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Anthocyanin and Flavonoid Production from *Perilla frutescens*: Pilot Plant Scale Processing Including Cross-Flow Microfiltration and Reverse Osmosis

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Extraction and concentration at a pilot plant scale of anthocyanins and flavonoids from *Perilla frutescens* var. *frutescens* harvested in the Guangzhou area of China were investigated. The study of extraction efficiency using mineral acids and organic acids showed that 0.01 mol/L nitric acid was the most suitable to extract flavonoids from this slightly red leaf cultivar. The red extract contained 12 mg/L (as cyanidin equivalent) anthocyanins and other flavones. The multistep process included cross-flow microfiltration (CFM) with a ceramic type membrane, reverse osmosis (RO), and rotating evaporation (RE). The filtration fluxes were high and constant for CFM (150 L/h/m^2 at 0.6 b) and for RO (22 L/h/m^2 at 40 b). The red extract was concentrated 9.4 times by RO and then 5.4 times by RE. It contained 422 mg/L anthocyanins, representing 77% of the total extracted anthocyanin. The proportion of flavonoids was found unchanged during processing. The concentrated extract showed a pH of 2.7, and its free acidity was found to be 46% of the acidity added for extraction, because of the buffering capacity of the extract. At the concentration level reached, a crystallized deposit occurred and was identified as tartrate.

KEYWORDS: ***Perilla frutescens*; polyphenolics; flavonoids; anthocyanins; membrane technology; pilot-scale; cross-flow microfiltration, reverse osmosis**

INTRODUCTION

Perilla frutescens (L.) Britt. (Lamiaceae) is an edible plant frequently used as one of the most popular spices and food colorants in some Asian countries such as China and Japan. The leaf and stem of the herb have been shown to have detoxicant, antitussive, antibiotic, anti-HIV, and antipyretic properties (*1*). The *P. frutescens* plant has been used as a traditional medicinal herb in China for centuries to treat various diseases including depression, anxiety, tumor (*2*), cough, bacterial and fungal infections, allergy (*3*, *4*), intoxication, and some intestinal disorders (*5*−*7*). Phenolic compounds are important components of *P. frutescens* (*8*). One interesting group among them is the anthocyanin family, because of their functional properties such as its attractive red coloring power and high solubility in aqueous mixtures that makes their incorporation into numerous aqueous food and nonfood formulations easy. In addition to their colorful characteristics, anthocyanins possess some positive therapeutic effects, mainly associated with their antioxidant properties (*9*, *10*). Because of the social trend toward the consumption of more natural origin products, instead of synthetic origin ones, anthocyanins have recently received increasing attention as natural extracts for several applications in various industry sectors for manufacturing food and nonfood products (*11*). Thus, new sources of natural pigments, such as anthocyanins, with functional and active properties are now desired. Schwarz et al. (*12*) reported the use of anthocyanins as natural food colorants and their potential health benefits regarding coronary heart disease and cancer prevention. Besides anthocyanins, *P. frutescens* contains other interesting phenolic compounds, such as phenolic acids and flavonoids that are supposed to exert a beneficial effect on human health (*13*, *14*).

Stability and colorpower of anthocyanins depend on their molecular structures and their intra- and intermolecular associations (*15*−*17*). Anthocyanins are also known for their heat sensitivity (*18*, *19*). Mild and health safe technologies to process plants containing anthocyanins need to be adapted to take into

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account the fragility of these compounds. The undesirable natural microbial load usually carried by these plants is also extracted with anthocyanins and other water-soluble compounds. Generally, heat treatments are applied to destroy microorganisms, but they have a negative effect on the color and antioxidant activities of anthocyanin content extracts (*20*). Therefore, bacteria free extracts have to be obtained by using softer technologies, such as membrane technology, whose uses have increased considerably in food and nonfood industries (*21*−*23*). Cross-flow microfiltration (CFM) is a "cold-operated process" that presents an interesting alternative to heat treatments for highly heat sensitive bioproducts, in particular to preserve their original functionalities and activities such as coloring, vitamin, and aromatic properties (*24*−*26*). In this study, CFM was thus applied to obtain a clarified, bacteria-free, functional, and active polyphenol water-soluble extract of *P. frutescens*. Reverse osmosis (RO) presents potential advantages in comparison to concentration by direct heat evaporation, which is still the most developed and widely used technique for liquid concentration. Apart from its lower energy consumption and capital costs, RO concentrated, in mild conditions and at a relatively high speed, several solutes in water medium if the osmotic pressure of the solutes is not too high. RO is designed when thermal damages are to be minimized and when intrinsic bioactivities have to be kept (*27*−*29*).

