

Agricultural Environment and Sustainability

Olive leaves as source material for novel products containing polyphenols

--Manuscript Draft--

Manuscript Number:	
Full Title:	Olive leaves as source material for novel products containing polyphenols
Short Title:	Olive leaves
Article Type:	Original Research Article
Section/Category:	Agricultural Resource Conservation & Sustainable Utilization
Keywords:	α - and γ -tocopherols; hydroxytyrosol; oleuropein; rutin
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Abstract:	The purpose of this study is to identify a method to process olive leaves that is sustainable, energy-efficient, and cost-effective, while also retaining high levels of polyphenolic compounds. The first step in our evaluations was the impact of washing and drying methods on the optimal process. Then, the rapid Folin-Ciocalteu assay was used to survey polyphenol contents following washing and drying. Upon identifying an optimal drying strategy, we measured specific polyphenols using ultra high-pressure liquid chromatography with quantitation by mass spectrometry. Further work is necessary to ascertain whether conjugates of polyphenols present in the leaves are released during drying. Overall, this polyphenol isolation method developed is straightforward and can be accomplished in a typical home kitchen to produce a high-quality polyphenol extract from olive leaves.
Additional Information:	
Question	Response
<p>Data Availability Statement</p> <p>To complete your submission you must select a statement which best reflects the availability of your research data/code. IMPORTANT: this statement will be published alongside your article. If you have selected "Other", the explanation text will be published verbatim in your article (online and in the PDF).</p> <p>(If you have not shared data/code and wish to do so, you can still return to Attach Files. Sharing or referencing research data and code helps other</p>	Data will be made available on request.

<p>researchers to evaluate your findings, and increases trust in your article. Find a list of supported data repositories in Author Resources, including the free-to-use multidisciplinary open Mendeley Data Repository.)</p>	
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April 11, 2026

Dear Editors,

We are pleased to submit a manuscript entitled “**Olive leaves as source material for novel products containing polyphenols**” by Scott W. Leonard, Bogdan “Beau” Caceu, and Maret G. Traber for consideration for publication in *Agricultural Environment and Sustainability*. We propose that our submission fits in the thematic area “Agricultural Resource Conservation and Sustainable Utilization”

We believe that our manuscript is unique in that the purpose of this study is to identify a method to process olive leaves that is sustainable, energy-efficient, and cost-effective, while also retaining high levels of polyphenolic compounds. Importantly, the leaf handling methods can be completed by the small farmer using readily available tools. We show our methods are useful by using technologically advanced techniques to test our outcomes.

We have prepared our manuscript (approximately 2300 words) with 3 figures, 1 table and 20 references.

Sincerely,

A handwritten signature in black ink that reads "Maret G. Traber".

Maret G. Traber, Ph.D.
Ava Helen Pauling Professor, Linus Pauling Institute
Professor Emeritus, College of Public Health & Human Sciences

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1 **Olive leaves as source material for novel products containing polyphenols**

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14 **Key Words:** α - and γ -tocopherols, hydroxytyrosol, oleuropein, rutin

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16 **Abbreviations:**

17 Dihydroxycoumarin (DHC), Folin-Ciocalteu (FC), multiple reaction monitoring (MRM), ultra-
18 high pressure liquid chromatography with mass spectrometry (UPLC-MS/MS), (m/z 611>303),

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21 **Abstract**

22 The purpose of this study is to identify a method to process olive leaves that is sustainable,
23 energy-efficient, and cost-effective, while also retaining high levels of polyphenolic compounds.
24 The first step in our evaluations was the impact of washing and drying methods on the optimal
25 process. Then, the rapid Folin-Ciocalteu assay was used to survey polyphenol contents following
26 washing and drying. Upon identifying an optimal drying strategy, we measured specific
27 polyphenols using ultra high-pressure liquid chromatography with quantitation by mass
28 spectrometry. Further work is necessary to ascertain whether conjugates of polyphenols present
29 in the leaves are released during drying. Overall, this polyphenol isolation method developed is
30 straight-forward and can be accomplished in a typical home kitchen to produce a high-quality
31 polyphenol extract from olive leaves.

