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Analytical Methods

Quantification of hesperidin in citrus-based foods using a fungal diglycosidase

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ABSTRACT

A simple enzymatic–spectrophotometric method for hesperidin quantification was developed by means of a specific fungal enzyme. The method utilises the diglycosidase α-rhamnosyl-β-glucosidase (EC 3.2.1.168) to quantitatively hydrolyse hesperidin to hesperetin, and the last is measured by its intrinsic absorbance in the UV range at 323 nm. The application of this method to quantify hesperidin in orange (*Citrus sinensis*) juices was shown to be reliable in comparison with the standard method for flavonoid quantification (high performance liquid chromatography, HPLC). The enzymatic method was found to have a limit of quantification of 1.8 μM (1.1 mg/L) hesperidin, similar to the limit usually achieved by HPLC. Moreover, it was feasible to be applied to raw juice, without sample extraction. This feature eliminated the sample pre-treatment, which is mandatory for HPLC, with the consequent reduction of the time required for the quantification.

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1. Introduction

The flavonoids are secondary plant metabolites that belong to the class of plant phenolics. Current interest in those compounds relies in their biological activities. They have been associated with lowered risk of certain cancers, and stroke. Moreover, antioxidant, anti-inflammatory, antimicrobial and antiviral properties have been demonstrated both *in vitro* and *in vivo* (Cano, Medina, & Bermejo, 2008; Salas, Céliz, Geronazzo, Daz, & Resnik, 2011). They also inhibit cytochrome P450 resulting in drug interactions (Doostdar, Burke, & Mayer, 2000). Their significance as nutraceuticals is widely recognised and constitute an important fraction of several dietary supplements (Tripoli, La Guardia, Giammanco, Di Majo, & Giammanco, 2007).

Because flavonoids are especially abundant in plant species from the genus *Citrus*, they have significant impact on nearly every aspect of citrus fruit production and processing. They are responsible of some unpleasant characteristics of the fruit juices, such as bitterness and turbidity (Manthey & Grohmann, 1996) and particularly hesperidin clogs the steel pipes of the citrus juice plants. Moreover, they are abundant in the by-products, mostly in peels (albedo + flavedo), accounting for 4–12% of the dry weight (Marín, Soler-Rivas, Benavente-García, Castillo, & Pérez-Álvarez, 2007). Its recovery from citrus industry by-products is challenging for two reasons: its bioactive properties and the reduction of the amount of residues. Worldwide industrial wastes may be estimated at more than 15×10^6 tons, as the amount of residue obtained from the fruits accounts for 50% of the original whole fruit mass (Bampidis & Robinson, 2006).

From citrus flavonoids, hesperidin is the most abundant in lemons, limes, sweet oranges, tangors and tangelos (~15 mg/100 g edible fruit) (Peterson, Dwyer, et al., 2006; Peterson, Beecher, et al., 2006). Owing to the importance of hesperidin for food and pharmaceutical industries, several efforts have been made for its extraction and purification. In parallel with the search of extraction procedures, hesperidin quantification has been attempted by several methods. Traditionally, it is performed by high performance liquid chromatography (HPLC) (Kelebek, Silli, Cnabas, & Cabaroglu, 2009). Time consumption, expense and sample pre-treatment requirements are some of the drawbacks of using such methodology. A recent work by Sims, Li, Torabi Kachoosangi, Wildgoose, and Compton (2009) reports the use of adsorptive striping voltammetric (AdSV) using multiwalled carbon nanotube modified electrodes for measuring the concentration of hesperidin in orange juice samples. In this work we developed an enzymatic–spectrophotometric technique to measure hesperidin.

2. Materials and methods

2.1. Chemicals and stock solutions

Standard flavonoids hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone 7-rhamnoglycoside) and hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) were purchased from Sigma Chemical (St.

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Louis). HPLC grade methanol was obtained from Avantor™ (Phillipsburg, NJ, USA, formerly Mallinckrodt Baker). Samples for the analysis were obtained from commercially available juices that claimed to be 100% sweet orange (*Citrus sinensis*) juice. The other chemicals were from standard sources.

