Scalbert and Williamson, 2000 ; Manach *et al.*, 2004). Un des flavonoïdes les plus étudiés est la quercétine. L'aglycon seul a une biodisponibilité de 24 ± 9% et les formes glycosylées ont une biodisponibilité de 52 ± 15 % (Hollman *et al.*, 1997). Dans notre étude, la biodisponibilité des composants de l'extrait de feuilles de cassis a été testée après une consommation journalière constante et sur 28 jours. Nous avons pu observer des changements au niveau plasmatique par rapport aux témoins, mais ces changements ne sont pas dose-dépendants sauf pour la myricétine et les phénols totaux. Vu la faible biodisponibilité des polyphénols, il serait intéressant de développer des techniques en vue d'augmenter cette biodisponibilité, et ce, dans le but d'augmenter l'action bénéfique de ces composés dans la prévention de pathologies.

Ce travail a aussi permis d'ouvrir d'autres voies éventuelles d'investigation comme l'utilisation d'extraits de cassis dans le traitement de l'obésité et des pathologies dérivantes comme le diabète de type II. Une autre approche, encore très peu étudiée, est l'étude des effets synergiques ou antagonistes des mélanges de polyphénols. A l'heure actuelle, les principales investigations concernant les effets santé des polyphénols sur modèle animal ou cellulaire sont réalisées avec des composés standards comme la quercétine ou le resvératrol, alors que l'on sait que les mélanges ne se comportent pas simplement comme une addition d'effets. Ayant un extrait végétal très bien caractérisé au niveau des flavonoïdes, des études sur les effets synergiques ou antagonistes pourraient être réalisées avec les principaux composants de l'extrait.

En conclusion, l'extrait de feuilles de cassis obtenu par une extraction au mélange acétone-eau-acide acétique possède de nombreuses qualités tant au niveau nutritionnel qu'au niveau de la protection santé. Cet extrait, riche en composés phénoliques, possède une haute activité antioxydante. Lors de cette étude, nous avons pu le tester sur divers modèles. Il peut agir tant au niveau extracellulaire qu'au niveau intracellulaire comme piégeurs de radicaux libres. Il peut également interagir sur l'activité de certaines enzymes dans divers processus comme l'inflammation et la motricité vasculaire.

Références bibliographiques

- Aldini G., Carini M., Piccoli A., Rossoni G., and Maffei Facino R., 2003. Procyanidins from grape seeds protect endothelial cells from peroxinitrite damage and enhance endothelium-dependent relaxation in human artery: new evidences for cardio-protection. Life Sciences, 73, 2883-2898.
- Anderson T.J., 2003. Nitric oxide, atherosclerosis and the clinical relevance of endothelial dysfunction. Heart Failure Reviews, 8, 71-86.
- André C.M., Ghislain M., Bertin P., Oufir M., Del Rosario Herrera M., Hoffman L., Hausman J.F., Larondelle Y., and Evers D., 2006. Andean potato cultivars (*Solanum tuberosum* L.) as a source of antioxidant and mineral micronutrients. Journal of Agricultural and Food Chemistry, 55, 366-378.
- Andriambeloson E., Kleschoyv A., Muller N., Beretz A., Stoclet J.C., and Andrianrsitohaina R., 1997. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. British Journal of Pharmacology, 120, 1053-1058.
- Andriambeloson E., Magnier C., Haan-Archipoff G., Lobstein A., Anton R., Beretz A., Stoclet J.C., and Andriantsitohaina R., 1998. Natural dieatry polyphenolic compounds cause endothelium-dependant vasorelaxation in rat thoracic aorta. The Journal of Nutrition, 128, 2324-2333.
- Arts I.C.W., Hollman P.C.H., Bueno de Mesquita H.B., Feskens E.J.M., and Kromhout D., 2001a. Dietary catechins and epithelial cancer incidence: The Zutphen Elderly Study. International Journal of Cancer, 92, 298-302.
- Arts I.C.W., Hollman P.C.H., Feskens E.J.M, Bueno de Mesquita H.B., and Kromhout D., 2001b. Catechin intake might explain the inverse relation between tea consumption and ischemic disease: The Zutphen Elderly Study. American Journal of Clinical Nutrition, 74, 227-232.
- Arts I.C.W., Jacobs Jr D.R., Gross M., Harnack L.J., and Folsom A.R., 2002. Dietary catechins and cancer incidence among postmenopausal women: the Iowa Women's Health Study (United States). Cancer Causes and Control, 13, 373-382.
- Ashraf M.Z., Hussain M.E., and Fahim M., 2004. Endothelium-mediated vasorelaxant response of garlic in isolated rat aorta: role of nitric oxide. Journal of Ethnopharmacology, 90, 5-9.
- Barouki R., 2006. Stress oxydant et vieillissement. Médecine/Sciences, 22, 266-272.
- Beattie J., Crozier A., and Duthie G.G., 2005. Potential health benefits of berries. Current Nutrition and Food Science, 1, 71-86.
- Benito S., Lopez D., Saiz M.P., Buxaderas S., Sanchez J., Puig-Parellada P., and Mitjavila M.T., 2007. A flavonoid-rich diet increases nitric oxide production in rat aorta. British Journal of Pharmacology, 135, 910-916.
- Benkhalti F., Leggssyer A., Gomez P., Paz E., Lopez-Miranda J., Perez-Jimenez F., and Boustani E., 2003. Effects of virgin olive oil phenolic compounds on LDL oxidation and vasorelaxation activity. Therapie, 58, 133-137.
- Benvenuti S., Pellati F., Melegari M., and Bertelli D., 2004. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes*, and *Aronia*. Food Chemistry and Toxicology, 69 (3), 164-169.