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33 Introduction

34 Olive leaves are an underutilized source of valuable polyphenolic compounds [1], which
35 can vary between olive cultivars [2]. The Moraiolo olive cultivar is a high polyphenolic
36 containing cultivar [3], that is grown at a local farm in the Willamette Valley, Oregon. The
37 leaves from these trees were harvested in summer for evaluation of methods for polyphenol
38 contents. Leaves were chosen because olive leaves have similar and sometimes higher
39 polyphenol contents than olives or olive oil [4]. Entire olive branches are pruned each year to
40 maintain the “open vase” shape of trees that is best for the health of the olive tree to promote air
41 flow, as well as for best olive production, due to light penetration inside the olive tree canopy.
42 While olives are harvested and milled in the fall during a very brief season, olive leaves can be
43 harvested at any time, year-round because olive trees are evergreen, making olive leaves always
44 available. The time of year the leaves are harvested has no impact on the content of the major
45 polyphenol, oleuropein [5, 6]. Thus, the use of olive leaves as a product would extend the season
46 for olive growers to one that is year-round.

47 This latter point concerning availability of leaves cannot be emphasized enough:
48 extending production from a few days (for the harvest and processing of olives into extra virgin
49 olive oil) to year-around could be significant for olive growers. This extended season could
50 significantly improve the economic sustainability of olive growers by (i) doubling the number of
51 value-added products they produce by adding production of shelf-stable, ground-olive leaf
52 powder that could be sold as an ingredient to the existing production of extra virgin olive oil, (ii)
53 providing a way to produce fresh batches of a product derived from the olive trees at any time of
54 the year, and (iii) making it possible to sell the shelf-stable ground-up olive leaf product to a
55 significantly higher number of professional customers the food and beverage industry.
56 Furthermore, olive growers can multiply their revenues and broaden their customer base by the
57 use of olive leaves to leverage the same production unit (olive tree or acres planted, etc.) into a

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5 58 much more efficient and economically sustainable orchard without any additional inputs (or only
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7 59 marginal inputs directly related to the processing and storage of the olive leaves).
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9 60 This project seeks to elevate leaves, which are a by-product of yearly pruning, and will
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11 61 up-cycle this by-product into a marketable value-added product. The purpose of this study is to
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13 62 identify a processing method of freshly picked olive leaves that yields the most sustainable,
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15 63 energy-efficient, and cost-effective procedure resulting in a product that retains high levels of
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17 64 polyphenolic compounds.
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19 20 65 **Methods** 21 22

23 24 66 *Leaf collection* 25

26 67 A sampling (approximately 1 kg) of young leaves from Moraiolo olive trees, a high
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28 68 polyphenol cultivar, was collected in July from an Oregon farm. These fresh cuttings were
29
30 69 transported to the Linus Pauling Institute (LPI) on the Oregon State University campus within an
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32 70 hour of harvesting from the trees. The leaves were weighed, frozen at -80 °C and stored until
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34 71 analyzed. Unprocessed leaves serve as a baseline for comparison of treatments to assess the
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36 72 recovery of polyphenols, and the distribution of specific polyphenols and tocochromanols.
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39 40 73 *Drying and Cleaning methods* 41

42 74 We tested oven drying (90 °C, or 37 °C) vs air drying (21 °C) to constant moisture, as
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44 75 described [6]. Moisture content of the powders used for analysis was measured by weighing the
45
46 76 samples before and after drying for the indicated times and temperatures.
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48 77 The next step was to evaluate cleaning methods since the leaf material will be used
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50 78 ultimately as a source for products intended for human consumption [7, 8]. A water rinse (1
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52 79 minute) was compared to a bleach (200 ppm total chlorine) rinse (1 minute for sanitizing the
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54 80 leaves) followed by a fresh water rinse (1 minute). Both steps (water only vs bleach followed by
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56 81 a water rinse) were followed by patting the samples dry. The optimized drying procedure (oven
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58 82 drying at 90 °C for 90 minutes) was then used to obtain dried leaves.
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5 83 A powder of each of the leaf samples was produced by grinding the leaves (~30 g fresh
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7 84 or ~15 g dried leaves) in liquid nitrogen in a Waring metal blender and stored less than 2 weeks
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9 85 at -80 °C until analysis.

