1   **Removal of pomace residues is critical in quantification of element concentrations**
2   **in extra virgin olive oil**

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16 **Highlights** (*maximum 85 characters, including spaces*)

- 17   • Ultrasonic extraction is applicable for elements analysis in pomace and olive oil.
- 18   • Pomace residue increases concentrations and affects distribution of elements in olive oil.
- 19   • Oil samples must be centrifuged prior to analysis to remove pomace residue.

20 **Graphical abstract**

22 **Abstract**

23 Analysis of elements in Extra Virgin Olive Oil (EVOO) is challenging due to the complex oil matrix as

24 well as very low element concentrations in oil. We postulate that inadequate sample preparation (in

25 particular, the presence of pomace residue in oil) may significantly contribute to variations in EVOO

26 element concentrations. In order to confirm this hypothesis, EVOO and pomace samples were analysed

27 for content of 29 elements by ultrasonic acid extraction and ICP-MS detection. Comparative analyses

28 of non-centrifuged and centrifuged EVOO samples confirmed significant influence of pomace residues

29 on element concentrations. Ranges of macronutrients (25–286 ng g⁻¹), micronutrients (0.3–1.1 ng g⁻¹)

30 and trace elements (0.001–0.2 ng g⁻¹) in centrifuged EVOO samples were up to 1000 times lower than

in non-centrifuged oils. We have shown that pomace residue, even when present in very small quantity (1 g of pomace in 1 L of oil), significantly altered concentrations and relative proportions of many elements in EVOO. Therefore, adequate sample pre-treatment (removal of pomace residue via centrifugation) is essential for determining real multi-element composition of olive oil. This is critical in application of EVOO multi-element composition in traceability studies and determination of geographic origin of olive oil.

Keywords: centrifugation, extra virgin olive oil, food analysis, food composition, ICP-MS, multi-elemental analysis, pomace, sample preparation, ultrasonic acid extraction

1. Introduction

Extra virgin olive oil (EVOO) is a substantial part of the traditional Mediterranean diet due to its beneficial properties (Therios, 2009). Polyunsaturated fatty acids, oxygen, temperature, light and metals such as Cu, Fe, Ni and Sn in oils affect the rate of oxidation responsible for oil deterioration (Choe & Min, 2006; Sikwese & Duodu, 2007). In addition, a number of elements (such as As, Cd, Cr, Hg and Pb) present in EVOO are of great interest because they are toxic and potentially carcinogenic to humans even at low concentrations (Tchounwou et al., 2012). It is therefore imperative to control the presence and amount of elements in EVOO in order to preserve its quality. In recent years elements determination in EVOO has gained importance for EVOO traceability (Benincasa et al., 2007; Camin et al., 2010a, 2010b; Dugo et al., 2004; Zeiner et al., 2005) and detection of oil adulteration (Cindric et al., 2007; Llorent-Martínez et al., 2011).

The presence of metals in olive oil depends on several factors. Plants accumulate elements (some being essential nutrients) from soil (Marschner, 2012), and they can be transferred into olive fruits (Therios, 2009). Non-nutrient elements can also be translocated from roots to olive fruits because the root selective capacity is relatively low (eg. Cd can be taken up via Zn transporters, Tavares et al. 2015, Saifullah et al. 2016). Depending on element characteristics, elements can be transferred from olive fruits into oil during the extraction process. EVOO can be contaminated by elements also during the production and storage processes (Lepri et al., 2011; Zeiner et al., 2010).

An overview of the literature (Table 1) shows a wide range of concentrations reported for each single element in various EVOO samples, from $\mu\text{g g}^{-1}$ (Bakircioglu et al., 2013; Cindric et al., 2007; Mendil et al., 2009; Zeiner et al., 2005) to ng g^{-1} (Bakkali et al., 2012; Beltrán et al., 2015; Cabrera-Vique et al., 2012), or even pg g^{-1} (Camin et al., 2010a, 2010b; Castillo et al., 1999). To the best of our knowledge, this discrepancy in the literature data was never discussed; hence, it remains unclear whether this is due to different EVOO origin and their different chemical compositions or perhaps due

to possible contamination during the production and storage. We hypothesize that a wide range of measured element concentrations in EVOO samples (Table 1) might be at least partly a consequence of incomplete separation of pure oil from pomace residue. Indeed, there is a lack of information on EVOO sample pre-treatment in most of the published papers, and only few articles reported an oil centrifugation step prior to EVOO analysis (Beltran et al., 2015; Cabrera-Vique et al., 2012), suggesting that in most circumstances the pomace residues were not separated from EVOO samples. On the other hand, there is also paucity of data on elements concentration in pomace, with only two publications found with the data on pomace element concentration (Llorent-Martínez et al., 2014; Beltrán et al., 2015).

