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Analysis of hesperetin enantiomers in human urine after ingestion of blood orange juice by using nano-liquid chromatography

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ABSTRACT

Hesperetin (HT) is a flavanone abundantly found in citrus fruits. It has been reported that HT possesses significant antioxidant, anticancer, anti-inflammatory and analgesic activities. This explains the necessity of developing new methods more powerful and sensitive for analyzing HT in biological fluids. Taking into account the chiral nature of HT, the study of the stereospecific kinetics of in vitro and in vivo metabolism and tissue distribution could be a useful tool for further understanding stereoselective biotransformations in human body. A simple nano-liquid chromatographic method for the determination of the enantiomeric composition of hesperetin in human urine was developed. Chiral separation was achieved using a 100 μm I.D. capillary, packed with phenyl-carbamate-propyl-β-cyclodextrin stationary phase, employing a mobile phase composed by a mixture of triethylammonium acetate buffer (1%, v/v, pH 4.5) and water/methanol (30:70, v/v) at room temperature. The detection was done by using on-column UV detector at 205 nm. Calibration curves were linear in the studied concentration range from 0.25 to 25 μg/mL ($r^2 > 0.999$). Precision assay was <4.5% and was within 3% at the limit of quantification (0.5 μg/mL). The recovery of 7-ethoxycoumarin (IS), R- and S-hesperetin was greater than 82.48%, utilizing a liquid–liquid extraction procedure. The developed method was successfully applied to the determination of hesperetin enantiomers in urine samples obtained from a male volunteer, after the ingestion of 1 L of a commercial blood orange juice.

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1. Introduction

Recently, the consumption of citrus fruits and juices have been widely promoted and encouraged by numerous international health organizations [1]. The well-established benefits of citrus products are mainly due to the presence of the most important class of secondary plant metabolites, namely flavonoids [2].

Flavonoids are naturally occurring polyphenolic compounds usually present in oranges, other citrus fruits and herbal products. They are conjugated with β-glycosides, e.g., in hesperidin the sugar is bonded at position 7 of the flavanone (3′,5,7-trihydroxy-4′-methoxyflavanone-7-rhamnoglucoside) (Fig. 1A). After juice ingestion, the rutinose sugar moiety is rapidly cleaved off during the transit in the gastrointestinal tract and liver. The aglycone molecule, hesperetin (±-3,5,7-trihydroxy-4′-methoxyflavanone, HT), a chiral flavonoid, exists in two enantiomeric forms due to the presence of an asymmetric carbon at position two (Fig. 1B). The 2S configuration is predominant in nature [3].

Hesperidin (Vitamin P) and hesperetin show evidence of antifungal, antioxidant, antibacterial, antiviral, anticancer, antiallergic, anti-inflammatory and analgesic [4–7] properties. All these health benefits easily explain all the research done till now and dealing with the study of hesperetin behavior in biological fluids [8–12]. However, these works did not take into account the chiral specificity of hesperetin. In view of the fact that adsorption, distribution, metabolism and elimination may be stereoselective [13], there is a need of specific analytical methods capable to separate HT enantiomers.

Despite several successful methods, including micellar electrokinetic chromatography (MEKC) [14], capillary electrophoresis (CE) [15] and high-performance liquid chromatography (HPLC) [16,17], have been used to enantioseparate hesperetin, only few papers dealing with the enantioselective identification and quantification of HT in biological fluids were reported [3,18]. Yáñez et al. developed a HPLC method for the separation of these chiral compounds using a commercial cellulose based silica column [3]. Recently, racemic hesperetin was resolved in its enantiomers by our

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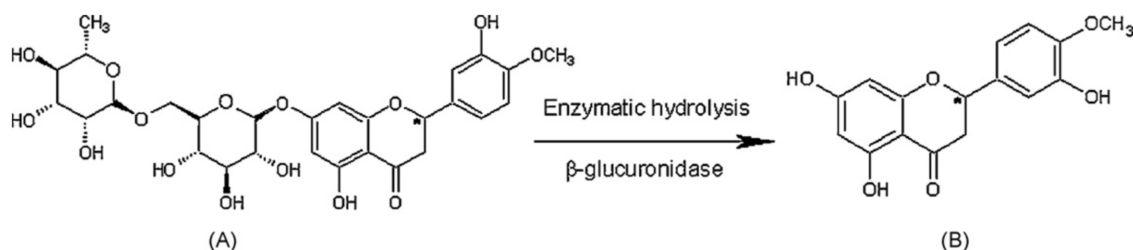

Fig. 1. Structures of hesperidin (A) and hesperetin (B).

group using nano-liquid chromatography (nano-LC) by means of a phenyl-carbamate-propyl-β-cyclodextrin stationary phase [19].

