

## Development of a Simple Green Extraction Procedure and HPLC Method for Determination of Oleuropein in Olive Leaf Extract Applied to a Multi-Source Comparative Study

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A simple, green and inexpensive water-based procedure was developed to extract oleuropein from olive leaf samples. Extraction was optimized in terms of the solvent, pH of the solvent, temperature and time of extraction. The experimental results revealed that deionised water adjusted to pH 3, at 60 °C for 4 h had the highest extraction efficiency. Samples were lyophilized and stored in tight containers at -18 °C until the analysis. Oleuropein was identified by comparing the retention time of the extracts with standard compound using UV detector at 280 nm. The method of analysis was based on RP-HPLC, with a mobile phase of water (adjusted to pH 3):acetonitrile (80:20 v/v) with a flow rate of 1 ml min<sup>-1</sup>. Linear dynamic range and limit of detection were found to be 50-900 µg ml<sup>-1</sup> and 9.5 µg ml<sup>-1</sup>, respectively. Intra- and inter-day precision of the method were calculated as RSD% of 1.2 and 5.7, respectively. Olive leaves gathered from various cultivars of Iran were analyzed by the method and results showed that olive leaves from Shiraz had the highest oleuropein content (about 13 mg g<sup>-1</sup>). The developed method can be used in industry for proper mass production of this compound that is of great significance in medicine as well as food and cosmetics industry.

**Keywords:** Oleuropein, Olive leaf, Extraction, HPLC

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### INTRODUCTION

Olive tree (*Olea europaea* L., Oleaceae) is one of the most important fruit trees in the Mediterranean countries [1-3]. The leaves and drupes of olive tree are rich in olive biophenols, such as oleuropein, verbascoside, ligstroside, tyrosol and hydroxytyrosol, which have exhibited antioxidant [4-5], and antimicrobial [6-7], properties. Although all parts of olive tree and its oil contain oleuropein [8], the leaves of olive tree are the richest source of this compound (60-90 mg g<sup>-1</sup> dry weight)

[9]. Oleuropein is a heterosidic ester of elenolic diterpene and 3,4-dihydroxyphenylethanol, containing a molecule of glucose, the hydrolysis of which yields elenolic acid glucoside and hydroxytyrosol [10]. Many molecules isolated from *O. europaea* fruits and leaves are thought to have been originated from oleuropein, *via* aglycon, by the opening of the elenolic acid ring with a final rearrangement into the secoiridoid compound, many forms of elenolic acid and simple phenolic compounds, such as hydroxytyrosol [11-12].

Phenolic compounds can be extracted by different methods from the leaves. Extracting procedures such as superheated liquid extraction using aqueous or organic solvents at a high pressure and temperature without reaching the critical point

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[13], supercritical fluid extraction [14], liquid-liquid extraction [15], derivatized polar extraction [16], fractionation by solid-phase extraction [17], dynamic acidified pressurized hot water extraction [18], and dynamic ultrasound-assisted extraction [19] have all been used to extract oleuropein and other phenolic compounds from olive leaves.

The level of phenolic compounds is a very important parameter in the evaluation of olive leaf extract (OLE) quality, since, phenols are closely related to both the OLE resistance to oxidation because of their antioxidative properties [3-5], and the typical bitter taste of OLE [2]. The concentration of oleuropein varies with the olive varieties. Therefore, it is of great relevance to assay oleuropein in OLE, both qualitatively and quantitatively. The level of the phenol content is determined by chromatography using diode array detector/mass spectrometry (DAD/MS) [16], gas chromatography (GC)-MS [20], reversed phase (RP) high performance liquid chromatography (HPLC)-MS [21], electrospray mass ionization spectrometry [22], GC-selective ion monitoring (SIM)-MS [21], reversed phase HPLC with fluorescence detector [7], as well as liquid chromatography-tandem mass spectrometry [23]. The analysis of oleuropein in olive oil [24], simultaneously with tyrosol in plasma [25], in the leaves [26-28], and branches [27-29] were performed by RP-HPLC [30-32].

The aim of this study was to develop and optimize a green, simple, reliable, reproducible, and inexpensive procedure for the extraction of oleuropein as a main phenolic component of

olive leaves using non-toxic extractants such as water at moderate temperature. Another aim of the study was to develop a simple and reliable HPLC method for the determination of oleuropein in the extract. Finally, this method was used for the analysis of olive leaf extracts from different areas of Iran to compare their oleuropein content.

