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Short communication

# Development and validation of an HPLC-PDA method for the determination of myrsinoic acid B in the extracts of *Rapanea ferruginea* Mez

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ABSTRACT

New, simple, rapid and precise HPLC-PDA method has been developed and validated for quantification of biomarker myrsinoic acid B in stem bark extracts of *Rapanea ferruginea* Mez. The method employs a Phenomenex C18 column (250 mm × 4.6 mm I.D., 5 μm) with acetonitrile:methanol:water (pH 2.6 with phosphoric acid) at 48:30:22 as mobile phase, at a flow rate of 0.7 mL $min^{-1}$ and photo diode array (PDA) detection at 270 nm. The validation data show that the method is specific, accurate, precise and robust. The method was linear, over a range of 5–100.0 μg $mL^{-1}$, with a limit of detection of 0.369 μg $mL^{-1}$ and limit of quantification of 1.233 μg $mL^{-1}$. The method has also shown consistent recoveries (average of 101.3% and 0.12% RSD) of the biomarker, with low intra and inter-day relative standard deviation (1.26% and 1.62%, respectively). The evaluated hydroethanolic extract and dry extract presented MAB values of 63.53 and 36.07 mg $g^{-1}$, respectively.



## 1. Introduction

*Rapanea* is a pan-tropical genus with 34 species in Brazil. Its distribution extends into Bolivia, Mexico, Argentina, Paraguay and Uruguay [1].

Diprenylated benzoic acids derivatives have been the main compounds isolated from this genus, including the myrsinoic acids (MA) A (3-geranyl-4-hidroxy-5-(3′-methyl-2′-butenyl)-benzoic acid), B (5-carboxy-2,3-dihydro-2-(1,5-dimethyl-1-hydroxy-4-hexenyl)-7-(3-methyl-2-butenyl) benzofuran), C ((2S), (3S)-6-carboxy-2,3-dihydro-3-hidroxy-2-methyl-2-(4′-methylpenta-3′-enyl)-8-(3″-methyl-2″-butenyl)-cromon), E (3,5-digeranyl-4-hydroxy)-benzoic acid (MAE) and F (5-carboxy-2,3-dihydro-2-(1′,5′-dimethyl-1′ *E*, 4′-hexadienyl)-7-(3″-methyl 2″-butenyl)-benzofuran) [2–7]. These compounds have shown anti-inflammatory and antinociceptive activity [4–9].

We have previously verified that a $CHCl_3$ extract of the stem bark of *R. ferruginea* Mez (Myrsinaceae) (syn. *Myrsine coriacea*) exhibits significant and potent antinociceptive action when evaluated in some pharmacological models of pain in mice, and that the principal compound in this extract, myrsinoic acid B, is one of the compounds responsible for this activity [8,9].

Although several studies on the chemical composition and pharmacological activity of the genus *Rapanea* can be found in literature [9–12], analytical methods for qualitative and quantitative analysis of plant extracts and derivatives as well as respectives biomarkers have not been reported yet. Therefore, pharmacological studies with the extracts derived from the stem bark of *R. ferruginea* indicate its potential use in the treatment of pain and inflammation, requiring the development and validation of methods for quality control of the extracts.

The present work has been performed in order to develop and validate an HPLC method for the determination of myrsinoic acid B (MAB) in stem bark crude extracts of *R. ferruginea* before and after drying by spray dryer, for subsequent use in quality control of dosage forms, with the aim of developing a new analgesic herbal medicine.

## 2. Experimental

### 2.1. Reagents and standards

All solvents were HPLC grade (Tedia, Fairfield, Ohio, USA) and were degassed by helium gas. The water was purified using a Milli-Q system (Millipore, Massachusetts, USA). All solutions were

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filtered through 0.45 μm membrane (Millipore, Massachusetts, USA). Aerosil® 200 was purchased from Degussa, Germany.

