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Analytical Methods

Quantitative analysis of pungent and anti-inflammatory phenolic compounds in olive oil by capillary electrophoresis

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ABSTRACT

The first CE procedure for the quantitative determination of pharmacologically relevant secoiridoids in olive oil, oleocanthal and oleacein, is described. Together with their precursors tyrosol and hydroxytyrosol they could be baseline separated in less than 15 min using a borax buffer with pH 9.5, at 25 kV and 30 °C. Method validation confirmed that the procedure is selective, accurate (recovery rates from 94.0 to 104.6%), reproducible ($\sigma_{max} \leqslant 6.8\%$) and precise (inter-day precision $\leqslant 6.4\%$), and that the compounds do not degrade quickly if non-aqueous acetonitrile is used as solvent. Quantitative results indicated a low occurrence of oleocanthal (0.004–0.021%) and oleacein (0.002–0.048%) in olive oil samples, which is in agreement to published HPLC data. The CE method impresses with its simple instrumental and methodological design, combined with reproducible and valid quantitative results.

1. Introduction

One of the characteristics of Mediterranean diet, which is associated with a lower occurrence of cardiovascular diseases and neurological disorders, is a reduced consumption of animal fats and the use of olive oil instead (Perez-Trujillo, Gomez-Caravaca, Segura-Carretero, Fernando-Gutierrez, & Parella, 2010) This vegetable oil represents a well-balanced source of glycerols (more than 98% of the total weight) and minor compounds such as alcohols, sterols, volatile constituents and antioxidants (El Riachy, Priego-Capote, Leon, Rallo, & Luque de Castro, 2011). The latter comprise phenolic acids and alcohols (tyrosol (abbreviated as p-HPEA) and hydroxytyrosol (3,4-DHPEA)), flavonoids, lignans and secoiridoids (oleuropein, ligstroside and derivatives). Antioxidants are most important for olive oil because they assure its shelf-life, and are relevant for organoleptic and pharmacological properties. Especially two secoiridoids have to be mentioned in this respect, oleocanthal (**1**) and oleacein (**3**; Fig. 1). They represent dialdehydic forms of decarboxymethyl elenoic acid (EDA) linked to tyrosol (oleocanthal is sometimes termed as p-HPEA-EDA) or hydroxytyrosol (3,4-DHPEA-EDA). Accordingly, they also can be seen as deacetoxy derivatives of the ligstroside- or oleuropein-aglycon.

Oleacein and particularly oleocanthal are responsible for the burning pungent sensation of certain olive oils (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003). Furthermore, oleocanthal showed potent anti-inflammatory effects *ex-vivo* comparable to ibuprofen (Beauchamp et al., 2005). The compound inhibits COX-1, COX-2, TNF-α and IL-1β activity, as well as NO production in macrophages (Scotece et al., 2012). The quantitative determination of both compounds is therefore of interest, but surprisingly few papers have been published on this topic. Phenolic compounds in olive oil were extensively studied by HPLC, HPLC–MS and 1H NMR, including a few quantitative investigations on **1** (Cicerale, Conlan, Barnett, & Keast, 2013; Herrero et al., 2011; Impellizzeri & Lin, 2006) or **1** and **3** (Karkoula, Skantzari, Melliou, & Magiatis, 2012). Olive oil phenols have also been assayed by capillary electrophoresis, but either "only" on a qualitative level (Carrasco-Pancorbo, Arraez-Roman, Segura-Carretero, & Fernandez- Gutierrez, 2006;

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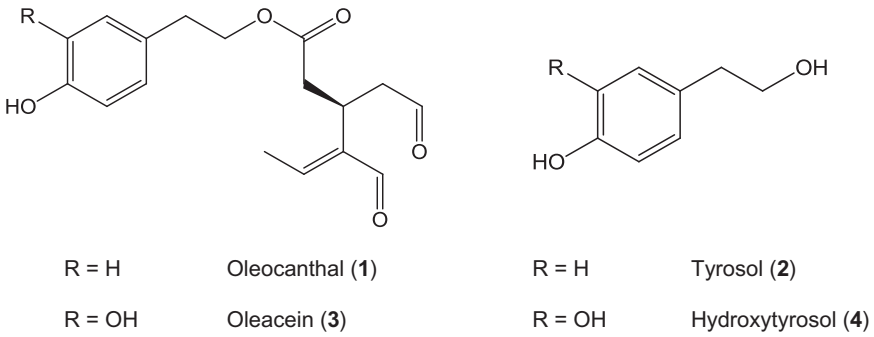

Fig. 1. Chemical structures of the studied compounds.

