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This is an **author produced version** of a paper published in:

Journal of the Science of Food and Agriculture 99.6 (2019): 3157-3167

**DOI:** https://doi.org/10.1002/jsfa.9531

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**Acid hydrolysis of saponin-rich extracts of quinoa, lentil, fenugreek and soybean to yield sapogenin-rich extracts and other bioactive compounds**

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**Abstract**

**BACKGROUND:** Typical hydrolysis times of saponins without considering the effect of time on the degradation of the targeted compounds, namely sapogenins, are generally assumed. When producing natural extracts, it should be considered that the performance of hydrolysis to yield a target compound might also affect the final composition of the extracts in terms of other bioactive compounds. In our study, saponin-rich extracts from fenugreek, quinoa, lentil and soybean were produced and their acid hydrolysis to produce sapogenin-rich extracts was performed at different times (0-6 h). Disappearance of saponins and appearance of sapogenins were analyzed by HPLC-DAD-MS and GC-MS, respectively. The impact of hydrolysis on phytosterols and tocopherol of the extracts was also evaluated.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.9531

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**RESULTS:** Fenugreek showed the highest content of saponins (169 g $kg^{-1}$), followed by lentil (20 g $kg^{-1}$ ), quinoa (15 g $kg^{-1}$) and soybean (13 g $kg^{-1}$). Hydrolysis during 1 h caused complete disappearance of saponins and the highest release of sapogenins. Hydrolyzed fenugreek and quinoa extracts contained the highest amounts of sapogenins and minor fractions of phytosterols and tocopherol. Hydrolyzed extracts of lentil and soybean contained a major fraction of phytosterols and a low fraction of sapogenins. In all cases, sapogenins decreased after 1 h of hydrolysis, phytosterols slightly decreased, and tocopherol was unaffected. Standards of diosgenin and oleanolic acid also showed this decreasing pattern under acid hydrolysis conditions.

**CONCLUSION:** Hydrolysis times of 1 h for saponin-rich extracts from the assayed seeds guarantee extracts with the maximum transformation to sapogenin-rich extracts, along with phytosterols and tocopherol, being fenugreek and quinoa seeds preferred.

**KEYWORDS**

saponins, sapogenins, phytosterols, tocopherol, acid hydrolysis, legumes

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## INTRODUCTION

Edible seeds are relevant sources of minor phytochemicals of interest, such as saponins, polyphenols, phytosterols, carotenoids, alkaloids or tocopherols, due to the bioactive properties described for such components. Within these compounds, saponins are widely found at relative high levels in many edible seeds.[1,2] Saponins consist of a hydrophobic aglycone backbone (sapogenin) linked to hydrophilic sugar chain/s. Depending on differences in the sapogenin structure or number of sugars linked, saponins can be diversely classified. According to the chemical structure of the sapogenin skeleton, saponins can be classified into steroidal or triterpenoid saponins. In addition, they can be divided attending the number of sugar chains as monodesmosidic, bidesmosidic, or tridesmosidic.[3] Triterpenoid saponins are commonly reported in seeds of legumes (alfalfa, soybean, chickpea, mung bean, kidney bean and lentil), quinoa, sunflower, or horse chestnut, as well as in other sources, such as ginseng roots, liquorice roots, spinach leaves, tea leaves, quillaja bark, sugar beet or alliums species. In the case of steroidal saponins, they are frequently found in seeds of fenugreek, tomato, or oats, as well as in other sources, such as yucca, yam, ginseng roots, asparagus, aubergine or capsicum peppers.[3,4]

Saponins, traditionally considered as antinutrients, have been under intense research over the last years due to their evidenced biological activities, such as immunostimulatory, hypocholesterolemic, antitumor, anti-inflammatory, antibacterial, antiviral, antifungal, or antiparasitic activities.[5–7] As recent examples, the anti-inflammatory activity of triterpenoid saponins from soybean have been shown in macrophages by downregulation on nitric oxide production.[8] Similarly, triterpenoid quinoa saponins have demonstrated to inhibit the release of inflammatory cytokines, as well as decrease the production of inflammatory mediators.[9] Quinoa saponins have also shown to inhibit triglyceride accumulation in adipocytes and suppress adipogenesis.[10] On the other hand, it was recently described that triterpenoid saponin extracts from lentil caused both hypocholesterolemic and prebiotic effect in rats.[11] Concerning

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steroid saponins, fenugreek extracts have shown to improve the serum lipid, bile acid efflux, anti-peroxide activities and lipid area of liver tissue in rats, consequently ameliorating dyslipidaemia by accelerating cholesterol metabolism, inhibiting cholesterol synthesis, and facilitating reverse cholesterol transport.[12] Fenugreek saponins are also known for their pro-apoptotic and anticancer properties.[13]

Unfortunately, saponins are poorly absorbed during the digestion process and exhibit low bioavailability due to diverse limiting factors such as low membrane permeability, chemical transformations, or colonic fermentation.[14] On the contrary, most sapogenins have demonstrated superior bioavailability, and some bioactivities of sapogenins are even higher than their former saponins, due to more favorable chemical properties caused by the lack of the sugar chain.[14–18] As examples, Choi, Jung, Lee & Park[19] demonstrated that hederagenin and oleanolic acid, obtained from the hydrolysis of the saponin rich fraction of the plant *Akebia quinata,* exhibited a stronger anti-edema and analgesic effect than their former kalopanaxsaponin A. Furthermore, some sapogenins can even display a certain biological effect that was not shown by their former saponins. For instance, Gurfinkel & Rao[20] found that the aglycones soyasapogenol A and B showed almost complete suppression of cell growth, while soyasaponin A1, A2, and I, had no effect on cell growth. Similarly, Uemura et al.[21] also found that a hydrolized fenugreek extract significantly inhibited triglyceride accumulation in HepG2 cells, while the saponin fraction had no effect. Therefore, a way to increase some specific bioactivities of saponins or even reveal novel bioactivities might be achieved by their previous transformation to sapogenins.

Hydrolysis of saponins is the known procedure for the conversion of saponins to sapogenins. In this sense, saponins can be hydrolyzed by chemical (by acid or alkali), enzymatic and microbial methods, leading to the formation of sapogenins, prosapogenins, sugar residues or monosaccharides depending on the hydrolysis method and conditions performed.[22] In general,

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it seems that enzymatic methods are less effective than chemical hydrolysis, making the acid hydrolysis of saponins the preferred procedure, which has been more extensively used and explored.[23]

On the one hand, it is important to consider that during chemical hydrolysis of saponins, specific conditions such as temperature, time of reaction, and type of hydrolysing agent could affect the stability of saponins, the yield on final products, and the generation of artifacts.[24,25] Specifically, prolonged heating with an organic acid can cause artifact formation, low yields and low selectivity.[26,27] Additionally, the abundance and diversity of the final products can change significantly depending on the structural complexity of saponins or sapogenins, the nature of the attached side chains, and the attachment positions of sugar moieties to the aglycone, resulting in a wide variety of these compounds.[3] Despite these circumstances, it is frequently found that the hydrolysis of saponins is performed under common and general conditions, without previous detailed evaluation of the ideal conditions of hydrolysis for the specific saponins of interest in order to obtain sapogenin-rich extracts. As example, typical hydrolysis conditions have been summarized in Table 1. In general, 2-3 h of reaction time, temperatures around 100 ºC, and 2 M HCl solutions are the most frequently used conditions. Longer times of hydrolysis up to 4, 8 or 72 h have also been found. It should be considered that excessive times of hydrolysis might lead to a potential degradation of sapogenins, together with unnecessary energy consumptions.[26,27] Therefore, considering the abovementioned hydrolysis parameters, at least the effect of time during the hydrolysis of saponins to produce sapogenins should be considered.