It is well known that the use of chromatographic columns with lower I.D. may be useful to achieve better results in terms of efficiency, resolution and sensitivity.

Passing from 4.6 mm (classical column) to 100 μm (capillary column) sensitivity will increase of about 2000 folds. This has been theoretically demonstrated and it was accounted for a reduction of peak dilution, due to the lower flow rate used with columns of small I.D. [20]. This feature, together with the low costs owing to the use of minute amounts of chiral stationary phase and the reduced volumes of mobile phase, proposes nano-LC as an alternative or complementary separation technique to the classical ones such as HPLC [21].

In the present work we describe the quantification of hesperetin enantiomers in urine of a healthy volunteer by using nano-LC. Samples were collected after juice intake (0–24 h). An enzymatic procedure was applied to make the cleavage of the glycoside moiety of hesperidin. Afterwards, a liquid–liquid extraction method was performed to recovery analytes. The stereospecific study was carried out using a phenyl-carbamate-hydroxyl-β-cyclodextrin based stationary phase prepared using a procedure previously described [22].

To the best of our knowledge, this is the first study concerning the determination of the enantiomeric composition of hesperetin in biological fluids by nano-LC.

2. Experimental

2.1. Chemicals and reagents

Racemic hesperetin, hesperidin, 7-ethoxycoumarin, β-glucuronidase H. pomatia Type-HP-2 were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade methanol, glacial acetic acid, triethylamine (TEA), HCl, ascorbic acid, sodium hydroxide and ethylacetate were from Carlo Erba (Milan, Italy). Distilled water was deionized by using a Milli-Q system (Millipore, Bredford, MA, USA). Oranfrizer® Blood Orange Juice (BOJ) was purchased from a local supermarket (Rome, Italy). The squeezed and pasteurized BOJ was obtained from Sicilian organic culture (blood oranges and mandarins).

2.2. Capillary column preparation

Fused silica capillaries, 100 μm I.D. × 375 μm O.D. were purchased from Composite Metal Services (Hallow, Worcestershire, UK) and packed with the cyclodextrin based chiral stationary phase (CD-CSP). The CD-CSP was synthesized by grafting 2-hydroxypropyl-β-CD onto silica gel (5 μm) following an earlier described method [22,23]. Capillaries were packed in our laboratory following a previous published procedure [24]. Briefly, one end of the capillary was connected to a mechanical temporary frit, Valco (Houston, Texas, USA) to retain the packing material. The other end of the capillary was joined to a stainless steel HPLC pre-column (5 cm × 4.1 mm I.D.), which was used as reservoir for the slurry. A series 10 LC pump, PerkinElmer (Palo Alto, CA, USA) was used for the packing procedure. Few milligrams of LiChrospher 100RP-18, 5 μm, 100 Å (Merck, Darmstadt, Germany) were suspended in 1 mL of acetone. The empty capillary was packed with this mixture for about 10 cm.

The slurry was then removed from the reservoir and the capillary flushed with water in order to completely eliminate the organic solvent. The frit was done by sintering the reversed phase particles for 6 s at 700 °C with a laboratory made heating wire, flushing continuously with water. The temporary frit was removed and the excess of packing material was eliminated by washing with water. Afterwards, the column was packed with the chiral stationary phase suspended in MeOH/i-PrOH mixture (1:1, v/v) for 22.0 cm. Finally the column was packed again with the RP-18 particles to prepare the second frit, following the same procedure above discussed. The detection window was prepared by eliminating the polymide layer with a razor. The capillary was cut at the desired length and equilibrated with the mobile phase using the LC pump at 35 MPa. The effective and total lengths of the column were 24.0 and 34.0 cm, respectively.

