



Recovery and stability of oleuropein and other phenolic compounds during extraction and processing of olive (*Olea europaea* L.) leaves

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Abstract

Polyphenols in olive leaves, especially oleuropein, are of great interest to researchers, household consumers and commercial entities due to many health benefits of these compounds. Various processing and extraction methods were investigated to evaluate stability and recovery of oleuropein and other polyphenols from olive leaves. Brief thawing of frozen leaf samples (5 minutes) caused a sharp reduction in extractable oleuropein levels (57.7%), and 53.5% loss in oleuropein occurred when frozen leaf powder was thawed for only 2 minutes. Simple drying of fresh leaves at room temperature (25°C) fully preserved oleuropein and verbascoside levels while drying at an elevated temperature of 60°C resulted in losses at various levels of all polyphenols studied. While extraction in 80% methanol is the most effective method for olive leaf polyphenols for laboratory use, boiling of dried leaves was also a very efficient method for extracting oleuropein and verbascoside that gave 96 and 94% recoveries of these compounds, respectively, when compared with the methanol extract. Oleuropein was quite stable in aqueous extracts for 7 days when stored at room temperatures but degraded after 17 days. Other polyphenols were less stable in aqueous extracts and started to show some degree of degradation after 7 days (little change occurred during the first 24 h storage at room temperature) and were completely degraded after 17 days. On the other hand, oleuropein and other polyphenols in methanol extract were quite stable for 30 days when stored at room temperature. The studies provide important information for efficient and effective processing and extraction of olive leaf polyphenols for research, home and commercial use.

Key words: *Olea europaea*, oleuropein, verbascoside, polyphenols, olive leaves.

Introduction

Lower incidences of coronary diseases and some forms of cancer in Mediterranean countries such as Greece, Italy and Spain are generally attributed to high consumption of olive oil¹⁻³. It is also well known that a majority of health benefits credited to olive oil are in fact due to the presence of a variety of phenolic compounds⁴⁻⁸. Phenolic compounds that are mostly polar in nature find their way in olive oil because it is pressed out like a juice and is not extracted by lipophilic solvents that are commonly used for the extraction of other cooking oils such as corn, canola and soybean oils⁸.

Oleuropein is the major component of olive polyphenols and is extensively studied for health benefits concerning a variety of ailments such as blood pressure, cancer, heart problems and an array of viral and bacterial diseases⁹⁻¹³. Oleuropein is most abundant in developing fruits but its concentration sharply declines when fruits begin to mature¹⁴. Thus, olive oil which is pressed from mature fruits contains very small amounts of oleuropein¹⁵⁻¹⁸. On the other hand, oleuropein is the most abundant polyphenol in olive leaves and health benefits of leaf extracts are well documented^{1, 14, 19-22}. Thus to harness the benefits of olive polyphenols several preparations of olive leaves and their extracts are sold in the market at premium prices.

Several extraction and processing methods to optimize oil and polyphenols extractions for laboratory research and commercial

preparations have been investigated^{7, 21, 23-28}. However, there is a paucity of knowledge regarding stability of oleuropein and other polyphenols in leaves during processing and extraction of olive leaves for home consumers to benefit from valuable polyphenols at reasonable costs. In addition, during the course of research regarding the role of olive polyphenols in developmental processes we observed large inconsistencies in results even with slight improper handling of samples. A preliminary survey of commercial leaf products also showed inconsistencies between the claimed and actual levels of oleuropein in some products indicating problems in controlling stabilities or recoveries during extraction and processing procedures. These studies were therefore conducted to provide guidance regarding recovery and stability of oleuropein and other polyphenols of olive leaves, during processing and extraction of olive leaves by researchers, home consumers and by commercial entities.