- Blasa M., Candiracci M., Accorsi A., Piacentini M.P. and Piatti E., 2007. Honey flavonoids as protection agent against oxidative damage to human red blood cells. Food Chemistry, 104, 1635-1640.
- Bors W., Heller W., Michel C. and Saran M., 1990. Flavonoids as antioxidants:determination of radical-scavenging efficiencies. Methods of Enzymology, 186, 343-355.
- Bosetti C., Bravi F., Talamini R., Parpinel M., Gnagnarella P., Negri E., Montella M., Lagiou P., Franseschi S., and La Vecchia C., 2006. Flavonoids and prostate cancer risk : a study in Italy. Nutrition and Cancer, 56, 123-127.
- Bosetti C., Rossi M., McLaughlin J.K., Negri E., Talamini R., Lagiou P., Montella M., Ramazzotti V., Franceschi S., and La Vecchia C., 2007. Flavonoids and the risk of renal cell carcinoma. Cancer Epidemiology, Biomarkers and Prevention, 16, 98-101.
- Bravo L., 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Review, 56, 317-333.
- Bruneton, 1999. Plantes médicinales (3ème édition). Pharmacognosie, Phytochimie, Plantes médicinales. Editions médicinales internationales, éditions Tec & Doc Lavoisier.
- Burns J., Gardner P.Y., O'Neil J., Crawford S., Morecroft I., McPhail D.B., Lister C., Matthews D., MacLean M.R., Lean M.E.J., Duthie G.G., and Crozier A., 2000. Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. Journal of Agricultural and Food Chemistry, 48, 220-230.
- Cacace J.E., and Mazza G., 2002. Extraction of anthocyanins and other phenolics from black currants with sulfured water. Journal of Agricultural and Food Chemistry, 50, 5939-5946.
- Cai H., and Harrison D.G., 2000. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circulation Research, 87, 840-844.
- Cao G., Sofic E., and Prior R.L., 1997. Antioxidant and prooxidant behaviour of flavonoids: structure-activity relationships. Free Radical Biology and Medicine, 22: 749-760.
- Cao G., Russell R.M., Lischner N., and Prior R.L., 1998. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. Journal of Nutrition, 128, 2383-2390.
- Chan T., Galati G., and O'Brien P.J., 1999. Oxygen activation during peroxidase catalysed metabolism of flavones or flavanones. Chemico-Biological Interactions, 122, 15-25.
- Chavan U.D., Shahidi F., and Naczk M., 2001. Extraction of condensed tannins from beach pea (Luthyrus maritimus L.) as affected by different solvents. Food Chemistry, 75, 509–512.
- Chrubasik S., 2000. Pain therapy using herbal medicines. Gynakologe 2000, 33, 59-64.
- Cillard J., Morel I., Cillard P., Lescoat G., and Gicquel M., 1990. Flavonoids as free radical scavengers. Flavonoids in Biology and Medicine III: Current Issues in Flavonoid Research; N.P. Das; National University of Singapore, 143-160.
- Cillard J., and Cillard P., 2006. Mécanismes de la peroxydation lipidique et des anti-oxydations. Oléagineux, Corps Gras, Lipides ; 13, 24-29.

- Commenges D., Scolet V., Renaud S., Jacqmin-Gadda H., Barberger-Gateau P., and Dartigues J.F., 2000. Intake of flavonoids and risk of dementia. European Journal of Epidemiology, 16, 357-363.
- Cook N.C., and Samman S., 1996. Flavonoids-Chemistry, metabolism, cardioprotective effects, and dietary source. Nutritional Biochemistry, 7, 66-76.
- Corder R., Douthwaite J.A., Lees D.M., Khan N.Q., Viseu dos Santos A.C., Wood E.G., and Carrier M.J., 2001. Endothelin-1 synthesis reduced by red wine. Nature, 414, 863-864.
- Cosentino F., and Luscher T.F., 2002. Les mécanismes moléculaires de la dysfonction endothéliale dans le diabète. Flammarion Médecine-Sciences, journées de diabétologie 2002.
- Counet C., and Collin S., 2003. Effect of the number of flavonols units on the antioxidant activity of procyanidin fractions isolated from chocolate. Journal of Agricultural and Food Chemistry, 51, 6816-6822.
- D'Archivio M., Filesi C., Di Benetto R., Gargiulo R., Giovannini C., and Masella R., 2007. Polyphenols, dietary sources and bioavailability. Annali Dell'Istituto Superiore di Sanità, 43, 348-361.
- De Nigris F., Willimas-Ignarro S., Lerman L.O., Crimi E., Mansueto G., D'Armiento F.P., De Rosa G., Sica V., Ignarro L.J., and Napoli C., 2005. Beneficial effects of pomegranate juice on oxidation-sensitive genes and endothelial nitric oxide synthase activity at sites of perturbes shear stress. Proceedings of the National Academy of Sciences, 29, 4896-4901.
- Declume C., 1989. Anti-inflammatory evaluation of hydroalcoholic extract of black currant leaves (Ribes nigrum). Journal of Ethnopharmacology, 27, 91-98.
- Deplanque D., 2003. Physiopathologie de l'ischémie cérébrale. Encyclopédie Médico-Chirurgicale, Editions Scientifiques et Médicales Elsevier SAS, Paris, Neurologie, 17-045-A-80, 1-10.
- Di Carlo G., Mascolo N., and Izzo A.A., 1999. Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sciences, 65, 337-353.
- Diebolt M., Bucher B., and Andriantsitohaina R., 2001. Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. Hypertension, 38, 159-165.
- Duthie G., and Crozier A., 2000. Plant-derived phenolic antioxidants. Current Opinion in Lipidology, 11, 43-47.
- Edirisinghe I., Burton-freeman B., Varelis P., and Kappagoda T., 2008. Strawberry extract caused endothelium-dependent relaxation through the activation of PI3 kinase/Akt. Journal of Agricultural and Food Chemistry, 56, 9383-9390.
- Ehala S., Vaher M., and Kaljurand M., 2005. Characterization of phenolic profiles of northern European berries by capillary electrophoresis and determination of their antioxidant activity. Journal of Agricultural and Food Chemistry, 53, 6484-6490.
- Fernandez-Pachon M.S., Villano D. Troncoso A.M., and Garcia-Parrilla M.C., 2006. Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with anioxidant activity. Analytica Chimca Acta, 563, 101-108.
- Flesch M., Schwarz A., and Bohm M., 1998. Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. American Journal of Physiology: Heart and Circulatory Physiology, 275, 1183-1190.

