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Analytical Methods

Optimization and validation of a rapid liquid chromatography method for determination of the main polyphenolic compounds in table olives and in olive paste

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ABSTRACT

A high performance liquid chromatography method, coupled to diode-array and fluorescence detectors, with a previous solid-liquid extraction, has been developed for the simultaneous detection and quantification of polyphenolic compounds in table olives and in olive paste. The effects of extraction variables have been studied by response surface methodology. The best conditions were extraction with 100% methanol (2 mM NaF) during 30 min for table olives, and 91% methanol (2 mM NaF) during 40 min for olive paste. Chromatographic separation of 26 polyphenols from different families was optimized. This method provides high linearity, in all cases higher than 98.65%, and high sensitivity whose detection limits ranged between 0.08 and 1.11 μg/mL. The validated method has been applied for the determination of polyphenols in table olive and olive paste samples. The intra-day and inter-day assay repeatability, in the analysis of real samples was less than 7.6 and 11%, respectively.

1. Introduction

The olive tree (*Olea europaea*), whose origin is associated to Syria, Palestine and Israel zones, is widely cultivated in Mediterranean climate characterizated by dry and hot summers. Table olives are fermented fruit of *Olea europaea* L., whose production, practically, is located in the Mediterranean zone. The total world production is estimated at 2,660,500 tons per year (IOC, International Olive Council, 2015), and Spain is the main table olives producer. In this country, commonly, olive fruit is treated with a diluted sodium hydroxide solution, during Spanish style process, to reduce oleuropein, which is a polyphenol responsible for bitterness of olive fruit. Tyrosol (*p*-HPEA) and hydroxytyrosol (3,4 DHPEA) derivatives concentrations increase during this process. Then, the fruits are washed to eliminate the alkali excess and placed in NaCl brine solution where fermentation takes place.

Table olives contain polyphenolic compounds which provide numerous and known healthy benefits because of their antioxidant (Kountouri, Mylona, & Andrikopoulos, 2007; Malheiro et al., 2014), cardioprotective, and anticarcinogenic activity. The major phenolic compounds present in table olives and in olive fruits are tyrosol and hydroxytyrosol. The concentration of polyphenolic compounds depends on the olive varieties, stages of maturity, industrial fermentation process and storage conditions, among others. Furthermore, polyphenols compounds contribute to nutricional, sensory and, commercial characteristics which are appreciated by the consumers.

Most investigations about phenolic content have been focused on olive oil while analytical methods for more complex matrices, as olive paste or table olives, have hardly ever been developed. Some procedures, based on extraction, separation and quantification by HPLC, have been proposed for the determination of phenolic compounds in table olives. Jerman, Trebse, and Mozetic Vodopivec (2010) used an ultrasound-assisted method for the extraction of olive oil phenols followed by HPLC-DAD-FLD-MS/MS for determination of nine polyphenols in 70 min, optimized all extraction variables, such as sonication time, temperature, extraction steps and, solvent composition, which methanol absolute were chosen, obtaining high recoveries much better than conventional agitation. Sahin and Samli (2013), used a response surface methodology to obtain the best combination of several

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ultrasonid-assisted extraction parameters of olive oil waste polyphenols: solvent concentration of EtOH, ratio of solid to solvent and extraction time. Furthermore, some researchers have developed chromatographic method for the only determination of tyrosol, hydroxytyrosol in plasma (Rodríguez-Gutiérrez, Wood, Fernández-Bolaños, Duthie, & de Roos, 2011), cosmetic products based on olive extracts (Miralles, Chisvert, & Salvador, 2015) and of oleuropein in olive leaves (Al-Rimawi, 2014) as well as they used a previous reversed-phase dispersive liquid-liquid microextraction for oleuropein preconcentration (Hashemi, Raeisi, Reza Ghiasvand, & Rahimi, 2010).

Vinha et al. (2005) developed an extraction of Portuguese olive fruits polyphenols procedure using methanol and the extract was purified using solid-phase extraction with ISOLUTE C18 cartridges and the eluates were analyzed by DAD in 66 min. Dagdelen, Tümen, Musa Özcan, and Dündar (2013) evaluated the phenolic profiles of olive fruits from different varieties at different ripening stages. Machado, Felizardo, Fernandes-Silva, Nunes, and Barros (2013) also used water/formic acid and methanol as mobile phases and a flow rate of 1.0 mL/min with a total run time of 80 min and preparation sample procedure based on extraction, vacuum evaporation and re-dissolution steps. Finally, Pistarino, Aliakbarian, Casazza, Paini, and Cosulich (2013) studied the effect of culture and temperature over the polyphenolic compounds during the fermentation of black olives.

The phenolic fraction of olives has been the focus of several studies based on the optimization of irrigation of olive trees to improve the relation between production and quality (Ben Brahim, Gargouri, Marrakchi, & Bouaziz, 2016; Gómez-Rico et al., 2007). Other researches are focused in the study of the different variables which affect the fermentation processes of table olives (Charoenprasert & Mitchell, 2012) in order to increase the quality and nutritional values. In all cases, the most common methods for the analysis of phenolic fraction in olive fruits are HPLC coupled to UV–Vis, or diode-array detection (DAD) and the run time is higher than 50 min.