Different sample preparation and extraction techniques have been used for element analyses in vegetable oils, including liquid-liquid extraction (Dugo et al., 2004), wet ashing (Lo Coco et al., 2003), dry ashing combined with acid extraction (Brkljača et al., 2013), microwave digestion (Angioni et al., 2006; Bakkali et al., 2012; Benincasa et al., 2007; Cindric et al., 2007; Mendil et al., 2009; Zeiner et al., 2010), simple dilution with a suitable solvent (Anthemidis et al., 2005; Castillo et al., 1999; De Souza et al., 2005; Jimenez et al., 2003), acid extraction in vortexed samples (Ni et al., 2016), and ultrasonic acid extraction (Camin et al., 2010b; Trindade et al., 2015). Among them, microwave digestion is the most commonly used extraction technique; however, it does not allow determination of elements at very low levels because of the limited amount of sample that can be processed (Lepri et al., 2011). In contrast, the ultrasonic acid extraction method can overcome the limits of other preparation techniques and offer a simple, quick and sensitive approach for extraction of metals from oil. However, ultrasonic acid extraction was never validated because there are no EVOO Certified Reference Materials (CRM) and no appropriate standards available. Additionally, the efficiency of ultrasound acid extraction was never compared to the microwave digestion method.

Therefore, in the first part of the paper, we validated the ultrasonic acid extraction method for elements determination in olive oil and pomace. Recovery of elements from pomace using ultrasonic acid extraction was evaluated by comparison with the microwave digestion method. Further, the method was applied to measure 29 elements in selected Croatian EVOO and the corresponding pomace samples. In order to test the hypothesis that pomace residue, when present in olive oil, can significantly influence element concentrations and their relative ratios, we conducted two experiments with pomace and EVOO samples: i) we compared non-centrifuged and centrifuged olive oils and ii) conducted experiments with addition of a known quantity of pomace into pure (centrifuged) EVOO samples.

The main aim of the present research was to assess whether centrifugation of EVOO samples may be a crucial pre-treatment step in their preparation for multi-element analysis. This analytical improvement could be a vital step in EVOO traceability studies.

2. Material and Methods

2.1. Sampling of olive fruits

Olive fruits from *Olea europaea* L. cv ‘Oblica’ were harvested in October 2016 from olive groves located along the coast of Croatia. In each olive grove, 2 to 3 kg of fruits were harvested from nine healthy trees and immediately transported to the laboratory. The ripening degrees (RD, from green to ripe) were assessed; ripening index (RI) was between 0.16 and 4.73. The ripening index was determined from one hundred olive fruits randomly selected from each olive batch (Uceda & Frías, 1975).

2.2. Olive processing, oil extraction and pomace separation

Olive fruits were processed within 48 h after harvesting. Prior to processing and in order to remove adhering dust, olives were rinsed with tap water, placed in 1% v/v acetic acid for 5 min, and finally rinsed two times with deionised water for 5 min each. Samples of 1 kg of olives were processed using a

laboratory setup (Abencor, MC2 Ingenieria y Sistemas, Sevilla, Spain) consisting of a hammer crusher, vertical thermostated olive paste mixers, and a centrifuge (Jukić Špika et al., 2015). Between each batch of olives collected from different olive groves along the Croatian Adriatic coast and geographic origin, the laboratory equipment was washed with hot tap water, laboratory soap and kitchen sponge, and finally rinsed several times with deionised water.

The obtained paste was malaxed for 35 min at 26 ± 2 °C. After centrifugation and decantation, the obtained oil samples were stored in plastic bottles (previously washed in 10% v/v HNO₃ and rinsed with Milli-Q® water) and stored in dark at 18 ± 2 °C. According to the protocol defined by ISO (2004a, 2004b, 2007), all olive oil samples were determined to be EVOO. Pomace samples were collected with a clean stainless steel spoon, oven dried at 65 °C for 48 h, and then ground in a stainless steel mill (A11 Analytical Mill, IKA, Staufen, Germany) and passed through a 2 mm stainless steel sieve.

2.3. Microwave digestion of olive oil and pomace

Plastic bottles containing 20 mL of EVOO were centrifuged at 3500 g for 5 min. In order to avoid any disturbance of the pomace precipitated on the bottom of the bottles, EVOO supernatant was carefully removed with a pipette using acid-cleaned tips. Extra virgin olive oil samples were weighed (0.5 g) and digested in a mixture of 5 mL HNO₃ (69% v/v Fluka TraceSELECT®, Steinheim, Germany) and 1 mL of Milli-Q® water in a microwave oven (Multiwave GO, Anton Paar, Graz, Austria, maximum power 850 W). A microwave-assisted heating program included 20 min of ramp time and 10 min of hold time at 180 °C.

Ten pomace samples (0.05 g) were digested in a mixture of 5 mL HNO₃ (69% v/v Fluka TraceSELECT®, Steinheim, Germany) and 2 mL of Milli-Q® water in a microwave oven (Multiwave GO, Anton Paar, Graz, Austria) with a heating program including 20 min of ramp time and 10 min of hold time at 160 °C. A risk of explosion during oil and pomace digestion limited the mass of sample and necessitated the use of a Multiwave GO microwave that is suitable for highly reactive samples.

2.4. Ultrasonic acid extraction of olive oil and pomace

Sixteen centrifuged or non-centrifuged EVOO and ten pomace samples (the same that were digested in microwave oven as described in paragraph 2.3) were extracted and analysed according to the method of Camin et al. (2010b), with minor modifications. EVOO samples (15 g) or pomace samples (0.5 g) were transferred into 50 mL polypropylene (PP) falcon bottles. In the case of centrifuged oil, EVOO samples were transferred by a pipette as specified above. After weighing, 15 mL of acid extraction solution consisting of 0.2% v/v HCl (30% v/v Suprapur® Merck, Darmstadt, Germany) and 2% v/v HNO₃ (69% v/v Fluka TraceSELECT®, Steinheim, Germany) was added. The mixtures were thoroughly vortexed for 30 s and then placed in an ultrasonic bath (1100 W, 50 Hz) for 5 min. Mixtures were then centrifuged at 3500 g for 5 min; in case of EVOO samples, the upper oil phase was accurately removed by aspiration. Finally, acid solutions (the lower aqueous phase in case of EVOO samples) were transferred into clean PP vials and subjected to ICP-MS analysis.