2.3. Instrumentation and chromatographic conditions

The nano-LC experiments were carried out using a laboratory assembled instrumentation. The system included an Accela TM micro-LC pump, Thermo Electron Corporation (San Jose, California, USA) which was controlled by a software system (Xcalibur 2.0). The flow rate was reduced from μL/min to nL/min using a static splitting device. For this purpose, a stainless steel T piece, Vici Valco (Houston, Texas, USA) was connected at one end to the pump through a 50 cm × 130 μm I.D. PEEK tube. The other two ends were connected to the injector valve by means of a stainless steel tube of 7 cm × 500 μm I.D. and to the waste using a 50 cm × 50 μm I.D. fused silica capillary. The flow rate was estimated connecting the end of the capillary column to a micro-syringe through a teflon tube and measuring the mobile phase volume after 5 min; during the present work the flow rate was fixed at 400 nL/min.

Sample injection was done by using a 100 nL 4 port micro-volume injection valve, Vici Valco (Houston, Texas, USA). On-column detection was carried out at 205 nm with a Spectra 100 photometric UV–vis detector, Thermo Separation Products (San Jose, California, USA). Data were collected with a Shimadzu CR5A Chromatopac integrator (Kyoto, Japan).

All chromatographic experiments were carried out at room temperature; the mobile phase consisted on a mixture of MeOH/water (70/30, v/v) and 1% triethylamine acetate buffer pH 4.5 (TEAA).

2.4. Standard solutions

Stock solutions of hesperetin and internal standard (7-ethoxycoumarin) were prepared by dissolving a proper amount of the compounds in methanol to a final concentration of 1 mg/mL. The prepared IS solution was further diluted with methanol to make

a working internal standard solution of 100 μg/mL. These standards were protected from light and stored at −20 °C between uses, for no longer than 1 month.

Standard mixtures used for calibration curves were prepared daily from the stock solution of hesperetin by sequential dilution with blank biological fluid to yield target concentrations of 0.25, 0.5, 1, 2.5, 5, 10 and 25.0 μg/mL.

2.5. Sample preparation and analysis

To the samples (0.5 mL), 50 μL of 100 μg/mL IS, 100 μL of 0.1 N HCl and 1 mL of ethylacetate were added. The mixture was vortexed for 1 min, ultrasonicated for 1 min and centrifuged at 3000 rpm for 8 min. The organic layer of supernatant was collected and the extraction procedure was repeated three times.

All the supernatant fractions were combined and evaporated to dryness under a nitrogen stream. The residue was reconstituted with 500 μL of mobile phase, vortexed for 1 min and centrifuged at 3000 rpm for 5 min, in order to ensure the complete removal of insoluble urine components. Finally, the supernatant was injected into the chromatographic system for quantification.

2.6. Precision and accuracy

The precision and accuracy studies were performed with replicate assays ($n = 6$) in the same day (within-run precision) and over 3 consecutive days (between-run precision) at three different concentration levels of hesperetin (0.5, 5, 25 μg/mL).

The precision was evaluated calculating the relative standard deviation (RSD) of the enantiomer concentrations. While accuracy was estimated based on the mean percentage error of measured and actual concentration.

2.7. Recovery

The recovery for hesperetin enantiomers from urine was assessed ($n = 3$) at 0.5, 5.0 and 25.0 μg/mL for each enantiomer and the recovery of the internal standard was evaluated at the concentration used in sample analysis (100 μg/mL). Known amounts of hesperetin and 7-ethoxycoumarin were added into 0.5 mL of blank urine to give the above concentrations. The samples were treated as described under Section 2.4 and analyzed by nano-LC. The extraction efficiency was determined by comparing the peak areas of HT enantiomers and IS in urine samples with those obtained analyzing standard solutions at the same concentrations in mobile phase (without extraction).

2.8. Urinary excretion of hesperetin in human

After 5 days of a citrus free diet and an overnight fast, a healthy volunteer drank 1 L of commercial BOJ (Oranfrizer®, Scordia Catania, Italy) in less than 10 min early in the morning. The volunteer was a male subject, non-smoker, with no history of gastrointestinal disease. He had stable food habits and was not vegetarian.

The juice used in the present study was a high quality juice. It underwent to a pasteurization treatment with aseptic packaging, and for this reason it had short sell-by dates (1–2 months), and had to be stored under refrigeration (4 °C). Furthermore, it had to be also consumed in less than 3 days after opening.

The determination of the enantiomeric composition of hesperidin and hesperetin in the juice was quantified. As expected, flavanone aglycone was not detected either because absent or present at a lower concentration than the LOQ. The analysis revealed the presence of two peaks corresponding to the two diastereoisomers of hesperedin at a concentration of 112.81 and 599.55 mg/L of (2R)- and (2S)-hesperedin, respectively.