11 86 *Analytical measurements*

14 87 To assess total polyphenolic contents, olive leaf powders were extracted, as described [9,
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16 88 10]. Briefly, powdered leaf samples were mixed with 50:50 methanol:water for 1 minute.
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18 89 Following sonication for 10 minutes, centrifugation, and transfer of the upper phase to a new
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20 90 tube, total phenolics were quantitated in the extract using the FC assay with gallic acid as a
21
22 91 standard; absorbance was measured at 755 nm using a UV/VIS spectrophotometer [11, 12].
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24 92 Experiments were carried out a minimum of three times; representative results are shown.

27 93 Targeted ultra-high pressure liquid chromatography with quantitation by mass
28
29 94 spectrometry (UPLC-MS/MS) was used to analyze major olive leaf phenolic compounds in the
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31 95 extracts obtained from fresh and dried leaves, isolated as described above [13]. Phenolic extracts
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33 96 of the olive leaf powders were diluted with 5% acetonitrile and injected using a Waters Acquity
34
35 97 UHPLC coupled to a Waters Xevo TQD mass spectrometer (Milford, MA). Separation was
36
37 98 performed with a Waters Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µM particle size)
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39 99 using gradient mobile phase delivery of acetonitrile containing 0.1% formic acid (mobile phase
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41 100 B) and water containing 0.1% formic acid (mobile phase A), as detailed [13] (with the exception
42
43 101 that mobile phase B was changed from methanol to acetonitrile). Phenolic compounds
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45 102 (hydroxytyrosol and oleuropein) were quantitated by comparison to authentic standards (Sigma
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47 103 Aldrich, St Louis MO) with syringic acid as an added internal standard, as detailed [9].
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49 104 Dihydroxycoumarin (DHC) was added as a positive mode internal standard. Rutin and DHC
50
51 105 were monitored in positive mode ESI, all other compounds were monitored in negative mode.
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53 106 Analytes were detected using multiple reaction monitoring (MRM) of the following transitions:
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55 107 hydroxytyrosol (m/z 153>123), oleuropein (m/z 539>377), rutin (m/z 611>303), DHC (m/z
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108 179>133), and syringic acid (m/z 197>121). Sample peaks were quantitated by comparison to
109 authentic standard compounds and adjusted for responses using the internal standard.

110 To analyze α - and γ -tocopherols in the various olive leaf powders, a modified version of
111 the method previously reported was used [14]. Approximately 50 mg leaf powder was saponified
112 with alcoholic KOH in the presence of 1% ascorbic acid, followed by extraction with hexane.
113 The hexane extract was then dried under nitrogen, resuspended in methanol, and injected into the
114 HPLC system. This system includes a Waters 2695 separations module equipped with a 50 μ L
115 sample loop. Tocopherols were detected using a Bioanalytical Systems Inc. LC-4B
116 amperometric electrochemical detector with a glassy carbon working electrode and a silver
117 chloride reference electrode. The column used was a Phenomenex Synergi Hydro-RP (150 \times 4.6
118 mm, 4 μ m particle size) with a Phenomenex Security Guard precolumn (4 \times 3.0 mm). The
119 mobile phase, delivered isocratically, consisted of 99:1 methanol with 0.1% lithium perchlorate,
120 and the total run time was 10 minutes. The electrochemical detector operated in the oxidizing
121 mode at a potential of 500 mV with a full recorder scale set at 500 nA. Peak areas were
122 integrated using the Waters Empower software package, and tocopherols were quantified by
123 comparison to authentic standards. Quality control was maintained using a sample with known
124 values.

125 *Statistical Analyses*

126 Statistical significance was determined using GraphPad Prism 10 software (La Jolla,
127 CA). Multiple comparisons were performed when a significant interaction was found using
128 Dunnett's multiple comparisons test, using fresh leaves as a comparator. All tests were two-
129 sided. Differences were determined to be significant when $P \leq 0.05$.

131 **Results**

132 *Drying process*

133 Olive leaves were weighed, washed briefly and blotted dry. The leaves were then either
134 kept at room temperature (21 °C), or in the oven at 37 °C or 90 °C. Samples were taken at
135 intervals for weighing. At 24 h, the leaves dried at 21 °C or in the oven at 37 °C were only
136 partially dried (**Figure 1A**). By contrast, the moisture content was reduced from ~54% to <3%
137 by rapidly drying the olive leaves in an oven for 90 minutes at 90 °C.

