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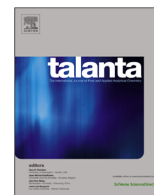

Evaluating the potential of LC coupled to three alternative detection systems (ESI-IT, APCI-TOF and DAD) for the targeted determination of triterpenic acids and dialcohols in olive tissues

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ABSTRACT

Herewith the development of a rapid and powerful LC methodology (with three different detectors) is presented to determine triterpenic acids and dialcohols in extracts from *Olea europaea* tissues (olive skin, pulp and leaves). After the proper optimization of the LC, DAD and MS conditions and the comprehensive characterization of the behavior of each analyte in ESI and APCI (with accurate *m/z* signals and, in ESI, with MS/MS data too), the method was fully validated. DAD, ESI-IT MS and APCI-QTOF MS were used as detection systems to give different alternatives to carry out the accurate determination of these analytes, evaluate their analytical performance, advantages and drawbacks, and check whether the quantitative results achieved by the three platforms were in good agreement. ESI-IT MS gave the lowest detection limits (3–455 µg/L) followed by APCI-QTOF MS (22–408 µg/L); in contrast, DAD (83–600 µg/L) had the widest dynamic range. The RSD values for *inter-day* repeatability were found below 11.82% in all the cases. No statistically significant differences were found among the quantitative results from the three detectors. Olive leaves showed the highest concentration levels of ursolic acid (1.8 mg/g), erythrodiol (1.6 mg/g) and uvaol (1.2 mg/g), whereas the olive skin was the richest matrix in terms of maslinic (80 mg/g), betulinic (0.20 mg/g), and oleanolic (26 mg/g) acids. Concentration values of triterpenic acids were established by first time for skinless olive pulp, and were found around 65, 1.2, 55 and 4.4 µg/g for maslinic, betulinic, oleanolic and ursolic acids, respectively.

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1. Introduction

Over the last years, a rich in fruits and vegetables diet has been associated to a lower incidence of diseases related to chronic damage and growth dysregulation, such as obesity, diabetes, cancer or cardiovascular disease. This is largely due to the phytochemicals found in food plants at different concentrations levels [1]. Some of these compounds are triterpenoids, an important group of natural products with numerous biological effects, which are being used as ingredients in dietary supplements, medicines and healthcare products [2,3]. In particular, pentacyclic triterpenes have been identified as the main components of medicine plants [4], and have shown, among others, analgesic, hepatoprotective, anti-tumor, anti-diabetic, anti-inflammatory and antioxidant effects [5–8]. They are secondary plant metabolites which arise from cyclization of squalene, and have a common skeleton of five 5 or 6-membered cycles substituted by different functional groups [9]. Such substances are excreted by plants as protection agents, so they use to be part of the cuticular waxes that surround plant surfaces (leaves, stems, flowers and fruits) [10].

Olea europaea is a valuable source of this kind of compounds, since different triterpenic acids and alcohols have been described in olive industry-related products (olive leaves, fruits, oil and pomace) [11–13]. Bioactive properties of pentacyclic triterpenes from *Olea europaea* have been systematically reviewed by different authors [14–19], and some protocols for obtaining their pure extracts have been patented [20–23]. Obtaining these components from olive-industry by-products could be a way to economically upgrade the sector. The use of these compounds as ingredients in new products leads to the need of developing appropriate methods for their determination in a growing variety of samples (raw materials or final products). The optimization of these methods, which can be implemented in routine laboratories to ensure the safety and quality of these new products, represents a considerable challenge.

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In recent years, in parallel with the discovery of the biological effects of triterpenoids, many studies have been carried out trying to achieve the best possible determination procedure. Their extraction from vegetal tissues is the first step to be optimized in order to achieve an accurate quantification; in this regard, different strategies have been evaluated [24,25]. In the case of *Olea europaea* tissues, after water removal, analytes of interest have been extracted with ethyl acetate in a Soxhlet apparatus [26], by maceration with ethanol [27], by solid–liquid extraction with a mixture of methanol/ethanol (1:1, v/v) [28] or by microwave assisted extraction with ethanol/water (80:20 v/v) as extractant mixture [29]. Other extraction techniques, such as ultrasonic assisted extraction [30] or supercritical fluid extraction [31], have been also applied to different plants.

Triterpenoid fraction in plant matrices is quite complex, in particular because the coexistence of some structural isomers, therefore, their quantification is quite difficult, making almost mandatory the use of a separation technique before their detection. In any case, interesting examples which do not imply the use of a previous separation can be found. For instance, discrimination and quantification of oleanolic and ursolic acids in plant matrices has been achieved using two-dimensional nuclear magnetic resonance spectroscopy [26]. Nevertheless, as stated before, in most of the proposed methods, their separation becomes the key to the success of the analytical process, since commonly used detectors are not capable of distinguish them. So, the analysis of prepared extracts has been commonly made with multiple separation techniques coupled to different detectors. Gas chromatography coupled to flame ionization detectors [27,32,33] or MS [34,35] has been extensively employed to this end. As a way of overcoming the tedious derivatization process (necessary step to increase the volatility of the triterpenoids) liquid-based methods such as capillary zone electrophoresis [36] or liquid chromatography have been also developed. As far as LC is concerned, it has been coupled to photodiode array [37], evaporative light scattering [38] or MS detectors whether for identification or quantification purposes. Fluorescence detection has been also used coupled to LC, but it requires a previous derivatization step [39–41]. In mass spectrometry, different interfaces (ESI, APCI and APPI [42]) and analyzers (IT [43], Q [28], QqQ [29], QTOF [38] and Orbitrap [44]), both in positive and negative polarities, have been employed.