The objective of this work was to investigate the new feasibility of obtaining concentrates of *P. frutescens* leaves extracted by a multistep technology, including the following: (i) acidified water extraction of *P. frutescens* dried leaves and stems, (ii) CFM of the *P. frutescens* extract, and (iii) concentration of the CFM permeate by successive RO and rotating evaporation (RE). Membrane performances and chemical characteristics of the various intermediate processed products were studied during the course of the process operating under fixed technological conditions. It is the first time that CFM and RO have been used to extract and concentrate anthocyanin products at pilot-scale to our knowledge.

MATERIALS AND METHODS

Plant Material. *P. frutescens* (L.) Britt. var. *frutescens* was harvested in Guangzhou, China, in June 2005. The plant (leaves and part of stems) was freeze-dried at South China Agricultural University to avoid functional polyphenolics degradation in plant samples. Samples were packed in sealed plastic bags and sent to France by speed post to be processed at CIRAD (Montpellier, France).

Laboratory Scale Polyphenol Extraction. *P. frutescens* powdered samples were extracted with citric-, acetic-, nitric-, sulfuric-, and hydrochloric-acidified water, respectively, at a ratio of 1 g/100 mL for 4 h at room temperature under gentle stirring. Samples were paper filtered before analysis.

Extraction−Concentration Process of *P. frutescens* at Pilot Plant Scale. A multistep process including coupled membrane-based separation technology and RE was used for the first time to extract polyphenolics from the *P. frutescens* plant. During the different steps of the concentration process, using CFM, RO, and RE techniques, samples of *P. frutescens* phenolic extracts were taken and analyzed. The total anthocyanin content was measured by high-performance liquid chromatography (HPLC), as cyanidin equivalents, and OD_{530} readings were directly taken from an aliquot of the product processed during the course of the concentration steps.

First Step: Extraction. On the basis of previous experimental results obtained with other types of plants processed at the pilot plant scale, we tested some material/water (m/v) ratios in the range of 1/20 to 1/200. The best and more practical one that minimized technical problems was found to be 1/100 for *P. frutescens*. The raw material was not converted into powder before water soaking to avoid further membrane processing problems: fine material particles transfer during gross prefiltration, extraction of undesirable macromolecules, and partial clogging of the CFM membrane. Entire pieces of dry leaves (0.6 kg) were therefore soaked overnight with 60 L of deionized water, acidified with nitric acid (0.01 mol/L), at room temperature (15−20 °C), in a large stainless steel container (0.9 m diameter, 1 m height) with very slight stirring at the beginning of the soaking to wet any part of the dry plant. The *P. frutescens* extract was roughly prefiltered, using a nylon cloth filter, to separate the soaked material from a cloudy reddish extract. The red extract recovered (59 L) was used for successive clarification and concentration steps.