On the other hand, it is important to remark that within the research field of the production of natural extracts, such as saponin-rich extracts, it should be considered that the performance of hydrolysis to yield a target compound might also affect the final composition of the extracts in terms of other bioactive compounds of interest. This is because natural extracts are

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complex mixtures of a wide diversity of phytochemicals, and the subsequent bioactive properties of the extracts are due to the combination or synergism of diverse compounds, making it difficult to attribute the effects to a single compound. Therefore, after performing a hydrolysis procedure to reach sapogenin-rich extracts from edible seeds, it should be taken into account the impact of the hydrolysis conditions on other coexisting compounds in the seed extracts, in order to understand the subsequent bioactive effects of the extracts or to preferably attribute specific bioactivities to specific compounds.

The aim of the present study was to obtain saponin-rich extracts from edible seeds, such as fenugreek (as typical source of steroidal saponins), and quinoa, lentil and soybean (as typical sources of triterpenoid saponins). Then, the acid hydrolysis of the extracts was performed at different times in order to assess the optimal time to produce sapogenin-rich extracts from each seed. Finally, the impact of the hydrolysis time on the composition of the extracts in both the target compounds, namely sapogenins, and other minor compounds, such as phytosterols and tocopherol was evaluated.

## MATERIALS AND METHODS

### Reagents and materials

Seeds of red quinoa (*Chenopodium quinoa* L.), soybean (*Glycine max* L.) and peeled red lentil (*Lens culinaris medicus*), were purchased from Hijo de Macario Marcos S.L (Salamanca, Spain). Seeds of fenugreek (*Trigonella foenum-graecum)*, were from Murciana de Herboristeria (Murcia, Spain).

Oleanolic acid standard was from Extra synthese. Protodioscin, diosgenin, hederagenin, soyasaponin I and soyasapogenol B from glycine max, β-sitosterol and tocopherol were from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany).

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**Production of saponin-rich extracts**

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 ≤ 100 μm. Samples were stored in dark and dry conditions until further analysis. Extractions were based on Navarro del Hierro et al.[1] with modifications. Briefly, samples were extracted with methanol 1:10 (w/v) by direct sonication (Branson SFX 250 Digital Sonifier®, Branson Ultrasonics, USA) with an ultrasonic probe (1/2" diameter, output sonication amplitude of 60%) for 15 min. The mixture was then centrifuged at 3400 ×g for 10 min. Supernatants were collected and defatted by addition of the same volume of hexane. This mixture was centrifuged at 3400 ×g for 10 min. Then, the supernatant was removed and the methanolic bottom phase was evaporated under vacuum at 45 ºC. This saponin-crude extract was concentrated to saponin-rich extract by *n*-butanol. To perform this procedure, the saponin-crude extract was solubilized with water at 50 mg/mL and added an equal volume of water-saturated *n*-butanol. This mixture was centrifuged at 3400 ×g for 5 min, and the supernatant of *n*-butanol was collected. This last procedure was repeated three times. All the supernatants were collected and evaporated under vacuum at 45 ºC. The obtained saponin-rich extracts were kept at 4 °C until further use. Extractions were performed in duplicate for each seed.

**Acid hydrolysis of saponin-rich extracts**

The saponin-rich extracts were hydrolyzed with HCl (2 M) at a ratio of sample to acid solution of 1:50 (w/v). Individual hydrolysis reactions were performed at 0, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 h at 100 ºC. Immediately after each reaction time, the hydrolyzed samples were freeze-dried and solubilized in methanol for further analysis. Hydrolysis were performed in duplicate for each seed extract and for each time of hydrolysis.

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The same procedure of acid hydrolysis was performed in duplicate with commercial standards of sapogenins, that is, oleanolic acid and diosgenin.

**Analysis of saponins by HPLC-DAD-MS**

A HPLC system (Agilent Infinity 1260) with DAD detection was used for the analysis of the samples. Separation was carried out on an ACE 3 C18-AR column (150 mm x 4.6 mm, 3 µm particle size) protected by a guard column. A gradient elution was performed using water with 0.05% TFA (phase A), and acetonitrile with 0.05% TFA (phase B). The method was as follows: 0 min: 95% A; 20 min: 5% A; 45 min: 5% A; 46 min 95% A; 50 min: 95% A. The flow rate was constant at 0.4 mL/min, and the column temperature was kept at 25 ºC. The injection volume was 20 µL, and UV spectra were recorded from 190 to 700 nm, whereas the chromatograms were registered at 210 nm.

Identification of saponins was performed in an Agilent 6120 HPLC-MS by pure commercial standards or by comparison with mass spectra from the literature. The same flow and gradient conditions previously described were used for the HPLC-MS analyses. The rest of the conditions were as described by Mad et al.[28]: MS ionization mode with APcI+/- between 30 and 1500 Da range mass. Nebulizing/drying gas at a flow rate of 5 L/min, 350 ºC and 20 psi. The vaporizer temperature was set at 250 ºC, the capillary voltage was set to 2000 and 4000 V, and fragmentor voltage was set at 40 V. Spectra were obtained over m/z 300-1500.

Quantitation of saponins was performed by calibration curves obtained from commercial standards whenever possible. Protodioscin was used for saponins from fenugreek extracts and soyasaponin I was used for saponins from quinoa, lentil and soybean extracts.

**Analysis of sapogenins and other minor compounds by CG-MS**

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Sapogenin profiles of hydrolyzed extracts were analysed by previous derivatization of samples with bis(trimethylsilyl) trifluoroacetamide (BSTFA), with ergosterol as internal standard, and heating at 75 ºC for 1 h. The derivatized extracts were analysed by GC-MS (Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) comprising a split/splitless injector, an electronic pressure control, a G4513A autoinjector, and a 5975C triple-axis mass spectrometer detector. The column used was an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm phase thickness). Helium was used as carrier gas at 2 mL/min. The injector temperature was 260 °C, and the mass spectrometer ion source and interface temperatures were 230 and 280 °C, respectively. The sample injections (1 μL) were performed in splitless mode. The oven temperature at 50 ºC was held for 3 min and increased at a rate of 15 ºC/min to 310 ºC, being held for 25 min. The mass spectra were obtained by electronic impact at 70 eV. The scan rate was 1.6 scans/s at a mass range of 30−700 amu. Identification of compounds was performed by the NIST MS Data library, the mass spectra according to literature, or according to those of pure commercial compounds whenever possible. Quantitation of sapogenins was performed by relative response factors with respect to the internal standard of ergosterol. Response factor of diosgenin was used for sapogenins from fenugreek extracts; oleanolic acid and hederagenin for sapogenins from quinoa extracts; and soyasapogenol B for sapogenins from lentil and soybean extracts.

