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Quantification of isoorientin and total flavonoids in *Passiflora edulis* fruit pulp by HPLC-UV/DAD

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ABSTRACT

A method is reported for the quantification of isoorientin (using a standard addition method) and total flavonoids (expressed as rutin, using the external standard method) in passion fruit pulp (*Passiflora edulis* Sims f. *flavicarpa* Degener, Passifloraceae). Extraction of flavonoids was optimized by experimental design methodology, and quantitative analysis was performed by high-performance liquid chromatography with photo-diode array detection (HPLC-UV/DAD). The method was developed and validated according to ICH requirements for specificity, linearity, accuracy, precision (repeatability and intermediate precision), LOD and LOQ. Rutin was chosen as standard for the quantification of total flavonoids in order to propose a HPLC method feasible for routine analysis of the flavonoids in the passion fruit pulp. The passion fruit pulp contained 16.226 ± 0.050 mg L^{-1} of isoorientin and 158.037 ± 0.602 mg L^{-1} of total flavonoid, suggesting that *P. edulis* fruits may be comparable with other flavonoid food sources such as orange juice or sugarcane juice.

1. Introduction

Passiflora is the largest and most important genus of the family Passifloraceae, comprising about 500 species, distributed mostly in warm temperate and tropical regions [1]. *Passiflora edulis* (passion fruit) is native of Brazil, the largest producer of this species in the world, where it is known popularly as "maracujá" and is widely cultivated, mainly for the use of its pulp in the food industry (processed juices and candies) [2].

Previous studies have described the presence of flavonoids as the major constituents of *P. edulis*, mainly *C*-glycosylflavones [1,3]. The flavonoids schaftoside, isoschaftoside, isoorientin, orientin, isovitexin, luteolin-6-*C*-chinovoside and luteolin-6-*C*-fucoside have been identified in the fruit [4], but there are no reports of validated methods for the quantification of flavonoids in the passion fruit pulp. Recently, the volatile composition of some *Passiflora* species, including *P. edulis*, was studied by HS-SPME–GC–MS (headspace solid phase microextraction gas chromatography–mass spectrometry [5]). In fact, most of the data in the literature still focus on the compounds in *Passiflora* leaves due to their pharmacological effects on the central nervous system and their use as a herbal medicine [3,6–8]. However, investigations about the potential of the passion fruit pulp as a nutritional source of flavonoids or possibly even as a functional food are also ongoing.

This paper reports on the development and validation of a method for quantification of flavonoids in the passion fruit pulp (*P. edulis*), using HPLC-UV/DAD (high-performance liquid chromatography-ultraviolet diode array detector). The quantitative analysis followed two strategies. The first focused on the determination of total flavonoids using standard rutin (Fig. 1), with a view to applying it in routine analyses of total flavonoids in *P. edulis*. The second procedure is more specific for the quantification of isoorientin, the compound used as a dependent variable in the optimization of flavonoid extraction by an experimental design methodology.

2. Material and methods

2.1. Samples

Fruits of *P. edulis* Sims f. *flavicarpa* Degener were collected in the city of Bauru, state of São Paulo, Brazil in April 2008. The plant material was identified by Dr. Luís Carlos Bernacci (Herbarium IAC, Campinas-SP, Brazil) and deposited in the herbarium of the Agronomic Institute of Campinas, São Paulo, Brazil (voucher number IAC 49929). The pulp was separated from the seeds by sieving, stored in jars, and frozen immediately at −20 °C prior to its use.

2.2. Chemicals and standards

The methanol (J. T. Baker, Phillipsburg, NJ, USA) and acetonitrile (Tedia, Fairfield, OH, USA) used were of HPLC grade. Formic acid was purchased from Merck (Darmstadt, Germany) and analytical grade

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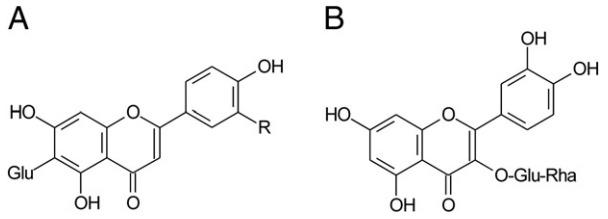

Fig. 1. Structure of flavonoids studied in this work: (A) isoorientin, R = OH and isovitexin, R = H (B) rutin. Glu: glucose; Rha: rhamnose.

ethanol (99.3% v/v) from Quemis (Diadema, Brazil). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

The standards isoorientin and isovitexin (both with purity ≥ 99%) were obtained from Carl Roth (Karlsruhe, Germany) and rutin (purity 95%) from Sigma-Aldrich (Steinheim, Germany).