To prepare standard solutions for enzymatic assays, flavonoids were solubilised in dimethylformamide (180 mM) and suspended in water. To adjust pH, 100 mM citric acid–sodium phosphate (pH 3), sodium citrate (pH 5), sodium phosphate (pH 6 and 8) and Tris–HCl buffers (pH 7.5–9), and 100 mM Na_2CO_3 (pH 10) were used. Spectra (250–800 nm) of analytes were obtained using a USB4000 spectrophotometer (Ocean Optics). The intensity of the hesperetin (in 50 mM sodium phosphate buffer pH 8) peak maximum at 323 nm was plotted against hesperetin concentration to produce the calibration curve. To prepare stock solutions (180 mM) for HPLC assays, flavonoids were solubilised in dimethylsulphoxide (DMSO). Standards were prepared by diluting stock solutions in elution solvent.

2.2. High performance liquid chromatography (HPLC)

Hesperidin was quantified by high performance liquid chromatography (HPLC) using a KONIK-500-A series HPLC system attached to a KONIK UVIS 200 detector. The column was a reversed-phase LiChroCART® 125–4 (12.5 cm length, 4 mm internal diameter) LiChrospher® 5 μm, RP 18 (pore size 100 Å). The elution consisted of an isocratic flow of 50%$_{v/v}$ methanol and 50%$_{v/v}$ water at a flow rate of 1.0 ml/min at 40 °C. Peak areas and extinction coefficient were calculated from chromatograms of authentic standards detected at 285 nm. Hesperidin extraction was performed twice placing samples (100 μl) with 400 μl of 50%$_{v/v}$ methanol and 50%$_{v/v}$ DMSO for 1 h at 50 °C. Then, the samples were centrifuged (10,000 rpm, 10 min), and the supernatants were collected and suitably diluted in elution solvent. Triplicate samples were used.

2.3. Enzyme assays

Hesperidin samples [standards, orange juice, and solvent extracted samples (see sample treatment for HPLC)] were prepared using 50 mM sodium phosphate buffer pH 8 and incubated with *Acremonium* sp. DSM 24697 α-rhamnosyl-β-glucosidase, which catalyses the following reaction:

$$\text{Hesperidin} + H_2O \xrightarrow{\alpha\text{-Rhamnosyl}-\beta\text{-glucosidase}} \text{Hesperetin} + \text{Rutinose}$$

The model used for the quantification method assumes that hesperidin concentration is low enough for the reaction to be completed. Therefore, initial hesperidin concentration is stoichiometrically equivalent to the increment of hesperetin concentration at the end of the enzymatic reaction:

$$\text{Hesperidin}_{\text{initial}} = \Delta\text{Hesperetin}$$

The enzyme was produced and purified as described before (Mazzaferro, Piñuel, Minig, & Breccia, 2010). The reaction was performed in a peltier cell (Quantum) for temperature control under stirring, and UV–visible spectra were continuously recorded (one measurement every 3 s) at 284, 323, 450 and 600 nm in a USB4000 spectrophotometer (Ocean Optics). The intensity of the peak at 323 nm was plotted as a function of time, which in fact represents the increase in the aglycone concentration during the reaction. To determine the kinetics of hesperidin hydrolysis, the enzyme was incubated with 27 μM hesperidin in 50 mM sodium phosphate buffer pH 8 and initial velocities were measured. One unit of α-rhamnosyl-β-glucosidase activity was defined as the amount of enzyme required to release 1 μmol of aglycone (as hesperetin) per min at 60 °C. To quantify hesperidin concentration, 1.4×10^{-3} U/ml α-rhamnosyl-β-glucosidase was used and reaction was allowed to proceed up to completion. Triplicate samples were assayed.

2.4. Statistical methods

Calibration curves for hesperidin and hesperetin were performed at the wavelengths of maximum absorbance of both compounds (284 and 323 nm). Triplicates of 10 concentration levels of each standard were used in the range 0–30 μM. Data were fitted using the least-squares method according to Beer–Lambert equation:

$$A = \epsilon lc$$

where: A, absorbance; ε, molar extinction coefficient ($M^{-1}\,cm^{-1}$); l, path length (cm); and c, molar concentration.

