

## Short communication

# Validated high-performance liquid chromatographic method utilizing solid-phase extraction for the simultaneous determination of naringenin and hesperetin in human plasma

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## Abstract

Naringenin and hesperetin, the aglycones of the flavanone glucosides naringin and hesperidin occur naturally in citrus fruits. They exert a variety of pharmacological effects such as antioxidant, blood lipid-lowering, anticarcinogenic and inhibit selected cytochrome P-450 enzymes resulting in drug interactions. A specific, sensitive, precise, and accurate solid-phase extraction high-performance liquid chromatographic (HPLC) assay for the simultaneous determination of naringenin and hesperetin in human plasma was developed and validated. After addition of 7-ethoxycoumarin as internal standard, plasma samples were incubated with  $\beta$ -glucuronidase/sulphatase, and the analytes were isolated from plasma by solid-phase extraction using C<sub>18</sub> cartridges and separated on a C<sub>8</sub> reversed phase column with methanol/water/acetic acid (40:58:2, v/v/v) as the eluent at 45 °C. The method was linear in the 10–300 ng/ml concentration range for both naringenin and hesperetin ( $r > 0.999$ ). Recovery for naringenin, hesperetin and internal standard was greater than 76.7%. Intra- and inter-day precision for naringenin ranged from 1.4 to 4.2% and from 1.9 to 5.2%, respectively, and for hesperetin ranged from 1.3 to 4.1% and from 1.7 to 5.1%, respectively. Accuracy was better than 91.5 and 91.3% for naringenin and hesperetin, respectively.

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## 1. Introduction

Flavonoids are phenolic compounds ubiquitous in foods of plant origin [1]. They occur naturally as glucosides and consist of flavones, flavonols, isoflavones and flavanones [2]. Naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside) and hesperidin (3',5,7-trihydroxy-4'-methoxyflavanone-7-rhamnoglucoside) (Fig. 1), also called citrus flavonoids, are the main flavanone glucosides naturally occurring in citrus fruits, such as grapefruit (*Citrus paradisi*) and orange (*Citrus sinensis*) [3,4]. Citrus flavonoids exert antioxidant [5], blood lipid-lowering [6,7] and anticarcinogenic activities [8]. They also alter the pharmacokinetics of a variety of clinically used drugs resulting in drug interactions by inhibiting selected cytochrome P-450 enzymes, such as CYP1A2 and CYP3A4 [9].

Different studies indicate that the flavanone glucosides naringin and hesperidin are hydrolyzed in the gastrointestinal tract before absorption of their aglycones naringenin and hesperetin which are recovered in plasma as glucuronides and sulphoglucuronides [10–12]. The conduct of pharmacokinetic studies in humans after oral administration of naringin and hesperidin requires a specific and sensitive analytical method for the simultaneous determination of their aglycones naringenin and hesperetin in plasma samples. A number of high-performance liquid chromatographic (HPLC) methods have been reported for the quantitative determination of either naringenin [10,13–15] or hesperetin [16] in biological fluids. Recently, two HPLC methods combined with UV detection [3] or electrochemical detection [17], for the simultaneous determination of naringenin and hesperetin in plasma have been described. However, these methods are not sufficiently sensitive, or there was no internal standard and are not directly applicable for the simultaneous quantitative determination of naringenin and hesperetin in human plasma.

