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Ultrasound-assisted extraction and bioaccessibility of saponins from edible seeds: quinoa, lentil, fenugreek, soybean and lupin

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Abstract

The efficient production of saponin-rich extracts is of increasing interest due to the bioactive properties that have being demonstrated for these compounds. However, saponins have a poor bioavailability. In this respect, the knowledge about the bioaccessibility of saponins as a first step before bioavailability has been scarcely explored. In this study, the production of ultrasound-assisted extracts of saponins from edible seeds (quinoa, soybean, red lentil, fenugreek and lupin) was carried out with ethanol, ethanol:water or water. Extraction yield, total saponin (TSC), fat and total phenolics content (TPC) were determined. Then, the bioaccessibility of saponins after the in vitro gastrointestinal digestion of the extracts was determined and the effect of TPC and fat in the extracts on bioaccessibility was evaluated.

The highest saponin-rich extracts were obtained by ethanol, being fenugreek and red lentil the richest extracts (12% and 10%, respectively). Saponins from ethanol:water extracts displayed variable bioaccessibility (from 13% for fenugreek to 83% for lentil), but a bioaccessibility closer to 100% was reached for all ethanol extracts. Correlation studies showed that TPC of the extracts negatively affected the bioaccessibility of saponins, whereas fat of the extracts enhanced this parameter.

As summary, ultrasound-assisted extraction is shown as an efficient method for obtaining saponin-rich extracts from edible seeds, being ethanol the most advantageous solvent due to the richness of saponins and the successful bioaccessibility from these extracts, likely caused by the co-extracted fat with ethanol. Regardless of the extracts, phenolic compounds or fat may hinder or enhance the bioaccessibility of saponins, respectively. Additionally, an adequate balance between saponins to lipids has shown to be relevant on such an effect.

Keywords: ultrasound-assisted extraction; saponins; edible seeds; legumes; gastrointestinal digestion; bioaccessibility; lipids; polyphenols

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Abbreviations Used

EY	Extraction yield
E	Ethanol extract
E:W	Ethanol:Water extract
FC	Fat content
GAE	Gallic acid equivalents
TPC	Total phenolic content
TSC	Total saponin content
UAE	Ultrasound-assisted extraction

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1. Introduction

Saponins constitute a wide group of structurally related compounds consisting of a triterpenoid or steroid non-polar aglycone (also known as sapogenin) attached to one or more hydrophilic oligosaccharide moieties through an ether or ester glycosidic linkage. Such combination of polar and non-polar structural elements confers them foaming and emulsifying properties. Saponins are largely distributed in the plant kingdom and are mainly found in the seeds, leaves, roots, fruits and stems. Triterpenoid saponins have been identified in legumes (soybean, lentils, alfalfa and chickpeas, among many others), quinoa seeds, ginseng roots, quillaja bark or liquorice roots, whereas steroid saponins have been found in fenugreek seeds, yucca, ginseng roots, asparagus or oats (Güçlü-Üstündağ & Mazza, 2007; Makkar, Siddhuraju & Becker, 2007).

Although saponins have traditionally been recognized as antinutrients due to their hemolytic activity, their inhibitory activity of digestive enzymes, or their effect on the permeability of the small intestinal mucosal cells, current research is being focused on saponins and sapogenins as bioactive compounds in view of an increasing evidence on their hypocholesterolemic, anti-inflammatory, antitumor, immunomodulatory, antibacterial, antiviral, antifungal and antiparasitic activities (Singh, Singh, Singh, & Kaur, 2017). Taking into consideration the potential of these molecules as bioactive agents, great efforts are being made to obtain saponin-rich extracts from non-conventional extraction methods, as conventional technologies (maceration, Soxhlet extraction, serial exhaustive extraction or hydrodestillation) are time-consuming, require high purity solvents and present low extraction selectivity and efficiency (Nguyen, Pham, Bowyer, Altena, & Scarlett, 2016). Among the most studied non-conventional technologies for the extraction of bioactive compounds from plant materials, ultrasound-assisted extraction (UAE) has been successfully developed for such purpose thanks to significantly reduced extraction times, energy consumption and higher extraction efficiency, although it has not been sufficiently explored in saponin extraction (Cheok, Salman, & Sulaiman, 2014). Both direct and indirect sonication has been applied on the extraction of saponins from different varieties of ginseng roots in order to evaluate the effect of

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water, methanol and buthanol on the yield of total saponins and ginsenosides (Wu, Lin, & Chau, 2001). Nonetheless, the influence of other green solvents, such as ethanol or water, and their combinations on the total saponin content of the final UAE extracts has been scarcely evaluated, being the studies rather recent (Champa, Whangchai, Jaturonglumlert, Nakao, & Whangchai, 2016; Ha et al., 2006). In the specific case of saponin extraction from edible seeds, the UAE is also novel and scarce (Wani, Bishnoi, & Kumar, 2016).

However, the major challenge in developing saponin-rich extracts for their use as functional ingredients, is their limited gastrointestinal absorption and, consequently, their poor bioavailability (Navarro del Hierro et al., 2018). In any oral-taken compound, its aqueous solubility in the intestinal lumen is one of the key properties that modulates its bioavailability. Generally, saponins are hydrosoluble thanks to the hydrophilic sugar chain(s) in their structures. Moreover, the amphiphilic nature of these molecules grants them the capacity to self-micellate, which increases their dispersion in the aqueous media for further absorption by enterocytes (Böttcher & Drusch, 2017). However, the good solubility of saponins should not be generalized for all saponins, since variable results of water solubility depending on the type of saponin have been described (Güçlü-Üstündağ & Mazza, 2007; Navarro del Hierro et al., 2018). On the other hand, due to the lack of sugar moieties in sapogenins, the aglycones have shown improved chemical properties compared to their precursor saponin that enhance their permeability and bioactivity, such as a lower molecular weight, higher lipophilicity or lower molecular flexibility (Gao, Basu, Yang, Deb, & Hu, 2012). However, due to these properties, the solubility of sapogenins in water is considerably lower than its corresponding glycoside. Therefore, the importance of the solubility of saponins and sapogenins on their final bioavailability seems to be relevant, and it could be determined through a preliminary study of bioaccessibility. This term refers to the amount of a compound that is released during digestion to a potentially absorbable form (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009; Kamiloglu et al., 2015). In the specific case of saponins, bioaccessibility might be defined as the fraction of total ingested saponins that remains solubilized and stable before cell absorption

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(Navarro del Hierro et al., 2018). Nonetheless, studies regarding the bioaccessibility of saponins are quite limited. Serventi et al. (2013) reported values of bioaccessibility in the range of 30-91% for different types of soybean and chickpea saponins incorporated in bread formulations, concluding that bile salts modulated their solubility either positively or negatively depending on the type of saponin. Other authors have evaluated the effect of cooking time on the bioaccessibility of soyasaponins from lentils, reporting values of 9-10% as well as a correlation between these two factors (Sagratini et al., 2013). The variable results in the described studies might be related to certain factors (type of saponin, temperature, salt concentration and pH of the aqueous phase) that condition the size and structure of micelles, as stated by diverse authors (Mitra & Dungan, 2001; Oakenfull, 1986). In addition to that, Martin et al. (2016) have recently demonstrated that the co-digestion of a marigold extract with olive oil enhanced the bioaccessibility of triterpenoid compounds present in such extract, which are molecules chemically analogous to the typical triterpenoid sapogenins. Thus, not only physicochemical factors during the digestion might influence the bioaccessibility of saponins, but also other components found in the extracts would affect either positively or negatively the potential absorption of saponins.

