In vitro permeability of saponins and sapogenins from seed extracts by the parallel artificial membrane permeability assay (PAMPA): effect of *in vitro* gastrointestinal digestion

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ABSTRACT

The permeability of saponins and sapogenins from fenugreek and quinoa extracts, as well as dioscin and diosgenin, was evaluated by the parallel artificial membrane permeability assay (PAMPA). The effect of the digestion process on permeability was determined, previous development of a gastrointestinal process coupled to PAMPA. Saponins from both seeds displayed a moderate-to-poor permeability ($> 1 \times 10^{-6}$ cm/s), although the digestion enhanced their permeability to values in the order of 10^{-5} cm/s ($p < 0.001$). Sapogenins exhibited a similar permeability to that of saponins, although the digestion enhanced the permeability of sapogenins from quinoa ($1.14 \pm 0.47 \times 10^{-5}$ cm/s), but not from fenugreek ($2.33 \pm 0.99 \times 10^{-6}$ cm/s). An overall positive impact of co-existing lipids on the permeability was evidenced. PAMPA is shown as a useful, rapid and easy tool for assessing the permeability of bioactive compounds from complex matrices, but the previous gastrointestinal process being a relevant step.

Keywords: PAMPA, saponins, quinoa, fenugreek, gastrointestinal digestion

1. Introduction

Saponins are a wide group of structurally diverse compounds broadly distributed in plants and consist of one or more hydrophilic sugar residues linked through an ether or ester glycosidic linkage to a hydrophobic triterpenoid or steroid skeleton, known as sapogenin or aglycone.[1] Saponins can be widely found in leaves, roots, seeds, fruits and legumes, although steroid-like saponins are commonly found in medicinal plants, such as fenugreek, ginseng, asparagus, *Allium* species, or agave, whilst triterpenoid-like saponins are commonly found in cultivated crops, such as quinoa, soybean, beans, lentils, sunflower seeds, or liquorice roots.[2,3] Concerning sapogenins, the natural presence of these compounds in these sources is very limited, thus the hydrolysis of saponins is required for the release of the aglycone.[4]

A large number of bioactivities have been recently described for both saponins and sapogenins, including immunostimulatory, hypocholesterolemic, hypotriglyceridemic, antitumor, anti-inflammatory and antibacterial, among others.[5] However, the extent of activity exerted by bioactive compounds in general, and specifically by saponins and sapogenins, is closely linked to their bioavailability and cellular uptake after gastrointestinal digestion. In case of saponins, a general poor bioavailability and low intestinal permeability has been described for these compounds. The intestinal permeability of saponins is mainly affected by their physicochemical properties, which mainly include solubility, molecular weight, chemical stability and the total number of hydrogen bonds.[5] In this sense, the number of sugar units is key for determining the solubility of saponins, as it increases with the number of sugar residues. Nevertheless, an increase in the number of sugar units inevitably leads to an increase in the molecular weight of the saponin (generally 700 to 2500 Da), which in turn causes a detrimental effect on its permeability.[6,7,8] Sapogenins, on the contrary, have more favorable molecular weights for a better permeability (generally 400 to 600 Da), but due to their higher lipophilicity, their solubility in the aqueous gastrointestinal tract is considerably lower than their former saponin. However, despite the fact that the solubility of sapogenins is worse than that of saponins, their permeability and bioavailability seem to be higher, thanks to the reduced number of sugar moieties, hydrogen-bonding capacity and molecular flexibility.[9] Such general better bioavailability described for sapogenins has been related to a superior bioactivity of these molecules compared to their precursor saponins.[5,6]

However, it is important to remark that the low or high permeability of saponins and sapogenins, respectively, should not be generalized when these compounds are only a fraction of complex natural

extracts, which is a frequent situation in the study and production of saponins products. This is because, within natural extracts, other co-existing compounds of the extracts might impact, either positively or negatively, on the parameters that take place during the gastrointestinal process, such as dispersion, solubility, bioaccessibility, permeability and, hence, the final bioavailability of the target compounds.[10,11] Therefore, instead of assuming a general poor bioavailability, the determination of the permeability of bioactive compounds, when these compounds are obtained as complex rich extracts, would be necessary in order to contribute to the validation of the potential of these extracts.

The *in vitro* determination of the permeability of compounds as well as the prediction of their oral bioavailability is generally performed by cell cultures, being the Caco-2 monolayers models the most popular.[12] Such assay allows to evaluate both active and passive transport of molecules and to assess the bidirectional permeability of a compound, from the apical to the basolateral side or vice versa. However, despite the generally recognized high reliability and robustness of Caco-2 models, its use as a high throughput tool is very limited due to the long cell growth cycle and high implementation costs. Therefore, the need for implementing high throughput and low cost models that maintain a good correlation with the *in vivo* permeability has led to the development of artificial membranes as alternative models for gastrointestinal permeability simulation.[13] In this way, the most known and used *in vitro* assay that employs artificial membranes is the parallel artificial membrane permeability assay (PAMPA), which has been extensively used in the pharmaceutical field for the preliminary screening of potential drug candidates. PAMPA is a fast, simple and low-cost method used to predict the passive transcellular absorption of compounds, which is the most common transport of compounds in the gastrointestinal tract. It measures the ability of a compound to diffuse from a donor compartment to an acceptor compartment through a PDVF membrane that is coated with a lipophilic mixture containing phospholipids and cholesterol, which mimics the basic composition of the membrane of a cell.[14,15] This versatile and cost-effective method has been validated in numerous works by comparing the permeability values of drugs with those obtained by more complex models such as Caco-2 or even rat intestine models.[16–18] However, due to the pharmaceutical background of the PAMPA method, its use has been mostly limited to pure compounds as potential drugs, and therefore, the evaluation of the permeability of compounds that are contained in complex matrices, such as natural extracts, is quite scarce. The permeability of bioactive flavonoids and iridoids from extracts of *Vitex agnus-castus* and *Silybum marianum* were assessed by PAMPA models and validated through Caco-2

assays,[16,19,20] as well as furanocoumarins, alkaloids, flavonoid glycosides and flavonolignans from extracts of *Angelica archangelica*, *Waltheria indica*, *Pueraria montana* or *Silybum marianum*, respectively.[15,21] These uses of PAMPA are of great interest, suggesting it as a novel, relevant and interesting tool to be explored for the screening of nutraceuticals or food ingredients with bioactive purpose.