2.3. Sample preparation

Flavonoid extraction from the passion fruit pulp was optimized using a two-level full factorial 2^3 (8 runs) experimental design performed in triplicate and in random order on three different days. Experiments were conducted to evaluate the influence and interaction of the solvent (methanol or ethanol), percentage of the solvent in water (100% or 60%), and range of extraction times (1.5 or 3.0 min) on the extraction. STATISTICA 7.0 software was used to calculate the effects and to plot the Pareto chart and the estimated response surface.

The pulp extracts were prepared by sonicating 10.0 mL pulp with 30.0 mL of the solvent at room temperature. The extracts were centrifuged at 1000 rpm, 25 °C for 20 min, after which the supernatant was evaporated to 2.0 mL in a rotary evaporator. The resulting aqueous solution was purified by solid phase extraction, using Sep-Pak C_{18} cartridges (400.0 mg, Waters Associates, Milford, MA, USA), which were preconditioned with 5.0 mL of methanol and 5.0 mL of water. The flavonoid fractions were obtained by elution with 2.0 mL of methanol 60%, and the final volume was adjusted to 2.0 mL with methanol 60% in a volumetric flask. The extracts were filtered through a 0.45 µm Millex-HV PVDF membrane (Millipore, New Bedford, MA, USA) prior to HPLC analysis. The samples were prepared and analyzed in triplicate.

2.4. HPLC-UV/DAD and LC-MS/MS analysis

The HPLC-UV/DAD analyses were carried out on a Waters Alliance 2695 (Milford, MA, USA) liquid chromatograph connected to a model 2996 (DAD) diode array detector and controlled by Waters Empower software. The separation was performed using a Symmetry® C_{18} column (250 mm long × 4.6 mm i.d.; 5 µm) supplied by Waters, Milford, MA, USA, preceded by a guard column (2.0 cm long × 4.0 mm i.d.; 5 µm), containing the same stationary phase. The samples were injected automatically (10.0 µL). The column and guard column were thermostatically controlled at 40 °C and a 0.8 mL min^{-1} flow rate was applied, using a linear gradient of 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B). The optimized gradients employed in passion fruit extracts were: 0–10 min, 12%–16% B in A and 10–30 min, 16–20% B in A. The chromatogram was monitored at 330 nm, and UV spectra of individual peaks were recorded in the range of 200–400 nm. Data were processed using Waters Empower software.

The LC-MS/MS analysis were carried out on a Waters Micromass Quattro Ultima Platinum triple quadrupole mass spectrometer coupled to an Alliance 2690 liquid chromatograph (Waters, Milford, MA, USA). The column and the chromatographic condition were the same as those used for the HPLC analysis. The LC effluent was split using a T-splitter to produce a flow of 0.2 mL min^{-1}. The quadrupole mass spectrometer was equipped with a Z-spray source for electrospray ionization (ESI) in the negative mode. Collision energy was 18 eV. Capillary and cone voltages were set at 3.2 kV and 50 V, respectively, and the temperature source was kept at 125 °C while desolvation temperature was held at 250 °C. Nitrogen was used both as cone and desolvating gas at a flow rate of 88 L h^{-1} and 502 L h^{-1}, respectively.

2.5. Validation procedure

Validation was performed according to the guidelines of the International Conference of Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use [9], which establish the evaluation of parameters: specificity, linearity and range, accuracy, precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ).

2.5.1. Specificity

In order to examine the specificity in the determination of isoorientin and total flavonoids, the purity of the peaks was checked using DAD (λ = 200–400 nm). Sections of the spectra corresponding to the upslope and downslope of each peak were overlapping, and peaks were considered pure when there was a coincidence between the two spectral sections.

2.5.2. Linearity

The linearity was determined by the correlation coefficients of the analytical curves generated by injections of the working solutions at five concentration levels. In the method for quantification of isoorientin in passion fruit, the analytical curves were constructed in triplicate, spiking the passion fruit pulp with volumes of the stock solution of isoorientin (300.0 mg L^{-1} in methanol 80%), which resulted in final concentrations of: 5.0, 10.0, 20.0, 40.0, and 80.0 mg L^{-1}. To verify the linearity of the total flavonoids method, an external standard method was employed, using a stock solution of rutin (500.0 mg L^{-1}) prepared in methanol 80%. Five working solutions with concentrations of 50.0, 100.0, 150.0, 200.0, and 250.0 mg L^{-1} were prepared by diluting the standard stock solution. The analytical curves were built by triplicate injections.