Phytosterols and tocopherol of hydrolyzed extracts were analysed following the same procedure described for sapogenins, including previous derivatization of samples with BSTFA and subsequent analysis by GC-MS. Quantitation was performed by relative response factors of standards of tocopherol and β-sitosterol with respect to the internal standard of ergosterol.

**Statistical analysis**

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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.

## RESULTS AND DISCUSSION

### Identification and quantification of saponins from seed extracts

The HPLC-DAD analysis of the seed extracts is shown in Figure 1. The identification of the main saponins of the extracts was performed by HPLC-MS-EPI. The mass spectra recorded in the positive ionisation mode exhibited the $[M+H]^+$ or $[M+H-H2O]^+$ ion, typical of many saponins possessing a labile hydroxyl group in the molecule.[29] Additionally, the mass spectra showed typical signals due to the subsequent losses of glucoside units up to yield the typical signal of the sapogenin, which allowed the tentative identification of individual saponins. Furthermore, the comparison of spectra with those of commercial standards or those previously described in the literature for saponins was also used to facilitate the identification.
The main recorded ions of the mass spectra of saponins from the extracts are shown in Table 2. In case of fenugreek, up to nine compounds were tentatively identified as saponins. Most of these saponins produced the distinctive fragment ions of the typical sapogenin at m/z 415 or 417, which might correspond to the sapogenin fragment of diosgenin, gitogenin, tigogenin or sarsasapogenin. All these sapogenins have been described in saponins of fenugreek seeds.[30] Therefore, following all these criteria, the compounds from 1 to 9 were tentatively identified as steroidal saponins. Additionally, in the case of fenugreek extracts, it was possible to tentatively elucidate the attached sugar units.
The quinoa seeds extracts showed the presence of three tentative saponins. However, co-elution of saponins occurred, since ion fragments of different quinoa sapogenins were found

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within the mass spectra of the same peak. Thus, compound 2 was tentatively identified as saponins containing oleanolic acid and serjanic acid; whereas compound 3 corresponded to saponins with serjanic acid and hederagenin as sapogenins. Phytolaccagenic acid was the only sapogenin found for compound 1.

For lentil extracts, three saponins were tentatively identified containing soyasapogenol B as sapogenin (compounds 1-3). Under positive ionization, these saponins produced the characteristic fragment ions of soyasapogenol B at m/z 441, 423 and 405. For compound 1, it was identified as soyasaponin I, according to the retention time and mass spectra of commercial standard. Additionally, compound 4 was tentatively identified as a likely soyasapogenol A saponin due to the m/z 477 and 459, more typical of these sapogenins.

Similar to lentil extracts, three soybean saponins were tentatively identified as soyasapogenol B types for the soybean extracts. However, compared to lentil, the soybean extracts did not show the presence of soyasaponin I, although both seeds have been previously described as sources of soyasaponin I.[31,32]

It is important to remark that different or diverse saponin profiles have been described in the scientific literature for extracts obtained from the assayed seeds, but the comparison is complex, due to the great variability in the studies concerning the extraction procedures, subsequent purification steps of the extracts, or analytical methods.

For comparative purposes, the identified saponins of the four extracts were tentatively quantitated by HPLC-DAD. The highest content of total saponins was found in fenugreek ($169.6 \pm 2.2$ g kg$^{-1}$ of extract), followed by lentil ($20.3 \pm 0.3$ g kg$^{-1}$ of extract), quinoa ($15.2 \pm 0.4$ g kg$^{-1}$ of extract) and soybean ($13.0 \pm 1.9$ g kg$^{-1}$ of extract). A comparison of the obtained values in this study with those described in literature is complex, as most of the studies that quantitate saponins of these seeds report the total saponin content of the seeds instead of the extracts, not making it possible to appropriately evaluate the richness of saponins in our

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extracts. Additionally, most of the studies used the traditional spectrophotometric method for total saponin contents in reaction with sulfuric acid-vanillin, which is not comparable with the specificity of the HPLC method. 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.[33–35] Concerning a recent study about the production of saponin-rich extracts by ultrasound assisted extraction where ethanol was used instead of methanol, it was found that fenugreek extracts contained 130 g of saponins $kg^{-1}$ of extract, whereas quinoa extracts contained 3 g of saponins $kg^{-1}$ of extract.[36] It could be concluded that the saponin enrichment of the extracts obtained with methanol in the present study was higher, at least for fenugreek and quinoa. This would suggest that methanol, as used in the present study, might be a more suitable alcohol for the ultrasound-assisted extraction of saponins in comparison with ethanol.

**Hydrolysis of the saponin-rich extracts to sapogenins**

The rate of hydrolysis of saponins of the four extracts was evaluated during 6 h of reaction (Figure 2). It was observed a progressive decrease of the saponin content up to 1 h of hydrolysis for all the extracts. From this time on, the saponin level kept constant for fenugreek (mean value of 7 g saponins $kg^{-1}$ extract) and quinoa (mean value of 5 g saponins $kg^{-1}$ for extract) extracts, whereas almost a complete disappearance of saponins was observed for lentil and soybean. Therefore, the obtained results suggested that hydrolysis times closer to 1 h might be enough for a complete disappearance of saponins from the considered extracts.

Different sapogenins were released and identified during the hydrolysis of the four extracts (Figure 3 and Table 3). Up to five different sapogenins were tentatively identified for the hydrolyzed fenugreek extract: the steroidal diosgenin, along with sarsasapogenin and neotigogenin, and two steroidal sapogenin acetate molecules. In the case of the hydrolyzed

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quinoa extract, the sapogenins oleanolic acid, hederagenin, serjanic acid and phytolaccagenic acid were clearly detected. Finally, only the expected sapogenin from soyasaponins was successfully detected as soyasapogenol B for the hydrolyzed lentil and soybean extracts.

The total amount of sapogenins for each time of hydrolysis was quantitated. The rate of release of total sapogenins of the extracts is shown in Figure 4. It was observed a progressive increase of sapogenins content up to 1 h of hydrolysis for all the extracts, time at which the maximum amount of sapogenins was detected, in agreement with the time of maximum hydrolysis of saponins. At this time of hydrolysis, the highest total amount of sapogenins was found for quinoa extract (around 40 g $kg^{-1}$ of extract) and followed by fenugreek extract (around 30 g $kg^{-1}$ of extract). Considerably lower levels were detected for soybean (around 7 g $kg^{-1}$ of extract) and lentil (around 4 g $kg^{-1}$ of extract) extracts. Therefore, after the same conditions of hydrolysis, the richest extract in sapogenins was found to be the quinoa extract, despite the fact that such extract did not contained the highest amount of saponins initially, but the fenugreek extract instead. It was suspected that total saponins in the extracts were not completely detected by the used analytical conditions, probably due to potential co-elutions of saponins with other compounds such as polyphenols.