In any case, it is important to remark that the evaluation of the permeability of compounds by PAMPA studies is performed by just solubilizing the samples in an aqueous donor media, which is mostly a buffering aqueous solution. However, this simple media is far from reproducing the physiological complex composition, conditions and changes that occur in the gastrointestinal tract during the digestion process and that should take place before any membrane permeability is produced. Therefore, previous to perform a PAMPA assay, and especially for the use of this tool for food purposes, we consider that the *in vitro* gastrointestinal digestion of the matrix should be performed. This is because, on one hand, chemical and physical transformations of the target compounds might take place during the gastrointestinal digestion that might impact in the final permeability, as it has been effectively shown in the specific case of saponins.[5] On the other hand, and as previously exposed, other co-existing components of the extracts might impact on the dispersion and solubility of the target compounds in the digestion medium,[10] and hence, on permeability, as already shown for saponins.[11] Finally, the own components of the gastrointestinal fluids might contribute to the overall permeability acting as absorption enhancers, as it has been reported for bile salts or the own mixed micellar structures that are formed after digestion.[22]

Therefore, taking into account all these approaches, the aim of this work was to perform a comparative study of the permeability of saponins and sapogenins from natural extracts by the PAMPA assay, with and without previous digestion of the extracts, by previously developing a gastrointestinal process coupled to PAMPA. Fenugreek extract or hydrolyzed fenugreek extract were used as examples of steroid-type saponins or sapogenins extracts, respectively; whereas quinoa extract or hydrolyzed quinoa extract were used as example of triterpenoid-type saponins or sapogenins extracts, respectively. Furthermore, the permeability of two commercial standards of saponins and sapogenins, namely dioscin and diosgenin, was also evaluated.

2. Materials and methods

2.1 Chemicals

Seeds of red quinoa (*Chenopodium quinoa*) were purchased from Hijo de Macario Marcos (Salamanca, Spain) and seeds of fenugreek (*Trigonella foenum-graecum*) were from Murciana de Herboristeria (Murcia, Spain).

Diosgenin, 1,7-octadiene, L-α-Phosphatidylcholine from egg yolk Type XVI-E ≥99%, cholesterol, pancreatin from porcine pancreas, Dulbecco's Phosphate Buffered Saline (PBS), pepsin, Amano lipase A from *Aspergillus niger*, bile salts, phosphatidyl choline from dried egg yolk, sodium chloride, calcium chloride, Trizma base, maleic acid, HCl and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Dioscin was from Cymit Quimica S.L (Barcelona, Spain). All solvents used were HPLC grade, including methanol, hexane, 1-butanol, ethyl acetate, dimethyl sulfoxide (DMSO) and acetonitrile, which were from Macron (Gliwice, Poland).

2.2 Preparation of the saponin-rich extracts

Seeds were ground in a knife mill (Grindomix GM200, Retsch, Haan, Germany) at 10000 rpm for 1 min and the resulting powder was sieved in a vertical sieve (CISA Cedacería Industrial, Barcelona, Spain) until obtaining fractions with a particle size between > 100 μm and ≤ 250. The subsequent extraction was based on Herrera et al.[4] with modifications. Samples was extracted with methanol at a ratio of sample to solvent of 1:10 (w/v) for 15 min by direct sonication (Branson SFX250 Digital Sonifier, Branson Ultrasonics, Danbury, CT, USA) with an ultrasonic probe (1/2" diameter, output sonication amplitude of 60%) at 20 kHz. Then, the mixture was centrifuged at 4500 rpm for 15 min. The supernatant was defatted with hexane at a ratio of 1:1 (v/v) by vortex agitation for 1 min and centrifuged at 4000 rpm for 10 min. The methanolic phase was collected and evaporated under vacuum. In order to achieve a further enrichment in the compounds of interest, the dried residue was extracted with water and 1-butanol. Briefly, miliQ water was added to the dried residue at a ratio of sample to solvent of 1:20 (w/v). Once solubilized, 1-butanol was added to the mixture at a ratio of water to 1-butanol of 1:2 (v/v), vortexed for 1 min and centrifuged at 4000 rpm for 10 min. The top phase was collected and the bottom phase was extracted again under the same proportions and conditions. Both collected phases were dried under vacuum and the resulting extract was stored at -20 ºC until further use.

2.2 Preparation of the sapogenin-rich extracts

In order to produce sapogenin-rich extracts, the previously obtained saponin-rich extracts were acid-hydrolyzed as described by Herrera et al.[4] with modifications. Briefly, the saponin-rich extracts were heated at 100 ºC with HCl solution (2 mol L^{-1}) at a ratio of sample to acid solution of 1:50 (w/v) for 1 hour. After, the mixture was ice-cooled for 5 min and liquid-liquid extracted with ethyl acetate at a ratio of 1:1 (v/v) by vortex agitation for 1 min and centrifuged at 4500 rpm for 5 min. The top phase was collected and the bottom phase was extracted again with the same volume of ethyl acetate under the described conditions. Both collected phases were dried under N_2 stream and the resulting extract was stored at -20 ºC until further use.