2.5. Multi-element analysis by HR ICP-MS

The multi-element analysis of prepared extracts was performed by high resolution inductively-coupled plasma mass spectrometry (HR ICP-MS) using an Element 2 instrument (Thermo, Bremen, Germany). The typical instrumental conditions and measurement parameters are presented in Table S1.

Mass calibration of HR ICP-MS was performed using multi-element solution containing the following elements: B, Ba, Co, Fe, Ga, In, K, Li, Lu, In, Rh, Sc, Tl, U and Y. The standards for multi-element analysis were prepared by appropriate dilution of a multi-element reference standard (Analytika, Prague, Czech Republic) containing As, Ba, Be, Bi, Cd, Co, Cr, Cs, Cu, Fe, Li, Mn, Mo, Ni, Pb, Se, Sr, Ti, Tl, V and Zn to which the single element standard solutions of Rb (Aldrich, Milwaukee, WI, USA), Sb (Analytika, Prague, Czech Republic), Sn (Analytika, Prague, Czech Republic) and U (Aldrich, Milwaukee, WI, USA) were added. Standard solution containing P (Fluka Analytical, Buchs, Switzerland) and S (Sigma-Aldrich, Steinheim, Germany) was prepared separately.

159 A multi-element reference standard (Analytika, Prague, Czech Republic) containing Ce, Dy, Er, Eu,
160 Gd, Ho, La, Lu, Nd, Pr, Sm, Tb, Tm, Y and Yb was used for rare earth elements determination,
161 whereas a multi-element reference standard (Fluka, Steinheim, Germany) containing Na, K, Ca and Mg
162 was used for major elements determination.

163 All samples were analysed for total concentration of 29 elements. Following isotopes were
164 measured at low resolution (LR): ^7Li , ^{85}Rb , ^{95}Mo , ^{111}Cd , ^{120}Sn , ^{133}Cs , ^{205}Tl and ^{208}Pb ; at medium
165 resolution
166 (MR): ^{23}Na , ^{24}Mg , ^{31}P , ^{32}S , ^{42}Ca , ^{47}Ti , ^{51}V , ^{52}Cr , ^{55}Mn , ^{56}Fe , ^{59}Co , ^{60}Ni , ^{63}Cu , ^{66}Zn , ^{86}Sr , ^{89}Y , ^{121}Sb , ^{138}B
167 a, ^{139}La and ^{140}Ce ; and at high resolution (HR): ^{39}K (for details, see Fiket et al., 2017). Indium (^{115}In) at
168 concentration of $1\text{ }\mu\text{g L}^{-1}$ was used as an internal standard, and quantification was performed using the
169 external calibration method. All lab-ware was washed in 10% v/v HNO_3 (65% v/v pro analysis,
170 Kemika, Zagreb, Croatia) for at least 24 hours and rinsed with Milli-Q® water.

171 2.6. Comparisons between centrifuged and non-centrifuged extra virgin olive oil

172 In order to test the influence of pomace on the elemental composition of EVOO, 16 different samples
173 were subdivided into two aliquots each: one centrifuged as above, and the other non-centrifuged (in the
174 latter case, flow of pomace into the falcon bottles was allowed, and no visible pomace residues
175 remained in the original plastic bottles). After non-centrifuged EVOO samples were transferred to
176 falcon bottles and before adding acid extracting solution, the relative amount of pomace within each
177 sample was estimated after centrifugation at 3500 g for 1 min. The precipitated pomaces at the bottom
178 of the falcon bottles were clearly distinguishable by their brown colour in comparison with the green-
179 yellow EVOO colour, and were photographed. Pomace volume was calculated, assuming a truncated
180 cone (according to the falcon geometry), using ImageJ (Rueden et al., 2017). Finally, the centrifuged
181 and non-centrifuged EVOO samples were extracted with HNO_3 and HCl mixture as described above.
182 The non-centrifuged EVOO samples were divided into three groups (differing roughly by a factor of 10

183 in the estimated relative volume of pomace): high, medium and low pomace content, corresponding to,
184 respectively, 100-340, 17-42 and 0.1-0.2 of relative volume units. For each group, the results were
185 presented as box-and-whisker plots (Frigge et al., 1989) of ratio of element concentrations between
186 non-centrifuged (NC) and centrifuged (C) samples.

187 *2.7. Elements determination in extra virgin olive oil spiked with a known amount of pomace*

188 To further test the influence of pomace, 15 g of EVOO samples were spiked with 0.0015 g or 0.0150 g
189 pomace added with a stainless steel lab-spoon (corresponding to 0.1 and 1 g pomace in 1 kg of EVOO).
190 As pomace corresponding to the EVOO used in this experiment was not available, we used one of the
191 pomace samples prepared in this study. For each spiking, four independent samples were extracted with
192 HNO₃ and HCl mixture as described above.

