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A versatile, stability-indicating and high-throughput ultra-fast liquid chromatography method for the determination of isoflavone aglycones in soybeans, topical formulations, and permeation assays

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ABSTRACT

There is a growing interest in the pharmaceutical field concerning isoflavones topical delivery systems, especially with regard to their skin care properties and antiherpetic activity. In this context, the present work describes an ultra-fast liquid chromatography method (UFLC) for determining daidzein, glycitein, and genistein in different matrices during the development of topical systems containing isoflavone aglycones (IA) obtained from soybeans. The method showed to be specific, precise, accurate, and linear (0.1 to 5 $\mu g\,mL^{-1}$) for IA determination in soybean acid extract, IA-rich fraction obtained after the purification process, IA loaded-nanoemulsions, and topical hydrogel, as well as for permeation/retention assays in porcine skin and porcine esophageal mucosa. The matrix effect was determined for all complex matrices, demonstrating low effect during the analysis. The stability indicating UFLC method was verified by submitting IA to acidic, alkaline, oxidative, and thermal stress conditions, and no interference of degradation products was detected during analysis. Mass spectrometry was performed to show the main compounds produced after acid hydrolysis of soybeans, as well as suggest the main degradation products formed after stress conditions. Besides the IA, hydroxymethylfurfural and ethoxymethylfurfural were produced and identified after acid hydrolysis of the soybean extract and well separated by the UFLC method. The method's robustness was confirmed using the Plackett-Burman experimental design. Therefore, the new method affords fast IA analysis during routine processes, extract purification, products development, and bioanalytical assays.

1. Introduction

The use of soybeans is of interest from scientific and economic points of view, basically due to the presence of isoflavones [1]. These compounds have demonstrated beneficial health effects, especially for preventing heart disease, osteoporosis, cancer, and diabetes [2], plus reducing climacteric symptoms in menopausal and postmenopausal women [3]. Moreover, isoflavones act against acute virus infections [4], and have many beneficial effects on the skin [5,6], demonstrating a growing interest on their topical use in the pharmaceutical and cosmetics field.

In general, isoflavones are incorporated into topical products in their glycoside forms [7]. In some cases, this may limit the biological action of these conjugated forms due to the low possibility of penetration through different skin layers or mucosa tissues [7]. Therefore, to get the isoflavones' desired effects, their aglycone forms such as daidzein, glycitein, and genistein, should preferably be used [7,8]. However, it is important to highlight that the isoflavone aglycones (IA) are less hydrosoluble than the conjugated forms, and this may limit their incorporation into traditional topical delivery systems [7]. To allow better use of IA in formulations and enhance their penetration, some alternative technologies have been studied, such as nanoemulsions [9-12].

During products development, routine analysis, or biological assays, it is essential to use an adequate method for quantification of the compounds of interest. In this way, a number of analytical methods has been reported for isoflavones analysis [13,14].

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Nevertheless, high-pressure liquid chromatography (HPLC) with ultraviolet-visible (UV–vis) detector is, undoubtedly, the method of choice applied in this field [13,14]. Although the HPLC methods have some advantages when it comes to analyzing isoflavones in terms of specificity, sensitivity, and straightforward operation, they require a relatively long period of time, normally from 20 min [15] to 65 min [16]. To overcome this lengthy amount of time, the use of high-throughput liquid chromatography technologies has been reported in the last decade for isoflavone analysis, reducing the chromatographic time to less than 10 min [17–21]. These techniques allow the use of short columns, packed with <3 μm particles, supporting elevated pressures, thus reducing the analysis time, solvent consumption, and, consequently, the environmental impact [22].

Among the high-throughput methods reported in the literature for isoflavones analysis, ultra-performance liquid chromatography (UPLC) and ultra-fast liquid chromatography (UFLC) are cited for their determination in soybeans cultivars [21], phytoestrogen-rich plants [17,23,24], soy bits [17], soymilk [25], texturized soy protein [20], and soy-based nutritional supplements [26]. However, none of them have reported the use of UFLC or UPLC for pharmaceutical or cosmetic formulation containing isoflavones and for permeation/retention studies of these compounds on skin or mucosa tissues.

Recently, our research group reported a stability-indicating HPLC method for routine analysis of IA present in soybeans after acid hydrolysis, as well as in nanoemulsions containing the extract [27]. The method has been reported as a new approach for isoflavones analysis, since it can be used to indicate the stability of these compounds in different stress conditions, but it has one big drawback: the total analysis time is quite long (30 min) as compared with the new scenario of chromatographic methods [17,20–26]. Therefore, the development of a fast and reliable method to analyze IA in complex biological matrices, even in the presence of their degradation products, becomes a new challenge in the current chromatographic systems for isoflavones analysis.

In this context, the aim of this work was the development and validation of a stability-indicating UFLC method for the determination of daidzein (DAID), glycitein (GLY), and genistein (GEN) applied to different assays, such as routine analysis of soybeans, monitoring the purification process, development of nanoemulsions and hydrogels containing IA, and their permeation/retention assay in porcine esophageal mucosa and porcine skin. The matrix effect for all of these applications was assessed to demonstrate the versatility and reliability of the UFLC method for IA analysis. Mass spectrometry analysis was performed to show the main products formed after acid hydrolysis of the soybeans, demonstrates the method specificity, and suggests the main degradation products formed after stress conditions. In addition, the most complex matrix used in this study (soybean acid extract) was also analyzed by a previously validated HPLC method to show the advantages in time and resolution of the new method being proposed.

2. Material and methods

2.1. Chemicals and materials

Isoflavone standards, daidzein, glycitein, and genistein ($\geq 95\%$, $\geq 98\%$, and $\geq 98\%$ of purity, respectively) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Methanol and acetonitrile liquid chromatography grade were obtained from Merck (Darmstadt, Germany). Egg-lecithin (Lipoid E-80®) and medium chain triglycerides (MCT) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). The soybeans (cultivar BRS 262) were obtained from *Empresa Brasileira de Pesquisa Agropecuária* (EMBRAPA), kindly donated by SEMEL seeds (São Paulo, Brazil). Porcine ears and porcine esophageal mucosa were obtained from a local slaughterhouse.

2.2. Apparatus and analytical conditions

2.2.1. UFLC analysis

The UFLC analysis was performed on a Shimadzu Prominence system device coupled to a photodiode array (PDA) detection and an automatic injector controlled by LC-Solution Multi-PDA software (Kyoto, Japan). The stationary phase was a Shim-pack XR ODS column (Shimadzu, 100×2.0 mm i.d.; particle size, 2.2 μm) guarded by an in-line pre-column filter Ultra KrudKatcher (Phenomenex, USA). The mobile phase consisted of (A) trifluoroacetic acid 0.1% (v/v) and (B) acetonitrile. The gradient elution was 20–25% B (0–2 min), 25–30% B (2–3 min), 30–35% B (3–4 min), and 35-20% B (4–6 min). The column was re-equilibrated with 20% B for 2 min before the next analysis. The flow rate was a gradient of 0.35 mL min^{-1} (0–1.5 min), 0.35–0.5 mL min^{-1} (1.5–2 min), 0.5 mL min^{-1} (2–3 min), 0.5-0.35 mL min^{-1} (3-4 min), and 0.35 mL min^{-1} up to 8 min. The wavelength was adjusted to 260 nm, injection volume of 3 μL, and the analysis was carried out at 55 °C.

2.2.2. HPLC analysis

The HPLC analysis was carried out as described by Yatsu et al. [27], and was performed on a Shimadzu LC-20AT system (Kyoto, Japan), coupled to PDA detector controlled by LC-Solution Multi-PDA software. The stationary phase was a Synergi-Fusion-RP column (Phenomenex, 150×4.6 mm i.d.; particle size, 4.0 μm), protected with a C-18 guard column. The mobile phase consisted of (A) acidified Milli-Q water (0.01% trifluoroacetic acid) and (B) acidified acetonitrile (0.1% trifluoroacetic acid). The gradient elution was: 20–25% B (0–10 min), 25–30% B (10–15 min), 30–35% B (15–23 min), 35% B (23–26 min), and 35-20% B (26–30 min), maintained at a flow rate of 1.0 mL min^{-1}. The wavelength was adjusted to 260 nm, injection volume of 10 μL, and the analysis was carried out at 40 °C.