138 *Total polyphenols as a measure of processing effectiveness*

139 The total polyphenolic contents, as assessed by the FC method, was used as a means to
140 evaluate the efficacy of the drying process (**Figure 1B**). Both room temperature drying and
141 drying in a 37°C oven were found to be ineffective at preserving the polyphenols; major losses
142 were observed in samples dried for 6 or 24 h at either 21 °C or 37 °C (**Figure 1B**). By contrast,
143 the oven drying for 90 minutes at 90°C prevented the losses observed in the other drying
144 approaches (**Figure 1B**, comparisons are all done per dry weight). Importantly, a bleach (200
145 ppm) rinse prior to drying the leaves had no impact on the total polyphenolic contents (**Table 1**).
146 In fact, the oven drying at 90 °C improved the extraction of the total polyphenols.

147 *Individual polyphenols in olive leaves*

148 Individual olive polyphenols were quantitated in olive leaves that were rinsed with
149 bleach, with water, then dried at 90 °C for 90 minutes. The powders made from the leaves were
150 then extracted and measured using targeted UPLC-MS/MS (**Figure 2**). All of the quantitated
151 polyphenolics showed a similar pattern—there was substantial benefit to completely drying the
152 leaves prior to extraction. Oleuropein was a major constituent of the olive leaf polyphenols and
153 the optimized drying method improved recovery of this important polyphenol. Oleuropein was
154 approximately 25-30% of the total polyphenols, while hydroxytyrosol also represented a

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155 substantial fraction of the polyphenols (7-8%). Very little of the rutin was extractable with the
156 processed we used from the fresh or fresh frozen leaves.

157 By contrast to the polyphenols, both α - and γ -tocopherols were present in the fresh
158 leaves, but both decreased by approximately 50% following either drying method; bleach had no
159 further effect (**Figure 3**).

160 **Discussion**

161 The purpose of this study was to develop a protocol, which would allow a small farmer to
162 be able to recover valuable bioactive components from olive leaves. Currently, leaves are a waste
163 product on the commercial olive orchard. Initial investigations included optimizing drying
164 methods. The simplest available methods include air drying at room temperature, then grinding,
165 and packaging for long shelf life. However, we found that slow drying was not effective in
166 maintaining the polyphenol concentrations. The 90 °C drying process preserved the polyphenols
167 effectively. This temperature is approximately 200°F, a temperature that can be achieved in a
168 household oven. However, oleuropein is a glycoside, which can be hydrolyzed to
169 hydroxytyrosol. Importantly, hydroxytyrosol is subject to oxidative damage and loss during
170 processing. Thus, hydroxytyrosol is a useful comparator between methods to evaluate potential
171 oxidation of the polyphenols, since drying methods can alter the polyphenol contents of the olive
172 leaves [15].

173 Concerns for processing included the observation that the leaves need some level of
174 cleaning, since the leaf material will be used ultimately as a source for products intended for
175 human consumption [7, 8]. This aspect prompted us to investigate rinsing the leaves with a
176 bleach water solution (200 PPM total chlorine) for 1 minute prior to drying. Since hypochlorous
177 acid does consume polyphenols [16], it was important to investigate potential losses. We found
178 that the one-minute rinse in dilute bleach solution had essentially no impact on the polyphenols
179 (Figure . However, the other bioactive compounds we investigated, tocopherols (Figure 3),
180 were greatly impacted.

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181 The reduction by 50% of both the both α - and γ -tocopherol concentrations by drying
182 were surprising. Previously, tocopherols were not oxidized by hypochlorous acid when low
183 density lipoproteins were oxidized by hypochlorous acid [17, 18]. However, recent studies have
184 shown that this is a controversial point [19]. This observation may have important implications
185 for use of dry forage for cattle or horses as there is concern about the adequacy of vitamin E
186 intake by horses eating dried hay (The Horse Report, UC Davis 2018). Further, vitamin E has
187 been found to decrease with drying in forage samples [20].