- Fitzpatrick D.F., Fleming R.C., Bing B., Maggi D.A., and O'Malley R., 2000. Isolation and characterization of endothelium-dependent vasorelaxing compounds from grape seeds. Journal of Agricultural and Food Chemistry, 48, 6384-6390.
- Franck T., Kohnen S., Grulke S., Neven P., Goutman Y., Peters F., Pirotte B., Deby-Dupont G. and Serteyn D., 2008. Inhibitory effect of curcuminoids and tetrahydrocurcuminoids on equine activated neutrophils and myeloperoxidase activity. Physiological Research, 57, 577-587.
- Furchgott R.F., and Zawadski J.V., 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature, 288, 373-376.
- Galati G., Chan T.S., Wu B. and O'Brien P.J., 1999. Glutathione-dependent generation of reactive oxygen species by the peroxidase-catalysed redox cycling of flavonoids. Chemical Research in Toxicology, 12, 521-525.
- Galati G., Teng S., Moridani M.Y., Chan T.S. and O'Brien P.J., 2000. Cancer chemoprevention and apoptosis mechanisms induced by dietary polyphenolics. Drug Metabolism and Drug Interactions, 17, 311-349.
- Galati G., and O'Brien P.J., 2004. Potential toxicology of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radical Biology and Medecine, 37, 287-303.
- Galley H.F., and Webster N.R., 2004. Physiology of the endothelium. British Journal of Anaesthesia, 93, 105-113.
- Garbacki N., Kinet M., Nusgens B., Desmecht D., and Damas J., 2005. Proanthocyanidins, from *Ribes nigrum* leaves, reduce endothelial adhesion molecules ICAM-1 and VCAM-1. Journal of Inflammation, 2, 1-12.
- Geleijne J., Launer L.J., Van der Kuip D.A.M., Hofman A., and Witteman J.C.M., 2002. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. American Journal of Clinical Nutrition, 75, 880-886.
- Geleijne J., and Hollman P.C.H., 2008. Flavonoids and cardiovascular health: which compounds, what mechanism? American Journal of Clinical Nutrition, 88, 12-13.
- Ghedria K., 2005. Les flavonoïdes: structure, propriétés biologiques, rôle prophylactique et emplois en thérapeuthique. Phytothérapie, 4, 162-169.
- Gilgun-Sherki Y., Melamed E., and Offen D., 2001. Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 40, 959-975.
- Girodon F., Blache D., Monget A.L., Lombart M., Brunet-Lecompte P., Arnaud J., Richard M.J., and Galan P., 1997. Effect of two-year supplementation with low doses of antioxidant vitamins and/or minerals in elderly subjects on levels of nutrients and antioxidant defence parameters. Journal of the American College of Nutrition, 4, 357–365.
- Goli A.H., Barzegar M., and Sahari M.A., 2005. Antioxidant activity and total phenolic compounds of pistachio (Pistachia vera) hull extracts. Food Chemistry, 92, 521–525.

- Goodwin J.L., Uemura E., and Cunnick J.E., 1995. Microglial release of nitric oxide by the synergetic action of β-amyloid and IFN-γ. Brain Research, 692, 207-214.