Validation of the method was performed using replicates of hesperidin standards for seven levels of concentration ($n = 22$) in the range 0–10 μM. The data were adjusted to a linear equation using the least-squares method:

$$Y = ax + b$$

where: y, hesperidin concentration as quantified by the enzymatic–spectrophotometric method; x, concentration of the standards; a, slope; b, intercept. The tested hypothesis were:

$$H_0 : a = 1 \text{ versus } H_1 : a \neq 1$$

$$H_0 : b = 0 \text{ versus } H_1 : b \neq 1$$

The error of the parameters was calculated according to Draper and Smith (1981) and a t-test was performed with a 95% confidence. The data of the citrus juice samples determination by different methods was analysed using a t-test for comparison of the means of paired samples with a 95% confidence (Massart et al., 1997, chap. 8).

3. Results

3.1. UV–visible spectra of hesperidin and its aglycone, hesperetin

To identify the substrate used in this catalysis, hesperidin, and the reaction product, hesperetin, the UV–visible spectra of these compounds at different pH values were measured. The spectra were shown to be different in the entire pH range studied (pH 3–10) (Fig. 1a and b). Fig. 1c shows the spectra of hesperidin, hesperetin and a mixture of both at pH 8. At pH close to neutrality and alkaline (pH 6–10) the maximum absorbance for hesperidin was found to be at 284 nm. Hesperidin showed a baseline caused by light dispersion. Even when hesperidin solubility in water (0.324 mM) is higher than the concentration used in this study (40 μM), dissolution velocity was previously demonstrated to be slow, with 37% dissolution after 15 min in water (Mauludin & Müller, 2008). Hesperetin maximum absorbance was at 323 nm, with a shoulder around 280 nm. The spectrum of samples containing hesperidin (39 μM) and a low amount of hesperetin (5 μM) showed that the spectra of both compounds are additive (Fig. 1c).

3.2. Calibration curves

To quantify the enzyme activity, calibration curves for hesperidin and hesperetin at 284 and 323 nm were performed. Absorbance at 284 nm was shown to be similar for hesperidin and hesperetin, with molar extinction coefficients (ε) of $4.1 \times 10^3\ M^{-1}\,cm^{-1}$ and $6.8 \times 10^3\ M^{-1}\,cm^{-1}$, respectively. However, since hesperidin did not show an absorbance peak at 323 nm, the hesperidin and hesperetin calibration curves at this wavelength were markedly differ-