Materials and Methods

Sampling and initial processing of samples before extraction:

Leaf samples for various extractions and processing experiments were collected from 5-6 year old trees planted at the USDA-ARS south farm in Weslaco, Texas. Fully expanded leaves were hand picked and brought on ice to the laboratory to process for different experiments. Portions of leaves were immediately stored at -80°C

(Revco, Model ULT2186-3-A36) and other portions were dried either at room temperature (25°C) or in convection ovens (Blue M, Model M01450A and Precision Scientific model STG 40) at various temperatures. Leaf samples were dried for various lengths of time and then immediately stored at -80°C until used for further processing or extraction. For thawing experiments, leaves were frozen immediately after harvesting and were stored at -80°C until used for thawing at room temperature for different lengths of time. Thawed samples were again placed back at -80°C until needed for extraction. For the 2 min thawing experiment, the frozen samples were first pulverized in liquid nitrogen, the powder was thawed for two min at room temperature and then immediately extracted. All plant samples (including dried at 25-130°C) were powdered according to our standard pulverization technique in liquid nitrogen reported previously²⁹. In drying experiments, moisture was determined for each sampling point and used to correct extraction results by accounting for the amount of water lost during drying.

Commercial products were stored at room temperature and directly extracted and analyzed. The following products were tested in the preliminary survey: ‘Olive Leaf’ (premium extract, standardized) by Nature’s Way Product, Inc., Springville, Utah, USA; standardized ‘Olive Leaf’ extract by Rexal, Inc., Boca Raton, Florida, USA; Herbals ‘Olive Leaf Extract’ by Bluebonnet Nutrition Corporation, Sugar Land, Texas USA; ‘Olive Leaf’ herbal supplement by General Nutrition Corporation, Pittsburgh, Pennsylvania, USA; liquid phyto-caps ‘Olive Leaf’ by Gaia Herbs, Inc., Brevard, North Carolina, USA. These products, however, will not be identified next to our analytical results because it is not the policy of USDA-ARS to endorse or disapprove commercial products.

Extraction methods: Powdered leaf samples (0.5 g) were extracted either in 50 ml of 80% methanol or in same volume of boiling water; in one experiment they were extracted in a coffee maker. For extraction in the coffee maker 1.0 g of leaf powder was extracted with 100 ml of water. This gave about 80-90 ml of extract which was adjusted to 100 ml giving the same weight volume ratio as in other extracts. Details of extraction method in 80% methanol have been described before¹⁴. For extraction in boiling water, frozen powder was weighed frozen and then immediately transferred to boiling water containing glass beads that kept plant particles stirring during extraction. Boiling was continued for a period of 10 min and then the extracts were placed on ice and filtered through a glass wool plug in a glass funnel. All commercial products were extracted in 80% methanol by our standard extraction method.

Quantitative analyses: All extracts were filtered through 0.45 µm filters before analyses by HPLC which were performed using the Waters (Milford, MA) Alliance HPLC system (Model 2695) equipped with photodiode array detectors (Model 2996). Details of quantitative HPLC analyses have been described previously¹⁴. Oleuropein, luteolin-7-O-glucoside, verbascoside and luteolin-4-O-glucoside standards were purchased from Extrasynthese, Genay, France. Solvents for HPLC were purchased from Fisher Scientific, Pittsburgh, PA, USA. All other chemicals were obtained through Sigma-Aldrich, St. Louis, MO, USA.

Statistical methods: A minimum of three replicate extractions were performed for each plant sample, and each extract was analyzed at least three times by HPLC analysis. Statistical analyses were conducted on treatment means using the t-test procedure of InStat® software, Version 3.0, (GraphPad, San Diego, CA) as described before¹⁴.