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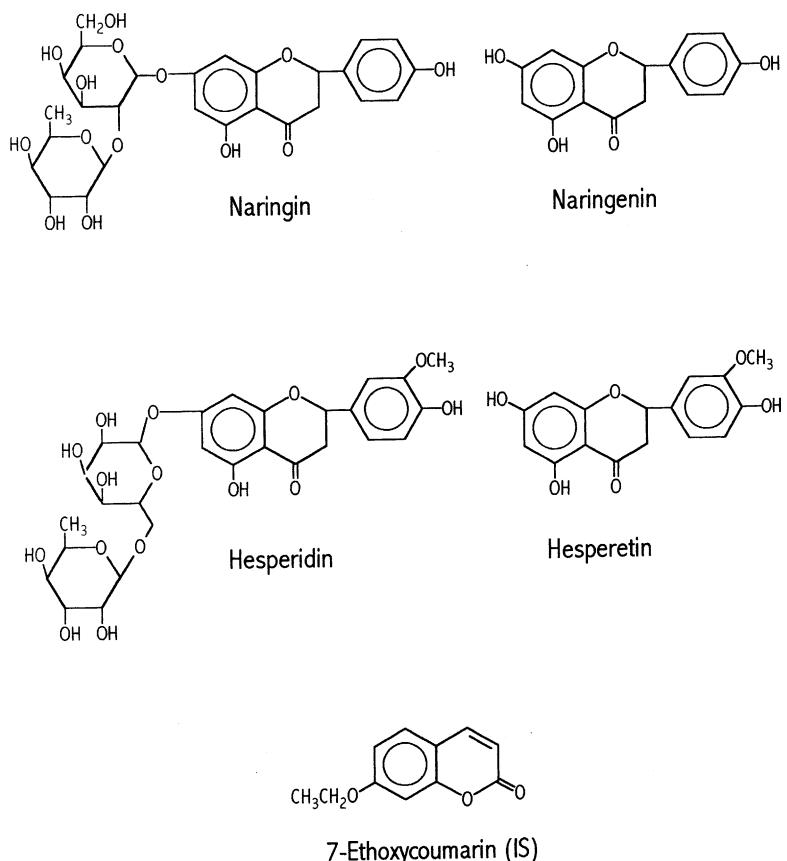


Fig. 1. Chemical structures of citrus flavanone glucosides naringin and hesperidin and their corresponding aglycones naringenin and hesperetin and the IS (7-ethoxycoumarin).

Therefore, the objective of the present investigation was to develop and validate a solid-phase extraction HPLC method for the simultaneous determination of naringenin and hesperetin in plasma samples, after enzymatic hydrolysis of their conjugates, suitable to conduct pharmacokinetic studies after the intake of the flavonoid glucosides naringin and hesperidin, either as pure substances or in foods that contain them.

## 2. Experimental

### 2.1. Chemicals and reagents

Naringenin (4'-5,7-trihydroxyflavanone), 95%, hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone), 95%, internal standard 7-ethoxycoumarin and  $\beta$ -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). Bakerbond C<sub>18</sub> cartridges, 3 ml 500 mg, were supplied from J.T. Baker (Deventer, The Netherlands). All other chemicals and solvents used were of analytical grade.

### 2.2. Chromatographic conditions

The analyses were performed using a high-performance liquid chromatographic 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- $\mu$ l loop (Rheodyne, Cotati, CA, USA) and an integrator (model 4290). Separation was performed on a Macherey Nagel Nucleosil C<sub>8</sub> analytical column (5  $\mu$ m particle size, 250  $\times$  4.6 mm i.d.), proceeded by a guard column (20  $\times$  4.6 mm i.d.) dry packed with pellicular ODS material (37–53  $\mu$ m).

The mobile phase consisted of methanol/water/acetic acid (40:58:2, v/v/v) and was filtered through a 0.45- $\mu$ m pore size nylon filter (Alltech) and degassed by ultrasonic treatment before use. The HPLC system was operated isocratically at a flow rate of 1 ml/min at 45 °C and the detector was set at 288 nm. The integrator attenuation was 2 and the chart speed was 0.1 cm/min.

### 2.3. Standard solutions

Stock solutions of naringenin, hesperetin and the internal standard 7-ethoxycoumarin were prepared daily

by dissolving appropriate amounts of the compounds in methanol to achieve concentrations of 400 µg/ml for each compound. Appropriate dilutions of the stock solution of naringenin and hesperetin were made with methanol/water (1:1) to prepare the working solutions containing 0.5, 1.25, 2.5, 3.75, 5, 7.5, 10, 12.5, and 15 µg/ml of naringenin and hesperetin. The stock solution of internal standard was further diluted with methanol/water (1:1) to give the internal standard working solution containing 25 µg/ml 7-ethoxycoumarin.

