Original Article

# Comparative study of *Passiflora* taxa leaves: II. A chromatographic profile

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A B S T R A C T

Popularly known as passion fruit, some species of the genus *Passiflora* are widely used in folk medicines, such as sedatives and tranquilizers in many countries. Although these plants are employed for the same purposes, research with different species of *Passiflora* has indicated their heterogeneous chemical compositions. Since different chemical compositions can result in varying degrees of therapeutic efficiency, quality control based on the chemical constituents of each species is essential. To that end, the aim of this study was to compare pharmacognostically species of *Passiflora* in order to establish a chromatographic profile for the quality control of drugs in herbal medicines containing passion fruit. The study was conducted by collecting samples of leaves from twelve *Passiflora* taxa (*i.e.*, ten species and two forms of *P. edulis*) – *P. actinia*, *P. alata*, *P. amethystina*, *P. capsularis*, *P. cincinnata*, *P. edulis* f. *flavicarpa*, *P. edulis* f. *edulis*, *P. incarnata*, *P. morifolia*, *P. urnifolia*, *P. coccinea*, and *P. setacea* – from different locations and obtaining their chromatographic profiles *via* thin-layer chromatography and high-performance liquid chromatography. Both methods used the flavonoid *C*-glycosides isoorientin, orientin, vitexin, and isovitexin as reference compounds and could ultimately establish specific profiles for each species. The chromatographic analyses discussed here can be used to assist in determining the quality and authenticity of herbal drugs derived from *Passiflora* species.

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## Introduction

Belonging to Passifloraceae family, the genus *Passiflora* encompasses nearly 600 species (Ulmer and MacDougal, 2004). Popularly known as passion fruit, in various countries some of these species are widely used in folk medicines, typically as sedatives and tranquilizers (Conrado et al., 2003). Although *Passiflora* species are employed for the same purposes, investigations have indicated their heterogeneous chemical compositions (Wohlmuth et al., 2010; Li et al., 2011; Zucolotto et al., 2012). Since different chemical compositions can result in varying degrees of therapeutic efficiency, it is critical to identify and differentiate species of the genus.

To achieve such identification and differentiation, pharmacobotanical tools and chromatographic analysis can offer great insights, particularly in regard to *Passiflora* species (Wosch et al., 2015). However, with these means, once original plant material is processed into a powder or when contaminants are present with similar morphoanatomic characteristics, which often occurs

accidentally (Veiga et al., 2005), pharmacobotanical tools cannot sufficiently authenticate species (Sucher and Carles, 2008). In such cases, it is necessary to use analytical methods to assess the qualitative and quantitative composition of each plant's chemical constituents.

To that end, chromatographic analysis can contribute to developing chemical profiles that aid in distinguishing species. The significance of applying chromatographic analysis also extends to secondary metabolites, which represent a chemical interface between a plant and its surrounding environment and whose synthesis is often affected by environmental conditions. As such, both seasonal and daily intraplant, intraspecies, and interspecies differences can affect the total content or relative proportions of secondary plant metabolites, if not both. Even in the presence of a genetic control, chemical constituents can be expressed in different ways owing to the interaction of biochemical, physiological, ecological, and evolutionary processes, which are considered to largely compromise any constancy in the concentration of secondary metabolites (Gobbo-Neto and Lopes, 2007).

Since environmental factors affect the final content of secondary metabolites in medicinal plants, they can also significantly affect the quality and therapeutic value of herbal preparations. To obtain

products composed of consistent, reproducible therapeutic properties, the quality control and standardization of herbal medicines are essential. Though both processes consist of several steps, sourcing and pinpointing the quality of raw materials are perhaps the most crucial. For that reason, yet also because too little information is available about most plants to guarantee their quality, efficacy, and safety, research on the quality control of medicinal plants is particularly important (Calixto, 2000).

According to Calixto (2000), standardizing and controlling the quality of raw plant material and related products intended for therapeutic use can be achieved by using analytical techniques such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), each employed alone or in combination with the other. At the same time, within the genus *Passiflora*, *C*-glycosylated flavonoids are the most studied class of chemicals, whose wide distribution in the genus and exhibition of both qualitative and quantitative differences among species are excellent chemical markers (Qimin et al., 1991; Bokstaller and Schmidt, 1997). Thus, the objective of our study was to analyze twelve *Passiflora* species by using TLC and HPLC, thereby enabling effective quality control for its leaves, all in order to contribute to the quality of herbal products containing passion fruit.

## Materials and methods

### Plant materials

We collected 32 samples from twelve *Passiflora* species on different days in the Brazilian states of Paraná, Santa Catarina, Rio Grande do Norte, and the Distrito Federal. One of the authors (DCI) performed botanical identification, and vouchers were deposited in different herbaria, according to Box 1 of Wosch et al. (2015).

### Processing of samples

Once collected, leaves were dried at 50 °C and ground in an analytical mill. The dried, ground samples were standardized in particle sizes equal to or less than 300 mm/μm.

### Thin-layer chromatography

To extract, 1 ml of 60% ethanol was added to 200 mg of each sample. The mixtures were vortexing for 10 s, and the samples were extracted for 10 min with ultrasound equipment. After decanting,
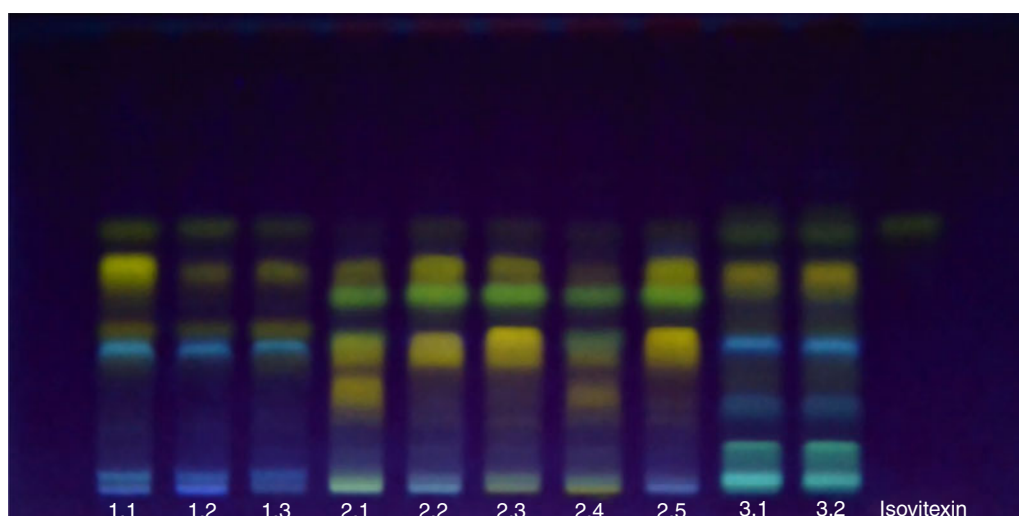


