SUPPLEMENTARY MATERIAL

Determination of trans-resveratrol in *Solanum americanum* Mill. by HPLC

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Abstract

Solanum americanum Mill. is a plant that belongs to the Solanaceae family, its respective ripe fruit is dark purple. Ripe *Solanum americanum* Mill. fruits were submitted to physico-chemical characterization, and their *trans*-resveratrol contents were quantified by high performance liquid chromatography. Such determination was executed with fruits at different stages of ripeness and freeze-stored fruits as well. *In natura* ripe fruit pulp and peel presented average *trans*-resveratrol amounts of 1.07 µg and 0.7960 µg *per* gram of sample, respectively. These amounts are significantly higher when compared to freeze-stored fruit (0.1353 µg of *trans*-resveratrol *per* gram of sample,) and to other berries. All ripe fruits showed significant amounts of total anthocyanins and total antioxidants. Thus, for the first time, *trans*-resveratrol has been identified and quantified in *Solanum americanum* Mill. fruit samples.

Keywords: 3,4,5-trihydroxystilbene; polyphenol; anthocyanins; stilbene; antioxidant; composition.

Experimental

Reagents

Resveratrol (3,4,5-trihydroxystilbene, *trans*-isomer purity >99%) was obtained from Sigma Alderich[®] (USA) and HPLC reagents methyl alcohol from J. T. Baker[®] (Mexico), ethylic alcohol from J. T. Baker[®] (Mexico), acetonitrile from Panreac[®] (Spain), bi-distilled water purified with Milli-Q (Millipore, USA) were used.

Equipment

A thermostatic bath (Fisatom[®]), a vacuum rotary evaporator (Fisatom[®]), a vacuum pump model TE-058 (Tecnal[®]), a refrigerated cold bath model TE-184 (Tecnal[®]) and an analytical balance model BK 200 (Labstore[®]) were used. Chromatographic separation was carried out using a chromatographic workstation (Termo[®]) with a ChromQuest managing model made up of a 4-way reciprocating piston pump model 240, a Rheodyne injection valve model 8096 (50 µL loop) and a diode array detector (DAD). Chromatographic separation was performed on a C-18 column (Phenomenex, 250 mm x 4.6 mm, 5 µm particle size).

Plant Material

Solanum americanum Mill. fruit were harvested in the city of Califórnia, Paraná, Brazil. A voucher specimen has been deposited in the Botanical Departament, Federal University of Minas Gerais (BHCB: 179995). The samples were taken to the Food Biochemistry Laboratory at the State University of Maringá, Paraná, Brazil and cleaned and stored in plastic package in 100 g portions. *In natura* fruit at different ripeness stages were separated and one lot was submitted to analysis and another lot was stored at -8 °C for 30 days until analysis.

Sample preparation

Solanum americanum Mill. fruit were divided into three different stages of ripeness (green, semi-ripe and ripe). The skin (5 g) was separated from the pulp (5 g) by hand. *In natura* and thawed fruit from each stage of ripeness were analyzed separately for determination of *trans*-resveratrol. Only ripe *in natura* and frozen fruit were submitted to other analyses.

Physical chemical characterization

To evaluate the quality of ripe *Solanum americanum* Mill. fruit, the following parameters were measured: color, using a reflectance colorimeter from Konica Minolta Sensing

(model CR-10), mass (g) using a Labstore (model BK 200) analytical balance and fruit diameter, measured by hand with a pachymeter.

The soluble solid (SS) content was determined with a digital refractometer (ATAGO, Pocket PAL-1); the results were expressed in °Brix. The titratable acidity (meq/L) and pH (Hanna Instruments Model HI 221) were measured in ripe fruit juice and the soluble solids/total ash (SS/TA) ratio was determined. The moisture, total ash, protein, ascorbic acid and mineral content (AOAC 1998), total sugar and total reducing sugar (Lane & Eynon 1934), lipids (Bligh & Dyer 1959) were also determined.

