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Article *in* Journal of Food and Drug Analysis · November 2013

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Research Article

Development and validation of a simple reversed-phase HPLC-UV method for determination of oleuropein in olive leaves

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ARTICLE INFO

Article history:
Received 13 August 2013
Received in revised form
4 October 2013
Accepted 7 October 2013
Available online xxx

Keywords:
HPLC
Method development
Oleuropein
Olive leaves
Validation

ABSTRACT

A simple, precise, accurate, and selective method is developed and validated for the determination of oleuropein, which is the main phenolic compound in olive leaves. Separation was achieved on a reversed-phase C_{18} column (5 μm, 150 × 4.6 mm inner diameter) using a mobile phase consisting of acetonitrile/phosphate buffer pH 3.0 (20:80, v/v), at a flow rate of 1.0 mL/minute and UV detection at 280 nm. This method is validated according to the requirements for new methods, which include accuracy, precision, selectivity, robustness, limit of detection, limit of quantitation, linearity, and range. The current method demonstrates good linearity over the range of 3–1000 ppm of oleuropein, with $r^2 > 0.999$. The recovery of oleuropein in olive leaves ranges from 97.7% to 101.1%. The method is selective, in that oleuropein is well separated from other compounds of olive leaves with good resolution. The method is also precise—the relative standard deviation of the peak areas of replicate injections of oleuropein standard solution is <1%. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust and rugged. The low limit of detection and limit of quantitation of oleuropein when using this method enable the detection and quantitation of oleuropein at low concentrations.

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1. Introduction

Phenolic compounds are plant secondary metabolites that play important roles in disease resistance and protection against pests [1,2]. Phenolic compounds are a complex and important group of naturally occurring products in plants and are present in the Mediterranean diet, which includes table olives and olive oil [2]. Many phenolic compounds are present in both olive (*Olea europaea* L.) fruit and leaves. These phenolic compounds includes, among others, hydroxytyrosol, tyrosol, rutin, verbascoside, luteolin-7-glucoside, and oleuropein. There are many techniques reported for the analysis of phenolic compounds in plants [3,4]. Oleuropein (whose structure is shown in Fig. 1), which is a secoiridoid, is the major and most abundant phenolic compound in olive leaves and fruits and is responsible for the characteristic bitterness of the olive fruit [5]. The concentration of oleuropein can reach up to 140 mg/g (14%) on a dry matter basis in young olives and 60–90 mg/g of dry matter in the leaves [5]. Olive leaves with

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http://dx.doi.org/10.1016/j.jfda.2013.10.002

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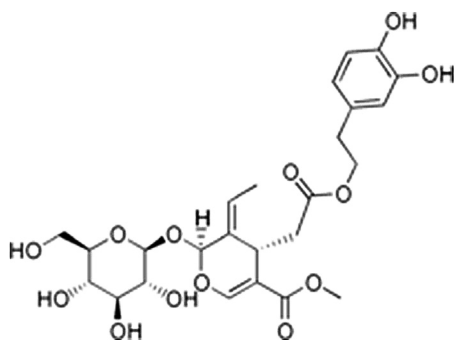

Fig. 1 – Structure of oleuropein, the major phenolic compound in olive leaves.

