http://dx.doi.org/10.21577/0103-5053.20180020

J. Braz. Chem. Soc., Vol. 29, No. 5, 1123-1129, 2018
Printed in Brazil - ©2018 Sociedade Brasileira de Química

Article

An Environmentally Friendly Procedure to Obtain Flavonoids from Brazilian Citrus Waste

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Currently, most food waste is used as animal feed and this process does not take advantage of the available chemical composition. A Brazilian example is the citrus fruit processing wastes (CFPW), which have begun to draw attention due to their biological importance. In order to access the main compounds of this matrix, an efficient and environmentally friendly procedure was tested. From this extract, flavonoids as naringenin, hesperitin, chrysoeriol, sinensetin, 3,5,6,7,3',4'-hexamethoxyflavone, nobiletin, 5-methoxysalvigenin, 3,5,6,7,8,3',4'-heptamethoxyflavone, 3,5,6,7,4'-pentamethoxyflavone and isosakuranetin were identified by high performance liquid chromatography (HPLC-UV), liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-nuclear magnetic resonance (LC-NMR). This study describes the potential use of citrus waste as a source of biologically active compounds. The extraction method proposed for this work was not expensive and the flavonoids were obtained in large amounts, thus, this extraction method is being developed using pilot plant scale-up techniques and will soon be available to the industry at a low cost.

Keywords: citrus waste, polymethylated flavonoids, polymethoxylated flavonoids, biomass, biorefinery

Introduction

Nowadays, despite remarkable technological and scientific advancements, there are still problems associated with them. Some impacts can be observed over the last decades, such as global warming, acid rain, smog in highly industrialized areas, groundwater pollution, among others. Waste has been generated globally more quickly than the planet can process.[1] Biomass represents an heterogeneous and chemical complex resource, which can generate energy and provide other high valuable products.[2,3] Within the different sources of biomass, food waste is noteworthy as it is produced in large quantities due to our inefficient food supply chain.[4,5] However, due to its large volume and wide range of chemical compounds, it is considered of high added value for energy and chemicals.[4] Currently, most food waste is used as animal feed, but this practice is considered underutilized because this process does not take full advantage of its chemical characteristics.[4] The main reason to develop advanced practices that convert waste materials into high value products is their significant amount of functional molecules such as carbohydrates, proteins, triglycerides, fatty acids, phenolic compounds, etc.[4]

Brazil is known for its orange production and is the world leader in this area (around 16 million tons of fruit *per* year).[6] São Paulo State is the main citrus producer and is responsible for 72% of Brazil's orange production.[7] Citrus fruit crops are the most abundant in the world, exceeding 94.8 million tons and generating around 15.6 million metric tons of waste annually.[5] Recent research has shown that orange waste can provide useful chemicals and other high value products, whose exploitation could reduce costs, generating profits and increasing their competitiveness.[5,8-10]

Citrus fruit processing wastes (CFPW) mainly consist of peel (albedo and flavedo), seeds and fruit pulp remaining after juice extraction; altogether they represent 50% of the fruit weight. The main compounds that are found in this biomass are water content (80%), soluble sugars, cellulose and hemicellulose (bioethanol production),[11] pectin (carrier for oral drug delivery systems),[12] *D*-limonene

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This paper is part of the PubliSBQ Special Issue "IUPAC-2017" (http://publi.sbq.org.br/).

(bio-solvent),[13] ethanol (fuels) and flavonoids,[4,8-10] making it an interesting source to explore. Recovering and valuing by-products has become a necessity when dealing with sustainable development and environmental protection.[10]

Earlier publications have described various methods to determine citrus flavonoids in fruit and orange juice,[14] however, few papers have reported the determination of flavonoids in CFPW.[15] These studies motivated us to develop a quick and sensitive method to determine flavonoids in the Brazilian CFPW, aiming to apply them into the control of plagues in agriculture.

Experimental

Chemicals and reagents

Solvents used for extraction were of analytical grade. Acetonitrile, methanol and ethanol HPLC grade were purchased from Tedia (Rio de Janeiro, Brazil) and Merck (Darmstadt, Germany). Tetra-deuterated methanol (CD_3OD) with 99.8% of deuterium (Acros Organics) and methanol LC-MS grade (JT Baker) were purchased from national suppliers. Deionized water was obtained from a Milli-Q water purification system (Millipore Corporation, Watford, UK).

