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Analytical Methods

Preparative isolation of oleocanthal, tyrosol, and hydroxytyrosol from olive oil by HPCCC

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ABSTRACT

For the provision of oleocanthal (OLC), a phenolic compound with very promising pharmacological properties, isolation from olive oil is a very important option. Due to the compound's sensitivity to decomposition upon exposure to oxygen and light, a very gentle isolation method has been developed under use of high performance countercurrent chromatography (HPCCC). By partition of olive oil between hexane and methanol, an extract enriched in phenolics was prepared and subjected to a two-step HPCCC separation under use of heptane–EtOAc–MeOH–H_2O mixtures in normal-phase and reverse phase mode, respectively. With this method, the isolation of tyrosol, hydroxytyrosol, and the mixture of (3S,4E)- and (3S,4Z)-OLC was achieved in approx. 70 min for each step. By one- and two-dimensional NMR-experiments and LC–MS, the equilibrium of (3S,4E)- and (3S,4Z)-OLC in such olive oil extracts has unambiguously been proven for the first time.

1. Introduction

The contribution of extra virgin olive oil (EVOO) to the health benefits of the Mediterranean diet has been objective of broad research since the 1970ies (Bach-Faig et al., 2011; Perez-Jimenez et al., 2005). The impact of phenolics in EVOO became evident by the fact that other seed oils with similar concentrations of monounsaturated fatty acids do not show health benefits comparable to EVOO (López-Miranda et al., 2010). Approximately 40 phenolics from the major classes of phenolic acids and alcohols, lignans, flavonoids, and secoiridoids have been detected in olive oil (Cicerale, Lucas, & Keast, 2012). Most abundant are the alcohols tyrosol and hydroxytyrosol as well as the secoiridoids oleuropein, ligstroside, and their derivatives (Goulas, Charisiadis, Gerothanassis, & Manganaris, 2012). One of these substances, oleocanthal (OLC, Fig. 1), has gained a lot of scientific interest during the recent years. It was isolated for the first time in 1993 (Montedoro et al., 1993) and later it was shown to be responsible for the oropharyngeal irritation which is triggered by many EVOOs. This irritation is similar to sensations after intake of the well-known anti-inflammatory drug ibuprofen (Andrewes, Busch, de Joode, Groenewegen, & Alexandre, 2003; Beauchamp et al., 2005). OLC elicits this irritation via the same mechanisms as ibuprofen (Bennett & Hayes, 2012; Peyrot des Gachons, Uchida, & Bryant, 2011) and mimics the anti-inflammatory activities of ibuprofen via the inhibition of COX-1 and COX-2 enzymes at almost equimolar concentrations (Beauchamp et al., 2005; Lucas, Russell, & Keast, 2011). Recently, several other potentially health promoting activities of OLC were described (Parkinson & Keast, 2014), such as:

(1) The enhancement of amyloid-β clearance from the brain (Abuznait, Qosa, Busnena, El Sayed, & Kaddoumi, 2013) and the modulation of the fibrillization of tau protein (Monti, Margarucci, Riccio, & Casapullo, 2012), both important mechanisms in neurodegenerative diseases.
(2) An inhibition of cell viability of and induction of apoptosis in HT-29 colon cancer cells, inhibition of proliferation, migration and invasion of MCF-7 and MDA-MB-231 breast cancer and PC-3 prostate cancer cell lines, and an anti-angiogenic effect (Elnagar, Sylvester, & El Sayed, 2011; Khanal, Oh, Yun, et al., 2011), stimulating further interest in cancer research.
(3) A decrease in LPS-induced iNOS protein expression in chondrocytes, which with the anti-inflammatory effects of OLC might be promising in joint-degenerative diseases (Iacono, Gómez, Sperry, et al., 2010).

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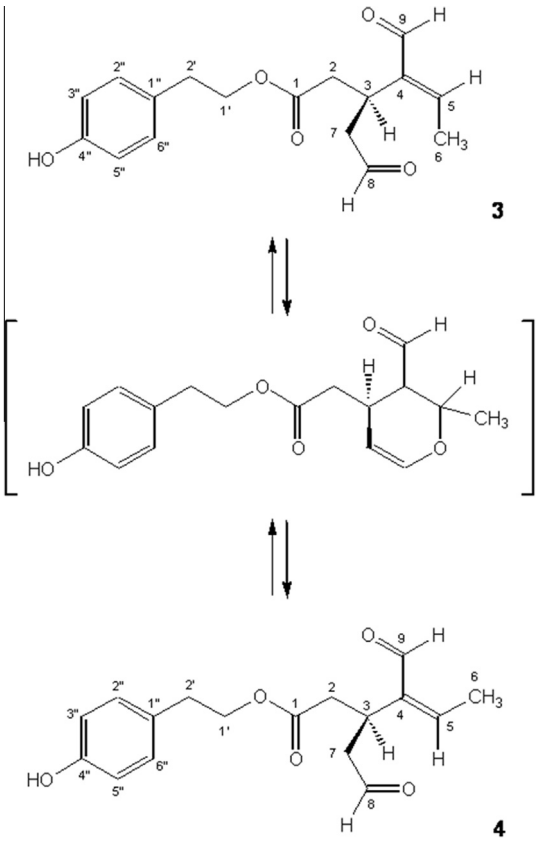

Fig. 1. The structure of oleocanthal. Proposed mechanism for the interconversion of E/Z isomeric enones **3** and **4** via a Michael addition/elimination process.