In order to obtain a more detailed information about the evolution of the release of sapogenins during hydrolysis, the specific course of individual sapogenins is detailed in Table 4. It was observed that the highest amount of individual sapogenins was also detected at 1 h of hydrolysis for most sapogenins. At 1 h of hydrolysis, the fenugreek extract consisted mainly of diosgenin as major sapogenin (around 20 g $kg^{-1}$ of extract), followed by sarsapogenin and neotigogenin (around 4 g $kg^{-1}$ of extract, for each sapogenin). The quinoa extract consisted of similar amounts of oleanolic acid, serjanic acid and phytolaccagenic acid, (around 10 g $kg^{-1}$ of extract, for each sapogenin), whereas the minor sapogenin was hederagenin (around 4 g $kg^{-1}$ of extract). In the case of lentil and soybean, as already shown in Figure 4, the only sapogenin

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detected was soyasapogenol B, whose evolution and maximum levels can be observed in Figure 4.

It is important to remark that, according to Figure 4 and Table 4, the sapogenin levels trended to decrease after 1 h of hydrolysis, being not able to measure such contents in lentil and soybean extracts after longer times of hydrolysis. This would suggest that under the assayed conditions of hydrolysis of saponins, the released sapogenins would be degraded at longer times of reaction. In order to deepen into this theory, additional experiments were performed by exposing pure standards of oleanolic acid and diosgenin, that is, the main sapogenins of the extracts, to the same hydrolysis conditions. As shown in Figure 5, both sapogenins trended to decrease during the hydrolysis, especially during the first and second hour of reaction. Thus, after 6 h of hydrolysis, the initial amount of oleanolic acid decreased around 30% and closer to 60% in the case of diosgenin. This result confirmed that the major sapogenins released from the extracts might be susceptible to the simultaneous degradation during the course of hydrolysis. Additionally, the obtained results would suggest that this effect might depend on the type of sapogenin, being the steroidal sapogenin more susceptible to degradation than the terpenoid sapogenin. In fact, Peng et al.[37] described a marked decrease in the yield of diosgenin after 6 hours of being exposed to 0.8 M sulphuric acid, which was attributed to the conversion of diosgenin to 25-spirosta-3, 5-dienes.

In order to understand how this observed effect might condition the evaluation of the hydrolysis of the saponins from the extracts, we tentatively evaluated the degree of hydrolysis of saponins by considering both the disappearance of saponins and appearance of sapogenins. Taking into account the disappearance of saponins as estimator, a hydrolysis closer to 100% might be suggested, according to Figure 2. Considering the appearance of sapogenins as an estimator, we firstly considered that each mol of saponin might release one mol of sapogenin after a complete hydrolysis. Then, the tentative molecular weight of each detected saponin

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and the molecular weight of its corresponding sapogenin was considered, according to Table 2. In general, it was estimated that approximately 500 g of sapogenin should be released per kilogram of saponin if a complete hydrolysis occurs. Thus, when the hydrolysis of saponins was estimated from the appearance of sapogenins as estimator, it was found that the hydrolysis of saponins was 35% for fenugreek, 43% for lentil, 72% for soybean and superior to 100% for quinoa. Therefore, this showed that the estimation of hydrolysis of saponins by appearance of sapogenins might not reflect the estimation of hydrolysis of saponins by disappearance of saponins. This lack of coherence might be related to the previously demonstrated degradation of sapogenins (Figure 5) simultaneous to their realease, suggesting that it would not be possible to estimate a degree of hydrolysis of saponins by the appearance of sapogenins. Further determinations to confirm these results, as well as additional exploration of variables of the hydrolysis process would be necessary in order to evaluate the optimal conditions that preserve the sapogenin molecules once they are released, so that an efficient process is guaranteed. In the case of the present study, we showed that, by performimg typical conditions of hydrolysis reported in the scientific literature, namely HCl 2M at 100 ºC, degradation of sapogenins took place even during the initial moments of reaction, and that hydrolysis times above 1 h caused the loss of the maximum reached amount of sapogenins. This was considered a relevant finding, since the performance of prolonged times of hydrolysis of saponins can be found in the scientific literature without any previous verification of the impact of the hydrolysis conditions on the final products, namely sapogenins (Table 1).

As previously discussed, it should also be taken into consideration that maximizing the yield of sapogenins after hydrolysis is a key factor for obtaining sapogenin-rich extracts when a specific stronger bioactive effect is desired, compared to its former original extract. As examples, Uemura et al.[21] found that a hydrolized saponin fraction of a fenugreek extract,

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mainly in the form of diosgenin, significantly inhibited triglyceride accumulation in HepG2 cells, while the saponin fraction had no effect. An improved bioactive effect compared to the former saponins was also demonstrated for hederagenin and oleanolic acid released from the saponin rich fraction of the plant *Akebia quinata*.; as well as from the aglycones soyasapogenol A and B compared to soyasaponins.[19,20] Therefore, these evidences clearly suggest that higher effect of some specific bioactivities may be achieved if saponin-rich extracts are submitted to hydrolysis, but, according to the contribution of the present study, such hydrolysis conditions must be optimized in order to guarantee the highest contents of aglycones and, likely, the highest bioactivity improvement. Further assays will be performed in future studies in order to evaluate the bioactive effects of the former saponin-rich extracts as compared to the resulting sapogenin-rich extracts obtained in the present research.

**Composition of the hydrolyzed extracts in sapogenins, phytosterols and tocopherol**

Along with the bioactive interest of sapogenins released during the hydrolysis of the seed extracts, it is relevant to consider that these extracts are complex matrices that might contain a wide diversity of other compounds of bioactive interest. Specifically, phytosterols and tocopherols are compounds which have been described in the assayed seeds, and whose bioactive importance is well known. Therefore, the production of seed extracts with the simultaneous presence of saponins, phytosterols and tocopherols might be of interest. However, when saponin-rich extracts are hydrolyzed in order to obtain sapogenin-rich extracts, the impact of the hydrolysis on the rest of the bioactive compounds has been scarcely considered. Since we considered of interest this issue so that a wider bioactive potential could be given to the hydrolyzed extracts, the evolution of minor compounds during the hydrolysis, namely phytosterols and tocopherol, was also evaluated.

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As shown in Figure 6, all the extracts initially contained variable amounts of phytosterols and tocopherol. Thus, in the case of phytosterols, the extracts of lentil and soybean were the richest in these compounds (around 10 g $kg^{-1}$ of extract), whilst fenugreek and quinoa extracts displayed the lowest contents (around 3 g $kg^{-1}$ of extract). In the case of tocopherol, all the extracts showed similar initial values (around 2 g $kg^{-1}$ of extract). However, when the extracts were hydrolyzed for the maximum release of sapogenins from saponins, namely 1 h, phytosterols slightly trended to decrease for most extracts (lentil and soybean), and disappeared in the case of fenugreek. As for tocopherol, its content remained almost constant at the different assayed times of hydrolysis (Figure 6).

