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Chemical assessment and antioxidant capacity of pepper (*Capsicum annuum* L.) seeds

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ABSTRACT

Capsicum annuum L. is reported to be the most widely cultivated species. Recently, waste of vegetable processing, like seeds, has been the subject of many studies as an attempt to find new, alternative and cheap resources of bioactive compounds with application in several industries. Despite their chemical, biological and ecological importance, *C. annuum* seeds are still poorly studied. To improve the knowledge on the metabolic profile of this matrix, a targeted metabolite analysis was performed in "sweet Italian" and "Reus long pairal" pepper seeds. Sterols, triterpenes, organic acids, fatty acids and volatile compounds were determined by different chromatographic methods. The antioxidant activity was assessed against DPPH·, superoxide and nitric oxide radicals. A concentration-dependent activity was noticed against all radicals. Acetylcholinesterase inhibitory capacity was also evaluated, but no effect was found. Data provide evidence of great similarities between "sweet Italian" and "Reus long pairal" pepper seeds. The present study indicates that *C. annuum* seeds are a potential source of valuable bioactive compounds that could be used in food industry.

1. Introduction

Vegetables are present in almost every diet throughout the world, because they are good sources of starch, dietary fibre, protein, lipids, and minerals. In addition to their nutritive value, they contain phytochemicals with antioxidant properties and are beneficial to human health (O'Sullivan et al., 2010).

Pepper (*Capsicum annuum* L.) is widely cultivated in Asia, Africa, and Mediterranean countries. *C. annuum* fruits have numerous uses in culinary preparations that make this one of the most important vegetables. There are several varieties, characterised by different sizes and shapes. Their maturation process is distinct and for human consumption their fruits are collected in different stages of maturation. The green fruits (more immature) and the red ones are the most used in culinary preparations. Depending on the flavouring intensity and texture, the culinary application changes from vegetable (bell pepper) to spice (chili pepper) or colourant (paprika). The colour of sweet bell peppers is the major factor associated with consumer purchasing decisions, green and red fruits being the most consumed.

Fresh peppers have been recognised as an excellent source of vitamins C and E, provitamin A, carotenoids and phenolic compounds, metabolites with well-known antioxidant activity (Materska and Perucka, 2005; Sun et al., 2007). These compounds have positive effects on human health, such as potential action against certain cancers, prevention of gastric ulcer, stimulation of the immune system, prevention of cardiovascular diseases and protection against age-related macular degeneration and cataracts (Materska and Perucka, 2005; Sun et al., 2007). Peppers have been shown to prevent the oxidation of cholesterol and docosahexaenoic acid (Sun et al., 2007).

Recently, attention has been focused on the use of by-products and wastes of food processing, as well as on under-utilised agricultural products. The problems related to industrial waste are becoming harder to solve, and more effort is needed to develop the nutritional and industrial potential of these non-used or under-utilised raw materials. Only a small part of the plant material is used directly for human consumption (El-Adawy and Taha, 2001), most of it has to be processed.

Seeds from several species are usually considered as an industrial by-product. Nevertheless, this vegetal material often constitutes a promising source of bioactive compounds that can be used for its nutritional properties and biological potential (Shukla et al., 2009; Pereira et al., 2009b; Okoko, 2009; Taveira et al., 2010; Marathe et al., 2011; Sasipriya and Siddhuraju, 2012). One alternative route could be its use in dietary supplements and in food fortification, since it is generally assumed that consumers prefer natural functional ingredients (Taveira et al., 2010). Other possibilities comprise the use in food preservation due to the antimicrobial properties (Taveira et al., 2010) and the exploitation

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by the pharmaceutical industry because of the biological properties (Pereira et al., 2009a,b; Suwannaphet et al., 2010).

One of the agricultural by-products or wastes of pepper processing industry is the seed. *C. annuum* seeds are separated from the pods and discarded before eating or after processing the flesh, being abundant, cheap and readily available residues. Nevertheless, there is a lack of information about the potential use of this material and no information exists with respect to its safety and toxicological effects. However, the seeds and central core of *C. annuum* may contain some capsaicin, which, when eaten, causes severe irritation and hot sensation to mouth, tongue and throat. The consumption in great amounts of this compound over extended period of time can cause chronic gastritis, kidney damage, liver damage and neurotoxic effects (Johnson, 2007).

