**Butia spp. (Areceae) LC-MS-Based Metabolomics for Species and Geographical Origin Discrimination**

Jessica Fernanda Hoffmann, † Ivan Ricardo Carvalho, † Rosa Lia Barbieri, § Cesar Valmor Rombaldi, † and Fabio Clasen Chaves* †‡§

†Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Caixa Postal 354, CEP 96010-900 Pelotas, RS, Brazil
§Embrapa Clima Temperado, Caixa Postal 403, CEP 96001-970 Pelotas, RS, Brazil

ABSTRACT: The metabolic variability of fruit from Butia spp. (Areceae) genotypes from different geographical locations was characterized using untargeted metabolomics by liquid chromatography–mass spectrometry (LC-MS) followed by multivariate data analyses. Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) from LC-MS data sets showed a clear distinction among Butia catarinensis, Butia odorata, Butia paraguayensis, and Butia yatay. The major metabolites that contributed to species discrimination were primary metabolites including sugars and organic acids and specialized metabolites such as tetrahydroxy-trans-stilbene and rutin. B. odorata fruit from Tapes, RS, Brazil, showed a high content of organic acids and flavonoids, whereas B. odorata fruits from Capão do Leão, RS, Brazil, showed a high sugar content. The results demonstrate that LC-ESI-qToF-MS-based metabolic profiling coupled with chemometric analysis can be used to discriminate among Butia species and between geographical origins of B. odorata and to identify primary and specialized metabolites responsible for the discrimination. 

KEYWORDS: metabolic variability, bioactive compounds, native fruit, metabolomics, LC-ESI-qTOF-MS, multivariate analysis

## INTRODUCTION

Brazil has the planet’s largest biodiversity with many unexplored fruit-bearing plants distributed in different biomes. The Pampa Biome (subtropical and temperate climates) has a number of underutilized native fruit species possessing commercial potential, especially for smallholders and family farmers, for new product development focusing on emerging markets for nutritional and functional foods. In southern Brazil, there are eight native species of Butia; the species Butia odorata, Butia yatay, Butia paraguayensis, and Butia catarinensis are the most predominant. Butia witeckii is the most recently described species and is considered to be most closely related to B. yatay and B. paraguayensis, differing from these species in fruit size and weight, endocarp, and inflorescence among other morphological characteristics.

Butia palms are used in landscaping, and their dried leaves are used in the making of hats, brooms, baskets, and other crafts. The fruits are fibrous, have an acid–sweet taste, intense flavor and aroma, and are rich in phenolic compounds, carotenoids, vitamin C, and potassium. They are usually consumed fresh, or the pulp is used to produce juices, liqueurs, sweets, jellies, and ice cream. The uses of these palms have been evident since prehistoric times, and their utilization, like that of most native fruit species, has occurred from natural populations without any systematic cultivation. In addition, the areas occupied by naturally occurring “butiazais” are subject to protection by the State Forestry Code and, therefore, management and commercial exploration of Butia spp. is limited.

To conserve and characterize the variability of Butia, research institutions maintain germplasm banks. The accessions belonging to these collections need to be evaluated for agronomic, technological, phytochemical, and biological potential to identify genotypes with superior quality and commercial potential. The knowledge of variability (molecular, phenological, morphological, and chemical) provides support for the establishment of conservation strategies, incorporation in regional production systems, sustainable management, and use.

Several studies have been developed resulting in an increase in scientific knowledge about Butia palm trees. However, these studies have primarily focused on agronomic, morphological, and molecular characterization of Butia spp. as well as some isolated preliminary chemical characterization of the fruit. With recent developments in analytical techniques, such as liquid chromatography coupled to mass spectrometry (LC-MS), it is possible to simultaneously detect hundreds of metabolites and thus compare the differences and similarities among samples. This nontargeted approach has been used to obtain information on the metabolic variability in germplasm banks, to differentiate species, and to establish the geographic origin of different fruits. Moreover, metabolomics can be used for chemical classification of plants by chemotaxonomy. Differentiation between different species of Butia based on their metabolomic profiling has not been carried out, and their speciation has been based predominantly on morphological characters and their regional occurrence. Thus, the aim of this

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study was to evaluate the metabolic variability of Butia spp. genotypes using untargeted metabolomics by LC-ESI-qToF-MS.

**MATERIAL AND METHODS**

Sample Collection and Preparation. This study was divided into two experiments. In the first, the interest was to evaluate differences among *B. catarinensis* Noblick and Lorenzi, *B. odorata* (Barb. Rodr.) Noblick, *B. paraguayanus* (Barb. Rodr.) L.H. Bailey, and *B. yatay* (Mart.) Becc. In the second, differences among genotypes of *B. odorata*, the predominant species in southern Brazil, from different geographical origins were examined. *Butia* species were identified and classified by R. L. Barbieri on the basis of morphological characteristics according to the guidelines of Lorenzi et al. *B. catarinensis* fruits were collected in Laguna, SC, Brazil (28°28′37″ S, 48°46′51″ W, and altitude of 2 m), *B. paraguayanus* fruits in Rondinha, RS, Brazil (27°49′41″ S, 52°54′35″ W, and altitude of 440 m), and *B. yatay* fruits in Giruá, RS, Brazil (28°01′42″ S, 54°20′9″ W, and altitude of 429 m). For *B. catarinensis*, *B. paraguayanus*, and *B. yatay*, a pool of 50 ripe fruits from three plants of each species was collected. For *B. odorata*, fruits were collected from 57 genotypes. Fruits of *B. odorata* were collected in two locations in the state of Rio Grande do Sul (RS), in the municipalities of Capão do Leão (31°52′00″ S, 52°21′24″ W, and altitude of 13 m) and Tapes (30°31′22.34″ S, 51°21′35.23″ W, and altitude of 10 m). Fifty ripe fruits from each of 38 genotypes were collected in Capão do Leão, and 50 ripe fruits from each of 19 genotypes were collected in Tapes for a total of 57 genotypes of *B. odorata*.

