ANIMAL AND PLANT NUTRITION, HEALTH, AND SAFETY

Development and Validation of a UPLC–ESI(-)–MS/MS Methodology for the Simultaneous Quantification of Hesperidin, Naringin, and their Aglycones in Chicken Tissue Samples

Eirini Baira and Ioanna Dagla
National and Kapodistrian University of Athens, School of Health Sciences, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Panepistimiopolis, 15771 Athens, Greece
Eleni Siapi and Panagiotis Zoumpoulakis
National Hellenic Research Foundation, Institute of Biology, Medicinal Chemistry and Biotechnology, 48 Vassileos Constantinou, 11635 Athens, Greece
Anthony Tsarbopoulos
National and Kapodistrian University of Athens, Medical School, Department of Pharmacology, Panepistimiopolis, 15771 Athens, Greece
Panagiotis Simitzis, Michael Goliomytis, and Stelios G Deligeorgis
Agricultural University of Athens, Faculty of Animal Science and Aquaculture, Department of Animal Breeding and Husbandry, 75 IeraOdos, 11855 Athens, Greece
Alexios-Leandros Skaltsounis
National and Kapodistrian University of Athens, School of Health Sciences, Faculty of Pharmacy, Department of Pharmacognosy and Natural Products Chemistry, Panepistimiopolis, 15771 Athens, Greece
Evagelos Gikas[1]
National and Kapodistrian University of Athens, School of Health Sciences, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Panepistimiopolis, 15771 Athens, Greece

Background: **The dietary supplementation of livestock with antioxidants to improve the meat quality represents an active research area of high commercial impact. In order to investigate the optimal dosing, analytical methodologies need to be developed in various tissues to evaluate which concentration does remain in the tissue.** ***Objective:*** **We aimed to develop and validate a sensitive and specific methodology for the simultaneous quantitative determination of hesperidin, naringin, hesperetin, and naringenin in chicken tissue samples employing ultra-performance LC–tandem MS.** ***Methods:*** **Lipid extraction using cold chloroform was performed followed by protein precipitation by cold acetone. Chromatography was performed on a C18 column using a ternary gradient of water, acetonitrile, and isopropanol–acetonitrile–acetone (58+40+2, v/v) as the mobile phase. Detection was performed by electrospray ionization in negative ion mode with the selected reaction monitoring technique.** ***Results:*** **Calibration plots exhibited good linearity ($r^2 > 0.99$) over the concentration range from 0.125 to 25 µg/g tissue for the four analytes, and the lower LOQ for the four analytes was 0.125 µg/g tissue. The repeatability as percent relative SD and precision as percent accuracy were <20 and >80%, respectively.** ***Conclusions:*** **The developed methodology was applied for the quantitative determination of hesperidin, naringin, hesperetin, and naringenin in tissue samples after dietary supplementation with 1.5 g/kg hesperidin and 1.5 g/kg naringin in Ross 308 broiler chickens.** ***Highlights:*** **This is the first methodology to access naringin, naringenin, hesperidin, and hesperetin in chicken tissue. It involved simple sample preparation, and the mass spectrometry based detection ensures high specificity and sensitivity.**

Flavonoids are a group of polyphenolic phytochemicals that act as antioxidants that sequester free radicals and reactive oxygen species (1, 2). The two main flavanones, hesperidin and naringin, occur naturally in oranges (31–43.2 mg hesperidin/100 g) and grapefruit (11–14.5 mg naringin/100 g; (3, 4).

Hesperidin is a flavanone glycoside, which is normally found in highly nutritious foods, such as oranges, tangelos, tangerines, grapefruits, and other citrus fruits. It presents a broad spectrum of biological significance, including antioxidant, anti-inflammatory, anticarcinogenic, and antiallergic activities (5). It is metabolized in the small intestine, affording the aglycone bioflavonoid hesperetin (6). Takumi et al. (7) has reported the tissue distribution of hesperetin after its dietary administration to rats for 4 weeks.

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[1]Corresponding author's e-mail: vgikas@pharm.uoa.gr
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According to his research, the highest concentration level was found in the liver and the second highest in the aorta (7).