### 2.2. Plant materials

Stem bark, leaves, branches and fruits of native specimens obtained from vegetative propagation of authentic *R. ferruginea* were collected in Blumenau (Santa Catarina, Brazil) in June/2008 and identified by Prof. Oscar Benigno Iza. A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC, Brazil) under number HBR 52715. Plant materials were dried in oven with air circulation at 35 °C, for seven days, powdered and sieved.

### 2.3. Preparation of hydroethanolic extract

The stem barks hydroethanolic extracts were prepared by dynamic maceration with ethanol–water 70:30 (v/v), at a plant:solvent ratio of 10% (w/v), stirred for 6 h at 330 rpm and then filtered. The extracts were kept in an amber flask.

To evaluate the profile of the different parts of the plant, leaves, branches, fruits and stem barks were dried in oven with air circulation at 35 °C for seven days, powdered and sieved. Only particles of 0.5 mm were used, at a plant:solvent ratio of 20% (w/v). Alcoholic extracts were prepared by maceration with 96% ethanol for 7 days, and then filtered. The extract was concentrated under reduced pressure and kept in an amber flask.

### 2.4. Dry extract

The dry extract from the barks was obtained through the spray drying technique, using a Büchi Mini *Spray Dryer* B-290. The concentrated extract was dispersed with 10% of the final solution volume (g) of ethanol, the excipient (Aerosil® 200) was added, and the volume (g) was completed with purified water. The drying conditions were inlet temperature of 130 °C, pressure of 15 mm Hg and flow of 7 mL $min^{-1}$.

### 2.5. HPLC analysis

A Shimadzu LC-10AD LC system (Shimadzu, Tokyo, Japan), consisting of a binary pump and a Shimadzu SPD-M10A photo diode array detector, SIL-10A auto-sampler and software Class VP (version 5.33), was used. The injections (20 μL) were carried out on a Phenomenex (Torrance, California, USA) Luna C18 5μ Fusion RP 100 Å (250 mm × 4.6 mm) conditioned in a Shimadzu CTO-10A column oven equilibrated at 35 °C. For method development, different solvent systems were tried out in isocratic and gradient conditions: 50:30:20, 50:40:10, 50:20:30, 43:43:14, 35:45:20, 35:48:17, 40:42:18, 40:45:15, 41:41:18, 45:30:25, 45:35:20, 48:35:17, 48:38:14 and 40:40:20 acetonitrile–methanol–water (pH 2.6, phosphoric acid). Flow of 0.7 at 1.0 mL $min^{-1}$ was tested. The analyses were monitored at 230 and 270 nm. However, none of them demonstrated better results then the isocratic method with mobile phase, that has consisted of a mixture of acetonitrile–methanol–water (pH 2.6, phosphoric acid) (48:30:22, v/v/v), at a flow rate of 0.7 mL $min^{-1}$.

At least six individual injections of Standard Solution were performed before all measurements to assess the suitability parameters, including resolution, theoretical plates, asymmetry and repeatability of the peak area.

#### 2.5.1. Sample preparation

Hydroethanolic extract was diluted at 1:10 in mobile phase and filtered through a 0.45 μm cellulose regenerated membrane filter.

#### 2.5.2. Standard solution

Myrsinoic acid B (1 mg) was dissolved in 10 mL of mobile phase (100 μg $mL^{-1}$) by sonication during 5 min to prepare a freshly working solution, which was used to validate the method.

### 2.6. Method validation

The method was validated according to the ICH guidelines [13] and Brazil's legislation [14]. To determine the linear relationship between peak areas and concentration of myrsinoic B acid, seven solutions with concentration at the range of 5.0–100.0 μg $mL^{-1}$ were analyzed in triplicate and all solutions were injected three times. The solutions were prepared by addition of MAB at hydroalcoholic extracts. The linearity was evaluated by adding increasing concentrations of the standard in a fixed concentration of the sample solution. The linearity equations were calculated by linear regression analysis, using Excel 5.0 software.