The present study aims to evaluate the effect of solvent (ethanol, aqueous ethanol or water) during UAE on the total saponin content of extracts from edible seeds (quinoa, lentil, fenugreek, soybean and lupin), as well as on the extraction of other compounds of interest, such as fat and total phenolic content. The subsequent *in vitro* gastrointestinal digestion of the extracts was performed, in order to assess the bioaccessibility of saponins of the extracts, and the effect of other components in the extracts on such parameter.

2. Materials and Methods

2.1. Reagents and materials

Seeds of red quinoa (*Chenopodium quinoa*), soybean (*Glycine max*), peeled red lentil (*Lens culinaris*) and lupin (*Lupinus albus*), as sources of triterpenoid saponins, were purchased from Hijo

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de Macario Marcos (Salamanca, Spain). Fenugreek (*Trigonella foenum-graecum*), as source of steroidal saponins, were from Murciana de Herboristeria (Murcia, Spain).

Oleanolic acid, gallic acid, quillaja bark saponins, diosgenin and vanillin were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Folin-Ciocalteu's reagent and sodium carbonate salt were from Panreac (Barcelona, Spain).

Trizma, maleic acid, Amano lipase A from *Aspergillus niger*, pepsin, pancreatin from porcine pancreas, bile salts, phosphatidyl choline from egg yolk were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Ultrasound-Assisted Extraction (UAE)

Seeds were ground in a knife mill (Grindomix GM200 RETSCH) at 10000 rpm for 1 min. The resulting powder was sieved in a vertical sieve (CISA Cedacería Industrial, España) in order to obtain fractions with a particle size ≤ 250 μm. Extraction was carried out by direct sonication (Branson SFX250 Digital Sonifier, Branson Ultrasonics, USA) with an ultrasonic probe (1/2" diameter). Samples were extracted with either ethanol, water or ethanol:water (1:1, v/v) at a ratio of sample to solvent of 1:10 (w/v) for 15 min, with a sonication output amplitude of 60%. The temperature during the extraction process was kept under 75 ºC and extractions were performed at least in duplicate. The mixture was then centrifuged at 3400 x g for 10 min. Supernatant of samples extracted with ethanol and ethanol:water was dried under vacuum using a rotary evaporator, whilst the aqueous fraction was lyophilized. Extraction yield (EY) was estimated and expressed as g of UAE extract per 100 grams of seed.

For the subsequent characterization of the extracts, the following treatment was performed. Hexane and methanol were added to the UAE extracts at a ratio 1:1:0.03 (v/v/w). Samples were vortexed and centrifuged at 14129 x g for 3 min. The upper hexane fraction was collected and the extraction of the sample with hexane was repeated. The two collected fractions of hexane were dried in order

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to assess the total fat content gravimetrically (expressed as g of fat per 100 g of UAE extract). The lower methanol fraction was dried and the total saponin content was determined.

2.3. Total saponin content

Total saponin content (TSC) of the UAE extracts was determined using a spectrophotometric method as described by Ncube et al. (2011) with minor modifications. Briefly, the dried methanolic extracts previously obtained were prepared at 2 mg/mL in methanol. Aliquots of 125 µL were transferred to vials, followed by 125 µL of freshly prepared vanillin in ethanol (0.8%, w/v) and 1.25 mL of sulphuric acid in water (72%, v/v). A control sample using methanol was also prepared. Samples were vortexed and heated at 60 ºC for 10 min. Vials were cooled in ice for 5 min and absorbance was measured at 520 nm using a UV–vis spectrophotometer (Genesis 10UV, Thermo Scientific) against the control sample containing methanol. The TSC was obtained from a standard curve of oleanolic acid (for lentil, lupin, quinoa and soybean) or diosgenin (for fenugreek) ranging from 100 to 1500 µg/mL, which were prepared under the same conditions as previously stated for samples. Oleanolic acid was used as a representative standard of terpenoid saponins, whereas diosgenin was used as a representative standard of steroid saponins. Results were expressed as g of total saponins per 100 g of UAE extract. Determinations were done in duplicate.

2.4. Total phenolics content

Total phenolics content (TPC) was measured using the Folin-Ciocalteu assay developed by Singleton et al. (1999) with minor modifications. Briefly, 10 µL of UAE extracts at 5 mg/mL (for ethanolic extracts, ethanol was used as solvent, whereas in the ethanol:water and water extracts, the solvent chosen was water), 600 µL of MiliQ water and 50 µL of Folin-Ciocalteu reagent were mixed and allowed to stand for 1 min at room temperature. Next, 150 µL of a 20% sodium carbonate solution were added, followed by 190 µL of MiliQ water. A control sample was also prepared. After incubation at room temperature for 1 hour in darkness, the absorbance of the

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mixture was read at 760 nm using either water or ethanol as blanks. The results were expressed as g of gallic acid equivalents (GAE) per 100 g of UAE extract using a standard curve of gallic acid (ranging from 12.5 to 1000 μg/mL). Determinations were done in triplicate.

2.5. In vitro gastrointestinal digestion

The *in vitro* digestion model was based on Martin et al. (2016) with slight modifications. For gastric digestion, 100 mg of the UAE extract (ethanol or ethanol:water) from each seed, and 10 mg of lecithin were mixed with a gastric solution (14 mL) at pH 2.5 containing NaCl (150 mM) and $CaCl_2$ (6 mM). This mixture was shaken in an orbital incubator at 200 rpm and 37 ºC for 1 min to allow the dispersion of the components. The gastric digestion was initiated by the addition of 3 mL of a fresh extract of gastric enzymes containing 170 mg of gastric lipase and 15 mg of pepsin in gastric solution, prepared by stirring for 10 min. Gastric digestion was performed for 45 min. The final pH after gastric digestion was 4.5. For intestinal digestion, a solution simulating a biliary secretion at pH 7.5 was previously prepared (0.1 g of lecithin, 0.25 g of bile salts, 0.5 mL of 325 mM $CaCl_2$ solution, 1.5 mL of 3.25 M NaCl solution, and 10 mL of Trizma-maleate buffer 100 mM pH 7.5). All these components were homogenized for 1 min at 3500 rpm (Ultra-Turrax IKA T18). At the end of gastric digestion, this biliary secretion was immediately added, and the whole medium was shaken in the orbital incubator for 1 min at 200 rpm and 37 ºC. Finally, fresh pancreatin extract (0.5 g of pancreatin in 3 mL of Trizma-maleate buffer 100 mM pH 7.5), stirred for 10 min and centrifuged at 2688 x g for 15 min was added. Intestinal digestion was continued for 60 min. The final pH of intestinal digestion was 7.