After collection, fruits were kept in a freezer (−20 °C) until analysis. For physicochemical analysis, fruits were manually depulped, and approximately 100 g of sample was ground in a ball mill with liquid nitrogen to a fine powder. For the metabolic profile by LC-ESI-qToF-MS, samples were lyophilized.

Physicochemical Analysis. Color, Soluble Solids, pH, and Acidity. Fruit skin color (measured in nine fruits per genotype) was determined using a colorimeter (Minolta Chroma Meter, CR 300) in the CIELab color system. The values of *a* and *b* were used to calculate hue angle (hue = tan−1 b/a).

Soluble solids (SS) content was determined by refractometry, and results were expressed as °Brix. For analysis of the pH and acidity (AC), 1 g of sample was diluted with 40 mL of distilled water. The pH was determined with a pH meter (Hanna Instruments HI2221). For analysis of the acidity, the mixture was titrated with NaOH (0.1 M) to pH 8.1. Results were expressed as milligrams of citric acid equivalent (CAE) per 100 g of fresh weight (fw). Analyses were performed according to AOAC methods.13

Vitamin C. Vitamin C content was determined using the titrimetric method with 2,6-dichlorophenolindophenol reagent. One gram of ground sample was mixed with 15 mL of extracting solution (3% metaphosphoric acid–8% acetic acid solution). The mixture was homogenized and centrifuged at 6600g for 15 min. Four milliliters of the supernatant was diluted with 2 mL of the extracting solution and 50 mL of water. This solution was titrated with a 2,6-dichlorophenolindophenol solution (0.01%). The titration end point was defined when the solution turned a persistent (15 s) pink color. Results were expressed as milligrams of L-ascorbic acid equivalents per 100 g of fw.

Carotenoid Content. Total carotenoid content analysis was carried out according to AOAC method 970.64.13 Fifteen milliliters of extraction solution (hexane:acetone/ethanol/toluene 10:7:6.7) was added to 2.5 g of ground sample in a polyethylene tube (50 mL) protected from light and stirred for 1 min by vortexing. Then, 1 mL of potassium hydroxide in methanol (10% w/v) was added, and the mixture was stirred for 1 min and then subjected to hot saponification (30 min in a water bath at 56 °C). The mixture stayed at room temperature for 1 h, and then 15 mL of hexane and 29 mL of sodium sulfate solution (10% w/v) were added. After 1 h, the absorbance was measured at 450 nm (Jenway 6705 UV–vis). A calibration curve (0–20 μg mL−1) was prepared using β-carotene (Sigma-Aldrich) in hexane. Results were expressed as milligrams of β-carotene equivalents per 100 g of fw.

Preparation of Extracts. Two grams of ground sample was homogenized with 20 mL of methanol for 1 min in an IKA Ultra Turrax (digital T8) and subsequently centrifuged for 10 min at 9900g (Eppendorf, 5430) at 15 °C. The supernatant was separated and used as a crude extract to determine total phenolic content, flavonoid content, and total antioxidant capacity (DPPH and ABTS).

Phenolic Compounds Content. Folin–Ciocalteu reagent was used to determine the total phenolic compounds content according to the method of Beskow et al. A calibration curve (0–250 μg mL−1) was prepared using gallic acid (Sigma-Aldrich) in methanol. Absorbance was measured at 725 nm, and results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fw.

Flavonoid Content. Flavonoid content was determined according to the method of Zhishen et al.14 Five hundred microliters of the extract was mixed with 2 mL of distilled water and 150 μL of sodium nitrite (5% w/v) and allowed to react for 5 min. One hundred and fifty microliters of aluminum chloride (10% w/v) was added and allowed to react for 6 min, followed by the addition of 1 mL of sodium hydroxide (1 M) and 1.2 mL of distilled water. Absorbance was measured at 510 nm. A calibration curve (0–150 μg mL−1) was prepared using (+)-catechin (Sigma-Aldrich) in methanol. Results were expressed as milligrams of (+)-catechin equivalents per 100 g of fw.

Radical Scavenging Assay. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacities of crude extract were measured according to a method previously reported by Pereira et al.15 The scavenging capacity of the extracts was expressed as percentage (%) DPPH and ABTS radical remaining according to the equation

\[
\% \text{ inhibition} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100
\]

where *A*<sub>control</sub> is the absorbance of the control (containing all reagent except the sample) and *A*<sub>sample</sub> is the absorbance of the sample.

Preparation of Extracts for Metabolic Profiling by LC-ESI-qToF-MS. The lyophilized fruit pulp sample was divided into three subsamples of 100 mg each, and 1 mL of ice-cold sample extraction solution (75% methanol and 0.1% formic acid) and 10 μg of reserpine mL−1 (as internal standard for relative quantification) were added. The samples were vortexed, sonicated in a water bath at room temperature for 15 min, and centrifuged (9000g, 15 min), and the supernatants were collected. The extraction process was repeated once.16 The supernatants were combined, filtered through a 0.2 μm nylon membrane, and stored at −80 °C until analysis.