Urine was collected each hour between 0 and 24 h after intake in acidic washed containers. At the end of each collection, a urine sample volume of 10 mL was kept frozen at −20 °C until analysis. A proper quantity of sodium azide (stabilizing agent) was also added at a concentration of 1 mg/mL.

Hesperetin conjugates were hydrolyzed by incubating 0.5 mL of urine samples under continuous shaking with 50 μL of 1 mol/L of sodium acetate buffer (pH 4.8), 50 μL of 0.1 M of ascorbic acid and 25 μL of β-glucuronidase/sulphatase for 18 h at 37 °C.

The amount of hesperetin found in urine was obtained from the cleavage of flavanone conjugates with glucuronic acid, sulphates and sugars [8].

2.9. Data analysis

Stereospecific quantification was based on calibration curves constructed using peak height ratio (PHR) of hesperetin to internal standard, against hesperetin concentrations using unweighted least squares linear regression.

3. Results and discussion

3.1. Chromatographic conditions

The optimization of the chiral separation of hesperetin in terms of resolution and selectivity using different chromatographic conditions was already done in a previous work [19]. The best separation of HT enantiomers and IS was achieved using a mobile phase based on a mixture of 1% (v/v) (TEAA) pH 4.5 in 30/70 (v/v) water/methanol. As can be observed in Fig. 2A–C, no interfering peaks co-eluting with the compounds of interest were recorded. The confirmation of elution order was done analyzing orange juice and urine sample after ingestion of juice. The results were compared to those reported in literature where these samples contained stereochemically enriched S(−)hesperidin or S(−)hesperetin [3].

The retention times of R- and S-hesperetin in urine sample were approximately 9 and 12 min, respectively. The internal standard eluted at approximately 7 min (Fig. 2B). Comparing these data with those reported by Yáñez et al. [18] it can be concluded that even if similar resolution factors have been obtained, the proposed nano-LC method is faster than the conventional HPLC one employing a commercial cellulose based CSP (analysis time about 30 min).

3.2. Method validation

3.2.1. Sensitivity and linearity

The limits of detection (LOD) and quantification (LOQ) were estimated at a signal-to-noise ratio of 3 and 10, respectively. The LOD value was 0.1 μg/mL for each enantiomer. The LOQ value was 0.5 μg/mL with a between-day RSD of 0.39 and 1.24% for R- and S-hesperetin, respectively and a bias of −1.82 and −2.01% for R- and S-hesperetin, in that order (Table 1).

Good linear relationships ($r^2 = 0.9990$ and 0.9996 for (R) and (S)-hesperetin, respectively) were demonstrated plotting the PHR of the hesperetin enantiomers to the internal standard against the corresponding urine concentrations of hesperetin enantiomers, over a range of 0.25–25 μg/mL. Mean regression lines of $y = 0.0557x + 0.0077$ for R-hesperetin and $y = 0.0487x - 0.0031$ for S-hesperetin. were obtained.

3.2.2. Precision, accuracy, and recovery

Within- and between-run precision (RSD%) calculated during replicate assays ($n = 6$) of hesperetin enantiomers were <4.5% over the studied range of concentrations (Table 1). The intra- and inter-run bias assessed for hesperetin enantiomers varied between −2.01

