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Development and evaluation of an enriched natural antioxidant preparation obtained from aqueous spinach (*Spinacia oleracea*) extracts by an adsorption procedure

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Abstract

Spinach (*Spinacia oleracea*) leaves contain antioxidant flavonoids, in particular spinacetin and patuletin. An adsorption procedure for the recovery of these flavonoids from a crude spinach extract was developed and evaluated. Four different resin and charcoal adsorbents, chosen for their high affinity for polyphenolic compounds, were tested in an open batch system. All proved to be very efficient in adsorbing polyphenols. However, while a major portion of the flavonoids could be desorbed from the resin matrices under the experimental conditions imposed by an industrial context, most of these compounds stayed irreversibly bound to the charcoal matrices. The use of a polymeric resin matrix, to purify a spinach crude extract, allowed the removal of almost 80% of the initial extract dry mass, while keeping the flavonoid content nearly constant. Using the nitro blue tetrazolium (NBT) and the DPPH tests to evaluate the antioxidant character of these preparations, it was determined that the spinach flavonoids, as well as the crude aqueous or resin-purified extracts, exhibit high antioxidant activities. Moreover, the antioxidant efficiency of the spinach polyphenol extracts was significantly increased by the elimination of a major part of non-phenolic components.

Keywords: *Spinacia oleracea*; Flavonoids; Patuletin; Spinacetin; Charcoal; Polymeric resins; Antioxidant activity; NBT-test; DPPH-test; HPLC

1. Introduction

In biological systems, an antioxidant is any substance that, when present at low concentration compared to those of oxidizable substrates (lipids, proteins, DNA or carbohydrates) significantly delays or prevents oxidation of these substrates (Frankel & Meyer, 2000).

Antioxidants are widely used to protect oxidizable goods such as cosmetics, pharmaceuticals, processed food or plastics from damage caused by reactive oxygen species. They play a major role in the food industry, to minimize rancidity, to delay the emergence of potentially toxic oxidative products, to protect and to stabilize colours, aroma, and nutritional quality and to increase self life of food products (Brand-Williams, Cuvelier, & Berset, 1995; Fukumoto & Mazza, 2000; Moure et al., 2001; Nair, 1999).

Some synthetic antioxidant additives, such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), are potentially dangerous for human health. Hence there is a growing interest in the use of alternative plant-based natural antioxidants (Larson, 1997; Moure et al., 2001).

Natural antioxidants constitute a broad range of compounds, including phenolic or nitrogen species and carotenoids (Bergman, Varshavsky, Gottlieb, & Grossman, 2001). They occur in higher plants where they may

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function as reducing agents, free radical scavengers, quenchers of singlet oxygen or complexants of pro-oxidant transition metals (Pratt, 1992). They suppress the levels of reactive oxygen intermediates and thus play an important role in the defence mechanisms of plants (Lomnitski et al., 2000; Nair, 1999).

Spinach is an important dietary vegetable. Cultivated globally, it is an important raw-material in the food processing industry (Aritomi & Kawasaki, 1984; Gil, Ferreres, & Tomas-Barberan, 1999).

The biological activities of spinach polyphenols have been reported (Edenharder, Keller, Platt, & Unger, 2001; Gil et al., 1999; Lomnitski et al., 2000). Results indicate significant antioxidant activity, principally involving the spinach flavonoids, as these constitute the major water-soluble polyphenols found in this vegetable (Herrmann, 1995).

Given the possibility of increasing the commercial value of spinach wastes, methods for obtaining highly enriched antioxidant polyphenol extracts were investigated, taking into account ecological restraints as well as economic considerations imposed by the industrial context.

A preliminary goal in this study was to optimize the conditions for extraction of aqueous polyphenols from fresh spinach leaves. The extract obtained exhibited significant antioxidant activity, but the yield of polyphenolic components in the dry matter was quite low. Moreover, many other compounds, such as proteins, sugars or metals, remained in the extract. These do not contribute to the antioxidant activity of the preparation and may even act as prooxidants.

In order to eliminate these compounds and to recover the antioxidant polyphenols, an adsorption procedure, easy to apply under industrial conditions, was developed and is described here.

Polyphenols can usually be purified by adsorption–desorption processes using various solid matrices. Two types of such matrices commonly used in the food industry are: charcoals and polymeric Amberlite (polystyrene or acrylic) resins (Nair, 1999; Pyong-Su, Hon-Yong, Hang-Won, & Hang-Rae, 1993; Weinand & Dedardel, 1994). Therefore, two representatives of each type were employed in an open batch procedure to evaluate their efficiencies in recovery of polyphenols from spinach extract. The charcoals were selected according to their particle size, while the polymeric resins chosen – a crosslinked polystyrene (XAD 16HP) and an aliphatic acrylic resin (XAD 7HP)-belong to the two resin classes most likely to adsorb polyphenolic compounds (Nair, 1999; Pyong-Su et al., 1993; Weinand & Dedardel, 1994).