Naringin is a flavanone glycoside found in grapes and citrus fruits. It possesses the distinct bitter taste of grapefruit juice. The biological activities of naringin are related to its antioxidant nature. Moreover, naringin has been found to influence and modify several molecular signaling pathways in metabolic syndrome, obesity, and related cardiovascular health issues, and it also inhibits the tumor necrosis factor-α–mediated inflammatory process and tissue damage in liver and vasculature (8). Naringenin, the aglycone of naringin, has also been demonstrated to exhibit antioxidant (9) and antiulcer (10) effects. Previous studies report tissue distribution of naringin or naringenin after a single intravenous dose or per oral administration (11–13) and after repeated dosing (14) in rat models.

Nevertheless, to the best of our knowledge, there have so far been no reports about the simultaneous quantitative determination of hesperidin, naringin, hesperetin, and naringenin in chicken tissue samples after dietary supplementation with hesperidin and naringin. Therefore, the aim of the current study was to describe a validated LC–tandem MS (MS/MS) methodology for the determination of the four analytes in chicken tissue samples for pharmacokinetic studies. LC–MS/MS has been demonstrated to be the most valuable tool for pharmacokinetic studies because of its higher sensitivity and specificity compared with other analytical tools (15–17). The bioanalytical methodology developed was validated in accordance with U.S. Food and Drug Administration (FDA) guidelines (18) and the European Medicines Agency (EMA) Committee for Medicinal Products for Human Use (CHMP) guidelines for bioanalytical methodologies (19) considering the specificity, linearity, recovery, matrix effect, repeatability precision, accuracy, and lower LOQ.

Materials and Methods

Chemicals and Reagents

All solvents used were of LC–MS grade. Acetonitrile and water were purchased from Fluka/Riedel-de Haën (Switzerland). Glacial acetic acid, methanol, 4-iodophenol, chloroform, and acetone were purchased from Sigma-Aldrich (Steinheim, Germany). Naringin was purchased from Alfa Aesar GmbH & Co KG (Germany) and Hesperidin from TSI Europe NV (Belgium).

Samples and Study Design

Forty-nine 1-day-old Ross 308 broiler chickens were housed in a controlled environment and randomly divided into three groups. The lighting program consisted of 23L:1D on arrival and was decreased to 18L:6D at day 7, remained constant until day 35, and thereafter gradually increased to 23L:1D at slaughter, with access to feed in mash form and water *ad libitum*. The experimental groups consisted of 15 chickens given diets supplemented with 1.5 g hesperidin per kilogram of feed and 17 chickens given diets supplemented with 1.5 g naringin per kilogram of feed. The control group consisted of 17 chickens that were fed with commercial basal diets. The administration of naringin and hesperidin started from the 11th day of age until slaughter at the age of 42 days. Chicken carcasses were then chilled at 4°C for 24 h. Tissue samples were frozen at −80°C until subsequent analysis.

All experimentation was carried out in strict accordance with the guidelines of "Council Directive 86/609/EEC Regarding the Protection of Animals used for Experimental and Other Scientific Purposes." The protocol was approved by the Bioethical Committee of the Agricultural University of Athens (Permit No. 20/20032013).

Instrumentation

The analysis was performed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to an Accela ultra-high-performance LC (UHPLC) system. The UHPLC system was equipped with an Accela quaternary pump, an Accela autosampler with tray temperature control, a vacuum degasser and a temperature-controlled column compartment. The mass spectrometer parameters were optimized for the selected reaction monitoring mode to achieve optimal sensitivity and selectivity. The mass spectrometer operated in the negative ion mode using the following conditions: sheath gas, 35 (arbitrary units); source voltage, 3 kV; auxiliary gas, 30 (arbitrary units); S lens radio frequency level, 60 (%); and capillary temperature, 350°C.

The Orbitrap resolution was set at 30 000 full width at half maximum, and the isolation width was set at 2 amu. A reversed-phase INTERCHIM ultra-performance LC (UPLC) C18 column (1.7 µm particle size, 2.1 mm × 100 mm) was used for the chromatographic separation. The column compartment temperature was maintained throughout all experiments at 40°C, and the flow rate was 0.3 mL/min. The mobile phase gradient consisted of solvent A, aqueous 0.1% glacial acetic acid (v/v); solvent B, acetonitrile; and solvent C, isopropanol–acetonitrile–acetone (58+40+2, v/v/v). A gradient elution methodology has been employed as follows: 0–0.1 min, 95% A to 5% B to 0% C; 0.1–1.1 min, 80% A to 20% B to 0% C; 1.1–3.1 min, 70% A to 30% B to 0% C; 3.1–5.3 min, 50% A to 50% B to 0% C; 5.3–5.8 min, 0% A to 0% B to 100% C; and 5.8–12 min, 95% A to 5% B to 0% C. The autosampler was kept at 4°C, and the injection volume was 10 µL. The chromatographic data was acquired and processed using ThermoXcalibur software (version 2.1).