2.2.3. Mass spectrometry (MS)

The MS analysis was performed on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometer (Waters, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Waters, MA, USA). The chromatography parameters were similar to those of the UFLC analysis; nevertheless TFA was changed for acid formic. The ESI conditions were: capillary voltage 3000 V, sample cone 30 V, source temperature 120 °C, desolvation temperature of 300 °C, cone gas flow 70 L.h^{-1} and desolvation gas flow of 350 L/h. Detection was performed in positive ion mode (ESI+) in the m/z range 50–300. Software used to control spectrometer, data acquisition and data processing was MassLynx (v 4.1). The molecular ions $[M+H]^+$ monitored during the analysis were m/z 255, 285, and 271, correspondingly of DAID, GLY, and GEN, respectively. The criteria for fragments selection were intensity and specificity being selected three fragments for compound in accordance with Wu et al. [28].

2.3. Solutions

2.3.1. Stock and reference solutions

A stock solution (30 μg mL^{-1}) of DAID, GLY, and GEN was prepared in methanol by weighing approximately 3.0 mg of the compounds into a 100 mL calibrated volumetric flask and diluting

to volume. The reference solutions were prepared by the stock solution dilution with acetonitrile 50% (v/v).

2.3.2. Matrices solutions

2.3.2.1. Soybean acid extract (SAE). Soybeans were previously grinded and defatted by exhaustive extraction with *n*-hexane in a Soxhlet apparatus. The extraction of isoflavones from defatted soybean material was performed in a Soxhlet using 80% ethanol at the temperature of 70–80 °C for 4 h (1:10, w/v). Afterward, 1.3 M hydrochloric acid (HCl) was added and the mixture was heated at temperature of 80 °C for 2 h under reflux. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 μm and analyzed.

2.3.2.2. Isoflavone aglycones-rich fraction. To obtain the isoflavone aglycones-rich fraction (IAF), the soybean acid extract was evaporated under reduced pressure to remove ethanol, leaving only an aqueous phase. Liquid-liquid partition with salting-out procedure was performed adding 20% (w/v) potassium chloride into the aqueous phase. The mixture was partitioned with ethyl acetate and washed with water three times to neutralize the partitioned fraction, that was subsequently evaporated under reduced pressure, and subjected to silica gel column. The ethyl acetate fraction was eluted successively with a gradient system with *n*-hexane: methylene chloride: ethyl acetate of increasing polarity. Six new fractions were collected, chloroform was added on fraction 4 and the isoflavone aglycones precipitated. The residue was filtrated, dried, and resuspended in methanol. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 μm and analyzed.

2.3.2.3. Nanoemulsions and hydrogels. Blank nanoemulsions (NE_B) or IAF loaded-nanoemulsions (NE_{IAF}) were composed with absence or presence of 0.1% (w/w) of IAF. The other compounds of formulations were medium chain triglycerides 8.0% (w/w), egg lecithin 2.0% (w/w), polysorbate 80 1.0% (w/w) and water up to 100%. The formulations were obtained by means of spontaneous emulsification procedure as firstly described by Yatsu et al. [27]. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 μm, and analyzed.

The hydrogels containing NE_B or NE_{IAF} were prepared incorporating the nanoemulsions into carbomer-hydrogel at a final concentration of 0.5% [11]. For this, Carbopol 940® was dispersed in the nanoemulsion and the mixture was stirred at room temperature and neutralized with triethanolamine to give a hydrogel with a pH of approximately 7.0. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 μm, and analyzed.

2.3.2.4. Porcine skin layers and esophageal mucosa. Full thickness skin was excised from the outer region of the porcine ear. After the removal of subcutaneous fat, the skin was cut into circle pieces, and the separation of stratum corneum was made by the tape stripping process. The first stripped tape was discarded, while the following 14 tapes were placed in test tubes and used for the stratum corneum analysis. The remaining layer (epidermis/dermis) was reduced to tiny pieces and placed in different test tubes. To extract the IA from skin layers, 5 mL of methanol were added and the samples were maintained in an ultrasound bath for 30 min, as previously reported by Vargas et al. [12].

The porcine esophageal mucosa was separated from the muscular layer by cutting the loose connective fibers with a scalpel. The remaining mucosa was then perforated into tiny pieces, and placed in test tubes. For extraction procedure, 5 mL of methanol were added on the tubes and the samples were maintained in an ultrasound bath for 30 min. An adequate aliquot was filtered by membrane of 0.22 μm and analyzed.

2.3.2.5. Receptor fluid for permeation studies. The chosen solution to be the receptor fluid for the permeation/retentions studies was 30% ethanol in phosphate buffer pH 7.4, as reported by Vargas et al. [12]. An adequate aliquot was filtered by membrane of 0.22 μm and analyzed.

2.4. Validation of UFLC method

The developed method was validated according to the official guidelines. For pharmaceutical products (nanoemulsions and hydrogel) it was used the ICH specifications [29], while for biotechnological products (derivative from soybeans) and biological matrices (skin layers and mucosa tissue) it was used the FDA and EMEA recommendations [30,31]. The results were analyzed by Student's t test and analysis of variance (ANOVA) using a significance level of $\alpha = 0.05$.

2.4.1. Assessment of the matrix effect

The analyses of matrix effects (ME) were performed as reported by Watanabe et al. [32] and Yatsu et al. [27]. The slopes obtained in standard curves of IA standards diluted in the mobile phase were compared with the slopes obtained in standard curves of IA-spiked in each matrix. The studied matrices were SAE, IAF, NE_B, H-NE_B, porcine epidermis/dermis skin layers (P.E/D), porcine stratum corneum layer after *tape stripping* method (P.SC), porcine esophageal mucosa (P.EM) and receptor fluid (RF). Three standard curves were obtained, in three consecutive days, by plotting the peak area versus the concentration of the isoflavone aglycone standards (0.1, 1.0, 2.0, 4.0, 5.0 $\mu g\,mL^{-1}$) in acetonitrile 50% (v/v) and in the matrices solutions. Five replicates were analyzed for each concentration level.

The matrix effect was calculated based on the following equation: $ME\% = 100 \times [1 - (S_m/S_s)]$, where S_m = slopes of the standard curves of the isoflavone standards in the mobile phase and S_s = slopes of the standard curves of the isoflavone standards in the matrix.

2.4.2. Specificity

The interference of the matrix composition was determined by the injection of samples containing only the matrices, and samples containing matrices spiked with DAID, GLY and GEN at the concentrations of 2 $\mu g\,mL^{-1}$ each one. The IA present in the different matrices were identified based on their UV spectra between 200 and 400 nm, their electrospray ionization (ESI+) mass spectra, and their retention times, with respect to the reference materials. The stock solutions of standards were also submitted to a forced degradation as reported by Yatsu et al. [27]. Hydrochloric acid (HCl) was added to the standard solutions to achieve the final concentration of 0.5 M to verify the acid hydrolysis. Sodium hydroxide (NaOH) was added to the standard solutions to achieve the final concentration of 0.5 M to verify the basic hydrolysis. After a pre-determined period of time, both stress solutions were neutralized with base and acid, respectively. The oxidative degradation was induced by storing the sample solutions in 30% hydrogen peroxide (H_2O_2). All these solutions were kept in 25 °C and 60 °C and protected from light to prevent the interference of photolytic degradation. After pre-determined times, the samples were diluted in mobile phase and analyzed using a PDA detector determining the peak purity of isoflavones and MS analysis was also performed for the degradation peaks obtained.