2.3 *In vitro* gastrointestinal digestion

The *in vitro* digestion model was based on Navarro del Hierro et al.[11] but modifications were needed for being coupled to PAMPA assays. For the gastric digestion, 25 mg of fenugreek extracts (saponins or sapogenins) or 11.2 mg of quinoa extracts were mixed with a gastric solution (2.2 mL) at pH 2.5 containing NaCl (150 mM), $CaCl_2$ (6 mM) and HCl (4 mM). In the case of digestions of the commercial standards, the amount digested was equivalent to the content of saponins or sapogenins in the extracts. The mixture was gently stirred in a water bath at 37 °C for 1 min to allow the dispersion of the components. The gastric digestion started after the addition of 0.45 mL of a fresh extract of gastric enzymes containing gastric lipase (16 mg/mL) and pepsin (5 mg/mL) in gastric solution previously stirred for 10 min. The reaction was performed for 45 min. For the intestinal digestion, a solution simulating a biliary secretion at pH 7.5 was previously prepared (6.3 mg of lecithin, 10.6 mg 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 stirred for 5 min. At the end of gastric digestion, 1.9 mL of this biliary secretion were immediately added, and the whole medium was stirred for 1 min at 37 °C. The intestinal digestion was initiated by the addition of 0.45 mL of a fresh pancreatin extract at 15.6 mg/mL in trizma-maleate buffer, which had been previously stirred for 10 min and centrifuged at 4000 rpm for 15 min. The reaction was performed for 60 min.

2.4 Parallel artificial membrane permeability assay

The PAMPA experiment was based on Piazzini et al.[16] with modifications. The assay was carried out in 96-well multiScreen-IP PAMPA filter and transport receiver plates (Millipore Corporation, Cork, Ireland). First,

10 µL of a lipid mixture containing L-α-Phosphatidylcholine (10 mg/mL) and cholesterol (8 mg/mL) in 1,7-octadiene solution were added to the PVDF filter of each well. Immediately after the artificial membrane was coated, 300 µL of donor solutions were added to each well of the donor plate. For the undigested samples, donor solutions were prepared at 5 mg/mL for both fenugreek extracts or 2.25 mg/mL for both quinoa extracts in PBS (containing 5% DMSO, v/v). For the commercial standards, donor solutions were prepared at equivalent concentration of saponins or sapogenins to that in the extracts. For the digested samples, the donor solutions consisted of the digestion medium added to the wells immediately after the intestinal digestion concluded, being the concentration of the fenugreek and quinoa extracts the same as for the undigested samples. Next, 300 µL of either PBS (5% DMSO, v/v) (for undigested samples) or trizma-maleate buffer (for digested samples) were added to each well of a multiScreen transport receiver plate. Finally, the donor plate was placed upon the receiver plate, ensuring that the underside of the membrane was in contact with the buffer. The resulting sandwich was incubated at room temperature for 4 hours.

The permeability of the compounds was calculated using the formula described by Chen et al.[23]:

$$P_e = \frac{-ln\left[1 - C_A(t)/C_{equilibrium}\right]}{A \times (1/V_D + 1/V_A) \times t}$$

$$C_{equilibrium} = [C_D(t) \times V_D + C_A(t) \times V_A]/V_D + V_A$$

where P_e is the effective permeability (cm/s). A = effective filter area = f × 0.3 cm^2, where f = apparent porosity of the filter = 1, V_D = donor well volume = 0.3 mL, V_A = acceptor well volume = 0.3 mL, t = incubation time = 14400 s, $C_A(t)$ = compound concentration in acceptor well at time t, and $C_D(t)$ = compound concentration in donor well at time t.

2.6 Determination of saponins by HPLC-DAD

At the end of the PAMPA experiment, donor and acceptor solutions from at least 3 wells were individually collected and directly analyzed according to Herrera et al.[4] for the quantitative determination of the saponins and dioscin. Initial obtained saponin-rich extracts were also analyzed. A high-performance liquid chromatography (HPLC) system (Agilent Infinity 1260, Agilent Technologies Santa Clara, CA, USA) with diode array detection (DAD) was used. Separation was carried out on an ACE 3 C18-AR column (150mm × 4.6 mm, 3 μm particle size) protected by a guard column (Advanced Chromatography Technologies Ltd,

Aberdeen, Scotland). A gradient elution was applied using water with 0.05% trifluoroacetic acid (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 flowrate 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. The chromatograms were registered at 205 nm. The identification and quantification of saponins was performed according to Herrera et al.[4]

2.7 Determination of sapogenins by GC-MS-FID

At the end of the PAMPA assay, donor and acceptor solutions from at least 15 wells were collected together for the extraction of sapogenins for the sapogenins and diosgenin determination. Donor and acceptor solutions were extracted with ethyl acetate at a ratio of 1:1 (v/v). Then, the mixture was vortexed for 1 min and centrifuged 10 min at 4000 rpm. The top phase was collected and the bottom phase was extracted again with the same volume of ethyl acetate under the described conditions. Both collected phases were dried under N_2. The resulting residue was derivatized with bis(trimethylsilyl) trifluoroacetamide (BSTFA) at 75 ºC for 1 h and then analysed by GC-MS-FID (Agilent 7890A, Agilent Technologies) according to Herrera et al.[4] for the determination of sapogenins in both donor and acceptor compartments. Initial obtained sapogenin-rich extracts were also analyzed.

The equipment consisted of 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 temperature of the oven started at 50 ºC and was held for 3 min, increased to 310 ºC at a rate of 15 ºC/min and 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. The identification and quantification of sapogenins was performed according to Herrera et al.[4]

2.8 Statistical analyses

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 (ANOVA). Differences were considered significant at $P \leq 0.05$.

3. Results and discussion

3.1 Permeability of saponins from the saponin-rich extracts

3.1.1 Permeability of saponins from undigested extracts

First, the permeability of saponins was established by the traditional method of determination of permeability in PAMPA, that is, solubilizing the extract in an aqueous buffer solution, which is also added to the acceptor compartment.

The effective permeability values of total saponins from the two extracts is shown in Figure 1A. Saponins from the fenugreek extract had a P_e of $1.63 \pm 0.55 \times 10^{-6}$ cm/s, while the P_e of saponins from the quinoa extract was $9.40 \pm 6.7 \times 10^{-7}$ cm/s, not being statistically different the permeability between these steroid-like (fenugreek) and triterpenoid-like (quinoa) saponins.