The samples were separated during the sample preparation protocol using a Mikro 200R centrifuge (Hettich Lab Technology, Germany). Samples were dried using a GeneVac HT-4X EZ-2 series evaporator Lyospeed ENABLED (Genevac Ltd, United Kingdom). Bulk tissue homogenization was performed by a Kinematica Polytron PT 1200C homogenizer (Brinkmann, Westbury, NY), whereas microhomogenization was achieved employing an IKA T 10 basic ULTRA-TURRAX (IKA-Werke GmbH & Co. KG, Staufen, Germany).

Preparation of Standard Solutions, Calibration Curves, and Quality Control Samples

All analyte solutions were stable for at least one month under refrigeration at 5°C. Stock solutions of naringin, naringenin, hesperidin, hesperetin, and 4-iodophenol [internal standard (IS)] were prepared in methanol at 1 mg/mL and stored at 4°C. The stock solution of IS was daily diluted with the initial mobile phase, i.e., 0.1% glacial acetic acid (v/v) solvent – acetonitrile 95+5 (v/v), to prepare the working solution (2 µg/mL), while stock

solutions of all compounds were also appropriately diluted with the initial percentage of the mobile phase. Thereafter, stock and diluted solutions of the compounds were appropriately mixed to produce six combined spiking solutions of all compounds with concentrations of 0.125, 1.25, 5, 10, 17, and 25 μg/g tissue. To overcome instrument signal enhancement or suppression, matrix-matched calibration standards were produced with 40 μL each of these combined solutions and were added to 0.020 g chicken tissue sample to construct the calibration curves.

Preparation of prespiked calibration curves.—The tissue samples were thawed to room temperature. For prespiked calibration standards, 0.020 g homogenized chicken tissue was combined with 100 μL IS working solution (2 μg/mL) and 40 μL calibration standards, with concentrations ranging from 0.125 to 25 μg/g tissue added. Then, appropriate volumes of a 0.1M ammonium acetate buffer adjusted to pH = 5 using acetic acid were added to reach a final ratio of 1:10 tissue:solution (w/v) for the homogenization. The samples were then homogenized for 5 min. One milliliter cold chloroform was added to the homogenizer mixture, and the samples were kept at −20°C for 10 min to achieve total lipid extraction. After the removal of the lipid phase (the upper layer has been aspirated and transferred to a new Eppendorf tube), 1 mL cold acetone was added for the protein precipitation, and then flavonoids were extracted by mixing with a vortex mixer for 1 min. The extracts were centrifuged at 13 500 rpm at 4°C for 10 min. After phase separation, the aqueous layer (acetone) was decanted to a second container (Eppendorf tube), and an additional 0.2 mL cold acetone was added to the tissue pellet. Samples were mixed with a vortex mixer and centrifuged as previously described. The supernatants were mixed to a new Eppendorf vial discarding the pellet and evaporated to dryness under vacuum for 90 min at 50°C (GeneVac centrifugal evaporator). The residue was reconstituted with 0.2 mL methanol–water (50+50, v/v) and centrifuged at 12 500 rpm for 8 min at 4°C, and then the supernatant was transferred to an autosampler vial with insert. A 10 μL injection volume was utilized for analysis by LC–MS/MS. Three prespiked calibration curves were utilized for the determination of SE.

Preparation of postspiked calibration curves.—The process was repeated in a similar manner, but in this case, the flavonoid solutions and the IS solution were added after the homogenization and protein precipitation procedures. Three postspike calibration curves were utilized for the determination of SE.

Preparation of QC samples.— In order to assess the method performance, four QC samples [low (LQC), 0.5 μg/g; medium (MQC), 2.5 μg/g; high (HQC), 12.5 μg/g] and the lower LOQ (LLOQ; 0.125 μg/g) were utilized. A total of six replicate fortified test portions were prepared for each QC sample concentration.