Calibration standard samples were freshly prepared in 1 ml of human plasma by spiking control pools with 20 µl of the naringenin and hesperetin working solutions and 20 µl of the internal standard working solution to yield concentrations corresponding to 10, 25, 50, 75, 100, 150, 200, 250, and 300 ng/ml of plasma.

#### 2.4. Quality control samples

Volumes of 25 ml of human plasma were spiked with appropriate volumes of naringenin and hesperetin stock solutions to obtain quality control samples containing 50, 100 and 200 ng/ml of naringenin and hesperetin. These 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 plasma samples as quality controls for the purpose of checking recovery of analytes in the daily analyses of plasma samples.

#### 2.5. Sample preparation

Plasma samples (1 ml) spiked with naringenin, hesperetin and internal standard were incubated with 100 µl of 1 mol/l of sodium acetate buffer (pH 5) and 40 µl of β-glucuronidase/sulphatase (crude preparation from *H. pomatia*, Sigma) for 18 h at 37 °C [12]. The hydrolyzed plasma samples were diluted with 2 ml of phosphate buffer (0.1 mol/l, pH 2.4), then they were applied to the extraction C<sub>18</sub> 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 5.0 ml of 10% methanol in 0.01 M HCl and then with 3.0 ml of 0.01 M HCl and purged with air. Naringenin, hesperetin 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 dissolved in 100 µl of mobile phase and a 20 µl volume was injected into the chromatographic system for quantification.

### 3. Results and discussion

#### 3.1. Chromatographic separation and plasma interferences

Reversed-phase HPLC methods with C<sub>18</sub> or C<sub>8</sub> columns are usually used for the separation of naturally occurring

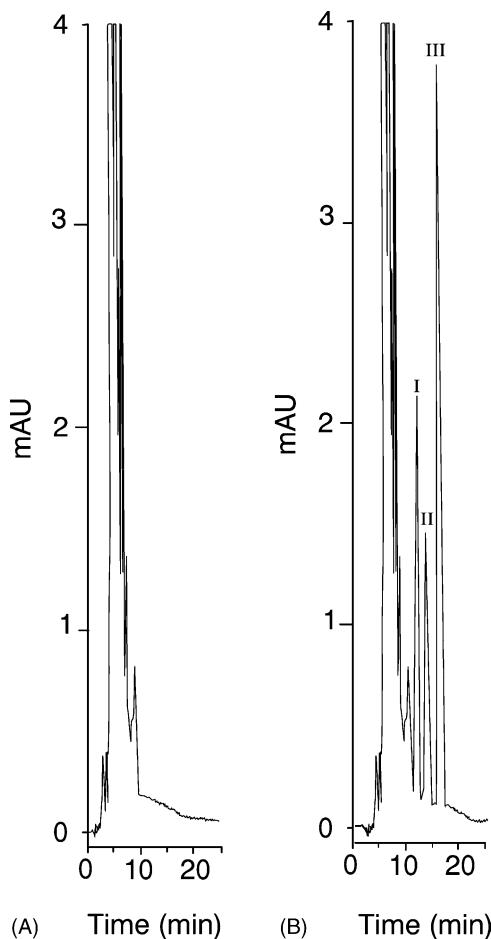


Fig. 2. Examples of chromatograms: (A) extract of 1 ml hydrolyzed flavanone-free human plasma; and (B) extract of a hydrolyzed plasma sample obtained from a volunteer, who followed a 1 week citrus-free diet, 10 h after a single oral dose of 300 mg of naringin and 300 mg of hesperidin, containing 54.2 ng/ml of total naringenin and 38.4 ng/ml of total hesperetin. Peaks: (I) naringenin, (II) hesperetin, and (III) internal standard (7-ethoxycoumarin).