Taking into account both the described initial composition of the extracts and the changes occurred after 1 h of hydrolysis, the global composition of the hydrolyzed extracts could be grouped by seeds due to a similar pattern observed between them, as shown in Figure 6. Thus, the final composition of the hydrolyzed fenugreek extract in the considered bioactive compounds, consisted mainly of sapogenins and a minor fraction of tocopherol. Similarly, quinoa extracts consisted mainly of sapogenins, and a minor fraction of phytosterols and tocopherol. On the contrary, the hydrolyzed extracts of lentil and soybean were quite similar between them, but quite different when compared to fenugreek and quinoa extracts, since they were mainly characterized by a major fraction of phytosterols, followed by sapogenins and tocopherol. Therefore, concerning the aim of producing sapogenin-rich extracts where sapogenins are the major bioactive compounds, the most interesting extracts would be those of fenugreek and quinoa, since those of lentil and soybean might be characterized by a higher content of other different bioactive compounds, mainly phytosterols. Nevertheless, lentil and soybean extracts might also be of interest if phytosterols-rich extracts containing minor fractions of sapogenins are desired.

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Regardless of these differences between seeds, it is interesting to remark the relevance of the hydrolysis procedure as a tool to obtain extracts with higher total level of bioactive compounds under the form of sapogenins, plus phytosterols or tocopherol. Thus, as shown in Figure 6, and comparing the initial composition of the extracts (0 h) with that after 1 h of hydrolysis, the sum of total considered bioactive compounds increased closer to 9 folds for quinoa extract and 5 folds for fenugreek extract. However, further studies would be necessary in order to explore other saponin extraction-concentration procedures that would allow obtaining higher initial levels of saponins in the extracts and further yield higher levels of sapogenins in the hydrolyzed extracts.

Finally, the impact of longer times of hydrolysis in the composition of bioactive compounds is also shown in Figure 6. Thus, for all the extracts, the sum of total bioactive compounds decreased after 4 h of hydrolysis compared to 1 h (a decrease of around 50% for fenugreek, 60% for quinoa, 20% for lentil, and 40% for soybean). This effect was mainly due to the decrease in the level of sapogenins, since the level of phytosterols and tocopherol was not remarkable. Therefore, these results would suggest that sapogenins might be more labile during the hydrolysis conditions assayed than the other considered compounds.

As a brief remark, it should be taken into consideration that, as well as the content of the described bioactive compounds can be modified during the course of the hydrolysis, other diverse and undesired compounds can be formed. This might be due to the complex chemical components of the extracts that can also suffer different chemical reactions when subjected to high temperatures and strong acids. Therefore, further studies would be necessary to reach a characterization of the hydrolysed extracts as full as possible.

## CONCLUSIONS

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Saponin-rich extracts obtained from edible seeds as fenugreek, quinoa, lentil and soybean by ultrasound-assisted extraction contain interesting saponins of either triterpenoid or steroid nature for potential development of food ingredients. Their transformation to sapogenin-rich extracts can be easily performed by acid hydrolysis, but a maximum time of hydrolysis of 1 h is suggested to guarantee the maximum level of sapogenins, since longer hydrolysis times would lead to lower sapogenin yields due to degradation of these compounds. Additionally, when acid hydrolysis is performed to produce sapogenin-rich extracts, other minor compounds of the extracts, as phytosterols and tocopherol, can be kept almost unaffected in the final product.

These findings contribute to the development of technologies to produce natural extracts with increased bioactive potential for food ingredients, as the chemical hydrolysis might be to transform saponin-rich extracts to sapogenin-rich extracts with stronger bioactive effects.

**Conflict of interest:** The authors declare that they have no conflict of interest.

**ACKNOWLEDGEMENTS**

This work was supported by the Ministerio de Economía y Competitividad, Spain (AGL2016-76736-C3-1-R) and the Community of Madrid, Spain (ALIBIRD-CM S2013/ABI-2728). Teresa Herrera thanks the Community of Madrid for her contract (Fondo Social Europeo, Programa Operativo de Empleo Juvenil e Iniciativa de Empleo Juvenil YEI). Joaquín Navarro del Hierro thanks the Ministerio de Educación, Cultura y Deporte for funding his research with a FPU predoctoral contract (FPU 15/04236). The authors would like to acknowledge the support of the Chromatography Laboratory from the Interdepartmental Investigation Service (SIdI-UAM) for the HPLC-MS analyses.

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**Figure Legends**

**Figure 1.** HPLC-DAD chromatograms showing the identified saponins from extracts of a) fenugreek, b) quinoa, c) lentil and d) soybean. Each number correspond to those shown in Table 2.

**Figure 2.** Evolution of the saponin content of the seed extracts (g saponins $kg^{-1}$ of extract) during the hydrolysis time.

**Figure 3.** GC-MS chromatograms showing the identified sapogenins from extracts of a) fenugreek, b) quinoa, c) lentil and d) soybean after 1 h of acid hydrolysis. Each number correspond to those shown in Table 3. Letters correspond to "a", tocopherol, and "b" phytosterols.

**Figure 4.** Evolution of the sapogenin content of the seed extracts (g sapogenins $kg^{-1}$ of extract) during the hydrolysis time.

**Figure 5**. Remaining amount of sapogenin standards (%) after being exposed to hydrolysis conditions at different times. (*) Significant differences compared to the remaining amount of sapogenin at time 0 ($p \leq 0.05$).

**Figure 6**. Total composition (g $kg^{-1}$) of the hydrolyzed extracts in sapogenins, phytosterols and tocopherol at initial (0 h), optimal (1 h) and long times (4 h) of hydrolysis of the extracts.

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**Table 1. Examples of acid hydrolysis conditions of different sources of saponins**

| Sample | Time (h) | Temperature (ºC) | Acid medium | Reference |
|---|---|---|---|---|
| Soybean | 3 | 100 | 2 N HCl in ethanol | 38 |
| | 1-12 | 100 | 3 N $H_2SO_4$ in dioxane/water | 24 |
| | 72 | 85 | 2 N HCl in methanol | 39 |
| *Panax notoginseng* | 3 | 55 | Acetic acid 25% | 40 |
| Medicago | 8 | | 2 N HCl in metanol | 27 |
| *Dioscorea zingiberensis* | 1-7 | 120 | $H_2SO_4$ | 37 |
| *Chenopodium quinoa* | 2 | 90 | 1 N HCl in dioxane/water | 41 |
| | 2 | 110 | 6 N HCl | 42 |
| | 2 | 100 | 2 N HCl in aqueous methanol | 43 |
| Legumes | 3 | refluxed | HCl in methanol 5% | 35 |
| Lentil seed | 3 | refluxed | Acetyl chloride in methanol 5% | 44 |
| *Primulaceae plants* | 4 | refluxed | 2 N HCl in ethanol | 45 |

