DETERMINATION OF OLEUROPEIN IN HERBAL PREPARATIONS AND *OLEA EUROPAEA* L. EXTRACTS BY HPLC

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ABSTRACT

Oleuropein is a secoiridoide monoterpene. It is found primarily in all parts of the *Olea europaea* L. tree, mostly in its unripe fruit and leaves. It has a number of beneficial effects on the human body, being readily absorbed from the gastrointestinal tract due to its hydrophilic properties.

The paper presents a simple, rapid and precise method for the assay of oleuropein in herbal formulations and the European Olive leaf extract. Chromatographic separation was performed using a C-18 column with an acetonitrile/water 20/80% vol. mixture as the mobile phase. The method has a wide range of linearity between 0.05 and 1.5 mg/mL, very good repeatability of retention time, and accuracies between 95.8 and 103.3%. Its limit of detection is 0.04 mg/mL. The developed method was tested on two herbal formulations which are commercially available in Poland.

Keywords: oleuropein, *Olea europaea* L., herbal products, high performance liquid chromatography

INTRODUCTION

Oleuropein (fig. 1) is a monoterpene with an O-glycosidic bond-linked sugar molecule [1].

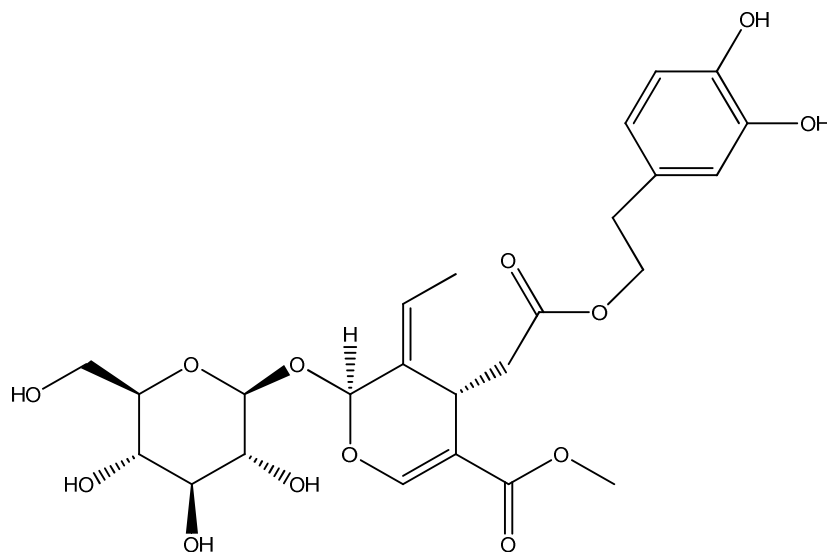[*] Corresponding author: Marcin Szymański; e-mail: mszymanski@ump.edu.pl

Fig. 1. Oleuropein

As well as hydroxytyrosol, oleuropein is responsible for the pungent and bitter taste of extra virgin olive oil [2].

Both oleuropein and olive leaf extract have a number of proven pharmacological properties, such as antioxidative [3, 4], anti-inflammatory [5], hypotensive and cardioprotective [6-8], anti-sclerotic [4, 9-14], anti-cancer [15-20], antibacterial [21-25], hypoglycaemic [26-27], antiviral [28-31] properties.

Oleuropein may be assayed by HPLC as specified in the Polish Pharmacopoeia VIII with 1% aqueous glacial acetic acid (phase A) and methanol (phase B) as the mobile phase. The oleuropein content is calculated by comparing the oleuropein peak area in the test solution chromatogram and that in the standard solution chromatogram. The calculation formula is the following [32]:

$$X = (A_2 \, m_2 \, p \, 8)/(A_1 \, m_1)$$

A_1 – oleuropein peak area in the test solution chromatogram,
A_2 – oleuropein peak area in the standard solution chromatogram,
m_1 – mass of the test substance, grams,
m_2 – mass of oleuropein in the standard solution, grams,
p – percentage of oleuropein in the oleuropein standard.

EXPERIMENTAL

Chemicals and reagents

Oleuropein from ChromaDex, USA and solvents: methanol (HPLC grade) from POCh, Poland, and acetonitrile from J. T. Baker, USA, were used in the investigation.

Formulations tested

Two products registered in Poland as dietary supplements (labelled S1 and S2) and European olive leaves collected in Greece in 2009 (labelled L) were used.

S_1 – capsules with a content of 400 mg of dry *Oleae folium* 6:1 extract per 1 capsule, as declared by the manufacturer.

S_2 – capsules with a content of 380 mg of dry olive leaf extract, as declared by the manufacturer.