- Gorman A.M., McGowan A., O'Neill C., and Cotter T., 1996. Oxidative stress and apoptosis in neurodegeneration. Journal of the Neurological Sciences 139, 45-52.
- Gosh D., and Scheepens A., 2009. Vascular action of polyphenols. Molecular Nutrition and Food Research, 53, 322-331.
- Häkkinen S., Heinonen M., Kärenlampi S., Mykkänen H., Ruuskanen J., and Törrönen R., 1999. Screening of selected flavonoids and phenolics acids in 19 berries. Food Research International, 32, 345-353.
- Halliwell B., and Aruoma O.I., 1993. DNA and free radicals. FEBS Letters 335-1, 140-141.
- Halliwell B., 1994. Free radicals and antioxidants: a personal view. Nutrition Reviews, 52: 253-265.
- Halliwell B., 2000. Lipid peroxidation, antioxidants and cardiovascular disease: How should we move forward? Cardiovascular Research 47, 410-418.
- Harborne J.B., and Williams C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry, 55, 481-504.
- Hayoz D., and Silacci P., 2006. Biologie de la cellule endothéliale, apport des modèles expérimentaux. Sang Thrombose Vaisseaux, 18, 318-324.
- He F.J., Nowson C.A., and MacGregor G.A., 2006. Fruit and vegetable consumption and stroke: meta-analysis of cohort studies. Lancet, 367, 320-326.
- Heiberg N., Mage F., and Haffner K., 1992. Chemical composition of 10 black currant (*Ribes nigrum*) cultivars. Acta Agriculturae Scandinavica Section B, 42, 251-254.
- Heinonen I.M., Meyer A.S., and Frankel E.N., 1998. Antioxidant activity of berry phenolics on human low density lipoprotein and liposome oxidation. Journal of Agricultural and Food Chemistry, 46, 4107–4112.
- Hertog M.G.L., Freskens E.J.M., Hollman P.C.H., Katan M.B., and Kromhout D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet, 342, 1007-1011.
- Hertog M.G.L., Kromhout D., Aravanis C., Blackburn H., Buzina F., Fidanza R., Giampaoli S., Jansen A., Menotti A., Nedeljkovic S., Pekkarinen M., Simic B.S., Toshima H., Freskens J.M., Hollman P.S.H., and Katan M.B., 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. Archives of Internal Medicine, 155, 381–386.
- Hertog M.G.L., Sweetnam P.M., Fehily A.M., Elwood P.C., and Kromhout D., 1997. Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly Study. American Journal of Clinical Nutrition, 65, 1489-1494.
- Hirvonen T., Virtamo J., Korhonen P., Albanes D., and Pietnen P., 2000. Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers. Stroke, 31, 2301-2306.
- Hirvonen T., Virtamo J., Korhonen P., Albanes D., and Pietinen P., 2001. Flavonol and flavone intake and the risk of cancer in male smokers (Finland). Cancer Causes and Control, 12, 789-796.

- Ho C.T., Osawa T., Huang M.T., and Rosen R.T., 1994. Food phytochemicals for cancer prevention. II. Teas, spices, and herbs. Austio, TX: American Chemical Society.
- Hollman P.C.H., Hertog M.G.L., and Katan M.B., 1996. Analysis and health effects of flavonoids. Food Chemistry, 57, 43-46.
- Hollman P.C.H., Van Trijp J.M.P., Mengelers M.J.B., De Vries J.H.M., and Katan M.B., 1997. Bioavailability of the dietary antioxidant flavonol quercetin in man. Cancer Letters, 114, 139-140.
- Hollman P.C.H., and Katan M.B., 1999. Dietary flavonoids: Intake, health effects and bioavailability. Food and Chemical Toxicology, 37, 937-942.
- Horie T., Mizuma T., Kasai S., and Azawu S., 1988. Conformational change in plasma albumin due to interaction with isolated rat hepatocytes. American Journal of Physiology, 254, 465-470.
- Howard L.R., Clark J.R., and Brownmiller C., 2003. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. Journal of the Science of Food and Agriculture, 83, 1238- 1247.
- Hsieh T.C., Juan G., Darzynkiewicz Z., and Wu J.M., 1999. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21(WAF1/CIP1), and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G2. Cancer Research, 59, 2596–2601.
- Hu M., 2007. Commentary: Bioavailability of Flavonoids and Polyphenols: Call to Arms. Mol. Pharmaceutics, 4, 803–806.
- Huang D., Ou B., and Prior R. L., 2005. The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, 53, 1841–1856.
- Ignarro L.J., Byrns R., Buga G.M., Wood R.S., and Chaudhuri G., 1987. Pharmacological evidence that endothelium-derived relaxing factors is nitric oxide: use of pyrogallol and superoxide dismutase to study the endothelium-dependent and nitric oxide elicited vascular smooth muscle relaxation. Journal of Pharmacology and Experimental Therapeutics, 244, 1-9.
- Ignarro L.J., Byrns R.E, Sumi D., De Nigris F., and Napoli C., 2006. Pomegranate juice protects nitric oxide against oxidative destruction and enhances the biological actions of nitric oxide. Nitric oxide, 15, 93-102.
- Jochmann N., Lorenz M., Krosigk A., Martus P., Böhm V., Baumann G., Stangl K., and Stangl V., 2008. The efficacy of black tea in ameliorating endothelial function is equivalent to that of green tea. British Journal of Nutrition, 99, 863-868.
- Karim M., McCormick K., and Kappagoda C.T., 2000. Effects of cocoa extracts on endothelium-dependent relaxation. Journal of Nutrition, 130, 2105S-2108S.
- Kim H.P., Son K.H., Chang H.W., and Kang S.S., 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. Journal of Pharmacological Sciences, 96, 229-245.
- Knasmüller S., Nersesyan A., Misik M., Gerner C., Mikulits W., Ehrlich V., Hoelzl C., Szakmary A., and Wagner K.H., 2008. Use of conventional and –omics based methods for health claims of dietary antioxidants: a critical overview. British Journal of Nutrition, 99 (E-suppl. 1), ES3-ES52.