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**Table 2. HPLC-MS characterization of the seed extracts**

| Peak | Rt. time | Main mass fragments (m/z)[a] | Tentative saponin |
|---|---|---|---|
| Fenugreek | | | |
| 1 | 13.5 | *1035*, 889, 757, 595, 433, 415 | Diosgenin-Glu-Glu-Xyl-Rha |
| 2 | 13.8 | *1049*, 873, 711, 579, 415, 397 | Diosgenin-GlcA-Glu-Xyl-Rha |
| 3 | 14.1 | *1031*, 885, 723, 577, 431, 415 | Diosgenin-Rha-Glu-Rha-Glu |
| 4 | 14.5 | *903*, 741, 595, 433, 415 | Gitogenin-Glu-Rha-Glu |
| 5 | 15.0 | *1049*, 873, 711, 579, 417 | Sarsasapogenin-GlcA-Glu-Xyl-Glu |
| 6 | 15.5 | *887*, 755, 579, 433, 415 | Diosgenin-Xyl-GlcA-Rha |
| 7 | 19.4 | *871*, 579, 417 | Sarsasapogenin-Rha-Rha-Glu |
| 8 | 20.6 | *871*, 739, 575, 417 | Sarsasapogenin-Xyl-Rha-GlcA |
| 9 | 21.1 | *871*, 739, 575, 473, 437, 419 | Unknown saponin |
| Quinoa | | | |
| 1 | 14.6 | *867*, 613, 517, 499, 481, 453, 435 | Phytolaccagenic saponin |
| 2 | 15.0 | *867*, 593, 497, 483, 465, 439, 421 | Oleanolic saponin + Serjanic saponin |
| 3 | 15.3 | *867*, 613, 501, 483, 465, 455, 437 | Serjanic saponin + Hederagenin saponin |
| Lentil | | | |
| 1 | 17.6 | *963*, 797, 599, 441, 423, 405 | Soyasaponin I |
| 2 | 19.0 | *923*, 797, 591, 441, 423, 405 | Soyasapogenol B saponin |
| 3 | 19.4 | *865*, 677, 593, 441, 423, 405 | Soyasapogenol B saponin |
| 4 | 20.6 | *867*, 695, 579, 477, 459, 441, 423 | Soyasapogenol A saponin |
| Soybean | | | |
| 1 | 19.5 | *1013*, 811, 631, 469, 441, 423 | Soyasapogenol B saponin |
| 2 | 20.6 | *1039*, 865, 811, 631, 477, 441, 423 | Soyasapogenol B saponin |
| 3 | 21.1 | *1035*, 865, 811, 631, 579, 519, 479, 441, 423 | Soyasapogenol B saponin |

[a] Ion fragment under cursive refers to $[M+H]^+$

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**Table 3. GC-MS characterization of the sapogenins from the hydrolyzed seed extracts**

| Peak | Rt. time | Main mass fragments (m/z) of the TMS-derivatized compounds | Tentative sapogenin |
|---|---|---|---|
| Fenugreek | | | |
| 1 | 20.9 | 559, 396, 282, 139 | Steroid sapogenin acetate |
| 2 | 21.0 | 559, 487, 396, 282, 139 | Steroid sapogenin acetate |
| 3 | 22.0 | 488, 416, 374, 345, 255, 139 | Sarsasapogenin |
| 4 | 22.4+22.5 | 486, 471, 414, 372, 282, 243, 187, 139 | Diosgenin |
| 5 | 22.7 | 488, 473, 416, 374, 345, 255, 187, 139 | Neotigogenin acetate |
| Quinoa | | | |
| 1 | 24.5 | 601, 585, 482, 320, 203, 189 | Oleanolic acid |
| 2 | 25.6 | 673, 570, 320, 203 | Hederagenin |
| 3 | 27.4 | 629, 526, 364, 247, 187 | Serjanic acid |
| 4 | 29.0 | 717, 614, 364, 247, 187 | Phytolaccagenic acid |
| Lentil | | | |
| 1 | 26.2 | 584, 306, 291, 278 | Soyasapogenol B |
| Soybean | | | |
| 1 | 26.2 | 584, 306, 291, 278 | Soyasapogenol B |

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**Table 4. Evolution of the content (g $kg^{-1}$ of extract) of individual sapogenins during the hydrolysis time of fenugreek and quinoa extracts**

| | Hydrolysis time (h) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.5 | 1 | 2 | 4 |
| Fenugreek extract | | | | | |
| Steroid sapogenin acetate | 0.2 | 1.8 | 1.8 | 2.5 | 1.2 |
| Steroid sapogenin acetate | 0.1 | 0.9 | 0.9 | 0.8 | 0.7 |
| Sarsapogenin | n.d. | 3.8 | 4.3 | 4.0 | 2.4 |
| Diosgenin | 0.4 | 17.7 | 21.1 | 18.0 | 9.4 |
| Neotigogenin acetate | n.d. | 2.7 | 4.1 | 1.9 | 1.7 |
| Quinoa extract | | | | | |
| Oleanolic acid | 2.2 | 8.1 | 12.1 | 10.5 | 6.7 |
| Hederagenin | n.d. | 2.2 | 3.8 | 2.9 | 1.1 |
| Serjanic acid | n.d. | 8.9 | 12.2 | 9.3 | 5.8 |
| Phytolaccagenic acid | n.d. | 7.5 | 10.0 | 6.5 | 2.0 |

n.d. not detected

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**Figure 1.** HPLC-DAD chromatograms showing the identified saponins from extracts of a) fenugreek, b) quinoa, c) lentil and d) soybean. Each number correspond to those shown in Table 2.