**Fig. 1.** Thin-layer chromatography profile of Samples 1.1–1.3 (*Passiflora actinia*), Samples 2.1–2.5 (*P. alata*), and Samples 3.1–3.2 (*P. amethystina*) using Eluent System 1, with isovitexin as the standard.
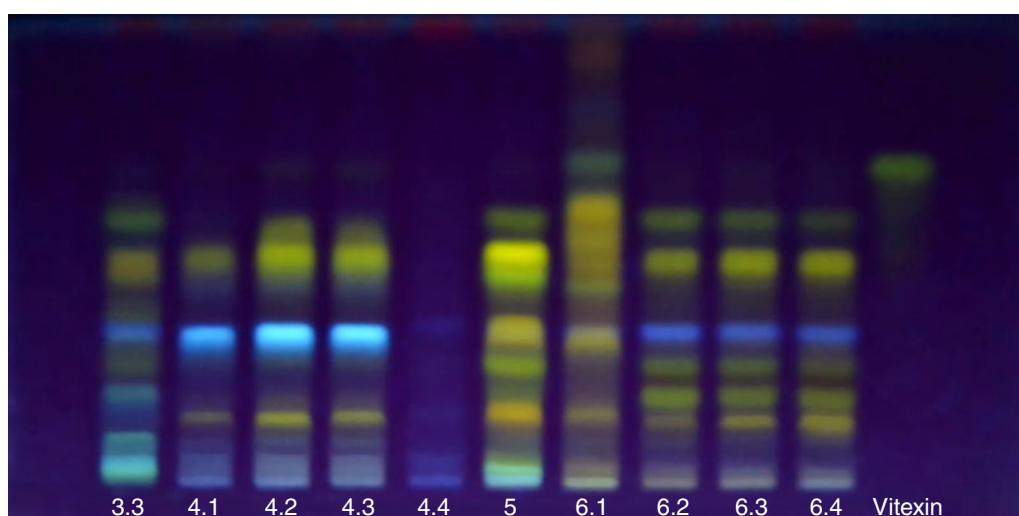


**Fig. 2.** Thin-layer chromatography profile of Samples 3.3 (*Passiflora amethystina*), Samples 4.1–4.4 (*P. capsularis*), 5 (*P. cincinnata*) and Samples 6.1–6.4 (*P. edulis* f. *flavicarpa*) using Eluent System 1, with vitexin as the standard.
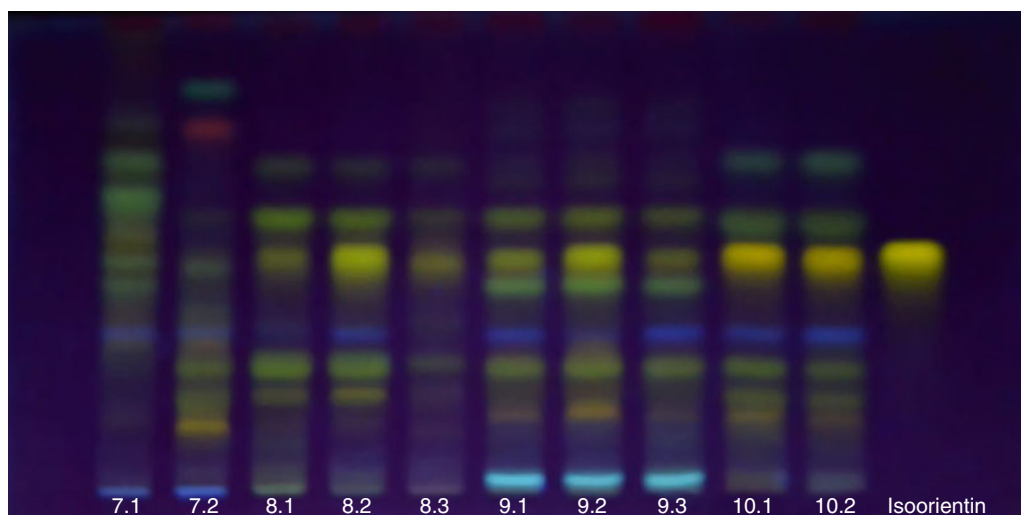
**Fig. 3.** Thin-layer chromatography profile of Samples 7.1–7.2 (*Passiflora edulis* f. *edulis*), Samples 8.1–8.3 (*P. incarnata*), Samples 9.1–9.3 (*P. morifolia*), and Samples 10.1–10.2 (*P. urnifolia*) using Eluent System 1, with isoorientin as the standard.

about 2 μl of the supernatant obtained was applied to chromatoplates in the form of bands. Silica gel 60 aluminum plates were used at 20 cm × 20 cm, without any fluorescence indicator for high-performance thin layer chromatography (HPTLC) (Merck®, Darmstadt, Germany, art. 5547).

The system was kept closed during analysis. Activated plates were held in an oven at 90 °C for 90 min, and the temperature and humidity were kept constant at 20 °C and 50%, respectively. The distance traveled by the eluent was standardized to 9 cm. Two different eluent systems (ES) were used: one of ethyl acetate, acetone, acetic acid, and water in a ratio of 6:2:1:1 (Gosmann et al., 2011) and the other – an adjustment of the mobile phase proposed by Wagner and Bladt (1996) – of ethyl acetate, acetic acid, formic acid, and water in a ratio of 10:1.1:1.1:2.5.

After the complete evaporation of the solvents, the plates were revealed with diphenylborioxietilamine 1% in MeOH and later with 5% polyethylene glycol 4000 in EtOH (Wagner and Bladt, 1996). Observations were made under UV 365 nm.

### High-performance liquid chromatography

To extract, 8 ml of 60% ethanol was added to 200 mg of the sample. The mixture was vortexing for 15 s, and the samples extracted for 30 min in ultrasound equipment. The extract was filtered, and the volume was completed to 10 ml with 60% ethanol. Samples were filtered through a Millex® LCR with membrane filter PTFE of 0.45 μm, packed in amber glass bottles at 4 °C until the time of analysis, and at that time, injected at 20 mg/ml.

Adapted from the method proposed by Muller et al. (2005), our method of determining flavonoid isovitexin in *Passiflora actinia* extracts was developed and validated by our research group. Later, the mobile phase consisted of a gradient of 0.5% acetic acid in MilliQ water (A), methanol (B), and acetonitrile (C) at 0 min with 75% (A), 15% (B) and 10% (C), at 25 min with 62% (A), 20% (B) and 18% (C), and at 30 min with 75% (A), 15% (B) and 10% (C). The flow rate was 1 ml/min for a running time of 30 min with a detection wavelength of 340 nm.

Analyses were performed with Varian ProStar Gradient equipment, a ProStar 230 pump, and a photodiode array detector 335 (Column Kromasil® 100, 5 μm C-18 [250 mm × 4.6 mm in diameter]).
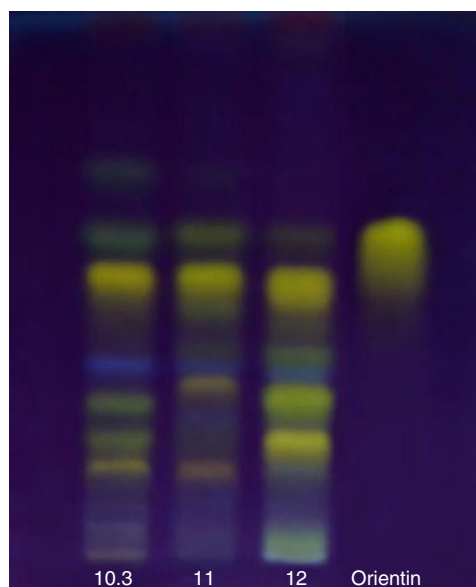


**Fig. 4.** Thin-layer chromatography profile of Samples 10.3 (*Passiflora urnifolia*), Sample 11 (*P. coccinea*), and Sample 12 (*P. setacea*) using Eluent System 1, with orientin as the standard.