Control digestions following the same procedure but in absence of extracts were also performed in order to determine the bioaccessibility of saponins as described in the following section.

2.5.1. Determination of bioaccessibility of total saponins

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At the end of digestion, the digestion medium was submitted to centrifugation at 2688 x g for 40 min (5810R Eppendorf Iberica, Madrid, Spain). After centrifugation, an upper aqueous phase (micellar phase, MP) and a minor precipitated phase were obtained. Aliquots from both the digestion medium (before centrifugation) and the MP were taken. The saponin content in the digestion medium and the MP was determined spectrophotometrically following the same procedure as described previously, although vanillin solution was prepared at 0.08% in ethanol (w/v). Additionally, standard curves of quillaja saponins diluted in either the control digestion medium or the control MP (ranging from 10 to 1500 µg/ml) were used to quantify the saponin content of the digestion medium or the MP, respectively. Absorbance was measured at 520 nm against a reagent blank containing Trizma-Maleic buffer and determinations were performed at least in triplicate. Finally, the bioaccessibility of total saponins was determined as:

Bioaccessibility (%) = (mg of saponins in MP/mg of saponins in digestion medium) x 100

2.6. Statistical analysis

Statistical analyses were performed by means of the general linear model procedure of the SPSS 24.0 statistical package (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance. Differences were considered significant at $p \leq 0.05$. Post-hoc Tukey's tests were performed in order to establish significant differences. Pearson's correlation tests were conducted for additional analyses.

3. Results and Discussion

3.1. Extraction yield and total saponin content

After performing the UAE of the edible seeds, the first parameter evaluated was the EY, defined as the amount of crude dried extract obtained from ground sample (Table 1). Regardless of the seed, the use of water as extraction solvent provided the highest EY compared to the ethanol and ethanol:water extractions ($p \leq 0.001$). These results may be attributed to the higher solubility of

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certain compounds, such as proteins and carbohydrates, in water and aqueous ethanol mixtures than in pure ethanol (Zieliński & Kozłowska, 2000), a solvent which may in turn enhance the extraction of other non-polar components. On the other hand, regardless of the solvent, the highest EY was obtained for soybean ($p \leq 0.001$), being the EY of lupin, quinoa, fenugreek and lentil similar. Thus, taking into account both factors, namely solvent and seed, soybean extracted with water led to the highest EY (mean value 61.3 g 100 g^{-1}), whereas the ethanol extract of lentil displayed the lowest EY (mean value 4.5 g 100 g^{-1}). Therefore, the obtained results suggest a great variability in the EY among the assayed edible seeds by UAE depending on the own physicochemical properties of the seeds and the used solvent.

It is important to remark that a higher EY does not precisely imply an increased concentration in bioactive compounds, that is, saponins. Therefore, an analysis of TSC of the extracts was assessed. It is important to remark that the spectrophotometric method used for TSC was merely done with the purpose of comparing between extracts. In order to precisely quantify the amount of total saponins, more sensitive and specific chromatographic methods would be required. This is due to the spectrophotometric method not being specific enough for saponins, what leads to other compounds in the extracts possibly interfering during the absorbance measurement. In any case, taking into account that the spectrophotometric method is the most popular and frequent assay used for the screening of TSC, it can be considered valid for comparison purposes between extraction conditions (Cheok, Salman & Sulaiman, 2014). The TSC of extracts is shown in Table 1. Regardless of the seed, the TSC found in all the extracts where ethanol was used as extraction solvent was significantly higher than the ethanol:water extracts, which were in turn higher than the ones extracted with water ($p \leq 0.001$) (Table 1). On the other hand, regardless of the extraction solvent used and considering only the seed factor, fenugreek displayed the highest TSC (mean value 9.5 g 100 g^{-1}), whereas soybean and lupin displayed the lowest values (mean value around 3 g 100 g^{-1}) ($p \leq 0.001$). Lentil and quinoa displayed intermediate contents of total saponins (mean value around 6 g 100 g^{-1} and 4 g 100 g^{-1}, respectively). Therefore, considering both the solvent and the

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seed factor, the ethanol extracts of fenugreek and lentil were found to be the richest in TSC (around 13 and 11 g 100 g^{-1}, respectively), followed by quinoa, lupin and soy (around 6, 5 and 4 g 100 g^{-1}, respectively), whilst the TSC of all water extracts ranged from 2 to 0.2 g 100 g^{-1}. Therefore, the obtained results suggest that the UAE of saponins from edible seeds would be enhanced by less polar solvents than water. To the best of our knowledge, the effect of different solvents on the total saponin yield of UAE extracts has only been explored in fenugreek seeds (Wani et al., 2016). These authors evaluated different concentrations of ethanol (20–100%, v/v) and extraction times (40, 50 and 60 min), achieving the maximum extraction of diosgenin with an 80% ethanol solution for 50 min (1.3 g diosgenin/100 g of seed powder). No other data has been found for the rest of the seeds studied in this work.

Moreover, the comparison between the values obtained in this study and others in the literature has proven to be complex, as most of the authors describe the TSC per grams of seed instead of grams of extract, not making it possible to appropriately evaluate the richness of saponins in our extracts. In any case, it was generally observed that the highest initial expected content in saponins of the seeds, led to the richest extracts in terms of the saponin content (Benichou, Aserin, & Garti, 1999; Muzquiz, Ridout, Price, & Fenwick, 1993; Price, Curl, & Fenwick, 1986). In the specific case of seed extracts described in other studies by other procedures different to UAE, Gee et al. (1993) reported values between 0.3 to 1.4 g of saponins 100 g^{-1} in defatted methanolic extracts of quinoa by Soxhlet extraction. The obtained results in the present study for TSC of quinoa extracts were superior, but it is not possible to state that it might be only due to the different procedure used as UAE or the used methodology for TSC quantification. Araya-Cloutier et al. (2017) have recently reported in 80% methanolic extracts of soybean obtained by pressurized solvent extraction a TSC of 6 to 9 g 100 g^{-1}, expressed as soyasaponin Bb equivalents. The TSC obtained for the soybean extracts by UAE in the present study were lower. As for fenugreek extracts, most of the consulted literature, which is rather old, describes the hypocholesterolemic effect of saponin-rich fractions of the seed, but do not specify the pureness of the extracts in these compounds. As far to our