2.4.3. Linearity, precision, and accuracy

The linearity of the method for each matrix was evaluated by regression analysis using the least square method. Three standard curves were obtained, in three consecutive days, by plotting the peak area *versus* the concentration of the isoflavone aglycones standards (0.1, 2.0, 4.0, 6.0, 8.0, and 10.0 μg mL^{-1}) in acetonitrile 50% (v/v) and (0.1, 1.0, 2.0, 3.0, 4.0, and 5.0 μg mL^{-1}) in matrices. Each concentration level was analyzed in six replicates. The limit of detection (LOD) and the limit of quantitation (LOQ) of the method were calculated based on the standard deviation of the intercept and on the slope of the standard curves.

The intra-day precision (repeatability) was determined by analyzing IA at three levels (0.1, 2.0, 4.0 μg mL^{-1}) in the presence of matrices, and with six determinations per concentration, during the same day under the same experimental conditions. Inter-day precision (intermediate precision) values were obtained by assaying IA samples of the same concentration levels on three different days. The standard deviation and the relative standard deviation (RSD) were calculated for each level.

The accuracy was evaluated by adding known amounts of IA standards at three different concentration levels (0.1, 2.0, 4.0 μg mL^{-1}) to the post extraction matrices. At each level, samples were prepared in six replicates and analyzed. The accuracy was evaluated as the standardized correlation between the measured value and the theoretical value, as follows: RE%=[(mean calculated concentration – theoretical value)/theoretical value] x 100.

2.4.4. Robustness

The robustness in each matrix was investigated by the Plackett-Burman design. The factors, analyzed in low levels (−1) and high levels (+1), were: column oven temperature (53; 57 °C), initial organic composition (19; 21%), initial flow rate (0.34; 0.36 mL min^{-1}), and TFA concentration (0.08; 0.12%). The four factors selected were tested with eight experiments designed in accordance with Heyden et al. [33]. The responses evaluated were the percentages of IA in the matrices obtained in relation to the standard solutions. After the calculation of the effects for each parameter (by the sums of the responses of the positive and negative levels), the statistical interpretation (*t*-test) allowed determination of the similarity or difference of the results. The DAID, GLY, and GEN standards and the samples were analyzed under identical experimental conditions, and for this reason no additional experiments were necessary. A half-normal probability plot for the effects in combination with the dummy factors was used to estimate the error and identify significant effects.

2.4.5. Recovery of IA after extraction from porcine skin and mucosa

Before the extraction procedure of porcine skin layers or mucosa, the matrices were spiked with isoflavone aglycones leading a theoretical concentration of 0.1, 2, and 4 μg mL^{-1}. Methanol was added in each matrix test tube, and the samples were maintained in an ultrasound bath for 30 min, filtered through a 0.22 μm membrane and analyzed by UFLC.

2.4.6. IA stability in matrices

The stability of IA-spiked matrices was determined after 48 h of storage at ambient temperature. The stability of these solutions was studied by performing the analysis and observing any change in the chromatographic pattern, compared with freshly prepared solutions.

2.4.7. System suitability

The system suitability test was also carried out to evaluate the adequacy of the system for the analysis. The parameters measured were peak area, retention time, theoretical plates, tailing factor, and resolution between DAID, GLY, and GEN.

2.5. Method application

The determination of DAID, GLY, and GEN in SAE and IAF were performed as described in sections 2.3.2.1 and 2.3.2.2, respectively. The IA determination in NE_{IAF} and H-NE_{IAF} was carried out as described in section 2.3.2.3.

The permeation/retention studies for IA were evaluated using Franz type diffusion cells, which presented a surface area for diffusion of 1.77 cm^2 and a receptor volume of 10.0 mL. The excised circular porcine ear skin and esophageal mucosa, prepared as described in section 2.3.2.4, were previously dipped in PBS pH 7.4 solution during 30 min. Then, they were mounted in a Franz-type diffusion cell between the donor and receptor compartments, with the inner part facing the upper inside portion of the cell. The bathing solution was kept under a controlled temperature (32 ± 1.0 °C) and stirred at 650 rpm. About 400 μL of NE_{IAF} were placed in the donor compartment, maintaining the sink conditions for the assay. At the end of experiment (8 h), an aliquot of RF was withdrawn for analysis and the skin or mucosa was removed from the cell and cleaned using a cotton swab. Next, the skin layers and mucosa were treated as described in section 2.3.2.4 to extract the IA, and analyzed by the UFLC method.

3. Results and discussion

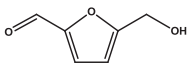
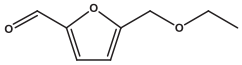
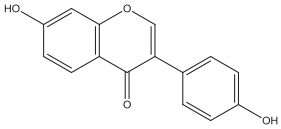
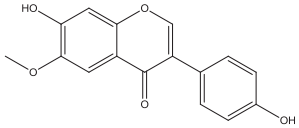
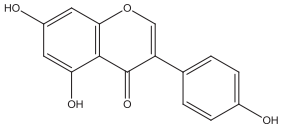
3.1. UFLC method development and advantages

For optimization studies of pharmaceutical formulations and permeation assays through skin or mucosa, numerous samples must be analyzed during the tests. Consequently, it is often necessary to develop an easy, fast, and reliable method to quantify the compounds of interest.

The UFLC was chosen in this study to achieve IA separation with good resolution in a short time. The choice of an appropriate column and other factors were evaluated considering the main chromatographic parameters obtained for the major compounds present in the SAE, since this sample was the most complex matrix used in the present work (Table 1). The greatest IA separation, with high resolution between all peaks, was achieved with a C18 column packed with 2.2 μm particle size. To allow a rapid and satisfactory separation of the main products, the use of solvent gradient, high temperature, and gradient of flow rates was needed. As previously reported by our research group, higher temperatures is a useful tool for reducing analysis time since mobile phase viscosity is significantly reduced and this, in turn, decreases the pressure and peak width [27]. The most effective separation occurred with a gradient of 0.1% (v/v) trifluoroacetic acid in water and acetonitrile. UV detection was at 260 nm, since all IA have good absorption at this wavelength.

The soybean extract was submitted to acid hydrolysis because isoflavone glycosides are the predominant forms in soybeans, but the activities and higher skin permeation are mainly credited to the aglycone forms [34,7]. However, when soy derivative products such as foods or extracts are submitted to acid conditions, other compounds could be obtained. For instance, the presence of sugars under extreme pH conditions and high temperatures usually induce the Maillard reaction, or caramelization process. Both phenomena can result in the production of furanic aldehydes, such as hydroxymethylfurfural (HMF) and furfural [35]. Recently, our research group had isolated HMF and ethoxymethylfurfural (EMF) from soybeans after acid hydrolysis in an ethanolic media [36]. These compounds are reported as toxic [37,38], and for this

Table 1
Chemical structure for the main products obtained after acid hydrolysis of soybeans, as well as their UFLC retention times, UFLC peak parameters, maximum UV absorption (λ max), and molecular ion $[M+H]^+$ acquired by MS analysis.

Compound	Chemical structure	Retention time	Theoretical plates	Tailing factor	Resolution	λ ($_{max}$)	$[M+1]^+$
HMF		0.92	10046.42	2.03	-	229/282	127
EMF		2.87	14104.95	1.38	17.86	229/281	155
Daidzein		3.63	21105.32	1.27	8.93	248/301	255
Glycitein		4.07	32900.98	1.14	4.98	256/319	285
Genistein		5.05	63837.17	1.13	11.5	260	271

HMF: hydroxymethylfurfural; EMF: ethoxymethylfurfural.