LC-ESI-qToF-MS Analysis. The LC-ESI-qToF-MS analysis was performed on a Prominence UFLC system (Shimadzu, Japan) coupled to a quadrupole time-of-flight mass spectrometer (Impact HD, Bruker Daltonics, Bremen, Germany). Separation of metabolites was performed using a Bidentate C18 column (100 × 2.1 mm, MicroSolv Technology Corp., Leland, NC, USA). Mobile phases were 0.1% aqueous formic acid (pH 2.8; solvent A) and acetonitrile (solvent B). The gradient program was set as follows: started at 5% B, increased linearly to 90% B at 15 min, and maintained for 3 min at 90% B; returned to 5% B in 2 min and maintained at 5% B for an additional 6 min at a flow rate of 0.2 mL min−1. The injection volume was 10 μL. All samples were injected in duplicate.

Parameters for MS analysis were set using negative and positive ionization modes with spectra acquired over a mass range from 32 to 1200. The parameters were as follows: capillary voltage, 20 kV; nebulizing gas pressure, 2 bar; collision RF, 300 Vpp; transfer time, 120 μs; and prepulse storage, 8 μs. Collision energy intensity was adjusted for automatic MS/MS experiments according to m/z ratios: m/z 100, 15 eV; m/z 500, 55 eV; m/z 1000, 50 eV, using nitrogen as collision gas.

Data Processing and Statistical Analysis. The MS data were analyzed using Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany). ProfileAnalysis software (version 2.0, Bruker Daltonics) was used for processing (data mining, alignment, and normalization) of LC-MS/MS records. The negative ionization data matrices
comprising lists of peaks characterized for each sample by retention
time (RT), m/z, and intensity were obtained, and total area sum
normalization and Pareto scaling (the square root of the standard
deviations) were performed for each sample.

To explore the metabolite multidimensional data set, both
unsupervised principal component analysis (PCA) and supervised
partial least-squares discriminant analysis (PLS-DA) multivariate
statistical methods were used after an analysis of variance (ANOVA,
p ≤ 0.05). A variable importance score (VIP) >1.0 was chosen to
select the most discriminating variables. Multivariate analyses
were performed in MetaboAnalyst 3.0.

After statistical analysis, significant peaks were subject to the
identification process. The elemental composition of each compound
was selected according to accurate masses and isotopic pattern through
Smart Formula tools (Bruker Compass DataAnalysis), which provides
a list of possible molecular formulas by combining accurate mass and
isotopic distribution reflected in their error (ppm) and mSigma values,
respectively. Tentative metabolite identification was performed by
matching the accurate m/z values and MS² fragmentation patterns
with data from databases (METLIN, KEGG compounds, PubChem,
Mass bank, Maven, FooDB, and ReSpect) and reference literature with
a mass accuracy window of 5 ppm. The identities of malic acid,
succrose, citric acid, hydroxybenzoic acid, chlorogenic acid, catechin,
syringic acid, epicatechin, caffeic acid, vanillic acid, rutin, quercetin,
p-coumaric acid, ferulic acid, luteolin, hesperetin, and pinocembrin
were confirmed with external standards (Sigma-Aldrich).

Relative quantification was performed by comparison to internal
standard (reserpine), except for epicatechin (y = 57.8x + 545.4, R² =
0.9990) and rutin (y = 177.4x + 731.4, R² = 0.9998), which were
quantified using an external standard calibration curve (39–5000 ng
mL⁻¹).

## RESULTS

### Physicochemical Characteristics of Butia spp.

**Butia** spp. fruit can be yellow, green, orange, redish-orange, bright
red, or purple. Mature **B. paraguayensis** and **B. catarinensis**
fruits were more reddish, whereas **B. odorata** and **B. yatay** had
yellow fruit (Figure 1). pH values varied from 3.3 (**B. yatay**
and **B. odorata**) to 4.0 (**B. catarinensis**). Fruits of **B. catarinensis**
were the least acidic (0.4% of citric acid), whereas fruits of **B. odorata**
were the most acidic (1.9% of citric acid). Soluble solids content
varied from 10.9 °Brix (**B. yatay**) to 13.7 °Brix (**B. catarinensis**).
Fruits of **B. catarinensis** showed the highest SS/AC ratio (Table 1).
These fruits presented lower acidity and higher soluble solids when compared with the other species in
this study. Vitamin C content varied from 20.2 mg of L-ascorbic
acid 100 g⁻¹ fresh weight (fw). mg of L-ascorbic acid equivalents 100 g⁻¹ fw. mg β-carotene equivalent 100 g⁻¹ fw. mg gallic acid equivalent
100 g⁻¹ fw. mg catechin equivalent 100 g⁻¹ fw. % inhibition.