Studies about the chemical composition and biological properties of *C. annuum* seeds are scarce. Existing reports concern to sterols (Itoh et al., 1977; Matthäus and Özcan, 2009; Piironen et al., 2003; Whitaker and Lusby, 1989), fatty acids (Marion and Dempsey, 1964; Matthäus and Özcan, 2009), volatiles (Pino et al., 2003) and antioxidant activity (Sim and Sil, 2008).

For this study we have selected the seeds of two varieties for which the fruits are traditionally collected and commercialised in distinct maturation stages: peppers from "Reus long pairal" variety are commercially available at a more mature stage (red), while peppers from "sweet Italian" variety are commercialised more immature (green). To our knowledge, this is the first study about the metabolic profile of *C. annuum* seeds from "Reus long pairal" and "sweet Italian" varieties and to evaluate some of their bioactivity. With this work we aimed to contribute to the knowledge of *C. annuum* seeds for possible application in several industries. Following these purposes, sterols, triterpenes, organic acids, fatty acids and volatile compounds profiles were established by distinct chromatographic procedures. In addition, the antioxidant and acetylcholinesterase inhibitory capacities were checked by microassays.

2. Materials and methods

2.1. Standards and reagents

All chemicals used were of analytical grade. The standard compounds were purchased from various suppliers: citric, ascorbic, oxalic, malic, aconitic and fumaric acids, hexanal, octanal, 6-methyl-5-hepten-2-one, 2-isobutyl-3-methoxypyrazine, benzaldehyde, benzophenone, β-sitosterol, campesterol, betulin and stigmasterol were from Sigma–Aldrich (St. Louis, MO, USA), fatty acid methyl esters kit were purchased from Supelco (Bellafonte, PA, USA) and methyl jasmonate (internal standard), (*E*)-2-octenal, 1-octen-3-ol, myrtenal and heptanal were from SAFC (St. Louis, USA). Menthol was from Fluka (Buchs, Switzerland). 1,1-Diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitrotetrazolium blue chloride (NBT), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), sulphanilamide, acetylcholinesterase (AChE) from electric eel (type VI-s, lyophilized powder), acetylthiocholine iodide (ATCI) and sodium nitroprusside dehydrate (SNP), toluene, methanol and acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO, USA). *N*-(1-Naphthyl)ethylenediamine dihydrochloride, ethanol, potassium dihydrogen phosphate and sulphuric acid were obtained from Merck (Darmstadt, Germany). Sodium sulphate anhydrous and isooctane were purchased from Panreac Química SA (Barcelona, Spain). Potassium hydroxide was from Pronalab (Lisboa, Portugal) and boron trifluoride (BF_3) 10% methanol solution from Supelco (Bellafonte, PA, USA). Water was deionised using a Milli-Q water purification system (Millipore, Bedford, MA).

2.2. Seed samples

Dried seeds of "Reus long pairal" and "sweet Italian" *C. annuum* varieties were purchased from the market (Semillas Fitó, Spain) and used without any pre-treatment. The seeds were ground into powder (mean particle size lower than 910 μm).

2.3. Sterols and triterpenes

2.3.1. Alkaline hydrolysis

Alkaline hydrolysis was performed to obtain the free compounds. The conditions used for saponification and extraction were adapted from those proposed by Lopes et al. (2011), with some modifications. Briefly, 0.5 g of seeds was saponified by refluxing with 20 mL of 1 M ethanolic KOH for 1 h, at constant temperature (80 °C). The mixture was then centrifuged at 4000 rpm during 5 min, and the supernatant was decanted to a separation funnel to extract the unsaponifiable fraction with *n*-hexane (3 × 10 mL). The organic phase was evaporated to dryness in a rotary evaporator (Büchi R-114), and the residue was dissolved in 1 mL of methanol and then filtered through a 0.45 μm polytetrafluoroethylene membrane (PTFE) (Millipore, Bedford, MA). This procedure was performed in triplicate.