flavonoid glucosides and their aglycones in crude plant materials, food products and biological fluids. Mobile phases consisting of acetonitrile/water as well as methanol/water are generally used. The chromatographic conditions described in this assay were arrived at after investigating several mobile phases and internal standards. The addition of acetic acid to the solvent system used (methanol/water/acetic acid 40:58:2, v/v/v) as well as the column temperature (45 °C) were important factors for improving separation of naringenin, hesperetin and internal standard in plasma and giving symmetrical and sharp peaks. Typical chromatograms obtained from extracts of a hydrolyzed flavanone-free plasma and hydrolyzed plasma sample obtained from a volunteer, who followed a 1 week citrus-free diet, 10 h after a single oral dose of 300 mg of naringin and 300 mg of hesperidin, containing 54.2 ng/ml of total naringenin and 38.4 ng/ml of total hesperetin, are illustrated in Fig. 2. The measured plasma levels of flavanones were comparable to those

reported in previous studies after the intake of orange and grapefruit juice, taking into account the different dosing of flavanone glycosides naringin and hesperetin [3,12]. No endogenous plasma components or flavanone metabolites elute at the retention time of naringenin, hesperetin or internal standard. Naringenin, hesperetin, and internal standard were eluted in 11.6, 14.0, and 16.9 min, respectively.

### 3.2. Sample preparation

Different attempts to extract naringenin, hesperetin, and internal standard by liquid–liquid extraction from plasma co-extracted endogenous plasma components which severely interfered with analytes causing difficulties with quantification. These results led us to investigate solid-phase extraction for sample pretreatment utilizing C<sub>18</sub> cartridges. A solution of 10% methanol in 0.01 M HCl was used to wash the cartridges, after the application of plasma samples incubated with  $\beta$ -glucuronidase/sulphatase, to obtain good recoveries for all analytes. The method developed finally gave clean chromatograms without interfering peaks at the retention time of naringenin, hesperetin and internal standard.

### 3.3. Linearity

The linearity of the method was demonstrated over the concentration range of 10–300 ng/ml of both naringenin and hesperetin, 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 naringenin/internal standard or hesperetin/internal standard (*y*) versus the naringenin or hesperetin concentrations (ng/ml) in spiked plasma samples (*x*). 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  $y = -0.00484 (\pm 0.00909) + 0.00408x (\pm 0.00006)$  (*r* = 0.999) and  $y = 0.00580 (\pm 0.01009) + 0.00398x (\pm 0.00006)$  (*r* = 0.999) for naringenin and hesperetin, respectively.

### 3.4. Recovery from plasma

The absolute recovery of naringenin, hesperetin, and internal standard was assessed by direct comparison of peak heights from extracts of plasma samples incubated with  $\beta$ -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 naringenin were  $77.3 \pm 5.8\%$ ,  $76.7 \pm 2.6\%$ , and  $77.4 \pm 1.6\%$  at the 50, 100, and 200 ng/ml concentrations, respectively and for hesperetin were  $80.8 \pm 6.2\%$ ,  $79.3 \pm 2.7\%$ , and  $79.6 \pm 1.8\%$  at the 50, 100, and 200 ng/ml

Table 1

Intra- and inter-day accuracy and precision for naringenin and hesperetin in quality control samples in human plasma incubated with  $\beta$ -glucuronidase/sulphatase

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	Accuracy (%) <sup>a</sup>	Precision (%R.S.D.) <sup>b</sup>
Naringenin			
Intra-day			
50	46.9	93.8	4.2
100	94.6	94.6	1.9
200	188.7	94.4	1.4
Inter-day			
50	46.8	93.6	5.2
100	92.4	92.4	2.4
200	183.0	91.5	1.9
Hesperetin			
Intra-day			
50	46.5	93.0	4.1
100	94.6	94.6	1.9
200	184.7	92.4	1.3
Inter-day			
50	47.6	95.2	5.1
100	94.4	94.4	2.5
200	182.6	91.3	1.7

<sup>a</sup> Accuracy: found concentration expressed in % of the nominal concentration.