large amounts as a result of pruning or defoliation of olive fruits prior to processing were shown to be a good source for oleuropein [6,7]. Oleuropein has several pharmacological effects including antioxidant, anti-inflammatory, anticancer, antiviral, antimicrobial, and antiatherogenic [5]. In this respect, determination of the concentration of oleuropein in olive leaves is important. Therefore, a sensitive, accurate, precise, and selective method is required to determine the concentration of oleuropein in olive leaves. Additionally, the method should be sensitive with a low limit of detection (LOD) and limit of quantitation (LOQ), where low concentrations of oleuropein can be determined as the concentration of oleuropein in olive leaves vary from season to season and can be low (lower than 0.5% based on dry matter). The objectives of this work are therefore to develop and validate a sensitive, selective, precise, accurate, robust, rugged, and linear (with wide dynamic range) method for determination of oleuropein in olive leaves. High-performance liquid chromatography (HPLC) with a UV detector and isocratic elution method were used in the current work for oleuropein analysis in olive leaves. The method is simple: the reversed-phase mode is used with isocratic elution and a UV detector, which is available in most analytical laboratories. Validation of the method will be conducted in accordance with the requirements of new methods: linearity and range, accuracy, precision, selectivity, robustness, LOD, and LOQ. In the scientific literature, many methods have been used for the determination of oleuropein in olive fruits and leaves using infrared [5], gas chromatography [8], and HPLC [2,5,9–12]. However, the HPLC-UV method (presented here), to the best of our knowledge, has not been reported so far.

2. Methods

2.1. Chemicals

Acetonitrile HPLC grade was obtained from J.T Baker (Phillipsburg, NJ, USA). Acetic acid and oleuropein standard (HPLC grade) were purchased from Merck (Darmstadt, Germany).

2.2. Apparatus

An HPLC system (Merck Hitachi Lachrome Elite HPLC system, Tokyo, Japan) with an L-2130 pump, an L-2200 autosampler, L-2300 column oven, and L-2490 UV detector was used. The Ezochrom Elite software (Agilent Technologies, Santa Clara, CA, United States) was used. The C_{18} column [5 μm, 150 × 4.6 mm inner diameter (I.D.)] is from Waters Corporation (Milford, MA, USA).

2.3. HPLC conditions

The chromatographic analysis was performed on a LiChroCart, HPLC-cartridge Purospher STAR RP-18 endcapped (5 μm, 150 × 4.6 mm I.D.) (Waters Corporation). UV detection was used at 280 nm, isocratic elution was used at a flow rate of 1.0 mL/min, and injection volume was set to 20 μL.

2.4. Preparation of the mobile phase and standard solutions

The mobile phase was prepared by mixing 200 mL acetonitrile with 800 mL water for HPLC, and addition of 1 mL acetic acid.

Stock standard solution of oleuropein with a concentration of 1000 ppm was prepared by dissolving 100 mg oleuropein in 100 mL acetonitrile. Six solutions of oleuropein of varying concentrations (3 ppm, 5 ppm, 100 ppm, 300 ppm, 500 ppm, and 800 ppm) were prepared from the stock standard solution by dilution using mobile phase as the diluent. These solutions were used for linearity and range study of the method. For recovery of oleuropein, three solutions of oleuropein spiked in blank (distilled water) at three concentrations (5 ppm, 100 ppm, and 1000 ppm) were prepared. The solutions used for the recovery study were also used for precision study.

To determine the LOD and LOQ of oleuropein using this method, solutions with low concentrations that are expected to produce a response of 3–20 times baseline noise were prepared. LOD is selected as the concentration of oleuropein that gives a signal/noise (S/N) ratio of 3–10, whereas LOQ is selected as the concentration that gives an S/N ratio of 10–20.

3. Results and discussion

3.1. Method development

Preliminary studies involved trying C_8 and C_{18} reversed-phase columns, and testing of several mobile phase compositions were conducted for the separation of oleuropein from other compounds present in olive leaves with good chromatographic parameters (e.g., minimized peak tailing, good symmetry, and good resolution between oleuropein and adjacent peaks). A C_{18} column (5 μm, 150 × 4.6 mm I.D.) as a stationary phase with a mobile phase of acetonitrile/water (20:80, v/v) containing 0.1% of acetic acid at a flow rate of 1.0 mL/min and a detection wavelength of 280 nm afforded the best separation of oleuropein. The acetic acid in the mobile phase gives sharp peaks for oleuropein, whereas the mobile phase without acetic acid gives very broad peaks (low theoretical plates) with very poor resolution. Fig. 2A shows a chromatogram of a standard solution of oleuropein with a retention time of about 16 minutes (Fig. 2A). Fig. 2B shows a chromatogram of oleuropein in a sample of olive leaves obtained from Palestine.