### Spectral analysis

Spectral analyses were performed using the Star Chromatography Workstation for data acquisition within Varian ProStar Gradient HPLC.

### Standards

Standards were isovitexin from ChromaDex®, vitexin, isoorientin, and orientin were graciously provided by Dr. Maique W. Biavatti at Universidade Federal de Santa Catarina, Brazil. Solutions were prepared at 1 mg/ml in methanol to isovitexin, isoorientin, and orientin, as well as methanol and water, in a ratio of 1:1 (v/v) to vitexin. For TLC, no further dilution was performed, and for HPLC, the same solubilizing solvents were used for the required dilution.
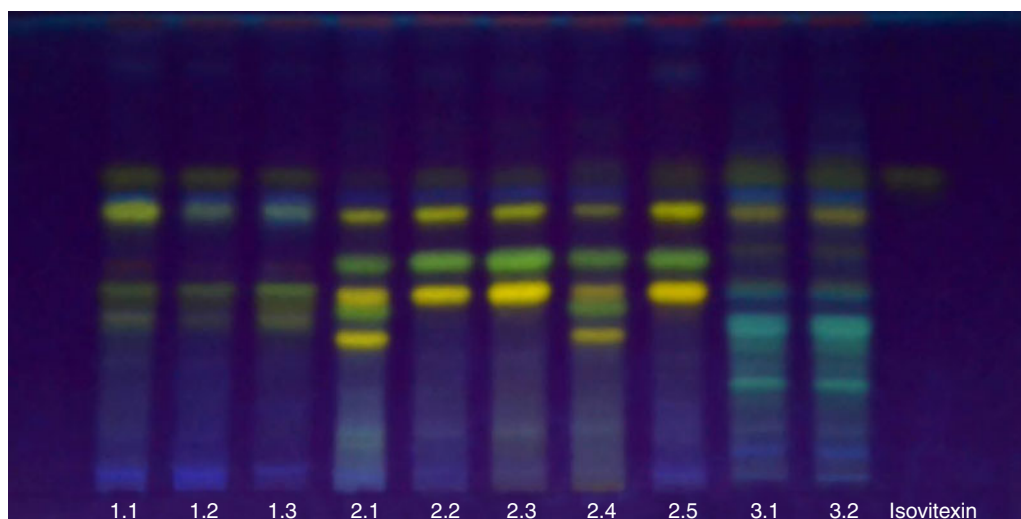
**Fig. 5.** Thin-layer chromatography profile of Samples 1.1–1.3 (*Passiflora actinia*), Sample 2.1–2.5 (*P. alata*), and Sample 3.1–3.2 (*P. amethystina*) using Eluent System 2, with isovitexin as the standard.

## Results

### Thin-layer chromatography

The results obtained with ES 1 appear in Box 1 and Figs. 1–4, whereas those obtained with ES 2 appear in Box 2 and Figs. 5–8. Both ES 1 and ES 2 allowed the observation of differences among samples. With ES 2, however, since the bands became more defined, it was possible to display more bands, which facilitated the comparison among species profiles (Figs. 5–8).

The three specimens analyzed for the species *P. actinia* (Samples 1.1–1.3) showed similar chromatographic profiles. An apparent quantitative difference was observed in the second band of Sample 1.1 (ES 1), which presented a more intense yellow color (Figs. 1 and 5). According to $R_f$ values and color, this band seemed to correspond to the standard isoorientin.

The samples analyzed for *P. alata*, by contrast, revealed qualitative differences (Figs. 1 and 5). However, those samples could be divided into two groups based on the similarity of their profiles: on the one hand, Samples 2.1 and 2.4, and on the other, Samples 2.2, 2.3, and 2.5. The chief difference between the groups is the presence of a yellow spot (ES 1, $R_f$ 0.22) or of two bands (ES 2, $R_f$ 0.39 and 0.33) in Samples 2.1 and 2.4, respectively, that were absent in the others.

Samples of *P. amethystina* (3.1, 3.2, and 3.3) were very similar (Figs. 1, 2 and 5, 6).

By presenting bands with hardly any detectable staining intensity, Sample 4.4 of *P. capsularis* stands out from the other three of the species, which were similar (Figs. 2 and 6).

The single sample of *P. cincinnata* studied showed numerous bands on both ES 1 and ES 2 (Figs. 2 and 6).

Whereas the first sample of *P. edulis* f. *flavicarpa* (6.1) differed entirely from the others by showing four bands on ES 1, all with a $R_f$ (0.93, 0.81, 0.70, 0.59) greater than the largest of the other three samples (Samples 6.2–6.4), the others were quite similar (Figs. 2 and 6). Most bands in Samples 6.2–6.4 were not present, or at least indistinguishable from Sample 6.1 (Fig. 2).

The two specimens analyzed for *P. edulis* f. *edulis* showed starkly different profiles. Whereas bands of Sample 7.1 presented a yellow-to-green band concentrated in the upper region of the plate and with higher $R_f$ values, bands with yellow coloring from Sample 7.2 had lower $R_f$ values (Figs. 3 and 7). Moreover, as Fig. 3
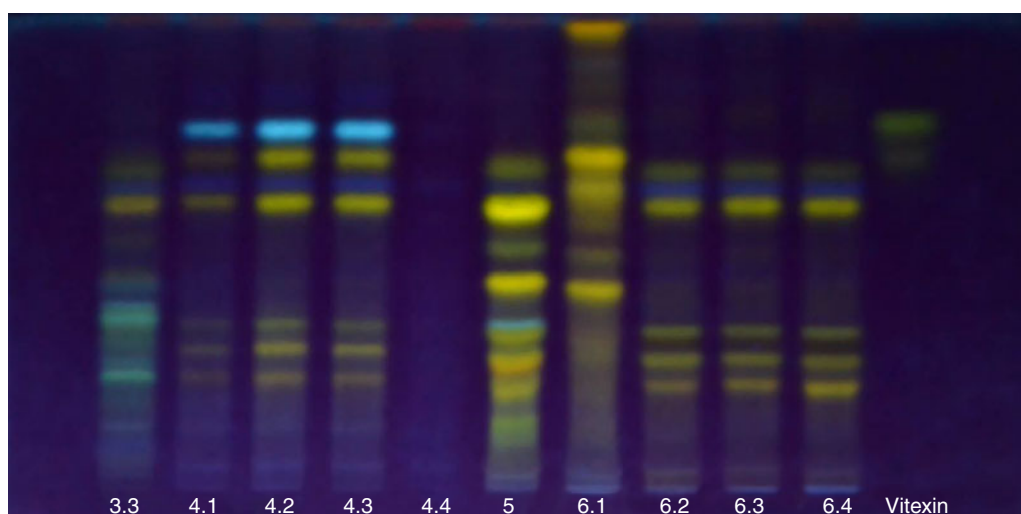