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knowledge, only Sauvaire, Ribes, Baccou, & Loubatieères-Mariani (1991) and Benichou et al. (1999) have described the saponin content of fenugreek extracts obtained by solid-liquid extraction or Soxhlet, being these values 22 g 100 g^{-1} and 11 g 100 g^{-1}, respectively. These data are similar to the obtained values of TSC for the fenugreek extracts in the present study, what targets this seed as an important source for the production of saponin-rich extracts of steroid nature. Nevertheless, further studies would be necessary to precisely quantify the total amount of saponins by more sensitive chromatographic methods and confirm the observed trends in this preliminary study. Finally, no data has been found in the scientific literature regarding the saponin content of specific extracts obtained from red lentil or lupin.

3.2. Fat content

Generally, when it comes to the extraction of saponins from plant materials, it is very frequent to previously perform a defatting process to obtain a purer extract. However, in the specific case of the assayed seeds, the presence of fat in the extracts might be of interest due to two reasons. On the one hand, a nutritional point of view should be considered. Thus, a high fat content of unsaturated fatty acid profile has been described for the seeds studied in this work. For example, Al-Jasass & Al-Jasser (2012) found that fenugreek seeds contained unsatured fatty acids mainly (93%), being the linoleic acid the major one (34%). In quinoa seeds, linoleic, oleic, and α-linolenic acids accounted for 52, 23, and 8%, respectively, of the total fatty acids (85%) (Wood, Lawson, Fairbanks, Robison, & Andersen, 1993). Similar values have been reported for red lentil, soybean and lupin (Gharibzahedi, Mousavi, Jafari, & Faraji, 2012; Liu, Brown, & Orthoefer, 1995; Uzun, Arslan, Karhan, & Toker, 2007). Therefore, the production of saponin-rich extracts from seeds by UAE without a defatting process might allow to obtain potential bioactive extracts due to saponins, together with the nutritional or bioactive properties of lipids of unsaturated nature in the same extract. On the other hand, the co-existence of lipids during the gastrointestinal digestion of diverse bioactive compounds has demonstrated to be an interesting approach to enhance the bioaccessibility

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and bioavailability of diverse compounds, especially those of poor solubility (Gupta, Kesarla, & Omri, 2013). Despite it has been assumed that the aqueous solubility of saponins is good, it cannot be generalized and seems to be quite variable, especially when the proportion of free sapogenins increases, due to the poor solubility of sapogenins (Gao et al., 2012; Navarro del Hierro et al., 2018). Therefore, the study of the influence of the fat content (FC) of the extracts on the bioaccessibility of saponins was approached in the present study, as it will be discussed in following sections.

On the basis of the FC shown in Table 1, it can be observed that, considering only the solvent factor, the ethanol extracts of all the studied seeds displayed the highest content in fat ($p \leq 0.001$). Additionally, although the values were significantly minor, the ethanol:water and water extracts also contained certain amount of fat. These results indicated that UAE might favor the extraction of fat even in presence of polar solvents such as water, likely due to the strong action of cavitation bubbles on the disruption of cell walls, enhancing the mass transfer of the cell contents. Nevertheless, this should be confirmed by comparative extraction of the seeds with the same solvents but without application of UAE. On the other hand, regardless of the extraction solvent used and considering only the seed factor, soybean displayed the highest FC (mean value 46.0 g 100 g^{-1}), followed by lupin (mean value 24.2 g 100 g^{-1}), quinoa (mean value 23.6 g 100 g^{-1}), fenugreek (mean value 16.5 g 100 g^{-1}) and lentil (mean value 12.6 g 100 g^{-1}) ($p \leq 0.001$). Therefore, when considering both factors, the soybean ethanolic extract showed the highest FC (76%), whereas the quinoa ethanol:water extract showed the lowest FC (3%).

It is important to remark that the co-extraction of fat of the seeds by UAE did not seem to negatively affect the extraction of saponins. On the contrary, the UAE with ethanol extracted both the highest content of fat and the highest content of saponins in each of the seeds. Therefore, the ethanol extracts from all the assayed seeds might result in great interest due to the appealing fatty acid profile of the extracted fat and the TSC.

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3.3. Total phenolics content

Polyphenols are widespread minor compounds that play an important role on the bioactivity of plant extracts. Although most of the assayed edible seeds are not considered relevant sources of polyphenols, the enrichment in these compounds after UAE has been scarcely explored for the assayed seeds. Additionally, diverse studies have shown that the presence of polyphenols might influence the gastrointestinal behavior of other components, such as proteins, carbohydrates or lipids, either positively or negatively . Thus, polyphenols can form insoluble complexes with proteins, associate with carbohydrates, inhibit digestive enzymes, or modify the emulsification of lipids during digestion; and hence, the digestibility and bioavailability can be modified either positively or negatively (Jakobek, 2015; Ozdal, Capanoglu, & Altay, 2013). However, the relationship between polyphenols and the bioaccessibility of saponins has not been previously explored. Due to these reasons, the content in TPC of the extracts from the five seeds has been determined, and the study of their influence on the bioaccessibility of saponins will be discussed in following sections.

The TPC of the extracts was performed spectrophotometrically by the Folin-Ciocalteu method. Similar to the previous explanation for TSC, it is important to remark that the used spectrophotometric method for TPC, which is not totally specific for phenolic compounds, was only done with the purpose of comparing between extracts, although in order to precisely measure the amount of total phenolic compounds, more sensitive and specific chromatographic methods would be needed. The TPC as g of GAE per 100 g of extract is shown in Table 1. By considering the solvent factor, it was observed that the ethanol:water extractions granted the highest TPC ($p \leq 0.001$), regardless of the seed. Additionally, there were not statistical differences between water or ethanol as extraction solvents ($p = 0.689$). The solubility of phenolic compounds in different solvents used during the extraction plays an important role on the final recovery of polyphenols from different plant materials. Frequently, ethanol and water mixtures are used for the extraction of phenols from plant materials due to the fact that aqueous ethanol mixtures can dissolve a wide range

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of phenols (both the hydroxylated and methoxylated compounds) (Alothman, Bhat, & Karim, 2009). In agreement, the obtained results showed that mixtures of ethanol:water enhanced the extraction of polyphenols from the assayed seeds by UAE. In this respect, it is interesting to remark that the influence of the polarity of the solvent on the extraction of TPC and TSC was different. Thus, ethanol:water mixtures favor the obtention of polyphenol-rich extracts, whereas pure ethanol is preferable for obtaining saponin-rich extracts.