Second Step: Separation−Clarification by CFM. The CFM pilot plant used was built on single-stage continuous feed and bleed loop configuration (TIA, Bollène, France). It was equipped with two stainless steel positive pumps. A frequency−speed trimmer controlled the rotating speed of these pumps. One pump was used to continuously feed the filtration loop with the prefiltered *P. frutescens* extract. The other was used to maintain a constant cross-flow velocity of the extract (CFM retentate) through the channels of the membrane during microfiltration. An on-line electromagnetic flow meter (Krohne, France) controlled the retentate velocity (4.5 m/s). The filtration loop was equipped with a membrane cartridge containing the ceramic membrane. The multichannel ceramic membrane used was a P 19-60 (Membralox) industrial type membrane, 800 mm long, 0.2 μm average pore size, with a total filtration surface of 0.304 m^2 (Pall-Exekia, Tarbes, France). A temperature-monitoring device, using two heat exchangers placed on the feeding line and on the filtration loop, was used to control the product temperature during the operation (20 °C). Two pressure sensors along with a pressure relief valve were positioned on the filtration loop to keep constant the transmembrane pressure set at 0.6 bar during the operation. The feed volume (retentate) was then reduced toward the end of the CFM cycle to collect a maximum volume of permeate.

Before performing every trial, the membrane was washed according to an industrial washing procedure using successive cleaning agents sodium hydroxide (10−20 g/L) at 70 °C and nitric acid (5−10 g/L) at 50 °C. Afterward, the permeate flow, measured with deionized water at 25 °C and at dP = 0.6 bar, was about 720 L/h/m^2.

Third Step: Concentration by RO. The RO module was built on the same stainless steel frame as that of the microfiltration module to allow continuous or simultaneous concentration of the CFM permeate obtained. The RO membrane was of an industrial type, SW 30-2540 composite polymeric membrane, packed in a spiral-wound configuration (Filmtec), with 2 m^2 of filtration surface. A three-piston high-pressure pump maintained the operational pressure at 40 bar, using a relief pressure valve. Before performing each RO trial, the membrane was washed according to the washing procedure using successive sodium hydroxide (pH 10) and nitric acid (pH > 4) treatments at room temperature. Afterward, the initial flow of permeate was measured with deionized CFM water at 20 °C (25 L/h/m^2 at $P = 40$ bar).

The CFM permeate obtained (48.5 L) was separated into a RO retentate (*P. frutescens* concentrated extract) and a RO permeate (pure water). The process was kept going at a constant transmembrane pressure of 40 bar until the volume of the RO retentate reached the value of the dead volume of the RO unit (3 L). The RO permeate was collected for RO flow measurement and was recycled for preparing the extraction solution for another *P. frutescens* extraction cycle.

The total volume of RO permeate was also used to follow the volume reduction factor (VRF) at time t (VRF_t) of the concentrated extract, according to the following formulas:

$$VRF_t = V_i/V_c = V_i/(V_i - V_w)$$

where V_i is the initial total volume of CFM permeate processed, V_w is the volume of extracted RO water at time t, and $V_c = V_i - V_w$ is the calculated volume of concentrated extract (RO retentate) at time t.

Rotating Evaporator Pilot Plant Unit. The RO concentrate was then concentrated using RE. A 10 L capacity evaporation flask was placed in a water bath maintained at 60 °C and put under 30 mbar reduced pressure (liquid ring pump attached). The product was concentrated until its VRF_t reached a final value of about 50.

Acidity. The acidity and pH of the *P. frutescens* extracts were measured using a pH meter (Tacussel, France). An aliquot of 20 mL of aqueous extract was titrated with sodium hydroxide 0.1 mol/L.

Color Measurement. The global color of *P. frutescens* extracts obtained at the pilot plant scale was measured as optical density (OD) at 530 nm with a UV-1605 spectrophotometer (Shimadzu, Japan) of the extracts without modifying the solution pH.

Calculation of Monomeric Anthocyanin Content. The extract was paper filtered. Two aliquots were taken as follows: one was set at pH 1.0 using a chloride potassium buffer (0.05 M), and the other was set at pH 4.5 using an acetate sodium buffer (0.8 M). The buffer volumes were generally the same to bring the two aliquots at the corresponding pH. The monomeric anthocyanin pigments content in dry *P. frutescens* material was expressed as shisonin equivalent, according to the formula published in the literature (*30*):

$$\text{anthocyanin (mg/g)} = (OD_{solution} \times MW_{anthocyanin} \times DF \times 1000)/\epsilon_{anthocyanin}$$

where $OD_{solution} = (OD_{\lambda_{max}} - OD_{700nm})_{pH1.0} - (OD_{\lambda_{max}} - OD_{700nm})_{pH4.5}$; $MW_{anthocyanin} = 811$ for shisonin, the second major anthocyanin compound in *P. frutescens* extract; $\epsilon_{anthocyanin} = 30175$ for shisonin (*31*); and DF = dilution factor.