**Fig. 6.** Thin-layer chromatography profile of Samples 3.3 (*Passiflora amethystina*), Samples 4.1–4.4 (*P. capsularis*), Sample 5 (*P. cincinnata*), and Samples 6.1–6.4 (*P. edulis* f. *flavicarpa*) using Eluent System 2, with vitexin as the standard.
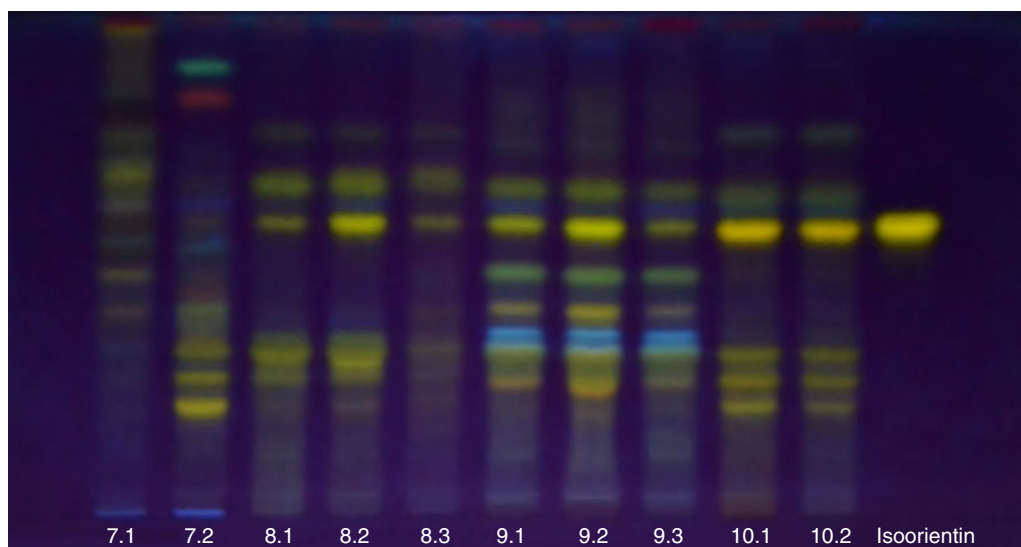
**Fig. 7.** Thin-layer chromatography profile of Samples 7.1–7.2 (*Passiflora edulis* f. *edulis*), Samples 8.1–8.3 (*P. incarnata*), Samples 9.1–9.3 (*P. morifolia*), and Samples 10.1–10.2 (*P. urnifolia*) using Eluent System 2, with isoorientin as the standard.
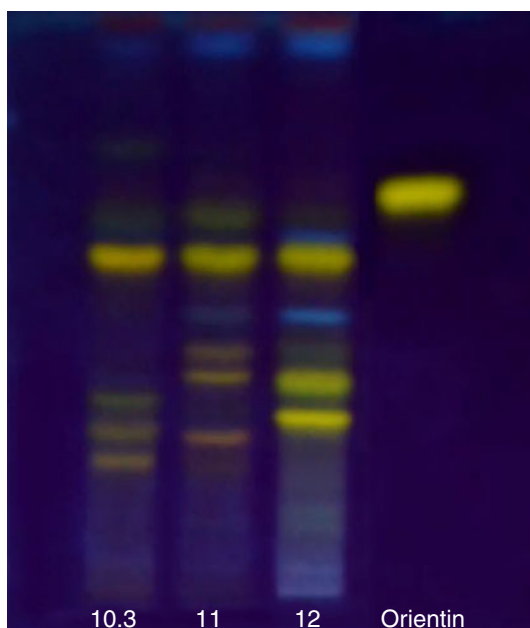


**Fig. 8.** Thin-layer chromatography profile of Sample 10.3 (*Passiflora urnifolia*), Sample 11 (*P. coccinea*), and Sample 12 (*P. setacea*) using Eluent System 2, with orientin as the standard.

shows, with ES 1 Sample 7.2 revealed two bands not found in any other sample analyzed (green and red, $R_f$ 0.86 and 0.77, respectively).

Similar profiles were found for Samples 8.1, 8.2 and 8.3 of *P. incarnata* (Figs. 3 and 7). A lower intensity of staining of bands appeared in Sample 8.3, by contrast, thus suggesting that they have a lower concentration of substances viewable in thin-layer chromatography methods. No significant differences were observed between the profiles of *P. morifolia* (Samples 9.1–9.3) and *P. urnifolia* (Samples 10.1–10.3), as Figs. 3, 4, 7, 8 illustrate.

Lastly, marked differences were observable among specimens of *P. coccinea* (11) and *P. setacea* (12). In terms of the band with lower $R_f$ values, two were orange and one was orange-to-blue for *P. coccinea*, and two were yellow for *P. setacea* in ES 1 (Fig. 4).

### High-performance liquid chromatography

#### Comparison of profiles of each species

Most specimens of each species analyzed by HPLC coupled to a diode array detector showed similar qualitative profiles and were therefore superimposable (Figs. 9 and 10). HPLC allows the detection of qualitative differences already identified in the flavonoid profile of some samples when compared to other specimens of the same species observed with TLC. Such differences were observed in *P. alata*, with samples divided into two groups according to the similarity of their profiles (Samples 2.1 and 2.4 and Samples 2.2, 2.3, and 2.5), as well as in Sample 6.1 of *P. edulis* f. *flavicarpa* (Fig. 9) and two samples of *P. edulis* f. *edulis* (Fig. 10).

With all samples processed in the same manner and injected in the same amounts and concentrations, HPLC allows a quantitative comparative analysis of samples with greater reliability than that achieved with TLC.

Sample 4.4 of *P. capsularis* presented the most significant quantitative variation (Fig. 9). Overlapping of the chromatograms obtained for this species allowed the visualization of peaks in 4.4 at the same retention times of those found in other samples (Fig. 11). Given its lower sensitivity, TLC did not permit such visualization.

Sample 8.3 of *P. incarnata* showed pronounced quantitative differences compared to Samples 8.1 and 8.2, chiefly by displaying less intense peaks (Fig. 10). Other variations observed were less significant.

#### Comparison of species by coinjection with standard

Coinjections with standards—that is, at least one flavonoid glycoside—allowed a correlation of some peaks in each profile, as summarized in Box 3.

Peaks were colored according with the flavonoids identified by coinjection: blue for isovitexin, pink for vitexin, green for isoorientin, and purple for orientin (Figs. 12 and 13). Partially colored peaks were partially co-eluted with standards, whereas bicolored peaks were co-eluted with two standards.

