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Short communication

# A validated solid-phase extraction HPLC method for the simultaneous determination of the citrus flavanone aglycones hesperetin and naringenin in urine

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**Abstract**

A simple, specific, precise, accurate, and robust HPLC assay for the simultaneous analysis of hesperetin and naringenin in human urine was developed and validated. Urine samples were incubated with β-glucuronidase/sulphatase and the analytes were isolated by solid-phase extraction using C18 cartridges and separated on a C8 reversed phase column using a mixture of methanol/water/acetic acid (40:58:2, v/v/v) at 45 °C. The method was found to be linear in the 50–1200 ng/ml concentration range for both hesperetin and naringenin ($r > 0.999$). The accuracy of the method was greater than 94.8%, while the intra- and inter-day precision for hesperetin was better than 4.9 and 8.2%, respectively and for naringenin was better than 5.3 and 7.8%, respectively. Recovery for hesperetin, naringenin and internal standard 7-ethoxycoumarin was greater than 70.9%. The method has been applied for the determination of hesperetin and naringenin in urine samples obtained from a male volunteer following a single 300 mg oral dose of each of the corresponding flavanone glycosides hesperidin and naringin. The intra- and inter-day reproducibility through enzyme hydrolysis was less than 3.9% for both total (free + conjugated) hesperetin and naringenin. Stability studies showed urine quality control samples to be stable for both hesperetin and naringenin through three freeze–thaw cycles and at room temperature for 24 h ( error ≤ 3.6%).


*Keywords:* Hesperetin; Naringenin; Citrus flavanones; HPLC determination

## 1. Introduction

Flavonoids are a group of naturally occurring polyphenolic compounds that are ubiquitous in all vascular plants and are widely used in the human diet [1]. They are usually present almost exclusively in the form of β-glycosides, and they can be divided on the basis of their molecular structure into four main groups: flavones; flavonols; flavanones, and isoflavones [2]. In contrast to other flavonoids, flavanone glycosides such as hesperidin (3',5,7-trihydroxy-4'-methoxyflavanone-7-rhamnoglucoside) and naringin (4', 5,7-trihydroxyflavanone-7-rhamnoglucoside) (Fig. 1) have a more restricted distribution, specifically in citrus fruits. Thus, hesperidin occurs naturally in orange fruits (*Citrus sinensis*), while naringin is the main flavonoid found in grapefruits (*Citrus paradisi*) [3,4].

Different studies have revealed a variety of pharmacological activities that citrus flavonoids possess, such as antioxidant [5,6], blood lipid and cholesterol lowering [7–9], anti-inflammatory [10,11], and anti-carcinogenic [12–14]. Hesperidin and naringin are hydrolyzed in the gastrointestinal tract by the enzymes of intestinal bacteria followed by absorption and conjugation of their aglycones, which subsequently are excreted in urine mainly as sulphoglucuronides and to a lesser extent as free aglycones [15–21].

A number of HPLC methods have been developed for the determination of hesperetin [22] and naringenin [17,23–25] in biological fluids, either alone or in combination [16,20,21]. However, some of these methods are time-consuming, some are expensive, some are using gradient elution, or there is no internal standard and are not directly applicable for the simultaneous quantification of hesperetin and naringenin in human urine. Therefore, the