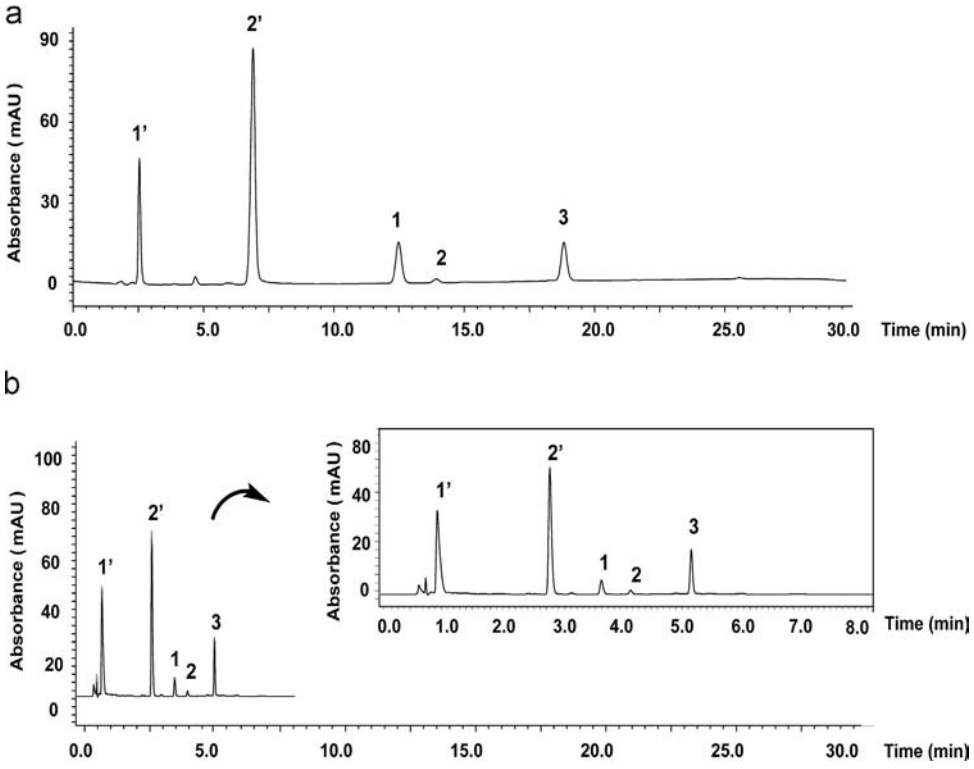


Fig. 1. Representative chromatographic profiles of soybean acid extract obtained by (a) HPLC and (b) UFLC methods at 260 nm. Where (1′) HMF, (2′) EMF, (1) DAID, (2) GLY, and (3) GEN.

reason, the purification of acid extracts becomes essential before they can be used in pharmaceutical products. Consequently, the development of an analytical method that enables IA quantification and the detection of HMF and EMF comes to be necessary to make sure these toxic compounds are removed from the extract. In this way, the UFLC method proved to be capable of separating the toxic compounds from the IA and is, therefore, suitable for the quality control of the purification processes.

In addition, to demonstrate the advantage of time-related analysis and reduced solvent consumption, the SAE was analyzed by the UFLC method and an HPLC method recently reported by our research group [27]. The representative chromatographic separations for both methods are shown in Fig. 1. As can be observed, the UFLC method greatly shortens the analysis time by up to three times that of the HPLC method, while maintaining the resolution between all peaks. Moreover, it is important to highlight that the

environmental impact and cost are minimized, since the UFLC flow rate was 0.5 mL min^{-1}, half that of the HPLC value.

Comparing the UFLC method described herein with other ultra-fast methods reported in the literature, it is possible to observe that the total run time is a little longer than the methods reported by Kiss et al. [24] and Fiechter et al. [21], which showed the IA analysis in soybeans and phytoestrogen-rich plants with chromatographic time of under 5 min. However, it is important to note that the separation of HMF, EMF, DAID, GLY, and GEN with high resolution was herein achieved, even with the difference in polarity between the furfural compounds and isoflavones.

3.2. UFLC method validation

3.2.1. Assessment of the matrix effect

Method validation is the process by which the compounds of interest are reliably quantified independently of the matrix [31]. For this reason, during the development of an analytical and bioanalytical method, it is essential to consider the effect of all matrices involved, which can be easily detected by comparing the response obtained from a standard solution with that from a spiked pre-treated sample [27]. When the response range is between -20% < ME% < 20%, the matrix effects is deemed low; when it is between −50% < ME% < -20% or 20% > ME% > 50%, it is considered medium, and when it is between ME% < −50% or ME% > 50%, it is considered high [39].

The matrix effects for each IA present in the SAE, IAF, NE_{IAF}, H- NE_{IAF}, P. E/D skin layer, P. SC layer after tape stripping process, RF and P. EM, were expressed by ME% and are presented in Table 2. The data indicate that samples exhibited low matrix effects (ME% < 12.42) for isoflavones determination, when analyzed according to Niessen et al. [39]. Furthermore, when IA spiked-matrices were analyzed by UPLC-MS/MS, no interfering signals (m/z) were found in the same retention time of DAID, GLY, and GEN. Therefore, the proposed method dispenses with the need for clean-up pre-treatment of the samples and is classified as a fast and simple method for analyzing IA in complex matrices.

3.2.2. Specificity

The chromatographic separation of IA standards at 260 nm is shown in Fig. 2A. The retention times for DAID, GLY, and GEN peaks were 3.60, 4.07, and 5.05 min, with characteristic λ_{max} (maximum UV absorption) at 248/301, 256/319 and 260 nm (Fig. 2B), and MS/MS spectra with characteristic molecular $[M+H]^+$ ions *m/z* 255 for DAI, *m/z* 285 for GLY, and *m/z* 271 for GEN (Fig. 2C). The main fragmentation ions were in accordance with those reported by Wu et al. [28], in which the characteristic retro-Diels-Alder fragments m/z 137 for DAID, m/z 167 for GLY, and m/z 153 for GEN were significantly present.

The UFLC method specificity was assessed by injecting blank matrices and evaluating the peak purity of each IA spiked-matrix. No matrix-related interference was found, showing that the IA peaks were free from any co-eluting substance and demonstrating that the proposed method is specific for the simultaneous analysis of IA in all of the assessed matrices (Fig. 3A).

Moreover, forced degradations were done to provide stability-indicating properties. A stability-indicating method is defined as an analytical method that accurately quantifies the compounds of interest without interference from degradation products. In the present work, the IA standards were submitted to acidic, alkaline, neutral, oxidative, and thermal stress conditions. No significant changes were observed in the isoflavones after stress exposure in acid, oxidative or neutral conditions at 25 °C and 60 °C during 6 h of exposure. On the other hand, when the IA were submitted to the

Table 2
Linearity data of the isoflavone aglycone standards and the matrix effect for each matrix studied.

Matrix	Compound	Equation	R^2	LOD	LOQ	ME (%)
				μg mL^{-1}		
Standards	Daid	y=19866x-337.73	0.999	0.10	0.35	–
	Gly	y= 21752x+142.0	0.999	0.09	0.32	–
	Gen	y=36619x+355.32	0.999	0.10	0.32	
SAE	Daid	y= 20071x-325.95	0.998	0.08	0.28	1.02
	Gly	y= 22006x-624.98	0.998	0.09	0.32	1.15
	Gen	y= 37002x-298.39	0.998	0.08	0.28	1.04
IAF	Daid	y= 19486x+2760.6	0.993	0.17	0.57	−1.95
	Gly	y= 21953x+104.11	0.988	0.21	0.71	0.92
	Gen	y= 36988x+3127.8	0.996	0.12	0.38	1.00
	Daid	y= 19389x-195.21	0.993	0.17	0.58	2.40
NE_B	Gly	y= 21567x-119.18	0.995	0.13	0.45	0.85
	Gen	y= 35753x+1182.1	0.994	0.15	0.50	2.36
H-NE_B	Daid	y= 19722x-106.42	0.998	0.08	0.27	−0.73
	Gly	y= 21986x-177.26	0.998	0.08	0.26	1.06
	Gen	y= 36700x+181.32	0.998	0.08	0.28	0.22
P. E/D	Daid	y= 20003x-312.91	0.998	0.09	0.31	0.68
	Gly	y= 22079x+54.060	0.998	0.08	0.26	1.48
	Gen	y= 36669x+576.41	0.998	0.10	0.32	0.14
P. SC	Daid	y= 19709x-1007.9	0.995	0.14	0.48	-0.80
	Gly	y= 21605-640.460	0.995	0.13	0.44	-0.68
	Gen	y= 37172x-2245.4	0.998	0.09	0.32	1.49
P. EM	Daid	y= 19722x-24.812	0.998	0.10	0.33	0.72
	Gly	y= 21886x+144.67	0.997	0.10	0.33	-0.62
	Gen	y= 36757+939.10	0.997	0.10	0.33	-0.38
RF	Daid	y= 22682x-489.67	0.998	0.08	0.28	12.42
	Gly	y= 23718x+44.349	0.998	0.09	0.30	8.29
	Gen	y= 39345x-988.07	0.999	0.09	0.39	6.93