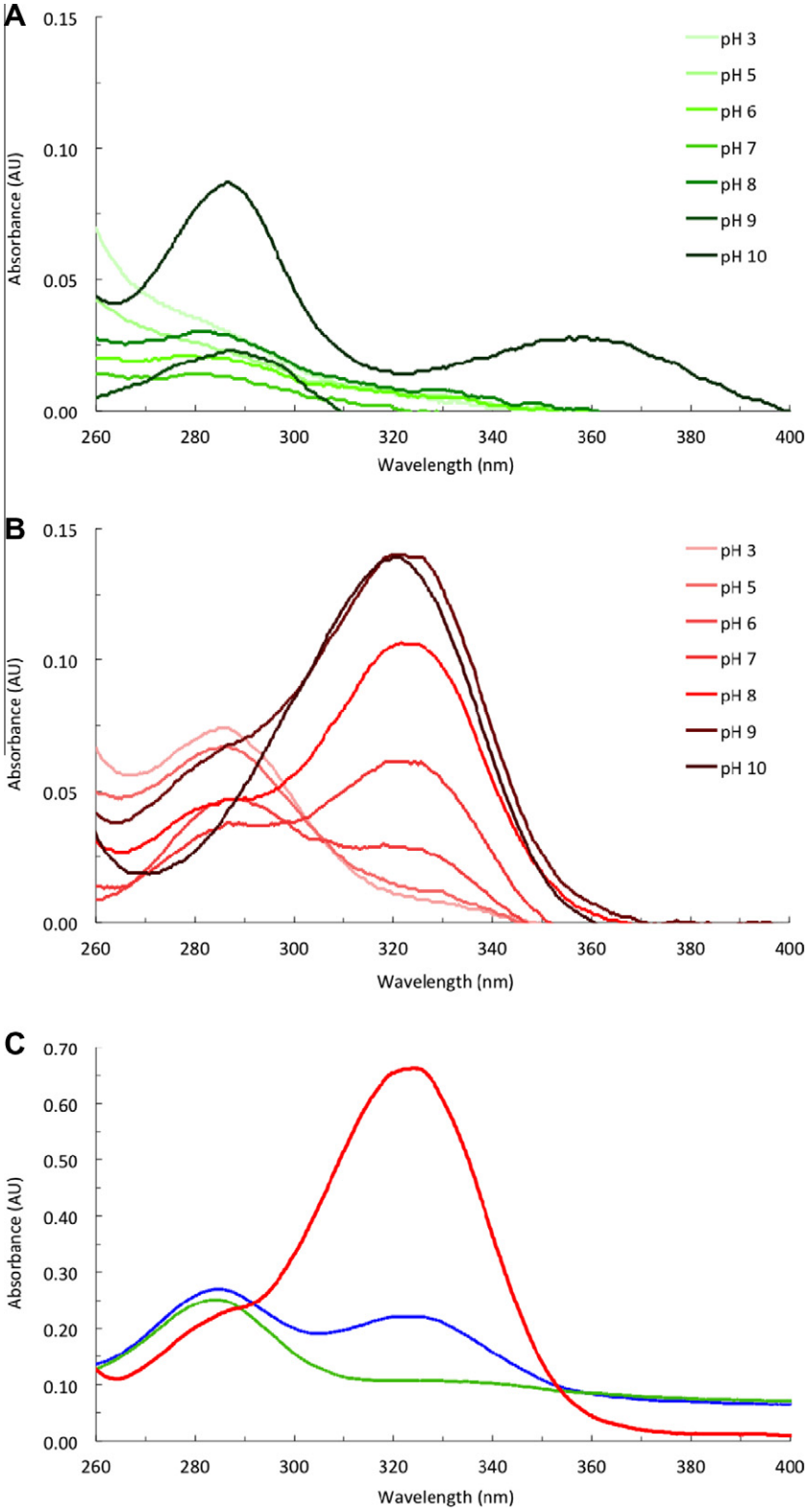

Fig. 1. UV–visible spectra at different pH values of (a) 5 μM hesperidin, and (b) 5 μM hesperetin; (c) UV–visible spectra of (–) in red for 32 μM hesperetin, (–) green for 40 μM hesperidin and (–) blue for 39 μM hesperidin + 5 μM hesperetin in 50 mM sodium phosphate buffer (pH 8). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

ent (Fig. 2). Hesperetin absorbance versus its concentration (up to 27 μM) yielded a linear plot ($R^2 = 0.99$), while hesperidin absorbance was not found to be significant at equimolar concentrations. From this calibration curve a ε value of 22. $1 \times 10^3\ M^{-1}\ cm^{-1}$ was obtained for hesperetin.

3.3. Enzymatic assay of α-rhamnosyl-β-glucosidase

In our previous work we reported the production and characterisation of the novel enzymatic activity α-rhamnosyl-β-glucosidase (EC 3.2.1.168, Mazzaferro et al., 2010). The enzyme is specific for