HPLC Analyses. Anthocyanins and flavones were analyzed by HPLC using a diode array detector (DAD; Agilent Technologies, France). The detection was set at 530 nm for anthocyanins and at 325 nm for flavones and other phenolic compounds. The separation column was a 250 mm × 4.6 mm i.d., 5 μm, RP 18 Satisfaction column (Cil Cluzeau, France). The binary solvent system was composed of 10:90 formic acid/water (solvent A) and 10:90 formic acid/acetonitrile (solvent B). The linear solvent gradient started with an initial mobile phase of 94% A and 6% B, to a mobile phase of 76% A and 24% B during 45 min. The washing cycle of the column used a mixture of 70% A and 30% B for 10 min. The flow rate for both analysis and washing cycles was set at 0.8 mL/min.

HPLC-MS Analyses. HPLC-MS was used to confirm the chemical structures and the identities of anthocyanin and flavone molecules considered in this study. The molecules were analyzed by HPLC equipped with a Waters-Alliance 2690 DAD detector equipped with a 250 mm × 2 mm i.d., 5 μm, Merck LiChrospher 100-RP 18 column, coupled with an LCQ-Advantage ion trap mass spectrometer (Thermo Electron S. A., Courtaboeuf, France). The mobile phase consisted of (A) water and formic acid (98:2, v/v) and (B) water, acetonitrile, and formic acid (18:80:2, v/v). The gradient method started at 0.25 mL/min from 94 to 50% (A) over 55 min. The heated capillary and voltage were maintained at 175 °C and 2 kV, respectively. The full-mass scan spectra from *m*/*z* 100 to 1000 were collected. All mass spectrometry data were acquired with a positive ionization mode.

Mineral Analysis. Mineral analyses of elements (Ca, K, Na, etc.) were performed with an inductive coupled plasma (ICP) spectrometer equipped with a coupled charge device detector (Varian Vista). Solid samples were dissolved using 10% aqueous hydrochloric acid and filtered before analysis.

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-300 spectrometer in DMSO-d_6 solutions. ^{13}C resonance multiplicities were established via the acquisition of distortionless enhancement by polarization transfer spectra.

RESULTS AND DISCUSSION

Anthocyanins are generally extracted with slightly acidified medium, either water or alcohol-based solutions (*32*−*34*). Besides anthocyanins, acidic alcohol extracted chlorophyll pigments and other undesirable components. Our *P. frutescens* cultivar from Guangzhou, China, did not show fully red leaves as some endogenous *P. frutescens* cultivars grown in Japan did. When fresh, the leaves of our *P. frutescens* samples showed reddish on the lower side, while the upper side was greenish. Coextracted chlorophyll pigments gave a faded yellow-brown color, which readily turned brown by degradation into pheophitins in the acidic medium.

Table 1. Acid Effect on the Efficiency of *P. frutescens* Anthocyanin Extraction

acid	acid molarity (mol/L)	pH of the extraction solution: before extraction	pH of the extraction solution: after extraction	measured OD_{530}	anthocyanin content[a]
citric	0.05	2.4	2.6	0.796	3.4
	0.1	2.2	2.4	1.000	3.5
	0.2	2.1	2.2	1.528	5.0
acetic	0.175	2.7	3.5	0.515	4.0
	0.7	2.4	3.0	0.771	4.0
	1.4	2.3	2.7	1.072	4.0
sulfuric	0.0125	1.8	2.1	1.254	3.7
	0.025	1.7	1.8	1.574	4.0
	0.05	1.5	1.5	2.069	4.7
nitric	0.01	2.2	3.4	0.549	3.5
	0.02	1.7	2.4	1.132	4.1
	0.05	1.6	1.6	1.796	4.6
hydrochloric	0.01	2.3	3.7	0.522	3.5
	0.02	2.0	2.7	0.861	3.5
	0.05	1.5	1.7	1.283	3.6

[a] Shisonin equivalent, mg/g.