SAE: soybean acid extract; IAF: isoflavone aglycone-rich fraction; NE_B: blank nanoemulsion; H-NE_B: hydrogel containing NE_B; P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after *tape stripping* process; P. EM: porcine esophageal mucosa; RF: receptor fluid; R^2=determination coefficient; LOD=limit of detection; LOQ=limit of quantitation; ME: matrix effect.

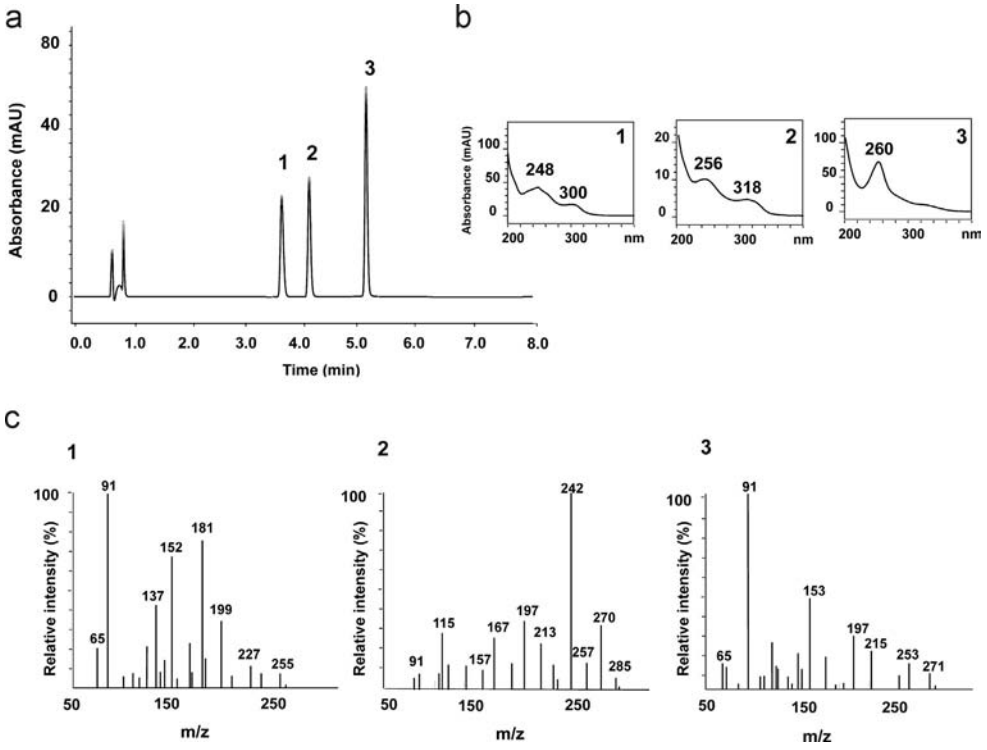


Fig. 2. Analysis of isoflavones (1) DAID, (2) GLY, and (3) GEN by (a) UFLC method, (b) UV–vis absorption spectra; (c) MS/MS spectra measured in positive mode.

alkaline condition at 60 °C for 6 h (Fig. 3B), DAID degraded more than 50%, GEN more than 20%, and GLY more than 15%.

However, only the degradation products of DAID and GEN could be observed in the developed UFLC method. To enhance the knowledge about the possible degraded products and confirm the purity of the isoflavones, the peaks were analyzed by PDA and MS detector following the stress process. The degradation products D1, D2, D3, D4, D5, and D6 had retention times of 1.80, 4.33, 4.63, 4.75, 5.29, and 5.75 min, respectively. In addition, they were well separated from the other ones in the chromatographic system, resulting in a good resolution (>1.4 between all peaks). Evaluation of DAID, GLY, and GEN purity after the stress condition demonstrated that these peaks were free from any co-eluting substance. The maximum UV spectra of the main degraded peaks showed λmax: 255 nm for D1, λmax: 236/289 nm for D2, λmax: 230/277 nm for D3, λmax: 240/283 nm for D4, λmax: 260/290/322 nm for D5, and λmax: 260/318 nm for D6. Furthermore, the MS spectra of three out of six degradation products were compared with the literature data, and the results showed that D1, D2, and D3 have the same precursor ions $[M+H]^+$, m/z 255, 259, and 273, than the degradation products reported by Yatsu et al. [27]. However, more studies are needed on the other three degradation products to propose their identities.

3.2.3. Linearity, precision, and accuracy

The results for linearity are shown in Table 2. Analysis of the determination coefficients demonstrated that the method is linear for all of the standard compounds within the tested range. The confidence interval observed in the *t*-test on the intercepts and the graphic examination of the residuals also demonstrated the absence of constant systematic errors (data not shown).

The LOD and LOQ calculated by standard curves are also presented in Table 2. The lower LOQ (LLOQ) measurement is a great concern in the validation methods, which are used to quantify low concentrations of drugs in biological matrices. The LLOQ was fixed at 0.1 μg mL^{-1} for all isoflavones in all assessed matrices, since it was the lowest IA concentration that could be determined with acceptable precision and accuracy (RSD < 15%).

The precision and accuracy of post-extraction spiked-matrices were evaluated by analyzing the IA at concentrations of 0.1 μg mL^{-1} (lowest concentration), 2.0 μg mL^{-1} (medium concentration), and 4.0 μg mL^{-1} (highest concentration) for each sample. The intra-day precision data for IA showed a relative standard deviation (RSD) value between 0.04–3.96 for the analytical assays (NE, H-NE, RF), between 0.02–8.40 for the bioanalytical assays (P.SC, P. E/D, P. EM), and between 0.09–6.21 for the biotechnological products (SEA, IAF). The inter-day precision data for IA showed a RSD value between 0.17–5.12 for the analytical assays, between 0.66–8.00 for the bioanalytical assays, and between 0.96–10.70 for the biotechnological products. The accuracy results for IA in all matrices were within the 90.37% to 104.81% range. Despite the complexity of the different matrices, the UFLC method can be considered precise and accurate according to official guidelines.

3.2.4. Robustness

A model's robustness refers to its ability to remain unaffected by small, deliberate variations in the analysis conditions [33]. A multivariate approach using design of experiments is often recommended in robustness testing since a number of different factors can be analyzed concurrently with a reduced number of experiments [40].

The responses after assessing the Plackett-Burman design are the percentage of DAID, GLY, and GEN in the samples in relation to the standard solutions in each experiment. As shown in Fig. 4, no significant factors were revealed for all analyses as the calculated *t*-values were lower than the *t*-critical values ($\alpha=0.05$). Thus, there were no significant changes in the assay results in terms of the percentage of IA contents with the changes made in the experimental conditions, thereby demonstrating the proposed method's robustness.