- Knekt P., Järvinen R., Reunanen A., and Maatela J., 1996. Flavonoid intake and coronary mortality in Finland: a cohort study. British Medical Journal, 312, 478-481.
- Knekt P., Järvinen R., Seppänen R., Heliövaara M., Teppo L., Pukkula E., and Aromaa A., 1997. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. American Journal of Epidemiology, 146, 223-230.
- Knekt P., Kumpulainen J., Järvinen R., Rissanen H., Heliövaara M., Reunanen A., Hakulinen T., and Aromaa A., 2002. Flavonoid intake and risk of chronic diseases. American Journal of Clinical Nutrition, 76, 560-568.
- Koechlin-Ramonatxo C., 2006. Oxygen, oxidative stress and anti oxidant supplementation, or an other way for nutrition in respiratory diseases. Nutrition Clinique et métabolisme, 20, 165-177.
- Kohnen S., Franck T., Van Antwerpen P., Zouaoui Boudjeltia K., Mouithys-Mickalad A., Deby C., Moguilevsky N., Deby-Dupont G., Lamy M., and Serteyn D., 2007. Resveratrol inhibits the activity of equine neutrophil myeloperoxidase by a direct interaction with the enzyme. Journal of Agricultural and Food Chemistry, 55, 8080-8087.
- Kolluru G.K., Siamwala J.H., and Chatterjee S., 2010. eNOS phosphorylation in health and disease. Biochimie, doi: 10.1016/j.biochi.2010.03.20.
- Kris-Etherton P.M., Lefevre M., Beecher G.R., Gross M.D., Keen C.L., and Etherton T.D., 2004. Bioactive compounds in nutrition and health-research methodologies for establishing biological function: The antioxidant and anti-inflammatory effects of flavonoids on artherosclerosis. Annual Review of Nutrition, 24, 511-538.
- Landbo A.K., and Meyer A.S., 2001. Enzyme-assisted extraction of antioxidative phenols from black currant juice residue (*Ribes nigrum*). Journal of Agricultural and Food Chemistry, 49, 3169-3177.
- Landolfi R., Mower R.L., and Steiner M., 1984. Modification of platelet function and arachidonic acid metabolism by bioflavonoids. Structure-activity relations. Biochemical Pharmacology, 33:1525-1530.
- Laurin D., Masaki K.H., Foley D.J., White L.R., and Launer L.J., 2004. Midlife dietary intake of antioxidants and risk of late-life incident dementia: The Honolulu-Asia aging study. American Journal of Epidemiology, 159, 959-967.
- Lebranchu Y., 2000. Lipides et dysfonctionnement de l'endothélium. Néphrologie, 21, 373-376.
- Lee K.W., Kim Y.J., Lee H.J., and Lee C.Y., 2003. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. Journal of Agricultural and Food Chemistry, 51, 7292-7295.
- Leikert F.J., Räthel T.R., Wohlfart P., Cheynier V., Vollmar A.M., and Dirsch V.M., 2002. Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. Circulation, 106, 1614-1617.
- Le Marchand L., Murphy S.P., Hankin J.H., Wilkens L.R., and Kolonel L.N., 2000. Intake of flavonoids and lung cancer. Journal of the National Cancer Institute, 92, 154-160.
- Li H., Xia N., Brausch I., Yao Y., and Förstermann U., 2004. Flavonoids from artichoke (*Cynara Scolymus L.*) up-regulated endothelial-type nitric oxide synthase gene expression in human endothelial cells. The Journal of Pharmacology and Experimental Therapeutics, 310, 926-932.