Sample collection and pre-treatment

The CFPW was obtained from a Brazilian company (Agroterenas). Sweet oranges were grown in crops in Santa Cruz do Rio Pardo/São Paulo, Brazil. They were harvested at maturity, used for juice production and the orange waste obtained after this process was used as biomass. The biomass was dried at 40 °C in an airflow oven (Technal) for nine days and grounded using an industrial blender (Fiochi) to produce an average particle size of 14 mesh.

Extracting compounds

Flavonoids extraction was done from 240 g of dry CFPW sample in 750 mL of ethanol using an Ultra-Turrax homogenizer (IKA, Ultra-Turrax-T10 basic, 20,000 rpm) for 2 min. The extract was vacuum filtered through filter paper and the resulting solid was extracted once again with 350 mL of ethanol using Ultra-Turrax for 2 min. The whole filtrate was concentrated in a rotary evaporator (Buchi, R-114 and R-200) at 40 ºC, and the recovered solvent was reused in other extractions. This whole procedure was performed in triplicate.

In order to concentrate the flavonoids, the resulting extract was processed by solid phase extraction, before high performance liquid chromatography (HPLC) analysis. For this purpose, 50 mg of extract was loaded in a C-18 SPE cartridge (Chromabond, 6 mL/500 mg) that was previously activated with methanol, followed by 10 mL of water/methanol 8:2 (fraction 1) and 10 mL of methanol 100% (fraction 2). These processes were carried out in quintuplicate. The extract was concentrated under vacuum and stored at room temperature.

HPLC analysis

HPLC analysis was performed by using an Agilent Technologies Liquid Chromatography 1200 Series configured with a degasser G1322A, quaternary pump G1311A, autosampler G1329A, column oven G1316A, UV detector G1314B and coupled with a 1260 Infinity Fraction Collector analytical scale G1364C. A commercial octadecylsilane (C18) column (ZORBAX Eclipse XDB; 250 × 4.6 mm ID, 5 μm, Agilent) equipped with a guard column (4 × 3 mm i.d., 5 μm) was used for the screening analysis of CFPW. To control the HPLC system, the acquired and processed data collection was performed using the Agilent Technologies OpenLAB CDS ChemStation Edition Workstation M8301AA.

Chromatographic conditions

Approximately 2 mg of fraction 2 (separated on SPE cartridge) were dried and dissolved in 2.0 mL of a mixture of methanol/water (1:1), and the solutions were filtered through a PVDF membrane syringe filter (25 mm, 0.45 μm, Tedia Brazil) prior to HPLC analyses to optimize all chromatographic conditions. The mobile phase consisted of a combination of A (water), B (methanol) and C (acetonitrile). A gradient program was used for HPLC-UV as follows: 20% A, 40% B and 40% C in the beginning, linear gradient to 0% A, 50% B and 50% C over 30 min and finally back to the initial conditions (equilibrating time was 10 min for each analysis). The injection volume for all samples was 15 μL. Flavonoids were monitored at 260 nm at a flow rate of 1 mL min^{-1}. After chromatographic optimization for efficient and reproducible compound separation, major peaks (with higher intensity) were selected to be collected to increase their concentrations so as to detect them satisfactorily. This was achieved using an automatic collector system after the UV detector. This is a good technique for the reproducible and selective concentration of analytes and can be used as an interface between HPLC and identification techniques such as nuclear magnetic resonance (NMR). Therefore, a more concentrated sample was prepared using the same

methodology described above and 20 μL of a 20 mg mL^{-1} solution was collected on vials (15 mL capacity, Agilent) using a threshold-based absorbance set at 1000 mAU. The collection took place using 20 injections and the concentrated analytes were then dried and post-analyzed by NMR and mass spectrometry (MS).

NMR measurements

^{1}H NMR spectra was recorded on a Bruker Avance 400 spectrometer at 400 and 100 MHz for ^{1}H and ^{13}C, respectively. A nuclear magnetic field was obtained for the isolated compounds. The samples were diluted with 500 μL of CD_3OD and transferred to a 5 mm NMR tube. The chemical shifts are expressed in δ (ppm) and coupling constants (*J*) are given in hertz (Hz).