## EXPERIMENTAL

### Reagents and Plant Materials

Organic solvents used for leaf extraction were of analytical grade and purchased from E. Merck AG (Darmstadt, Germany). Acetonitrile used for HPLC analyses was of chromatographic grade and purchased from E. Merck AG (Darmstadt, Germany). Ultrapure water used for the preparation of solutions, dilutions, and mobile phase was produced by a Milli-Q system (18.2 M, Millipore, Bedford, MA, USA). Standard oleuropein was purchased from Roth (Germany). Oleuropein stock solution was prepared by dissolving 100 mg oleuropein in 100 ml deionised water and stored at 2-8 °C. The standard working solutions at the concentration of the calibration range were prepared by serial dilutions of stock solutions with deionised water. Olive leaves used in this research were collected from different areas of Iran, including Roudbar, Behshahr, Shiraz, Amol, Sarpol-e-Zahab, Paveh, Kermanshah, and Kerman whose geographical and climatic characteristics are shown in Table 1.

**Table 1.** Climatic Characteristics of Olive Leaf Cultivars and Their Oleuropein Content in Iran

| Cultivars      | Mean annual temp. (°C) | Annual rainfall (mm) | Altitude (m) | Geographical latitude (min:s) | Geographical longitude (min:s) | Oleuropein content (mg g <sup>-1</sup> ) <sup>a</sup> |
|----------------|------------------------|----------------------|--------------|-------------------------------|--------------------------------|---|
| Sarpol e Zahab | 20.4                   | 467.0                | 545          | 34:27                         | 45:52                          | 6.1 ± 0.7   |
| Kermanshah     | 14.4                   | 450.8                | 1322         | 34:17                         | 47:07                          | 6.4 ± 1.4   |
| Kerman         | 16.9                   | 142.0                | 1754         | 15:30                         | 56:58                          | 10.2 ± 1.0  |
| Shiraz         | 17.8                   | 344.7                | 1488         | 29:36                         | 52:32                          | 13.0 ± 1.9  |
| Paveh          | 14.9                   | 778.4                | 1485         | 35:03                         | 46:20                          | 7.4 ± 1.8   |
| Amol           | 20.0                   | 780.0                | 2370         | 36:28                         | 52:23                          | 6.5 ± 3.7   |
| Behshahr       | 17.7                   | 567.0                | -14          | 36:45                         | 53:44                          | 9.6 ± 0.3   |
| Roodbar        | 15.5                   | 650.0                | 338          | 36:44                         | 49:25                          | 9.0 ± 0.9   |

<sup>a</sup>Oleuropein content of olive leaf extract measured as mg of oleuropein per gram of dried leaf powder.

### Instrumentation

An HPLC system comprising of a Breeze System P1525 pump (Waters, USA) binary pump P1525 equipped with a manual injector (Rheodyne, Rohnert Park, CA, USA) and a 20- $\mu$ l loop, coupled to a Breeze Dual Lambda System UV2487 detector ( $\lambda = 280$  nm) was utilized. The whole process was computer-controlled by the Breeze program through the Breeze controller. Chromatographic separation was performed on a reversed-phase Nucleosil<sup>®</sup> 100-5 C18 (length 250 mm, i.d. 4.6 mm) column (Perker Style, Ireland) equipped with an ACE 5 C18 (50  $\times$  4.6 mm, Scotland) pre column.

### HPLC Analysis of the Oleuropein and OLE

An isocratic elution was used for the analysis of oleuropein. The mobile phases used were acetonitrile: water with different ratios to obtain optimum condition for the analysis of oleuropein in OLE. The flow-rate was set at 1 ml  $\text{min}^{-1}$  at all conditions. At the end of each run, usually set at 10 min, the column was left to be washed for an additional time of 5 min, giving a total chromatographic analysis time of 15 min. All mobile phases were vacuum-filtered through a 0.2- $\mu$ m PTFE membrane filter (Sartorius, Germany) and degassed in an ultrasonic bath prior to HPLC analysis. The column was maintained at ambient temperature throughout all the experiments. The injection volume was 20  $\mu$ l. Each standard solution and sample solution was analyzed in triplicate.