Ecological and dietary restraints must be considered for the entire production process. Hence, ethanol – an abundant green solvent – approved for food use – was the only organic solvent used in the process.

At every step of the open batch procedure, the efficacy of the purification was assessed by the determination of flavonoid content and of antioxidant activity.

2. Materials and methods

2.1. Chemicals

Fresh spinach leaves from local commercial production were used. All solvents were HPLC grade and were purchased from Carlo Erba Reagenti (methanol, acetonitrile, ethyl acetate) or from *S.d.S. France* (ethanol, phosphoric acid). All the other chemicals were obtained from Sigma. The reference compounds were prepared by hydrolysis of flavonol glycosides provided by Professors Aritomi (Kumamoto University, Japan) and Tomas-Barberan (CEBAS Murcia, Spain). For the quantitative analysis, a larger quantity of patuletin and spinacetin was prepared from spinach leaves. Their chemical structures were verified by ^{1}H-NMR and ^{13}C-NMR analyses.

2.2. Characteristics of the adsorbent matrices used for flavonoid purification

The characteristics of the matrices evaluated are as follows:

- Charcoals:
  - NORIT ROX 0.8 (NORIT, France), extruded activated carbon, particle size >600 μm
  - Acticarbone S (CECA S.A., France), activated carbon in fine powder form; particle size <400μm
- Resins:
  - AMBERLITE XAD 16HP (Rohm & Haas, France), macroporous polystyrenic crosslinked polymer used as white beads; pore size: 200–320 Å
  - AMBERLITE XAD 7HP (Rohm & Haas, France), macroreticular aliphatic acrylic crosslinked polymer used as white translucent beads; pore size: 300–450 Å.

2.3. Preparation of the aqueous spinach extract

About 2 kg of ground fresh spinach leaves were homogenized for 1 h in 2 l of acidified water (pH = 2; phosphoric acid), previously heated up to 70 °C in a laboratory reactor equipped with an outer heating system. The extract was then separated from its solid matter by centrifugation and filtration and stored at 4 °C. The adsorption experiments were carried out within 24 h. The average pH of this aqueous preparation was 4.5. A sample of this crude extract was used as the reference for analysis of the different purification fractions (see below).

2.4. Procedure for spinach extract purification

The adsorption experiments were carried out in duplicate at room temperature in an open batch-system (Fig. 1). The adsorbent/spinach extract ratio was 1/3. After adsorption, the matrices were washed with water. The solutions obtained from the adsorption and washing steps were mixed to constitute fraction 1. A desorption step was then performed by bathing the matrices in 75% aqueous ethanol. The eluent constituted fraction 2. A last washing of the matrices with pure ethanol gave fraction 3. In each fraction, the flavonoid content and the antioxidant activity were assayed.

2.5. Flavonoids HPLC analysis

2.5.1. General

To determine the flavonoid content of the various samples, spinach flavonoid aglycones were analyzed by HPLC. These aglycones were obtained by acid hydrolysis of the conjugated forms present in the samples. The hydrolysis conditions were optimized for complete and unambiguous recovery of the active flavonol-3-O-glycosides.

2.5.2. Sample preparation

The extract or the purified fractions were subjected to acid hydrolysis (1 M HCl) at 100 °C during 60 min, in the presence of ascorbic acid (1 g/l) to prevent oxidation of the flavonoids. The aglycones were then isolated by liquid/liquid extraction with ethyl acetate. Next, the organic fraction containing the aglycones was reduced to dryness under vacuum, and the residue was dissolved in methanol for analysis.

2.5.3. HPLC procedure

20 μl of the hydrolyzed sample were analyzed on a Nucleosyl column (RP C18 AB; 250 × 4; 5 μm particle size; Macherey & Nagel) under gradient conditions. The mobile phases were: (A) distilled water containing triethylamine (1 ml/l), phosphoric acid 85% (1 ml/l) and KH_2PO_4 (1 g/l); (B) HPLC grade acetonitrile. The HPLC programme began with 20% B for 7 min. Hereafter, the gradient reached 25% B at 25 min and 55% B at 40 min. Finally, the gradient was converted back to the initial 20% B at 50 min.

The flow rate was 1 ml/min and optical absorption at 370 nm was monitored using a diode-array detector.