The LOD and LOQ were calculated based on standard deviation of the *y*-intercepts of regression lines [13,14]. The data were evaluated using ANOVA ($P < 0.05$).

The accuracy of the method was measured through the analyte recovery test [13,14] in triplicate, regarding the linearity of the method. Standard concentrations of about 25, 50 and 75 μg $mL^{-1}$ for MAB were added to the diluted matrices samples (hydroethanolic extract).

Repeatability (intra-day) and intermediate precision (inter-day) were determined through analysis (triplicate) of the sample (hydroethanolic extract) at three levels (25, 50 and 75 μg $mL^{-1}$) of the marker, and the %RSD (relative standard deviation) was determined. The intermediate precision was determined over a period of three days.

The robustness of the method, related to the variation in retention time, area, and myrsinoic acid B assay in the sample (hydroethanolic extract), was evaluated by changing the mobile phase flow (0.6, 0.7 and 0.8 mL $min^{-1}$), oven temperature (34, 35 and 36 °C), pH of acidified water (2.5, 2.6 and 2.7) and mobile phase ratio (47:30:23, 48:30:22 and 49:30:21, v/v/v). On each condition the extract solution and the standard solution (both at 50 μg $mL^{-1}$) were injected (triplicate). The data were evaluated using an analysis of variance (ANOVA) single factor ($P < 0.05$).

## 3. Results and discussion

Myrsinoic acids A and B (MAA, MAB) found in *R. ferruginea* are relevant for their therapeutic effect, mainly as anti-inflammatory and analgesic compounds. As these myrsinoic acids are not available commercially, they were isolated by our research group and characterized [3,9], with purity of 99.18% and 73.25% for MAB and MAA, respectively through PDA (Fig. 1a and b). Because MAA is oil, and is chemically instable and hard to purify, it is not suitable for use as an analytical standard, therefore the analytical method was developed and validated for MAB, which is the main compound in the stem barks of *R. ferruginea*. It is easy to purify and proved to be a high purity compound.

In this work, a sensitive and time-saving method was developed for qualitative analysis of myrsinoic acids found in *R. ferruginea* and validated for quantitative analysis of MAB. The method showed a satisfactory separation of the MAB, the main component in the *R. ferruginea* extracts (hydroethanolic extracts), with good resolution within a short space of time using an isocratic method (Fig. 1c). Similar chromatographic profile was observed for dried extract. Peak 1 is MAB, peak 2 was isolated and identified as myrsinoic acid C (MAC) [3] and peak 3 is MAA. It was also observed that MAB is more predominant than the other compounds in the stem barks extracts.

**Table 1**
Percentage of recovery, to evaluate the accuracy of the method.

| Constituent | Spiked ($\mu g\,mL^{-1}$) | [a]Found ($\mu g\,mL^{-1}$) (RSD%) | Recovery (%) | Mean (%) | RSD (%) |
|---|---|---|---|---|---|
| Myrsinoic acid B | 25.50 | 25.56 (1.01) | 101.39 | 101.27 | 0.12 |
| | 51.01 | 51.60 (1.02) | 101.16 | | |
| | 76.51 | 77.47 (1.03) | 101.25 | | |

[a] $n = 3$; triplicate injection.

**Table 2**
Validation results for intra- and inter-day precision.

| Component | Concentration level ($\mu g\,mL^{-1}$) | [a]Method precision (average of RSD%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Level 1 | | Level 2 | | Level 3 | |
| | | Intra-day | Intra-day | Intra-day | Intra-day | Intra-day | Intra-day |
| Myrsinoic acid B | 25–50–75 | 1.58 | 1.95 | 0.71 | 1.76 | 1.50 | 1.16 |

[a] $n = 3$; triplicate injection in three different days.