Preparation of standard calibration curve in solvent.—A standard calibration curve was constructed in methanol–water (50+50, v/v, which has been found to afford the same chromatographic quality peak data as 0.1% acetic acid in 5% acetonitrile used for the construction of the matrix matched samples) using the following concentrations: 0.125, 1.25, 5, 10, 17, and 25 μg/mL, with the IS being added at the 0.4 μg/mL level. The peaks were similar in terms of peak symmetry and tailing, whereas the chromatographic peak areas were also comparable (no more than 5% difference). Therefore, the two reconstitution systems were deemed as equivalent. One replicate was constructed for each level of the calibration curve. The samples were analyzed randomly, and a blank sample was used after each level to avoid carry-over effect.

Sample Preparation of Unknown Chicken Tissue Samples

The unknown samples were preprocessed according to the methodology described, and a 10 μL injection volume was analyzed by LC–MS/MS.

Method Validation

A full method validation was performed according to the FDA (18) and the EMA CHMP guidelines for bioanalytical methodologies (19) by evaluating the specificity, linearity, recovery, matrix effect, repeatability, precision, accuracy, and LLOQ. All parts of the validation comply with both guidelines, as they enforce similar requirements. Minor departures from the validation requirements (less than 1%) that were observed for the validation data of naringin and naringenin were not considered as crucial because they did not essentially alter the conclusions or the applicability domain of the methodology, and they were considered as fit for purpose for the methodology described. The samples have been analyzed within 5 days from the animal sacrifice, and care has been taken to process each sample immediately after its thawing. The substances were stored as dried extracts to −80°C, and upon reconstitution, they were immediately analyzed. Under such conditions, the stability of the substances is not compromised in our experience. No issues were identified whatsoever concerning the method's robustness.

Results and Discussion

Method Development

UPLC–electrospray ionization (ESI)(-)–MS/MS optimization.—The mass spectrometric parameters were explored based on three parameters: sensitivity, repeatability, and the number of scans to decide whether the ion trap or the orbitrap should be used for the quantitation. In order to compare the sensitivity between the two ion traps, the Fourier Transform Mass Spectrometer based Orbitrap and the linear ion trap, four calibration curves were constructed, and comparisons were based on solvent calibration standard solutions. The concentration levels used were those of the corresponding curves used for the linearity study. For both traps, the MS/MS mode using the deprotonated ion as precursor was employed (Table 1). The results have been evaluated based on the ratio of the slopes for each analyte. The linear ion trap exhibited 10-fold higher sensitivity compared with the Orbitrap analyzer. Concerning the repeatability as well as the number of scans, it was found that the two analyzers did not differ significantly. Thus, the linear ion trap has been chosen as the analyzer employed for the quantification based on the advantage of higher sensitivity. The increased sensitivity of the linear ion trap over the Orbitrap is considered a result of the much larger capacity in terms of available physical space of the former.

Chromatographic optimization.—The chromatographic run has also been optimized in an effort to avoid any coelution during the analysis that would possibly compromise the analysis due to matrix effect (Figure 1). In order to attain reasonable peak shape,

Table 1. Equations of linear ion-trap based quantitation methodology for the analytes naringin, hesperetin, hesperidin, and naringenin[a]

Analyte	IT-based equation	R^2
Naringin	Y = 12456 (±723) X –2190 (±3076)	0.995
Hesperetin	Y = 22639 (±654) X +39128 (±25245)	0.994
Hesperidin	Y = 13943 (±547) X –11750 (±3456)	0.991
Naringenin	Y = 25173 (±468) X +62554 (±1849)	0.993

[a] The parameters in parentheses are the SEs of slope and intercept for each equation.

acid modification of the mobile phase was found to be required. The usage of formic acid afforded lower signal, and therefore acetic acid was employed as the aqueous phase modifier. The organic modifier was acetonitrile because the use of methanol failed to afford adequate separation of the flavonoids.

Method Validation Results

Specificity, carry-over, and linearity.—For the study of specificity, i.e., the ability to differentiate between target analytes and interference, six blank chicken tissue samples were analyzed. The samples were prepared as previously described. No interfering peaks could be detected at the expected retention time window for any of the four target analytes higher than the 20% of the LLOQ indicating the specificity of the method.