Carrasco-Pancorbo, Neusüß, Pelzing, Segura-Carretero, & Fernandez-Gutierrez, 2007; Monasterio, de los Angeles Fernandez, & Silva, 2013) or oleocanthal and oleacein were not among the quantified constituents (Carrasco-Pancorbo, Gomez-Caravaca et al., 2006). This indicates that till date no economic (compared to the use of NMR), validated and quantitative procedure for the simultaneous determination of these two seco-iridoids has been reported. CE should actually be an ideal approach for their analysis because compared to HPLC the techniques shows superior resolution power, versatility and speed (**1** and **3** are known to be rather instable in protic solvents; Bianco, Piperno, Romeo, & Uccella, 1999; Ganzera, 2008). Thus, in this study we developed and validated the first CE-assay for the qualitative and quantitative determination of these relevant secoiridoids, also including their precursors/degradation products tyrosol (**2**) and hydroxytyrosol (**4**).

2. Materials and methods

2.1. Samples, standards and reagents

Olive oil samples were purchased in different supermarkets in Innsbruck, Austria (OO-1 to OO-5). For the procurement of oleacein and oleocanthal the total polyphenol fraction (TPF) of olive oil was used as starting material (Keiler et al., 2013), while for their isolation column chromatography (CC) and preparative TLC were employed. Briefly, 250 mg of TPF were subjected to a silica gel column using mixtures of dichloromethane and methanol with increasing polarity for elution (0–10% methanol). From the dichloromethane/methanol fraction at 98/2 oleocanthal (11.1 mg) was isolated, while in the 97/3 eluate oleacein (19.8 mg) was obtained. For further purification normal phase preparative TLC was carried out using dichloromethane/methanol in the ratio 94/6 as mobile phase.

Identity and purity ($\geqslant$96%) of the isolated compounds were confirmed in NMR, MS and HPLC experiments. Standards ($\geqslant$98% pure) of tyrosol and 3-hydroxytyrosol were obtained from Sigma–Aldrich (St. Louis, MO, USA). All solvents (ethanol, methanol, dichloromethane, anhydrous acetonitrile, and hexane) and chemicals (borax, sodium hydroxide) required for CE-analysis were of p.A. quality and came from Merck (Darmstadt, Germany). Ultrapure water was produced by a Sartorius Arium 611 water purification system (Göttingen, Germany).

2.2. Sample preparation

The samples were prepared by liquid–liquid partitioning following the protocol described by Rios-Martin and Gutierrez-Rosales (2010), with slight modifications. In brief, 5 mL of an ethanol/water-mixture (8/2; v/v) was added to five gram olive oil, the mixture was vortexed for five min and then centrifuged (5000 rpm for 5 min). The polar extract (lower phase) was removed, the same extraction procedure repeated two more times and the combined solutions evaporated to dryness under reduced pressure (at max. 40 °C). In dry form the samples were stable for at least one month if stored at −20 °C. Prior to CE analysis they were dissolved in 5.00 mL anhydrous acetonitrile and washed two times with hexane (5 mL each) to remove lipids and pigments. Finally, the ACN phase was membrane filtered (Phenex-RC, 0.45 μm; Phenomenex, Torrance, CA, USA) and used for analysis.

2.3. Analytical method

Analytical experiments were performed on a 3D-CE system from Agilent (Waldbronn, Germany), equipped with autosampler, diode array detector (DAD) and temperature controlled column compartment. Separations were performed in fused-silica capillaries (50 μm i.d × 52 cm effective length) purchased from Polymicro Technologies (Phoenix, AZ, USA).

For best results an aqueous 45 mM borax (sodium tetraborate decahydrate) solution was used as buffer, with a pH of 9.5 (adjusted with 1 M NaOH solution). Applied voltage, temperature and detection wavelength were set to 25 kV, 30 °C, and 200 nm, respectively. All samples were injected in hydrodynamic mode (50 mbar for 3 s) and the required run time was 18 min. Between runs the capillary was flushed for 3 min with 0.01 N NaOH solution, 1 min water and 4 min buffer; new capillaries were rinsed with 0.1 N NaOH, 0.01 N NaOH and water (30 min each) prior to initial use. Before analysis all samples, buffers and washing solutions were membrane filtered.

2.4. Method validation

The developed CE-method was validated as required by ICH guidelines (International Conference on Harmonization guideline Q2(R1), 2005). Calibration curves were established by dissolving the reference compounds in non-aqueous acetonitrile and preparing individual concentration levels by serial dilution with the same solvent. Selectivity was assured by the respective peak-purity option in the operating software (chemstation, version B.04.03 SP1; Agilent). Sensitivity of the assay was concluded based on limit of detection (LOD) and limit of quantitation (LOQ), which represent concentrations equal to 3- and 10- times baseline noise. Accuracy was investigated by spiking one sample (OO-1) with known amounts of **1**–**4** prior to extraction, and then comparing the quantitative results with theoretically calculated ones. Repeatability was deduced from relative standard deviations for multiple injections of the same solutions below 5.0% for most compounds (see quantitative results), and intermediate precision investigated by assaying (i.e. extraction and analysis) the same sample (OO-1)