Mass spectrometer analysis

Electrospray ionization mass spectrometry (ESI-MS) spectra were obtained using an API 2000 triple quadrupole (QqQ) mass spectrometer (Applied Biosystems, Concord, Canada), equipped with an electrospray ionization (ESI) source. The software used to control all the MS parameters was the Analyst® Software, version 1.5.1 (AB Sciex, CA, USA).[17] Mass spectrometric analyses were performed using direct infusion of each of the previously isolated compounds (5 μg mL^{-1}) at 20 μL min^{-1} flow. Mass analyses were carried out in full scan mode ranging from 100 to 900 Da, and MS parameters were optimized for each compound. The compounds ionization was carried out using electrospray ionization (ESI) in positive (ESI+) and negative (ESI-) modes. The universal parameters used in all mass spectra were as follows: positive ion mode: nebulizing gas (GS1), heater gas (GS2) and curtain gas were set at 20, 20 and 30 a.u. (arbitrary units), respectively. Nitrogen gas was used for GS1, GS2 and curtain gas. The ion spray voltage was set at +4500 V. Analyte dependent parameters as declustering potential (DP), focusing potential (FP) and entrance potential (EP) were set at 60, 230 and 8 V, respectively. Negative ion mode: nebulizing gas (GS1), heater gas (GS2) and curtain gas were set at 30, 25 and 20 a.u. (arbitrary units), respectively. The ion spray voltage was set at –4500 V. Analyte dependent parameters as declustering potential (DP), focusing potential (FP) and entrance potential (EP) were set at –130, –220 and –10 V, respectively.

Results and Discussion

Our investigations initially focused on establishing the best extraction and separation conditions for flavonoids from CFPW. The method based on the ultra-turrax homogenizer and ethanol as an extraction solvent was selected as it ensures efficiency and it is simpler, faster and greener when compared to conventional ones.[16] The final chromatographic system for analyses of the ethanolic extract obtained after SPE clean-up was chosen after screening analysis using HPLC-UV. The optimal conditions consisted of a gradient program of a solvent system containing H_2O, MeOH and ACN, using a reverse-phase analytical C18 column. Ultraviolet and visible spectroscopy was one of the earliest techniques routinely used for flavonoid analysis due to the existence of two characteristic UV-Vis bands in flavonoids, Band I in the 300 to 550 nm range from the B ring, and band II in the 240 to 285 nm range from the A ring.[14] Thus, flavonoids were monitored at 260 nm and the concentration of the sample analyzed was 20.0 mg mL^{-1}. The extract at this concentration (20 μL) was injected 20 times in order to collect the peaks (Figure 1) at a sufficient concentration to obtain the NMR and MS spectra.

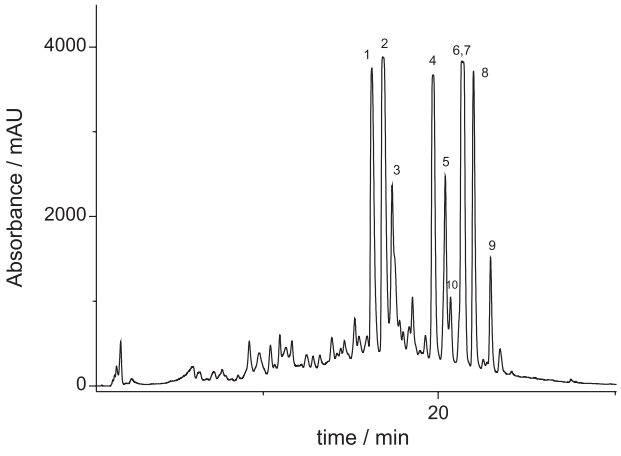

Figure 1. HPLC-UV chromatogram of ethanolic extract from citrus waste obtained after SPE clean-up. Peak numbers refer to compounds shown in Tables 1 and 2.

^{1}H NMR spectra of the selected peaks indicate the presence of naringenin (**1**), hesperitin (**2**), chrysoeriol (**3**), sinensetin (**4**), 3,5,6,7,3',4'-hexamethoxyflavone (**5**), nobiletin (**6**), 5-methoxysalvigenin (**7**), 3,5,6,7,8,3',4'-heptamethoxyflavone (**8**), 3,5,6,7,4'-pentamethoxyflavone (**9**) and isosakuranetin (**10**) (Tables 1 and 2, Figure 2).