On the other hand, regardless of the extraction solvent used and considering only the seed factor, fenugreek displayed the highest TPC (mean value 4.4 g GAE 100 g^{-1}), followed by lupin (mean value 2.5 g GAE 100 g^{-1}). Quinoa (mean value 1.3 g GAE 100 g^{-1}), soybean (mean value 1.3 g GAE 100 g^{-1}) and lentil (mean value 0.7 g GAE 100 g^{-1}) displayed similar contents of total phenolics ($p \leq 0.001$). Therefore, considering both evaluated factors, the ethanol:water and water extracts of fenugreek displayed the maximum TPC, whereas the soybean water extract displayed the lowest value. Djordjevic, Šiler-Marinkovic, & Dimitrijevic-Brankovic (2011) described similar values of TPC in lentil (2.1 g GAE 100 g^{-1}) and soybean (1.8 g GAE 100 g^{-1}) extracts obtained by magnetic stirring with 70% ethanol. Belguith-Hadriche et al. (2010) and Kaviarasan et al. (2007) reported a TPC of 7.5 and 7.8 g GAE 100 g^{-1} in fenugreek extracts, respectively, very similar values compared to the TPC value obtained in the fenugreek ethanolic extract of this study. Tsaliki, Lagouri, & Doxastakis (1999) reported a polyphenol content of 20.7 g GAE 100 g^{-1} in lupin seed flour extracted with hot methanol.

Taking into account these preliminary results, further studies would be necessary to precisely quantify the total amount of phenolic compounds by more sensitive chromatographic methods and confirm the observed differences in the extraction of TPC from the five different edible seeds.

3.4. Bioaccessibility of saponins

Due to the richness of the ethanol and ethanol:water extracts in TSC, their consequent *in vitro* gastrointestinal digestion and the study of the bioaccessibility of saponins from these extracts was

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carried out, whilst the water extracts were discarded. Additionally, the high content in fat and total phenolics observed in the ethanolic and ethanol:water extracts, respectively, contributed to the selection of both extracts in order to assess the effect of such components on the bioaccessibility of saponins.

To the best of our knowledge, the bioaccessibility of saponins from UAE extracts of fenugreek, lentil, lupin, quinoa and soybean has been assessed for the first time. According to Figure 1, the bioaccessible fraction of saponins from the ethanol:water extracts was reasonably variable depending on the seed. In increasing order, saponins from fenugreek extracts displayed the lowest bioaccessibility value (13%), followed by those of quinoa (38%), lupin (62%), soybean (72%) and lentil (83%) extracts. It is rather difficult to compare the obtained results with others in the literature, since the study of the bioaccessibility of saponins or saponin-rich extracts is relatively recent and, consequently, the available information is truly scarce. Only Serventi et al. (2013) and Sagratini et al. (2013) have reported bioaccessibility values of specific saponins from soybean-chickpea bread formulations (type A saponins: 30%, type B: 45 – 65%, type E: 86 – 91%, and type DDMP: 51 – 61%) and soyasaponin I from cooked lentils (approximately 10 %, variable depending on cooking time), respectively. Other authors have assessed the stability of steroidal saponins from asparagus during a simulated oral and gastrointestinal digestion (recovery range between 87 – 94%), although not in terms of bioaccessibility (Jaramillo et al., 2016).

In order to understand the wide variability obtained in the bioaccessibility of the assayed extracts and whether the obtained bioaccessibility values were affected by the components in the ethanol:water extracts, the influence of the TSC, TPC and FC values, as well as the TSC/FC ratio of the extracts were considered. Firstly, the TSC negatively correlated with the bioaccessibility, although it was not statistically significant ($p_{[E:W]}$ = 0.054) (Figure 2.a). Thus, a trend to a worse bioaccessibility might be suspected as the concentration of saponins of the extracts increased. Although it was not statistically significant and further studies would be necessary, a dose-effect on

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the bioaccessibility of saponins might be suggested, and might be considered a factor previously undescribed to take into account in the understanding of the gastrointestinal behavior of saponins.

On the other hand, a negative correlation was found between the TPC and the bioaccessibility of saponins ($p_{[E:W]}$ = 0.037) (Figure 2.b), which means that high levels of phenolic compounds in the extracts hindered the bioaccessibility of saponins. In this respect, it has been widely reported the ability of polyphenols to form complexes with other molecules such as proteins, leading to changes in the structural, functional and nutritional properties of both compounds, as well as a decreased solubility of polyphenol-protein complexes caused by low pH conditions (Ozdal et al., 2013). Furthermore, phenolic compounds may also interact and bind with dietary fiber during gastrointestinal digestion, either by hydrogen bonds, strong (covalent) interactions or physicochemical entrapment exerted by dietary fiber (Quirós-Sauceda et al., 2014). Similarly, the obtained results might suggest the formation of less soluble complexes between saponins and phenolic compounds during the gastrointestinal digestion, which would hamper the self-micellization of saponins in the aqueous medium. However, further studies in this respect would be necessary in order to confirm these preliminary observations and to elucidate the chemical nature of a potential phenolic-saponin interaction, since previous information about the relationship between polyphenols and saponins during the gastrointestinal process has not been found.

Concerning the FC of the different ethanol:water extracts, a lack of effect was found of this component on the bioaccessibility of saponins ($p_{[E:W]}$ = 0.494) (Figure 2.c). However, in order to get a deeper knowledge of the relationship between saponins and lipids during the gastrointestinal digestion, the ratio TSC to FC of the ethanol:water extracts was estimated and the correlation with bioaccessibility evaluated. Interestingly, a negative correlation was found ($p_{[E:W]}$ = 0.032) (Figure 2.d). Therefore, the higher the ratio saponin-to-fat, the worse the bioaccessibility. Thus, according to Figure 2.d, a ratio of TSC/FC superior to 1 caused a poor bioaccessibility (< 40%), whereas those samples with a ratio lower than 1 showed bioaccessibilities > 60 %. This might suggest a positive interaction between saponins and lipids that might enhance the bioaccessibility of saponins,

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although it would depend on the specific concentration of saponins and lipids, being preferable a higher concentration of lipids than saponins.