193 *2.8. Data assessment and analysis*

194 Validation parameters of the ultrasonic acid extraction method included efficiency (recovery in pomace
195 after ultrasonic acid extraction as compared to microwave digestion), precision (repeatability) and limit
196 of detection (LOD). We calculated precision as relative standard deviation (RSD) of 10 independent
197 analyses of the same pure (centrifuged) EVOO sample. The limit of detection (LOD) was calculated as
198 three times the standard deviation (SD) of the data for the procedural blank sample analysed 10 times.

199 Statistical analyses were performed using *t*-test and one-way ANOVA, and means were
200 compared by Tukey test at $p \leq 0.05$ (Sokal & Rohlf, 2010). The correlation coefficients were estimated
201 for a relationship between the pomace amount and element concentrations in the non-centrifuged
202 EVOO samples (Sokal & Rohlf, 2010).

203 **3. Results and discussion**

3.1. Comparison of ultrasonic acid extraction with microwave digestion for extra virgin olive oil and pomace

The most frequently used extraction method for determination of elements in pomace and olive oil is microwave digestion (Lepri et al., 2011). However, due to high content of organic matter and strong matrix reactivity, high pressure develops during digestion; hence, the quantity of sample that may be processed is limited. Usually no more than 0.5 g of oil sample is recommended to be digested in a microwave oven (Lepri et al., 2011). Due to very low concentrations of most trace elements in olive oil, the microwave digestion method resulted in poor detection limits (Table 1). When we applied microwave digestion for determination of elements in EVOO samples, only seven (Cu, K, Mg, Mn, P, Rb and S) out of 29 measured elements were found in detectable amounts (Table 2). Potassium showed the highest concentration ranging from 7 to 8 $\mu\text{g g}^{-1}$, whereas from 10- to 30-fold lower concentrations were found for Mg, P and S (ranging from 0.2 to 0.7 $\mu\text{g g}^{-1}$). Finally, Cu, Mn and Rb concentrations (ranging from 2 to 8 ng g^{-1}) were up to three orders of magnitude lower than K concentration. This is in line with the literature data (Table 1) where minimal concentrations reported after microwave digestion, even when ICP-MS was used as a detection technique, were commonly in the order of ng g^{-1} (Bakkali et al., 2012; Beltrán et al., 2015; Llorent-Martínez et al., 2011; 2014).

The ultrasonic acid extraction showed very low detection limits (Table 3); when it was applied to the same EVOO samples described above regarding microwave digestion, 28 out of 29 measured elements were determined successfully, with only Tl below the detection limit (Table 4). Comparison of microwave digestion with ultrasonic acid extraction (Table 2) showed similar results (no significant difference) for elements that could be determined by microwave digestion. However, comparison of the detection limits for microwave (Table 1) and ultrasonic acid extraction (Table 3) showed considerably higher sensitivity of ultrasonic acid extraction. The detection limits of ultrasonic acid extraction were below 0.2 ng g^{-1} for all trace elements, with the highest method sensitivity achieved for Cd, Ce, Co, Cs,

228 La, Tl and Y (LOD values of 1 pg g^{-1}) (Table 3). The LOD values we obtained were comparable with
229 the data obtained by ultrasonic extraction of EVOO using the same extraction method (Camin et al.,
230 2010a, b), and were much better than obtained in other vegetable oils using similar extraction methods
231 (Ni et al., 2016; Trindade et al., 2015).

232 Unlike elements concentration in EVOO, data on elements concentration in pomace are scarce
233 in the literature, and based only on the microwave digestion method (Beltrán et al., 2015; Llorent-
234 Martínez et al., 2014). Because the ultrasonic acid extraction method was never applied to pomace, and
235 no published report could be found on the influence of pomace residue on the elements concentration in
236 EVOO containing pomace residues, we needed to verify the efficiency of ultrasonic acid extraction for
237 pomace. Therefore, we calculated the relative recoveries (ultrasonic acid extraction compared to
238 microwave digestion of 10 pomace samples) for analysed elements (Table 3). The mean recoveries for
239 most elements (Ba, Ca, Cd, Co, Cs, Cu, K, La, Mg, Mn, Na, P, Pb, Rb, Sn, Sr, Ti, Tl, Y and Zn) were
240 high (80-100%), but for some elements (Ce, Cr, Fe, Li, Mo, Ni, S, Sb and V), they were lower (50-
241 70%), albeit acceptable, thus showing the efficiency of the ultrasonic acid extraction procedure for
242 pomace. The lower recoveries for some elements were also obtained by the same extraction procedures
243 while testing efficiency of the method with multi-element standard in oil and fat (Camin et al., 2010a).

244 When discussing the analytical methods for multi-elemental analyses of EVOO, it should be
245 emphasised that Certified Reference Materials (CRM) for olive oil do not exist. Therefore, the
246 validation of existing analytical methods was performed using various commercially-available multi-
247 element oil standards (Camin et al, 2010a,b). However, these oil standards are often not a good
248 substitute for vegetable oils, especially not for EVOO, due to different matrices. In contrast, it is
249 reasonable to assume a similar organic matrix in both pomace and EVOO; therefore, pomace should be
250 a better model for EVOO validation methodology in comparison to the commercially-available multi-

251 element oil standards. We may, thus, consider the recoveries obtained for pomace (Table 3) valuable in
252 validation of the method for elements determination in EVOO.