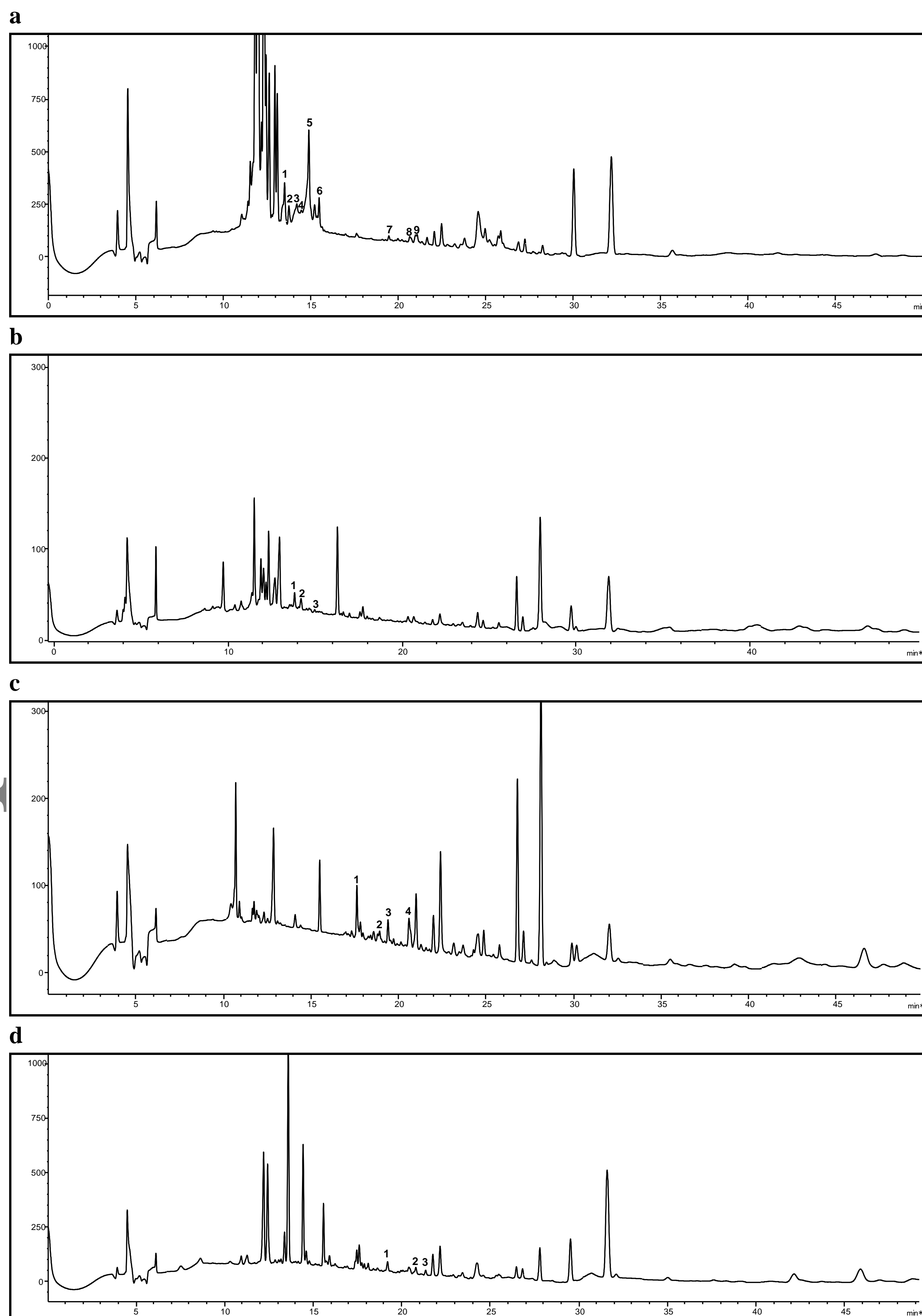


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**Figure 2.** Evolution of the saponin content of the seed extracts (g saponins kg-1 of extract) during the hydrolysis time.

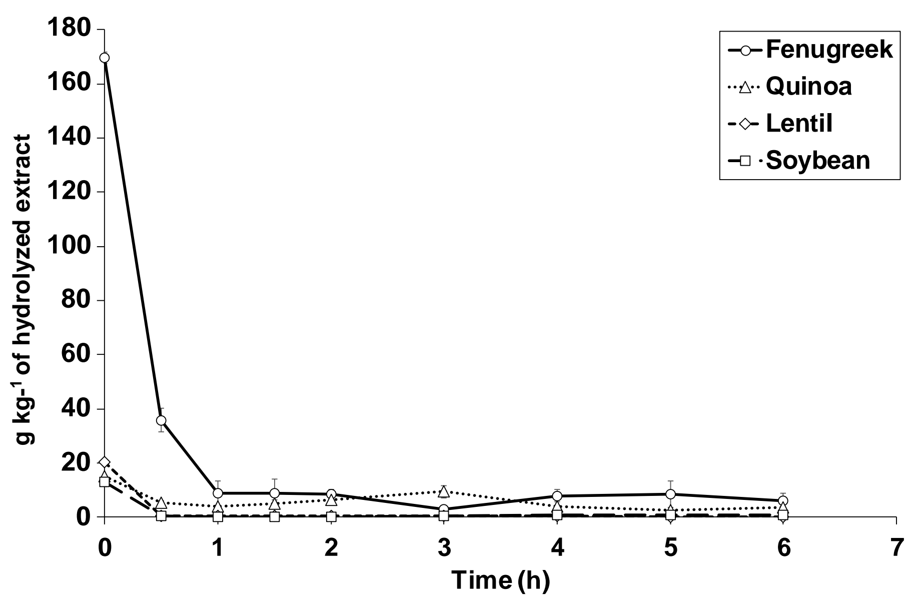


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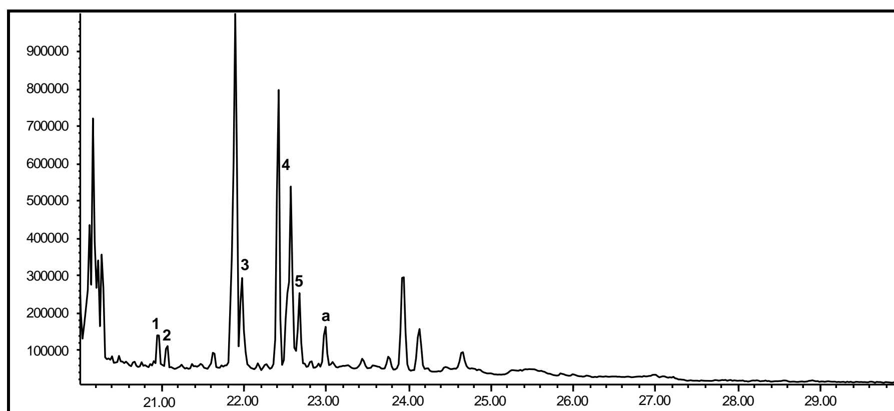
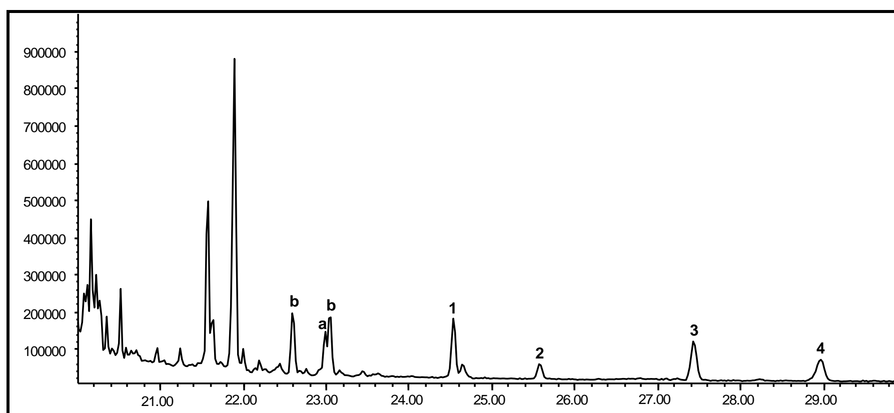
**Figure 3.** GC-MS chromatograms showing the identified sapogenins from extracts of a) fenugreek, b) quinoa, c) lentil and d) soybean after 1 h of acid hydrolysis. Each number correspond to those shown in Table 3. Letters correspond to “a”, tocopherol, and “b” phytosterols.