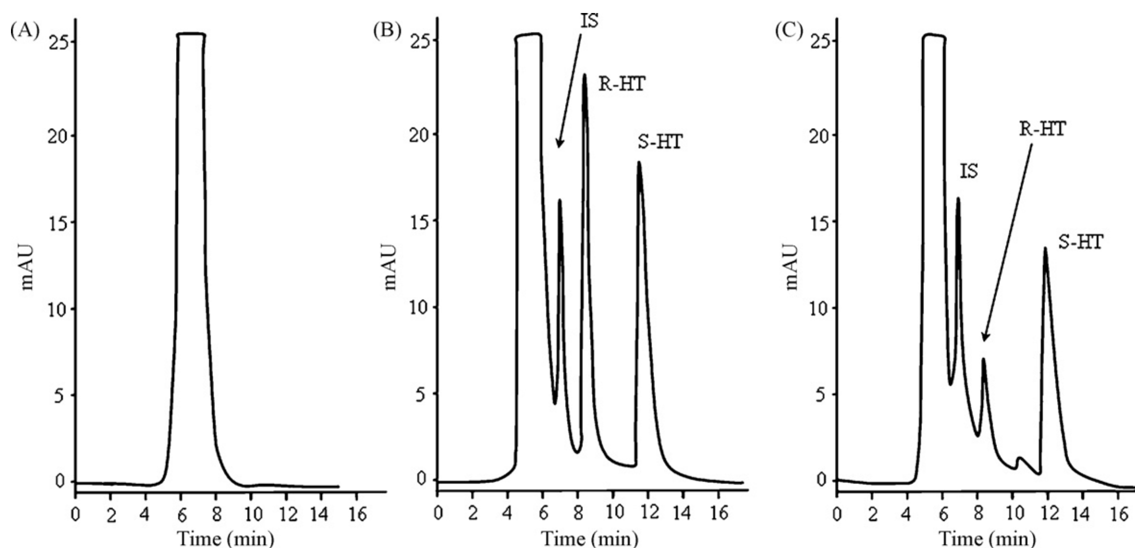

Fig. 2. Representative chromatograms of (A) hesperetin-free urine, (B) urine containing hesperetin at a concentration of 50 μg/mL and the internal standard (IS) at a concentration of 100 μg/mL, and (C) human urine sample containing hesperetin and IS 7 h after oral ingestion of juice. Experimental conditions: capillary column packed with phenyl-carbamate-2-propyl-β-CD, 100 μm I.D. × 22.0 cm packed length, 22.5 and 34.0 cm effective and total lengths, respectively; mobile phase, 1% (v/v) TEAA pH 4.5 in methanol/water 70/30 (v/v) flow rate 400 nL/min.

Table 1
Within and between-day precision and accuracy of the assay for hesperetin (HT) enantiomers in human urine (n = 6).

Concentration of each added enantiomer (μg/mL) (R-/S-HT)	Observed concentration				RSD (%)				Bias (%)			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	R-HT	S-HT	R-HT	S-HT	R-HT	S-HT	R-HT	S-HT	R-HT	S-HT	R-HT	S-HT
0.5	0.49	0.49	0.49	0.49	2.57	2.73	0.39	1.24	−1.98	−0.71	−1.82	−2.01
5.0	4.98	4.94	4.95	4.90	1.39	4.45	1.05	1.12	−0.32	−1.68	−0.94	−2.00
25.0	24.94	24.88	24.75	24.56	1.59	1.88	1.29	1.32	−0.25	−0.47	−0.99	−1.78

and −0.25% (Table 1). These data indicated that the developed nano-LC method is reproducible and accurate.

The absolute recovery of the two enantiomeric forms of hesperetin and the internal standard was evaluated by direct comparison of their peak area from extracts of flavanone-free urine samples which had been spiked with known concentrations of hesperetin. Recovery of internal standard was determined in the same solutions simultaneously.

The mean extraction efficiency for internal standard was 96.53% ± 1.86 (mean ± S.D.) and for hesperetin enantiomers in urines varied from 82.48 to 99.67% (Table 2).

3.3. Stereospecific pharmacokinetics of hesperetin in human

The developed nano-LC method was applied to the determination of hesperetin enantiomers in urine after oral administration of 112.81 mg of (2R)-hesperidin and 599.55 mg of (2S)-hesperidin to a healthy male volunteer after ingestion of 1 L juice. Even if such concentrations of HT could appear relatively high, it is noteworthy observing that similar levels can be easily ingested with the consumption of fresh squeezed or entire fruits. It has been shown that hesperetin is the most highly consumed flavonoid with an intake of 28.3 mg/day, equivalent to 30% of the total flavonoid [9,25].

Fig. 3 reports the concentration–time profile of S- and R-hesperetin in urine, after the administration of 1 L of BOJ. As can be observed, the hesperetin enantiomers appeared in urine 2 h after the juice ingestion, reaching the maximum concentration after 7 h and returned back at initial level in 24 h. As expected, following the oral administration of orange juice in the studied time, the two curves indicate that S-hesperetin was excreted at higher concentrations than R-hesperetin.