253 *3.2. Concentrations and ratios of elements in pomace and olive oil*

254 The concentration ranges of 29 elements measured in 10 different pomace and the corresponding
255 centrifuged EVOO samples (except for Tl, which was not detected in EVOO) are shown in Table 4.
256 The concentrations of elements obtained in Croatian EVOOs in this work (Table 4) are similar to the
257 concentrations obtained in Italian and other European EVOOs reported by Camin et al. (2010a, b)
258 (Table 1). Exceptions are K, Mg, Mn and Na, whose concentrations were about 10 times higher in
259 Croatian samples, and this could be related to different sample preparation and analytical methods, as
260 well as genotypic and geographic differences. However, concentrations of both macro and trace
261 elements in our samples (Table 4) were lower compared to the data reported in other studies (see Table
262 1) in which preparation steps did not explicitly include centrifugation and pomace separation from oil.
263 Some of the data presented in Table 1 were from Croatian olive oils (Brkljača et al., 2013; Cindric et
264 al., 2007; Zeiner et al., 2005) in which higher concentrations of all elements (except K) were obtained
265 compared with the present study (Table 3).

266 The range of element concentrations in pomace (Table 4) was in agreement with the scant
267 literature data (Llorent-Martínez et al., 2014; Beltrán et al., 2015). Depending on a particular element,
268 the range was from 330 to >100,000 times higher in pomace than in EVOO (Table 4).

269 Plant macronutrients Ca, Mg, K, P and S and the beneficial element Na were present in the
270 highest concentrations in both pomace and pure (centrifuged) EVOO (Table 4). This is consistent with
271 published observations regarding element concentrations in EVOO and olive pomace (Beltrán et al.,
272 2015), and also regarding composition of wheat germ oil (Brandolini & Hidalgo, 2012). On the other
273 side, micronutrients Cu, Fe, Mn and Zn were consistently present at concentrations lower than
274 macronutrients but higher than other trace elements not involved in plant metabolism (Table 4). This is

275 in agreement with the data on *Camellia* oil (Ni et al., 2016). The above observations are consistent with
276 the plant nutritional requirements and reflect the element concentrations in plant aboveground biomass
277 (Marschner, 2012). An exception would be Rb, present in concentrations similar to those of
278 micronutrients. Even though Rb is not involved in plant metabolism, it is a chemical analogue of K,
279 and plants can absorb it readily through the K pathways (White, 1997).

280 Although the element concentrations generally decreased in both pomace and EVOO in the
281 order: macro> micro>trace elements, the distribution pattern of single elements within each of this
282 categories was not the same in pomace and EVOO. Indeed, elements concentration in pomace
283 decreased in the order:

284 K>Ca>P>Mg>S>Na>Rb>Cu>Fe>Mn>Zn>Sr>Ni>Ba>Cr>Mo>Ti>Co>Sb>Cs>Pb>Sn>Li>Cd>V>Ce
285 =Ti>La>Y. In contrast, elements concentration in EVOO decreased in the order:

286 K>S>P>Ca>Mg>Na>Fe>Zn>Cu>Mn>Rb>Ni>Sr>Sn>Pb>Ba>Ti>Li>Mo>Cr>Sb>Y>Co>V>Ce>La>
287 Cs>Cd. Importantly, the proportions of different elements in pomace and EVOO differed widely (Table
288 4). These findings imply that the presence of pomace residue increases the concentration of elements in
289 EVOO and such an increase is element-specific. Hence, we calculated the expected increase of
290 elements concentration after adding 1 g of pomace (containing median elements concentration) into 1
291 kg of EVOO. The increase was the highest (50-80 times) for K, Rb, Sr, Ca, Cu, Mn and P, lower (10-
292 30 times) for Mg, Cr, Cs, Co, Ba, Zn and Fe, even lower (5-10 times) for Mo, Cd, Na, Sb, Ni and S,
293 and the lowest (less than 5-fold) for Ti, V, Ce, La, Pb, Li, Y and Sn (Table 4).

294 3.3. Evaluation of pomace influence on elements concentration in olive oil

295 In the process of developing the method for multi-element analysis of EVOO, we started with analyses
296 of non-centrifuged EVOO samples and obtained very inconsistent data from repeated analyses of the
297 same sample (data not shown). In order to quantify, not only theoretically (Table 4), the influence of
298 pomace residues on elements concentrations in EVOO we performed two additional experiments.

299 In the first experiment, we compared elements concentrations between non-centrifuged (NC)
300 and centrifuged (C) EVOO samples. In the group with estimated high pomace content, median NC-to-
301 C ratios were >1000 for K, P and Rb (Fig. 1). In the same group of EVOO samples, ranges of median
302 NC-to-C ratios for Ba, Ca, Co, Cs, Cu, Mg, Mn, Na, S, Sr and Zn were between 30 and 650, whereas
303 for Cd, Cr, Fe, Mo, Ni and Pb they were between 9 and 17. Finally, NC-to-C ratios for Ce, La, Li, Sb,
304 Sn, Ti, V and Y were small (between 1 and 4) (Fig. 1). The difference between non-centrifuged and
305 centrifuged EVOO samples was particularly pronounced for elements with concentrations in pomace
306 higher than 10,000 ng g⁻¹ (Ca, Cu, Fe, K, Mg, Mn, Na, P, Rb, S and Zn). In contrast, the small NC-to-C
307 ratios were generally observed for those elements whose concentration in pomace was below 100 pg g⁻¹
308 (Cd, Ce, Cr, La, Li, Pb and Sn) (Fig. 1).