### Spectral analysis

All peaks co-eluted with vitexin and isovitexin showed spectra with the same UV profile over the whole peak, thus indicating their
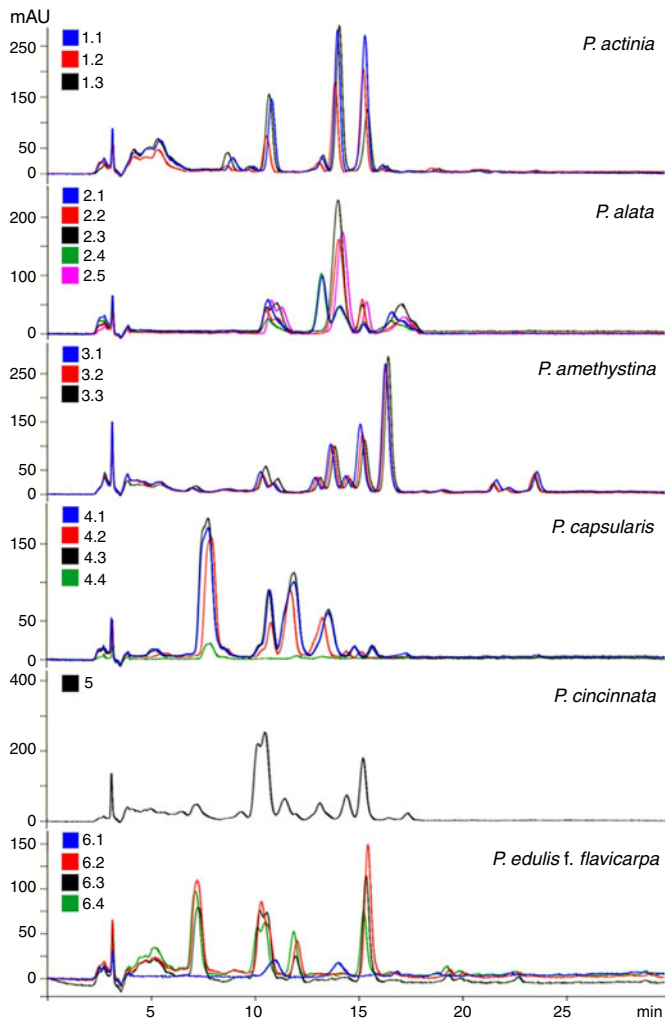
**Fig. 9.** Overlay of the chromatograms obtained by high-performance liquid chromatography for samples of *Passiflora actinia*, *P. alata*, *P. amethystina*, *P. capsularis*, and *P. cincinnata* compared to *P. incarnata* and *P. edulis* f. *flavicarpa*.

**Fig. 10.** Overlay of the chromatograms obtained by high-performance liquid chromatography for samples of *Passiflora edulis* f. *edulis*, *P. incarnata*, *P. morifolia*, *P. urnifolia*, *P. coccinea*, and *P. setacea*.

pureness. Regarding peaks co-eluted with isoorientin and orientin, purity varied according to the chromatogram analyzed.

For *P. actinia*, Peak 3 related to isoorientin and, though symmetrical, exhibited a shift in spectral profile from 276/348 to 278/341 nm at its right end. Peak 3, of *P. capsularis*, was also

comprised of two separate spectra, meaning that it did not correspond only to orientin.

Peak 2 from Sample 2.1 of *P. alata* showed a very similar spectrum to Peak 3 (275/343 nm for Peak 2 and 275/345 nm for Peak 3), thus suggesting that it could be a substance with a structure similar to that of vitexin.



**Fig. 11.** High-performance liquid chromatography profile of *Passiflora capsularis* Samples 4.4, with Sample 4.2 representing the other samples, in relation to thickness variation of leaf mesophyll and quantitative chemical composition (detection: 340 nm; bar = 50 μm).

**Box 1**

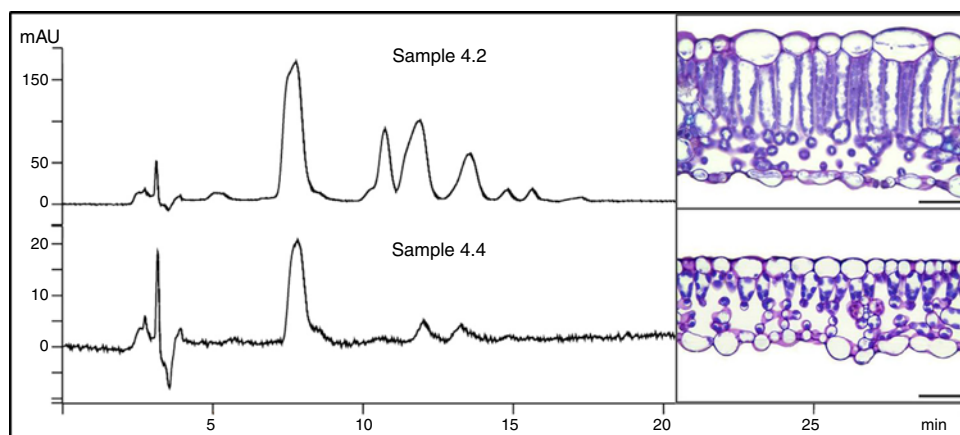Summary of results obtained with thin-layer chromatography using Eluent System 1 for twelve *Passiflora* species.

| Species | Sample (s) | Number of major bands | Color | $R_f$ [a] | Standards assigned |
|---|---|---|---|---|---|
| *P. actinia* | 1.1, 1.2 and 1.3 | 6 | Yel-Gn; yellow; yellow; BBe; yellow; Be | 0.57; 0.49; 0.35; 0.32; 0.28; 0.04 | Isoorientin, isovitexin |
| *P. alata* | 2.1 and 2.4 | 5 | Yel-Gn; yellow; Gn; Yel-Gn; Yel | 0.57; 0.49; 0.41; 0.31; 0.22 | Isoorientin, isovitexin |
| | 2.2, 2.3 and 2.5 | 5 | Yel-Gn; Be; yellow Gn; Yel | 0.57; 0.51; 0.49; 0.41; 0.31 | Isoorientin, isovitexin |
| *P. amethystina* | 3.1, 3.2 and 3.3 | 5 | Yel-Gn; Yel; BBe; Be; BGn | 0.57; 0.49; 0.32; 0.19; 0.10 | Isoorientin, isovitexin |
| *P. capsularis* | 4.1, 4.2 and 4.3 | 3 | Yel; BBe; Yel | 0.49; 0.33; 0.15 | Isoorientin |
| *P. cincinnata* | 5 | 6 | Yel-Gn; Yel; Yel-Gn; Oe; Yel-Gn; Oe | 0.57; 0.49; 0.44; 0.33; 0.26; 0.16 | isoorientin, isovitexin |
| *P. edulis* f. *flavicarpa* | 6.1 | 7 | Oe; Be; Yel-Gn; Oe; Yel; Oe; Oe | 0.93; 0.81; 0.70; 0.59; 0.43; 0.31; 0.15 | Isoorientin, isovitexin |
| | 6.2, 6.3 and 6.4 | 7 | Yel-Gn; Yel; BBe; Yel-Gn; Bn; Yel-Gn; Oe | 0.57; 0.49; 0.33; 0.26; 0.22; 0.19; 0.15 | Isoorientin, isovitexin |
| *P. edulis* f. *edulis* | 7.1 | 8 | Gn; Bn; Gn; Gn; Oe; Gn; Yel; BBe | 0.78; 0.75; 0.70; 0.62; 0.52; 0.49; 0.45; 0.34 | Vitexin |
| | 7.2 | 8 | Gn; Yel; Yel-Gn; Yel-Gn; BBe; Yel-Gn; Yel-Gn; Oe | 0.86; 0.77; 0.58; 0.47; 0.34; 0.27; 0.19; 0.14 | Isovitexin |
| *P. incarnata* | 8.1 and 8.2 | 6 | Yel-Gn; Yel-Gn; Yel; BBe; Yel-Gn; Yel | 0.69; 0.57; 0.50; 0.33; 0.27; 0.20 | Isoorientin, vitexin, isovitexin |
| | 8.3 | 5 | Yel-Gn; Yel-Gn; Yel; Yel-Gn; Yel | 0.69; 0.57; 0.50; 0.27; 0.21 | Isoorientin, vitexin, isovitexin |
| *P. morifolia* | 9.1, 9.2 and 9.3 | 9 | Yel; Yel; Yel; Yel; Yel-Gn; BBe; Yel-Gn; Oe; BGn | 0.80; 0.68; 0.58; 0.50; 0.44; 0.33; 0.27; 0.16 | Isoorientin, vitexin, isovitexin |
| *P. urnifolia* | 10.1, 10.2 and 10.3 | 7 | Gn; Yel-Gn; Yel; BBe; Yel; Yel; Oe | 0.71; 0.57; 0.50; 0.33; 0.27; 0.19; 0.16 | Isoorientin, isovitexin |
| *P. cocinea* | 11 | 6 | Yel; Yel; BBe; Be; Be; Oe | 0.59; 0.51; 0.35; 0.33; 0.26; 0.17 | Isoorientin, isovitexin |
| *P. setacea* | 12 | 6 | Yel; Yel; Yel; BBe; Yel; Yel | 0.59; 0.51; 0.38; 0.35; 0.30; 0.20 | Isoorientin, isovitexin |
| Standards | Isovitexin | 1 | Yel-Gn | 0.57 | |
| | Vitexin | 1 | Yel-Gn | 0.69 | |
| | Isoorientin | 1 | Yel | 0.50 | |
| | Orientin | 1 | Yel | 0.60 | |