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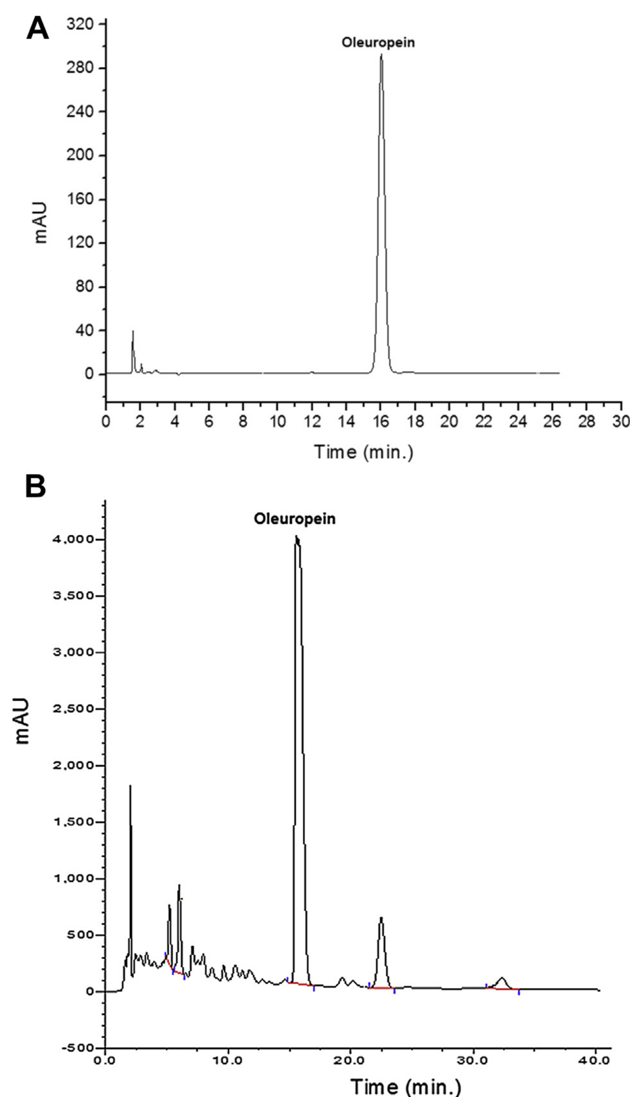

Fig. 2 – Chromatogram of oleuropein analyzed by the current method. (A) Standard of oleuropein. (B) Sample of olive leaves analyzed for oleuropein; other peaks that appear in the chromatogram are for other compounds present in the olive leaves. Mobile phase: acetonitrile/phosphate buffer pH 3.0 (20:80, v/v), flow rate 1.0 mL/min, injection volume 20 μL. Column: C_{18}, 5 μm (5 μm, 150 × 4.6 mm inner diameter), UV detection: 280 nm. [a]Peak asymmetry and theoretical plates of oleuropein peak in standard solution (A) are 1.02 and 3900, respectively. [b]Peak asymmetry, and theoretical plates of oleuropein peak in sample solution (B) are 1.09 and 3100, respectively.

3.2. *Method validation*

After method development, validation of the method for oleuropein was performed in accordance with the requirements for new methods that include accuracy, precision, selectivity, robustness, linearity and range, LOD, and LOQ.

3.2.1. *Linearity and range*

Linearity is the ability of a method to elicit test results that are directly proportional to the analyte concentration within a given range. Range is the interval between the upper and lower levels of analytes that have been demonstrated to be determined with precision, accuracy, and linearity using the method as described. A minimum of five concentration levels, along with certain minimum specified ranges are required. The acceptance criterion for linearity is that the correlation coefficient (r^2) should not be less than 0.990 for the least squares method of the analysis of the line [13].