Determination of the antioxidant activity, anthocyanins and phenolic compounds

For the total phenolic compounds, 5 g of sample were added to 25 mL/methanol. An aliquot of 0.1 mL was added to 5 mL of water and 0.5 mL of Folin-Ciocalteu reagent. After 3 min, 2 mL of 15% sodium carbonate were added and the volume was completed to 10 mL with water. The samples were stored in the dark for 2 h. The absorbance values were measured at 765 nm. Total anthocyanins were determined following Lee & Francis (1972): 5 g of sample with 100 mL of the solvent solution (70% ethanol acidified to pH 2.0 with 0.1% HCl) were homogenized for 2 min. After that, the volume was completed to 200 mL and stored in a refrigerator at 4 °C for 12 h. Next, the sample was filtered, yielding 50 mL of the filtrate, which was completed to 100 mL with solvent solution. Next, an aliquot of 2 mL was completed to 100 mL. The samples were stored in the dark for 2 h. The absorbance values were measured at 535 nm (Bucić-Kojić et al. 2007). The total antioxidant activity was determined by the DPPH free-radical scavenging method following Mensor et al. (2001): 5 g of sample were added to 25 mL/ethanol, absorbance was measured at 518 nm after 30 min and converted to percentage antioxidant activity (AA%) using the extract with ethanol as a blank, the DPPH solution with ethanol as a negative control and the following formula:

$$AA(\%) = 100 - \{ (Abs_{sample} - Abs_{blank}) \times 100 / Abs_{control} \} \quad (1)$$

Trans-resveratrol extraction

To extract *trans*-resveratrol, 25 mL of a 80:20 (v/v) ethanol/water solution were added to each of the pulp and skin samples and manually macerated for 5 min. Next, the samples were placed in a thermostatic bath at 60 °C for 30 min under agitation every 5 min. The samples were then filtered with filter paper and the volumes were completed to 50 mL, according to (Romero-Pérez et al. 2001) with modifications. The samples were concentrated in a rotary evaporator under vacuum for 30 min until almost completely dry. The residue was reconstituted with 0.2 mL of ethanol and completed to 10 mL with bi-distilled water. The samples were stored in the dark until purification.

Extract purification

The cleanup of extracts were carried out with described by Durst & Wrolstad (2001) with adaptations, using solid phase extraction at 50.0 mg octadecyl C18/18% cartridges with capacity for 1.0 mL. All steps were carried out with the aid of a peristaltic pump with a flow rate of 1mL/min. The cartridges were activated by eluting 10 mL of ethanol and 20 mL of water. Next, 10 mL of extract were eluted through the cartridge and the interferential was removed by eluting 10 mL of a 10% ethanol solution. The analyte was removed from the cartridge by eluting 1 mL of a 50:50 ethanol/water (v/v) solution and stored in the dark until analysis by HPLC.

Chromatographic conditions

There are no studies that have specific methodology for chromatographic quantification of *trans*-reveratrol in this berry. Extracts obtained from *Solanum americanum* Mill. fruit underwent chromatographic separation using different methodologies developed for separation of this compound in grapes and wine samples (Pezet et al. 1994; Soleas et al. 1997; Romero-Pérez et al. 2001; Souto et al. 2001; Sun et al. 2006; Cvejic et al. 2010; Liu et al. 2013).

The best chromatographic separation between *trans*-resveratrol and interfering compounds was obtained using: acetonitrile (A) and water (B) as the mobile phases at a flow rate of 1.0 mL/min and detection wavelength of 306 nm at room temperature as follows: linear gradient from 10% at 0 min (A), 17% at 5 min (A), 18% at 12 min (A), 22% at 22 min (A), 33% at 30 min (A), 38% at 45 min (A) and 100% at 58 min (A) following (Sun et al. 2006). Quantification was achieved by plotting an analytical curve at a significance level of 0.05 at 1.6 mg/L.

Statistical analysis

All determinations were performed in triplicate. Statistical analysis was done with R software package 2.15.1 (R. Development Core Team 2011) to assess significant differences between means. Tukey test ($P \leq 0.05$) was used to accesses significant differences between means for samples.

Table S1 Physical chemical parameters of ripe *in natura* and frozen *Solanum americanum* Mill. fruit.

Parameters	In natura SAM Fruit	Frozen SAM Fruit
	Mean±SD	Mean±SD
Mass (g)	0.27 ± 0.05 ^a	0.17 ± 0.04 ^b
Diameter (mm)	7.84 ± 0.81 ^a	5.30 ± 0.80 ^b
L	9.00 ± 0.45 ^a	8.40 ± 0.40 ^a
a	4.90 ± 1.68 ^a	3.03 ± 0.32 ^a
b	-0.80 ± 0.26 ^a	-1.23 ± 0.15 ^a
c	4.90 ± 1.70 ^a	2.56 ± 0.32 ^a
h	9.00 ± 0.45 ^a	12.8 ± 3.63 ^a

^{SD} Standard Deviation. Means followed by different letters in the same line are significantly different (P≤0.05). L: lightness; a: color are represented red at positive/green at negative; b: color are represented yellow at positive /blue at negative; C: chromaticity; h: Hue° angle.