2.6. Evaluation of antioxidant activity

2.6.1. Nitro blue tetrazolium (NBT) assay

The superoxide radical anion ($O_2^{\cdot -}$) scavenging properties of the samples were determined using the in vitro NBT assay; a xanthine–xanthine oxidase system (Giannopolitis & Reis, 1977; Nishibori & Namiki, 1998). The activities are expressed in terms of IC_{50}. The IC_{50} value is the antioxidant concentration required to inhibit 50% of the formazan formation.

2.6.2. DPPH radical method

The original method established by Brand-Williams et al. (1995) was adapted as follows: 100 μl of an antioxidant solution was mixed with 1.4 ml of a 60 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH·) solution in methanol and the absorption were read after 5 h at room temperature. The IC_{50} value corresponds to the antioxidant concentration which decreases the absorption at 515 nm to 50% of the control value (methanol only).

3. Results and discussion

3.1. Means of evaluation performed for the adsorption procedure

3.1.1. Evaluation of antioxidant activity

Several methods have been developed to evaluate the free radical-scavenging ability of antioxidants, such as flavonoids. These are based on a large variety of radical-generating systems and various methods have been used for oxidation end-point determination (Frankel & Meyer, 2000; Koleva, van Beek, Linssen, de Groot, & Evstatieva, 2002). The results of different evaluation tests depend on the specificity and test parameters of the method employed to analyze the progress of oxidation, including the degree of oxidation chosen as end-point (Frankel & Meyer, 2000). Generally, it is preferable to

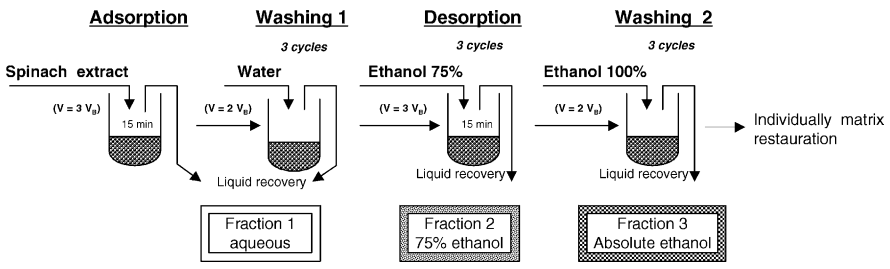

Fig. 1. Scheme of the successive steps in the procedure used to purify the spinach crude aqueous extract.

confirm the general trend of measured activities by one or more additional methods operating in the same antioxidant context (i.e. an environment containing hydrophilic or lipophilic radicals).

The antioxidant properties of the spinach extract were determined by two in vitro assays: the NBT and the DPPH assays. These reflect the ability of the compounds present to scavenge hydrophilic free radicals. With the NBT test one may quantify the abilities of molecules to scavenge superoxide radical anion ($O_2^{\cdot-}$) – an oxygen-centred free radical generated by a xanthine–xanthine oxidase system (Giannopolitis & Reis, 1977; Nishibori & Namiki, 1998). The superoxide radical anion is thought to be widely produced in biological media. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of highly reactive species (i.e. hydroxyl radicals) and the study of the scavenging of this radical is thus particularly important (Frankel & Meyer, 2000; Pietta, 2000).

The second test is representative of the methods employing model radicals to assess free radical scavenging activity. In this assay, the relatively stable DPPH· radical is reduced in an alcoholic solution by the present hydrogen-donating antioxidant (Brand-Williams et al., 1995; Frankel & Meyer, 2000; Koleva et al., 2002).

First, the activities of the standards (glycosides and glucuronides of flavonols), were evaluated, using these two test methods. Rutin, a well known antioxidant compound with a structure similar to those of the spinach flavonoids, was chosen as reference (Afanas'ev, Ostrachovitch, Abramova, & Korkina, 1995; La Casa, Villegas, Alarcon de la Lastra, Motilva, & Martin Calero, 2000; Saija et al., 1995). IC_{50} values are shown in Table 1.

The two methods employed did not always give the same results, due to the different reaction conditions. Generally, the glycosides – especially those derived from patuletin and spinacetin – showed an antioxidant activity comparable to that of rutin. The spinacetin glycoside exhibited less activity in the DPPH than in the NBT method. It appears that the supplementary methoxylation – and thus the presence of one less hydroxyl group in the spinacetin structure than in that of patuletin leads to an important decrease of activity, as reflected by the alcoholic test method (Fig. 2). This may be due to the fact that the methods are sensitive to different types of radicals, as indicated above. The results obtained agree with those reported by Gil et al. (1999) for the DPPH assay of different spinach flavonoid glycosides. Further, our results with these two test methods confirm those authors' findings that glucuronidation in the 4′-position is a structural factor very unfavourable to the antioxidant activity of the spinach flavonoids. Since the aim of this investigation was the development of a potent antioxidant preparation, subsequent efforts at purification were focussed upon the recovery of flavonol-3-O-glycosides.