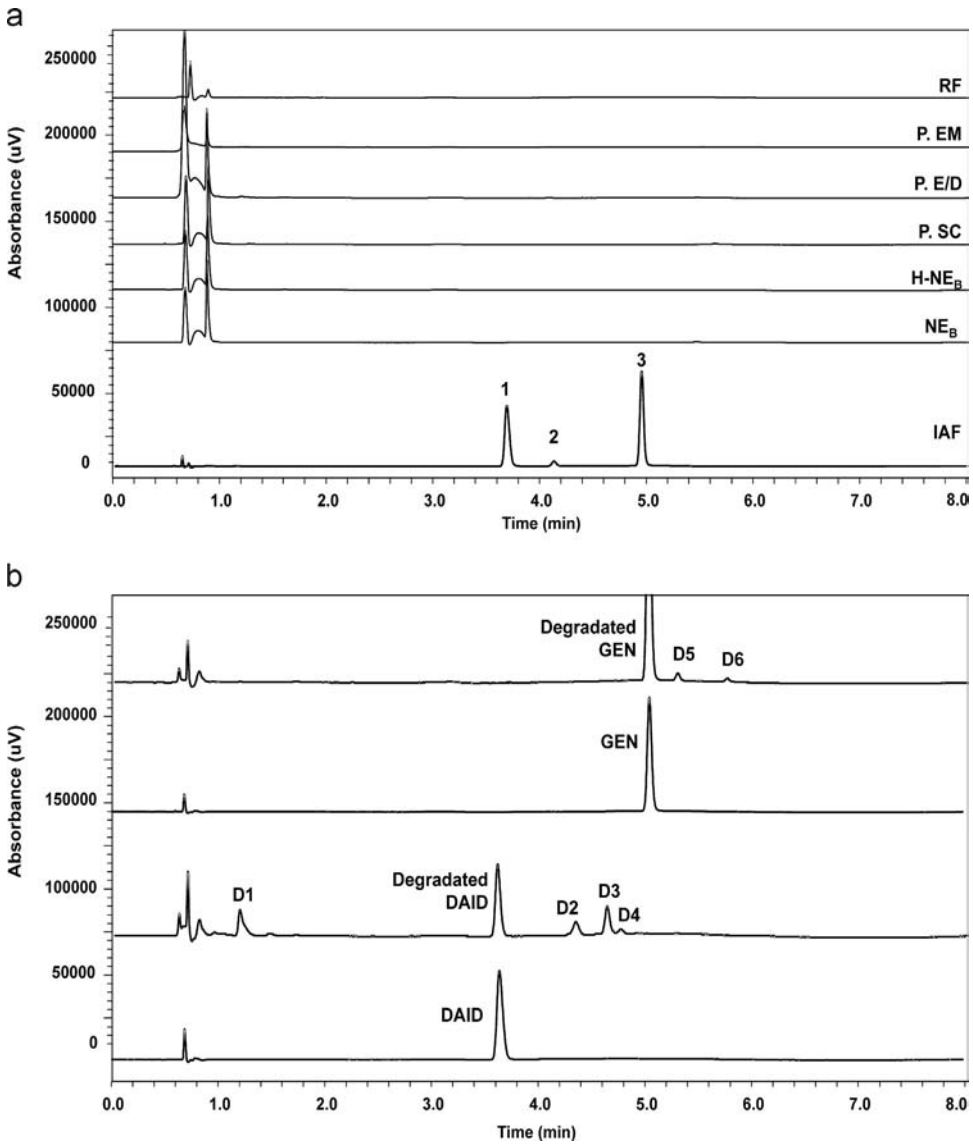


Fig. 3. Representative chromatographic profiles obtained in the specificity assay for the (a) different matrices, where, IAF: isoflavone aglycone-rich fraction, 1: DAID, 2: GLY, 3: GEN, NE_B: blank nanoemulsions, H-NE_B: topical hydrogel containing nanoemulsions, P. SC: porcine stratum corneum after tape stripping method, P. E/D: porcine epidermis/dermis skin layer, P. EM: porcine esophageal mucosa, RF: receptor fluid; and for the (b) degradation products of DAID (D1, D2, D3, D4), and GEN (D5, D6) obtained after alkaline hydrolysis of separated standard solutions of DAID and GEN.

3.2.5. *IA stability in matrices*

The stability evaluation results showed that the concentration of isoflavones in the different matrices remained constant after 48 h of storage at room temperature. The DAID, GLY, and GEN amounts in all matrices after this period of time were found to be between 99.45% and 101.99%.

3.2.6. *IA extraction from skin and mucosa*

The recovery data for IA quantification after extraction of previously spiked-matrices are shown in Table 3, and was within FDA recommendations for bioanalytical method validation [30]. Taken together, the recovery yields are highly satisfactory and demonstrated that 30 min was sufficient for the complete IA extraction from matrices, and no matrix components interfered during the procedure. Furthermore, IA recovery showed adequate precision in all assessed matrices (RSD < 8.3%).

3.2.7. *System suitability*

Routine analyses of the standard substances were performed under the developed experimental conditions. Parameter values and their variability (RSD, %) for each compound were: (i) DAID analysis: 3.6 (0.13) min for migration time, 20166 (2.11) for theoretical plates, and 1.28 (0.40) for tailing factor; (ii) GLY analysis: 4.07 (0.09) min for migration time, theoretical plates 32122 (1.95), and tailing factor 1.23 (0.81); (iii) GEN analysis: 5.05 (0.09) min for migration time, 60526 (2.06) for theoretical plates, and 1.16 (0.74) for tailing factor. The resolution between DAID and GLY peaks was 4.9 (0.99) and 11.39 (0.85) between GLY and GEN. The parameters indicate that the system is suitable for the analysis.

3.3. *Method application*

As a last objective of this work, the UFLC method was applied to determine the IA amount in the real samples. The IA content in SAE,