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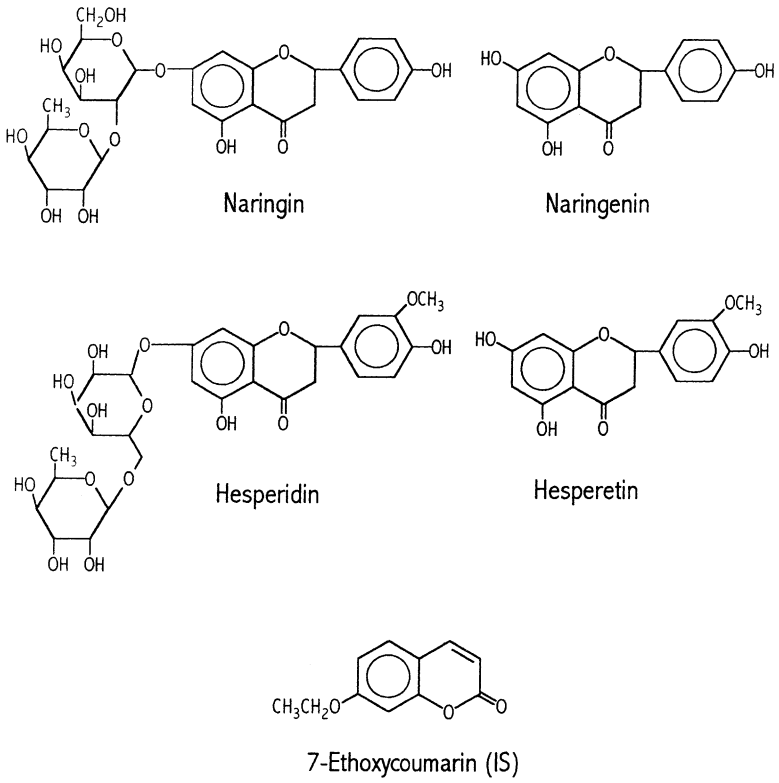


Fig. 1. Chemical structures of citrus flavanone glycosides naringin and hesperidin and their corresponding aglycones naringenin and hesperetin and 7-ethoxycoumarin (IS).

objectives of the present study were to develop and validate a solid-phase extraction HPLC-UV method for the simultaneous determination of hesperetin and naringenin in human urine, which can be used to conduct pharmacokinetic studies after the intake of the dietary flavanone glycosides, hesperidin and naringin, either as pure substances or in citrus fruits that contain them.

## 2. Experimental

### 2.1. Chemicals and reagents

Hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone), 95%, naringenin (4'-5,7-trihydroxyflavanone), 95%, internal standard 7-ethoxycoumarin and β-glucuronidase/sulphatase (crude solution from *Helix pomatia*, type HP-2, G7017) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol, acetonitrile and acetic acid were obtained from Merck (Darmstadt, Germany). Sep-Pak C18 cartridges, 3 ml 500 mg, were supplied from Waters (Milford, Ireland). All other chemicals and solvents used were of analytical grade.

### 2.2. Chromatographic conditions

The development and validation work was carried out on a HPLC system (Varian, Palo Alto, CA, USA) consisting of two high-pressure solvent delivery pumps (Model 2510), a static high-pressure mixer (Model 2584), a variable wavelength UV-Vis detector (Model 2550), a manual injector with a 20 μl loop (Rheodyne, Cotati, CA, USA) and an integrator (Model 4290). Separation was performed on a Macherey Nagel Nucleosil C8 analytical column (5 μm particle size, 250 × 4.6 mm i.d.), proceeded by a guard column (20 × 4.6 mm i.d.) dry packed with pellicular ODS material (37–53 μm).

The HPLC system was equilibrated isocratically with the mobile phase consisting of methanol/water/acetic acid (40:58:2, v/v/v), at a flow rate of 0.9 ml/min at 45 °C. The injection volume was 20 μl and the chromatographic peaks were determined at 288 nm. The integrator attenuation was eight and the chart speed was 0.1 cm/min.

### 2.3. Standard solutions

Stock solutions of hesperetin, naringenin, and the internal standard 7-ethoxycoumarin were prepared daily by dissolving appropriate amounts of the compounds in methanol to achieve final concentrations of 400 μg/ml for each compound. Intermediate solutions, containing both flavanone aglycones, were prepared by mixing aliquots of respective stock solutions (final concentrations 60 μg/ml for both hesperetin and naringenin). Appropriate dilutions of the intermediate solutions of hesperetin and naringenin were made with methanol/water (1:1) to prepare the working solutions containing 2.5, 3.75, 5, 10, 20, 30, 40, 50, and 60 μg/ml

of hesperetin and naringenin. The stock solution of internal standard was further diluted with methanol/water (1:1) to give the internal standard working solution containing 70 μg/ml of 7-ethoxycoumarin.