The acidified water solution was less efficient than acidic alcohol solutions to extract anthocyanins, as previously observed (*31*), but acidified water avoided extraction of other undesirable coloring matter, such as chlorophylls. Within the objective of scaling up to a semi-industrial level this innovative process that was applied for the first time to *P. frutescens* plants for the extraction of anthocyanins and other phenolic compounds, it seemed safer to develop this technology on the use of aqueous solutions rather than alcoholic-based ones for the extraction step. Therefore, we did not consider further using acidic alcohol solutions for extraction and decided to use acidified water for pilot plant operations.

Effect of the Nature of Acids Used for Extraction. Both organic and mineral acids were tested for the aqueous extraction step of dried *P. frutescens* (**Table 1**). The monomeric anthocyanin content was calculated with the formulas given above. Direct OD readings at λ_{530nm} (OD_{530}) of samples of *P. frutescens* extracts gave a measure of their red color intensity but not of their true anthocyanin content.

Direct readings of *P. frutescens* extracts showed that the higher the acid concentration used, the higher the OD_{530}, whatever the type of acid used. These readings showed that mineral acids gave redder colored extracts than organic acids, when used at the same acid molarities. This color intensity was the result of two additive effects: pH and extraction efficiency of the acids used. After extraction, the pH of the extract generally increased, as compared to the initial pH of the acidified solution prepared. Some buffering effect, probably due to coextracted components (salts of endogenous organic acids), might be responsible for this pH increase.

Acetic acid seemed to have the optimal anthocyanin extraction power (4 mg/g), whatever its molarity. Citric acid extracted more anthocyanins when its molarity was increased from 0.05 to 0.2 M (3.5−5 mg/g, respectively). The extract with citric acid was redder than the one obtained with acetic acid, because of the lower pH values of the citric acid extract. Generally, organic acids had to be used at higher concentrations (13.5−54 g/L for citric acid) than mineral acids to extract the same amount of anthocyanins. Considering that the extract has to be further concentrated by the process (up to 50 times), concentrated *P.*

frutescens extracts will contain too much citric acid. This may lead to some technical problems because citric acid will reach its maximum solubility in water (60% w/w at 30 °C) before reaching the maximum concentration level of the extract. Acetic acid may produce a strong undesirable off-flavor. Moreover, when mineral acids or high acid molarities were used, this pH did not increase too much. Therefore, extracts obtained with mineral acids appeared redder than those obtained with organic acids, on the same acid concentration basis, because of the lower pH value. Therefore, for these technical and final product quality reasons, use of these organic acids was no longer considered for the multistep concentration process.

Hydrochloric acid extracted the lowest level of the range of anthocyanin content observed for mineral acids. The anthocyanin content of hydrochloric acid extracts was 3.5 mg/g, whatever the acid molarity used, even when this molarity was set at a minimum value (0.01 mol/L) to avoid too much hydrochloric acid in the final concentrated extract. This acid did not seem very efficient in extracting anthocyanins from *P. frutescens*. For technical reasons, hydrochloric acid should be avoided when working with stainless steel machineries. Thus, it was not selected for further trials at the pilot plant scale. Sulfuric acid seemed to be equivalent to nitric acid, in terms of anthocyanin extraction power (3.7−4.7 mg/L), whatever the molarity tested. The highest red intensity was observed with the sulfuric acid, because of the pH of the extract. Nitric acid appeared to be as efficient at the lowest molarity, 0.01 mol/L, as sulfuric acid for anthocyanin extraction (3.5 mg/L). Nitric acid is ordinarily recommended for cleaning stainless steel built-in pilot plant units, even at the industrial scale. Nitric acid at 0.01 mol/L gave a final extraction pH of 3.4, which was a more convenient working condition than with a more acidic medium (pH2.1), given by sulfuric acid at 0.0125 mol/L. Therefore, we decided to use nitric acid for processing trials at the pilot plant scale.