In order to understand the extent of permeability of a compound, a general categorization has been well stablished in the literature. In general terms, a compound has a high permeability or good absorption if the P_e is higher than 1×10^{-5} cm/s, a moderate absorption if the P_e is lower than 1×10^{-5} cm/s and higher than 1×10^{-6} cm/s, and a low absorption if the P_e is lower than 1×10^{-6} cm/s.[24,25] Considering this classification, it could be stablished that saponins from the fenugreek and quinoa extracts exhibited a low to moderate permeability when the PAMPA assay was performed under traditional conditions.

The direct comparison of these results with those in the literature is quite difficult, since as far as we are concerned, no previous studies describing the permeability of saponins from these or other plant sources have been conducted by the PAMPA method. Nevertheless, a couple studies have assayed the permeability of saponins by Caco-2 cultures. On the one hand, Stockdale, Challinor, Lehmann, De Voss, & Blanchfield[26] have recently evaluated the permeability of steroidal saponins from *Chamaelirium luteum* and found that three of those saponins exhibited a moderate permeability, being the P_e values very similar to those from the fenugreek saponins in the present study; while those with more than two sugar units were found to have low permeability. On the other hand, Hu, Reddy, Hendrich, & Murphy[27] evaluated the permeability of the triterpenoid-like soyasaponin I and found that this saponin had a very limited absorption, which lied between

the range of 9×10^{-7} to 3.6×10^{-6} cm/s, depending on its concentration. The values reported by these authors are very much alike the P_e obtained for the triterpenoid saponins from the quinoa extract.

Therefore, this study shows for the first time values of permeability of saponins from complex natural extracts of either fenugreek or quinoa by the *in vitro* PAMPA assay, suggesting their apparent poor membrane permeability, regardless of their chemical structure as steroid or triterpenoid saponins.

3.1.2 Effect of the gastrointestinal digestion on the permeability of saponins

Concerning the alternative artificial membrane assays such as PAMPA, and as far to our knowledge, there is an inexistent literature regarding the coupling of a gastrointestinal digestion to PAMPA. Therefore, previous to perform such assay, it was necessary to develop a modified gastrointestinal digestion protocol to maintain the integrity of the artificial membrane.

3.1.2.1 Development of in vitro gastrointestinal digestion model coupled to PAMPA

The gastrointestinal digestion model of Navarro del Hierro et al.[11] was initially tested to evaluate the compatibility with the membranes in the PAMPA plate. However, it was found that the volume of a control digestion medium added to the donor well completely permeated to the acceptor wells after a short while, which meant a loss of membrane integrity. In order to assess which component of the digestion medium was responsible for the disruption of the PAMPA membrane, we tested each of the individual components at their final concentration in the digestion medium. The integrity of the membrane was confirmed by visually comparing the volume of the donor wells to a control donor well filled with PBS. By ascending order of complexity, the tested components were: gastric solution; gastric solution + lipase; gastric solution + lipase + pepsin, and progressively increasing such complexity until reaching the mixture of gastric solution + lipase + pepsin + Trizma Maleic buffer + pancreatin + lecithin + bile salts. It was with the introduction of bile salts when the membrane visually and clearly disrupted, so we assumed that this component was the one whose concentration in the digestion medium needed to be modified. The disruption of membranes by bile salts has also been described in the literature, due to interactions occurring between them and the phospholipid bilayer.[22]

Subsequently, the effect of decreasing concentrations of bile salts of the digestion medium on the integrity of the membranes was tested, ranging from the initial concentration at 12.65 mM to as low as 0.32 mM. The

criteria to choose this range of concentration were to reduce the amount of bile salts as needed but always within *in vivo* physiological values. Therefore, the relevant reference of Riethorst et al.[28] was taken. These authors described the range of the concentration of bile salts that were found in the human duodena during fasted and fed state conditions from 20 volunteers. Such values varied from 0.03 mM to 36.18 mM at fasted state and from 0.74 mM to 86.14 mM at fed state. In our study, we observed that the concentration of bile salts that did not visually disrupt the PAMPA membrane was 1/16 from the initial one, that is, 0.79 mM. Therefore, even though this concentration might seem low, it is still within the range of the concentration of bile salts that can be found in the human duodena during fed state conditions,[28] so it was adopted to allow to perform the PAMPA assays under physiological concentrations.

After adopting this modification of the bile salts concentration, it was necessary to take into account the physiological ratio that exists between bile salts and phospholipids within the biliary secretions *in vivo*. According to Riethorst et al.[28], such ratio has a mean value of approximately 3 under fed state conditions. Therefore, the concentration of lecithin of the media was reduced to 0.26 mM accordingly.

This final modified gastrointestinal model was tested on PAMPA by a control digestion medium and the volume of the donor well completely remained within the wells during a whole time of incubation for 4 hours.

3.1.2.2 Permeability of saponins from digested extracts

The effective permeability values of saponins from the two extracts submitted to gastrointestinal digestion is shown in Figure 1A. Very interestingly, it was observed a generalized significant effect of the digestion process on the increase of the permeability of saponins ($p < 0.001$). Focusing on the permeability of each of the extracts, the P_e of saponins from the fenugreek extract submitted to digestion was $1.14 \pm 0.19 \times 10^{-5}$ cm/s, which was 7 folds higher than the permeability of these saponins under traditional conditions of PAMPA ($p < 0.001$). Saponins from the quinoa extract displayed a P_e value of $1.57 \pm 0.33 \times 10^{-5}$ cm/s, which was 16 folds higher than the permeability of these saponins under traditional conditions ($p = 0.002$). In this case, saponins from quinoa exhibited a significantly higher permeability than those from fenugreek when submitted to gastrointestinal digestion ($p = 0.039$), although such difference was not especially remarkable. Therefore, according to the permeability classification previously described, saponins from both extracts

would be considered to have a high permeability and good absorption if an *in vitro* gastrointestinal digestion is previously performed.