Calibration standard samples were freshly prepared in 1 ml of flavanone-free urine by spiking with 20 μl of the hesperetin and naringenin working solutions and 20 μl of the internal standard working solution, to yield concentrations corresponding to 50, 75, 100, 200, 400, 600, 800, 1000, and 1200 ng/ml of hesperetin and naringenin in urine.

### 2.4. Quality control samples

Volumes of 25 ml of the flavanone-free human urine samples were spiked with appropriate volumes of hesperetin and naringenin stock solutions to obtain quality control samples containing 200, 400 and 800 ng/ml of hesperetin and naringenin. The samples were divided into aliquots of about 2 ml into one-dram vials capped tightly, and placed at −20 °C pending analysis. These samples were used in the analysis of urine samples as quality controls for the purpose of checking recovery of analytes in the daily analyses of urine samples.

### 2.5. Sample preparation

Sample preparation was performed as described previously for plasma samples [26], with a slight modification in the washing process which resulted in cleaner chromatograms. Flavanone-free urine samples (1 ml) spiked with hesperetin, naringenin, and internal standard were incubated under continuous shaking with 100 μl of 1 mol/L of sodium acetate buffer (pH 5), 100 μl of 0.1 M of ascorbic acid and 40 μl of β-glucuronidase/sulphatase (crude preparation from *Helix pomatia*, Sigma) for 18 h at 37 °C. The hydrolyzed urine samples were diluted with 2 ml of phosphate buffer (0.1 M, pH 2.4), centrifuged for 10 min at 3500 rpm, then they were applied to the extraction C18 cartridges (3 ml, 500 mg), preconditioned successively with 6 ml of methanol and 6 ml of 0.01 M HCl, and allowed to run through. The cartridges were washed successively with 5 ml of 10% methanol in 0.01 M HCl and then with 15 ml of distilled water and purged with air till complete drying of the aqueous phase. Hesperetin, naringenin, and internal standard were eluted with 1.5 ml (3 × 0.5) of acetonitrile. The eluate was evaporated to dryness at 45 °C with the aid of a gentle stream of air. Finally, the residue was redissolved in 100 μl of mobile phase and a 20 μl volume was injected into the chromatographic system for quantification.

### 2.6. Urine collection

To one healthy male volunteer, who followed a one week citrus-free diet and 24 h vegetable-free diet, a single oral dose containing 300 mg of each hesperidin and naringin was administered along with 250 ml of water. Following dose administration urine samples were collected for 48 h in six timed intervals (0–3, 3–6, 6–9, 9–15, 15–24 and 24–48 h). At the end of each collection period the volume of urine was measured and a 10 ml urine sample was kept frozen at −20 °C until analysis. The urine samples had been thawed and then were homogenized by vortex for 30 s and a 1 ml urine volume was then sampled as quickly as possible to avoid sedimentation of any solids.

## 3. Results and discussion

### 3.1. Chromatographic separation and urine interferences

Usually, the chromatographic separation of flavonoid glycosides and their aglycones is carried out using C18 or C8 reversed-phase analytical columns. The chromatographic conditions described in this assay were arrived after exploring C18 and C8 columns and different mobile phases consisting of aqueous mixtures of methanol, acetonitrile, or tetrahydrofuran in different ratios. The better chromatographic separation of the analytes was achieved using a C8 reversed-phase column with methanol/water/acetic acid (40:58:2, v/v/v) as the mobile phase. The addition of acetic acid in the mobile phase, which suppresses the ionization of phenol groups, as well as the column temperature (45 °C) were found to improve further the separation of the analytes and the symmetry of the chromatographic peaks. Typical chromatograms obtained after β-glucuronidase/sulphatase treatment of flavanone-free urine and urine sample obtained from a male volunteer 10 h after a single oral dose of 300 mg each of the flavanone glycosides hesperidin and naringin, are illustrated in Fig. 2. No interfering peaks due to urine components or metabolites elute at the retention time of hesperetin, naringenin, or internal standard. Naringenin, hesperetin and internal standard were eluted in 13.1, 16.1, and 19.2 min, respectively.