To evaluate the linearity of the method, different calibration standards of oleuropein were analyzed by HPLC-UV, and the responses are recorded. A plot of the peak areas of the oleuropein versus concentration (in ppm) was found (Fig. 3) to be linear in the range of 3–1000 ppm with $r^2 > 0.995$. This result demonstrates the linearity of this method over a wide dynamic range.

3.2.2. *Accuracy (percentage recovery)*

The accuracy of an analytical method measures the agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and the value found (i.e., accuracy is a measure of the exactness of an analytical method). Accuracy is measured as the percent of analyte recovered after spiking samples in a blank. To document accuracy, a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations, three replicates for each) are collected. Accuracy is performed at three concentrations covering the range of the method. At each level studied, replicate samples are evaluated. The relative standard deviation (RSD) of the replicates provides the analysis variation and gives an indication of the precision of the test method. Moreover, the mean of the replicates, expressed as a percentage of label claim, indicates the accuracy of the test method. The mean recovery of the assay should be within 100 ± 5.0% at each concentration over the studied range [14–16].

For determination of the percentage recovery of oleuropein in olive leaves, it is spiked in distilled water followed by an analysis using HPLC-UV. The average recovery for each level has been calculated by the proportion of the area of the peak of oleuropein resulting from the spiked solution to the area of the peak that resulted from a standard solution. The average

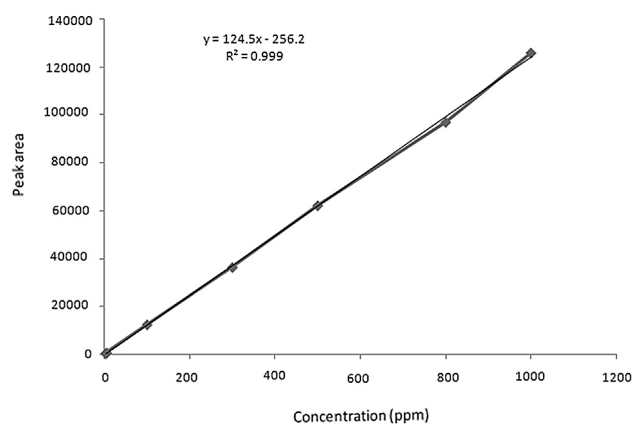

Fig. 3 – Calibration curve for oleuropein determination by the current method (area vs. concentration in ppm).

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recovery and the RSD for each level have been calculated. Results have shown that the current method has a good recovery (from 97.7% to 101.1%) for oleuropein at the three concentration levels studied (5 ppm, 100 ppm, and 1000 ppm), and with an RSD lower than 1.0% (Table 1).

3.2.3. *Precision*

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the RSD for a statistically significant number of samples. There are two types of precision: repeatability and intermediate precision (ruggedness).

(1) Repeatability. This is the closeness of agreement between mutually independent test results obtained with the same method on identical test materials in the same laboratory by the same operator using the same equipment within short intervals of time. It is determined from a minimum of nine determinations covering the specified range of the procedure (e.g., three levels, three repetitions each). RSD for replicate injections should not be greater than 1.5% [17].

Repeatability of the current method for determination of oleuropein was evaluated by calculating the RSD of the peak areas of six replicate injections of three standard solutions with three concentrations (5 ppm, 100 ppm, and 1000 ppm), which was found to be less than 1.0% (data not shown). These results show that the current method for determination of the oleuropein is repeatable.

(2) Intermediate precision (ruggedness). The intermediate precision (also called ruggedness) of a method measures the repeatability of the result obtained with the same method, on the same sample, in the same laboratory, but conducted by different operators and in different days. The intermediate precision of the current method was evaluated by calculating the % recovery of oleuropein at three concentration levels (5 ppm, 100 ppm, and 1000 ppm) by another analyst in a different day. Results of this study showed that the % recovery obtained by the second analyst is comparable to that obtained by the main analyst and ranges from 98.6% to 102.4% (data not shown), indicating that this method is rugged.

