Food Chemistry 169 (2015) 169–179

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Fast simultaneous determination of prominent polyphenols in vegetables and fruits by reversed phase liquid chromatography using a fused-core column

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ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form 16 July 2014
Accepted 30 July 2014
Available online 7 August 2014

Keywords:
Functional quality
HPLC-DAD
Food
Ultrasound-assisted extraction
Response surface methodology

ABSTRACT

A reversed-phase high-performance liquid chromatography method with photodiode array detection has been developed enabling the joint determination of 17 prominent flavonoids and phenolic acids in vegetables and fruits. A multi-segmented gradient program using a fused-core column for the separation of several phenolic classes (phenolic acids and flavonoids) has been optimised. The influence of extraction conditions (sample freeze-drying, ultrasound extraction, solvent composition and extraction time) has been also optimised using response surface methodology with tomato samples as a model. Complete recoveries (76–108%) were obtained for the phenolic compounds present in tomato. The developed method provided satisfactory repeatability in terms of peak area (RSD < 2.9%) and retention time (RSD < 0.2%) both for standards and real samples. Detection limits ranged between 3 and 44 μg kg^{-1} for the detected polyphenols. This method is recommended for routine analysis of large number of samples typical of production quality systems or plant breeding programs.

1. Introduction

Regular consumption of fruit and vegetables has been associated with reduced risk of certain types of cancer, cardiovascular diseases and other functional declines associated with ageing and modern lifestyle. It seems that phenolic compounds may play an important role in the antioxidant activity found in these products (Liu, 2003). Several epidemiological studies have shown a direct relationship between the intake of fruits, vegetables and their products, which are rich in polyphenols, and a protective effect against these diseases (Arts & Hollman, 2005; Petti & Scully, 2009).

Polyphenols are secondary plant metabolites widely distributed in plant tissues, being usually accumulated in fruit skins (Torres, Davies, Yañez, & Andrews, 2005). The polyphenol profile and concentration depend largely on the species considered. For instance, in tomato (*Solanum lycopersicum* L.), chlorogenic acid is the main phenolic acid, and the main flavonoids are rutin, naringenin and myricetin (Helmja, Vaher, Püssa, Raudsepp, & Kaljurand, 2008; Martínez-Valverde, Periago, Provan, & Chesson, 2002; Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003). On the other hand, in bell pepper the main flavonoids and phenolic acids are quercetin and luteolin glycosides; onion accumulates quercetin and its glycosides; eggplant chlorogenic and ferulic acids; orange hesperidin and naringenin glycosides, etc. (Miean & Mohamed, 2001; Sakakibara et al., 2003).

Consumers are aware of the functional characteristics of agricultural food products, and more consumers choose foods considering their healthy characteristics. Thus, there is an increasing attention in the development of new antioxidant-rich varieties via breeding programs (Goldman, 2011). A great effort has already been done in the case of carotenoids, and right now phenolic compounds are receiving more attention. In order to develop breeding programs to achieve this target or to develop quality controls of food products, it is necessary the presence of rapid and inexpensive analytical procedures for the quantitation of the main flavonoid and phenolic acids present in each species.

Several analytical methods have been published for the determination of these compounds in food samples. The most widely used are based on reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with UV–vis detection and/or mass (LC–MS) or tandem mass spectrometry (LC–MS/MS) (Barros et al.,

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http://dx.doi.org/10.1016/j.foodchem.2014.07.151
0308-8146/

2012; De Paepe et al., 2013; Helmja et al., 2008); however, the number of published studies dealing with LC–MS and LC–MS/MS techniques and the possibility of access to these technologies for most laboratories are so far limited.

Most of the chromatographic procedures developed for the simultaneous measurement of phenolic acids and flavonoids in foods require long analysis times (1 h or more per sample) (Merken & Beecher, 2000; Sakakibara et al., 2003), or they are focused on a single or a few groups of phenolic structures (Mattila & Kumpulainen, 2002; Repollés, Herrero-Martínez, & Ràfols, 2006). In addition, the most above-reported RP-HPLC methods did not have taken full advantage of recent advances in LC instrumentation (Nováková & Vlčková, 2009). Indeed, it could be beneficial to further improve chromatographic performance in terms of throughput and/or resolution particularly when numerous complex food extracts have to be analysed. In this context, several analytical strategies related to column technology have been developed in HPLC, including the use of monolithic supports, packed columns with sub-2 μm particles operating at ultra-high pressure (UHPLC) or with core–shell or fused-core particles. Advantages of this latter technology are the ability to reach high peak efficiency (even at higher flow rates) without the necessity to use instrumentation and consumables of higher costs required for sub-2-μm particles (McCalley, 2010). However, only few works have used this core–shell particle technology for the analysis of phenolic compounds, mostly focused in tea samples (Rostagno et al., 2011).