The reasons that might explain why such enhancement in the permeability was observed are complex, being likely related to multiple factors. First, it is known that the amphiphilic nature of saponins causes them to self-assembly as micelle-like aggregates in water, forming aggregates of about 50 molecules for certain types of saponins.[29] These large structures might negatively interfere with the permeability of the saponins contained in such aggregates due to the size they can reach. The formation of these structures might be one of the reasons to explain why the permeability of saponins is moderate to low when assayed under simple aqueous solutions as the traditional PAMPA assays.

On the other hand, during the digestion process, vesicles, emulsion droplets, lamellar and micellar structures are naturally formed thanks to the presence of bile salts and phospholipids. Thus, the increased micellar surface might allow saponins to incorporate into those micellar structures, favoring their dispersion and preventing them from aggregation, and consequently, enhancing their permeability.[30] Additionally, concerning bile salts, it is well-known that they act as drug absorption enhancers due to their double ability to act as permeation-modifying and drug-solubilizing agents. They can partition into the membrane and increase its fluidity and permeability, as well as associate with phospholipids and cause a dissociation of integral membrane proteins, resulting in the breakdown of the cell membrane.[22] In fact, this undesirable effect of bile salts was observed in the current study, as already explained, which led to the necessary reduction of the used bile salts concentrations for the gastrointestinal digestion model prior to PAMPA assays. Therefore, despite the considerably low concentration of bile salts used in the digestion medium, it could be thought that they might be, at least partially, enhancing the permeation of compounds through the PAMPA membrane without causing an irreversible disruption of it.

Concerning other reasons that might be involved in the improvement of the permeability of saponins after gastrointestinal digestion, it is important to remark that the assayed extracts also contained a lipid fraction, as shown in Table 1. This fraction was previously determined according to Navarro del Hierro et al.[11] for total lipids, and analyzed following the same GC-MS procedure described for sapogenins (for free fatty acids, monoglycerides and diglycerides). Thus, the total lipid content was around 9% and 53% for fenugreek and quinoa extracts, respectively. Under physiological conditions, gastrointestinal digestion of lipids leads to main lipid products in the form of free fatty acids and monoglycerides. These lipid products also cause an

increase in the production of micellar structures by forming mixed micelles of bile salts and phospholipids and, consequently, increasing the micellar surface.[10]

Therefore, in order to evaluate if the lipid co-existing components of the extracts were partially responsible for the increased permeability of saponins, we tested the permeability of the saponin standard dioscin, a steroid-like saponin representative of the fenugreek-like saponins, in absence and presence of lipids. A mixture of triglycerides (in the form of extra virgin olive oil) and oleic acid were used in order to properly mimic the lipid composition of the fenugreek extracts (Table 1). The proportions of all the components were the same as those in the saponin-rich extract from fenugreek, according to Table 1. The assay was only performed under traditional conditions of PAMPA, because under digestion conditions the co-elution of the dioscin peak with other components of the digestion medium made it unable to be identified. As shown in Figure 2A, the permeability of dioscin without lipids was $7.38 \pm 1.00 \times 10^{-9}$ cm/s, whereas the addition of lipids significantly enhanced the permeability of dioscin to $1.30 \pm 0.33 \times 10^{-8}$ cm/s ($p = 0.015$). Even though in both cases the permeability of this saponin would be considered very low, it was confirmed that this lipid mixture was able to increase the permeability of dioscin up to 78% under the proportions studied. Gleeson et al.[31] have also proposed several candidates as intestinal permeation enhancers, including oleic acid and medium-chain fatty acids as caproic acid. Other authors have confirmed the enhancement effect of oleic acid, among other fatty acids, on the permeability of drugs across the skin[32] and very recently across the buccal mucosa[33]. Although we demonstrated that oleic acid and triglycerides were able to improve the permeability of dioscin, even without previous digestion, additional studies assessing the effect on the permeability of saponins by other fatty acids, as well as different proportions would be considered of relevance. Additionally, it is important to remark that the positive effect of lipids on the permeability of dioscin was observed by the simple co-existence of these compounds within the aqueous solution of the saponins. This would support the idea that the co-existing compounds of the natural extracts, or co-excipients,[10,34] should be considered relevant in the contribution of the final permeability of the target bioactive compounds.

As summary, the obtained results confirm the relevance that the gastrointestinal process has on the permeability of bioactive compounds such as saponins when performed under *in vitro* PAMPA assays, suggesting that the lack of mimicked digestion conditions might lead to the underestimation of the effective permeability of these compounds. Additionally, the impact of co-existing components, as lipids, in the permeability improvement of saponins is evidenced. This might have additional technological and economic

advantages, since the useful natural co-excipients of the extracts might be an alternative to the development of specific, complex and costly formulations that are frequently produced for natural extracts in order to enhance the final bioavailability and bioactivity of the desirable bioactive compounds.

3.2 Permeability of sapogenins from the sapogenin-rich extracts

3.2.1 Permeability of sapogenins from undigested extracts

Prior to the determination of the permeability of sapogenins, the acid hydrolysis of the saponin-rich extracts was performed to produce extracts with a relevant content of sapogenins (Table 1). The effective permeability values of total sapogenins from the two extracts under traditional PAMPA conditions is shown in Figure 1B. Sapogenins from the fenugreek extract had a P_e of $1.64 \pm 0.48 \times 10^{-6}$ cm/s, while the P_e of sapogenins from the quinoa extract was $3.36 \pm 1.80 \times 10^{-6}$ cm/s, not being statistically different the permeability between the steroid-like and triterpenoid-like sapogenins. There were not either any significant differences between the permeability of saponins and sapogenins of both seeds (Figure 1A and 1B), suggesting that these compounds would exhibit a similar permeability under traditional PAMPA conditions, which is not presumably affected by the molecular and chemical (hydrophilicity/lipophilicity) differences between saponins and sapogenins when found in these extract matrices. Therefore, considering the classification previously described, sapogenins from both fenugreek and quinoa extracts would be labelled as "moderately absorbed" when the PAMPA assay was performed under traditional conditions.