The MAB calibration curves proved to be linear over the proposed range (5.0–100.0 $\mu g\,mL^{-1}$), as shown by linear regression coefficients ($r^2$) of 0.9997 for the compound studied, demonstrating an acceptable data fit to the regression line ($y = 56404x - 38295$). Statistical analysis of linear regression obtained from a $F_{calc}$ 67176.10 as compared to $F_{crit}$ ($3.51 \times 10^{-35}$) demonstrates the statistical significance of method linearity. Residual analysis is developed to assess whether the regression model has been fitted to the data, which corresponds to an appropriate model. In plotting the values in the residue graph was observed that a curvilinear effect fits into the quadratic model, and the observed y values are very close to the predicted $y$ values [15].

The sensitivity of the method for the compound was expressed as the slope of the analytical curve, and by LOQ and LOD values of 1.238 and 0.369 $\mu g\,mL^{-1}$, respectively.

The accuracy of the standard in the spiked sample was evaluated at three levels: 25, 50 and 75 $\mu g\,mL^{-1}$ for myrsinoic acid B, with recoveries of 101.4, 101.2 and 101.2%, respectively. The method showed good accuracy at low, medium and high level concentrations for the prenylated benzoic acid, within the linearity of the method. An RSD value of <2.0% was observed in all determinations of the recovery test (Table 1). Both the intra and the inter-day study presented RSD lower than 2.0% [13]. These results show the high accuracy of the method (Table 2).

The robustness of the method was analyzed by little and deliberate variations of the oven temperature (34, 35 and 36 °C), mobile

**Table 3**
Chromatographic parameters from robustness studies.

| Parameters | [b]Average (RSD%) of myrsinoic acid B | | |
|---|---|---|---|
| | *Rt* | *Area* | $mg\,g^{-1}$ [a] |
| pH of aqueous phase | | | |
| 2.5 | 16.26 (0.07) | 3,128,637 (0.42) | 73.24 (0.42) |
| 2.6 | 16.18 (0.03) | 3,086,966 (0.99) | 73.24 (0.99) |
| 2.7 | 16.64 (0.53) | 3,217,327 (0.33) | 73.91 (0.33) |
| RSD (%) | 1.33 | 1.92 | 0.97 |
| $F_{calc}/F_{crit}$ | 6.95 | 6.28 | 1.16 |
| Oven temperature | | | |
| 34 °C | 16.65 (0.28) | 3,151,300 (0.71) | 73.90 (0.71) |
| 35 °C | 16.18 (0.03) | 3,086,966 (0.99) | 73.24 (0.99) |
| 36 °C | 16.15 (0.02) | 3,125,429 (0.22) | 73.06 (0.22) |
| RSD (%) | 1.50 | 1.09 | 1.00 |
| $F_{calc}/F_{crit}$ | 64.50 | 1.22 | 0.94 |
| Flow rate | | | |
| 0.6 mL min$^{-1}$ | 18.99 (0.46) | 3,608,897 (1.73) | 73.66 (1.73) |
| 0.7 mL min$^{-1}$ | 16.18 (0.03) | 3,086,966 (0.99) | 73.24 (0.99) |
| 0.8 mL min$^{-1}$ | 14.15 (0.06) | 2,691,791 (0.09) | 72.42 (0.09) |
| RSD (%) | 12.79 | 12.78 | 1.28 |
| $F_{calc}/F_{crit}$ | 1322.67 | 76.48 | 0.37 |
| Mobile phase proportion | | | |
| 47:30:23 | 17.93 (0.08) | 3,188,100 (0.39) | 72.89 (0.39) |
| 48:30:22 | 16.18 (0.03) | 3,086,966 (0.99) | 73.24 (0.99) |
| 49:30:21 | 15.12 (0.40) | 3,190,287 (1.47) | 73.00 (1.47) |
| RSD (%) | 7.48 | 1.86 | 0.95 |
| $F_{calc}/F_{crit}$ | 894.20 | 1.85 | 0.05 |

*Rt*, retention time.
[a] Myrsinoic acid B assay in the sample (hydroalcoholic extract).
[b] Triplicate injection.