- Lin J., Rexrode K.M., Hu F., Albert C.M., Chae C.U., Rimm E.B., Stampfer M.J., and Manson J.E., 2007. Dietary intake of flavonols and flavones and coronary heart disease in US women. American Journal of Epidemiology, 165 (11), 1305-1313.
- Lopez-Figueroza M.O., Caamano C., Marin R., Guerre B., Alonso R., Morano M.I., Akil H., and Watson S.J., 2001. Characterization of basal nitric oxide production in livings cells. Biochemica et Biophysica Acta, 1540, 253-264.
- Lopez-Revuelta A., Sanchez-Gallego J.I., Hernandez-Hernandez A., Sanchez-Yague J., and Llanillo M., 2006. Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress. Chemico-Biological Interactions, 161, 79-91.
- Lorenz M., Wessler S., Follmann E., Michaelis W., Düsterhöft T., Baumann G., Stangl K., and Stangl V., 2004. A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. The Journal of Biological Chemistry, 276, 6190-6195.
- Manach C., Scalbert A., Morand C., Rémésy C., and Jiménez L., 2004. Polyphenols: food sources and bioavailability. American Journal of Clinical Nutrition, 79, 727-747.
- Mariani E., Polidori M.C., Cherubini A., and Mecocci P., 2005. Oxidative stress in brain aging, neurodegenerative and vascular diseases: An overview. Journal of Chromatography B, 827, 65-75.
- Marin J., and Rodriguez-Martinez M.A., 1997. Role of vascular nitric oxide in physiological and pathological conditions. Pharmacology and Therapeutics, 75, 111-134.
- Marletta M.A., 1993. Nitric oxide synthase structure and mechanism. The Journal of Biological Chemistry, 268, 12231-12234.
- Martin S., and Andriantsitohaina R., 2002. Cellular mechanism of vasculo-protection induced by polyphenols on the endothelium. Annales de Cancérologie et d'Angéiologie, 51, 304-315.
- Mazza G., Kay C.D., Cottrell T., and Holub B.J., 2002. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. Journal of Agriculture and Food Chemistry, 50, 7731-7737.
- McKenna E., Smith J.S., Coll K.E., Mazack E.K., Mayer E.J., Antanavage J., Wiedmann R.T., and Johnson R.G., 1996. Dissociation of phospholamban regulation of cardiac sarcoplasmic reticulum Ca^{2+} ATPase by quercetin. The Journal of Biological Chemistry, 271, 24517-24525.
- Mendes A., Desgranges C., Cheze C., Vercauteren J., and Frelson J.L., 2003. Vasorelaxant effects of grape polyphenols in rat isolated aorta. Possible involvement of a purinergic pathway. Fundamuntal and Clinical Pharmacology, 17, 673-681.
- Mikkonen T.P., Määttä K., Hukkanen A.T., Kokko H.I., Törrönen A.R., Kärenlampi S.O., and Karjalainen R.O., 2001. Flavonol content varies among black currant cultivars. Journal of Agricultural and Food Chemistry, 49, 3274-3277.
- Mink P.J., Scrafford C.G., Barraj L., Harnack L., Hong C.P., Nettleton J.A., and Jacobs Jr. D.R., 2007. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. American Journal of Clinical Nutrition, 85, 895-909.

- Mladenka P., Zatloukalova L., Filipsky T., and Hrdina R., 2010. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. Free Radical Biology and Medicine, article in press, doi:10.1016/j.freeradbiomed.2010.06.010.
- Moon Y.J., Wang X., and Morris M.E., 2006. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. Toxicology In Vitro, 20, 187-210.
- Moyer R.A., Hummer K.E., Finn C.E., Frei B., and Wroldtad R.E., 2002. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium, Rubus,* and *Ribes*. Journal of Agricultural and Food Chemistry, 50, 519-525.
- Murad F., Forstermann U., Nakane M., Schmidt H., Pollock J., Sheng H., Matsumoto T., Warner T., Mitchell J., Tracey R., and Buechler W., 1992. The nitricoxide-cyclic GMP signal transduction pathway in vascular smooth muscle preparations and other tissues. The Japanese Journal of Pharmacology, 58, 150-157.
- Nakamura Y., Matsumoto H., and Todoki K., 2002. Endothelium-dependent vasorelaxation induced by black currant concentrate in rat thoracic aorta. Japanese Journal of Pharmacology, 89, 29-35.
- Ness A.R., and Powles J.W., 1997. Fruit and vegetables, and cardiovascular disease: a review. International Journal of Epidemiology, 26, 1-13.
- Ndiaye M., Chataigneau T., Andriantsitohaina R., Stoclet J.C., and Schini-Kerth V.B., 2003. Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism. Biochemical and Biophysical Research Communications, 310, 371-377.
- Ndiaye M., Chataigneau T., Chataigneau M., and Schini-Kerth V.B., 2004. Red wine polyphenols induced EDHF-mediated relaxations in porcine coronary arteries through the redox-sensitive activation of the PI3-kinase/Akt pathway. British Journal of Pharmacology, 142, 1131-1136.
- Nishida S., and Satoh H., 2004. Comparative vasodilating actions among terpenoids and flavonoids contained in *Ginkgo biloba* extract. Clinica Chimica Acta, 339, 129-133.
- O'Leary K.A., Day A.J., Needs P.W., Mellon F.A., O'Brien N.M., and Williamson G., 2003. Metabolism of quercetin-7-and quercetin-3-glucuronides by an *in vitro* hepatic model: the role of human β-glucuronidase, sulfotransferase, catechol-*O*-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochemical Pharmacology, 65, 479-481.
- Ono Y., Hattori E., Fukaya Y., Imai S., and Ohizumi Y., 2006. Anti-obesity effect of *Nelumbo nucifera* leaves extract in mica and rats. Journal of Ethnopharmacology, 106, 238-244.
- Pantelidis G.E., Vasilakakis M., Manganaris G.A., and Diamantidis G., 2007. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. Food Chemistry, 102, 777–783.
- Pincemail J., Thirion A., Dupuis M., Braquet P., Drieu K., and Deby C., 1987. Ginkgo biloba extract inhibits oxygen species production generated by phorol acetate stimulated human leukocytes. Experientia, 43, 181-184.