2.5.3. Accuracy

Recovery experiments were performed to evaluate the accuracy of the methods. Passion fruit pulp samples were spiked with three concentration levels of isoorientin (20.0, 45.0, and 70.0 mg L^{-1}) prior to extracting the flavonoids. The same procedure was employed using the standard rutin at final concentrations of: 70.0, 150.0, and 250.0 mg L^{-1}. The spiked samples were analyzed in triplicate. Accuracy was expressed as the percentage deviation between the amount of standard found by HPLC analysis and the amount added at the three concentrations examined.

2.5.4. Repeatability and intermediate precision

Repeatability was estimated injecting in triplicate, on the same day, spiked samples containing three different concentrations of isoorientin (20.0, 45.0, and 70.0 mg L^{-1}) and also samples spiked with rutin (70.0, 150.0, and 250.0 mg L^{-1}). Intermediate precision was determined by analyzing, in triplicate, the same solutions employed in the repeatability test on two consecutive days. Precision was expressed in terms of relative standard deviation (RSD).

2.5.5. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were estimated experimentally by injecting standard solutions of isoorientin and rutin diluted in methanol 80% until the signal-to-noise ratio for the standards reached a 3:1 ratio for LOD and 10:1 for LOQ.

Table 1
Parameters used in the experimental design for optimization of the extraction of flavonoids from the passion fruit pulp.

Essays	Solvent	Proportion of solvent (%)	Extraction time (min)	Response (% area of isoorientin/total area of flavonoids)	Standard deviation
1	Methanol	60	1.5	18.12	0.11
2	Ethanol	60	1.5	19.40	0.12
3	Methanol	100	1.5	17.82	0.16
4	Ethanol	100	1.5	17.99	0.14
5	Methanol	60	3.0	18.08	0.09
6	Ethanol	60	3.0	19.13	0.14
7	Methanol	100	3.0	18.38	0.12
8	Ethanol	100	3.0	18.39	0.11

2.6. Quantitative analysis of flavonoids

The isoorientin in the passion fruit pulp was quantified by the standard addition method, using analytical curves constructed from samples spiked with a stock solution of isoorientin (300.0 mg L^{-1} in methanol 80%) to reach a final concentration in the range of 5.0 to 80.0 mg L^{-1}. This procedure was repeated in triplicate and the amount of isoorientin was calculated based on the peak area at $\lambda = 330$ nm.

Total flavonoids were determined by an external standard method, using rutin as reference. The standard solutions (50 to 250 mg L^{-1}) were prepared in methanol 80%. The total flavonoid content of each pulp sample was determined by adding up the areas at $\lambda = 330$ nm of all the peaks identified by UV/DAD as being flavonoids (see details in Section 3.2); therefore, the corresponding concentration of flavonoids was calculated based on the analytical curve. This procedure was repeated in triplicate.

3. Results and discussion

3.1. Optimization of the extraction procedure

The experimental design methodology was used to evaluate the extraction parameters and optimize the experimental conditions. This methodology is based on the use of an optimum and reasonable set of experiments, which allows all the experimental factors to be varied simultaneously, taking into account the possible interactions between the factors. The experimental design methodology is faster, more economical and effective than the traditional optimization procedures [10,11].

The optimization step was carried out using a two-level full factorial design, and the effect of the three factors on the high and low level was evaluated: type of solvent (methanol or ethanol), percentage of the solvent in water (100% or 60%) and range of extraction times (1.5 or 3.0 min). Minimum and maximum levels of each factor (Table 1) were chosen according to flavonoid extraction data in the literature [7,12], and also considering preliminary experiments using the passion fruit pulp. The relative area of the peak isoorientin (area of isoorientin peak at $\lambda = 330$ nm/total area of flavonoids at $\lambda = 330$ nm, Fig. 2) was used as a dependent variable, expressed in percentage.

Fig. 3 presents a Pareto chart showing the influence of each investigated factor on the response, as well as the possible cross effects among these factors. The results, considering the relative area of isoorientin as response, demonstrated that the effects of the type of