![Figure 1. Fruits of Butia spp.: (A) B. catarinensis (photograph by M. P. Eslabão); (B) B. paraguayensis (photograph by G. Heiden); (C) B. yatay (photograph by G. Heiden); (D) B. odorata (photograph by G. T. Beskow).](image)

| Table 1. Physicochemical Characteristics of Butia spp.* |
|-------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Characteristic                      | B. odorata (n = 171) | B. paraguayensis (n = 3) | B. catarinensis (n = 3) | B. yatay (n = 3) |
| °hue                                | 74.5 ± 0.6 b           | 50.1 ± 1.5 c            | 66.9 ± 3.0 bc            | 88.5 ± 3.8 a       |
| pH                                  | 3.3 ± 0.0 b           | 3.4 ± 0.0 b            | 4.0 ± 0.0 a            | 3.3 ± 0.0 b       |
| SS                                  | 12.5 ± 0.1 ns         | 11.7 ± 0.2            | 13.7 ± 0.3            | 10.9 ± 0.2       |
| AC                                  | 1.9 ± 0.0 a           | 1.0 ± 0.0 b            | 0.4 ± 0.0 c            | 1.3 ± 0.0 ab      |
| SS/AC                               | 7.1 ± 0.2 c           | 11.5 ± 0.3 b           | 32.6 ± 1.6 a           | 8.5 ± 0.2 c      |
| ASC                                 | 58.3 ± 2.4 a          | 56.7 ± 0.1 a           | 20.2 ± 0.1 b           | 64.6 ± 0.1 a      |
| CAR                                 | 10.7 ± 0.5 b          | 26.7 ± 0.5 a           | 11.8 ± 0.5 b           | 10.9 ± 0.2 b      |
| PHE                                 | 171.1 ± 2.6 ns        | 142.4 ± 2.8           | 160.8 ± 3.2           | 200.1 ± 1.9       |
| FLA                                 | 99.4 ± 4.1 ns         | 61.4 ± 0.6            | 100.0 ± 1.7           | 90.7 ± 0.6       |
| DPPH                                | 83.3 ± 1.4 a          | 57.4 ± 0.4 ab          | 52.1 ± 0.7 b           | 82.6 ± 1.0 ab     |
| ABTS                                | 67.8 ± 1.5 a          | 36.5 ± 0.4 b           | 33.2 ± 2.1 b           | 64.5 ± 0.4 a     |

*Results expressed as the mean ± standard error of the mean. Different letters within the same row indicate significant differences by Tukey’s test (p ≤ 0.05). ns, not significant at p ≤ 0.05. °hue, tonality; pH, hydrogen potential; SS, soluble solids; AC, acidity; SS/AC, soluble solids/acidity ratio; FIB, dietary fiber; ASC, ascorbic acid; CAR, total carotenoids; PHE, total phenolics; FLA, total flavonoids; DPPH and ABTS, antioxidant capacity.

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content varied from 142.4 mg GAE 100 g$^{-1}$ (B. paraguayensis) to 200.1 mg GAE 100 g$^{-1}$ (B. yatay). B. paraguayensis showed the lowest total flavonoid content (61.4 mg), whereas B. catarinensis showed the highest (100.1 mg). The lowest level of antioxidant capacity was observed in B. catarinensis fruit for both DPPH and ABTS radicals (52.1 and 33.2%).
respectively), and B. odorata had the highest antioxidant capacity (83.3 and 67.8% for DPPH and ABTS, respectively) (Table 1). It is reported in the literature that antioxidant capacity is strongly correlated with phenolic content. In this study, antioxidant capacity against the DPPH radical showed intermediate correlation with total phenolic content ($R^2 = 0.6433$) and flavonoid content ($R^2 = 0.5712$), and antioxidant capacity against the ABTS radical showed low correlation with total phenolic content ($R^2 = 0.4257$) and intermediate correlation with flavonoid content ($R^2 = 0.6116$).

**Nontargeted LC-MS Analysis of Butia spp. Multivariate Statistical Analysis.** Compared to the positive-ion mode, negative-ion MS spectra revealed higher sensitivity and more observable peaks, especially for organic acids, phenolic acids, and flavonoids (Figure S1, Supporting Information). Thus, all samples and metabolite identifications were carried out in the negative ionization mode (Figure S2). Figure 2A shows the PCA score plot for components 1 and 2. These components explained 77.7% of total variance. PCA showed clear differentiations among B. odorata, B. yatay, B. paraguayensis, and B. catarinensis species.

To obtain a more distinct separation of the samples and determine the metabolites contributing to the discrimination, PLS-DA was used on the LC-MS data set. For the PLS-DA model, both $Q^2$ max (0.86) and $R^2$ values (0.91) were higher in the permutation test than in the real model, suggesting good predictability and goodness of fit. Figure 2B shows the separation of species by PLS-DA.

**Key Components in Butia spp. Differentiation by PLS-DA.** VIP scores were used (VIP values > 0.96) to determine which compounds contributed to the separation between groupings (Figure 2C). Table 2 presents metabolites most useful in discriminating butia species.

Peaks with $[\text{M} - \text{H}]^-$ m/z 293.1239 (C$_{12}$H$_2$O$_9$, peak 5) and $[\text{M} - \text{H}]^-$ m/z 259.0608 (C$_{10}$H$_{12}$O$_5$, peak 7) were not identified, but were significant markers for differentiating B. catarinensis and B. yatay fruits, respectively.

Galabiose had a parent ion of $[\text{M} + \text{M} + \text{Cl} - 2\text{H}]^- m/z$ 719.2018 and fragment ions of m/z 377.0851 ($[\text{M} - \text{Cl}]^-$, 341.1089 ($[\text{M} - \text{H}]^-$), and 215.0318 (Figure 3F). Galabiose content varied from 42.8 μg g$^{-1}$ (B. yatay) to 190.5 μg g$^{-1}$ (B. odorata) (Figure 4). This disaccharide influenced the separation of B. odorata fruit from other species.