Results and Discussion

Oleuropein levels significantly degraded (53.5% loss) within two min of thawing the frozen olive leaf powder, and a 57.7% loss occurred within 5 min of thawing frozen leaf samples; 95.4% of oleuropein was lost if leaves were thawed for 30 min (Fig. 1). Notable losses in oleuropein also occurred when leaf samples were air-dried at above ambient temperatures (60°C) (Fig. 2). It is possible that inconsistencies in oleuropein levels among some commercial products might in part be due to such inadvertent discrepancies in handling processes, as quantities of oleuropein in products by vendors 4 and 5 (labeled “Leaf Extracts”) actually contained less oleuropein than simply dried olive leaf samples in our laboratory (Table 1, Fig. 1). For example, we determined that oleuropein content in fresh leaves was 36.4 mg/g fresh wt (see legend for Fig. 1) which comes to about 7.3% on a dry weight basis (50% moisture in fresh leaves when dried at room temperature) while these products contained only 4.9 and 5.9% oleuropein on dry weight basis (Table 1, and Figs. 2 and 3).

A sharp decline in oleuropein levels after brief thawing is probably due to mixing of oleuropein with oleuropein-degrading enzymes that are compartmentalized in fresh leaf cells. Such compartmentalization of oleuropein and oleuropein-degrading enzymes has recently been shown in olive fruit cells³⁰. This compartmentalization, perhaps, is the reason for maintaining high oleuropein levels in immature green fruits that decline drastically when fruits turn black and softer; possibly due to leakage during softening of olive fruits at maturity¹⁴. It is well known that freezing and thawing of plant tissue result in breakage of membranes^{31,32}, and hence mixing of compartmentalized compounds could occur from freezing and thawing as seen in our results (Fig. 1). Nearly a 60% percent loss in oleuropein levels within 5 min of thawing and

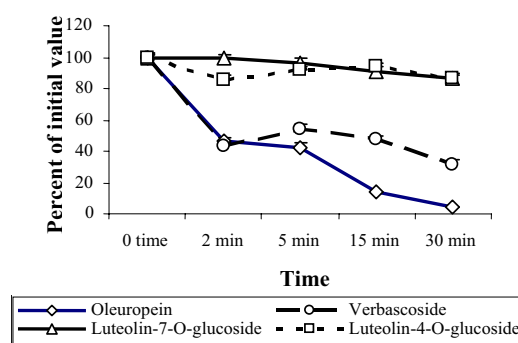


Figure 1. The effect of thawing frozen olive leaves for various lengths of time on the recovery of oleuropein and other polyphenols. Only 2 min samples were frozen powder of leaves. Standard error bars are given at each data point. All data points of oleuropein and verbascoside were significantly different from initial values at $p < 0.001$ based on t-test. Initial values (\pm standard error) of oleuropein, verbascoside, luteolin-7-O-glucoside and luteolin-4-O-glucoside content in frozen leaves were 36.4 ± 1.06 , 3.08 ± 0.38 , 3.01 ± 0.31 and 1.48 ± 0.15 mg/g fresh wt, respectively.

95% loss after 30 min thawing at ambient temperatures indicate the presence of a highly active enzyme system for degradation of this compound. Degradation of luteolin glucosides in olive leaves after freezing and thawing was much slower under the same conditions indicating either minimal mixing of these compounds with their degrading enzymes or that the enzymes responsible for their degradation were not very active (Fig. 1).

Taking cues from these results we were able to obtain highly consistent data on olive leaf polyphenols [initially, some inconsistencies were observed that were to small discrepancies in handling and processing] when the frozen leaf samples (stored at -80°C) were processed in a following manner. Frozen leaves were transferred from a freezer to a foam bucket containing dry ice (or vermiculite soaked with liquid nitrogen). Samples were pulverized in liquid nitrogen²⁹ and the powder was transferred to plastic tubes in a dry ice bucket (or vermiculite soaked with liquid nitrogen) with a spatula frozen in liquid nitrogen. Aliquots of frozen plant powder were weighed quickly using frozen spatulas on weighing boats that had been frozen in liquid nitrogen. Extraction solvent (mostly 80% methanol; occasionally boiling water was also used) was then immediately poured on the frozen powder before transferring to an extraction vessel¹⁴. While this processing method gave highly consistent results, it is strictly a laboratory method that cannot be conveniently adopted for consumer or commercial use, thus freezing olive leaves for extracting polyphenols by consumers or commercial entities should be avoided.