Similar to saponins, the direct comparison of these results with those in the literature is a hard task, since as far as our knowledge goes, only one study has described the permeability of the triterpenoid sapogenin betulinic acid under the PAMPA method.[35] These authors were only able to describe a moderate to poor permeability of betulinic acid derivatives. Nevertheless, a few studies have assayed the permeability of sapogenins by Caco-2 cultures. Stockdale et al.[26] have described a low-to-moderate permeability across the monolayers for halogenin, a steroidal sapogenin, and the authors suggested that its permeability was linked to a poor solubility in the buffer. Hu et al.[27] described a low permeability for the triterpenoid soyasapogenol B, which was between 0.3 to 0.6×10^{-7} cm/s, depending on its concentration. Qiang et al.[36] assessed the permeability of ursolic acid from a *Salvia officinalis* extract, a pentacyclic triterpene acid whose structure is very similar to the aglycones contained in quinoa. Very interestingly, the permeability exhibited by this compound ($2.5 \pm 0.4 \times 10^{-6}$ cm/s) was very similar to the permeability value described for the sapogenins in

the quinoa extract from our study (Figure 1B). Additionally, these authors did not find any difference between the permeability of pure ursolic acid and ursolic acid contained in the *Salvia officinalis* extract, suggesting that the rest of the compounds from the plant extract did not affect the permeability of this aglycone.

This study shows for the first time values of permeability of sapogenins from complex natural extracts of either fenugreek or quinoa by the *in vitro* PAMPA assay, suggesting their apparent moderate permeability, and being comparable to that of their former saponins.

3.2.1 Effect of the gastrointestinal digestion on the permeability of sapogenins

Following the gastrointestinal digestion protocol previously optimized, the digestion of the sapogenin-rich extracts from fenugreek and quinoa was performed. The effective permeability values of total sapogenins from the two extracts submitted to gastrointestinal digestion is shown in Figure 1B. Considering the statistical analysis of the effect of the digestion factor, and regardless of the seed, it was not observed a significant effect of the digestion process on the increase of the permeability of sapogenins when compared to the traditional conditions. However, when focusing on the permeability of each of the extracts, the P_e of sapogenins from the quinoa extract submitted to digestion ($1.14 \pm 0.47 \times 10^{-5}$ cm/s) was significantly superior, that is, 3 folds higher than the permeability of these sapogenins under traditional conditions ($p = 0.05$). On the contrary, sapogenins from the fenugreek extract displayed a P_e value of $2.33 \pm 0.99 \times 10^{-6}$ cm/s, which was not different to that under traditional conditions. Additionally, such value of permeability of digested fenugreek sapogenins was considerably lower than the P_e of sapogenins from quinoa under digestion conditions ($p = 0.031$).

Taking into account these P_e values of the digested extracts, sapogenins from quinoa would be considered to have a moderate permeability, whilst those from fenugreek would be less permeable and therefore exhibit a low permeability if an *in vitro* gastrointestinal digestion is previously performed. Therefore, in the case of sapogenins, the digestion process did not exceedingly improve the permeability of such compounds when compared to the traditional conditions, as it remarkably happened for saponins. Since it has been described that the major problem of the bioavailability of sapogenins is related to their poor solubility,[37] it could be hypothesized that the gastrointestinal digestion of the sapogenin-rich extracts did not extremely improve the dispersion and solubility of these compounds, especially in case of fenugreek extracts.

It can be thought that this lack of relevant effect of the digestion process might be, at least, partly related to the composition of these extracts, and more precisely, to their lipid profile, considering the role of lipids on the formation of micellar structures necessary for the solubilization of lipophilic compounds, as sapogenins are. According to Table 1, after hydrolysis of the saponin extracts, the lipid profile mainly consisted of FFA. This different lipid composition might lead to a different final proportion of lipid digestion products after digestion compared to the saponin-rich extracts. Whether this situation affected to the micellar structures formation, and did not properly allow a better dispersion and solubility of sapogenins, especially in the case of fenugreek, would need to be further studied.

In order to deepen into this, and as performed with the saponin dioscin, it was tested the permeability of its corresponding sapogenin as diosgenin under traditional PAMPA conditions, under a previous gastrointestinal digestion process, and under a previous gastrointestinal digestion process in which a mixture of lipids was added (Figure 2B). The same lipid mixtures as the used for dioscin was added, for comparative purposes (Figure 2A), but the proportions of all the components was the same as those in the sapogenin-rich extract from fenugreek, according to Table 1. In this case, and unlike dioscin, the assay under traditional conditions with the addition of the lipid mixture was not possible due to an inadequate solubilization of all the components in the solution. As shown in Figure 2B, the permeability of diosgenin under traditional conditions was $3.66 \pm 0.68 \times 10^{-7}$ cm/s, whereas the performance of a gastrointestinal digestion before PAMPA increased the permeability of diosgenin to $1.03 \pm 0.30 \times 10^{-6}$ cm/s ($p = 0.02$). Therefore, these results reveal that the digestion process alone is able to enhance the permeability of diosgenin when compared to that under traditional conditions. However, since this result was not evidenced for the hydrolyzed fenugreek extract (Figure 1B), this would suggest that the composition of the sapogenin-rich extract from fenugreek might be either hindering the permeability of aglycones after the digestion process, or enhancing the permeability of aglycones under traditional conditions, causing such permeability to be similar in both cases.

Very interestingly, the co-digestion of diosgenin with lipids improved two folds the permeability of this sapogenin up to $2.18 \pm 0.64 \times 10^{-6}$ cm/s when compared to the digestion without lipids ($p = 0.049$) (Figure 2B). Therefore, it was evidenced that the incorporation of lipid forms during the digestion process, such as FFAs and TAGs, enhance even more the permeability of diosgenin to a very similar value as the one obtained for the sapogenins from the fenugreek extract, which might support the hypothesis that the lipid

composition of such extract is mostly responsible for the P_e observed. Nevertheless, it remains unclear if the enhancement in the permeability due to the lipid forms is either consequence of a solubility improvement after digestion, or if such lipid forms are acting as permeation enhancers *in situ*, that is, directly in the PAMPA membrane, or a combination of both. Therefore, further studies would be of interest in order to assess these enhancement mechanisms and to approach strategies to improve the effective action of sapogenin-rich extracts or diosgenin products.