The structures of the isolated flavonoids were identified by ^{1}H NMR (Supplementary Information section), and confirmed by ESI-MS (Table 3) and by comparing them with the literature. Flavonoids **1**-**10** were previously isolated from sweet oranges (*C. sinensis*).[18-25]

The total concentration of flavonoids in the sample analyzed was 160.0 mg mL^{-1} and it was injected 20 times

Table 1. ^{1}H NMR chemical shifts for **1**-**5** (400 MHz, CD_3OD)

H	**1**	**2**	**3**	**4**	**5**
2	5.34 (dd, 12.90, 2.90)	5.32 (dd, 12.59, 3.02)			
3	3.11 (dd, 17.27, 12.90) 2.70 (dd, 17.27, 2.90)	3.07 (dd, 17.17, 12.59) 2.72 (dd, 17.17, 3.02)	6.67 (s)	6.66 (s)	3.93 (s) OMe
5				4.02 (s) OMe	4.00 (s) OMe
6	5.88 (d, 2.28)	5.88 (d, 2.29)	6.24 (d, 2.51)	3.87 (s) OMe	3.80 (s) OMe
7				4.02 (s) OMe	3.95 (s) OMe
8	5.90 (d, 2.28)	5.91 (d, 2.29)	6.50 (d, 2.51)	7.13 (s)	7.10 (s)
2'	7.32 (d, 8.74)	6.90 (dd, 8.2, 1.6)	7.54 (dd, 8.28, 2.26)	7.63 (dd, 8.53, 2.01)	7.79 (dd, 8.28, 2.26)
3'	6.82 (d, 8.74)	6.95 (d, 8.2)	6.96 (d, 8.28)	7.11 (d, 8.53)	7.13 (d, 8.28)
4'				3.92 (s) OMe	3.93 (s) OMe
5'	6.82 (d, 8.74)	3.85 (s) OMe	3.99 (s) OMe	3.95 (s) OMe	3.87 (s) OMe
6'	7.32 (d, 8.74)	6.93 (d, 1.6)	7.50 (d, 2.26)	7.52 (d, 2.01)	7.76 (d, 2.26)

Table 2. ^{1}H NMR chemical shifts for **6**-**10** (400 MHz, CD_3OD)

H	**6**	**7**	**8**	**9**	**10**
2					5.38 (dd, 12.80, 3.01)
3	6.70 (s)	6.63 (s)	3.96 (s) OMe	3.92 (s) OMe	3.10 (dd, 12.80, 17.07) 2.77 (dd, 17.07, 3.01)
5	4.03 (s) OMe	3.92 (s) OMe	4.12 (s) OMe	4.05 (s) OMe	
6	3.92 (s) OMe	3.86 (s) OMe	3.84 (s) OMe	3.95 (s) OMe	5.88 (d, 2.00)
7	3.89 (s) OMe	4.01 (s) OMe	4.03 (s) OMe	4.12 (s) OMe	
8	4.11 (s) OMe	7.10 (s)	3.94 (s) OMe	6.70 (s)	5.87 (d, 2.00)
2'	7.66 (dd, 8.53, 2.18)	7.95 (d, 9.16)	7.88 (dd, 8.53, 2.01)	8.01 (d, 9.03)	6.94 (d, 8.53)
3'	7.13 (d, 8.53)	7.08 (d, 9.16)	7.17 (d, 8.53)	7.14 (d, 9.03)	7.42 (d, 8.53)
4'	3.93 (s) OMe	3.89 (s) OMe	3.96 (s) OMe	3.91 (s) OMe	
5'	3.94 (s) OMe		3.94 (s) OMe	7.14 (d, 9.03)	7.42 (d, 8.53)
6'	7.54 (d, 2.18)		7.82 (d, 2.01)	8.01 (d, 9.03)	6.94 (d, 8.53)

(20 μL) in order to collect the peaks, which was converted into 172.7, 243.3, 56.0, 72.0, 90.0, 97.4, 48.7, 82.0, 39.3 and 73.3 μg of flavonoids **1**-**10**, respectively. Thus, the concentrations of these flavonoids were expressed as mg g^{-1} of citrus waste: 5.76, 8.11, 1.87, 2.4, 3.0, 3.25, 1.62, 2.73, 1.31 and 2.44 mg g^{-1}, respectively.