188 The olive leaf powder, or the olive leaf extract, could improve economic sustainability of
189 olive tree farming by increasing product diversification and by broadening the total addressable
190 market. The shelf-stable olive leaf powder could be marketed as a standalone food (e.g. as a
191 replacement for green tea, which also has antioxidants, but olive leaf tea is caffeine-free and it
192 has a much lower carbon food print compared to green tea which must be shipped from abroad).
193 Alternatively, the olive leaf powder or olive leaf extract could be marketed as a functional
194 ingredient to be used in other food and beverage products to impart polyphenolics.

195 In summary, rinsing freshly picked olive leaves in a bleach water solution (200 PPM) for
196 one minute, followed by blotting dry to remove surface water, then oven drying at 90 °C for 90
197 minutes is an effective method to preserve polyphenols in olive leaves. The leaves then can be
198 crushed and extracted to obtain polyphenols or other bioactive compounds.

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Acknowledgements

This study was supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2023-38640-39571 through the Western Sustainable Agriculture Research and Education program under subaward project number, G238-25-WA507. USDA is an equal opportunity employer and service provider.

Authorship

Authors have made substantial contributions to all of the following: The conception and design of the study (all), acquisition of data (SWL), analysis and interpretation of data (SWL, MGT); Drafting and revising manuscript (all); final approval (all).

AI or generative AI was not used in the generation of the text or the data in this manuscript.

Conflict of Interest Statement

SWL, MGT have no conflicts of interest to declare. BC is the owner of La Creole Orchards a farm growing olive trees and producing olive-related products.

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295 **Figure Legends**

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297 **Figure 1. Drying process has a major impact on polyphenol contents of olive leaf extracts.**

298 **A.** The moisture content of the leaves was not effectively reduced by drying at either 21 °C or 37
299 °C. **B.** Slow drying at either 21 °C or 37 °C did not preserve polyphenols. The concentrations
300 shown were adjusted for the dry weight of the sample. Statistical analyses: ANOVA, overall
301 $P < 0.0001$, Dunnett's multiple comparison test results: ****, $P < 0.0001$; ***, $P < 0.0005$; **,
302 $P = 0.0026$.

303
304 **Figure 2. Drying improves extraction of specific olive leaf polyphenols**

305 Statistical analysis: ANOVA, overall $P < 0.0001$, Dunnett's multiple comparison test
306 results: ****, $P < 0.0001$.

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309 **Figure 3. Drying depletes α - and γ -tocopherols from olive leaf extracts**

310 Drying causes major losses of both α - (upper panel) and γ - (lower panel) tocopherols
311 from olive leaves. Statistical analysis: ANOVA, α -tocopherol overall $P = 0.0002$, γ -tocopherol
312 overall $P = 0.0023$; Dunnett's multiple comparison test results: ***, $P = 0.0005$; **, $P = 0.0056$, *,
313 $P = 0.0427$.

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316 **Table 1. Total polyphenol contents of fresh and dried leaves**