The best method for processing and storage of olive leaves for extraction of oleuropein and other polyphenols is simply drying the leaves at room temperatures (25°C), that gave full recoveries of oleuropein and verbascoside and about 29 and 42% losses in luteolin-7-O-glucoside and luteolin-4-O-glucoside, respectively (Fig. 2). Thus, drying olive leaves at room temperature may be an adequate and the most convenient method for commercial or consumer purposes (also for researchers primarily interested in oleuropein) because oleuropein is the most important and the major polyphenol in olive leaves¹⁰; in fact, most commercial preparations of olive leaves or extracts only provide data for oleuropein content (Table 1). Considering that oleuropein levels matched perfectly with the claimed values on some commercial products (that are generally stored at room temperature for years) supports the view that simple drying and storage at room temperature would be ideal for taking advantage of health benefits from polyphenols in olive leaves (Table 1). The nearly 20% higher extractability of oleuropein and verbascoside in samples dried at room temperature is perhaps due to fineness of the leaf powder produced from dried leaves compared to frozen leaves under identical pulverizing conditions (Fig. 2). While simple drying of olive leaves seems ideal for most useful purposes, the frozen fresh procedure described above may still be the method of choice for researchers interested in luteolin derivatives or in total polyphenols profiles.

Drying of olive leaves under ambient conditions takes long time (> 2 days for complete drying), so it was tempting to investigate if drying time could be shortened at elevated temperatures without significant losses. Leaves placed at 60°C almost completely dried within 4 h but such treatment resulted in substantial losses of polyphenols (approximately 50% for most polyphenols), thus making the technique unsuitable for most purposes (Fig. 2).