Carry-over was evaluated by injecting a blank after a high concentration sample ($n=6$). The absence of carry-over effect was demonstrated as the injection of the blank samples showed no peaks higher than 20% of the LLOQ at the corresponding retention time of each analyte.

Calibration curves ranging from 0.125 to 25 µg/g tissue for the four analytes of interest were constructed and analyzed on three laboratory days. The calibration concentration levels were 0.125, 0.25, 0,5, 1, 2.5, 5, 10, 15, and 25 µg/g tissue. The linearity of the calibration curves was checked by regression analysis with no weighting, whereas the 0.0 point was neither forced nor included as an additional point. The fit of the regression was assessed by evaluating the correlation coefficient, R^2, as well as the back-calculated values under the constraint that they should not deviate more than 15% of the theoretical value, except the LLOQ, for which a value of 20% was allowed. Analysis of variance showed that the proposed model had a probability of nonrandom fitting with a value of $P<0.05$ (Supplemental Table 1), whereas the normality testing employing the Shapiro-Wilk and the Colmogorov-Smirnov tests along with the Q-Q plot showed that the residuals were normally distributed with no apparent trend owing to homoscedasticity. All calibration curves exhibited high correlation coefficients ($R^2>0.99$). The calibration curve data can be found in Table 1.

Table 2. Recovery for the four analytes determined after comparing the slope ratios of the calibration curve from prespiked samples and the calibration curve from postspiked chicken tissues samples and matrix effect determined by the comparison of slopes of the calibration curves prepared in methanol–water (50+50, v/v) and the corresponding ones prepared by the pre-spike samples

Analyte	% Rec.	Matrix effect
Naringin	0.94	0.99
Hesperidin	1.05	0.96
Naringenin	0.99	0.93
Hesperetin	1.04	0.95

Recovery matrix effect.—For the recovery study, three calibration curves from prespiked samples and three curves from postspiked samples were analyzed and compared. The data were averaged for each calibration curve point, leading to one curve for prespiked and one for postspiked samples for each analyte. The slopes of the two calibration curves were compared. As shown in Table 1, the slope ratios were found to be greater than 0.94 for each analyte. This showed that the extraction protocol was suitable for the analysis of the four analytes in chicken tissue homogenates (Table 2).

The matrix effect on instrument signal enhancement or suppression arises because of the influence of endogenous tissue components on the ionization process of the analytes. The matrix effect was investigated by comparing the slopes of a calibration curve prepared in methanol–water (50+50, v/v) and the calibration curve prepared by the prespiked samples. The slope ratios were found to be greater than 0.90 for each analyte, and it is therefore safely concluded that the matrix effect could not adversely affect the accuracy and precision of the methodology (Table 2).

LLOQ.—The LLOQ was defined as the lowest concentration of a calibration curve point at which both precision and accuracy were less than or equal to 20% in order for an analyte in a sample to be quantified reliably. The analyte signal of the LLOQ sample should yield a S/N of at least five times the signal of a blank sample. The noise was calculated as the SD of height at the expected retention time span of each target analyte

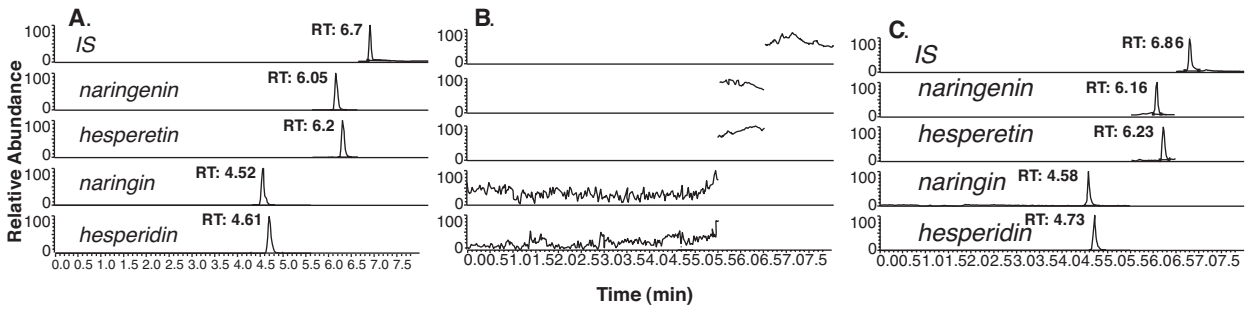

Figure 1. Representative chromatograms of (A) a standard solution at the MQC level, (B) a blank sample, and (C) a plasma sample spiked at the MQC level.

for the blank samples. The current assay exhibited LLOQ values for naringin, hesperidin, naringenin, and naringin at the 0.125 µg/g tissue level.