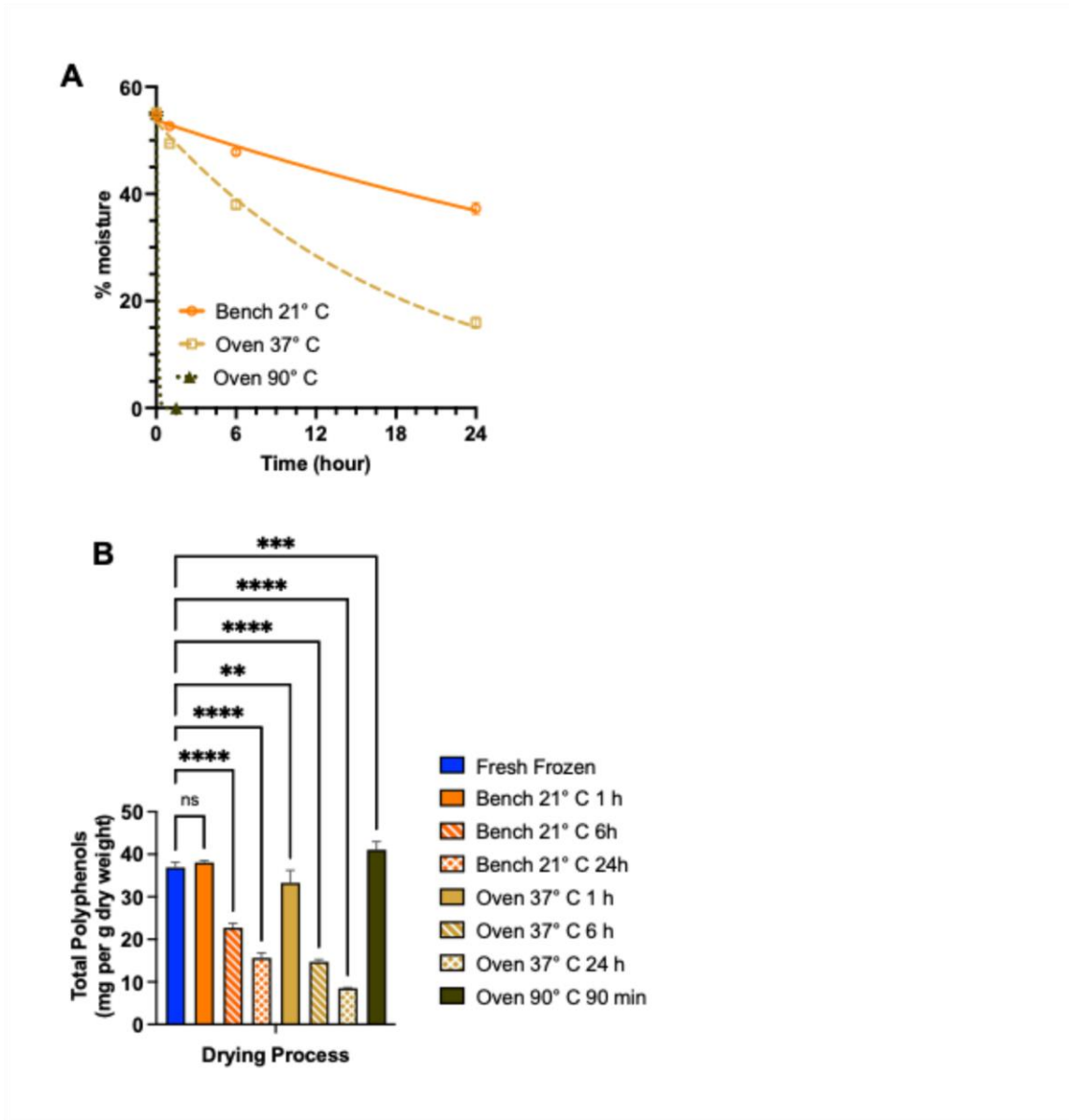
Process	Mean ± SEM	n=	P-value compared with fresh leaves*
	mg per g dry weight		
fresh picked leaves/stored -80°C	40.5 ± 1.1	5	-
wash H ₂ O/blot dry/stored -80°C	41.3 ± 0.5	5	ns
wash, dry 90 min @ 90°C/ stored -80°C	53.8 ± 0.5	5	=0.0003
bleach wash/dry 90 min @ 90°C/ stored -80°C	50.3 ± 0.4	5	=0.0036