### 3.2. Sample preparation

For the successful separation of analytes in a biological matrix, sample pretreatment prior to HPLC analysis is generally required. It is well known that urine samples have many endogenous substances that interfere with the chromatographic separation of analytes causing difficulties with their quantification. Different attempts utilizing liquid–liquid extraction were tested with disappointing results due to many interfering peaks which co-extracted from urine and which were severely interfered with the analytes. These unsuccessful results led us to investigate the solid-phase extraction technique for the selective elution of hesperetin, naringenin, and internal standard.

The solid-phase extraction conditions described in this method were arrived after investigating different solid-phase extraction supports. The better clean-up method was found to be by using Sep-Pak C18 cartridges which effectively

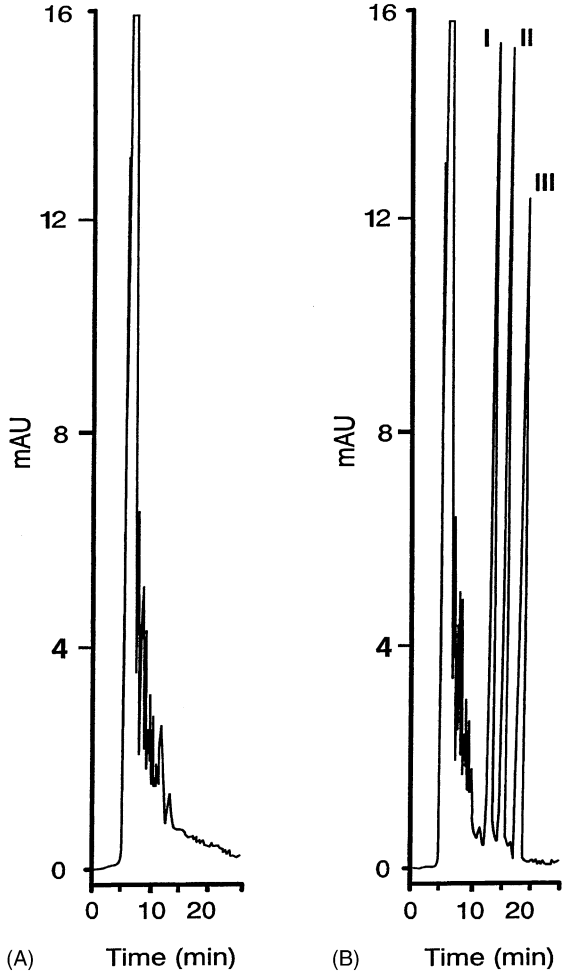


Fig. 2. Examples of chromatograms: (A) extract of 1 ml flavanone-free urine sample from a volunteer who followed a one week citrus-free diet; (B) extracts of a urine sample obtained from the same volunteer, after taking a single oral dose of 300 mg each of naringin and hesperidin. Peaks: I = naringenin; II = hesperetin and III = internal standard (7-ethoxycoumarin).

eliminated the interfering peaks and resulted in high recoveries of all analytes. The washing of C18 cartridges, with 5 ml of 10% methanol in 0.01 M HCl followed by 15 ml of distilled water, was found to be critical for giving clear chromatograms without interferering peaks at the retention time of hesperetin, naringenin, or internal standard.

### 3.3. Linearity

The linearity of the method was demonstrated over the concentration range of 50–1200 ng/ml of both hesperetin and naringenin, by assaying nine calibration standards and three quality control samples in triplicate on three separate occasions. Calibration curves were obtained by plotting the peak height ratios of hesperetin/internal standard or naringenin/internal standard ($y$) versus the hesperetin or naringenin concentrations (ng/ml) in spiked flavanone-free urine samples ($x$) resulted in straight lines over the concentration range. Their equations were calculated by using linear regression analysis. Calibration curves were established on each day of analysis and typical calibration curves had the regression equation of $y = 0.09667 + 0.00105\times$ ($r = 0.999$) and $y = 0.24673 + 0.00122\times$ ($r = 0.999$) for hesperetin and naringenin, respectively.