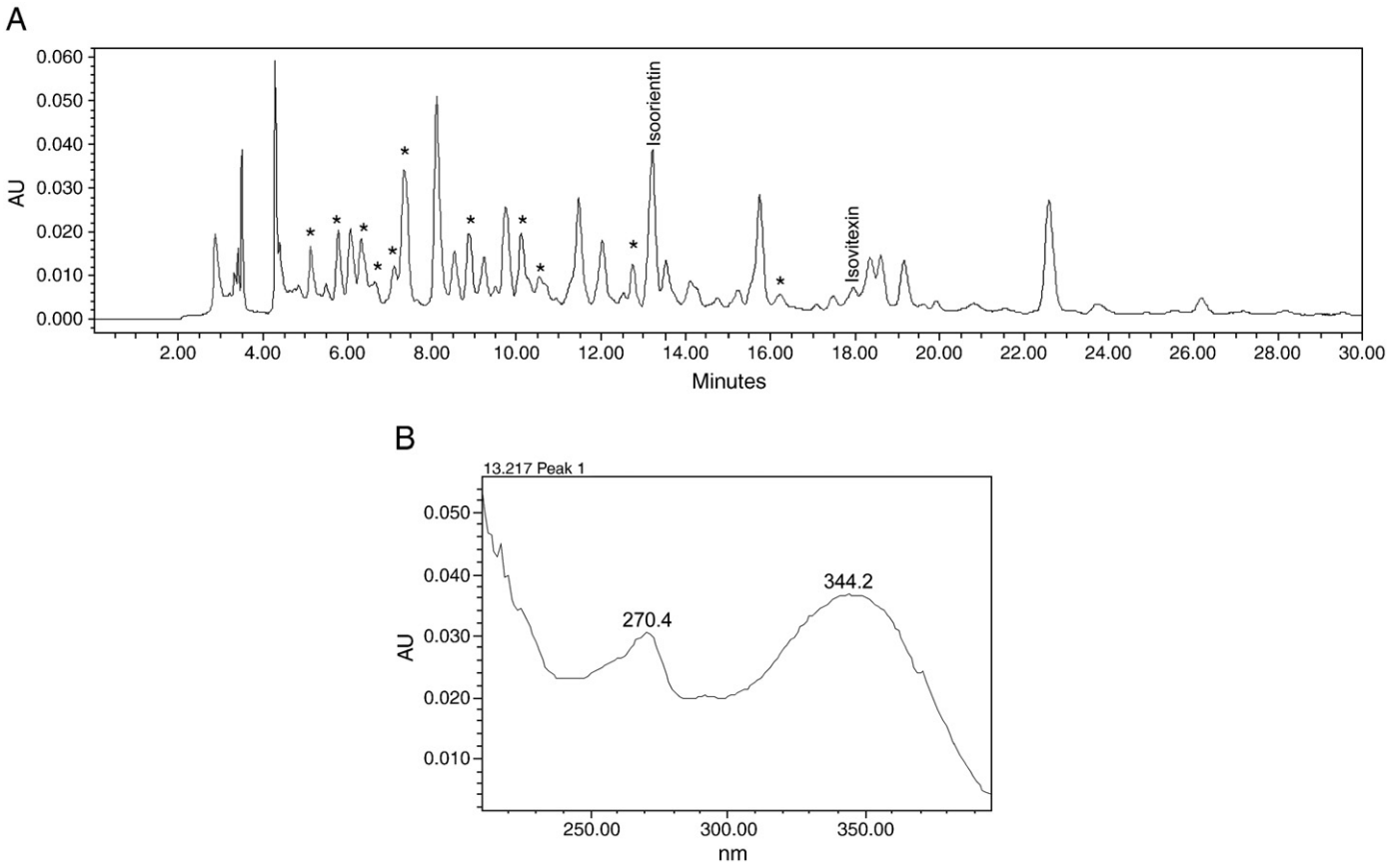

Fig. 2. (A) Typical HPLC-UV/DAD chromatogram ($\lambda = 330$ nm) of passion fruit (*Passiflora edulis* Sims f. *flavicarpa* Degener) pulp extract. Mobile phase: linear gradient of 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B); 0–10 min, 12%–16% B in A and 10–30 min, 16–20% B in A. For other chromatographic conditions, see Section 2.4. (*) peaks identified as flavonoids; (B) UV/DAD spectra of isoorientin (tr = 13.217 min).

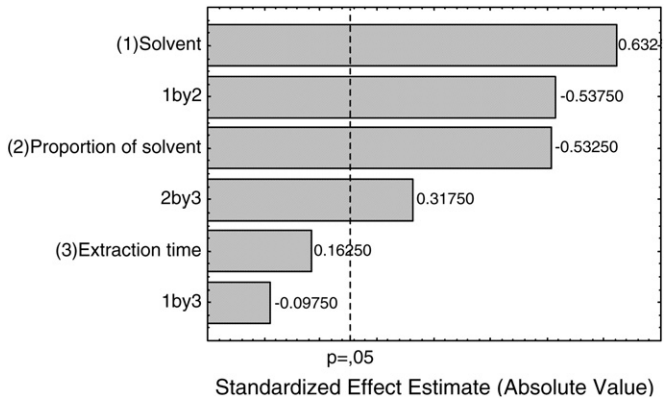

Fig. 3. Pareto chart showing the values of effects from variables using the relative isoorientin area (area of isoorientin peak/total area of flavonoids) as response.