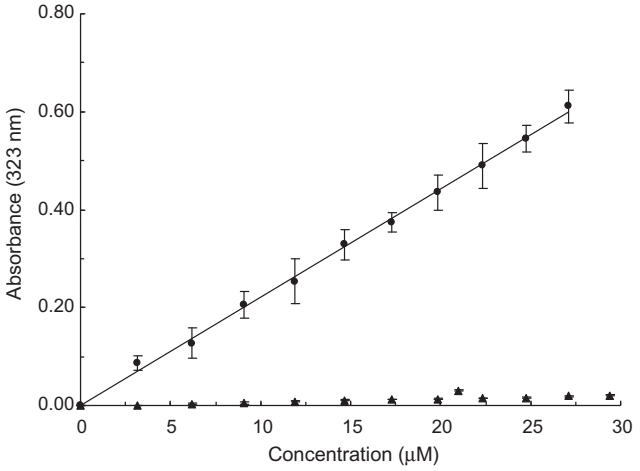

Fig. 2. Calibration curve of (▲) hesperidin and (●) hesperetin in 50 mM sodium phosphate buffer (pH 8) at 323 nm.

flavonoid 7-*O*-rutinosides, including hesperidin and eriocitrin, the predominant flavanones in citrus. The pH value for this assay was chosen taking into account the enzymatic activity and the hesperetin absorbance (323 nm) in the pH range 3–10 (Fig. 3). Since the enzyme is active in a broad pH range (>60% activity in the range 4.0–8.0) and, in view of the higher product absorption at neutral-alkaline conditions, the pH value 8.0 was selected for the enzymatic assays. Upon addition of α-rhamnosyl-β-glucosidase (1.4×10^{-3} U/ml) the glycosidic bond between the aglycone and the sugar moieties was hydrolysed yielding hesperetin and rutinose, with an increase in the absorbance (323 nm) associated with hesperetin (Fig. 4). In order to avoid interferences caused by hesperidin (Fig. 1c) or other particulate materials, light scattering was corrected by subtracting the absorbance at 450 nm. The absorbance of the enzyme was neglected since it does not contribute significantly to the 323 nm peak. Spectra did not change with temperature values ranging from 25 to 70 °C (data not shown). In order to reduce the time of the assay to a minimum, temperature was fixed at 60 °C because enzyme activity is near-optimal, and residual activity after 30 min is higher than 65% (Mazzaferro et al., 2010). Hesperidin in the concentration range 0–10 μM was shown to be hydrolysed to completion within 10 min.

Standards of hesperidin were incubated under stirring at 60 °C until absorbance was constant. Then, the enzyme was added, and allowed to hydrolyse the substrate to completion (Fig. 4b). Concentration was calculated from the absorbance difference (ΔA) between two straight lines parallel to the abscissa: the baseline obtained before enzyme addition, and other calculated using the data forming the plateau after completion of the reaction. Hesperidin concentration as estimated by the enzymatic method correlated well with hesperidin concentration, since linear regression ($R^2 = 0.99$) showed a slope of 0.98 (±0.02) and intercept at 0.11 (±0.09) (Fig. 5). The limit of quantitation, defined as the lowest quantifiable concentration at the curve at which both precision and accuracy were within the maximum tolerable coefficient of variation of 10%, was found to be 1.8 μM (1 mg/L) hesperidin.

3.4. Analysis of orange juices

The method shown above was found to be reliable for the commercially available substrate hesperidin in neat reaction mixtures. The measurements using natural substrates were performed using commercial orange juices that claim to be 100% sweet orange (*C. sinensis*) packed aseptically. The concentrations of hesperidin (Fig. 6) were similar to those reported previously by other authors (Peterson, Dwyer, et al., 2006). Since hesperidin extraction is mandatory for HPLC assays, natural samples were extracted with methanol-DMSO and analysed by both methods. First, there were not differences between samples quantified by the enzymatic method and HPLC. Then, extraction was demonstrated to be unnecessary when using raw juice, with the consequent reduction of the time required to complete the protocol.