Yel, yellow; Yel-Gn, greenish yellow; Oe, orange; Be, blue; BBe, bright blue; Gn, green; BGn, bright green; Bn, brown.

[a] Data are presented in order of appearance, from top to bottom, on the chromatographic plate.

**Box 2**

Summary of results obtained with thin-layer chromatography using Eluent System 2 for twelve *Passiflora* species.

| Species | Sample (s) | Number of major bands | Color | $R_f$ [a] | Standards assigned |
|---|---|---|---|---|---|
| *P. actinia* | 1.1, 1.2 and 1.3 | 7 | Yel-Gn; Be; Yel; Red; Yel-Gn; Yel-Gn; Be | 0.66; 0.62; 0.59; 0.47; 0.42; 0.36; 0.04 | Isoorientin, isovitexin |
| *P. alata* | 2.1 and 2.4 | 7 | Yel-Gn; Be; Yel; Gn; Yel; Gn; Yel | 0.66; 0.62; 0.59; 0.50; 0.42; 0.39; 0.33 | Isoorientin, isovitexin |
| | 2.2, 2.3 and 2.5 | 5 | Yel-Gn; Be; Yel; Gn; Yel; | 0.66; 0.62; 0.59; 0.50; 0.42 | Isoorientin, isovitexin |
| *P. amethystina* | 3.1, 3.2 and 3.3 | 9 | Be; Yel-Gn; Be; Yel; Yel; Yel-Gn; BGn; BGn; Be | 0.71; 0.66; 0.62; 0.59; 0.50; 0.42; 0.34; 0.22; 0.07 | Isoorientin, isovitexin |
| *P. capsularis* | 4.1, 4.2 and 4.3 | 7 | BBe; Yel; Be; Yel; Yel; Yel; Oe | 0.76; 0.70; 0.65; 0.61; 0.35; 0.31; 0.24 | Isoorientin, orientin |
| *P. cincinnata* | 5 | 10 | Yel; Be; Yel; Yel; Yel; Be; Yel; Oe; Yel; Yel | 0.68; 0.65; 0.61; 0.52; 0.45; 0.36; 0.33; 0.28; 0.21; 0.13 | Isoorientin, isovitexin |
| *P. edulis* f. *flavicarpa* | 6.1 | 8 | Oe; Yel; Yel; Yel; Yel; Yel; Oe; Yel | 0.98; 0.91; 0.86; 0.78; 0.71; 0.62; 0.50; 0.43 | Isoorientin, isovitexin |
| | 6.2, 6.3 and 6.4 | 7 | Yel; Be; Yel; Yel; Yel; Bn; Oe | 0.68; 0.65; 0.60; 0.34; 0.32; 0.29; 0.23 | Isoorientin, isovitexin |
| *P. edulis* f. *edulis* | 7.1 | 11 | Oe; Bn; Yel; Yel; Yel; Bn; Yel-Gn; Bn; Yel; Oe; Be | 0.98; 0.80; 0.75; 0.68; 0.61; 0.58; 0.55; 0.52; 0.48; 0.41; 0.33 | Vitexin, orientin |
| | 7.2 | 10 | Gn; Red; Be; Yel-Gn; Gn; Oe; Yel; Yel; Yel; Yel | 0.90; 0.84; 0.62; 0.58; 0.53; 0.44; 0.41; 0.33; 0.28; 0.22 | – |
| *P. incarnata* | 8.1 and 8.2 | 7 | Yel; Yel; Be; Yel; Yel; Yel; Yel | 0.77; 0.67; 0.62; 0.59; 0.37; 0.33; 0.27 | Isoorientin, vitexin, isovitexin |
| | 8.3 | 6 | Yel; Yel; Yel; Yel; Yel; Yel | 0.77; 0.67; 0.59; 0.37; 0.33; 0.27 | |
| *P. morifolia* | 9.1, 9.2 and 9.3 | 10 | Yel; Yel; Yel; Yel; Yel; Yel-Gn; Oe; BBe; Yel; Oe | 0.82; 0.75; 0.67; 0.63; 0.59; 0.50; 0.41; 0.36; 0.32; 0.26 | Isoorientin, vitexin, isovitexin |
| *P. urnifolia* | 10.1, 10.2 and 10.3 | 6 | Yel-Gn; Yel-Gn; Yel; Yel; Yel; Yel | 0.77; 0.65; 0.58; 0.34; 0.28; 0.23 | Isoorientin, isovitexin |
| *P. cocinea* | 11 | 7 | BBe; Yel-Gn; Yel; Be; Oe; Yel; Oe | 0.96; 0.64; 0.57; 0.48; 0.43; 0.39; 0.28 | Isoorientin, isovitexin |
| *P. setacea* | 12 | 8 | BBe; Yel-Gn; BBe; Yel; BBe; Yel-Gn; Yel; Yel | 0.96; 0.65; 0.62; 0.58; 0.48; 0.42; 0.37; 0.30 | Isoorientin, isovitexin |
| Standards | Isovitexin | | Yel, Yel-Gn | 0.66 | |
| | Vitexin | | Yel-Gn | 0.78 | |
| | Isoorientin | | Yel | 0.58 | |
| | Orientin | | Yel | 0.68 | |

Yel, yellow; Yel-Gn, greenish yellow; Oe, orange; Be, blue; BBe, bright blue; Gn, green; BGn, bright green; Red, red; Bn, brown.