The aim of this study is the development and validation of an analytical method for the quantitative determination of the main polyphenols in table olives, and in olive paste, by high performance liquid chromatography (HPLC) coupled with DAD and FLD. The combination of HPLC and FLD provides a fast and selective method with a run time of 30 min, and allow the analysis of more than 25 polyphenols in only one run. The extraction of polyphenols in olives table and in olive paste was optimized by using the experimental design and the response surface methodology (RSM).

2. Material and methods

2.1. Samples

The study was carried out with olive (*Olea europaea*, L.) cultivars (Arbequina, Carrasqueña and Manzanilla Cacereña varieties) collected in an experimental orchard from the Research Center "Finca La Orden" (Badajoz, Spain), situated within the limits of the olive-growing area "Tierra de Barros" (south-western of Spain), during the season 2014–2015. The olive orchards were composed by fifteen-years-old olive trees (plantation frame 6×6 m^2). The sampling of the olives, in perfect sanitary conditions, was carried out for green stage of maturation using the subjective evaluation of the skin and flesh colour, as proposed by Uceda and Frías (1975). After harvesting, the olive fruit samples were immediately transported to the laboratory in ventilated storage trays to avoid compositional changes.

To obtain olive paste, Arbequina and Carrasqueña olive varieties, previously deboned, were crushed with a hammer mill. Then, the obtained paste was stored away from the light in amber-colored glass bottles at – 80 °C until analysis (within 1 month).

For elaboration table olives, 50 kg of Arbequina, Carrasqueña or Manzanilla Cacereña olive varieties were immersed in 1.7% (w/v) NaOH solution during 11 h at room temperature (ca. 25 °C) until the alkali reached 2/3 of the flesh as measured from the epidermis to the pit. After this treatment, olives were washed several times with tap water to eliminate the excess of alkali. Three water changes at 4, 8 and 12 h were undertaken. Fermentation was undertaken in 8 L total capacity screw-capped plastic vessels containing approximately 4 kg of olives and 3 L of freshly prepared 10% (w/v) NaCl brine. All treatments were performed by duplicate and fermentation vessels were maintained at room temperature for an overall period of 4 months (112 days).

2.2. Reagents, solvents and phenolic standards

Polyphenol analytical standards were supplied as follows: apigenin-7-*O*-glucoside, cyanidin-3-*O*-glucoside, cyanidin-3-rutinoside, hydroxytyrosol (3,4 DHPEA), luteolin, oleuropein, procyanidin B1 (PB1), procyanidin B2 (PB2) and verbascoside by Extrasynthése (Genay, France). Apigenin, catechin, epicatechin, *t*-ferulic acid, gallic acid monohydrate, luteolin-7-*O*-glucoside, neochlorogenic acid, quercetin, quercetin-3-rutinoside, syringic acid, tyrosol (*p*-HPEA) and vanillic acid by Sigma-Aldrich Chemie (Steinheim, Germany). Caffeic acid, *p*-coumaric acid, sinapic acid, *o*-vanillin by Fluka Chemie (Steinheim, Germany). Vanillin by MERK-Schuchardt (Hohenbrunn, Germany).

Stock standard solutions were prepared by dissolving accurately weight of each compound in methanol. The concentration of each standard solutions were 32 μg/mL for luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside; 40 μg/mL for gallic acid, neochlorogenic acid, cyanidin-3-glucoside, PB1, catechin, cyanidin-3-rutinoside, vanillic acid, caffeic acid, PB2, syringic acid, epicatechin, vanillin, *p*-coumaric acid, *t*-ferulic acid, sinapic acid, *o*-vanillin, verbascoside, quercetin, luteolin and apigenin; 96 μg/mL for 3,4 DHPEA; 100 μg/mL and *p*-HPEA, quercetin-3-rutinoside and oleuropein. All solutions were kept in amber bottles, and they were stored at -20 °C in the darkness.

Acetonitrile and methanol HPLC grade were purchased from Fisher Scientific (Loughborough, UK). Formic acid was supplied by PANREAC (Barcelona, Spain), and sodium fluoride was supplied by Sigma-Aldrich Chemie (Steinheim, Germany).

2.3. Analytical procedures

2.3.1. Reversed-phase-HPLC analysis

Chromatographic analysis were carried out employing an Agilent 1100 model LC system (Hewlett-Packard, Waldbronn, Germany) equipped with an UV−Vis diode-array detector and with a rapid scan fluorescence spectrophotometer detector. Separation was performed using a Phenomenex (Phenomenex, Torrance, CA, USA) Gemini-NX C18 column (150×4.6 mm i.d., 3 μm particle size). The mobile phases were prepared from 0.1% (*v/v*) formic acid in water (eluent A) and 0.1% *(v/v)* formic acid in acetonitrile (eluent B). The gradient used was as follows: 0–1 min, 3% B isocratic; 1–30 min, linear gradient from 3% to 35% B; 30–33 min, linear gradient from 35% to 50% B; 33–34 min, linear gradient from 50% to 100%; 34–50 min, 100% B isocratic. Then, the gradient returned to 3% of eluent B and this composition was held during 3 min for re-equilibrate the column. The column temperature was set at 40 °C, the injection volume was set at 10 μL, and the flow rate was 1 mL min^{-1}. Quercetin and oleuropein were monitored by DAD and quantified at 255 nm, benzoic acids at 280 nm, cinnamic acids at 320 nm, flavones and quercetin-3-rutin at 350 nm, and anthocyanins at 515 nm. Fluorescence detection at 275/315 nm