### 3.4. Recovery from urine

The absolute recovery of hesperetin, naringenin, and internal standard was assessed by direct comparison of their peak heights from extracts of flavanone-free urine samples that had been spiked with known concentrations of hesperetin and naringenin and incubated with β-glucuronidase/sulphatase versus those found by direct injection of standards of the same concentration prepared in methanol/water (1:1). Recovery of internal standard was determined in the same solutions simultaneously. The mean recoveries for hesperetin were 74.0 ± 4.3, 73.1 ± 2.1, and 74.6% ± 1.9% at the 200, 400, and 800 ng/ml concentrations, respectively, and for naringenin were 70.9 ± 5.0, 72.4 ± 2.6, and 71.4% ± 1.8% at the 200, 400, and 800 ng/ml concentrations, respectively, ($n = 6$). Mean recovery of internal standard at 1400 ng/ml was 86.9% ± 1.3% ($n = 18$). The results provide evidence that there was no major loss during sample processing.

### 3.5. Accuracy and precision

Intra-day precision was determined by calculating the % R.S.D. for six determinations at each concentration of three quality control samples (200, 400, and 800 ng/ml) and was found to be less than 5.3% for both hesperetin and naringenin, respectively. Intra-day accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 95.5% for both flavanone aglycones (Table 1).

Inter-day psrecision and accuracy were assessed by assaying three quality control samples in triplicate on three separate occasions. Inter-day precision for hesperetin was 8.2% based on % R.S.D. values of 8.2, 3.7, and 2.1% for quality control samples containing 200, 400, and 800 ng/ml, respectively and for naringenin was 7.8% based on % R.S.D. values of 7.8, 3.5, and 2.2% for quality control samples containing 200, 400, and 800 ng/ml, respectively. Inter-day accuracy for hesperetin was found to be better than 95.5% and for naringenin was better that 94.9% (Table 1).

### 3.6. Limit of quantification

The limit of quantification, defined as the lowest quantifiable concentration on the calibration curve at which both accuracy and precision should be within the maximum tolerable CV of 20%, was deemed to be 50 ng/ml for both hesperetin and naringenin using a 1 ml flavanone-free urine sample.

Table 1
Intra- and inter-day accuracy and precision for naringenin and hesperetin in quality control samples in flavanone-free urine incubated with β-glucuronidase/sulphatase

| Nominal concentration (ng/ml) | Mean found concentration (ng/ml) | Accuracy[a] (%) | Precision[b] (R.S.D., %) |
|---|---|---|---|
| Naringenin intra-day | | | |
| 200 | 191.7 | 95.8 | 5.3 |
| 400 | 405.8 | 101.5 | 2.8 |
| 800 | 774.8 | 96.9 | 1.9 |
| Inter-day | | | |
| 200 | 189.7 | 94.9 | 7.8 |
| 400 | 403.9 | 101.0 | 3.5 |
| 800 | 773.5 | 96.7 | 2.2 |
| Hesperetin Intra-day | | | |
| 200 | 190.9 | 95.5 | 4.9 |
| 400 | 413.6 | 103.4 | 3.2 |
| 800 | 776.3 | 97.0 | 1.6 |
| Inter-day | | | |
| 200 | 190.9 | 95.45 | 8.2 |
| 400 | 408.5 | 102.1 | 3.7 |
| 800 | 774.6 | 96.8 | 2.1 |

[a] Accuracy: found concentration expressed in % of the nominal concentration.
[b] R.S.D.: relative standard deviation.