Parent ions observed at $[\text{M} - \text{H}]^-$ m/z 341.1103 were identified as sucrose confirmed by MS/MS with online databases and external standard. The fragment ion detected at m/z 179.0553 may be produced by the neutral loss of the hexose $[\text{M} - \text{H} - 162]^-$. Sucrose content varied from 151.7 to 227.9 μg g$^{-1}$ (Figure 4) and was responsible for the separation of B. catarinensis from the other species. This species had sweeter fruit than others, as indicated by the soluble solids content (Table 1).

Malic acid had a parent ion in negative mode of $[\text{M} - \text{H}]^- m/z$ 133.0147 and a fragment ion of m/z 115.0441 resulting from a loss of water (Figure 3B). The concentration of malic acid varied from 1975.4 μg g$^{-1}$ (B. catarinensis) to 3818.0 μg g$^{-1}$ (B. yatay) (Figure 4).

Citric acid had a parent ion in negative mode of $[\text{M} - \text{H}]^- m/z$ 191.0200 and fragment ions of m/z 129.0195, [M − H − CO$_2$ − 2H$_2$O]$^- 111.0089$, and 87.0090 (Figure 3G). Citric acid content varied from 295.8 μg g$^{-1}$ (B. catarinensis) to 549.0 μg g$^{-1}$ (B. odorata) (Figure 4). Malic and citric acid influenced the separation of B. yatay fruit from the other studied species.

Isopropylmalic acid was characterized by parent ion of $[\text{M} - \text{H}]^- m/z$ 175.0608, and fragment ions of [HCO$_2$ + CH$_3$]$^- m/z$ 115.0389 and m/z 85.0648 (Figure 3E) influenced the separation of B. catarinensis. The content varied from 19.9 μg g$^{-1}$ (B. paraguayensis) to 124.0 μg g$^{-1}$ (B. catarinensis) (Figure 4). B. catarinensis had malic and citric acid contents approximately 50% less than that of the other species, whereas isopropylmalic acid content in B. catarinensis was 84% greater than in other species tested.

Rutin was characterized by an $[\text{M} - \text{H}]^- m/z$ 609.1485, with a fragment ion at m/z 300.0290 derived from a loss of hexose and deoxyhexose (−308 Da) (Figure 3I). B. odorata fruit had the highest rutin content, which varied from 2.5 μg g$^{-1}$ (B. yatay) to 10.2 μg g$^{-1}$ (B. odorata) (Figure 4).

Tetrahydroxyl-trans-stilbene was characterized by an $[\text{M} - \text{H}]^- m/z$ 243.0661 and fragments ions of m/z 201.0555 and m/z 159.0455 (Figure 3H) and was approximately 95% higher in B. paraguayensis than other species. It is not possible to distinguish between cis and trans isomers by mass spectra; however, the cis isomer is highly unstable, and its presence in plants is highly unlikely. Tetrahydroxyl-trans-stilbene content varied from 9.8 μg g$^{-1}$ (B. catarinensis) to 254.0 μg g$^{-1}$ (B. paraguayensis) (Figure 4). To the best of our knowledge, this is the first time that this compound was reported in Butia spp.

**Nontargeted LC-MS Analysis for Butia odorata. Multivariate Statistical Analysis for B. odorata Fruits.** To verify how location influenced the metabolic profile, 57
Figure 5. Principal component analysis score plot (A), partial least-squares (PLS-DA) (B), and VIP scores by PLS-DA (C) derived from LC-MS data using negative electrospray ionization of 57 genotypes of Butia odorata fruit collected from two locations in RS, Brazil.

Genotypes of Butia odorata collected in Capão do Leão and Tapes, RS, Brazil, were evaluated by LC-ESI-qToF-MS. The first two principal components explained 35.3% of the total variation (Figure 5A). Two separate groups based on the location of Butia odorata genotypes were formed. For the PLS-DA model, both $Q^2_{\text{cum}}$ (0.83) and $R^2$ values (0.88) were higher in the permutation test than in the real model, suggesting good predictability and goodness of fit. Figure 5B shows the separation of two groups by PLS-DA analysis.

**Key Components in Butia odorata Differentiation by PLS-DA.** The compounds contributing to the separation of Butia odorata fruit from different locations are shown in Table 3. Metabolites that presented VIP scores >1 were selected and presented in Figure S3.

The ion [M + M + Cl − 2H]$^-$ $m/z$ 719.2018 (peak 2, Figure S3) and fragment ions of $m/z$ 377.0851 [M − Cl]$^-$, 341.1089 [M − H]$^-$, and 215.0318 were tentatively identified as galbolic acid, and fruits collected from Capão do Leão were rich in this compound.

Fumaric acid (peak 3, Figure S3) was characterized by an [M − H]$^-$ with $m/z$ 115.0040 and fragment ions of $m/z$ 84.9859 and $m/z$ 71.0146 resulting from a loss of methyl ester (−31 Da) and carboxylic acid (−44 Da), respectively, contributing to discrimination of fruit from Tapes.