solvent and percentage of the solvent in water are significant factors ($p<0.05$). On the other hand, the extraction time is not a relevant factor in the range considered. Moreover, the negative value of the effect for the proportion of solvent (−0.538) indicates that a proportion of 60% is more efficient for extraction, and the factor type of solvent showed a strong positive effect (0.632). Furthermore, the 1 by 2 interaction (type and proportion of solvent) was found to be statistically significant ($p<0.05$), demonstrating that ethanol 60% provided a higher extraction of isoorientin. This analysis can be confirmed by the response surface, plotting type *versus* proportion of solvent (Fig. 4): the response surface shows the optimal level for each variable, and the highest response in the flavonoid extraction of the passion fruit was obtained with solvent ethanol 60% and 1.5 min of sonication.

The optimization of the HPLC conditions for quantitative analysis was a difficult task due to the highly complex chromatographic profile, which showed several flavonoid peaks. HPLC conditions were selected first on the basis of previous studies of *Passiflora* leaves flavonoids [7], which proved to be unsatisfactory for the quantitative analysis of fruit flavonoids. Therefore, different gradient programming, flow rate (0.8; 1.0; 1.2 mL min^{-1}) and temperatures (35; 40; 45 °C) were tested. Detection at λ = 330 nm proved high sensitivity

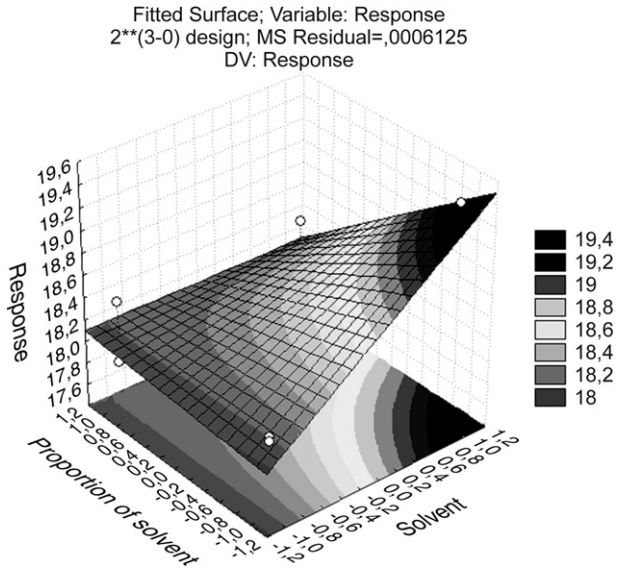

Fig. 4. Response surface estimated for the extraction of flavonoids from the passion fruit pulp, obtained by plotting type *versus* proportion of solvent.

for most passion fruit flavonoids, and the best results are illustrated by a typical chromatogram of the passion fruit pulp (Fig. 2).

3.2. Quantitative analysis of flavonoids in the passion fruit pulp

The flavonoid peaks were identified by their UV/DAD spectra due to their characteristic UV spectral pattern (Band I, λ_{max} around 300–350 nm and Band II, λ_{max} around 230–280 nm) [13]. This UV pattern allows for the selection of flavonoid peaks for quantitative analysis; hence, UV/DAD is an important alternative in the absence of a mass detector.

The peak purity of flavonoids was determined by overlapping the UV/DAD spectra in different regions of the same peak using the HPLC software. There was co-elution among some peaks considered flavonoids, which did not interfere in the quantification of total compounds.

The standard chosen for the validation process was isoorientin because it is the most abundant flavonoid identified in the passion fruit pulps. Its presence was confirmed by HPLC-UV/DAD and LC-MS/MS: characteristic fragment ions $[M–H-120]^-$ (m/z 327) and $[M–H-90]^-$ (m/z 357) of flavones *C*-glucosides were observed in the MS/MS spectra of isoorientin (Fig. 5) [14]. Only one additional flavone *C*-glucoside, isovitexin (Fig. 1), was also identified in the pulp extracts, and this compound was confirmed by LC-MS/MS analysis and comparison with an authentic standard (Fig. 5). However, as isovitexin is not a major peak and its chromatographic separation was not fully satisfactory (Fig. 2), this compound was not selected for the development of the analytical method. The standard addition method was the choice for the quantification of isoorientin since the passion fruit pulp is a complex sample matrix. Rutin (a widely available, low-cost flavonol *O*-glucoside) was chosen as the standard for the quantification of total flavonoids considering the proposition of an alternative analytical procedure suitable for the routine analysis of total flavonoids (*e.g.*, in the quality control in the food industry of the passion fruit pulp products), and also because only a few of *Passiflora C*-glycosylflavones are commercially available as analytical standards. Quantification of rutin was done by the external standard method, a simple procedure, specially if the analysis of a great number of plant samples is required. This approach has shown to be a valuable tool for the analysis of total flavonoids in *Passiflora* leaves extracts, including cultivation studies [7,15].

