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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

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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.

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).

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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 17 000*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$^{\bullet}$), 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$^{\bullet}$), 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.

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 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.

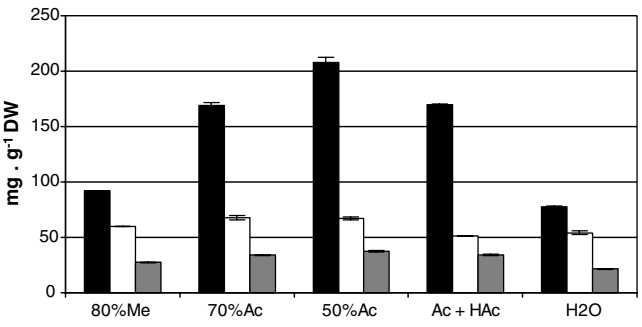

Fig. 1. Total phenolics (■ mg CAE g^{-1} DW) and antioxidant capacities (mg TE g^{-1} DW) measured with DPPH (□) and ABTS (▩) in leaves of black currant extracted with various solvents (80%Me: 80% methanol; Ac: 50 or 70% acetone; Ac + HAc: 70% acetone + 0.5% acetic acid).

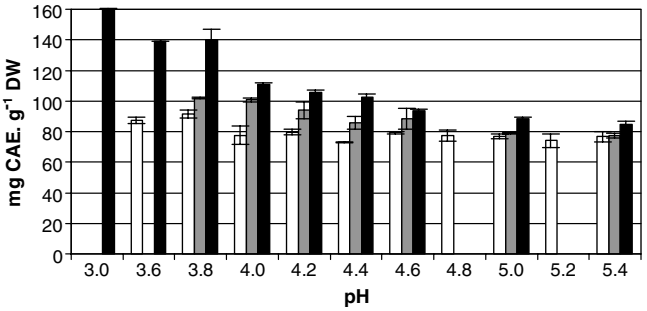

Fig. 2. Total phenolics (mg CAE g^{-1} DW) from leaves of black currant extracted with acetate buffers of various pH (3.0–5.4) and ionic strength: 0.05 M (□), 0.1 M (▩) and 0.2 M (■) of NaOH.

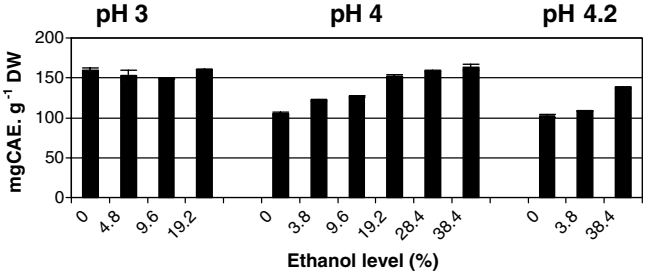

Fig. 3. Total phenolics (mg CAE g^{-1} DW) from leaves of black currant extracted with acetate buffers of pH 3, 4 and 4.2, and supplemented with various levels of ethanol (0–38.4%).

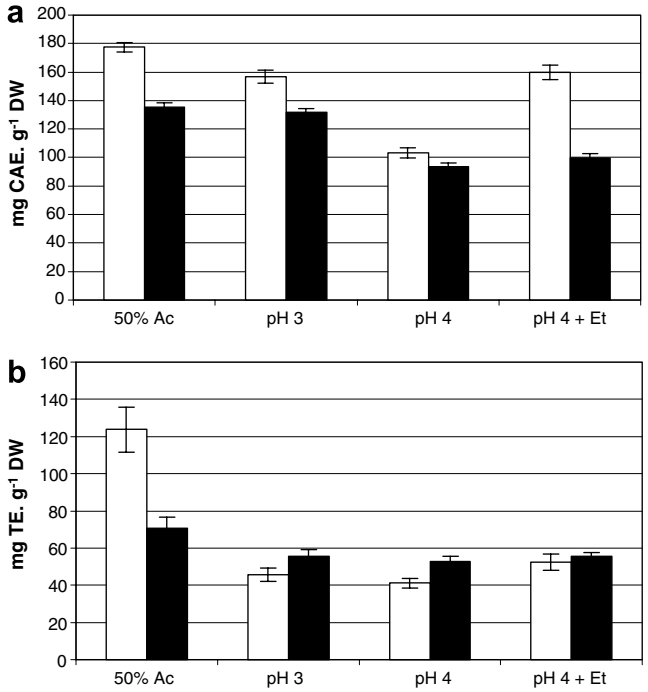

Fig. 4. Total phenolics (a, mg CAE g^{-1} DW) and antioxidant capacities (b, DPPH, mg TE g^{-1} DW) from leaves (□) and buds (■) of black currant extracted with various solvents (50% Ac: 50% acetone, acetate buffers at pH 3 and 4, supplemented or not with 38.4% ethanol (Et)).