Malic acid ([M − H]$^-$/$m/z$ 133.0140) and citric acid ([M − H]$^-$/$m/z$ 191.0197) influenced separation of the fruit from Tapes. These fruits were more acidic than fruit from Capão do Leão (data not shown). On the other hand, fruits from Capão do Leão were sweeter than fruits from Tapes and had high sucrone content (peak 5, Figure S3).

Furoic acid (peak 7, Figure S3) with a parent ion of [M − H]$^-$/$m/z$ 111.0090 and fragment ions of $m/z$ 67.0192 and $m/z$ 49.0095, resulting from a loss of carboxylic acid and water, 44 and 62 Da, respectively, was higher in fruit from Tapes.

Citramalic acid was characterized by an [M − H]$^-$ at $m/z$ 147.0300, with a fragment ion at $m/z$ 129.0189 derived from a loss of water (−18 Da) and $m/z$ 101.0242 derived from a loss of water and carbon monoxide (−46 Da), characteristic of carboxylic acids. Fruit from Tapes showed higher content for this compound.

Isopropylmalic acid (peak 10, Figure S3) with a parent ion of [M − H]$^-$/$m/z$ 175.0620 and fragment ion of $m/z$ 115.0389 and $m/z$ 85.0648, resulting from a loss of C$_4$H$_8$O$_3$ (60 Da) and C$_5$H$_8$O$_3$ (90 Da), was higher in fruit from Capão do Leão (Table 3).

(−)-Epicatechin (peak 12, Figure S3) had a parent ion of [M − H]$^-$/$m/z$ 289.0720 and a fragment ion of $m/z$ 245.0821, resulting from a loss of carboxylic acid. The identity of this compound was confirmed by MS/MS with online databases and an external standard. (−)-Epicatechin and (+)-catechin isomers were distinguished by retention time matching that of the external standards and not by mass spectrum. Fruit from Tapes showed higher (−)-epicatechin than fruit from Capão do Leão.

Peak 13 (Figure S3) had a parent ion of [M − H]$^-$/$m/z$ 623.1620 (C$_{25}$H$_{25}$O$_{16}$), with a fragment ion at $m/z$ 315.0510 derived from a loss of hexose and deoxyhexose (−308 Da). This compound is an isorhamnetin derivative ($m/z$ 315). Fruit from Tapes showed a high content for this compound (Table 3).

Peaks with [M − H]$^-$/$m/z$ 439.0810 (C$_{10}$H$_{16}$NO$_{12}$ peak 1), [M − H]$^-$/$m/z$ 293.1240 (C$_{12}$H$_{20}$O$_{8}$ peak 9), [M − H]$^-$/$m/z$ 259.1911 (C$_{14}$H$_{22}$O$_{8}$ peak 14), and [M − H]$^-$/$m/z$ 417.1555 (C$_{22}$H$_{22}$O$_{11}$ peak 11) were not identified but were significant markers for fruit from Capão do Leão.

**Qualitative Profile.** In the present work, a total of 39 semipolar compounds have been tentatively characterized (Table S1, Supporting Information). A total of nine known organic acids were identified in all Butia species tested. These compounds eluted between 1.9 and 5.7 min. The identified compounds were fumaric acid ([M − H]$^-$/$m/z$ 115.0040), malic acid ([M − H]$^-$/$m/z$ 133.0140), citric acid ([M − H]$^-$/$m/z$ 191.0200), furoic acid ([M − H]$^-$/$m/z$ 111.0090), aconitic acid ([M − H]$^-$/$m/z$ 173.0092), citraconic acid ([M − H]$^-$/$m/z$ 129.0195), citramalic acid ([M − H]$^-$/$m/z$ 147.0300), pantothenic acid ([M − H]$^-$/$m/z$ 218.1032), and isopropylmalic acid ([M − H]$^-$/$m/z$ 175.0620). Two sugars, galbose ([M − H]$^-$/$m/z$ 719.2002) and sucrose ([M − H]$^-$/$m/z$ 341.1090), were identified.

Phenolic acid derivatives were found in all four species of butiá. These compounds eluted between 3.1 and 7.7 min and were classified as hydroxybenzoic and hydroxycinnamic derivatives. Among the hydroxybenzoic derivatives, aglycones were detected including hydroxybenzoic acid ([M − H]$^-$/$m/z$ 137.0242), syringic acid ([M − H]$^-$/$m/z$ 197.0445), and vanillic acid ([M − H]$^-$/$m/z$ 167.0347), as well as one glycoside ester, hydroxybenzoic acid hexose ([M − H]$^-$/$m/z$ 315.0510).
Table 3. Compounds Differentiating Butia odorata Fruit Collected from Two Locations in RS, Brazil

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<tr>
<th>peak</th>
<th>RT (min)</th>
<th>m/z measured</th>
<th>error (ppm)</th>
<th>fragment ion m/z</th>
<th>energy of collision (eV)</th>
<th>molecular formula</th>
<th>mSigma</th>
<th>compound</th>
<th>VIP score</th>
<th>CAP</th>
<th>TAP</th>
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aRT, retention time; VIP, variable importance in the projection; CAP, fruit of B. odorata from Capão do Leão; TAP, fruit of B. odorata from Tapes.
bAn asterisk indicates significant difference by LSD test at p ≤ 0.05.