Linearity was checked based on the values of correlation coefficients of isoorientin and rutin analytical curves. All the curves showed a linear response with $r^2>0.999$, in the selected range, covering the recommended range of 70–120% [9]. Table 2 lists the LOD and LOQ values. The values of peak areas of the flavonoids below the LOQ were not used in the quantification of total flavonoids in extracts. These results suggest that the proposed HPLC method is sufficiently sensitive to determine flavonoids in the passion fruit pulp.

The precision of the method was estimated by measuring repeatability (intra-day, $n=3$) and intermediate precision (in three days, $n=3$) at three different levels. The relative standard deviation values for both parameters ranged from 0.1 to 3.7% (Table 3). Therefore, since all the values of RSD in the repeatability and intermediate precision estimates were below 5% (the value recommended by the ICH protocols), the methods were considered repeatable [9]. Recovery experiments were performed to evaluate the accuracy of the methods. Known amounts of the standards at three different concentrations levels were added to samples. As the passion fruit pulp samples already contained isoorientin, the area of its respective chromatographic peak obtained in the analysis of non-spiked samples was subtracted in the calculations of isoorientin recovery. Recoveries ranged from 84 to 91%, confirming the accuracy of the proposed methods (Table 3). Rutin was not found in the analyzed passion fruit pulp samples, so the good results of recovery from pulp samples spiked with rutin are indicative of the