Finally, regarding the permeability of saponins and sapogenins when considering the type of aglycone (triterpenoid or steroid), it seems that the differences between the two structures are only and clearly evidenced for the sapogenin-rich extracts when performing the gastrointestinal digestion, as the triterpenoid-like aglycones from quinoa exhibited a considerably higher permeability than the steroid-like ones from fenugreek. These differences were not remarkable between the two type of sapogenins in aqueous solution or between saponins from the two seeds in either aqueous solution or digested, suggesting that in general terms the permeability of saponins and sapogenins contained in the extracts of quinoa and fenugreek is not influenced by the type of structure of the aglycone. Besides, as this study assesses the permeability of only steroid-like standards (dioscin and diosgenin), the further evaluation of the permeability of other triterpenoid-like standards might be of interest in order to confirm if the structure of the aglycone is a relevant factor when considering the permeability of these compounds.

As summary, PAMPA is shown as a novel, useful, rapid and easy tool for assessing the passive membrane permeability of bioactive compounds from complex matrices for food purposes, such as natural extracts. However, the relevance that the gastrointestinal process has on the permeability of bioactive compounds when performed under *in vitro* PAMPA assays is evidenced. Additionally, the positive or negative impact of co-existing components of the extracts in the permeability is evidenced, being co-existing lipids pointed out as desirable compounds in the case of saponins and sapogenins. These preliminary *in vitro* results lay the foundations and the interest of evaluating the permeability of these extracts in cell or animal models to accurately determine the degree of intestinal absorption of their bioactive compounds saponins and sapogenins. Besides, further studies would be considered relevant in order to know which of the other components of the extracts might be responsible for an improvement or detrimental in the permeability of saponins and sapogenins in order to approach strategies to favor their co-extraction, as a novel technological tool to increase the efficacy of natural extracts.

Abbreviations used

DAG	Diglycerides
DMSO	Dimethyl sulfoxide
FFA	Free Fatty Acid
MAG	Monoglycerides
MCFA	Medium Chain Fatty Acid
OA	Oleic Acid
PAMPA	Parallel Artificial Membrane Permeability Assay
PBS	Phosphate Buffered Saline
P_e	Effective Permeability value
TAG	Triglycerides

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Notes

The authors declare no competing financial interests.

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606 **Figure Captions**

607

608 **Figure 1.** PAMPA effective permeability values, P_e (cm/s), of total saponins from saponin-rich extracts (A)
609 and total sapogenins from sapogenin-rich extracts (B) of fenugreek and quinoa under PBS 5% DMSO
610 conditions and under a previous gastrointestinal digestion. (*) Denote significant differences between the
611 two conditions ($p \leq 0.05$).

612

613 **Figure 2.** PAMPA effective permeability values, P_e (cm/s), of the compound dioscin under PBS 5% DMSO
614 conditions with and without the addition of a lipid mixture (A), and diosgenin under PBS 5% DMSO
615 conditions, under a previous gastrointestinal digestion and under a previous gastrointestinal digestion with
616 the addition of a lipid mixture (B). (*) Denote significant differences between the assays ($p \leq 0.05$).

617 Table 1. Saponin, sapogenin and lipid profile[1] (g/100 g of extract) of the different extracts from fenugreek and quinoa.

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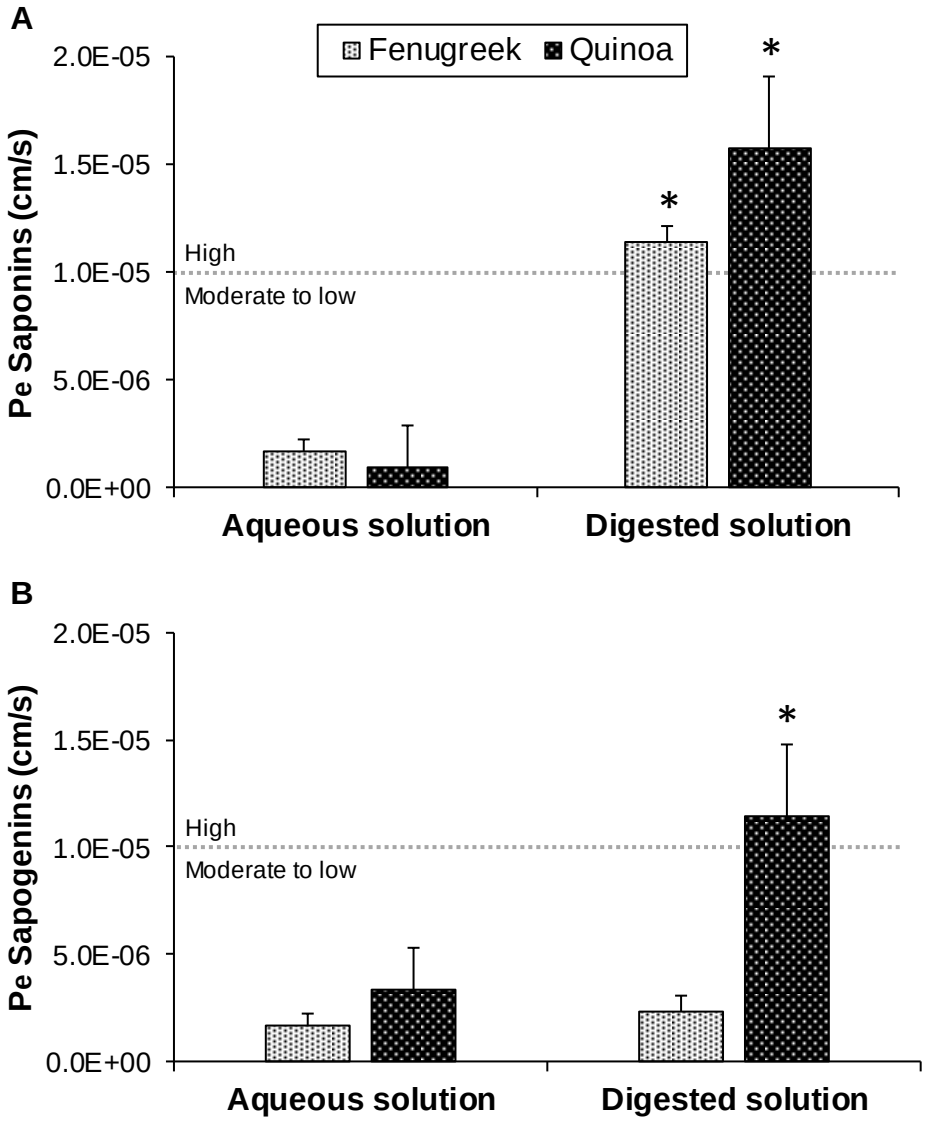
	Saponin-rich extracts		Sapogenin-rich extracts	
Compound	Fenugreek	Quinoa	Fenugreek	Quinoa
Total Saponins	31.1	7.9	2	2
Total Sapogenins	nd	nd	6.9	5.6
FFAs	2.6	23.6	35.9	61.9
MAGs	0.4	2.2	1.0	4.1
DAGs	nd	18.0	6.1	18.4
TAGs*	5.5	9.5	nq	nq