### Validation of the Assay Method

At the beginning and during every laboratory day, two standard oleuropeins at 300 and 800  $\mu\text{g ml}^{-1}$  levels, serving as system-suitability working standard solutions, were injected for assessing the performance of the chromatographic procedure in terms of retention time stability and signal sensitivity.

### Linearity

For the linearity study, 11 samples of aqueous oleuropein solutions (containing 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000  $\mu\text{g ml}^{-1}$  of oleuropein) were analyzed, and the linearity was checked by performing linear least-squares regression analysis of course from LOQ.

### Precision

The repeatability and reproducibility of the analyses were

evaluated by the overall chromatographic method at all levels in six replicates ( $n = 6$ ) and RSD calculation. In order to evaluate the within-day and between-day precision, six replicates of the standard solutions at three different concentrations (50, 400 and 900  $\mu\text{g ml}^{-1}$ ) were assayed within the same day and three separate days.

### Accuracy

The accuracy of the method was assessed by three quality control standards at three concentration levels, which were different from the calibration curve standards, *i.e.* 50, 400 and 900  $\mu\text{g ml}^{-1}$ , and was evaluated as the relative percentage error.

### Determination of LOD and LOQ

The limit of detection (LOD) is the minimum quantity or concentration that can be distinguished from zero. The limit of quantification (LOQ) is the minimum quantity or concentration that can be evaluated with a certain precision [33]. In this study, 3 and 10-criteria were applied for the calculation of LOD and LOQ values according to guidelines for the validation of analytical methods [33], using the following formula (1):

$$L = kh_{\text{max}}/S \quad (1)$$

where L stands for LOD or LOQ; k is a factor depending on the required limit, whose value is 3 for LOD and 10 for LOQ;  $h_{\text{max}}$  is the mean of maximal signal amplitude of a blank which is equal to 20 times of the peak width at half-height, on at least 5 blanks; S is the slope of a linear regression equation of calibration using 3 different concentrations. The values of limits of detection (LOD) and LOQ were measured according to J. Caporal-Gautier *et al.* [28], where LOD is considered at signal-to noise ratio of 3:1 and LOQ is defined as the concentration that gives rise to a signal, which is 10 times higher than the noise level. Correlation coefficients estimation of both chromatographic methods was made by the punctual procedure of linear regression studies described in statistical literature [34].

### Stability of the Analyte

Known concentrations of oleuropein were prepared and in

order to minimize possible degradation of the analyte, they were stored in dark-colored vials and kept refrigerated at 2-8 °C. Samples were drawn on days 1, 2, 3, 4, 5, 6, 14, 21 and 28 and assayed for their remaining oleuropein content. Logarithm of percent of the remaining oleuropein concentration was drawn against time to determine the first order stability constant of oleuropein.

### Proposed Extraction Procedure

Olive leaves were washed, dried under ambient temperature and milled to 30 mesh powders by an electrical mill, and then were kept at ambient temperature in a dark place. The solvents used for the extraction were deionized water adjusted to various pHs (in each solution containing water, pH was adjusted with HCl 0.1 N solution) at 40 °C, 60 °C and 80 °C, n-hexane at ambient temperature, deionized water/methanol at ratios of 10/90 and 50/50 (v/v), dichloromethane at ambient temperature, ethanol/deionized water at ratios of 80/20 (v/v) and 59/41 (v/v). A quantity of 50 g of olive leaves powder were macerated in 400 ml of the solvents at various times for the extraction. The extracts were then filtered through a Whatman No.1 filter (Whatman, UK) to separate coarse particles from the solutions by a Buchner funnel. The filtered extracts evaporated in a rotary evaporator at room temperature under vacuum (Rotovac, Heidolph, Germany). The concentrated extracts were lyophilized with an Eyela FD-81 freeze dryer. (Rikakikai Co., Ltd., Tokyo, Japan).

The lyophilized extracts were stored in a desiccator at 2-8 °C until use.