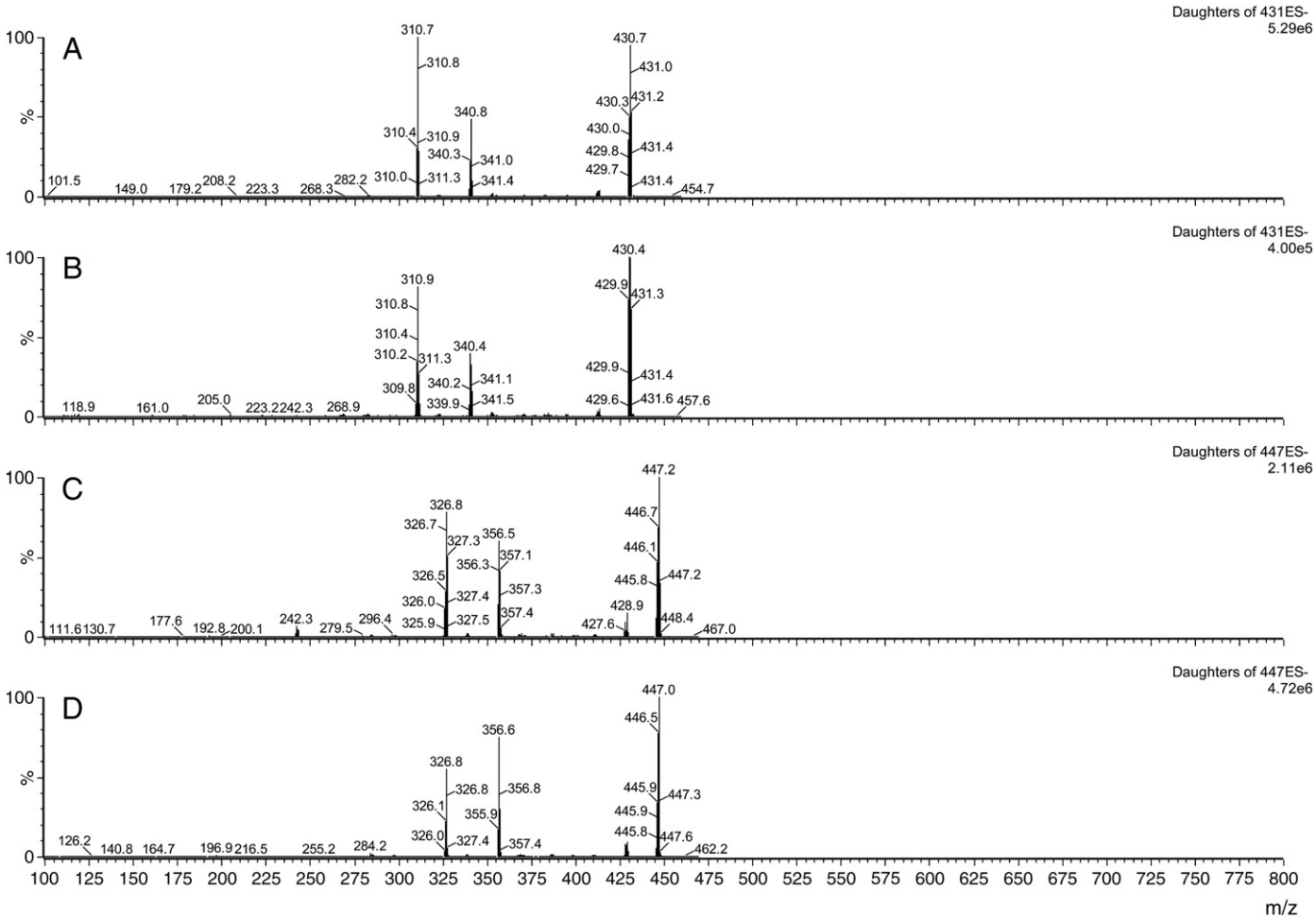

Fig. 5. LC-MS/MS spectra (ESI, negative mode) of (A) commercial standard of isovitexin; (B) peak from the passion fruit pulp extract identified as isovitexin (tr = 17.480 min in Fig. 2); (C) commercial standard of isoorientin; (D) peak from the passion fruit pulp extract identified as isoorientin (tr = 13.217 in Fig. 2). For LC-MS/MS conditions see Section 2.4.

Table 2
Parameters of the analytical curves, LOD and LOQ for flavonoids quantification by HPLC-UV/DAD.

Standard	Concentration range (mg L^{-1})	Equation ($y = a + bx$)	Correlation coefficients (r^2)	LOD	LOQ
Isoorientin	5.0–80.0	297,697.9 + 26,210.0x	0.99995	0.10 mg L^{-1} ($2.23 \cdot 10^{-4}$ mmol L^{-1})	0.4 mg L^{-1} ($8.92 \cdot 10^{-4}$ mmol L^{-1})
Rutin	50.0–250.0	−55,539.3 + 15,326.25x	0.99975	0.14 mg L^{-1} ($2.29 \cdot 10^{-4}$ mmol L^{-1})	0.57 mg L^{-1} ($9.34 \cdot 10^{-4}$ mmol L^{-1})

Table 3
Analytical performance data for the procedure applied to the quantitative analysis of flavonoids in the passion fruit pulp.

Standard	Concentration levels (mg L^{-1})	Recovery (%)[a]	Repeatability (1 day, $n = 3$) RSD (%)	Intermediate precision (3 days, $n = 3$) RSD (%)
Isoorientin[b]	20.0	87.04	0.65	3.43
	45.0	90.22	0.32	2.32
	70.0	91.13	0.78	3.81
Rutin[c]	70.0	86.22	0.11	2.25
	150.0	84.10	0.25	2.02
	250.0	87.67	0.16	3.73

[a] Mean value of 3 experiments in the same day.
[b] The amount of isoorientin originally present in passion pulp sample was subtracted for recovery calculation.
[c] Values obtained from experiments using the passion fruit pulp samples spiked with rutin.

good overall performance of the method (sample preparation and HPLC analysis).

The quantitative data (Table 4) indicated that the major flavonoid isoorientin represented 19% of the flavonoids in the passion fruit pulp. The total flavonoid content was calculated considering all the peaks with the characteristic flavonoid UV spectra [13], and the results were expressed as mg L^{-1} of rutin (Table 4). The total flavonoid content of the passion fruit pulp (~0.16 mg flavonoid mL^{-1} of pulp) was quite significant in comparison with other beverages that are sources of flavonoids, such as orange juice (~0.20 mg flavonoids mL^{-1} [16]) and sugarcane juice (~0.24 mg total flavonoids mL^{-1} juice (expressed as diosmin)), corresponding to ~0.40 mmol L^{-1} diosmin [17]. These data, however, should be considered only as indicative of the potential of the passion fruit pulp as a natural source of flavonoids and more extensive studies are required, including the analysis of several harvests from different geographical origins.

Table 4
Quantification of flavonoids content in the passion fruit pulp[a].

Sample	Total flavonoids, expressed as rutin		isoorientin	
	($mg\ L^{-1}$) ± s.d.	($mmol\ L^{-1}$) ± s.d.	($mg\ L^{-1}$) ± s.d.	($mmol\ L^{-1}$) ± s.d.
P. edulis pulp	158.037 ± 0.602	0.259 ± 0.001	16.226 ± 0.050	0.036 ± 0.001

[a] Mean values of 3 samples from the same harvest.