Repeatability, accuracy, and intermediate precision.—The repeatability (within-run), intermediate precision (between-run), and accuracy of the proposed methodology were examined by analyzing six replicates of four QC samples prepared at concentrations levels of 0.125, 0.5, 2.5, and 25 µg/g tissue. The results for the intermediate precision (three laboratory days) and repeatability were expressed as the relative SD (RSD, %). The exclusion criteria were set as RSD, % <20 for the LLOQ and <15 for the QC samples (Tables 3 and 4).

Accuracy was expressed as the %SE (%Er) between the mean concentration and the calculated concentration for each of the four QC levels. The exclusion criteria were set as %Er <20 for the LLOQ and <15 for the QC samples (Table 5).

The results showed the repeatability did not exhibit values more than 17.9% for the LLOQ and 15.31% for the QC samples, and the precision did not exhibit values more than 19.68% for the LLOQ and 15.06% for the QC samples. The %Er of the accuracy did not exhibit values more than 14.32% for the LLOQ and 15.77% for the QC samples. No trend has been found concerning accuracy, except for naringenin, which seems to show somewhat elevated values. Nevertheless, the results are within the error accepted by the regulatory bodies (or marginally out); therefore, the developed methodology can be considered fit-for-purpose. These results demonstrate that the method was accurate and precise for the quantitation of the four analytes in chicken tissue samples, although there is a small (less than 1%) discrepancy in the results compared with the proposed values from the regulatory bodies. Such a discrepancy is not expected to be crucial for the results because this is an exploratory study of the nutritional administration of flavonoids to chickens.

Stability of the analytes.—The stability of the analytes has been assessed as freeze-thaw (the tissue has been spiked at the LQC and HQC levels and subjected to three consecutive thaw cycles with a freezing lag of 20 h between them) and in-process stability (the samples remained for 12 h in the autosampler, and an injection was repeated every 1.5 h; Supplemental Table 2). The results were expressed as RSD, % at each level for each analyte, and the exclusion criteria were set as >15% RSD.

Table 3. Validation results from the intermediate precision of the proposed methodology after the analysis of six replicates of four QC levels at nominal concentrations of 0.125, 0.5, 2.5, and 25 µg/g tissue[a]

Analyte	LLOQ, %	LQC, %	MQC, %	HQC, %
Hesperidin	16.00	11.32	10.35	5.81
Naringin	19.68	12.95	15.06	4.99
Hesperetin	4.85	3.48	6.88	4.73
Naringenin	13.78	7.29	2.61	4.13

[a] The results are expressed as the RSD, %. The excluded criteria were set as RSD, % <20 for the LLOQ and <15 for the QC samples.

Table 4. Validation results from the repeatability of the proposed methodology after the analysis of six replicates of four QC levels at nominal concentrations of 0.125, 0.5, 2.5, and 25 µg/g tissue[a]

Analyte	LLOQ, %	LQC, %	MQC, %	HQC, %
Hesperidin	11.52	11.01	6.18	7.20
Naringin	17.90	9.19	7.22	7.11
Hesperetin	16.20	10.52	9.71	8.73
Naringenin	17.05	15.31	12.64	9.78

[a] The results are expressed as the RSD, %. The exclusion criteria were set as RSD, % <20 for the LLOQ and <15 for the QC samples.

Table 5. SE of the proposed methodology utilizing the results from four QC concentrations analyzed in replicate (n = 6) at nominal concentrations of 0.125, 0.5, 2.5, and 25 µg/g tissue[a]

Analyte	LLOQ, %	LQC, %	MQC, %	HQC, %
Hesperidin	11.78	6.30	4.09	4.33
Naringin	12.60	12.21	1.45	2.09
Hesperetin	1.45	15.76	13.87	14.68
Naringenin	14.32	14.44	14.50	15.77

[a] The results are expressed as the %SE (%Er) between the mean concentration and the nominal concentration. The excluded criteria were set as %Er <20 for the LLOQ and <15 for the QC samples.