4. Discussion

Here we have demonstrated the application of the *Acremonium* sp. DSM 24697 α-rhamnosyl-β-glucosidase, a fungal diglycosidase,

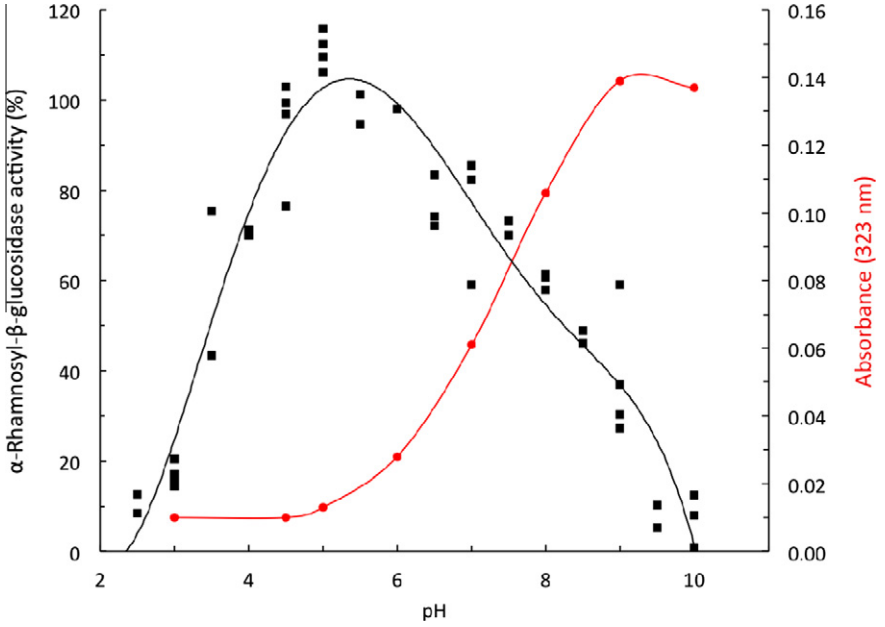

Fig. 3. (■) in black for α-Rhamnosyl-β-glucosidase activity (Mazzaferro et al., 2010) and (●) in red for hesperetin absorption (323 nm) displayed at different pH values. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