299.0070) characterized by the neutral loss of the glycosidic moiety (162 Da). Hydroxybenzoic hexose was identified in B. catarinensis and B. odorata fruits. Four hydroxyxynamic derivatives, chlorogenic acid ([M – H]− m/z 353.0871), caffeic acid ([M – H]− m/z 179.0348), p-coumaric acid ([M – H]− m/z 163.0403), and ferulic acid ([M – H]− m/z 193.0505) were identified. P-Coumaric acid was not detected in B. paraguayensis fruit.

Flavonoid compounds are divided into different classes. In butia fruits tested, flavan-3-ol, flavonol, flavone, flavanone, stilbene, and flavonoid glycoside were identified. The flavonoids eluted between 5.7 and 12.2 min. Three flavan-3-ols, catechin ([M – H]− m/z 289.0720), epicatechin ([M – H]− m/z 289.0720), and catechin/epicatechin dimer ([M – H]− m/z 577.1350), were identified by comparing retention times relative to external standards and MS/MS fragmentation patterns.

Flavonol was the predominant group of flavonoids with seven compounds identified. Four quercetin derivatives (main fragments at m/z 300.0271) were identified, including rutin or quercetin-O-rutinoside ([M – H]− m/z 609.1448), quercetin-O-glucoside ([M – H]− m/z 463.0885), quercetin-O-
malonylglucoside ([M – H]⁻ m/z 549.0881), and the aglycone quercetin ([M – H]⁻ m/z 301.0343). In addition, three kaempferol derivatives (main fragments at [M – H]⁻ m/z 285.0404 and [M – H]⁻ m/z 284.0335) were identified: kaempferol-O-rutinoside ([M – H]⁻ m/z 593.1513), kaempferol-O-glucoside ([M – H]⁻ m/z 447.0936), and kaempferol-O-acetylglucoside ([M – H]⁻ m/z 489.1041).

Luteolin ([M – H]⁻ m/z 285.0402) was the only flavone identified in butiá fruits and was found in all species. The identity of this compound was confirmed with an external standard. Additionally, the flavanones pinocembrin and hesperetin were identified. Pinocembrin ([M – H]⁻ m/z 255.0659) was not identified in B. paraguayensis fruit, whereas hesperetin ([M – H]⁻ m/z 301.0707) was only identified in B. odorata fruit. The identities of these compounds were confirmed by external standards. A flavonol glycoside tentatively identified as an isorhamnetin derivative ([M – H]⁻ m/z 623.1620) with a main fragment at m/z 315.0510 was identified. Finally, a stilbene was identified as tetrahydroxy-stilbene ([M – H]⁻ m/z 243.0667).

**DISCUSSION**

In relation to physicochemical characteristics, this study demonstrated that B. catarinensis fruits were the sweetest (13.7° Brix) and B. odorata fruits the most acidic with 1.9% citric acid equivalents (CAE). Other species such as B. capitata have been shown to have a mean acidity of 0.4% CAE, pH 3.018 and 9.3° Brix,19,20 whereas for B. erosipatha the pH ranged from 2.4 to 3.1, the acidity from 0.4 to 1.9% CAE,19 and the soluble solids content from 6.4 to 9.3° Brix.18

The ascorbic acid content found in butiá fruit (20.2–64.6 mg l-ascorbic acid 100 g⁻¹) was similar to that reported for green pea (40 mg), cauliflower (49 mg), orange (54 mg), kiwifruit (59 mg), strawberry (77 mg), and broccoli (112 mg).21 The variation in l-ascorbic acid content in this study was greater than previously found for B. odorata (23–63 mg), B. erosipatha (21–31 mg), and B. capitata (38–73 mg).3 Fruits of B. yatay, B. odorata, and B. paraguayensis tested contained more than the recommended daily intake of l-ascorbic acid for adult women (60 mg).

In this study, B. paraguayensis fruit had the highest carotenoid content (26.7 mg β-carotene equivalent 100 g⁻¹) among species tested. Carotenoids are considered health-beneficial compounds, as some carotenoids are precursors to vitamin A and have high antioxidant potential. These properties make fruits and vegetables rich in carotenoids interesting food sources because their consumption has been associated with a reduced incidence of many types of cancers and degenerative and cardiovascular diseases.22-24 Butia fruit tested in this study had higher carotenoid content (10.7–26.7 mg β-carotene 100 g⁻¹) than other commonly consumed fruits and vegetables such as carrot (18.3 mg), mango (131 mg), spinach (5.6 mg), lettuce (1.3 mg), and tomato (1.2 mg).25

B. odorata has been previously shown27 to have a total phenolic content >280 mg gallic acid equivalent (GAE) 100 g⁻¹. In this study, B. yatay fruit showed the highest phenolic content (200.1 mg GAE 100 g⁻¹), followed by B. odorata fruit (171.1 mg GAE 100 g⁻¹). For total flavonoids, B. catarinensis had the highest content (100.0 mg (+)-catechin equivalents 100 g⁻¹) and B. paraguayensis the lowest content (61.4 mg (+)-catechin equivalent 100 g⁻¹). Fruits and vegetables are the main source of phenolic compounds in the diet, and the regular consumption of these foods is increasingly associated with health benefits such as anticancer effects, which have been associated with the antioxidant capacity of these compounds.25