Table S2 Chemical parameters of ripe *in natura* and frozen *Solanum americanum* Mill. fruit.

Parameters	In natura SAM fruit	Frozen SAM fruit
	Mean±SD	Mean±SD
Total sugars (% glucose)	8.33 ± 1.63 ^a	5.84 ± 2.13 ^a
Total reducing sugars (% glucose)	6.12 ± 0.93 ^a	4.70 ± 1.26 ^b
Total soluble solids (°Brix)	15.53 ± 0.40 ^a	12.36 ± 0.11 ^b
Titrateable Acidity (meq L ⁻¹)	46.59 ± 6.50 ^a	42.77 ± 5.50 ^b
SS/TS	0.30±0.05 ^a	0.29±0.04 ^b
Proteins (g)	3.37 ± 1.20 ^a	3.30 ± 0.32 ^a
Lipids (g)	0.88 ± 1.08 ^a	0.87 ± 1.68 ^a
Moisture (%)	82.29 ± 0.38 ^a	79.17 ± 0.48 ^b
Total ash (%)	15.36 ± 1.19 ^a	12.00 ± 1.49 ^b
pH	4.47 ± 0.02 ^b	4.73 ± 0.06 ^a
Vitamin C (mg/100g)	6.59 ± 0.83 ^a	4.78 ± 0.16 ^b

^{SD}: Standard Deviation. Means followed by different letters in the same line are significantly different (P≤0.05).

Table S3 Biochemical compounds in ripe *in natura* and frozen *Solanum americanum* Mill. fruit.

Parameters	In natura SAM fruit	Frozen SAM fruit
	Mean±SD	Mean±SD
Total Anthocyanins (mg/100g)	2.51 ± 0.33 ^a	2.16 ± 0.03 ^a
Antioxidant Activity (% inhibition)	95.66 ± 0.93 ^a	94.07 ± 1.25 ^a
Phenolic Compounds (mg/100g)	488.25 ± 0.04 ^a	258.92 ± 0.03 ^b

^{SD}: Standard Deviation. Means followed by different letters in the same line are significantly different (P≤0.05).

Table S4 Mineral content of *in natura* ripe and frozen *Solanum americanum* Mill. fruit.

Minerals	<i>in natura</i> ripe SAM fruit mg/100 g Mean±SD	Frozen ripe SAM fruit mg/100 g Mean±SD
Magnesium	36.40 ± 0.03 ^a	21.40 ± 0.16 ^b
Calcium	35.50 ± 0.05 ^a	24.65 ± 0.05 ^b
Phosphorus	56.47 ± 0.17 ^a	19.8 ± 0.08 ^b
Nitrogen	205.7 ± 0.50 ^a	0.40 ± 0.80 ^b
Potassium	86.43 ± 0.13 ^a	24.97 ± 0.20 ^b
Iron	5.86 ± 0.05 ^a	1.32 ± 0.15 ^b
Copper	0.19 ± 0.01 ^a	0.13 ± 0.03 ^b
Manganese	0.32 ± 0.01 ^a	0.28 ± 0.01 ^b
Zinc	0.56 ± 0.01 ^a	0.18 ± 0.20 ^b

^{SD}: Standard Deviation. Means followed by different letters in the same line are significantly different (P≤0.05).

Table S5 *Trans*-resveratrol content in natura and frozen *Solanum americanum* Mill. fruit at different ripeness and storage stages.

Samples	Ripeness stages	<i>In natura</i> SAM fruit* Mean±SD	Frozen SAM fruit* Mean±SD
Skin	Green	nd**	nd**
	Semi-ripe	0.1753 ± 0.104 ^a	0.3446 ± 0.051 ^a
	Ripe	0.7960 ± 0.158 ^a	0.1353 ± 0.006 ^b
Pulp	Green	-	-
	Semi-ripe	0.3103 ± 0.073 ^a	0.1126 ± 0.056 ^b
	Ripe	0.2745 ± 0.209 ^a	0.1770 ± 0.077 ^a

^{SD}: Standard Deviation. *Values expressed in µg resveratrol g⁻¹ sample. **nd: non-detectable (below the method detection limit).

Figure S1 Chromatogram obtained at 298 nm. (1) *trans*-resveratrol presents in skin of *Solanum americanum* Mill. (2) Sample of Skin of *Solanum americanum* Mill. with standard.

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