*Calculated as the difference between total lipids and the sum of FFA+MAG+DAG

FFA = Free Fatty Acid; MAG = Monoglycerides; DAG = Diglycerides; TAG = Triglycerides; nd = not detected; nq = not quantified

[1]The concentration of each of the constituents is referred to the quantitation from a single extraction

621 **Figure 1.**



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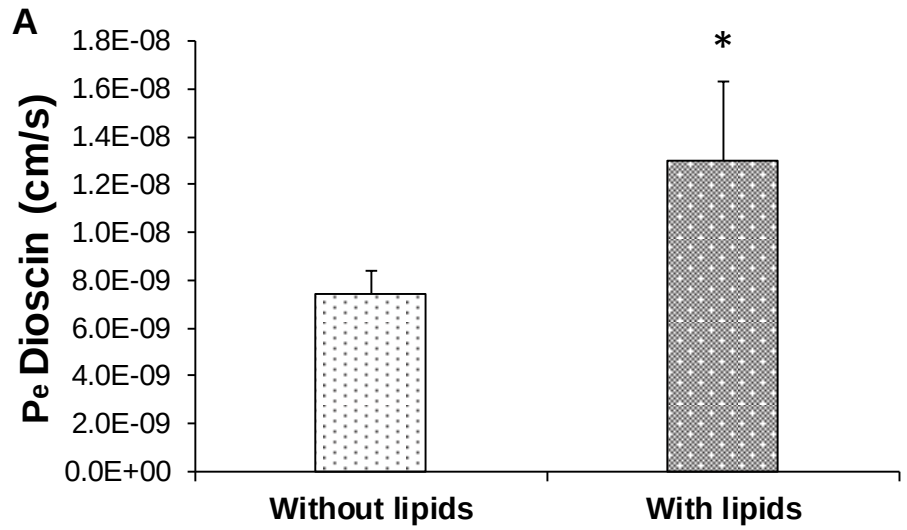
625 **Figure 2.**



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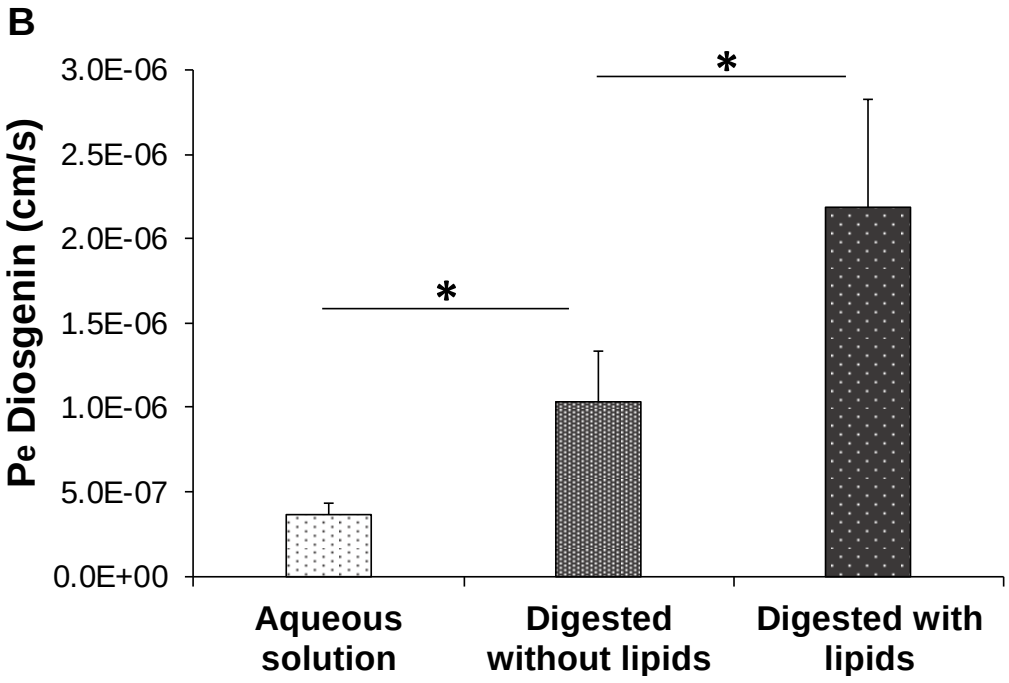

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629 **GRAPHIC FOR TABLE OF CONTENTS**

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