Until now, almost exclusively *in vitro* studies on the activities of OLC are available. For *in vivo* studies, higher amounts of the compound are needed. The synthesis of OLC requires about ten steps and the yields remain quite low (English & Williams, 2009; Smith, Sperry, & Han, 2007; Takahashi, Morita, & Honda, 2012; Valli et al., 2013). Thus, isolation of OLC from natural sources (olive oil, olive oil pomace, olive leaves, or olive oil mill waste water) is an important option. Previous methods are quite laborious and time-consuming due to the use of column chromatography and/or preparative HPLC (Abuznait et al., 2013; Agalias et al., 2007; Khanal et al., 2011; Paiva-Martins & Gordon, 2001). In addition, keto-enol tautomerism and decomposition upon exposure to oxygen and light has been reported for OLC (Caruso, Colombo, Patelli, Giavarini, & Galli, 2000; Cicerale, Conlan, Barnett, & Keast, 2013), which has to be considered in the development of efficient isolation procedures.

High performance countercurrent chromatography (HPCCC), as a liquid–liquid separation method without any adsorptive matrix, is an excellent tool for the isolation of natural products from different chemical classes – among them many phenolics (Yoon, Chin, & Kim, 2010). This technology allows full recovery of the sample under low solvent consumption. Peak tailing is minimized and separation times are short. Therefore, the risk of sample degradation is low as well (Marston & Hostettmann, 2006). Due to these advantages, we hypothesized that HPCCC can be a suitable tool for a fast and simple isolation of OLC from olive oil extracts. To the best of our knowledge, hydroxytyrosol is the only EVOO phenolic that has been isolated by partition chromatography until now – from an enriched phenol concentrate from olive oil mill waste water (Agalias et al., 2007).

Thus, the presented project studied the potential of HPCCC for an efficient isolation of OLC and other EVOO phenolics from olive oil.

2. Materials and methods

2.1. Chemicals

The oil for extraction was Greek extra virgin olive oil "Kloster Toplou" purchased from Hofer KG, Sattledt, Austria. The reference compounds tyrosol and 3-hydroxytyrosol were obtained from Fluka (Buchs, Switzerland) and Sigma (St. Louis, USA), respectively. The solvents MeOH, EtOAc, heptane, and acetonitrile were from Fisher Chemicals (Loughborough, UK) and VWR (Vienna, Austria).

2.2. Instrumentation

The extraction of olive oil was performed by sonification in a Branson 3150 ultrasonic bath (Dumbury, USA). High performance countercurrent chromatography (HPCCC) of the extract was performed on a Spectrum HPCCC instrument (Dynamic Extractions, Slough, UK) connected to an Agilent 1200 preparative system with 100 mL pump, multi wavelength detector (MWD) and fraction collector (Agilent Technologies, Santa Clara, USA). LC–MS analyses were performed on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap mass spectrometer via an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (UltraShield) using a 5 mm switchable probe (PA BBO 500SB BBF-H-D-05-Z, 1H, BB = 19F and 31P–15N) with z axis gradients and automatic tuning and matching accessory (Bruker BioSpin). The resonance frequency for ^{1}H NMR was 500.13 MHz and for ^{13}C NMR 125.75 MHz. A Shimadzu instrument with LC-20AD pump, SPD M20A diode array detector and SIL 20AC HT auto-sampler (Kyoto, Japan) was used for analytical HPLC on a Luna 5 µm C18(2) 100 Å (250 × 4.6 mm) column (Phenomenex, Torrance, USA).

2.3. Extraction of the phenolic compounds from EVOO for analytical purposes

The phenolic compounds were enriched by a modified method (based on Impellizzeri & Lin, 2006): 45 mL EVOO were mixed with 45 mL hexane and 75 mL MeOH for 1 min and the mixture was sonicated at 20 °C for 5 min. After partition, the methanolic phase was centrifuged at 3500 rpm for 10 min. The hexane phase was extracted once again with 75 mL MeOH. The combined MeOH phases were evaporated at 39 °C and 200 mbar. The resulting oily residue was extracted with 1 mL MeOH–water (1 + 1) for 1 min and then sonicated for 5 min. The mixture was treated with 5 mL hexane for 1 min and centrifuged at 13,000 rpm for 10 min. The aqueous MeOH layer was used for analysis.

2.4. Extraction of the phenolic compounds from EVOO for preparative purposes

A similar method was applied for enrichment of phenolic compounds for the preparative work. 0.5 L of EVOO was mixed with 0.625 L MeOH–hexane (4 + 1). After shaking for 1 min, it was additionally sonicated at 20 °C for 10 min. The solution separated into two layers. The lower layer was collected and the above process with 0.625 L MeOH–hexane (4 + 1) was repeated. All upper layers were combined and the solvent was evaporated under reduced pressure at 20 °C. In sum, 7.5 L of EVOO were extracted with this method to yield 110 g of oily extract in total, which were mixed with 50 mL of MeOH–H_2O (1 + 1) under sonication. Then, 150 mL hexane was added for partition. After the partition step, the mixture was centrifuged for 10 min at 3500 rpm. The lower layer was collected and centrifuged for another 10 min at 13,500 rpm.