This study showed the high quality and potential of the Brazilian CFPW considering the investigated compounds, which could serve as an inexpensive and readily available source of polymethoxylated flavonoids. These are usually found in citrus peel, but commercial juices are rich in polymethoxylated flavonoids because the industry processes the fruits with the peel. Flavonoids found in citrus juices are mainly their glycosylated derivatives, such as flavanone-O-glycosides and flavone-O- or -C-glycosides.[26] Additionally, the therapeutic potential use of citrus waste as it is a source of 10 bioactive flavonoids that are beneficial for human health and agriculture, as will be discussed later here.

In a review paper on flavonoid composition of citrus juices, Gattuso *et al.*[14] indicated that these compounds show a strong antioxidant and radical scavenging activity and appear to be associated with a reduced risk for certain chronic diseases, the prevention of some cardiovascular disorders and certain kinds of cancerous processes. They also discussed that flavonoids exhibit antiviral, antimicrobial and anti-inflammatory activities, beneficial effects on capillary fragility and an ability to inhibit

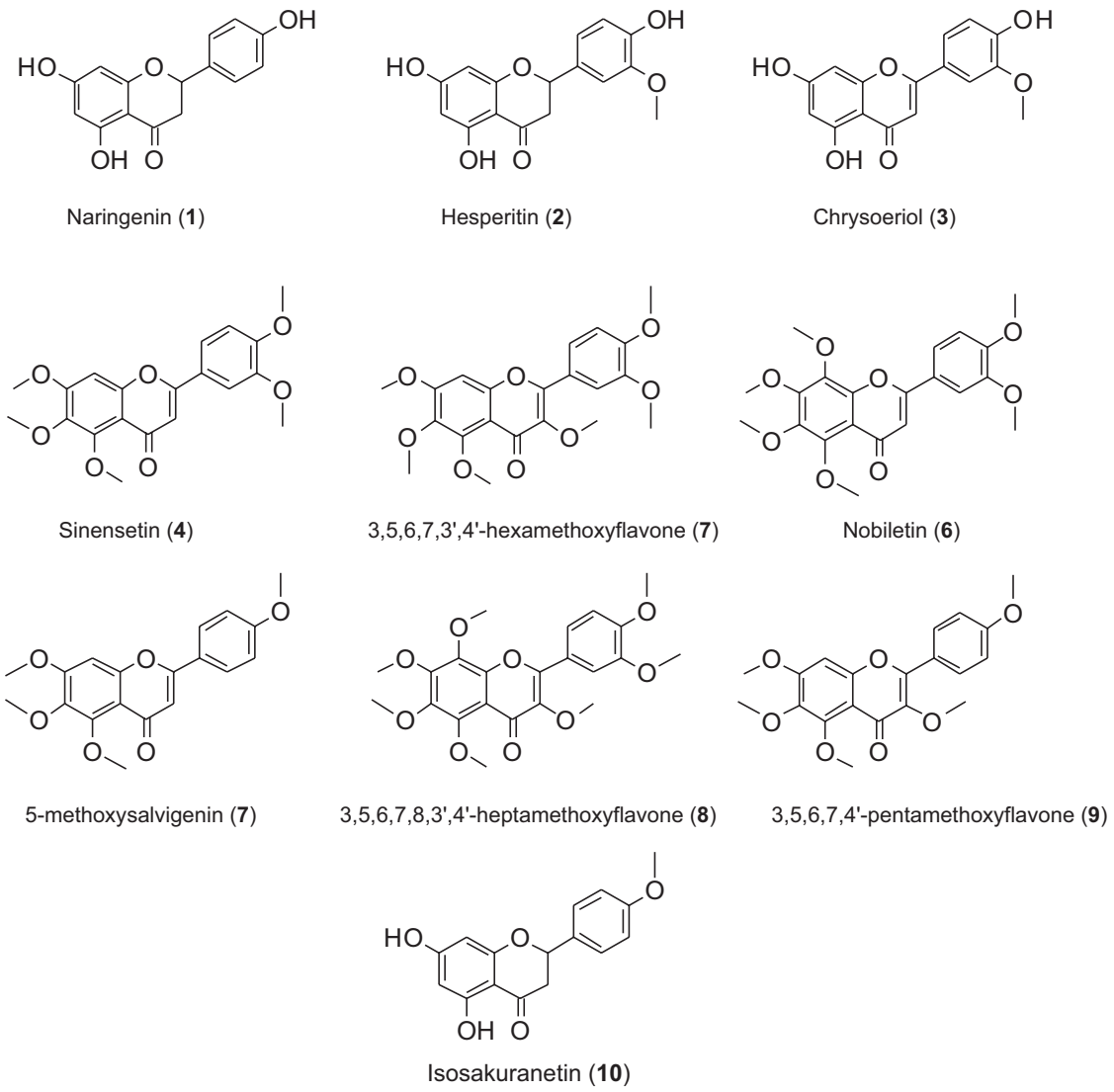

Figure 2. Flavonoid derivatives isolated from Brazilian citrus fruit processing wastes.