309 In the group with an estimated medium amount of pomace residues, the NC-to-C ratios were
310 >20 for Ba, K, P, Rb and Sr, between 5 and 19 for Ca, Co, Cr, Cu, Mg, Mn, Pb, S, Ti and Zn, and
311 between 1 and 3 for the other elements (Fig. 1). Finally, in the group with estimated low content of
312 pomace residues, the NC-to-C ratios were mostly around 1, except for Ca, Cr, K, Mg, Mn, P, Rb, Sn,
313 Sr and Zn (between 3 and 7) (Fig. 1).

314 In order to illustrate more clearly the pomace influence on elements concentration in EVOO, the
315 relationships between relative pomace residue content and elements concentration in the non-
316 centrifuged EVOO samples were presented in scatterplots (Fig. S1). In the group of samples with the
317 high pomace residue content, the positive and significant correlations (from 0.75 to 0.91; $p \leq 0.05$) were
318 found for many elements, particularly Ca, Cd, Co, Cu, Fe, K, Mg, Mn, Mo, P, S, V and Zn. No
319 correlation was found for Cs, Li, Sn and Ti. On the other hand, in the samples with the medium pomace
320 residue content, only Ce and Sb ($r = 0.80$ each; $p \leq 0.05$) showed significant positive r values, whereas
321 no correlation was found for samples with the low pomace content (Fig. S1). Therefore, our data

322 indicated that the discrepancy in the element concentrations between the non-centrifuged and
323 centrifuged samples was related to the amount of pomace residue and element concentrations in it.

324 To quantify the pomace influence on the element concentrations in EVOO (in the above
325 described experiment, the exact amount of pomace in EVOO was not known), we prepared the second
326 experiment whereby the EVOO samples were spiked with different amounts of pomace (0.1 or 1 g of
327 pomace per kg of EVOO) (Fig. 2). Although pomace added was not the same as pomace originally
328 present in non-centrifuged EVOO samples, it was the only way to perform a comparative experiment
329 with exactly known quantity of pomace in each EVOO sample. The addition of pomace at the level of
330 0.1 g kg^{-1} resulted in a small, but significant increase only in Cr concentration. In contrast, 1 g of
331 pomace added to 1 kg of EVOO increased significantly (up to 5 times) concentrations of most elements
332 (except Ce, La, Mo, Pb, Sb, Sn, Ti, V and Y). This increase in concentration was in agreement with the
333 increase predicted by calculations (Table 4), but for some elements (especially macro elements) it was
334 smaller. The reason for this difference is the fact that the EVOO sample used in this experiment
335 contained higher concentrations of macro elements (Fig. 2) than the median concentrations of the 10 oil
336 samples used in Table 4 calculations.

337 The results of this study indicate a clear impact of pomace residue on EVOO element
338 concentrations and distribution depending on both quantity and composition of pomace. In addition to
339 the change in concentration of individual elements, the unequal NC-to-C (non-centrifuged vs
340 centrifuged) ratios (Fig. 1) for various elements indicated that (i) influence of pomace differed for
341 various elements and (ii) it also changed the elements relative ratios (i.e. fingerprint) in EVOO. If such
342 a fingerprint would be used for EVOO traceability, which relies on the assumption that trace element
343 distribution in EVOO corresponds to the geographic origin, presence of pomace in EVOO may
344 interfere with such traceability application. Therefore, the improper sample pre-treatment and
345 preparation procedure and the analysis of non-centrifuged samples could lead to inaccurate data. The

346 centrifugation of EVOO samples is a crucial step in sample preparation as pomace (even when present
347 in small quantities) can influence the element concentration and the relative ratios of elements.

348 **4. Conclusions**

349 Ultrasonic acid extraction of both EVOO and pomace followed by ICP-MS detection allows
350 determination of a large number of elements at low pg g^{-1} levels, including macro, micro and trace
351 elements. The study reveals that pomace residues, when present in olive oil even at small quantities
352 (less than 1 g in 1 kg), significantly alter not just the concentrations, but also the relative ratios of
353 elements present in EVOO. Therefore, an adequate sample pre-treatment, including EVOO
354 centrifugation and pomace separation, is essential for application of EVOO multi-element composition
355 in traceability studies and determination of geographic origin of oil.

356 **Acknowledgments**

357 We thank Anton Paar GmbH for providing a Multiwave GO microwave oven.
358 Funding: This work was supported by the Unity through Knowledge Fund [“Role of soil properties and
359 environmental conditions in elemental and isotopic composition of the olive: basis for oil geographic
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473

474 **Table 1.** Comparison of literature data for elements determination in extra virgin olive oil (EVOO).

475 Data represent the ranges of concentrations (ng g⁻¹). For comparative purposes, some information on

476 the analytical methods used is also given, as well as the limits of detection.