- Pincemail J., Deby C., Thirion A., De Bruyn-Dister M., and Goutier R., 1988. Human myeloperoxidase activity is inhibited *in vitro* by quercetin. Comparaison with three radical compounds. Experientia, 44, 450-453.
- Pincemail J., Deby C., Drieu K., Anton R., and Goutier R., 1990. Flavonoids as “antioxidants”. Flavonoids in Biology and Medicine III: Current Issues in Flavonoid Research; N.P. Das; National University of Singapore, 161-179.
- Pincemail J., Meurisse M., Limet R., and Defraigne J.O., 1999. Espèces oxygénées activées, antioxydants et cancers. Medi-Sphere, 97, 29-33.
- Prior R.L., Wu X., and Schaich K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry, 53, 4290- 4302.
- Raffo A., Paoletti F., and Antonelli M., 2004. Changes in sugar, organic acid, flavonol and carotenoid composition during ripening of berries of three seabuckthorn (*Hippophae rhamnoides* L.) cultivars. European Food Research and Technology, 219, 360-368.
- Rice-Evans C.A., Miller N.J., and Paganga G, 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine, 20, 933-956.
- Rimm E.B., Katan M.B., Ascherio A., Stampfer M.J., and Willett W.C., 1996. Relation between intake of flavonoids and risk for coronary heart disease in male health professionals. Annals of Internal Medicine, 125, 384-389.
- Roberfroid M., 2002. Aliments Fonctionnels. Editions TEC & DOC, Collection Sciences & Techniques Agroalimentaires.
- Ross J.A., and Kasum C.M., 2002. Dietary flavonoids: Bioavailability, metabolic effects and safety. Annual Review of Nutrition, 22, 19-34.
- Rossi M., Garavello W., Talamini R., Negri E., Bosetti C., Dal Maso L., Lagiou P., Tavani A., Polesel J., Barzan L., Ramazzotti V., Franceschi S., and La Vecchia C., 2007a. Flavonoids and the risk of oral and pharyngeal cancer: a case-control study from Italy. Cancer Epidemiology, Biomarkers and Prevention, 16, 1621-1625.
- Rossi M., Garavello W., Talamini R., La Vecchia C., Franceschi S., Lagiou P., Zambon P., Dal Maso L., Bosetti C., and Negri E., 2007b. Flavonoids and risk of squamous cell esophageal cancer. International Journal of Cancer, 120, 1560-1564.
- Rusznyak S., and Szent-Gyorgyi A., 1936. Vitamin P: Flavonols as vitamins. Nature, 138, 27.
- Scalbert A., and Williamson G., 2000. Dietary intake and bioavailability of polyphenols. The Journal of Nutrition, 130, 2073S-2085S.
- Scalbert A., Johnson I.T., and Saltmarsh M., 2005a. Polyphenols: antioxidants and beyond. American Journal of Clinical Nutrition, 81 (suppl), 215-217.
- Scalbert A., Manach C., Morand C., and Rémésy C., 2005b. Dietary Polyphenols and the prevention of diseases. Critical Reviews in Food Science and Nutrition, 45, 287-306.
- Scarmeas N., Stern Y., Mayeux R., and Luchsinger J.A., 2006a. Mediterranean diet, Alzheiler disease, and vascular mediation. Archives of Neurology, 63, 1709-1717.

- Scarmeas N., Stern Y., Tang M.X., Mayeux R., and Luchsinger J.A., 2006b. Mediterranean diet and risk for Alzheiler's disease. Annals of Neurology, 59, 912-921.
- Schmitt C.A., and Dirsch V.M., 2009. Modulation of endothelial nitric oxide by plant-derived products. Nitric oxide, 21, 77-91.
- Sennequier N., and Vadon-Le Goff S., 1998. Biosynthèse du monoxyde d'azote (NO) : mécanisme, régulation et contrôle. Médecine/Sciences, 14, 1185-1195.
- Serteyn D., Grulke S., Franck T., Mouithys-Mickalad A., and Deby-Dupont G., 2003. La myéloperoxydase des neutrophiles, une enzyme de défense aux capacités oxydantes. Annales de médecine Vétérinaire, 147, 79-93.
- Sesso H.D., Gaziano J.M., Liu S., and Buring J., 2003. Flavonoid intake and the risk of cardiovascular disease in women. American Journal of Clinical Nutrition, 77, 1400-1408.
- Shahidi F., and Naczk M., 2004. Phenolics in Food and Nutraceuticals. CRC Press: Boca Raton, FL;131-155.
- Shiba Y., Kinoshita T., Chuman H., Taketani Y., Takeda E., Kato Y., Naito M., Kawabata K., Ishisaka A., Terao J., and Kawai Y., 2008. Flavonoids as substrates and inhibitors of myeloperoxidase : molecular actions of aglycone and metabolites. Chemical Research in Toxicology, 21, 1600-1609.
- Shin W.H., Park S.J., and Kim E.J., 2006. Protective effect of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats. Life Science, 79, 130-137.
- Singh M., Arseneault M., Sanderson T., Murthy V., and Ramassamy C., 2008. Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. Journal of Agricultural and Food Chemistry, 56, 4855-4873.
- Skiloba C.F., and Smith M.T., 2000. Potential health impact of excessive flavonoid intake. Free Radical Biology and Medicine, 29, 375-383.
- Skrede G., and Wrolstad R.E., 2002. Flavonoids and other polyphenolics in grapes and other berry fruit. In Functional Foods-Biochemical Processing Aspects; Shi J., Mazza G., Le Maguer M., Eds.; CRC Press: Boca Raton FL; Vol II, 71-130.
- Stevens A., and Lowe J., 1997. Histologie Humaine. De Boeck Edition.
- Stoclet J.C., Chataigneau T., Ndiaye M., Oak M.H., El Bedoui J., Chataigneau M., and Schini-Kerth V.B., 2004. Vacular protection by dietary polyphenols. European Journal of Pharmacology, 500, 299-313.
- Stratil P., Klejdus B., Kuban V., 2006. Determination of total Content of Phenolic Compounds and Their Antioxidant Activity in Vegetables – Evaluation of Spectrophotometric Methods. Journal of Agricultural and Food Chemistry, 54, 607-16.
- Strube M., Dragsted L.O., and Larsen J.C., 1993. Naturally occurring antitumourigens. I. Plant Phenols. Nordiske Seminar-og Arbejdsrapporter, 605. Copenhague, Denmark: Nordic Council of Ministers.
- Su M.S., and Silva J.L., 2006. Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (*Vaccinim ashei*) by-products as affected by fermentation. Food Chemistry, 97,447–451.
- Sun T., and Ho C., 2005. Antioxidant activities of buckwheat extracts. Food Chemistry, 90, 743–749.