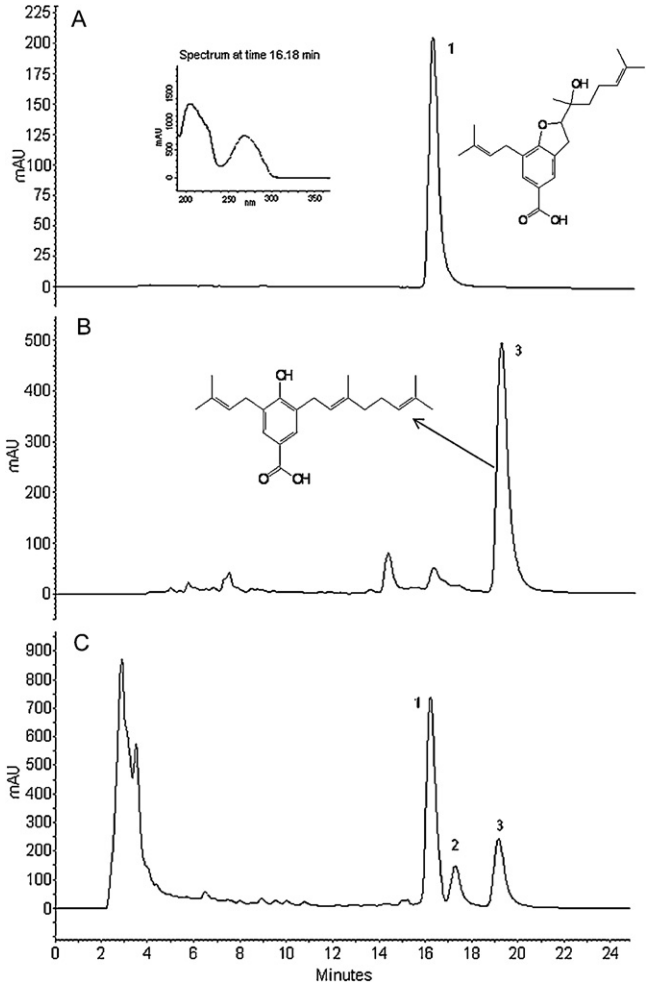


**Fig. 1.** Chromatograms for myrsinoic acid B (A), myrsinoic acid A (B) and hydroethanolic extract (C). For chromatographic conditions, see Section 2.