Reference	Bakkali et al. (2012)	Llorent-Martínez et al. (2011)	Beltrán et al. (2015)	Benincasa et al. (2007)	Zeiner et al. (2005)	Cindric et al. (2007)	Brkljača et al. (2013)	Castillo et al. (1999)	Bakircioglu et al. (2013)	Camin et al. (2010b)	Camin et al. (2010a)
Preparation method	MW [†] (HNO ₃ +H ₂ O ₂)	MW (HNO ₃)	MW (HNO ₃ +HCl+H ₂ O ₂)	MW (HNO ₃)	MW (HNO ₃ +HCl+H ₂ O ₂)	MW (HNO ₃ +H ₂ O ₂)	dry ashing + HNO ₃ extraction	EBE	UE, EBE	UE (HNO ₃ +HCl)	UE (HNO ₃ +HCl+H ₂ O ₂)
Detection technique	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-AES; ETA-AAS	ICP-AES, GF-AAS	ET-AAS	ICP-MS	ICP-AES	ICP-MS	ICP-MS
Country	Spain	Spain	Spain	Italy	Croatia	Croatia	Croatia	Spain	Turkey	Italy	Europe
Range of LODs for presented elements	0.007–0.038	0.5–40	not available	0.03–370	1–50	50–1630	0.2–150	0.25–50	not available	0.001–25	0.001–25
Ba		<0.5–28.4	9.9–22.4					100–700		0.550–2.49	<0.12–0.37
Ca			5.1–11.2	1800–15,000	1250–9000	1800–2200				0.395–0.950	100–133
Cd	1.1–7.1	<15		0.088–0.366			<0.6	3–4	10–78		<0.005
Ce										0.002–0.058	<0.0050–0.0081
Co	4.1–18.8	<1.5	7.9–11	0.056–0.413	<1.0–5400	1120–1150		140–180		0.003–0.006	
Cr	1.8–7.3	18–120	52–123	116–437	<1.0	<1		690–2000	12–1800		
Cs			0.2							0.003–0.012	<0.001–0.006
Cu	13.1–96.4	<1.5–130	14–47		40–4510	30–50	<6–10	140–280	244–1500	0.160–2.107	0.3–0.51
Fe		<40–200	0.5–1.2	89–1600	13,100–18,500	14,200–16,600	500–1300		1100–8800		
K			5.9–6.9		50–190	20–100				0.033–1.171	62–156
La										0.001–0.064	<0.0020–0.0052
Li			2.9–6.4							0.004–0.031	<0.008
Mg			0.6–0.9	56–1000	2900–3600	3200–3600				0.015–0.087	13–19
Mn	6.6–15.7	3.5–150	0.1–0.2	<9.2–12.7	<1.0–130	90–200		10–24	7–35	0.058–0.921	<0.2–0.003
Mo	2.1–15.6	<1.5	13.1–24.3								<0.050
Na			3.8–7.8		29,000–39,000	34,000–34,500				0.060–0.280	<20–34
Ni	3.4–17.9	<15	79.7–174	21.2–46.9	70–2300	1100–1900	<12	40–70	432–1200		<1
Pb	4.4–92.5	<0.8–5.0	48.3–74.2		<1.0	<1	<3–4	12–24	37–134	0.299–8.46	<0.1–0.1
Rb			0.8–2.6							0.036–0.628	
Sb	1.0–10.4	<1.5		0.194–0.411							
Sn	1.8–9.0		1.2–13.3								
Sr				<9.6–48.9						1.12–3.85	<0.3
Ti		<15									
Tl		<1.5						<500			<0.004
V		<2.0–5.0	13.8–18.4					170–460			<0.007–0.009
Zn			149–328		2800–4000	3000–3700			424–2500		8–12

[†]MW - microwave digestion; UE- ultrasonic extraction; EBE- emulsion breaking extraction

478 **Table 2.** Comparison of element concentrations in centrifuged extra virgin olive oil (EVOO) after
 479 microwave digestion with nitric acid (MW) or ultrasonic acid extraction (UAE) with the mixture of
 480 HNO₃ and HCl. Only the elements that could be determined after microwave digestion are presented.
 481 For each element, identical letters indicate no significant difference between MW and UAE ($p \leq 0.05$, t -
 482 test). Values are means $\pm$ SD, n = 4.

Element	MW	UAE
Cu (ng g ⁻¹)	2.3 $\pm$ 0.1 a	2.3 $\pm$ 0.1 a
K (μg g ⁻¹)	7.4 $\pm$ 0.4 a	8.0 $\pm$ 0.6 a
Mg (μg g ⁻¹)	0.24 $\pm$ 0.05 a	0.22 $\pm$ 0.01 a
Mn (ng g ⁻¹)	7.9 $\pm$ 0.4 a	8.5 $\pm$ 0.3 a
P (μg g ⁻¹)	0.70 $\pm$ 0.01 a	0.71 $\pm$ 0.02 a
Rb (ng g ⁻¹)	5.7 $\pm$ 0.1 a	5.5 $\pm$ 0.1 a
S (μg g ⁻¹)	0.35 $\pm$ 0.04 a	0.37 $\pm$ 0.01 a

483

484 **Table 3.** Limits of detection (LOD) and precision (repeatability) for elements in olive oil after
 485 ultrasound acid extraction, and recovery of elements in pomace determined by ultrasonic acid
 486 extraction versus microwave digestion.