- Swiderski A., Muras P., and Koloczeck H., 2004. Flavonoid composition in frost-resistant *Rhododendron* cultivars grown in Poland. Scientica Hortica, 100, 139-151.
- Tabart J., Kevers C., Pincemail J., Defraigne J.O., and Dommes J., 2006. Antioxidant capacity of black currant varies with organ, season, and cultivar. Journal of Agricultural and Food Chemistry, 54, 6271-6276.
- Tabart J., Kevers C., Sipel A., Pincemail J., Defraigne J.O., and Dommes J., 2007. Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and stability during storage. Food Chemistry, 105, 1268-1275.
- Tabart J., Kevers C., Pincemail J., Defraigne J.O., and Dommes J., 2009. Comparative antioxidant capacities of phenolic compounds measured by various tests. Food Chemistry, 113, 1226-1233.
- Tavani A., Spertini L., Bosetti C., Parpinel M., Gnagnarella P., Bravi F., Peterson J., Dwyer J., Lagiou P., Negri E., and La Vecchia C., 2006. Intake of specific flavonoids and risk of acute myocardial infarction in Italy. Public Health Nutrition, 9, 369-374.
- Teutter D., 2006. Significance of flavonoïds in plant resistance: a review. Environmental Chemistry Letters, 4, 147-157.
- Wallerath T., Poleo D., Li H., and Föstermann U., 2003. Red wine increases the expression of human endothelial nitric oxide synthase. Journal of the American College of Cardiology, 41, 471-478.
- Wallerath T., Li H., Gödtel-Ambrust U., Scharz P.M., and Föstermann U., 2005. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide, 12, 97-104.
- Wang H., Cao G., and Prior R.L., 1996. Total antioxidant capacity of fruits. Journal of Agricultural and Food Chemistry, 44, 701-705.
- Wang S.Y., and Lin H., 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry and strawberry varies with cultivar and developmental stage. Journal of Agricultural and Food Chemistry, 48, 140-146.
- Williamson G., Day A.J., Plumb G.W., and Couteau D., 2000. Human metabolic pathways of dietary flavonoids and cinnamates. Biochemical Society Transactions, 28, 16-22.
- Williamson G., and Santos-Buelga C., 2003. Methods in polyphenols analysis.
- Wink D.A., and Mitchell J.B., 1998. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radical Biology and Medicine, 25, 434-536.
- Wolfe K.L., and Liu R.H., 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. Journal of Agricultural and Food Chemistry, 55, 8896-07.
- Wolfe K.L., and Liu R.H., 2008a. Cellular antioxidant activity of common fruits. Journal of Agricultural and Food Chemistry, 56, 8418-26.
- Wolfe K.L., and Liu R.H., 2008b. Structure-activity relationships of flavonoids in the cellular antioxidant activity assay. Journal of Agricultural and Food Chemistry, 56, 8404-8411.
- Xu H.B., and Huang Z.Q., 2007. Icariin enhances endothelial nitric-oxide synthase expression on human endothelial cells in vitro. Vascular Pharmacology, 47, 18-24.

- Yao L.H., Jiang Y.M., Shi J., Tomas-Barberan F.A., Datta N., Singanusong R., and Chen S.S., 2004. Flavonoids in food and their health benefits. Plant Foods for Human Nutrition, 59, 113-122.
- Yochum L., Kushi L.H., Meyer K., and Folsom A.R., 1999. Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. American Journal of Epidemiology, 146, 943-949.
- Yun J.W., 2010. Possible anti-obesity therapeutics from nature - A review. Phytochemistry, 71, 1625-1641.
- Zadernowski R., Naczk M., Nesterowicz J., 2005. Phenolic acid profiles in some small berries. Journal of Agricultural and Food Chemistry, 53, 2118-2124.
- Zago A.S., and Zanesco A., 2006. Nitric oxide, cardiovascular disease and physical exercise. Arquivos Brasileiros de Cardiologia, 87, e227-e233.
- Zenebe W., Pechanova O., and Andriantsitohaina R., 2003. Red wine polyphenols induce vasorelaxation by increased nitric oxide bioactivity. Physiological Research, 52, 425-432.
- Zuo Y., Chen H., and Deng Y., 2002. Simultaneous determination of catechins, caffeine and gallic acids in green, oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta, 57, 307–316.