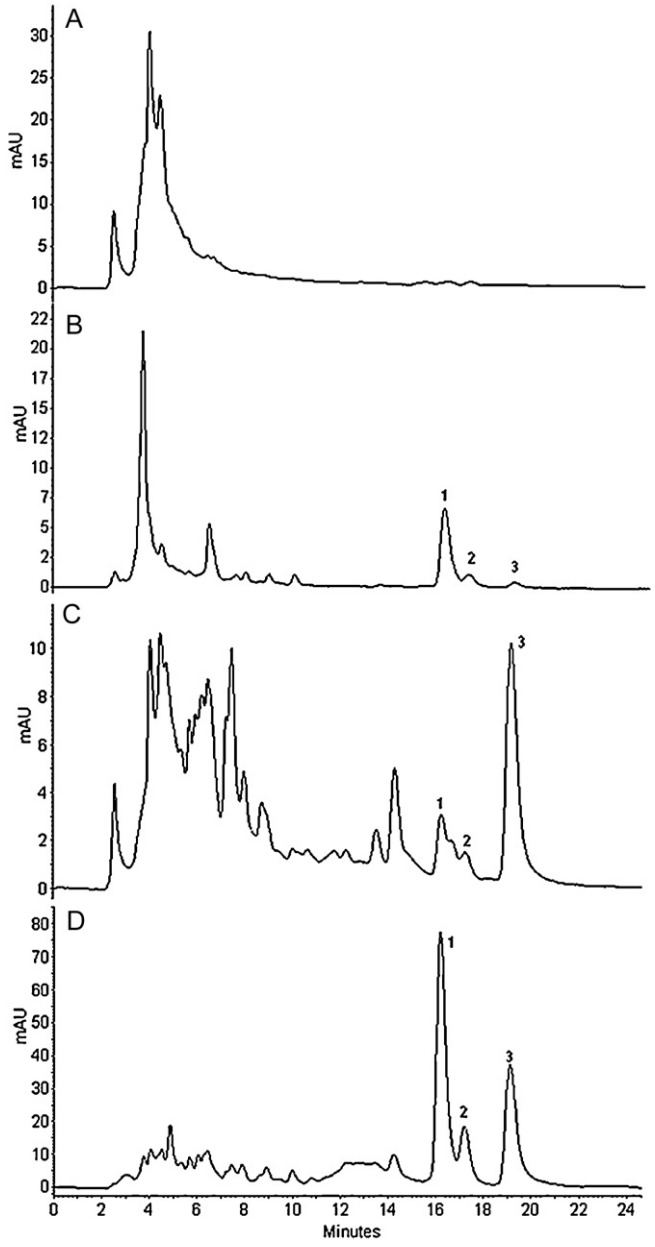


**Fig. 2.** Chromatograms for branch extract (A), leaf extract (B), fruit extract (C) and stem bark extract (D). For chromatographic conditions, see Section 2.

phase flow (0.6, 0.7 and 0.8 mL min$^{-1}$), pH of aqueous phase (2.5, 2.6 and 2.7) and mobile phase ratio (47:30:23, 48:30:22 and 49:30:21, v/v/v), as shown in Table 3. The robustness was estimated using the overall mean, standard deviation, %RSD for each variable. Although the calculated *F*-value was higher than the critical *F*-value for retention time and area, RSD was lower than 5% for the variables pH, temperature and mobile phase ratio. The present method showed to be robust for the assay of MAB present in the hydroethanolic extract.

The selected conditions (35 °C, pH 2.6, 48:30:22 and 0.7 mL min$^{-1}$ flow) presented good results for MAB analysis, as shown by the acceptable resolution about 5.0, peak asymmetry >1.5, theoretical plates of about 7000, purity peak of 0.99 through PDA detector and repeatability of area (RSD < 2.0%). The analytical method was also applied, both qualitatively and quantitatively, to other parts of the plant, including the branch extract (Fig. 2a), leaf extract (Fig. 2b) and fruit extract (Fig. 2c). The branch extract presented none of the compounds. The leaf extract presented all the compounds, with MAB being the predominant one, for which assay showed 12.03 ± 0.12 mg g$^{-1}$. MAA was majority in the fruit extract with co-elution of others compounds not identified near MAB and MAC for this sample. All compounds were observed on the stem bark extract (Fig. 2d), the MAB assay showed 204.33 ± 0.81 mg g$^{-1}$, 94% more than the leaf extract. These results confirmed the use of stem bark of *R. ferruginea* in folk medicine for the treatment of pain and inflammation [9,16] and justified the development of a quality control method to be applied on a future herbal medicine produced from *R. ferruginea* stem barks.

The concentrated hydroethanolic extract and dry extract of *R. ferruginea* stem barks presented values for MAB of 63.53 ± 0.36 and 36.07 ± 0.12 mg g$^{-1}$, respectively. The reduction of MAB on the dry extract is probably due to the addition of adjuvants in the drying process, but the chromatographic profile remained similar (Fig. 1c).

## 4. Conclusion

A simple, fast, selective, precise and accurate HPLC-PAD method has been developed for quantifying myrsinoic acid B in *R. ferruginea* extracts, and should be useful for the pharmaceutical industry in the analysis of hydroethanolic and dried stem bark extracts that may be further adapted to their pharmaceutical dosage forms.

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