3.2.4. *Selectivity*

Selectivity is the ability to assess unequivocally the analyte in the presence of other analytes and other components that may be expected to be present in the matrix or sample [18]. It is a measure of the degree of interferences from such components, ensuring that a response is due to a single component only. The selectivity of the current method was demonstrated by a good separation of oleuropein from other compounds present in olive leaves with good resolution (resolution between oleuropein peak and the adjacent peak is 2.6) Fig. 2 shows a chromatogram of oleuropein analyzed in olive leaves.

3.2.5. *Robustness*

Robustness measures how a method stands up to slight variations in the operating parameters of the method such as flow rate, wavelength, and % of mobile phase composition. The robustness of the current method was investigated by analysis of oleuropein (standard and sample) using the same method developed in this study but deliberately changing one chromatographic condition each time. The chromatographic conditions that were changed are (1) flow rate (0.8 mL/minute and 1.2 mL/minute vs. the original flow rate of 1.0 mL/min), (2) volume fraction of acetonitrile (18% and 22% vs. the original percentage of 20%), and (3) wavelength (278 nm and 282 nm vs. the original wavelength of 280 nm). Results have shown that separation is not affected by slightly changing the chromatographic conditions; the resolution between oleuropein and an adjacent peak remained at about 2.5. Additionally, the recovery of oleuropein at three concentration levels was not significantly affected by changing the chromatographic conditions (flow rate, % of acetonitrile, and wavelength; Table 2).

3.2.6. *LOD and LOQ*

LOD is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. It can be determined by preparing a solution that is expected to produce a response that is about 3–10 times the baseline noise. The solution is injected three times, and the S/N ratio for each injection is recorded. The concentration of the solution is considered an LOD if the S/N ratio is between 3 and 10. LOQ can be determined in the same manner but with an S/N ratio of 10–20.

The LOD and LOQ of oleuropein using this method were determined by preparing dilute solutions of oleuropein (1 ppm, 2 ppm, 3 ppm, 4 ppm, and 5 ppm) and injecting these solutions into the liquid chromatograph and recording the S/N ratio for oleuropein peak at each concentration. LOD was selected to be the concentration that gives a S/N ratio between 3 and 10, whereas LOQ was selected to be the concentration that gives a S/N ratio between 10 and 20. Results have shown that the LOD and LOQ of oleuropein are 3 ppm and 5 ppm, respectively. The low LOD and LOQ permit the determination of oleuropein in olive leaves at low concentrations.

Table 1 – Percent Recovery of oleuropein at three concentration levels (5 ppm, 100 ppm, and 1000 ppm).

		% recovery	Mean	SD	RSD (%)
Concentration (ppm)	5	98.5, 97.7, 99.1	98.4	0.70	0.71
	100	100.5, 101.1, 99.3	100.3	0.92	0.92
	1000	101.1, 100.7, 99.8	100.5	0.67	0.67

RSD = relative standard deviation; SD = standard deviation.

Table 2 – Robustness testing of the method for determination of oleuropein.

Parameter	% Recovery		
	Concentration (ppm)		
	5.0	100.0	1000.0
Flow rate (mL/min)			
0.80	99.7	101.3	100.5
1.2	101.5	99.1	100.5
% Acetonitrile			
18	101.1	99.6	99.8
22	99.3	98.6	98.5
Wavelength (nm)			
278	100.5	102.1	101.1
282	100.1	100.5	99.6

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4. Conclusions

A simple, accurate, precise, and selective HPLC method was developed and validated for the determination of oleuropein in olive leaves. The method is linear for the determination of oleuropein with a wide dynamic range (3–1000 ppm). This method is also accurate, where the % recovery of oleuropein is within 97.7–101.1%. The precision of the method is confirmed by the low RSD of replicate injections of oleuropein. The method shows a good separation of oleuropein from other compounds in olive leaves with good resolution. Low LOD and LOQ of oleuropein enable the detection and quantitation of oleuropein in olive leaves at low concentrations.

Conflicts of interest

All authors declare no conflicts of interest.

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