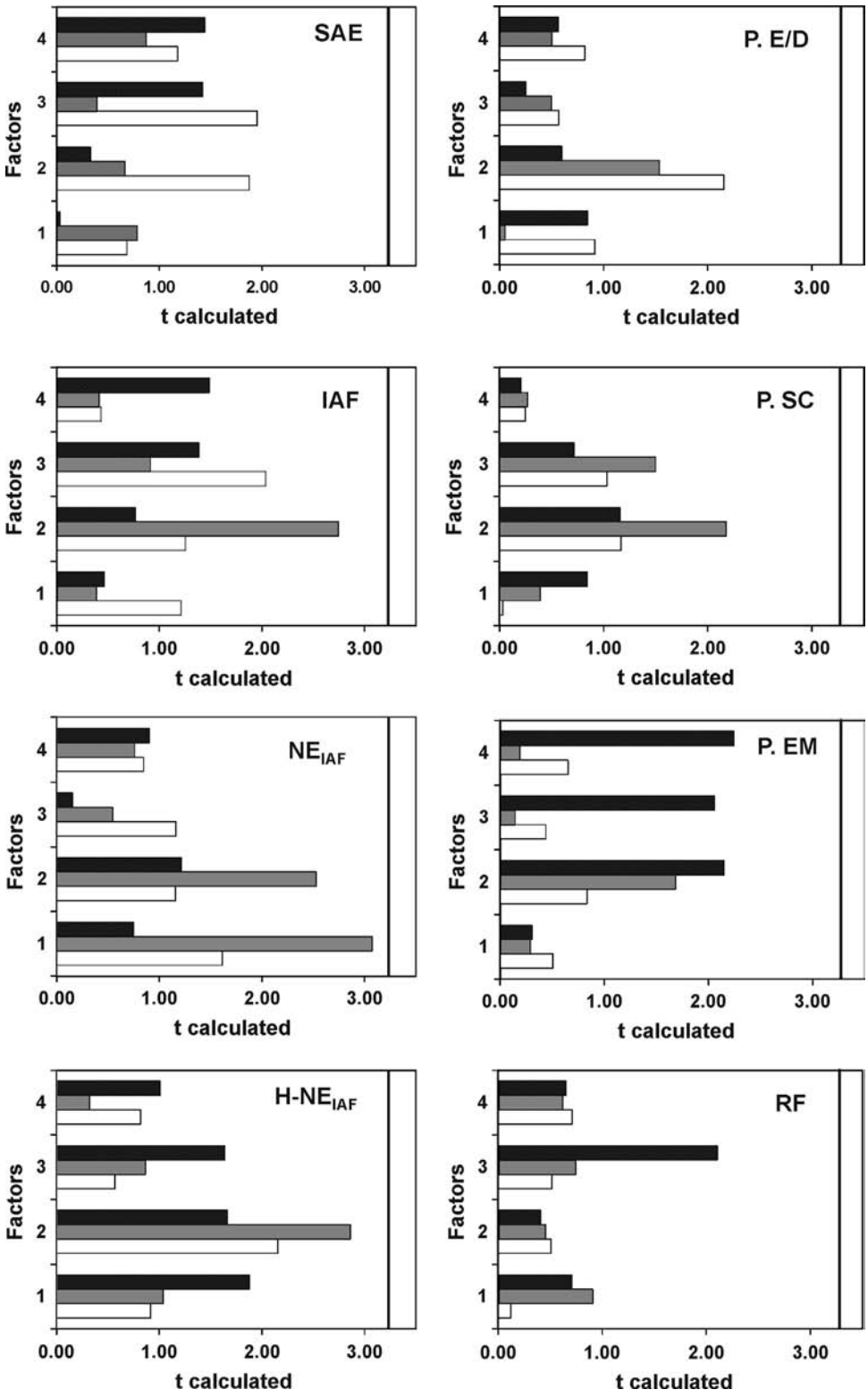


Fig. 4. Bar charts representing the *t*-calculated for quantitative determination (assay) of the investigated factors (1,2,3, and 4) in Plackett-Burman experimental design and their *t*-critical, represented by the vertical line, for each isoflavone in the matrices. Where, 1: column oven temperature, 2: initial flow rate, 3: TFA concentration, 4: initial organic composition, SAE: soybean acid extract, IAF: isoflavone aglycone-rich fraction, NE_{IAF} isoflavone aglycone rich-fraction loaded nanoemulsion, H-NE_{IAF} topical hydrogel containing nanoemulsions, P. SC: porcine stratum corneum layer after tape stripping method, P. E/D: porcine epidermis/dermis skin layer, P. EM: porcine esophageal mucosa, RF: receptor fluid and the bar charts: DAID (black columns), GLY (gray columns), and GEN (white columns).

IAF, NE_{IAF}, H-NE_{IAF}, receptor fluid, and in skin or mucosa layers are shown in Table 4, and the results indicate the precision of the method in all samples, in which the RSD was lower than 3.14% in the analytical assays and lower than 18.52% in the bioanalytical assays.

As previously reported by Yatsu et al. [27], the soybean cultivar used in this work had a total isoflavone aglycones content greater that had been reported for 14 different soybeans cultivars [21], demonstrating the importance of this sample for industrial

Table 3
Recovery data of the IA added in biological matrices.

Matrix	Nominal (μg mL^{-1})	Recovery (%) (RSD)		
		DAID	GLY	GEN
P. E/D skin layer	0.1	98.09 (8.3)	95.12 (3.7)	95.43 (5.2)
	2	97.94 (1.4)	99.45 (2.0)	99.93 (1.1)
	4	95.15 (2.8)	94.56 (2.3)	100.8 (8.1)
P. SC skin layer	0.1	101.8 (2.6)	99.60 (5.9)	99.80 (4.2)
	2	101.1 (1.6)	99.10 (1.8)	100.9 (2.6)
	4	101.8 (1.5)	100.0 (1.3)	102.0 (5.7)
P. E mucosa	0.1	98.81 (1.6)	97.92 (3.2)	99.13 (1.5)
	2	99.16 (2.3)	93.49 (3.1)	93.24 (0.3)
	4	98.24 (2.2)	95.49 (2.3)	96.32 (1.4)

P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after tape stripping process; P. E: porcine esophageal mucosa; RSD=relative standard deviation.

Table 4
Determination of the isoflavone aglycones in real samples.

Application of UFLC method	Mean of six replicates (RSD)			
	DAID	GLY	GEN	IA Total
Samples				
SAE (mg 100 g $^{-1}$ of DSS)	75.14 (2.49)	16.54 (2.66)	112.16 (0.40)	203.85 (1.16)
IAF (mg mg $^{-1}$)	0.45 (1.18)	0.043 (1.02)	0.39 (2.64)	0.89 (1.14)
NE_{IAF} (mg mL $^{-1}$)	0.44 (1.81)	0.037 (1.68)	0.37 (3.14)	0.86 (2.39)
H-NE_{IAF} (mg g $^{-1}$)	0.45 (1.40)	0.038 (1.18)	0.37 (1.47)	0.86 (1.42)
Skin retention				
P. SC (μg cm^{2} $^{-1}$)	0.47 (13.50)	LLQ	0.16 (16.25)	0.63 (14.87)
P.E/D (μg cm^{2} $^{-1}$)	0.24 (18.52)	LLQ	0.39 (14.50)	0.63 (16.50)
RF (μg cm^{2} $^{-1}$)	LLQ	LLQ	LLQ	–
Mucosa permeation				
P.E mucosa (μg cm^{2} $^{-1}$)	2.70 (10.16)	0.29 (11.44)	3.43 (10.78)	6.42 (10.80)
RF (μg cm^{2} $^{-1}$)	6.65 (9.87)	0.79 (18.02)	4.25 (18.10)	11.69 (15.30)

SAE: soybean acid extract; DSS: defatted soybean seeds; IAF: isoflavone aglycone-rich fraction; NE_{IAF}: isoflavone aglycone rich-fraction loaded nanoemulsion; H-NE_{IAF}: hydrogel containing isoflavone aglycone rich-fraction loaded nanoemulsion; P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after tape stripping process; RF: receptor fluid; LLQ: lower than limit of quantification; P. E: porcine esophageal mucosa;; RSD=relative standard deviation.

applications. To obtain the IA in a pure fraction, we described an appropriate method of purification and acquisition of DAID, GLY, and GEN from SAE, which allowed obtaining a fraction with 0.89 mg of IA per mg of powder (Table 4) and free from HMF and EMF (Fig. 3A IAF).

When this fraction was incorporated into nanoemulsions, the quantification of all IA showed adequate repeatability. This also occurred during the analysis of hydrogels containing the NE_{IAF}. Both formulations had successfully incorporated the IA, since their amount was 0.86 mg mL^{-1}, resulting in more than 96% of IA when compared with the 0.89 mg mL^{-1} initially added.

Regarding the *in vitro* skin permeation/retention study, no IA could be detected in the receptor fluid after 8 h of assay. Conversely, DAID and GEN could be quantified with adequate precision in the stratum corneum, as well as in the epidermis and dermis layers. Yet, the same was not observed with GLY, which was detected in all skin layers, but could not be quantified, because its peak areas were always lower than the LLOQ.

In the literature, no report was found concerning the validation of a method for simultaneous quantification of DAID, GLY, and GEN after skin permeation/retention assays. Among some studies comprising such matters, Vargas et al. [11] showed GEN quantification in the skin layers and receptor fluid after the cutaneous permeation/retention assay using a previously validated HPLC method [12]. On the other hand, for simultaneous IA quantification, Huang et al. [6] have reported *in vitro* and *in vivo* skin retention/permeation of DAID and GEN, without demonstrating the validation of the method used for the assessment. That being said, the present study reports, for the first time, a fast and validated method for skin studies involving all soybean isoflavone aglycones.