## RESULTS AND DISCUSSION

### Optimization and Validation of HPLC Method

The chromatographic conditions were developed and optimized using both oleuropein standards and real olive leaf samples. Because the absorptions of the oleuropein lay in the UV range with  $\lambda$  max of 280 nm, this wavelength was chosen for the detection. The experimental variables, optimized in order to obtain an appropriate separation of the analyte peaks in the chromatogram, were the composition and pH of mobile phase to recover oleuropein in a proper time and to separate it from all other components in the OLE. The number of theoretical plates calculated for the column used at each pH and composition of mobile phase, the resolution factor ( $R_s$ ) and analysis time were selected as criteria for optimization. The separation for which various combinations of water/acetonitrile (acidified to different pHs with HCl) was used, produced the best sensitivity, efficiency and peak shape. Results indicated that the optimum mobile phase composition and pH were deionized water/acetonitrile 80/20 (v/v), and 3, respectively. The chromatogram of the standard oleuropein solution is shown in Fig. 1.

The oleuropein peak can be well-separated from all the other peaks in OLE ( $R_s > 1.3$ ) as illustrated in Fig. 2.

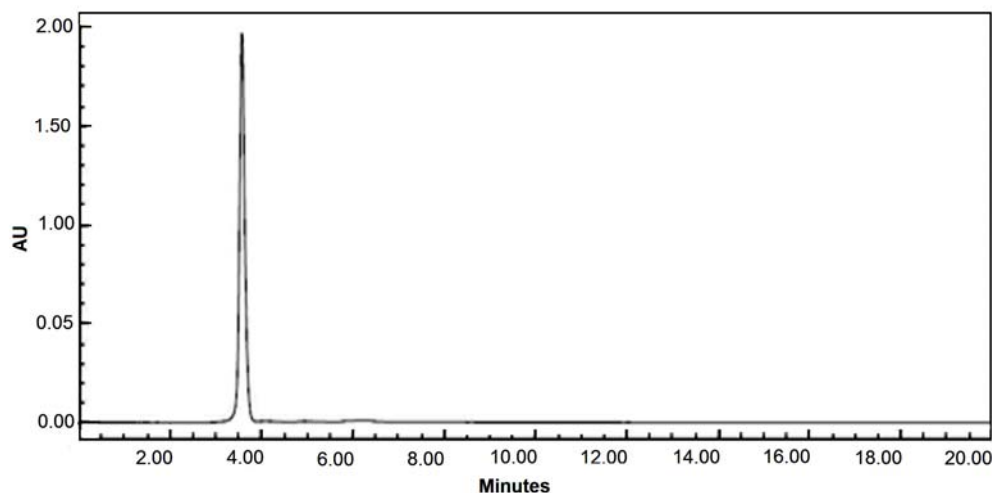


Fig. 1. Chromtogram of oleuropein standard solution (500 mg l<sup>-1</sup>).

## Development of a Simple Green Extraction Procedure

Oleuropein and other ingredients were identified on the basis of the elevation of AUC, after spiking the components that were assumed to be present with their known concentrations as shown in Fig. 2.

Under the chromatographic conditions used in this study, calibration curves exhibited good linearity from regression analysis ( $r^2 > 0.991$ ). The regression equation obtained was  $Y = 2633.8X + 9000.8$ . The calibration curve for the oleuropein was linear within the range of 50-1000  $\mu\text{g ml}^{-1}$ . The linearity of the calibration curve was verified by the correlation coefficient as well as visual inspection. The detection limit for oleuropein, defined as a signal-to-noise ratio of 3 [23], was 9.5  $\mu\text{g ml}^{-1}$ . The value was more sensitive than those reported in the literature using HPLC [13,15,23], super-heated liquid extraction [13], and dynamic ultrasound-assisted extraction [15]. The RSD% of retention times and peak areas were 3.9 and 5.7% for precision, 3.4 and 6.1% for reproducibility, and 4.5% and 5.8% for stability, respectively. The recoveries were within the range of 119.9-120.1% with an RSD% of 3.4%, demonstrating superior efficiency of the method in both recovery and accuracy. All the results revealed that the developed analytical method was reproducible with good precision.

### Effect of Extraction Solvent on Oleuropein Content

Various types of solvents were used to extract oleuropein from olive leaves. Higher oleuropein levels were observed regarding the solvent containing deionised water at 60 °C (Fig. 3a) because it is a water-soluble phenolic compound whose solubility can be increased by elevating the temperature.