The anthocyanin content calculated for the different acidic extraction conditions (3.4−4.7 mg/g, as shisonin equivalents) confirmed that this *P. frutescens* cultivar from Guangzhou was not as rich as the Japanese cultivar that showed 19.8 mg/g anthocyanin content of dry leaves (*35*).

Identification of Major Anthocyanins and Flavones. HPLC characterizes the different major phenolic compounds extracted from *P. frutescens* dry leaves. This analytical technique was used to check if the proportions of the major anthocyanins and of the major flavones extracted were modified or not by the nature of the acid used.

Identification of extracted *P. frutescens* flavonoids was confirmed by HPLC (retention times, R_t) and HPLC-MS analysis and UV/vis spectra (**Table 2** and **Figure 1**). These identifications were also matched with literature data (*36*). Four anthocyanin structures were not yet determined, but these compounds were used along with the others for process optimization, as they were followed by HPLC-DAD (R_t, UV/vis spectra) in every sample analyzed. The major compounds in our *P. frutescens* samples were selected for this comparative study. For each molecule family, six anthocyanins and six flavones, the total peak area and the percentage of individual peak area were calculated (**Table 3**). In these extraction trials, acid molarities were chosen to have the same pH for the extracting solution (pH 2). If we consider the percentages of the two major components extracted, either of the anthocyanin family or the flavone family, they appeared to be of the same magnitude, 52−58 and 20−23% for malonylshisonin and shisonin, respectively, and 33−35 and 36−40% for luteolin 7-*O*-diglucuronide and apigenin 7-*O*-diglucuronide, respectively. This indicated that the major phenol compounds extracted were not degraded during the extraction step, whatever the nature of the acid used. The major *P. frutescens* anthocyanins were acylated (*37*). Mazza et al. (*34*) reported that extraction with mineral acids degraded acylated anthocyanins. It seemed that in our case, *P. frutescens*

Table 2. Identification of Flavones and Anthocyanins Used for the Optimization of the Process Conditions

chemical names	HPLC R_t (min)	LC-MS *m/z*	HPLC-DAD (nm)
	flavones		
apigenin 7-*O*- caffeoylglucoside	16.8	595	329–(334), 272
scutellarein 7-*O*-diglucuronide	17.9	639, 287	334, 282
luteolin 7-*O*-diglucuronide	19.1	639, 287	348.3, 253
apigenin 7-*O*-diglucuronide	24.2	623, 271	339, 268
luteolin 7-*O*-glucuronide	27.6	463, 287	344
scutellarein 7-*O*-glucuronide	28.7	463, 287	334, 282
	anthocyanins		
malonylshisonin (cyanidin 3-coumaroyl-glucoside-5-malonylglucoside)	41.7	843, 287	526, 320, 281
shisonin (cyanidin 3-coumaroyl-glucoside-5-glucoside)	38.4	757, 287	521, 320, 282
unidentified anthocyanin 1	34.9		525, 320, 282
unidentified anthocyanin 2	33.5		528, 281
unidentified anthocyanin 3	32.4		526, 330, 281
unidentified anthocyanin 4	31.0		521, 280

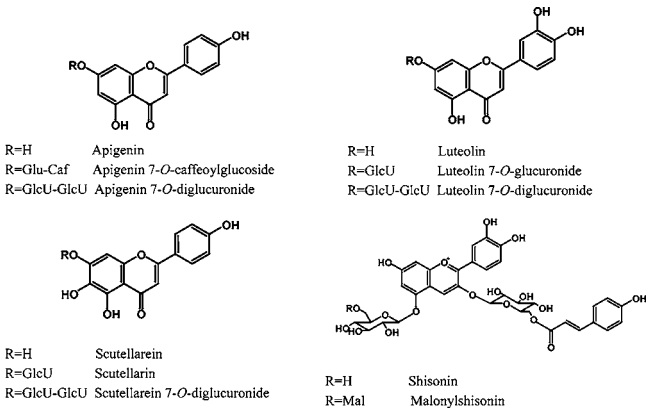

Figure 1. Structures of major flavonoids and anthocyanins in *P. frutescens* (from Guangzhou, China).