### 3.7. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method conditions. The robustness of the method was performed by evaluating small variations in mobile phase composition (±2%). Variation of the methanol content in the mobile phase in the 38–42% range significantly affects the elution behavior. Increasing the organic content in the mobile phase resulted in a decrease of the retention time for all analytes. However, the concentration of the quality control samples was not significantly affected by these changes (Table 2). Therefore, the method is robust with respect to small changes in mobile phase composition. Furthermore, the selectivity of the method over time was not significantly affected and more than 900 sample injections were performed without loss of column performance.

### 3.8. Quantitative analysis of urine samples

The present method was applied to perform the determination of the urine concentration of hesperetin and naringenin, after oral administration of 300 mg of each of their glycosides, hesperidin and naringin, to a healthy male volunteer. Fig. 3 illustrates the cumulative amount excreted in urine of total (free + conjugated) hesperetin and naringenin. The data showed that there is a delay of about 6 h for the urinary excretion of both flavanone aglycones, which might be attributed to their slow absorption from the gastrointestinal tract, due to prior hydrolysis of the flavanone glycosides hesperidin and naringin by the enzymes of intestinal bacteria. The fraction of the oral dose excreted in urine was 0.65% for hesperetin and 4.25% for naringenin, respectively. These values are comparable to those reported previously [16].

### 3.9. Reproducibility of enzyme hydrolysis

Intra-day reproducibility of total (free + conjugated) hesperetin and naringenin through enzyme hydrolysis was determined by subjecting six aliquots of two urine samples (obtained at 3–6 h and 15–24 h collecting periods) from a healthy male volunteer who received an oral administration of 300 mg of each of the corresponding glycosides, hesperidin and naringin. Accordingly, inter-day reproducibility of total (free + conjugated) hesperetin and naringenin through enzyme hydrolysis was determined by subjecting three aliquots on three separate occasions of the same two urine samples.

The samples were processed through the method (hydrolysis with β-glucuronidase/sulphatase and solid phase extraction) and the eluate of each sample was injected in the HPLC system. Based on absolute peak heights of hesperetin and naringenin, a R.S.D. of less than 10% was considered

Table 2
Robustness of the method for quality control samples by varying the organic content in mobile phase by ±2%

| Nominal Concentration (ng/ml) | Mean concentration found (ng/ml) normal mobile phase 40% MeOH | Mean concentration found (ng/ml) 42% MeOH | Difference from normal conditions (%) | Mean concentration found (ng/ml) 38% MeOH | Difference from normal conditions (%) |
|---|---|---|---|---|---|
| Naringenin[a] | RT = 13.1[b] | RT = 11.7 | | RT = 14.9 | |
| 200 | 190.8 | 191.8 | 0.5 | 188.7 | 1.1 |
| 400 | 399.4 | 400.1 | 0.2 | 395.8 | 0.9 |
| 800 | 783.8 | 786.4 | 0.3 | 781.7 | 0.3 |
| Hesperetin[a] | RT = 16.1 | RT = 13.8 | | RT = 18.7 | |
| 200 | 192.5 | 194.3 | 0.9 | 190.6 | 1.0 |
| 400 | 402.9 | 405.7 | 0.7 | 401.8 | 0.3 |
| 800 | 790.9 | 792.3 | 0.2 | 788.9 | 0.3 |
| Is[a] | RT = 19.2 | RT = 16.9 | | RT = 21.6 | |

[a] Each quality control sample was injected three times.
[b] Retention time (min).