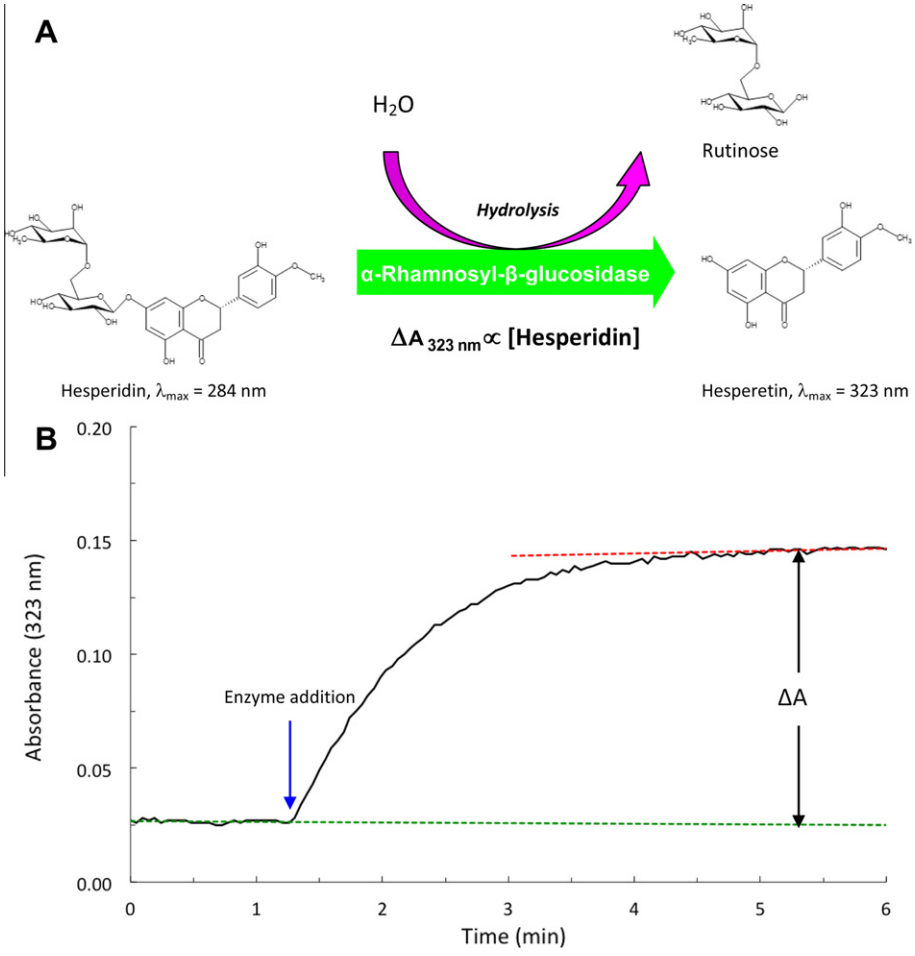

Fig. 4. (a) Hesperidin hydrolysis catalysed by the diglycosidase α-rhamnosyl-β-glucosidase (EC 3.2.1.168), and (b) time course of the reaction. Sample (suitably diluted to 3 ml with 50 mM sodium phosphate buffer pH 8) was placed in a quartz cuvette at 60 °C under magnetic stirring and the system was allowed to stabilize for approx. 1 min. Then, enzyme solution (10 μl) was added to a final concentration of 1.4×10^{-3} U/ml (blue arrow). Absorbance was recorded at 323 nm (and corrected by subtracting the absorbance at 450 nm) until it reached a plateau.

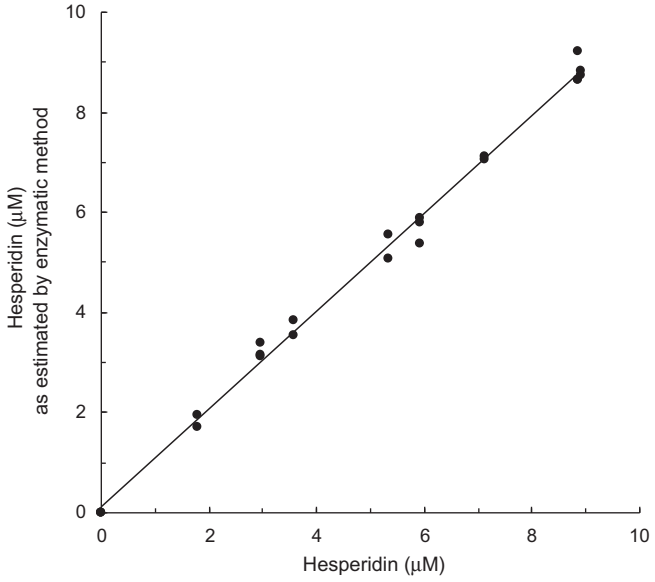

Fig. 5. Hesperidin quantified by the enzymatic–spectrophotometric method (vertical axis) in comparison with standard solutions (horizontal axis).

in combination with spectrophotometry for the measurement of hesperidin concentration in citrus-based foods. Previously, Lai, Gopalan, and Glew (1992) reported the use of the differential spectra of the glucosides picein and prunasin and their aglycones to online measure β-glucosidase activity. Likewise, hesperidin and its aglycone were shown to have distinctive UV–visible absorption spectra, with $\varepsilon_{323\mathrm{nm}}$ markedly different at alkaline pH; and could be used to quantify α-rhamnosyl-β-glucosidase activity using the slope of the curve shown in Fig. 4.

The critical point of the procedure is the selective and stoichiometric deglycosylation of hesperidin. Although several methods have been described for hydrolysis of hesperidin to yield hesperetin, to the best of our knowledge all of them lack the required characteristics. The patent documents US4150038A (Wingard, 1979) and EP2017272A2 (Hilmer, Ley, & Gatfield, 2008) described chemical methods for hesperetin production from commercial hesperidin. As common characteristics they have need of mid- to high-temperatures (from 50 to 160 °C), strong acids and reaction time of at least 1 h (up to 12 h). Due to the instability of flavonoids when kept at high temperatures and extreme pH values, a high amount of side products are obtained (Biesaga, 2011). Authors reached high hesperetin yield-up to 90%-although not acceptable for the purpose of hesperidin stoichiometric deglycosylation. Regarding enzymatic methods, the most common mechanism for hesperidin deglycosylation involves two enzymes from microbial sources (*Aspergillus* or *Penicillium* spp.), which act in a sequential mode