Metabolomics is currently considered one of the most popular approaches for the creation of profiles based on primary and specialized metabolites in plants and has been used for the identification of markers for the detection of physiological changes, genotype differences, geographic origin, and quality control.26,27 In this study, the compounds responsible for the discrimination among Butia species were both general and specialized metabolites. Primary metabolites include carbohydrates, amino acids, lipids, and organic acids, which are essential for normal growth, development, and reproduction of organisms. In this study, several organic acids and sugars known to be associated with the degree of ripeness of fruit were identified as markers of Butia spp. Sucrose is a source of energy and a signaling molecule in plants.28 Organic acids have strong organoleptic influence in fruits and vegetables, typically responsible for their sour taste, and are important at the cellular level with roles in energy production and the formation of precursors for amino acid biosynthesis and at the whole-plant level in modulating adaptation to the environment.29

Specialized metabolites are associated with plant fitness and their capacity to cope with environmental conditions and provide defense against herbivores, micro-organisms, and other pathogens. Additionally, specialized metabolites found in fruits and vegetables play relevant roles both in human health, because of their biological activities, and in influencing food color, flavor, and nutritional characteristics.30 The specialized metabolite profile is unique to individuals within a species or a close taxonomic group; however, it can be altered because biosynthetic pathways may be influenced by environmental conditions such as climate (temperature, light, and water), soil properties, and attack by pathogens and pests.31 In this study, several phenolic compounds were identified in butiá fruit, and a flavonol (rutin), a flavan-3-ol (epicatechin), a glycosylated flavonoid (isorhamnetin), and a stilbene (tetrahydroxy-trans-stilbene) were significant markers for discrimination of geographic origin and species. Flavonoids protect plants from UV-B radiation and are relevant for human health, acting as anti-inflammatory, antimicrobial, anti-tumor, and antiinflammasome agents.32 In plants, stilbenes pre-exist or are synthesized after a pathogen attack (phytoalexins). These compounds may also be involved as chemical signals in allelopathy or in response to oxidative stress generated by UV irradiation, suggesting that these compounds are important components of defense responses and may constitute indicators of disease resistance.33 For human health, several studies have reported that tetrahydroxy-trans-stilbene has potential activity against lung cancer, with antioxidant, antiaging, and antiangiogenetic properties.34

This is the first report of luteolin, pinocembrin, and hesperetin in butiá fruit. Luteolin has been previously identified in other fruits in the Arecaceae family, such as açai (Euterpe oleracea) and date palm (Phoenix dactylifera). It is reported that luteolin has high antioxidant, anti-inflammatory, and antimicrobial activities.35 Hesperetin occurs predominantly in citrus fruits and contributes to the antioxidant capacity of orange juice,36 whereas pinocembrin has anticancer properties.25

A metabolomic fingerprinting of date palm fruit (P. dactylifera) indicated flavonols (rutin and isorquercetin) and sugars (glucose, fructose, and sucrose) as biomarkers contributing to
the classification of date palm fruit.\textsuperscript{37} Similarly, an evaluation of the profile of semipolar compounds in four species of peppers (\textit{Capsicum} spp.) by LC-MS revealed differentiation among species based on acyclic diterpenoid glycosides, phenolic acids (coumaric acid and ferulic acid), and flavonoids (luteolin, apigenin, quercetin, and kaempferol).\textsuperscript{38} Discrimination of the geographic origin of Goji berry (\textit{Lycium barbarum}) by metabolite profiling was influenced by flavonoids and phenolic acids.\textsuperscript{39} Metabolomics followed by multivariate analyses were also useful in discriminating tea\textsuperscript{40} and blueberry\textsuperscript{41} samples.

This study demonstrated that the use of LC-MS followed by multivariate statistical analyses was a useful tool to differentiate \textit{Butia} spp. and provided biomarkers for discrimination among \textit{B. catarinensis}, \textit{B. odorata}, \textit{B. paraguayensis}, and \textit{B. yatay}. Organic acids were markers for \textit{B. yatay}, rutin and galabioside for \textit{B. odorata}, and sucrose and isopropylmalic acid for \textit{B. catarinensis}, which was sweeter and less acidic; tetradroxy-trans-stilbene was a marker for \textit{B. paraguayensis}. Currently, \textit{Butia} species are identified by morphological markers, which can be difficult and inconclusive. Thus, metabolomics can be a useful additional tool for the identification of species. However, to confirm the validity of the biomarkers identified in this study, future evaluations should be expanded to include more \textit{Butia} genotypes and species.

\textit{B. odorata} fruit from Tapes showed a high content of organic acids and flavonoids, whereas fruit from Capão do Leão had a high sugar content. A previous study using molecular markers observed less variation among populations than within populations of \textit{B. odorata}.\textsuperscript{42} To the best of our knowledge, this is the first time that a metabolomics approach has been used to reveal compositional differences among \textit{Butia} species and between fruits from the same species grown in different locations. Typically, outcrossing species have greater variation within individuals of the same population than among populations.\textsuperscript{43} Although genetic diversity measures were not included, this study identified metabolic markers that can be employed to assess \textit{Butia} population diversity and assist in species identification.

\section{ASSOCIATED CONTENT}

\subsection{Supporting Information}

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b03203.

Figures S1–S3 and Table S1 (PDF)

\section{AUTHOR INFORMATION}

\subsection{Corresponding Author}

*(F.C.C.) Phone: +55 53 32757284. E-mail: fabio.chaves@ufpel.edu.br.

\subsection{ORCID}

Fabio Clasen Chaves: 0000-0002-5773-0800

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\subsection{Notes}

The authors declare no competing financial interest.

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