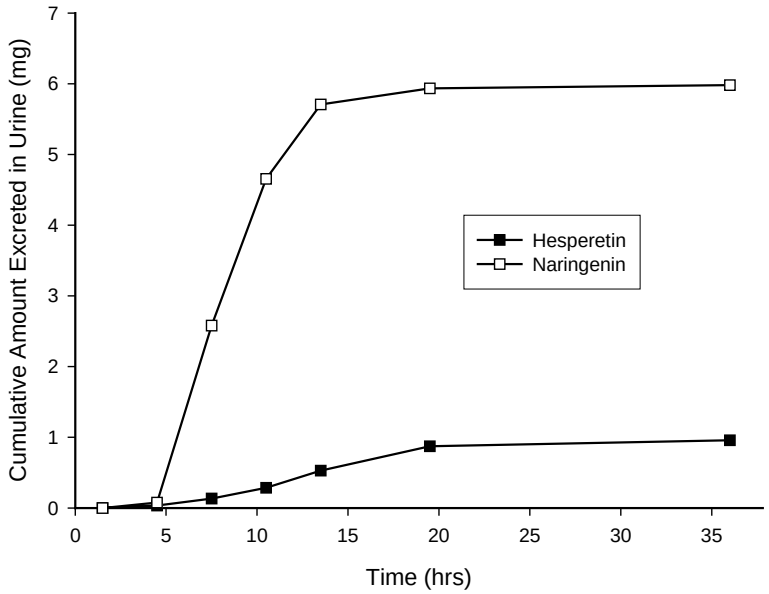


Fig. 3. Cumulative urinary excretion curves for hesperetin and naringenin after a single oral administration of 300 mg each of hesperidin and naringin.

to be suitable for acceptable reproducibility. The intra- and inter-day reproducibility for total hesperetin was less than 3.1 and 3.9%, respectively, and for total naringenin was less than 2.8 and 3.6%, respectively.

### 3.10. Stability

Freeze–thaw stability of free hesperetin and naringenin urine samples was determined by subjecting six aliquots of two QC samples of low and high concentration (200 and 800 ng/ml) to three consecutive freeze–thaw cycles and comparing the concentrations with those of freshly thawed QC samples.

Short-term stability of free naringenin and hesperetin urine samples at room temperature was determined by subjecting six aliquots of two QC samples of low and high concentration (200 and 800 ng/ml) to room temperature for 24 h and comparing the concentrations with those of freshly thawed QC samples.

An error of less than 10% from the expected concentrations for the test QC samples was considered to be acceptable for both stability tests. Stability data of naringenin and hesperetin QC samples are summarized in Table 3. Urine QC samples were found to be stable through all three freeze–thaw cycles and at room temperature for 24 h with percentage errors ranging from −1.8 to −3.6% and from −1.0 to −3.0% for hesperetin and naringenin, respectively.

### 3.11. Conclusion

The results presented in this study show that a solid-phase extraction reversed-phase HPLC-UV assay for the simultaneous determination of hesperetin and naringenin in human urine was developed and validated. The method is selective, precise, accurate, and robust and seems to be suitable for the quantitative determination of total (free and conjugated) hesperetin and naringenin in urine samples obtained in the conduct of pharmacokinetic studies in humans, after oral administration of their flavanone glycosides hesperidin and naringin. The determination of urinary flavanone aglycones hesperetin and naringenin may be useful biomarkers for citrus flavonoids intake.

Table 3
Stability of free naringenin and hesperetin urine QC samples

| Stability test | | Hesperetin | | Naringenin | |
|---|---|---|---|---|---|
| | | 200 ng/ml | 800 ng/ml | 200 ng/ml | 800 ng/ml |
| Freeze–thaw stability (3 cycles) | Mean ± S.D. | 193.6 ± 16.1 | 785.6 ± 19.1 | 195.8 ± 15.8 | 792.0 ± 23.7 |
| | R.S.D. (%) | 8.3 | 2.4 | 8.1 | 3.0 |
| | % Error[a] | 3.2 | −1.8 | −2.1 | −1.0 |
| Short-term stability (24 h) | Mean ± S.D. | 192.8 ± 14.5 | 784.8 ± 17.4 | 194.0 ± 11.7 | 790.4 ± 19.5 |
| | R.S.D. (%) | 7.5 | 2.2 | 6.0 | 2.5 |
| | % Error | −3.6 | −1.9 | −3.0 | −1.2 |

[a] % Error $= \frac{\text{Theoretical} - \text{Mean}}{\text{Theoretical}} \times 100$.

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