Standards and samples

A stock solution (5 mg/mL) was prepared by dissolving 0.0050 g of oleuropein in 1 mL of methanol. Standard solutions were prepared by serial dilution of the stock solution.

Appropriate weighed amounts of samples (S1, S_2, L) were added to conical flasks, approx. 8 mL of methanol was measured and the samples were placed in an ultrasonic bath at 30°C for 40 min. Subsequently, they were transferred without filtration to 10 mL volumetric flasks and the flasks were filled to the mark.

Equipment

A Hewlett Packard Liquid Chromatograph, model HP 1050 (Waldbronn, Germany) with a quaternary pump was used with a variable-wavelength UV detector operating at 254 nm and a Rheodyne model 7125 injection valve with a 20 µL sample loop. Separation was performed on a LiChrospher, RP-18 (5 µm, 250 mm x 4.6 mm I.D.) stainless steel column (Merck, Germany). Before use, the mobile phase was vacuum-filtered through a 0.45 µm cellulose filter and degassed with helium.

The water was distilled and then purified using a Milli-Q water purification system (Millipore, USA).

Analytical balance: Sartorius analytic A200S (USA).

Chromatography conditions

The mobile phase consisted of 20/80 vol. % of acetonitrile/water. The mobile phase flow rate was 1 mL/min. Peaks were monitored at 254 nm.

Analytical method validation parameters

Calibration curves

Calibration curves were obtained based on the results of chromatographic analysis of oleuropein standard solutions. The resulting solutions contained: 0.05; 0.10; 0.25; 0.50; 1.00; 1.50 mg/mL. Each standard solution was injected six times. Regression equations were calculated in the form of $y = ax + b$, where y and x were the peak areas and sample concentrations, respectively.

Repeatability

The repeatability of the chromatographic system was assessed under the chromatographic conditions previously selected by means of 10 replicate injections of the solution containing 0.10 mg/mL of oleuropein.

Limit of Detection and Quantification

The limit of detection (LOD) under the present chromatographic conditions is defined as a concentration of the analyte giving a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) is the lowest analyte concentration which can be assayed with the required precision and accuracy. Based on six parallel results obtained for each standard solution concentration, relative standard deviations were derived and LOQ for RSD = 10% was derived from the relationship between RSD and test glycoside concentrations.

Accuracy

The accuracy of the method was determined by recovery experiments. The recovery value was determined by adding known quantities of the test compounds to the test formulations (standard addition method). The recovery of the standards added (oleuropein) was measured three times for each concentration level, corresponding to approx. 120% of the analyte in the sample. Subsequently, they were assayed in the test formulations. Each sample was tested six times at each concentration level according to the above procedure.

RESULTS AND DISCUSION

Optimization of Chromatographic Analysis

Reversed-phase chromatography was suggested as a suitable procedure for determination of oleuropein in complex matrices, such as pharmaceutical formulations. The optimization of the chromatographic process involved, first, determination of the detection wavelength and next, selection of mobile phase parameters suitable for the total resolution of oleuropein as rapidly as possible, with no interference from other ingredients of the test samples.

An example of the chromatograms obtained for the oleuropein standard is shown in Figure 2. The retention times of oleuropein were 22.923 ± 0.022 min.

We note a very good peak symmetry and also the fact that retention of the test compounds is repeatable with very good precision (low RSD values).
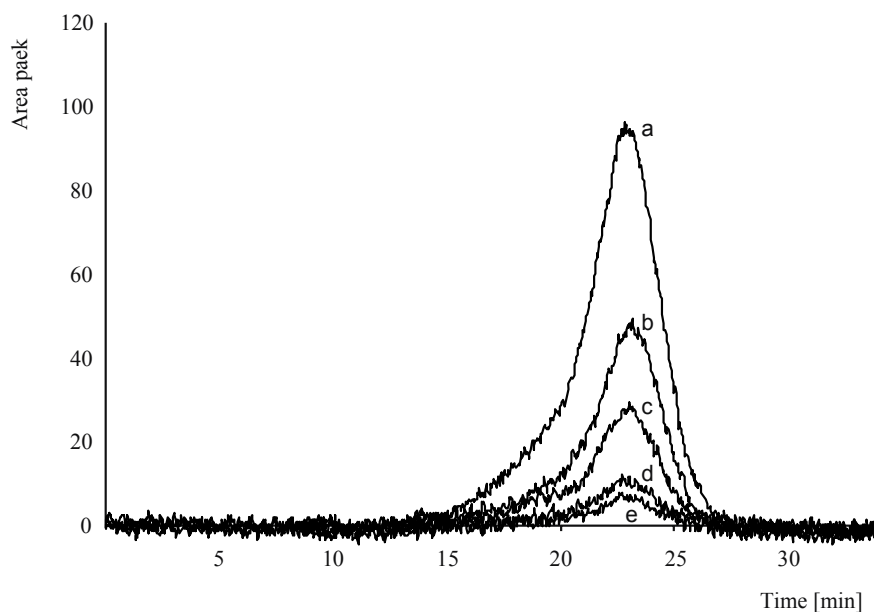
Fig. 2. Chromatograms of oleuropein for standard solutions with different analyte concentrations: a) 1.0; b) 0.5; c) 0.25; d) 0.10; e) 0.05 mg/mL