[a] Data are presented in order of appearance, from top to bottom, on the chromatographic plate.

**Box 3**
Standards attributed to particular species in high-performance liquid chromatography, by co-elution and UV absorption.

| Species | | Standards |
|---|---|---|
| *P. actinia* | | Isoorientin, vitexin, isovitexin |
| *P. alata* | | Isoorientin, orientin, vitexin, isovitexin |
| *P. amethystina* | | Isoorientin, vitexin, isovitexin |
| *P. capsularis* | | Isoorientin, orientin, vitexin, isovitexin |
| *P. cincinnata* | | Isoorientin, vitexin, isovitexin |
| *P. edulis* f. *flavicarpa* | | Isoorientin, isovitexin |
| *P. edulis* f. *edulis* | 7.1 | Isoorientin, vitexin |
| | 7.2 | Vitexin, isovitexin |
| *P. incarnata* | | Isoorientin, vitexin, isovitexin |
| *P. morifolia* | | Isoorientin, vitexin, isovitexin |
| *P. urnifolia* | | Isoorientin, isovitexin |
| *P. coccinea* | | Isoorientin, isovitexin |
| *P. setacea* | | Isoorientin, isovitexin |
| Estrela-do-cerrado | | Isoorientin, isovitexin |
| Rubiflora | | Isoorientin, isovitexin |
| Roseflora | | Isoorientin, isovitexin |
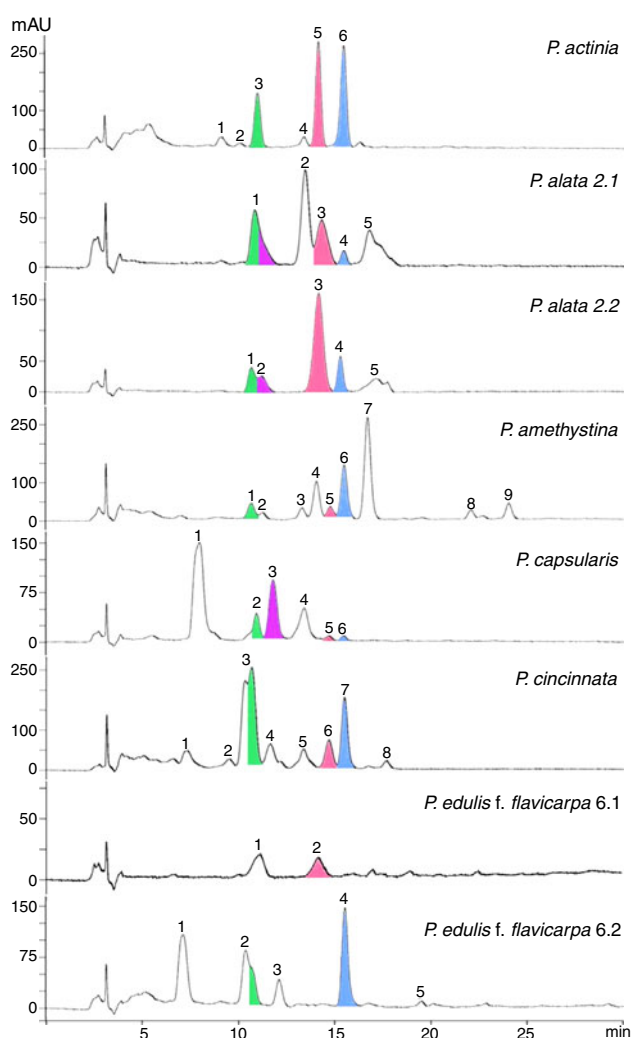


**Fig. 12.** Comparison of different chromatographic profiles obtained by high-performance liquid chromatography for the species *Passiflora actinia*, *P. alata* (Samples 2.1 and 2.2), *P. amethystina*, *P. capsularis*, *P. cincinnata*, and *P. edulis* f. *flavicarpa* (Samples 6.2 and 6.1). Peaks are identified by coinjection with standard: green for isoorientin, purple for orientin, pink for vitexin, and blue for isovitexin.
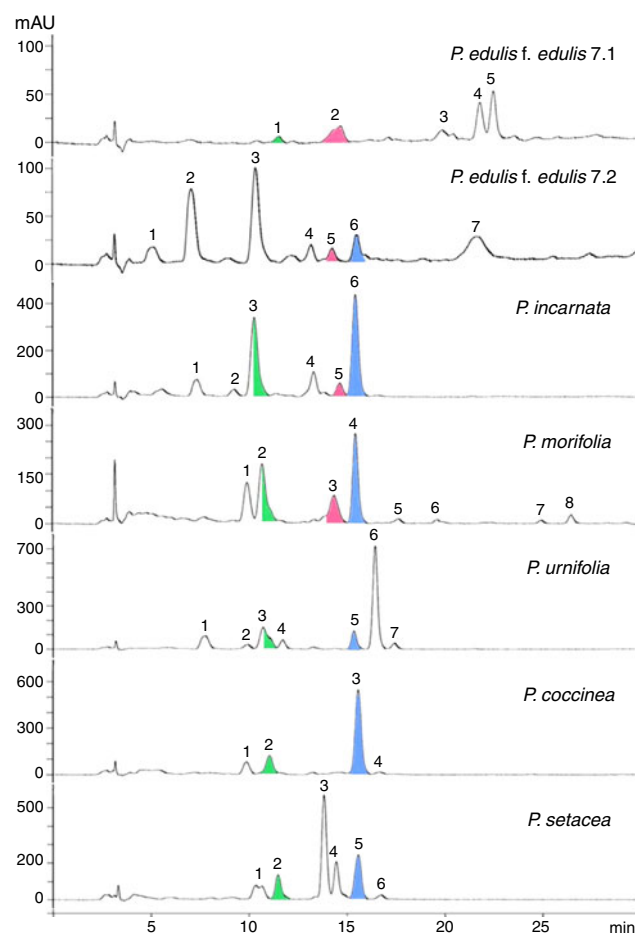


**Fig. 13.** Comparison of different chromatographic profiles obtained by high-performance liquid chromatography for the species *Passiflora edulis* f. *edulis* (7.2), *P. incarnata*, *P. morifolia*, *P. urnifolia*, *P. coccinea*, and *P. setacea*. Peaks identified by coinjection with standard: green for isoorientin, purple for orientin, pink for vitexin, and blue for isovitexin.

Peak 7, of *P. amethystina*, in being unidentified, presents a spectrum with maximum absorption at 277/343 nm, which was virtually identical to that of Peaks 5 (277/343 nm) and 6 (276/343 nm), thereby corresponding to vitexin and isovitexin, respectively.

Peaks 1–4 of *P. capsularis* were impure. Peak 1 probably represented a flavonoid, for its spectrum presented a maximum absorption at only 335 nm. The same occurred for Peak 4, which presented a maximum absorption at 318 and 336 nm. Peak 3 was contaminated with a substance, which caused it to show a spectrum with a maximum at 320 nm.

The profile presented for Peak 3 by Sample 6.2 of *P. edulis* f. *flavicarpa* shows a maximum absorption at only 279 nm, which is inconsistent with that of flavonoids.