3.1.2. HPLC determination of the spinach flavonoids

Spinach phytochemistry studies are reported for the isolation and identification of nearly 20 flavonoid compounds (Aritomi & Kawasaki, 1984; Aritomi, Komori, & Kawasaki, 1986; Bergman et al., 2001; Edenharder et al., 2001; Fang, Mabry, & Sanderson, 1989; Ferreres, Castañer, & Tomas-Barberan, 1997; Gil et al., 1999). Since the available standards did not allow the identification of all flavonol glycosides present in the extracts, it was decided to hydrolyse the samples in order to assay the major spinach flavonoid aglycones.

The HPLC analysis revealed that patuletin and spinacetin, exhibiting essentially identical UV spectra, were the main aglycones present in the spinach extracts (Fig. 2). Consequently, these two aglycones, previously purified in our laboratory, were used as references to evaluate the effectiveness of the purification procedure.

3.2. Effectiveness of the purification procedure

The results of the adsorption step are shown in Fig. 3. For all the absorbents tested, less than 5% of the crude extract flavonoids remained in fraction 1. This behav-

Table 1
Antioxidant activities determined by the NBT and the DPPH assays for several spinach-derived glycosides and for the rutin standard

Compound	NBT-IC_{50} (mM)	DPPH-IC_{50} (mM)
Patuletin-3-β-D-glycopyranosyl-(1 → 6)-β-D-glucopyranoside	0.69 ± 0.02	0.046 ± 0.006
Patuletin-3-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside	0.58 ± 0.01	0.083 ± 0.011
Patuletin-3-β-D-glycopyranosyl-(1 → 6)-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside	1.44 ± 0.01	0.067 ± 0.015
Spinacetin-3-β-D-glycopyranosyl-(1 → 6)-β-D-glucopyranoside	0.77 ± 0.04	1.41 ± 0.080
5,4′-dihydroxy-3,3′-dimethoxy-6:7-methylenedioxyflavone 4′-glucuronide	NA[a]	NA[a]
5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4′-glucuronide	NA[a]	NA[a]
5,7,4′-trihydroxy-3,6,3′-trimethoxyflavone 4′-glucuronide	1.96 ± 0.14	NA[a]
5,7,3′,4′-tetrahydroxy-3,6-dimethoxyflavone 4′-glucuronide	2.43 ± 0.17	2.67 ± 0.48
Rutin	0.63 ± 0.01	0.080 ± 0.005

[a] No activity at a maximal concentration of 1.5 g/l.

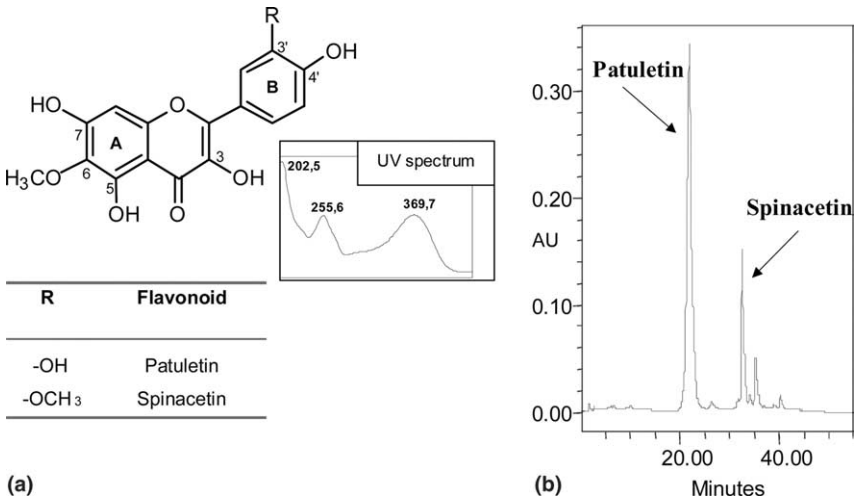

Fig. 2. (a) Chemical structure and UV-absorption spectrum of the spinach flavonoid aglycones, patuletin and spinacetin. (b) HPLC-chromatogram of the spinach aqueous crude extract after acid hydrolysis ($\lambda = 370$ nm).