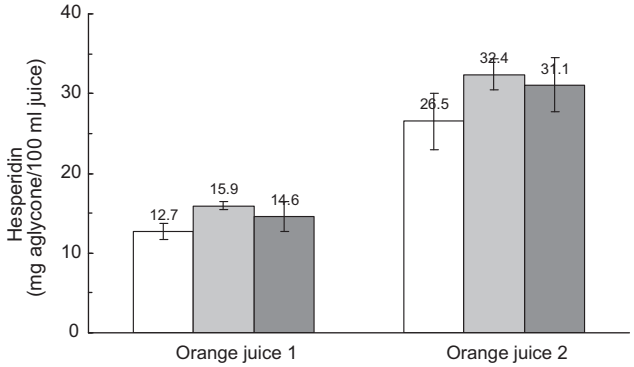

Fig. 6. Hesperidin quantification in two orange juices. Enzymatic method using (□) raw samples and (■) solvent extracted samples, (■) HPLC method using solvent extracted samples.

(Sarry & Gunata, 2004). First, an enzyme recognising the linkage between the two sugar moieties (α-rhamnosidase, EC 3.2.1.40) splits-off a rhamnose moiety (Manzanares, van den Broeck, de Graaff, & Visser, 2001; Orrillo, Ledesma, Delgado, Spagna, & Breccia, 2007). Then, a β-glucosidase (EC 3.2.1.21) hydrolyses the heterosidic linkage between the glucose moiety and the aglycone (Barbagallo, Spagna, Palmeri, Restuccia, & Giudici, 2004). The enzymatic cocktails containing both activities are commercialised under the terms "naringinase" and "hesperidinase". The use of these enzymes, which are rather promiscuous, does not provide the required selectivity for hesperidin deglycosylation in presence of the several glycosides present in fruits. The enzymatic activity α-rhamnosyl-β-glucosidase recently described for the first time by our group was demonstrated to be highly specific for flavonoid 7-*O*-rutinosides deglycosylation in one step (Mazzaferro et al., 2010). *Sporobolomyces singularis* glucosidase-like BglA protein was also shown to hydrolyse hesperidin to hesperetin (Li et al., 2008). However, the enzyme was previously shown to be promiscuous regarding the sugar moiety, and it was able to hydrolyse also monoglycoconjugates: β-galactosidase and β-glucosidase activities were detected. Because of its broad sugar specificity it was named as "β-hexosidase" (Ishikawa, Sakai, Ikemura, Matsumoto, & Abe, 2005).

Under the appropriate α-rhamnosyl-β-glucosidase concentration, temperature and pH, stoichiometric hydrolysis of hesperidin to hesperetin was performed in only 5–10 min and without generation of by-products. The traditional method for flavonoid determination in food material is HPLC based in UV–visible detection. Limits of quantitation (LOQ) are usually around 0.25 mg/L (Kanaze, Gabrieli, Kokkalou, Georgarakis, & Niopas, 2003), which are comparable with the LOQ obtained by the enzymatic–spectrophotometric method developed in the present work. The use of detectors such as diode array and electrospray ionisation mass spectrometry detection resulted in an increment of the resolution capacity for different compounds rather than an increase in the sensitivity. As an example, He et al. (2011) recently described the simultaneous determination of alkaloids, flavanones and hydroxycinnamic acids in citrus fruits using HPLC with a LOQ for hesperidin of 0.26 mg/L. On the other hand, the AdSV method was proven to lower the limit of quantitation of flavonoids to the nanomolar range (Sims et al., 2009; Temerk, Ibrahim, & Kotb, 2011). The number of recent reports dealing with hesperidin and related flavonoids detection and quantification gives account on the current interest in the development of analytical techniques for such compounds. Features such as sensitivity, robustness or simplicity make one or another superior for the application to a particular purpose.

The method developed in this work is fast, easy to perform and reliable, and can be adapted for high-throughput assays by means of a microplate reader. It can be applied within the citrus fruit industry as well as biochemical research.

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