Robustness.—In order to explore the method's resistance to small changes in the experimental conditions the % of acetic acid (0.09, 0.1, and 1.1%) in the aqueous part of the mobile phase, the concentration of 0.1M ammonium acetate in the extraction buffer (0.095, 1, and 1.05M in all cases adjusted to pH = 5 using acetic acid) and the flow rate of the chromatographic analysis (0.28, 0.3, and 0.32 mL/min) were changed. All analyses were performed at the MQC. The RSD, % values of the peak area were examined, and in every case, the change was less than 13.2%, indicating that the method affords consistent results after small deliberate changes in the aforementioned parameters.

Application of the validated UPLC–ESI(-)–MS/MS methodology on a pharmacokinetic study.—The utility of the validated methodology was evaluated for fitness by accessing tissue samples from chickens after dietary supplementation with hesperidin and naringin. Thus five chickens were administered with diets supplemented with 1.5 g hesperidin per kilogram of feed and five chickens with diets supplemented with 1.5 g naringin per kilogram of feed versus a control group, consisting of five chickens fed with commercial basal diets. Feed additives were supplemented until slaughter at the age of 42 days. The quantitation was based on matrix-matched calibration curves that were prepared for hesperidin, naringin, hesperetin, and naringenin on the day of the analysis. The results showed an average of 448.47 (±171.25) ng/g tissue for naringin and 1331.04 (±397.98 ng/g) for hesperidin [mean (±confidence limits)], whereas no measurable levels were found for naringenin and hesperetin (Figure 2). This initial result shows for the first time low absorption of the glucosides in contrast to the aglycones in tissues and needs to be verified with a larger cohort of experimental animals. It is well known that the glycosides could be absorbed actively by transporters as the sodium-dependent glucose transporter 1, whereas the aglycones are subjected to very fast metabolism, affording glucuronides and/or sulfates. Therefore, even if the aglycones were absorbed, they would have been metabolized rapidly, resulting in no detectable levels in the tissues.

Overall, a fully validated methodology was developed for the simultaneous quantitation of hesperidin, naringin, hesperetin, and naringenin in chicken tissue samples. The methodology was applied on chickens after dietary administration with

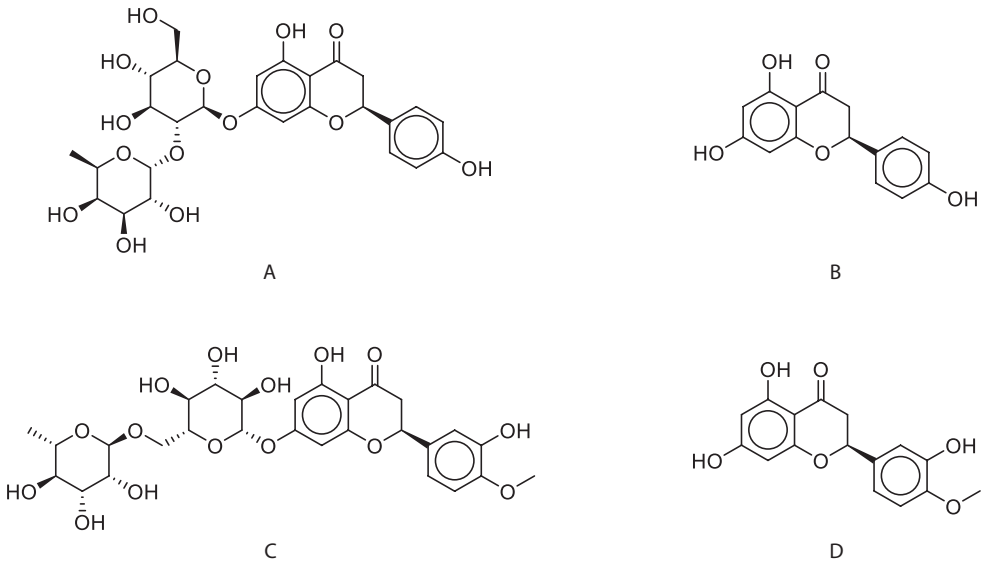

Figure 2. Chemical structures of the studied analytes (A) Naringin, (B) Naringenin, (C) Hesperidin, and (D) Hesperetin.

hesperidin and naringin. Only naringin and hesperidin were detected. More *in vitro* studies need to be done to investigate the metabolic fate of the aglycones in tissues.

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