In LC, a great variety of mobile phases has been used, mostly in isocratic methods, although some gradients has been also proposed for triterpenoids separation [38]. Because of the relatively low polarity of these compounds, organic solvents (methanol and/or acetonitrile) mixed with low proportions of water (usually acidified) have been commonly employed [28,43,45]; the effect of some modifiers such as cyclodextrins [46] or triethylamine [47] have been also tested. The use of neutral [48] and basic [29,49] chromatographic conditions has been reported in few communications, even though they have not been so commonly used.

The aim of this work has been to develop and validate a rapid and powerful analytical method for the determination of pentacyclic triterpenes (maslinic, betulinic, oleanolic and ursolic acids, erythrodiol and uvaol) offering different alternatives (in terms of detection systems) to carry out their accurate determination. Three detectors were selected: DAD, for being the more likely available one in a routine analysis olive oil laboratory; and two MS detectors (one of them with ESI interface and an analyzer of low resolution but very fast switching polarities and the other with an APCI source and a high resolution analyzer), since MS is continuously growing, has a great potential and is becoming a kind of mandatory. We evaluated their analytical performance, discussed their drawbacks and advantages, and checked whether the quantitative results obtained by the three platforms were in good agreement. To achieve this purpose, different olive tissues (olive skin, pulp and leaves) were selected and their triterpenoid content assessed. To the best of our knowledge, the triterpenes levels of one of the matrices under study have been never evaluated before.

2. Materials and methods

2.1. Chemicals and standards

All reagents were of analytical grade and used as received. Acetonitrile and methanol of LC–MS grade from Prolabo (Paris, France), and deonised water from a Millipore Milli-Q (Bedford, MA, USA) water purification system, were used for preparing chromatographic mobile phases. Ammonium formate and ammonium hydroxide from Sigma-Aldrich (St. Louis, MO, USA) were used as buffer components in the aqueous mobile phase. This phase was vacuum filtered with a Nylaflo™ 0.45 μm nylon membrane filter from Pall Corporation (Ann Arbor, MI, USA) before entering into the chromatographic system. Ethanol from J.T. Baker (Deventer, The Netherlands) was used for the extraction of the triterpenic compounds from the selected tissue samples. Pure standards of maslinic acid (MA), betulinic acid (BA), oleanolic acid (OA), ursolic acid (UA), erythrodiol (ER), and uvaol (UV) were all supplied by Sigma-Aldrich. A methanolic stock standard solution containing 200 mg/L of each compound was first prepared by dissolving the appropriate amount of each analyte in methanol and, then, serially diluted to working concentrations. All solutions were stored at −20 °C. All the samples and stock solutions were filtered through a Clarinert™ 0.22 μm nylon syringe filter from Agela Technologies (Wilmington, DE, USA) before injection into the instrument.

2.2. Samples and extraction procedure

Olive tissues samples were supplied by a local company. Olive skin (mix of varieties not specified by the supplier) was treated as received (it came dried from the olive mill). Olive pulp (Picudo *cv.*) was obtained manually, after peeling the olive fruits and removing their stones, and then, it was frozen to be further freeze-dried. Olive leaves (Picual *cv.*) were oven-dried at 35 °C until their weight remained constant. After water removal, pulp and leaves tissues were ground to powder before the extraction of triterpenic compounds.

Compounds of interest were isolated by ultrasonic assisted extraction according to the method described by Goulas and Manganaris [24], adapted from those of Lee et al. [50] and Li et al. [51]. Briefly, 0.5 g of dried tissue and 20 mL of ethanol were put inside a falcon tube which was left in an ultrasonic bath from J.P. Selecta (Barcelona, Spain) for 30 min. The ultrasonic bath characteristics were: 6 L of capacity, dimensions of 15, 30 and 14 cm of height, width and depth of usable bath, respectively, with a generator power of 150 W, a total power capacity of 360 W and a fixed frequency within the range 50–60 kHz. Afterwards, the tube was centrifuged at 9500 rpm for 5 min. Finally, the supernatant was evaporated to dryness and redissolved in 10 mL of methanol.

In order to evaluate the recovery percentage of the extraction system, the first part of the described procedure was repeated three times for each matrix, as follows: after taking the obtained supernatant from the first step, another 20 mL ethanol were added to the solid residue, being left into the ultrasonic bath for 30 min. This was repeated once more. In this way, we could establish the percentage of the total amount of each analyte which remained into the sample after going through the first extraction stage. Thus, when quantifying the analytes of interest in the samples, a correction factor (including the recovery and the dilution factor) was obviously applied to properly calculate the final concentration values of the compounds in the analyzed tissues.

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