317 One way ANOVA P<0.0001, *Dunnett's multiple comparisons are shown

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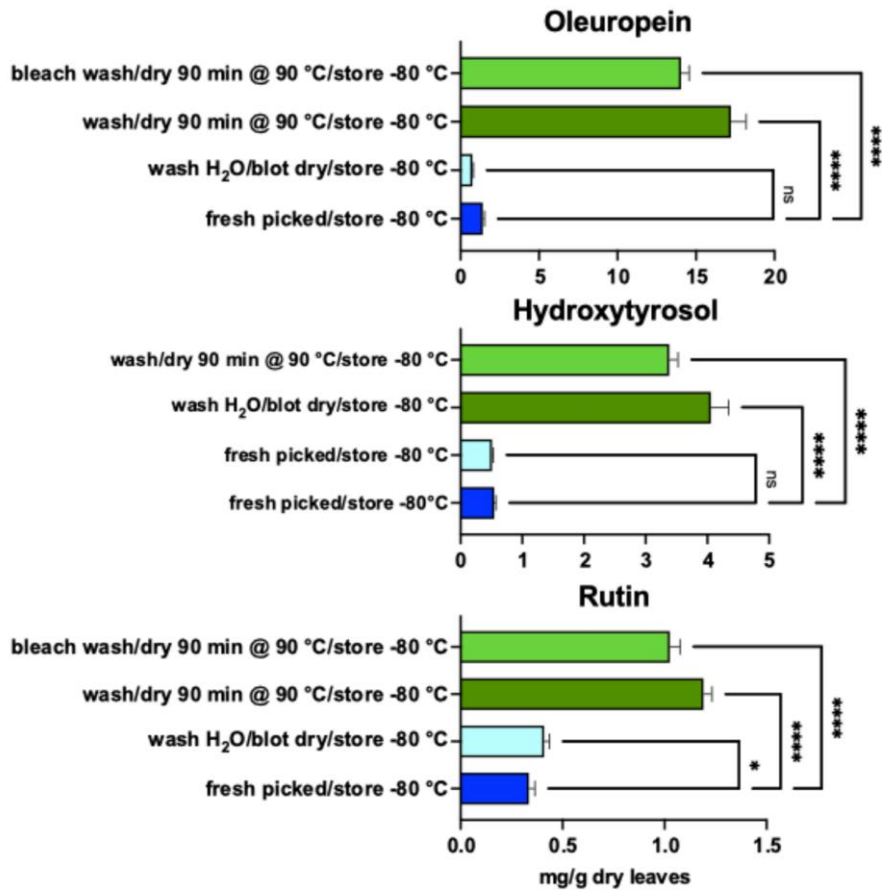
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319 **Figure 1. Drying process has a major impact on polyphenol contents of olive leaf extracts.**



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321 **Figure 2. Drying improves extraction of specific olive leaf polyphenols**

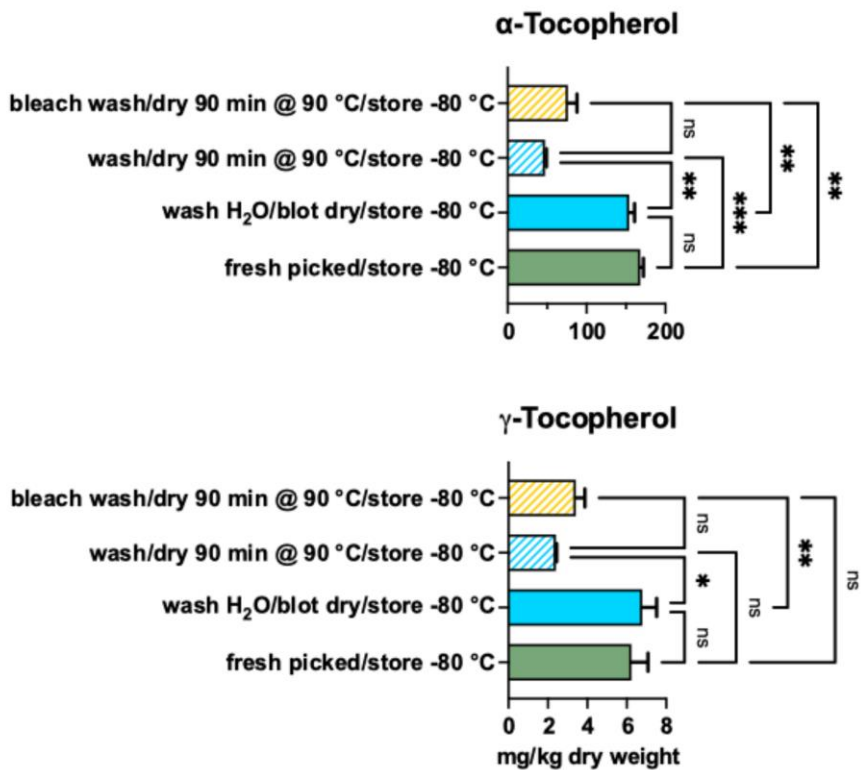


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Figure 3. Drying depletes tocopherols from olive leaf extracts



Declaration of Interest Statement

- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
- The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for this journal and was not involved in the editorial review or the decision to publish this article.
- The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

SWL, MGT have no conflicts of interest to declare. BC is the owner of La Creole Orchards a farm growing olive trees and producing olive-related products.