<sup>b</sup> R.S.D.: relative standard deviation.

concentrations, respectively (*n* = 6). Mean recovery of internal standard at 500 ng/ml was  $85.5 \pm 1.7\%$  (*n* = 18).

### 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 (50, 100, and 200 ng/ml) and was found to be less than 4.2% for both naringenin and hesperetin, respectively. Intra-day accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 92.4% for both flavanone aglycones (Table 1).

Inter-day precision and accuracy were assessed by assaying three quality control samples in triplicate on three separate occasions. Inter-day precision for naringenin was 5.2% based on %R.S.D. values of 5.2, 2.4, and 1.9% for quality control samples containing 50, 100, and 200 ng/ml, respectively and for hesperetin was 5.1% based on %R.S.D. values of 5.1, 2.5, and 1.7% for quality control samples containing 50, 100, and 200 ng/ml, respectively. Inter-day accuracy for naringenin was found to be 93.6, 92.4, and 91.5% for samples containing 50, 100, and 200 ng/ml, respectively, and for hesperetin was 95.2, 94.4, and 91.3% for samples containing 50, 100, and 200 ng/ml, respectively (Table 1).

### 3.6. Limit of quantification

The limit of quantification, defined as the lowest quantifiable concentration on the calibration curve at which both

Table 2

Robustness of the method for quality control samples by varying the column temperature by  $\pm 3^\circ\text{C}$ 

Nominal concentration (ng/ml)	Mean concentration found (ng/ml) (normal conditions, 45 °C)	Mean concentration found (ng/ml) (42 °C)	Difference from normal conditions (%)	Mean concentration found (ng/ml) (48 °C)	Difference from normal conditions (%)
Naringenin <sup>a</sup>	RT = 11.6 <sup>b</sup>	RT = 12.3		RT = 10.6	
50	49.5	47.7	3.6	49.8	0.6
100	100.8	101.0	0.2	100.2	0.6
200	198.4	197.9	0.3	197.9	0.5
Hesperetin <sup>a</sup>	RT = 14.0	RT = 14.8		RT = 12.6	
50	48.4	47.4	2.1	47.5	1.9
100	96.1	99.7	3.7	100.8	4.9
200	198.9	197.8	0.6	198.8	0.1
IS <sup>a</sup>	RT = 16.9	RT = 17.9		RT = 15.1	

<sup>a</sup> Each quality control sample was injected three times.<sup>b</sup> Retention time (min).

accuracy and precision should be within the maximum tolerable CV of 20%, was deemed to be 10 ng/ml for both naringenin and hesperetin using a 1 ml plasma sample. This limit of quantification is better or comparable to that reported previously [3,17] and is sufficient to determine plasma concentrations of naringenin and hesperetin in the conduct of pharmacokinetic studies.

### 3.7. Robustness

The robustness of the method was assessed by evaluating small changes in column temperature. Increasing the column temperature resulted in a decrease of retention time for all analytes. However, the concentration of the quality control samples are not significantly affected by these changes (Table 2). Therefore the analytical method is robust with respect to small changes in column temperature (range: 42–48 °C). Furthermore, the selectivity of the method is not significantly affected with aging of the column and analytical performance remained satisfactory after more than 800 sample injections.

### 3.8. Conclusion

In conclusion, a reversed-phase HPLC assay using solid-phase extraction with C<sub>18</sub> cartridges, for the simultaneous determination of naringenin and hesperetin in plasma samples incubated with  $\beta$ -glucuronidase/sulphatase was developed and validated. The method was precise, accurate, selective, robust, and sufficiently sensitive and seems suitable for the quantitative determination of plasma samples

obtained in the conduct of clinical pharmacokinetic studies of total naringenin and hesperetin after oral intake of their flavonoid glucosides to humans.

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