Element	Limit of detection for EVOO (LOD, ng g ⁻¹)	Precision for EVOO (RSD [†] , %)	Recovery for pomace (%)
Ba	0.02	10	97 ± 16
Ca	5.1	2	98 ± 7
Cd	0.001	9	100 ± 13
Ce	0.001	1	69 ± 13
Co	0.001	8	99 ± 9
Cr	0.005	6	83 ± 23
Cs	0.001	6	81 ± 22
Cu	0.05	2	103 ± 5
Fe	0.1	7	53 ± 8
K	2.1	3	98 ± 13
La	0.001	10	82 ± 25
Li	0.01	20	56 ± 18
Mg	2.0	1	103 ± 4
Mn	0.01	1	106 ± 5
Mo	0.003	7	57 ± 19
Na	2.1	5	96 ± 8
Ni	0.05	11	64 ± 26
P	1.0	1	84 ± 7
Pb	0.02	20	78 ± 25
Rb	0.009	1	103 ± 3
S	1.0	1	57 ± 11
Sb	0.002	15	60 ± 15
Sn	0.02	16	70 ± 20
Sr	0.01	2	93 ± 13
Ti	0.01	15	79 ± 20
Tl	0.001	7	108 ± 26
V	0.002	9	58 ± 16
Y	0.001	10	80 ± 13
Zn	0.2	10	89 ± 14

[†]RSD=relative standard deviation

487

Table 4. Median values and ranges of concentrations (ng g⁻¹) of elements in 10 pomace samples and corresponding centrifuged EVOO samples determined by ultrasonic acid extraction (UAE). Median value of ratios between concentrations in pomace and EVOO (4th column), and the calculated increase factor of concentration in EVOO (5th column) after simulated addition of 1 g pomace (characterised by elements median values and the range, 2nd column) into 1 kg of virtual EVOO (characterised by the elements median values and the range, 3rd column) are shown as well.

Element	Pomace (UAE)	EVOO	Ratio (Pomace / EVOO)	Increase factor
Ba	263 (160–837)	0.020 (0.01–1.07)	11,100	15
Ca	1,360,000 (1,240,000–2,000,000)	27.8 (7.14–212)	50,000	54
Cd	7.33 (4.33–9.03)	0.001 (0.001–0.002)	6360	9
Ce	2.99 (1.00–5.91)	0.002 (0.001–0.04)	2210	3
Co	39.9 (12.0–112)	0.003 (0.001–0.01)	16,100	18
Cr	98.8 (88.5–141)	0.005 (0.005–0.58)	18,400	22
Cs	17.7 (3.00–44.0)	0.001 (0.001–0.004)	12,700	19
Cu	12,500 (9060–14,700)	0.30 (0.06–1.99)	45,200	47
Fe	12,000 (10,300–17,600)	1.11 (0.19–2.57)	13,200	13
K	21,500,000 (13,400,000–27,700,000)	286 (7.28–1670)	103,600	83
La	1.82 (1.00–11.8)	0.001 (0.001–0.02)	1500	3
Li	7.42 (1.00–18.1)	0.01 (0.005–0.04)	367	2
Mg	643,000 (551,000–729,000)	24.6 (2.31–99.2)	26,200	29
Mn	10,700 (7600–13,200)	0.25 (0.05–7.18)	39,000	47
Mo	44.7 (40.9–63.2)	0.006 (0.003–0.01)	7860	10
Na	38,000 (14,000–134,000)	4.92 (1.74–315)	7860	9
Ni	408 (39.1–2590)	0.08 (0.05–0.14)	4080	7
P	1,160,000 (686,000–1,570,000)	28.2 (3.83–367)	54,900	46
Pb	16.2 (2.00–23.9)	0.02 (0.02–0.52)	536	2
Rb	14,100 (11,000–29,300)	0.24 (0.01–1.81)	102,000	66
S	400,000 (340,000–577,000)	89.4 (32.2–357)	4770	6
Sb	17.2 (3.50–98.5)	0.003 (0.002–0.01)	6500	7
Sn	8.40 (1.40–16.8)	0.02 (0.02–0.10)	330	2
Sr	1280 (1050–3530)	0.02 (0.01–0.17)	87,900	56
Ti	42.4 (32.0–335)	0.01 (0.004–0.05)	3010	4
Tl	2.99 (0.50–9.00)	<0.001	-	-
V	5.94 (2.99–6.01)	0.002 (0.002–0.01)	1730	4
Y	1.16 (0.77–2.43)	0.003 (0.001–0.02)	380	2
Zn	10,300 (7300–12,900)	0.88 (0.25–5.61)	11,400	14

495 **Figure captions**

496 **Fig. 1.** Box-and-whisker plots of ratio of element concentrations in non-centrifuged (NC) and
497 centrifuged (C) extra virgin olive oil (EVOO) samples. Sixteen different non-centrifuged EVOO
498 samples were divided into three groups based on estimated quantity of pomace residue (high, medium
499 and low). For each element and each group of ratios, median values (the line inside the box), 25-75%
500 interquartile range boxes and whiskers are shown. The ends of whiskers are representing the lowest
501 datum still within 1.5 times interquartile range of the lower quartile, and the highest datum still within
502 1.5 times interquartile range of the upper quartile. Outliers, defined as data outside the whiskers, are
503 presented as circles. Whisker are not shown if the 25% interquartile was equal to the lowest datum
504 (excluding the outliers), or if the 75% interquartile was equal to the highest datum (excluding the
505 outliers).

506 **Fig. 2.** Comparison of element concentrations determined by ultrasonic acid extraction with 2% v/v
507 HNO₃ and 0.2% v/v HCl in centrifuged extra virgin olive oil (EVOO) and in the same EVOO after
508 addition of 0.1 g kg⁻¹ or 1 g kg⁻¹ of pomace. For each element, different letters indicate significant
509 differences in concentration among the pure (centrifuged) and spiked EVOO samples ($p \leq 0.05$, Tukey
510 test). Values are means + SD, n = 4.