When the *in vitro* gastrointestinal digestion of the ethanol extracts was performed, a significant increase in the bioaccessibility of saponins from most of the studied seeds was observed when compared to the ethanol:water extracts (Figure 1). In increasing order, saponins from fenugreek displayed a bioaccessibility value of 88%, followed by those of lentil (92%), soybean (96%), quinoa (96%) and lupin (106%). Similarly to the ethanol:water extracts, we tried to explain these results taking into account the likely influence of TPC and FC of the extracts. In this respect, a lack of relationship was found between the bioaccessibility values and TSC ($p_{[E]}$ = 0.105), TPC ($p_{[E]}$ = 0.697), FC ($p_{[E]}$ = 0.323) or TSC/FC ($p_{[E]}$ = 0.185) (Figure 2). However, in order to understand which chemical component (TPC or FC) of all the extracts caused the great increase in the bioaccessibility of saponins of the ethanol extracts, we considered the chemical composition (TPC or FC) of both the ethanol and aqueous ethanol extracts together and studied its relationship with the bioaccessibility. A positive correlation was found between the FC and the bioaccessibility of saponins ($p_{[E\&E:W]}$ = 0.013). Thus, according to Figure 2.c, those samples with a FC ≥ 30% showed similar and high values of bioaccessibility of saponins (>70%), whereas samples with a FC < 5% showed variable values of bioaccessibility (from 13% to 83%). Therefore, these results might suggest that the co-existence of lipids during the gastrointestinal digestion of saponins might enhance the bioaccessibility, but a threshold of lipids might be necessary to guarantee a high bioaccessibility. Additionally, as already observed for the ethanol:water extracts, when all the extracts were considered together, the TSC/FC ratio of the extracts negatively correlated with the bioaccessibility ($p_{[E\&E:W]}$ < 0.001) (Figure 2.d). Therefore, the higher the ratio saponin-to-fat, the worse the bioaccessibility. We again found that a ratio of TSC/FC superior to 1 caused a poor bioaccessibility (<40%), whereas those samples with a ratio lower than 1 showed bioaccessibilities >60%, regardless of the solvent used for UAE. Taking into account all these results, it seems that either a threshold of lipids or a high ratio of lipids to saponins would be desirable in order to

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enhance the bioaccessibility of saponins. Further studies by using more sensitive methods for saponins and wider ranges of saponins/lipids concentrations than those used would be necessary in order to confirm these preliminary results.

The explanation of the role of the lipids on the bioaccessibility of saponins is complex because previous information has not been found. As already explained, despite it has been assumed that the aqueous solubility of saponins is good, it cannot be generalized and seems to be quite variable, probably due to a variable hydrophilic-lipophilic balance of the molecules depending on the chemical structure of the aglycone or glucosidic chain. Additionally, when the proportion of free sapogenins of the TSC increases, it might also cause a worse solubility and bioaccessibility of the TSC, due to the more hydrophobic properties of sapogenins compared to saponins. The origin of sapogenins in the digestive media might be due to either the natural content of the own extract or to the hydrolysis of saponins caused by the acidic conditions during the gastric phase, as suggested by diverse studies (Navarro del Hierro et al., 2018; J. R. Wang et al., 2014; Z. Wang, Kurosaki, Nakayama, & Kimura, 1994). The digestion of lipids releases mainly fatty acids and monoglycerides, which lead to the formation of micellar structures with bile salts and phospholipids, and consequently, an increase in the micellar surface. In this way, the increase in the available micellar structures might enable the inclusion of more hydrophobic compounds, such as sapogenins or less hydrophilic saponins, and hence, their bioaccessibility is enhanced. In this respect, and according to the obtained results, we hypothesized that a minimum of lipid micellar structures would be necessary to compensate a decreased solubility of saponins. Similarly, an increase in the bioaccessibility has been recently observed by ourselves for pentacylic triterpenes when co-digested with olive oil (Martin et al., 2016), which are compounds chemically analogous to the typical triterpenoid sapogenins of the saponins from the assayed seeds in the present study. Nevertheless, further studies would be necessary to deepen in the contribution of the sapogenins of the digested extracts on the obtained results.

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Finally, a negative correlation was found between the TPC and the bioaccessibility ($p_{[E\&E:W]}$ = 0.003), possibly due to the reasons stated earlier. Thus, according to Figure 2.b, a TPC value < 2 g GAE 100 g^{-1} of the extracts would be preferable for a proper bioaccessibility of saponins.

It is important to remark that no correlation was found between the TSC and the bioaccessibility of saponins when all the extracts were considered together ($p_{[E\&E:W]}$ = 0.970), although such correlation was indeed found in the case of the ethanol:water extracts (Figure 2.a). This means that the negative effect of the concentration of saponins on its bioaccessibility was only remarkable for the ethanol:water extracts, whereas for the ethanol extracts, the concentration of saponins did not influence their bioaccessibility. Since one of the major differences between ethanol and ethanol:water extracts was the FC, it could be thought that the negative effect-dose of saponins on their bioaccessibility might be compensated by other components, as lipids have significantly shown for the ethanol extracts.

4. Conclusions

The ultrasound-assisted extraction is shown as a cost-effective and rapid method for obtaining saponin-rich extracts from quinoa, soybean, red lentil, fenugreek and lupin. Ethanol is the preferred solvent in terms of the richness of total saponins, as well as for the co-extraction of other compounds of interest, such as lipids. Nevertheless, in order to confirm these preliminary results and precisely quantify the saponin richness of the extracts, further analysis by more sensitive methods would be necessary.

After the *in vitro* gastrointestinal digestion of the five seeds extracts Saponins from ethanol:water extracts display quite variable values of bioaccessibility depending on the seed, whereas almost a complete bioaccessibility of saponins is reached for the ethanol extracts from all seeds. It is concluded for the first time that the presence of other components in the extracts such as phenolic compounds or fat, may hinder or enhance the bioaccessibility of saponins, respectively. Additionally, an adequate balance between saponins to lipids seems to be relevant on such an effect.

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These results have important implications for broadening the knowledge on the gastrointestinal digestion of saponins, especially concerning the scarcely available information about the bioaccessibility of these molecules, which is a parameter with increasingly great relevance in the study of bioactive compounds.

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Notes

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Figure Captions

Figure 1. Bioaccessibility (%) of saponins from UAE extracts of edible seeds after *in vitro* gastrointestinal digestion. (*) Bars within the same seed are significantly different if $p \leq 0.05$.

Figure 2. Correlation (*r*) between bioaccessibility (%) and a) TSC (total saponin content); b) TPC (total phenolics content); c) FC (fat content); d) TSC/FC ratio, of ethanol (E) or ethanol:water (E:W) extracts of quinoa (E,▲; E:W,△); soybean (E,■; E:W,□); red lentil (E,–; E:W, ✕); fenugreek (E,●; E:W,○); lupin (E,◆; E:W, ◇).