2.3.2. HPLC-DAD analysis

Twenty microlitres of each hydrolysed extract was analysed on an analytical HPLC unit (Gilson), using a reverse phase Hypersil ODS (4.0 × 200 mm, 5 μm particle size; Thermo Scientific, Waltham, MA) column, according to Lopes et al. (2011). The mobile phase was methanol:acetonitrile (30:70). The flow rate was maintained at 0.8 mL/min. Detection was achieved using a diode array detector (Gilson). The compounds in each extract were identified by comparing their retention times and UV spectra with those of authentic standards. Quantification was achieved by measuring the absorbance recorded at 205 nm relative to external standards. This procedure was performed in triplicate.

2.4. Organic acids

2.4.1. Extraction

Organic acids extraction was performed according to a described procedure (Silva et al., 2002). Organic acids were extracted from 1.0 g of powdered seeds with 50 mL of H_2SO_4 0.01 N for 30 min under stirring (300 rpm). The obtained extracts were then filtered, evaporated to dryness under reduced pressure and redissolved in H_2SO_4 0.01 N (1 mL), followed by filtration and analysis by HPLC-UV (20 μL).

2.4.2. HPLC-UV analysis

The separation and quantification of organic acids was carried out in a system consisting of an analytic HPLC-UV unit (Gilson Inc., Middleton, WI) with an ion exclusion column (Nucleogel Ion 300 OA, 300 × 7.7 mm; Macherey-Nagel, Düren, Germany). Elution (70 min) was performed in isocratic mode with H_2SO_4 (0.01 N), at a flow rate of 0.2 mL/min. Detection was achieved with a UV detector set at 214 nm. Organic acids quantification was accomplished by measuring the absorbance recorded in the chromatograms relative to external standards. This procedure was performed in triplicate.

2.5. Fatty acids

2.5.1. Derivatisation

The procedure by Ribeiro et al. (2009) was followed. Briefly, esterified fatty acids in 0.01 g of powdered seeds were hydrolysed with KOH methanolic solution (11 g/L), at 90 °C, for 10 min. The free fatty acids originally present and those resulting from the alkaline hydrolysis were derivatised to their methyl ester forms with BF_3 methanolic solution (10%), at 90 °C, for 10 min. The methyl esters derivatives were extracted with isooctane and anhydrous sodium sulphate was added to assure the total absence of water. The resulting extract was evaporated to dryness under a stream of nitrogen and redissolved in isooctane. The assays were performed in triplicate.

2.5.2. GC-IT/MS analysis

The GC-IT/MS analysis was performed according to Ribeiro et al. (2009), with some modifications, in a Varian CP-3800 gas chromatograph (USA) coupled to a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. A VF-5 ms 30 m × 0.25 mm × 0.25 μm (FactorFour) column from VARIAN was used in the analysis. The injector port was heated to 250 °C, and injections were performed in split mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The oven temperature was set at 40 °C for 1 min, then increasing 5 °C/min to 250 °C, 3 °C/min to 300 °C, and held for 15 min. Ionisation was maintained off during the first 4 min, to avoid solvent overloading. The ion trap detector was set as follows: the transfer line, manifold and trap temperatures were 280, 50, and 180 °C, respectively. The mass ranged from *m*/*z* 50 to 600, with a scan rate of 6 scan/s. The emission current was 50 μA and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionisation time was 25,000 μs, with an ionisation storage level of 35 *m*/*z*. The injection volume for liquid extracts was 1 μL and the analysis was performed in Full Scan mode. The components were identified according to their retention indices relative to C8-C20 *n*-alkanes and mass spectra, which were compared with those of the NIST 05MS Library Database (Match and R. Match >80%), pure standards analysed under the same conditions and the NIST Chemistry Web-Book. The amounts of fatty acid methyl esters (FAMEs) present in the extract were calculated from the calibration curve of the respective FAME standard. The FAME values were then converted to the respective fatty acid contents.