Addition of more lipophilic solvents such as methyl or ethyl alcohol to the water decreases water efficiency in extracting oleuropein from the leaves. As can be seen clearly in Fig. 3a, the use of lipophilic solvents such as n-hexane and dichloromethane is of no help to extract oleuropein from the olive leaves. Figure 3b shows that optimum pH of extraction can be obtained at pH 3. The extraction of oleuropein at this pH is about 1.5 to 10 times more efficient than the other pHs examined in this study.

### Effect of Extraction Time on Oleuropein Extraction Yield

Olive leaves powder was macerated in deionised water at

60 °C (as the most efficient solvent that has been described earlier) at various times including 1, 2, 4, 6, 8 and 24 h. Extraction yields were calculated at each time as mg of oleuropein extracted from each gram of dried powder of olive leaf. Results showed that the maximum extraction yield was produced within 4 h. Beyond it, the extraction yield decreased which was probably due to the decomposition of oleuropein at 60 °C, because oleuropein is a phenolic molecule (catecholic structure) [30] that is very sensitive to oxidation. Considering the results at this stage, subsequent extractions were made within 4 h.

### Effect of pH on Oleuropein Extraction

To optimize the extraction of oleuropein by aqueous solvents, olive leaves powder was extracted with deionised water at 60 °C and various pH values. Higher yields of oleuropein were observed at an optimum pH of 3 that may be related to lower degradation of oleuropein at this pH [15,16], while the use of higher or lower pHs, caused a significant decrease in the yields of oleuropein.

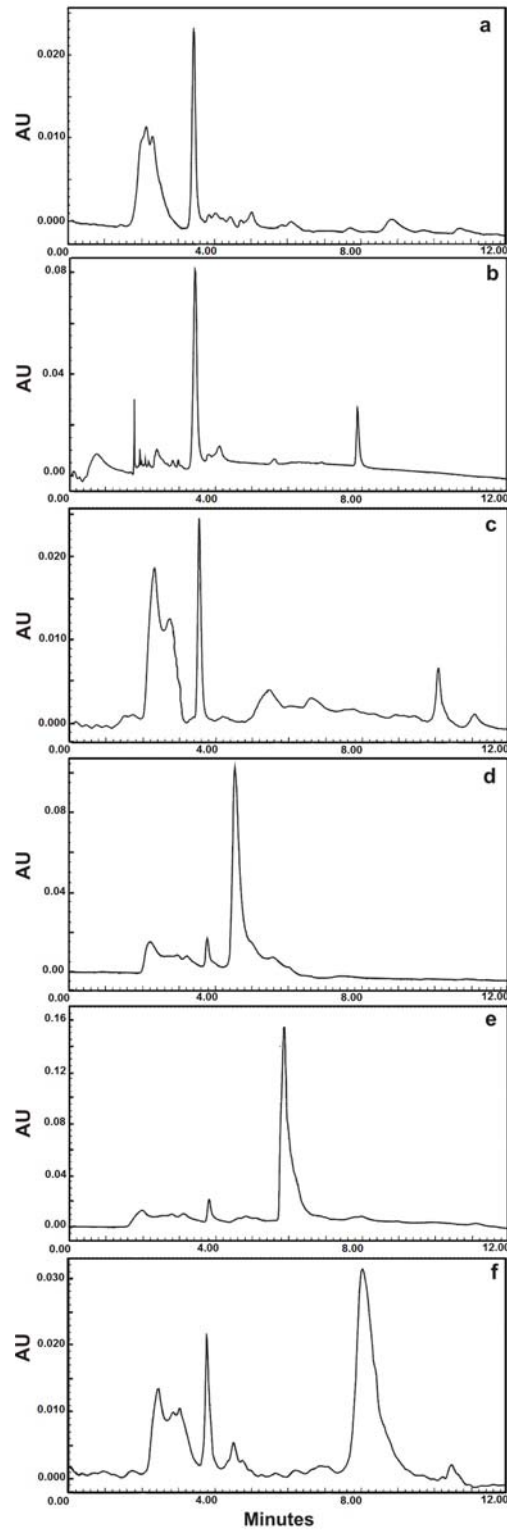
### Stability of Oleuropein Standard Solutions

The standard solution of oleuropein (500  $\mu\text{g ml}^{-1}$ ) was stored at 4 °C and in the dark. It was analyzed during one month of storage for its oleuropein content. On the basis of its calculated shelf-life, oleuropein was stable at least for a month under these conditions and could be used confidently in the analysis of oleuropein.