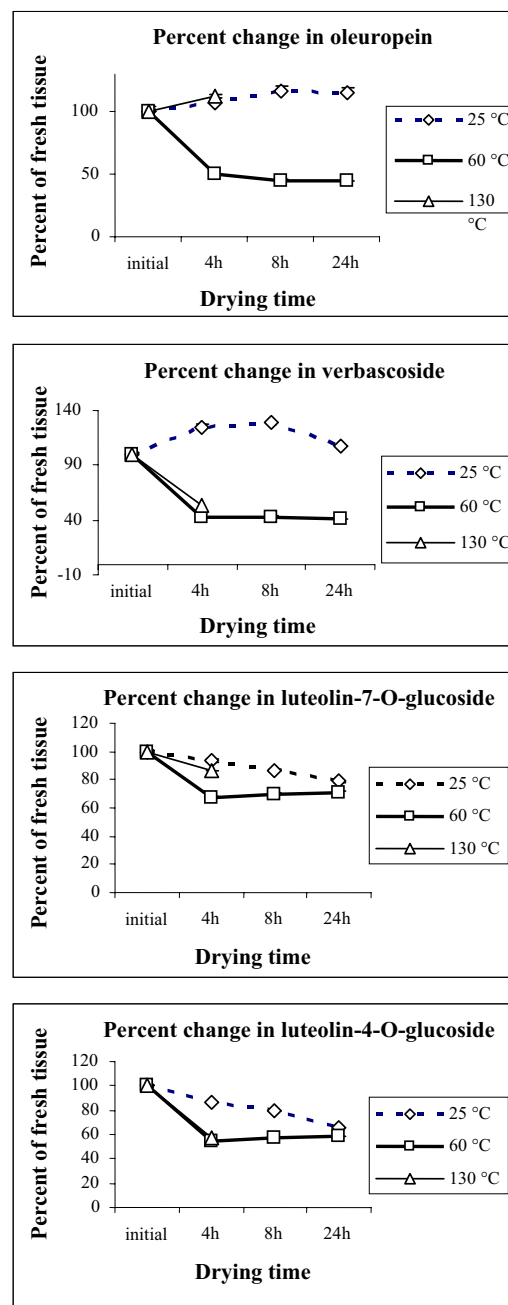


Figure 2. The effect of drying olive leaves at various temperatures on the recovery of oleuropein and other polyphenols. Standard error bars are given for each data point. All data points for leaves dried at 60°C are significantly different from initial values at $p < 0.001$ based on t-test. Except oleuropein, levels of all other polyphenols were significantly different from initial values in leaves dried at 130°C .

Possibly, some leakage in membranes might have occurred when fresh leaves were placed for drying at elevated temperatures (60°C), thus resulting in the rapid degradation of oleuropein and other polyphenols (Fig. 2). Similar degradation of polyphenols, except oleuropein, was also observed when leaves were dried at an even higher temperature of 130°C (Fig. 2). Preservation of oleuropein levels at 130°C might be due to early denaturation of oleuropein-degrading enzymes at such elevated temperatures. Drying at room

Table 1. Differences in oleuropein content claimed on various olive leaf products in the market and the values determined in our laboratory ¹.

Product	Oleuropein		Verbascoside	Luteolin-7-O-glucoside	Luteolin-4-O-glucoside	
	Claimed	Observed				
	% dry wt					
	Observed					
			mg/g dry wt			
Vendor-1 ²	10.00	10.20	102.01±0.78 ³	2.67±0.03	6.53±0.08	2.60±0.07
Vendor-2	20.00	8.48	84.79±5.03	1.98±0.11	5.35±0.32	2.05±0.20
Vendor-3	12.00	12.40	124.05±1.80	3.27±0.05	6.82±0.15	3.34±0.06
Vendor-4	6.00	4.92	49.22±0.91	0.81±0.01	4.50±0.07	2.13±0.03
Vendor-5	10.00	5.95	59.50±1.38	1.43±0.04	5.80±0.20	2.42±0.08

¹ Quantities of other detectable phenolic compounds are generally not provided by manufactures but are given here for general interest

² It is against USDA policy to endorse or discredit any commercial products, and therefore, the names of the manufacturers for each product are not given here. ³ Values of standard error of mean are given after ±.

temperature appears to be the most suitable method of processing olive leaves; although one may consider keeping leaves in an enclosed chamber with some desiccant (e.g., silica gel) to speed up the drying process.

To find a simpler method for extracting oleuropein and other polyphenols from olive leaves, we compared their contents in standard methanol extract with the extracts obtained through simple boiling or steam perfusion of air-dried olive leaf powder in a coffee maker. Simple boiling of olive leaf powder in pure water for 10 min was quite effective in extracting oleuropein and verbascoside; i.e., aqueous extract contained 96% of oleuropein and 94% of verbascoside found in the methanol extract (Fig. 3). Although the extractability of luteolin compounds was less than 50% in aqueous extracts, it still seems that simple boiling of olive leaf powder would be a useful method for all practical purposes to obtain most important olive polyphenols, such as oleuropein and verbascoside ¹⁰ [initial results from shorter boiling time (2 min) were not much different; data not provided here]. To test if lower amounts of luteolin compounds in boiled aqueous extract might be due to their degradation during boiling, we boiled pure standard compounds in water and the recovery of luteolin-4-O-glucoside and luteolin-7-O-glucoside were 96 and 84%, respectively, which cannot account for less than 50% recoveries from boiling leaf powders in water (Figs. 3 and 4). Steam perfusion of olive leaf powder in a coffee maker does not seem to be the most efficient way of extracting major polyphenols from olive leaves as the recoveries of oleuropein and verbascoside were approximately 60% of the alcoholic extract (Fig. 3), yet it may still be the most convenient way (in areas where olive leaves are very cheaply available) of extracting sufficient amounts of important polyphenols for individual needs.