Several extraction techniques have been also developed for the isolation of phenolic compounds, namely ultrasound-assisted extraction (UAE) (Jerman, Trebše, & Vodopivec, 2010), supercritical fluid extraction (Adil, Cetin, Yener, & Bayındırlı, 2007), microwave-assisted extraction (Li et al., 2012) and pressurized liquid extraction (Alonso-Salces et al., 2001). These techniques reduce considerably the consumption of solvents, increase the speed of the extraction process and simplify it. Amongst these, ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques. Despite of the large number of investigations made, there is still a great interest in the development of analytical procedures for an easy, inexpensive and quick extraction and determination of phenolic acids and flavonoids in vegetable and fruit and samples.

In this work, a methodology to separate and quantify simultaneously the most representative phenolic compounds in several vegetables and fruits, using an UAE protocol followed by RP-HPLC analysis with diode array detection has been developed to cover this demand. For this purpose, gradient elution conditions were optimised to achieve a rapid separation of phenolic compounds of interest. Additionally, the extraction procedure was also optimised using a response surface methodology (RSM) to obtain the optimum extraction conditions of tomato polyphenols by considering the effects of freeze-drying, MeOH-H_2O proportion in the extraction solvent, extraction time and ultrasound-assisted extraction. The optimised method was carefully validated and applied to the quantitation of different vegetable and fruit matrices.

2. Material and methods

2.1. Chemicals and reagents

The standards of phenolic compounds: gallic acid, caffeic acid, p-coumaric acid, trans-ferulic acid, benzoic acid, chlorogenic acid, (+)-catechin, kaempferol, quercetin, myricetin, naringenin, genistein, luteolin, apigenin, rutin, naringin and hesperidin were purchased from Sigma–Aldrich (Syeinheim, Germany). Butylated hydroxytoluene (BHT), formic acid and HPLC-grade methanol (MeOH) were also supplied by Sigma. HPLC-grade acetonitrile (ACN) was purchased from Panreac (Castellar del Vallés, Spain). Water was purified on a Milli-Q water system (Millipore, Molsheim, France). Stock solutions of polyphenols were prepared in a methanol/water mixture (80:20, v/v) at 500 mg L^{-1}, except for apigenin and hesperidin, which was prepared in a mixture of methanol/acetonitrile (70:30, v/v). All stock solutions were stored at −20 °C until their use and protected from light. Prior to injection, working solutions (25 mg L^{-1}), were prepared daily by dilution of stock solutions with mobile phase, and filtered through a 0.2 μm pore diameter PTFE filter.

2.2. Instrumentation and conditions

A 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with a quaternary pump, a degasser, a thermostatic autosampler, and a diode array detector (DAD), was used to separate the analytes. The analytical column was a fused-core Kinetex-XB C18 column (150 mm × 4.6 mm i.d.; particle size, 2.6 μm) from Phenomenex (Torrance, USA). The column and guard column were thermostatically controlled at 35 °C, the flow rate was kept constant at 0.9 mL min^{-1} and the sample injection volume was 10 μL. The mobile phase solvents consisted of water, ACN and MeOH, each of them containing 0.1% (v/v) of formic acid. Detection and quantification was performed at 255 nm (for genistein and rutin), at 280 nm (for gallic and benzoic acids, catechin, naringin and hesperidin), at 290 nm (for naringenin), at 320 nm (for caffeic, p-coumaric, ferulic and chlorogenic acids) and at 365 nm (for kaempferol, quercetin, myricetin, luteolin and apigenin). Each polyphenol UV–vis spectrum was also recorded using a DAD detector for the identification of the studied compounds. Peak purity was studied with the ChemStation Rev B.03.01 software (Agilent Technologies, Waldbronn, Germany). In addition, samples were spiked in order to corroborate the peak identification.

2.3. Plant material

Fresh tomatoes from an experimental line (“Fortuna-C”), two highly consumed cultivars (“Pera” and “Kumato®”) and an accession of a wild species related to tomato (*Solanum neorickii* D.M: Spooner, G.J. Anderson & R.K. Jansen, S.) were used. Standard cultivars commonly available at local markets were used for onion, celery, grape, green pepper (Italian type), red pepper (Lamuyo type), eggplant, muskmelon (Piel de sapo type), apple (cv. “Fuji”) and orange. Organic soy milk (13.2% peeled soy seeds blended in water) from a local supermarket was also analysed. For each sample, the edible part was processed. When the skin was included in the sample, it was previously washed with tap water. Sample was ground in an 1100 W blender until it was completely homogenised and stored at −80 °C until analysis. When required a SilentCrusher M homogenizer (Heidolph, Schwabach, Germany) was also used. “Fortuna-C” and *S. neorickii* accession were provided by the Genebank of the Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV, Spain). Other fruits and vegetables were purchased in local markets. Samples were extracted using the optimised extraction procedure.

In order to provide a supplementary quantification of free aglycones the extracts were also hydrolysed. For this purpose, a slight modification of the conditions reported by Hertog, Hollman, and Venema (1992) was adopted. HCl was added to the extracts at final concentration of 1.5 M and hydrolysis was performed at 90 °C for 90 min. Each sample was analysed twice. In order to discard negative effects of hydrolysis conditions on flavonoid aglycones recoveries after hydrolysis were studied in tomato, obtaining recovery values of 99% for quercetin and 76% for naringenin. These values