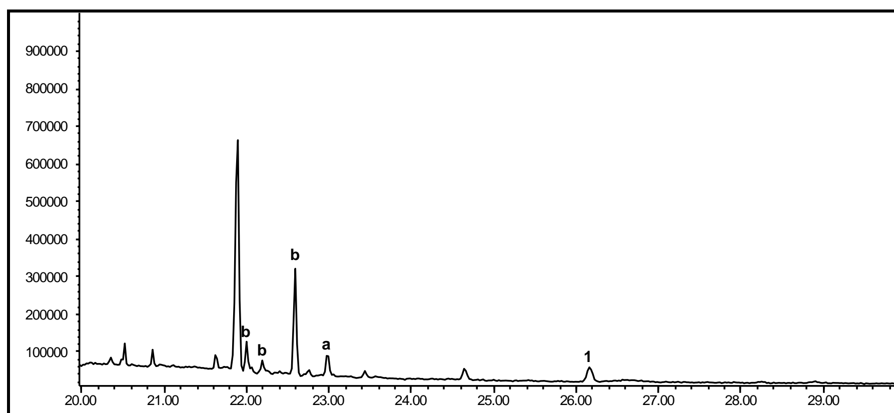
**a**




**b**




**c**




**d**

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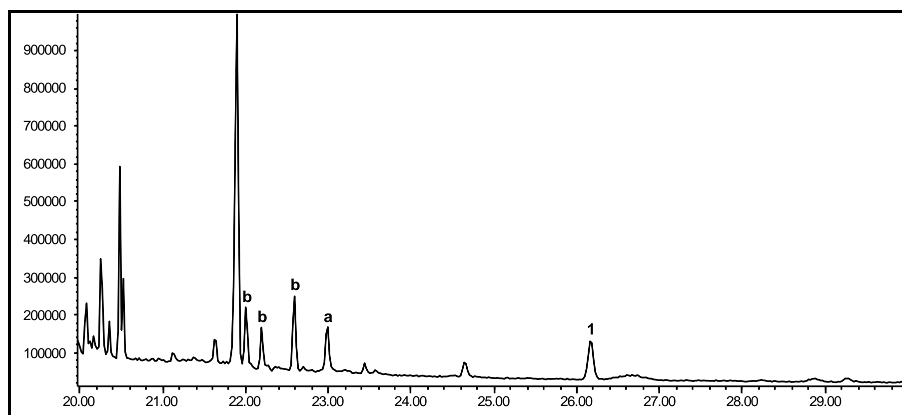


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**Figure 4.** Evolution of the sapogenins content of the seed extracts (g sapogenins kg-1 of extract) during the hydrolysis time.

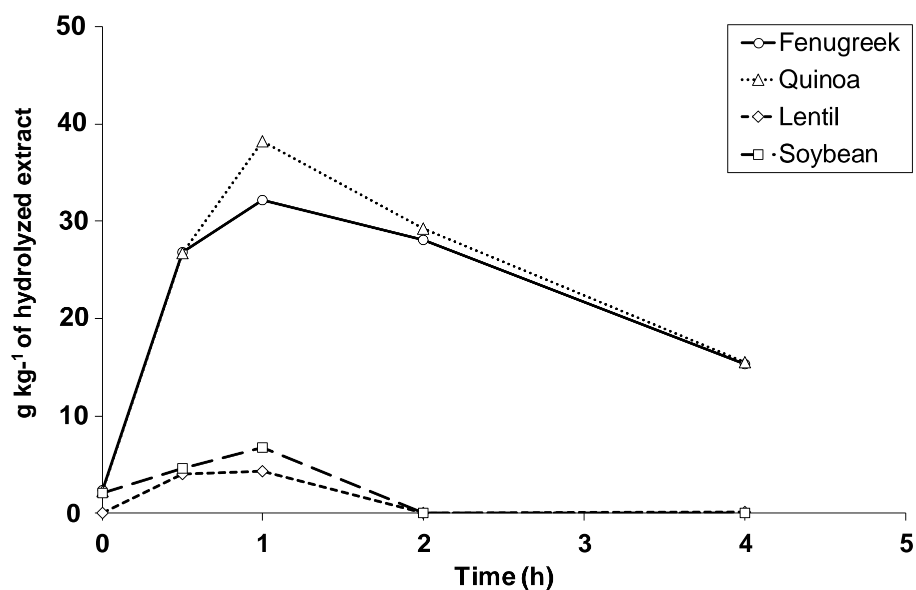


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**Figure 5.** Remaining amount of sapogenin standards (%) after being exposed to hydrolysis conditions at different times

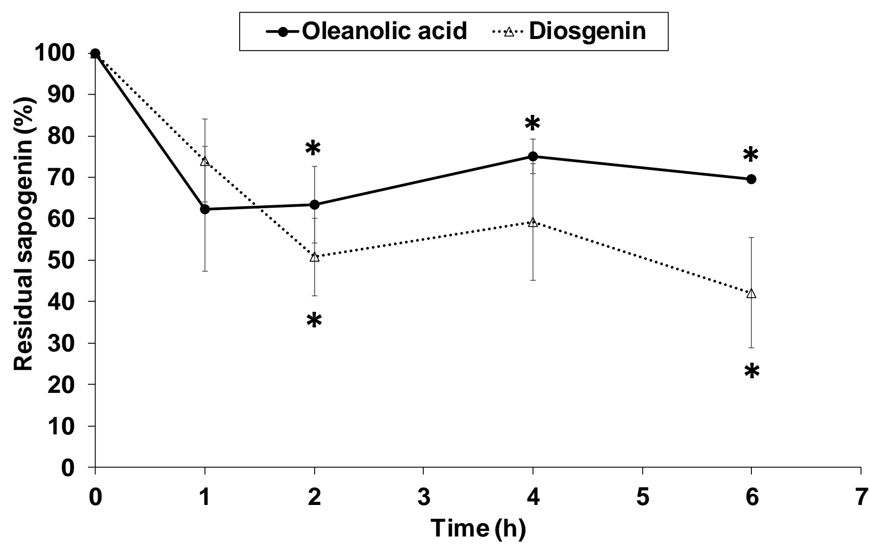


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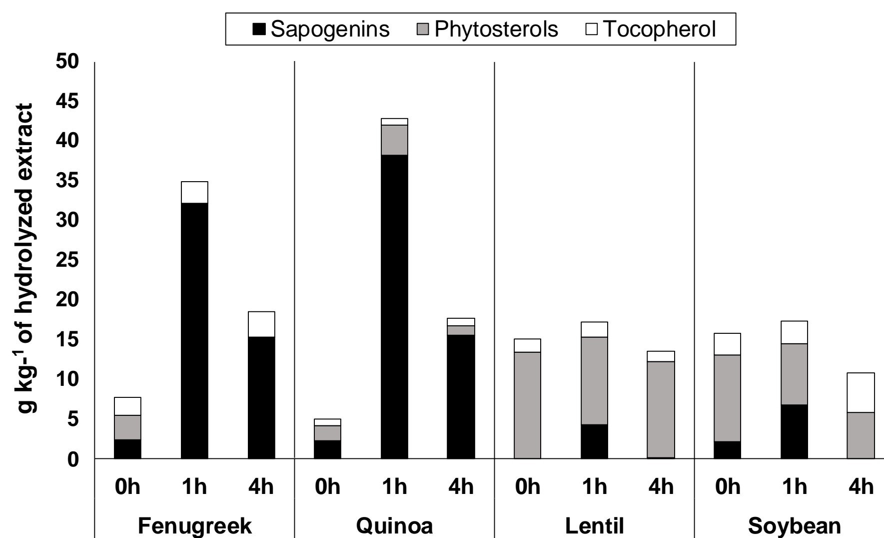
**Figure 6.** Total composition (g kg-1) of the hydrolyzed extracts in sapogenins, phytosterols and tocopherol at initial (0 h), optimal (1 h) and long times (4 h) of hydrolysis of the extracts.

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