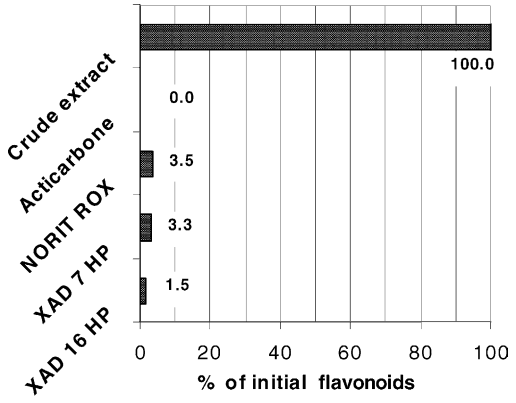

Fig. 3. Percentage of initial flavonoids detected in the aqueous fractions after adsorption on the different matrices studied compared to the crude extract arbitrarily set at 100%.

iour demonstrates that the four matrices studied efficiently adsorbed the molecules of interest.

By contrast, large differences between the charcoals and the polymeric resins were observed for the desorption step (Table 2).

Most of the flavonoids could not be released from the charcoals (99% for NORIT ROX; 97% for Acticarbone) by 75% ethanol. Even a pure ethanol rinsing did not improve their desorption, indicating that the flavonoids remained bound to the charcoals. Conversely, the major parts of the adsorbed patuletin and spinacetin 3-O-glycosides were released from the resins with application of 75% ethanol. Indeed, HPLC analyses revealed that about 90% of the flavonoids were desorbed from the XAD 7HP and even more than 95% from the XAD 16HP (Table 2). It is noteworthy that a rinsing with 100% ethanol was ineffective, as no flavonoids were detected in fraction 3.

By contrast, the use of a polystyrenic resin (XAD 16 HP) or an acrylic one (XAD 7 HP) appeared very effective for the recovery of antioxidant spinach flavonoids from an aqueous crude extract.

At the end of the process, the initial amount of dry matter introduced into the resin batch systems (3.1 g) was found to be decreased by more than 70% (0.9 g) in the case of XAD 16HP and by almost 80% (0.7 g) for the XAD 7HP. Therefore, these steps facilitated the purification of the crude extract while maintaining a similar flavonoid content but in more concentrated form. It was then necessary to verify that the antioxidant activity had been preserved in the extracts after purification.

3.3. Antioxidant activity

The antioxidant activity of the different fractions was compared to that of the crude spinach extract. Only the

Table 2
Evaluation of the flavonoid content and of the dry matter in the crude extract as well as in the different desorption fractions

	Flavonoid content (mg/fraction); (patuletin + spinacetin)	Dry matter (g/fraction)
Crude extract	68.8 ± 4	3.1
XAD 16HP 75% ethanol	66.8 ± 6	0.9
XAD 7HP 75% ethanol	62.9 ± 13	0.7
NORIT ROX 75% ethanol	2.02 ± 0.04	<0.005
Acticarbone 75% ethanol	3.36 ± 0.06	<0.005

Table 3
Evaluation, by two different test methods, of the antioxidant activities in the crude extract as well as in the two resin desorption fractions

	NBT-IC_{50} (g dry extract/l)	DPPH-IC_{50} (g dry extract/l)
Crude extract	7.22 ± 0.15	4.80 ± 0.32
XAD 16HP 75% ethanol	4.42 ± 0.79	2.18 ± 0.04
XAD 7HP 75% ethanol	3.60 ± 0.009	1.65 ± 0.11

two fractions from resin desorption showed a significant activity. In all other fractions (fractions 1 and 3 as well as the charcoal desorption fractions) no activity could be detected. These results confirm that the antioxidant activity of the spinach extract is mainly due to its flavonoid content.

As shown in Table 3, the relative antioxidant activity of the resin-purified fractions was enhanced significantly. In these fractions, the quantity of dry matter necessary to reach the IC_{50} value in the two test methods could be reduced to about 50%. The XAD 7HP resin gave better results than the XAD 16HP resin, as reflected in the loss of dry matter using the two acrylic resins (almost 80% *vs* 70% in the case of XAD 16HP).

In conclusion, polymeric resin matrices were used to isolate an enriched polyphenol fraction from a crude spinach extract. By comparison with the crude extract, the antioxidant efficiency of this fraction was significantly increased, essentially by concentrating the phenolic compounds present. Both resins gave acceptable results for obtaining an enriched antioxidant preparation from aqueous spinach extracts. However, the choice of the resin to be used in an industrial process would be also guided by technical (resin preparation and regeneration) and/or economic factors.

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