4. Conclusions

HPLC-UV/DAD proved suitable and reliable for the quantitative analysis of isoorientin as well as the quantification of total flavonoids in *P. edulis* pulp. The parameters evaluated here demonstrated that the methods offer good specificity, linearity, accuracy and precision within acceptable limits, while the LOD and LOQ confirmed the efficiency of the methodologies in quantifying low concentrations of flavonoids. All the criteria and parameters recommended for the ICH protocol [9] were respected in the validation procedures. The analytical method described for total flavonoids quantification using rutin as standard may be an alternative for routine analyses in view of its lower cost and the availability of commercial rutin.

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References

[1] K. Dhawan, S. Dhawan, A. Sharma, *Passiflora*: a review update, J. Ethnopharm. 94 (2004) 1–23.
[2] Instituto Brasileiro de Geografia e Estatística (IBGE), 2008, http://www.ibge.gov.br.
[3] C.A.M. Pereira, J.H.Y. Vilegas, Constituintes químicos e farmacologia do gênero *Passiflora* com ênfase a *P. alata* Dryander, *P. edulis* Sims e *P. incarnata* L. Rev. Bras. Med. 3 (2000) 1–12.
[4] U. Mareck, R. Galensa, K. Herrmann, Identizierung von passionsfruchtsaft in fruchtprodukten mitels HPLC, Z. Lebensm. Unters. Forsch. 191 (1990) 269–274.
[5] M. Pontes, J.C. Marques, J.S. Câmara, Headspace solid-phase microextraction–gas chromatography–quadrupole mass spectrometric methodology for the establishment of the volatile composition of *Passiflora* fruit species, Microchem. J. 93 (2009) 1–11.
[6] C.D. Birk, G. Provensi, G. Gosmann, F.H. Reginatto, E.P. Schenkel, TLC fingerprint of flavonoids and saponins from *Passiflora* species, J. Liq. Chrom. & Relat. Technol. 28 (2005) 2285–2291.
[7] C.A.M. Pereira, J.H. Yariwake, F.M. Lanças, J.N. Wauters, M. Tits, L. Angenot, A HPTLC densitometric determination of flavonoids from *Passiflora alata*, *P. edulis*, *P. incarnata* and *P. caerulea* and comparison with HPLC method, Phytochem. Anal. 15 (2004) 241–248.
[8] S.D. Müller, S.B. Vasconcelos, M. Coelho, M.W. Biavatti, LC and UV determination of flavonoids from *Passiflora alata* medicinal extracts and leaves, J. Pharm. Biom. Anal. 37 (2005) 399–403.
[9] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)—Validation of Analytical Procedures, Text and Methodology Q2 (R1), 2005, http://www.ich.org/cache/compo/363-272-1.html.
[10] S.L.C. Ferreira, W.N.L. dos Santos, M.A. Bezerra, V.A. Lemos, J.M. Sendra-Bosque, Use of factorial design and Doehlert matrix for multivariate optimisation of an on-line preconcentration system for lead determination by flame atomic absorption spectrometry, Anal. Bioanal. Chem. 375 (2003) 443–449.
[11] B. Barros Neto, I.S. Scarminio, R.E. Bruns, Como Fazer Experimentos: Pesquisa e Desenvolvimento na Ciência e na Indústria, Editora da Unicamp, Campinas, 2002.
[12] R. Colombo, F.M. Lanças, J.H. Yariwake, Determination of flavonoids in cultivated sugarcane leaves, bagasse, juice and in transgenic sugarcane by liquid chromatography-UV detection, J. Chromatogr. A 1103 (2006) 118–124.
[13] J.B. Harbone, Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 2 ed.Chapman and Hall, London, 1984.
[14] P. Waridel, J.L. Wolfender, J.B. Lachavanne, K. Hostettmann, Identification of the polar constituents of *Potamogeton* species by HPLC-UV with post-column derivatization, HPLC-MS^n and HPLC-NMR, and isolation of a new ent-labdane diglycoside, Phytochemistry 65 (2004) 2401–2410.
[15] M.C.H. Reimberg, R. Colombo, J.H. Yariwake, Multivariate analysis of the effects of soil parameters and environmental factors on the flavonoid content of leaves of *Passiflora incarnata* L, Rev. Bras. Farmacog. 19 (2009) 853–859.
[16] J. Peterson, J. Dwyer, Flavonoids: dietary occurrence and biochemical activity, Nutr. Res. 18 (1998) 1995–2018.
[17] F.C. Vila, R. Colombo, T.O. Lira, J.H. Yariwake, Microfractionation of flavones and antioxidant (radical scavenging) activity of *Saccharum officinarum* L, J. Braz. Chem. Soc. 19 (2008) 903–908.