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Table 1. Characterization of the ultrasound-assisted extracts from edible seeds extracted with different solvents

	EY ($g \cdot 100\ g^{-1}$)	TSC ($g \cdot 100\ g^{-1}$)	FC ($g \cdot 100\ g^{-1}$)	TPC ($g\ GAE \cdot 100\ g^{-1}$)
Quinoa				
Ethanol	$14.80 \pm 0.46^{g,x}$	$5.51 \pm 1.18^{c,x}$	$60.64 \pm 1.05^{b,x}$	$0.76 \pm 0.08^{gh,x}$
Ethanol:Water	$10.62 \pm 0.45^{hi,y}$	$4.43 \pm 0.53^{cd,x}$	$3.15 \pm 0.04^{e.y}$	$2.62 \pm 0.17^{d,y}$
Water	$34.00 \pm 0.27^{b,z}$	$0.26 \pm 0.02^{gh,y}$	$4.21 \pm 0.33^{e,y}$	$0.75 \pm 0.07^{g,x}$
Lentil				
Ethanol	$4.53 \pm 0.29^{j,x}$	$10.63 \pm 1.86^{b,x}$	$28.25 \pm 0.98^{d,x}$	$0.42 \pm 0.09^{h,x}$
Ethanol:Water	$11.76 \pm 0.80^{h,y}$	$3.28 \pm 0.84^{de,y}$	$4.91 \pm 0.54^{e,y}$	$1.18 \pm 0.07^{f,y}$
Water	$25.73 \pm 5.23^{c,z}$	$0.19 \pm 0.08^{h,z}$	$4.67 \pm 0.12^{e,y}$	$0.49 \pm 0.26^{gh,x}$
Fenugreek				
Ethanol	$9.35 \pm 0.17^{i,x}$	$12.90 \pm 0.91^{a,x}$	$41.51 \pm 1.01^{c,x}$	$1.80 \pm 0.11^{e,x}$
Ethanol:Water	$18.35 \pm 0.80^{ef,y}$	$8.99 \pm 0.72^{b,y}$	$3.52 \pm 0.51^{e,y}$	$7.14 \pm 0.4^{a,y}$
Water	$17.16 \pm 1.80^{f,y}$	$2.36 \pm 0.12^{ef,z}$	$4.63 \pm 0.37^{e,y}$	$4.29 \pm 0.19^{b,z}$
Soybean				
Ethanol	$23.19 \pm 0.06^{d,x}$	$4.08 \pm 0.7^{cd,x}$	$75.73 \pm 1.25^{a,x}$	$1.97 \pm 0.16^{e,x}$
Ethanol:Water	$24.05 \pm 1.44^{cd,x}$	$2.10 \pm 0.72^{ef,y}$	$36.74 \pm 6.31^{c,y}$	$1.20 \pm 0.18^{f,y}$
Water	$61.33 \pm 0.01^{a,y}$	$1.94 \pm 0.12^{efg,y}$	$25.75 \pm 0.56^{d,y}$	$0.55 \pm 0.06^{gh,z}$
Lupin				
Ethanol	$12.91 \pm 0.58^{gh,x}$	$4.55 \pm 0.36^{cd,x}$	$63.43 \pm 1.54^{b,x}$	$1.90 \pm 0.05^{e,x}$
Ethanol:Water	$20.41 \pm 1.46^{e,y}$	$1.97 \pm 0.56^{ef,y}$	$5.07 \pm 1.6^{e,y}$	$3.18 \pm 0.14^{c,y}$
Water	$23.53 \pm 0.34^{cd,z}$	$0.80 \pm 0.04^{fgh,z}$	$4.33 \pm 0.04^{e,y}$	$2.68 \pm 0.13^{d,z}$

a-i Different superscript letters within a column denotes statistically significant differences ($p \leq 0.05$)

xyz Different superscript letters within a column and within a same seed denotes statistically significant differences ($p \leq 0.05$)

EY (extraction yield); TSC (total saponin content); FC (fat content); TPC (total phenolics content)

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Figure 1.