In addition, the method was able to quantify IA in a mucosa permeation assay in both receptor fluid and tissue with satisfactory precision. This is an important finding, considering that isoflavone aglycones have shown anti-herpetic activities [4], requiring application on some mucosas to carry out their functions. The choice of porcine esophageal mucosa to investigate the permeation study was done in accordance with Consuelo et al. [41], since the authors showed that this membrane is a useful and practical substitute for buccal mucosa for *in vitro* permeability studies. Lastly, it is important to emphasize that this was the first time this application had been reported for isoflavone aglycones.

4. Conclusions

The overall results showed that the developed UFLC method is an excellent tool for determing isoflavone aglycones present in soybean acid extract, isoflavone aglycone-rich fractions, isoflavone aglycones loaded-nanoemulsions, topical hydrogel containing isoflavone aglycones loaded-nanoemulsions, porcine skin layers, porcine esophageal mucosa, and in a 30% ethanol solution in phosphate buffer pH 7.4 (receptor fluid for permeation studies). The method had a low matrix effect, without any interference from the matrices and degradation products, besides being simple,

quick, and able to quantify isoflavones with precision, robustness, and accuracy in different complex matrices.

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References

[1] K.I Chen, M.H. Erh, N.W. Su, W.H. Liu, C.C. Chou, K.C. Cheng, Appl. Microbiol. Biotechnol. 96 (2012) 9–22.
[2] P. Albertazzi, D.W. Purdie, Maturitas 42 (2002) 173–185.
[3] E. Poluzzi, C. Piccinni, E. Raschi, A. Rampa, M. Recanatini, F. De Ponti, Curr. Med. Chem. 21 (2014) 417–436.
[4] A. Andres, S.M. Donovan, M.S. Kuhlenschmidt, J. Nutr. Biochem. 20 (2009) 563–569.
[5] K. Miyazaki, T. Hanamizu, R. Lizuka, K. Chiba, Skin Pharmacol. Appl. Skin Physiol. 15 (2002) 175–183.
[6] Z.R. Huang, C.F. Hung, Y. Lin, J.Y. Fang, Int. J. Pharm. 364 (2008) 36–44.
[7] D. Schmid, F. Zulli, H.P. Nissen, H. Prieur, Cosmet. Toiletries 118 (2003) 71–74.
[8] D. Schmid, F. Zulli, Cosmet. Toiletries 117 (2002) 45–50.
[9] A.P. Cappra Silva, L.S. Koester, P. Mayorga, V. Linck Bassani, H. Teixeira, Pharmazie 62 (2007) 732–734.
[10] A.P. Silva, B.R. Nunes, M.C. De Oliveira, L.S. Koester, P. Mayorga, V.L. Bassani, H. F. Teixeira, Pharmazie 64 (2009) 32–35.
[11] B.A. Vargas, J. Bidone, L.K. Oliveira, L.S. Koester, V.L. Bassani, H.F. Teixeira, J. Biomed. Nanotechnol. 8 (2012) 1–7.
[12] B.A. Vargas, D.F. Argenta, G. Borghett, L.S. Koester, V.L. Bassani, H.F. Teixeira, J. Chromatogr. Sci. 50 (2012) 114–118.
[13] M.A. Rostagno, A. Villares, E. Guillamón, A. Gárcia-Lafuente, J.A. Martinéz, J. Chromatogr. A 1216 (2009) 2–29.
[14] D.L. Luthria, S.S. Natarajan, Planta Med. 75 (2009) 704–710.
[15] I.C. César, F.C. Braga, C.D.V. Soares, E.A. Nunan, G.A. Pianetti, F.A. Condessa, T.A. F. Barbosa, L.M.M. Campos, J. Chromatogr. B 836 (2006) 74–78.
[16] A.P. Griffith, M.W. Collison, J. Chromatogr. A 913 (2001) 397–413.
[17] B. Kledjus, R. Mikelová, J. Petrlová, D. Potesil, V. Adam, M. Stiborová, P. Hodek, J. Vacek, R. Kizek, V. Kuban, J. Chromatogr. A 1084 (2005) 71–79.
[18] M.A. Rostagno, M. Palma, C.G. Barroso, Anal. Chim. Acta 582 (2007) 243–249.
[19] B. Klejdus, J. Vacek, L. Lojková, L. Benesová, V. Kubán, J. Chromatogr. A 1195 (2008) 52–59.
[20] N. Manchón, M. D'Arrigo, A. García-Lafuente, E. Guillamón, A. Villares, A. Ramos, J.A. Martínez, M.A. Rostagno, Talanta 82 (2010) 1986–1994.
[21] G. Fiechter, I. Opacak, B. Raba, H.K. Mayer, Food Res. Int. 50 (2013) 586–592.
[22] P.V. Gaikwad, S.D. Sawant, M.R. Ghante, N.M. Munot, Pharmacie Globale IJPC 1 (2010) 1–3.
[23] B. Klejdus, J. Vacek, L. Benešová, J. Kopecký, O. Lapčík, V. Kubáň, Anal. Bioanal. Chem. 389 (2007) 2277–2285.
[24] B. Kiss, D.-S. Popa, D. Hanganu, A. Pop, F. Loghin, Rev. Roum. Chim. 55 (2010) 459–465.
[25] N. Toro-Funes, I. Odriozola-Serrano, J. Bosch-Fuste, M.L. Latorre-Moratalla, M. T. Veciana-Nogues, M. Izquierdo-Pulido, M.C. Vidal-Carou, Food Chem. 135 (2012) 2832–2838.
[26] G. Fiechter, B. Raba, A. Jungmayr, H.K. Mayer, Anal. Chim. Acta 672 (2010) 72–78.
[27] F.K.J. Yatsu, G.P.R. Pedrazza, D.F. Argenta, F. Barreto, M.C. Nemitz, H.F. Teixeira, L.S. Koester, V.L. Bassani, Food Anal. Method 7 (2014) 1881–1890.
[28] Q. Wu, M. Wang, W.J. Sciarappa, J.E. Simo, J. Agric. Food Chem. 52 (2004) 2763–2769.
[29] International Conference on Harmonization (ICH). Technical requirements for the registration of pharmaceutical for human use, Validation of Analytical Procedures: Text and Methodology Q2(R1). 2005, 1-13.
[30] Food and Drug Administration (FDA), Center for drug evaluation and research, Guidance for Industry: Bioanalytical Method Validation. 2013, 1-34.
[31] European Medicine Agency EMEA/CHMP/EWP/192217/2009 Guideline on bionalytical method validation. 2011, 1-22.
[32] E. Watanabe, Y. Kobara, K. Baba, H. Eun, Food Chem. 154 (2014) 7–12.
[33] Y.V. Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723–753.
[34] E.J. Zhang, K.A. Ming, Ng, K.Q. Luo, J. Agric. Food Chem. 55 (2007) 6940–6950.
[35] S.-C. Shen, J.S.-B. Wu, J. Food Sci 69 (2004) FCT273–FCT279.
[36] M.C. Nemitz, Processos de purificação do extrato de soja: obtenção de fração enriquecida em isoflavonas para incorporação em nanoemulsões visando uso tópico e isolamento de derivados furânicos (Master Dissertation), Universidade Federal do Rio Grande do Sul, Brazil, 2014.
[37] I. Severin, C. Dumont, A. Jondeau-Cabaton, V. Graillot, M.-C. Chagnon, Toxicol. Lett. 192 (2010) 189–194.
[38] C. Petisca, A.R. Henriques, T. Perez-Palacios, O. Pinho, I.M.P.L.V.O. Ferreira, J. Food Compos. Anal. 33 (2014) 20–25.
[39] W.M.A. Niessen, P. Manini, R. Andreoli, Mass Spectrom. Rev. 25 (2006) 881–899.
[40] M. Zeaiter, J.M. Roger, V. Bellon-Maurel, D.N. Rutledge, Trends Anal. Chem. 23 (2004) 157–170.
[41] I.D. Consuelo, Y. Jacques, G.-P. Pizzolato, R.H. Guy, F. Falson, Arch. Oral Biol. 50 (2005) 981–987.