### Quantification of Oleuropein in the Samples

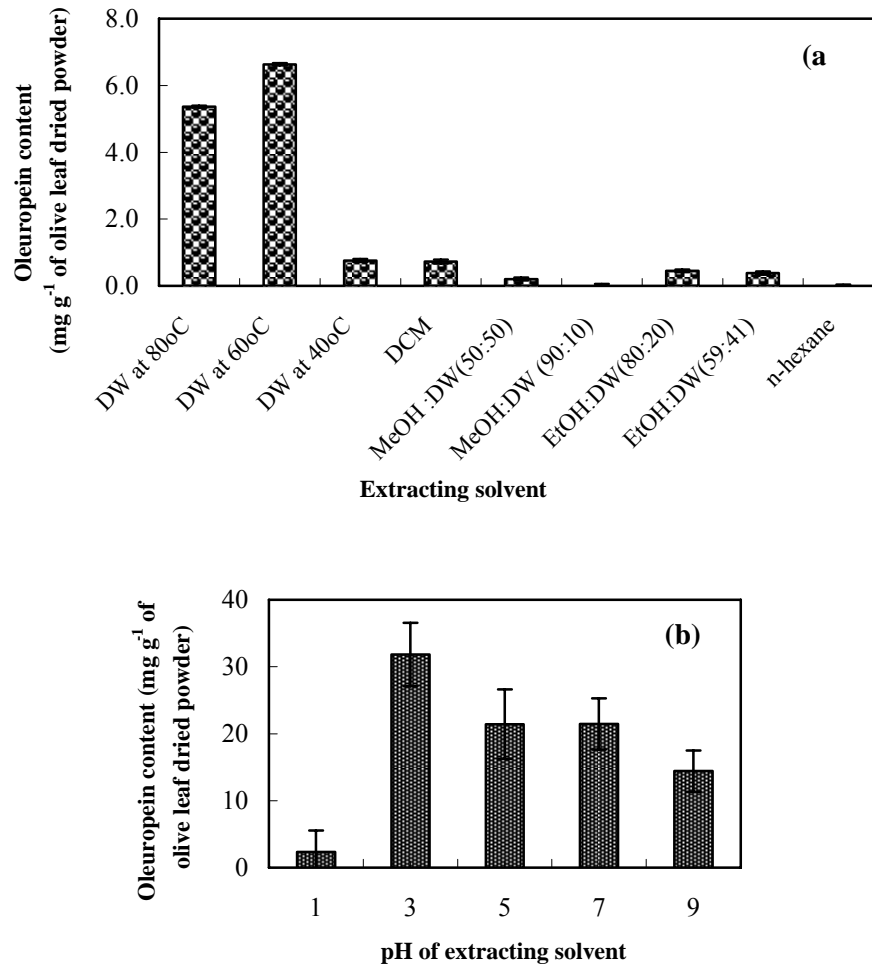
The developed method was subsequently applied to determine the oleuropein contents in different olive leaf samples prepared from various cultivars of olive tree in Iran. Representative chromatograms of the extracts of these olive leaf samples are shown in Fig. 4, and the observed yields of oleuropein have been summarized in the last column of Table 1.

These data indicate that the contents of oleuropein in olive leaves obtained from different geographic origins vary over a range from 6.1 to 13.0  $\text{mg g}^{-1}$  of olive leaf dried powder. It was found that the contents of oleuropein in 8 types of olive leaves cultivated in various provinces of Iran, whose climatic conditions have been summarized in Table 1, correlate with



**Fig. 2.** Chromatograms of a) unspiked extract; b) extract spiked with oleuropein (RT: 3.5 min); c) extract spiked with luteolin-7-glucoside (RT: 2.2 min); d) extract spiked by hydroxytyrosol (RT: 4.0 min); e) extract spiked by apigenen-7-o-glucoside (RT: 6.0 min); f) extract spiked by verbascoside (RT: 7.7 min).