Assay validations

Linearity of the method was evaluated in a concentration range from 0.05 to 1.5 mg/mL by injecting the standard solutions in the chromatography conditions listed above. A calibration curve for oleuropein was determined using the least-squares method. Table 1 shows the linearity range tested and the resulting curve parameters with the correlation coefficient, the measure of linearity.

Table 1. Calibration range and fitted parameters

Linearity range [mg/mL]	Slope	Intercept	Correlation coefficient
0.05 – 1.5	67.598	0.839	0.9991

In determination of oleuropein, the calibration curves were found to be linear in the concentration range in question and the correlation coefficients for the regression lines were 0.9991.

Repeatability, expressed as the relative standard deviation determined for the analyte, was the RSD.

The limit of detection for oleuropein was 0.04 mg/mL, and the limit of quantification was 0.12 mg/mL.

The method was used for determination of oleuropein in two herbal products available in Poland and in the leaf extract of *Olea europaea*. Preparation of

analytical samples is discussed in the experimental part. The results of analyses calculated from calibration curve equations are shown in Table 2.

Table 2. Oleuropein content in the supplements and *Olea europaea* L. leaves (n = 6)

	Content, as declared by the manufacturer	Mean mass	SD	RSD [%]
S 1 [mg oleurop./capsule]	400[a]	48.8	1.5	3.1
S 2 [mg oleurop./capsule]	380[b]	13.1	0.3	2.4
Olive leaf [mg oleurop./g dw[c]]	-	12.2	0.2	1.7

[a] content of dry *Olea europaea* L. (6:1) extract in 1 capsule;
[b] content of dry *Olea europaea* L. extract in 1 capsule;
[c] dw: dry weight.

To account for the inconsistencies and to verify the correctness of our method, an experiment was performed to determine analytical accuracy. A solution of the test product was prepared with analyte concentration x within the range of concentrations used for the calibration curve and three series of identically prepared solutions to which oleuropein with a known concentration, a, was added. The value of "a" for oleuropein in pharmaceuticals (S1, S2) and *Olea europaea* extracts (L) was 0.4 mg/mL. Three series of solutions with concentrations x + a were thus obtained. Determinations were performed for all the test products and both analytes (six independent analyses for each series of solutions in the same conditions). Four series of results were obtained, x and x + a, as listed in Table 3.

Table 3. Accuracy of oleuropein assay in the test products (n = 6)

sample	Analyte concentration c [mg/mL]	Analyte concentration (spiked with the standard) c+a [mg/mL]	Determined (c+a)-c [mg/mL]	Added a [mg/mL]	Recovery %
S1	1.0318	1.4233	0.3915	0.4	97.9
S1	1.0054	1.4186	0.4132	0.4	103.3
S1	1.0127	1.4114	0.3988	0.4	99.7
S2	0.2554	0.6386	0.3832	0.4	95.8
S2	0.2686	0.6575	0.3889	0.4	97.2
S2	0.2586	0.6579	0.3993	0.4	99.8
L	0.1123	0.5082	0.3959	0.4	99.0
L	0.1100	0.5057	0.3957	0.4	98.9
L	0.1134	0.5169	0.4035	0.4	100.9

The method accuracy is determined from recovery values for the quantities "a" of oleuropein added to pharmaceutical product solutions. The recovery values are close to 100%, within the range from 95.8 to 103.3 % for oleuropein.

The results shown in the tables confirm that the method for the assay of oleuropein in herbal preparations and *Olea europaea* extracts, developed by the present authors, has both high precision and accuracy.

CONCLUSIONS

A method for the assay of oleuropein in pharmaceutical products using HPLC is proposed. The method has a high precision ranging from 1.7 to 3.1; accuracy from 95.8 to 103.3, and very good repeatability with high precision, as indicated by the low coefficient of variation (below 0.1%). Its limit of detection is 0.04 mg/mL. The results show that the method may be used for controlling the content of oleuropein in pharmaceutical products.

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