Methanol extracts were stable for several days when kept at room temperature, but aqueous extracts showed significant degradation of luteolin-4-O-glucoside and luteolin-7-O-glucoside (about 45 and 30% loss respectively) within 7 days of storage at room temperature and were completely degraded after 17 days (Fig. 5). Thus, aqueous extraction of olive polyphenols would be a practical method for consumer and perhaps commercial use but prolonged storage at room temperature should be avoided.

These results provide important information for researchers to be extra vigilant while pulverizing, transferring and handling frozen olive leaf samples to obtain consistent data. For consumers, the

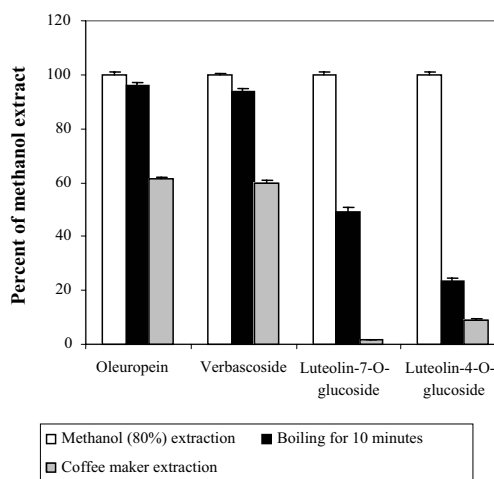


Figure 3. Recovery of oleuropein and other polyphenols in olive leaves by different extraction methods.

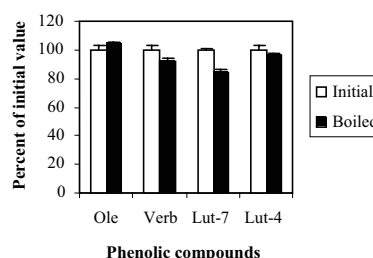


Figure 4. Effect of boiling standard oleuropein (Ole), verbascoside (Verb), luteolin-7-O-glucoside (Lut-7), luteolin-4-O-glucoside (Lut-4), for 10 minutes on their stability.

information should be useful because it is generally assumed safer to keep leaf samples frozen to preserve active ingredient but more often than not the leaf material is indiscriminately allowed to thaw before extracting as a tea which would result in substantial losses of important polyphenols such as oleuropein. Similarly, drying leaves in oven to speed up drying process could lead to unnecessary losses in useful polyphenols. Thus, a simple method of processing and extracting oleuropein from olive leaves described

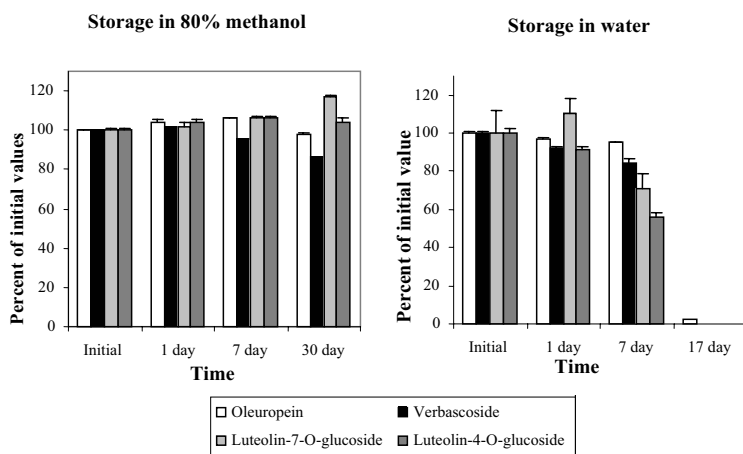


Figure 5. Stability/degradation of oleuropein and other olive leaf polyphenols during storage of alcohol (80% methanol) or aqueous leaf extracts at room temperature.

here should be useful for consumers, researchers and commercial entities.

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