## Development of a Simple Green Extraction Procedure

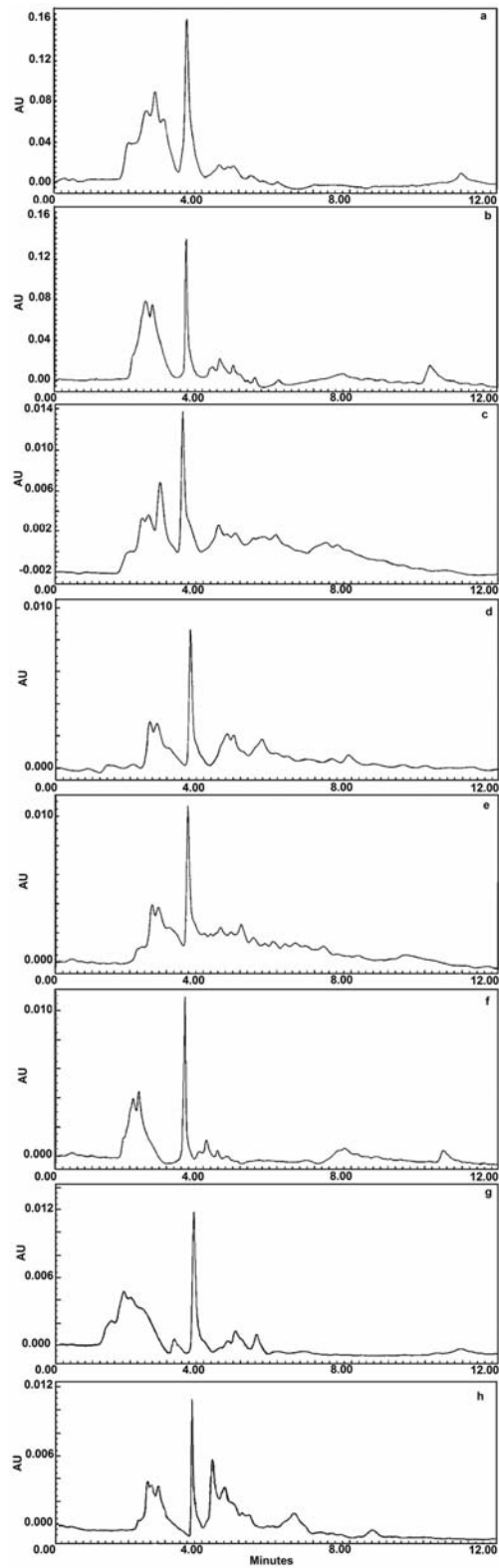


**Fig. 3.** Effect of extracting a) solvent and b) pH on extraction yield of oleuropein from olive leaves.

product origins. Higher levels of oleuropein in OLE were obtained from the leaves in Shiraz, Kerman, Behshahr and Roodbar that are located in the south and north of Iran. Cultivars located in the west of Iran showed a lower amount of oleuropein content. It seems that higher amounts of oleuropein content is obtainable at the annual mean temperature range of 15.5 to 18 °C, and the average annual rainfall does not correlate well with the amount of oleuropein. Therefore, one may assume that many other factors, such as the type of soil and its condition or content, are important parameters in the extraction of oleuropein from olive trees.

## CONCLUSIONS

Although there are many reports and patents for the separation of olive biophenols, particularly oleuropein, from olive leaves [31], there is little or no improvement in the leaching step. Laboratory-scale extraction methods have been reported using leachants, such as methanol/water mixtures [12,31,32,35], and hexane [36], and comparing maceration and Soxhlet extraction [29]. It is worth emphasizing that there is need to avoid using toxic leachants. In this study, a simple environment-friendly method of extraction based on water at



**Fig. 4.** Representative chromatograms of olive leaf extract of various cultivars, a) Amol, b) Behshahr, c) Kerman, d) Pave, e) Kermanshah, f) Sarpol-e-Zahab, g) Roudbar, and h) Shiraz.

## Development of a Simple Green Extraction Procedure

60 °C was developed to extract oleuropein from olive leaves. Optimum temperature, pH, and time of extraction were easily available. A simple, reliable and reproducible HPLC-UV method was developed and validated for the determination of oleuropein in OLE. The time of chromatographic analysis was short enough (about 15 min) to use the method in the quality control of pharmaceutical products containing OLE as a routine procedure compared with some other reports [37]. Analyses of OLE prepared from different areas of Iran to determine their oleuropein content showed that cultivars located in the north and south of Iran afforded the highest amount of oleuropein.

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