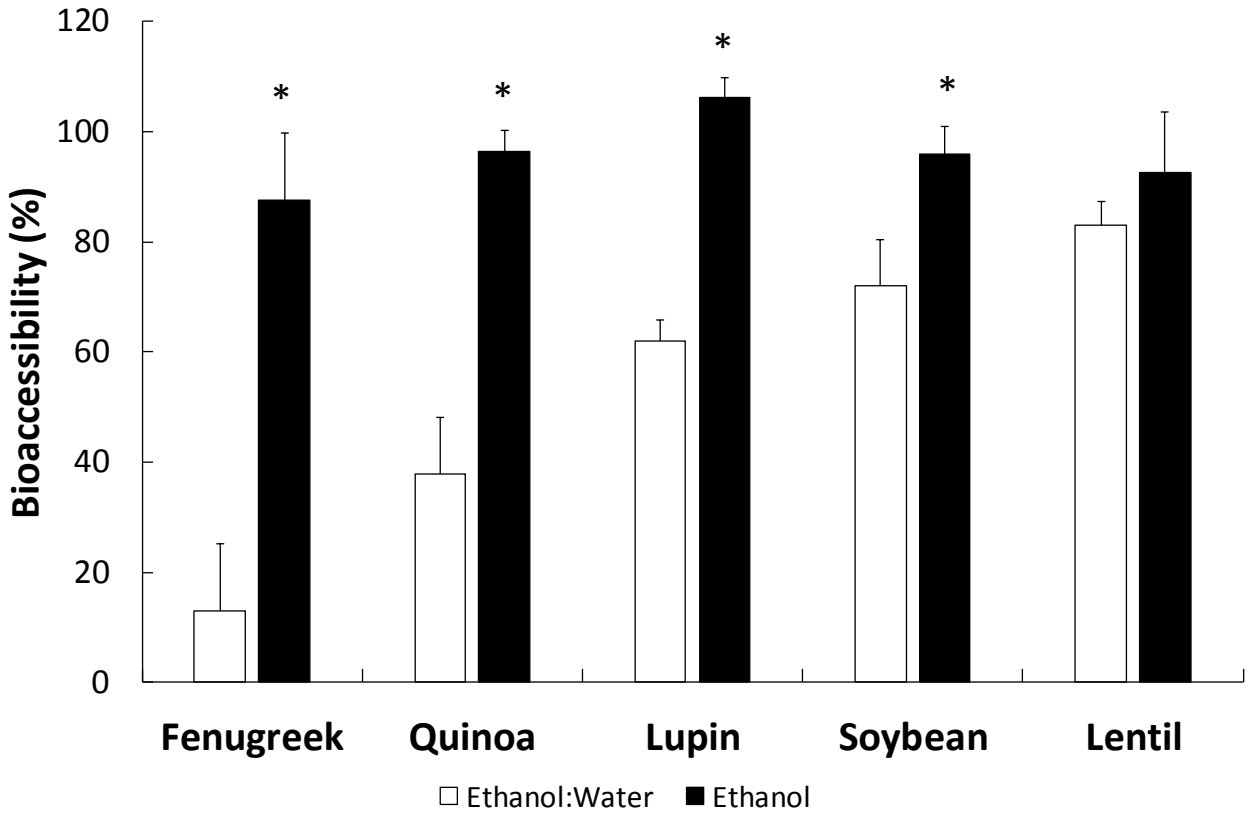

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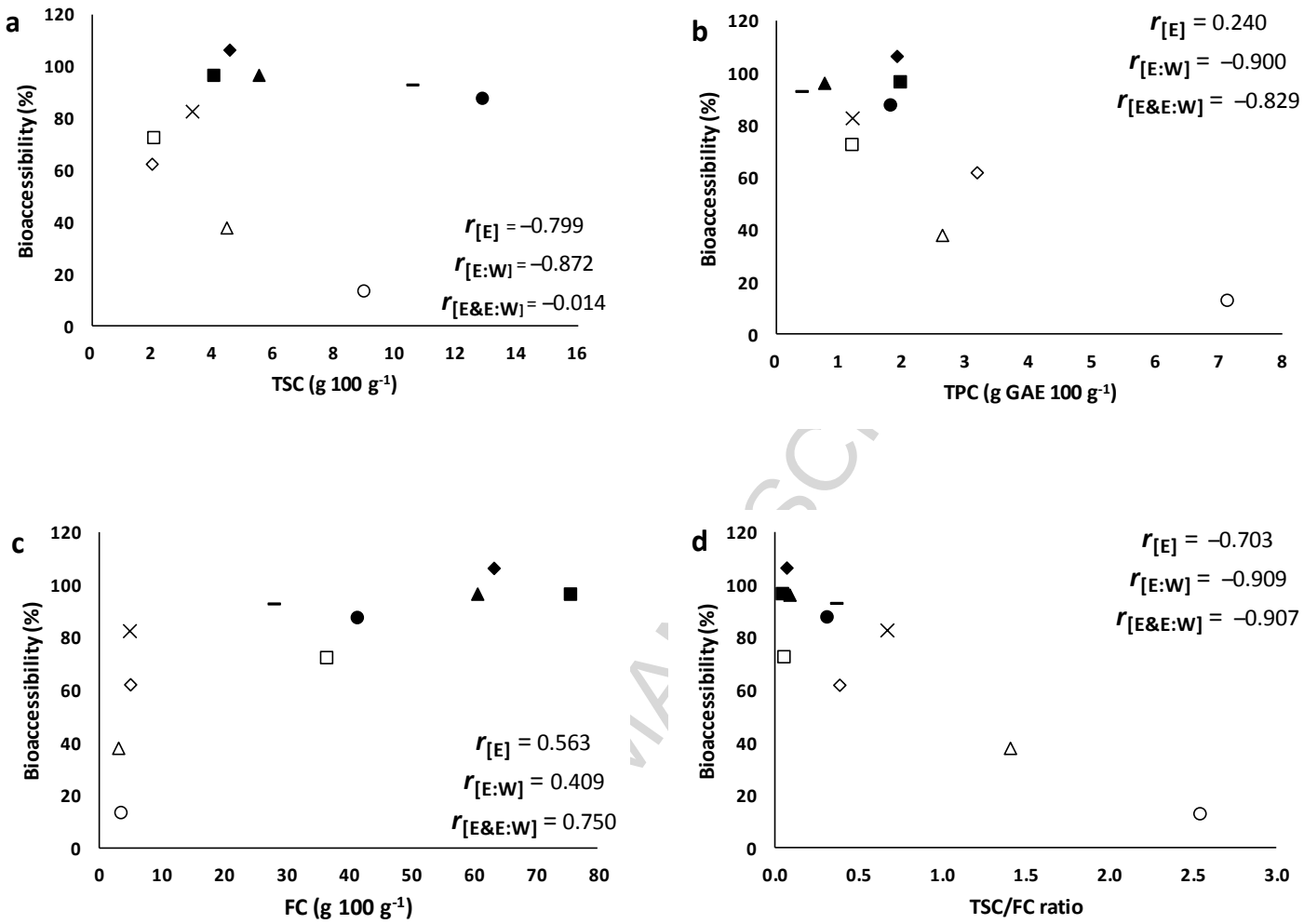
Figure 2.



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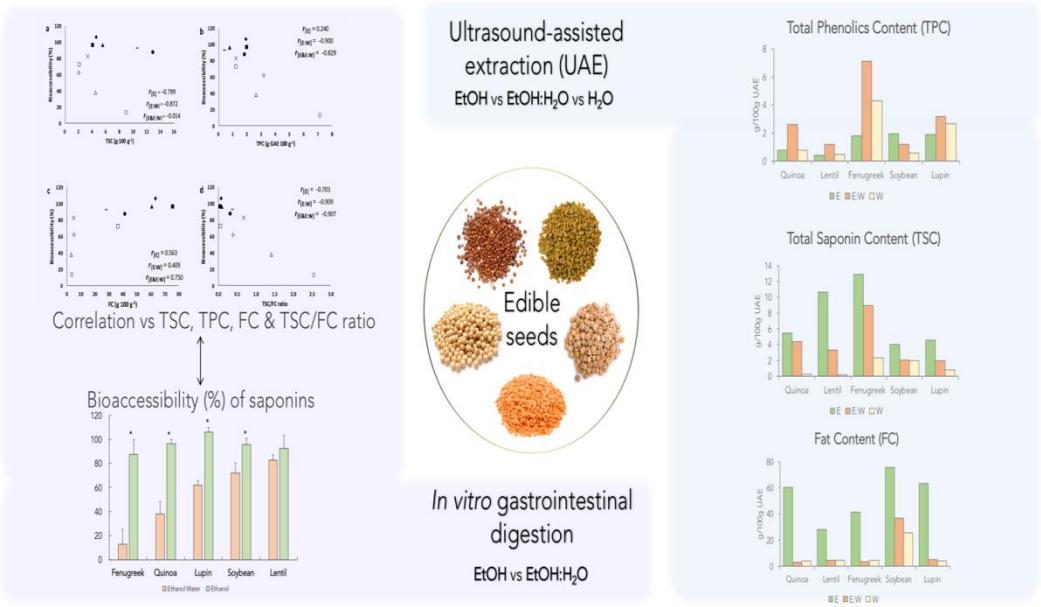
Graphical abstract



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Highlights

- Ethanol is preferred to obtain saponin-rich extracts of edible seeds by ultrasounds
- Bioaccessibility of saponins of ethanol:water extracts varies depending on the seed
- Bioaccessibility is almost complete for the ethanol extracts regardless of the seed
- Phenolic compounds of the extracts hinder the bioaccessibility of saponins
- A proper ratio saponins to lipids in the extracts enhances their bioaccessibility

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