Table 3. HPLC and MS characteristics of flavonoids from citrus biomass

HPLC peak	RT / min	MS ion	*m/z* (precursor)	Molecular formula	Identification
1	16.2	$[M - H]^-$	271.1	$C_{15}H_{12}O_5$	naringenin
2	16.9	$[M - H]^-$	301.1	$C_{16}H_{14}O_6$	hesperitin
3	17.3	$[M - H]^-$	299.2	$C_{16}H_{12}O_6$	chrysoeriol
4	19.8	$[M + Na]^+$	395.2	$C_{20}H_{20}O_7$	sinensetin
5	20.5	$[M + Na]^+$	425.2	$C_{21}H_{22}O_8$	3,5,6,7,3',4'-hexamethoxyflavone
6	21.4	$[M + Na]^+$	425.1	$C_{21}H_{22}O_8$	nobiletin
7	21.4	$[M + Na]^+$	365.2	$C_{19}H_{18}O_6$	5-methoxy-salvigenin
8	22.0	$[M + Na]^+$	455.1	$C_{22}H_{24}O_9$	3,5,6,7,8,3',4'-heptamethoxy-flavone
9	22.9	$[M + Na]^+$	395.2	$C_{20}H_{20}O_7$	3,5,6,7,4'-pentamethoxy-flavone
10	21.1	$[M + H]^+$	no	$C_{16}H_{15}O_5$	isosakuranetin

HPLC: high performance liquid chromatography; RT: retention time; MS: mass spectrometry.

human platelet aggregation, antiulcer and antiallergenic properties. A recent review on chemistry and pharmacology of *Citrus sinensis* revealed a similar therapeutic potential for citrus flavonoids.[26]

Considering the use of flavonoids as starting material to prepare green pesticides, for instance, some authors have described a number of successful cases.[27] An example of a pest that has been studied is *Xylella fastidiosa* that colonizes the xylem of plants causing diseases to various economically important crops such as citrus variegated chlorosis (CVC). A number of flavonoids were tested for *in vitro* activity on the growth of *X. fastidiosa* by our group. As a result, it was found that hesperidin, which is found in great amounts in cells of the mesophyll of the affected leaves with CVC, shows a moderate activity suggesting that it can act as a good barrier for small-size colonies from *X. fastidiosa*.[28,29] Therefore, the search for novel semisynthetic modifications of some flavonoids has attracted our attention. One strategy would be to promote metal chelation of flavonoids and study the biological potential of these interactions.

Thus, this study intends to contribute by responding to these needs, using citrus fruit as it is the most important fruit tree crop in the world with an annual production of more than 90 million tons. Considering this, the main reason to develop economic methods that convert the waste of this material into new insecticides is to promote more economical and less harmful agriculture in Brazil.

Conclusions

Citrus waste biomass is a widely abundant renewable feedstock, especially in Brazil. This study suggests that Brazilian citrus waste can be an attractive source of various flavonoids, which are only found regularly in about 10-12 Angiosperm families and are most significant in the Rutaceae, mainly in *Citrus*.[30] The rich diversity and quantity of flavonoids in this residual biomass opens up new opportunities in terms of producing commodities considering a greater perspective of process valorisation.[31] Our method using an ultra-turrax homogenizer and ethanol as an extraction solvent is an effective and green process, allowing for the recovery of the solvent after extracting the flavonoids. Thus, this method was developed on a preparative scale as a pilot study and will be available soon for industries at a low cost.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

The authors would like to thank the Brazilian agencies: National Council for Scientific and Technological Development (CNPq-INCT, 573742/2008-1), FAPESP-São Paulo Research Foundation (FAPESP-INCT, 08/57859-5; 13/12052-5, 14/50827-1), and CAPES-Coordination for the Improvement of Higher Education Personnel (2032/2014-07) and Agroterenas.

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Submitted: October 11, 2017
Published online: February 8, 2018