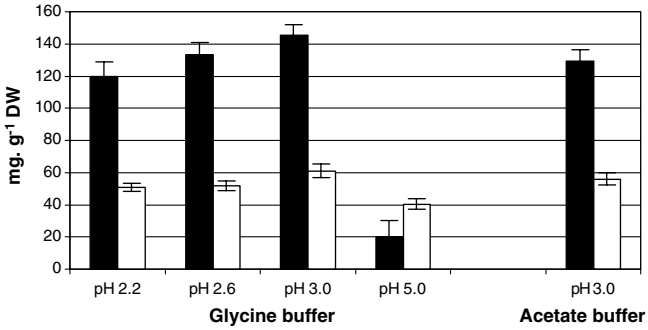

Fig. 5. Total phenolics (■, mg CAE g^{-1} DW) and antioxidant capacities (□, DPPH, mg TE g^{-1} DW) from buds of black currant extracted with various buffers: glycine at pH 2.2 to 5 and acetate, pH 3.

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 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).

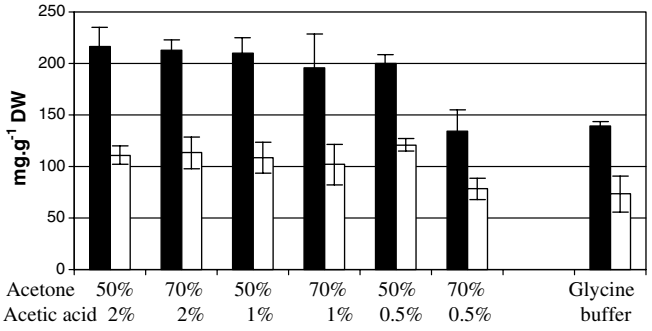

Fig. 6. Total phenolics (■, mg CAE g^{-1} DW) and antioxidant capacities (□, mg TE g^{-1} DW) from buds of black currant extracted with various acetone mixture (50% or 70% acetone with 0.5, 1 or 2% acetic acid) and glycine buffer, pH 3.

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

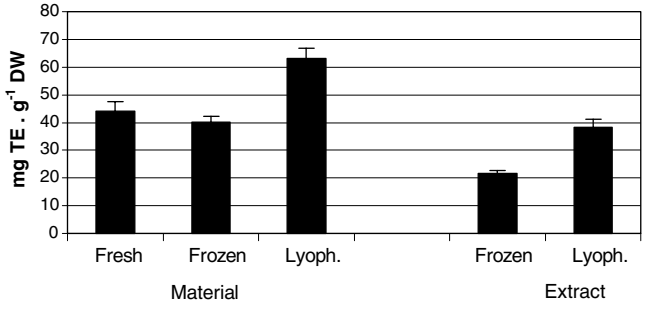

Fig. 7. Antioxidant capacities (DPPH, mg TE g^{-1} DW) of extracts (made with glycine buffer, pH 3) from buds of black currant: fresh material, frozen or lyophilised material stored for one week, extracts from fresh buds frozen or lyophilised for one week.

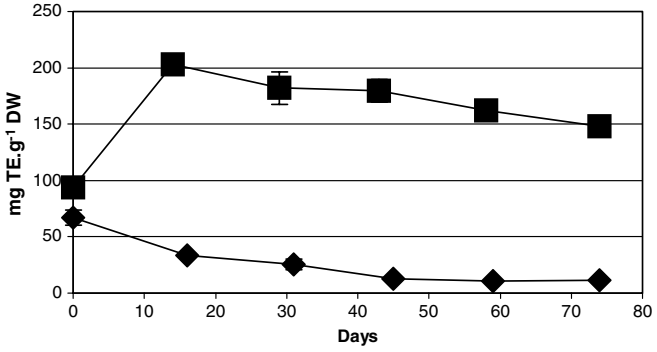

Fig. 8. Evolution of antioxidant capacities (DPPH, mg TE g^{-1} DW) of lyophilised extracts from buds of black currant made with glycine buffer, pH 3 (♦) or acetone mixture (■, 70% acetone, 2% acetic acid) and stored at 4 °C in darkness.

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 $ABTS^{\bullet}$ and $DPPH^{\bullet}$. 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.

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