Fig. 4 illustrates the cumulative urinary curves of the two enantiomers which were mainly excreted between 4 and 8 h after orange juice ingestion. As reported, the percentage excreted during this time (4–8 h) of the total excreted amount was 91.42 and 87.13% for S- and R-hesperetin. The relative urinary excretions from the BOJ of S- and R-hesperetin were 3.55 and 6.44%. These values were obtained calculating the ratio between the cumulative concentra-

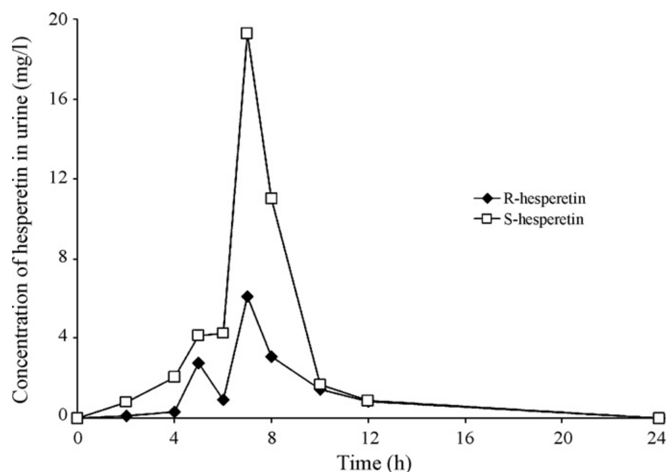

Fig. 3. R- and S-hesperetin concentration curves in urine after ingestion of blood orange juice. For experimental conditions see Fig. 2.

Table 2
Recovery of hesperetin enantiomers from human urine ($n = 3$).

Concentration of each added enantiomer (μg/mL) (R-/S-HT)	Recovery (%) ± S.D.		RSD (%)	
	R-HT	S-HT	R-HT	S-HT
0.5	85.65 ± 1.35	82.48 ± 0.90	1.57	1.10
5.0	94.92 ± 5.42	87.99 ± 5.03	5.71	5.69
25.0	99.67 ± 0.87	97.34 ± 0.69	0.87	0.71

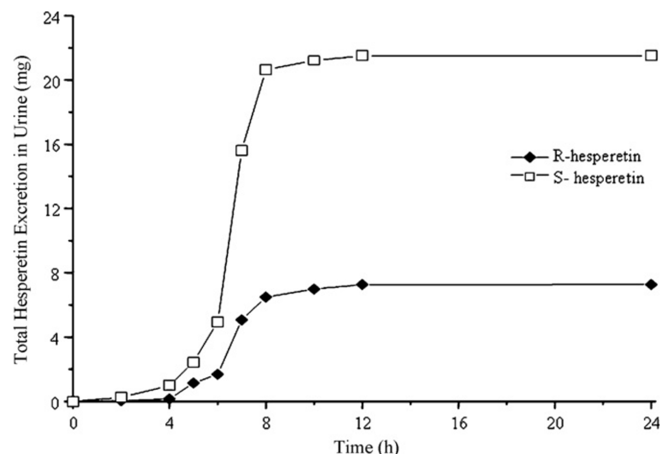

Fig. 4. Cumulative urinary excretion profile of hesperetin enantiomers 1 L of blood orange juice intake. For experimental conditions see Fig. 2.

tion of S- or R-hesperetin after 24 h and the concentration of S- or R-hesperidin ingested with the juice, respectively. As a result, the corresponding value for racemic hesperetin was 4.00% which was achieved considering the cumulative HT concentration after 24 h to the total concentration of hesperedin ingested. The data obtained in our study, are in accordance with previous published pharmacological studies in relation to the analysis of hesperetin in human urine [8,9,26].

4. Conclusion

We have developed a stereospecific nano-LC method for hesperetin analysis in human urine. The nano-LC system allows to achieve good and efficient enantiomeric separations at low costs and reduced environmental impact, due to the need of minute amounts of chiral stationary phase and low volumes of mobile phases. This method has been specifically developed for the fast quantitative determination of hesperetin enantiomers in urine. In fact the baseline resolution of the studied analytes was achieved in less than 12 min. The sensitivity, reproducibility and accuracy of the method were demonstrated, obtaining very satisfactory results.

Excretion of R- and S-hesperitin in urine was monitored within 24 h and it was observed that the flavanoid was almost completely eliminated in about 12 h. Due to the beneficial effects of hesperetin, its determination can be useful as biomarker for citrus juice intake.

This approach results quite simple, fast and cost effective compared to the mentioned HPLC method. All these features make it suitable and practical to use in pre-clinical and clinical applications where small biological sample requirements and timesaving are needed.

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