Only Peak 4 of *P. edulis* f. *edulis* (Sample 7.1) presented a spectrum unlike the others, with a maximum at 276 nm. Peak 2, although asymmetrical, showed the same spectra, with a maximum absorption at 274/346 nm.

For Sample 7.2, Peaks 4 and 7 showed only an absorption maximum in their spectra. Peak 3 of this sample showed a spectrum with absorptions at 280/339 nm, which were relatively distant from 275/355 nm—that is, the pattern presented for isoorientin—probably corresponding to a flavonoid with a retention time closer to that of isoorientin.

Peak 8 of *P. morifolia* did not show any characteristic spectral profile of flavonoids.

All other peaks not quoted exhibited two absorption maxima at wavelengths similar to those presented by the standards.

## Discussion

### Chromatographic analysis

As a method in the quality control of drug materials, TLC was chosen for being the simplest and most economical chromatographic technique for rapid separation and visual identification (Lopes, 2006). Both ES 1 and ES 2 allowed us to differentiate species by presenting a profile for each. ES 2 allowed the visualization of more bands, which greatly facilitated comparison. At the same time, all variations detected by HPLC were also observed with TLC. Similarly, the two eluent systems allowed the correlation of some bands with patterns used (Boxes 1 and 2), which was also possible with HPLC (Box 3).

For these reasons, the two methods developed can be used for the quality control of drugs and extracts of leaves of *Passiflora* species. Such methods allow the detection of qualitative and quantitative variables among samples of the same species or form. Regarding qualitative variations, samples of *P. alata* differed greatly and could be placed in two groups, whose chief difference was the presence of an additional peak, as in Samples 2.1 and 2.4. Similar profiles to the two profiles found in our study were also reported by Muller et al. (2005) and Madoglio (2011), the latter of whom showed that the peak co-eluted with standard vitexin was vitexin-2-*O*-rhamnoside.

The species *P. alata* presented a variability already well documented in the literature. Meletti et al. (2003) found a variation in its morphological and agronomic characters, whereas Bellon et al. (2009) detected its genetic variability, which is more pronounced in wild accessions due to the wide geographical distribution of the species. Such well-documented genetic variability could relate to the diversity in the chemical constitution of different *P. alata* species, and since different chemical constitutions cause different therapeutic efficiencies, the standardization of the plant extract and product drugs is necessary based on their chemical constitution. This became evident in our study by observing the chromatograms of the two forms of *P. edulis* (Figs. 3, 7 and 10) that showed significant chemical differences, as well as in the samples.

Zucolotto et al. (2012) studied the two forms of *P. edulis* and obtained a chromatogram for *P. edulis* f. *flavicarpa* that closely resembled that for our Samples 6.2–6.4 than for our Sample 6.1. Their chromatogram for *P. edulis* f. *edulis* was also closer to our Sample 7.1. On this point, the authors suggested that the results for the two forms of *P. edulis* were due to a wide range of inter- and intrachemical compositional forms.

Viana et al. (2003) detected genetic variability among the forms of *P. edulis*, which along with the results obtained in this study indicate the importance of identifying the form of the species, as well as the chemical characterization and standardization of herbal drugs and extracts of *P. edulis*. These drugs and extracts could form part of the constitution of teas, herbal remedies, or cosmetics, considering that the concentration and composition of phenolic substances correlate with biological activity (Colomeu et al., 2014).

Regarding quantitative variations, *P. capsularis* samples showed the most pronounced difference, which related to morphoanatomical variation. Sample 4.4 of *P. capsularis* showed peaks at far lower intensities than those of Samples 4.1–4.3 (Figs. 2, 6 and 9), as clearly shown in Fig. 11, in which the qualitative composition of Sample 4.4 greatly resembles to the composition presented by the others.

In addition to peaks with lower intensities, the mesophyll thickness of Sample 4.4 was narrower than that of one other sample due to environmental influences. Sample 4.4 was collected from an Atlantic forest, where it had grown in the shadow of trees, whereas the other samples were collected from an open space with direct exposure to the sun.

In addition to reporting the same morphoanatomic differences for *P. capsularis*, Tattini et al. (2000) showed that the species *Phyllyrea latifolia* grown in shady spaces under a thick forest of *Pinus pinea* or grown in the full sun of open dunes demonstrated the accumulation of flavonoids and glandular trichomes in the leaves. Those authors reported that the concentration of flavonoid glycosides showed a sharp increase in leaves collected from sunny places. Nevertheless, the composition of flavonoids remained unchanged, as in agreement with findings for *P. capsularis* in our work.

Such differences in concentration result from plant adaptations to different light intensities by regulating their physiological states and changing their primary and secondary metabolic pathways. In doing so, their anabolic and catabolic processes achieve their maximum functional status (Nobel, 1991).

*P. incarnata* samples also exhibited quantitative variations, as in Sample 8.3, which showed main peaks with lower intensities along with UV absorption. Unlike others of the species, Sample 8.3 was a dried extract provided by the herbal industry, while the other two samples were collected at a cultivation site. The detection of problems in commercial extracts is not uncommon, however. The importance of qualitative and quantitative standardization of flavonoids present in the drugs or extracts of *Passiflora* species, especially in preparations of *P. incarnata*, relies on the fact that flavonoids such as vitexin, isovitexin, orientin, and isoorientin contribute to the drug activity (Menghini and Mancini, 1988).

Unfortunately, it is not unusual to detect problems in commercial extracts. Silva et al. (2013) tested the effectiveness, regarding to the protection of fibroblasts against the effects of UV rays, five green tea commercial extracts used to enrich formulations cosmetic, compared to a fluid extract prepared according to the recommendations of the Brazilian Pharmacopoeia (2010). The authors observed that the EGCG content was much higher in the extract prepared according to the pharmacopoeia, being the only one to show significant antioxidant activity. It is evident, thus, the need to apply a more rigorous quality control for commercial extracts, covering the determination not only qualitative but also quantitative of their chemical markers.

Each species of *Passiflora* analyzed exhibited a distinctive chromatographic profile of its leaves' hydroalcoholic extracts, thereby making it possible to suggest a correlation between some bands in TLC and some peaks in HPLC of *C*-glycosylated flavonoids such as isoorientin, orientin, vitexin, and isovitexin. These compounds can therefore be used in the quality control of raw materials as chemical markers for authenticating and differentiating species with the proposed methods.

## Conclusion

The evaluation of the morphological and anatomical features of drugs derived from plants in the genus *Passiflora*, as shown previously (Wosch et al., 2015), when allied to chromatographic profiles, can contribute to the diagnosis and differentiation of species. Variations found within the same species or within and between forms underscore the importance of conducting pharmacognostic analysis and standardizing culture conditions of *Passiflora* species whose products are used for therapeutic purposes.

## Authors' contributions

LW (M.Sc. student) was responsible for collecting and identifying plant material, running the laboratory work, analyzing the data, and drafting the paper. DCI contributed to plant collection and

identification as well as to confecting the herbarium, whereas KCS (Ph.D. candidate) contributed to validating HPLC analyses. Lastly, CAMS designed the study, supervised the laboratory work, and read the manuscript critically. All authors read the final manuscript and approved its submission.

## Conflicts of interest

The authors declare no conflicts of interest.

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