Table 3. Effect of the Nature of Acid on the Extraction Efficiency of Flavonoids from *P. frutescens*

major flavonoids in crude *P. frutescens* extracts	acid (in mol/L) citric (0.4)	acetic (1.7)	sulfuric (0.005)	nitric (0.01)
	flavones			
apigenin 7-*O*- caffeoylglucoside	3.4[a]	4.9	6.1	6.8
scutellarein 7-*O*-diglucuronide	9.7	9.6	9.1	10.1
luteolin 7-*O*-diglucuronide	33.0	32.2	32.8	32.6
apigenin 7-*O*-diglucuronide	36.1	36.9	38.5	38.1
luteolin 7-*O*-glucuronide	3.9	7.3	2.8	3.5
scutellarein 7-*O*-glucuronide	13.7	9.1	10.7	8.9
	anthocyanins			
malonylshisonin	52.3	58.0	55.7	58.1
shisonin	23.4	20.3	21.3	20.3
unidentified anthocyanin 1	6.5	6.4	5.7	3.5
unidentified anthocyanin 2	6.3	6.0	6.8	7.4
unidentified anthocyanin 3	3.4	2.9	2.8	1.7
unidentified anthocyanin 4	2.9	2.4	2.8	2.7

[a] HPLC peak area percentage (standard deviation, ±5%).

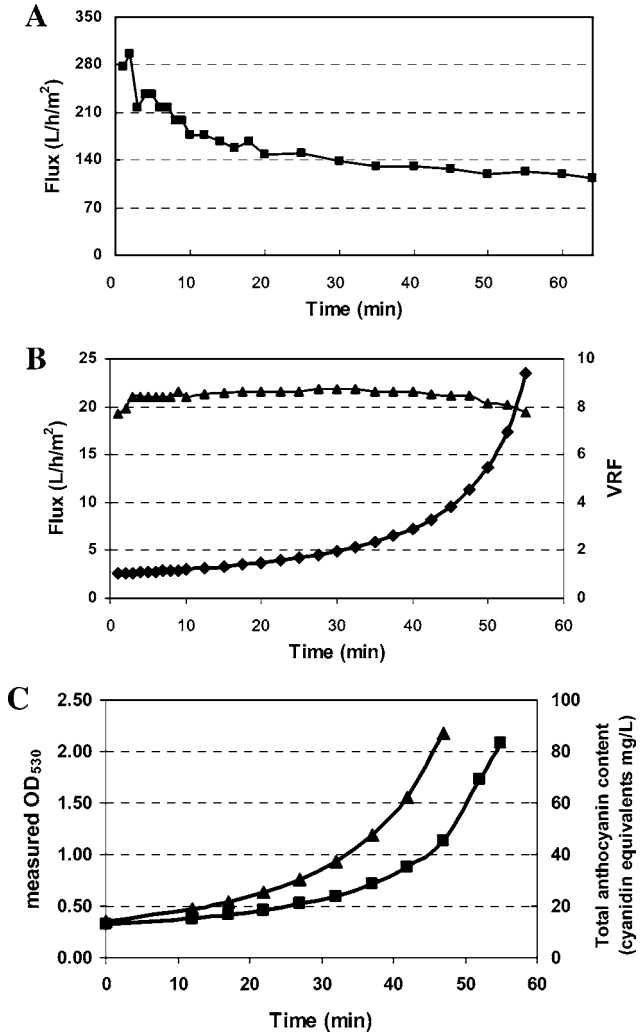

Figure 2. (**A**) Permeate flux of CFM of raw *P. frutescens* extract, (**B**) permeate flux (▲) and volumetric reduction factor (VRF) (◆) of RO concentration of CFM permeate *P. frutescens* extract, and (**C**) evolution with time of total anthocyanin content (◆) and measured OD (▲) of RO concentrates of *P. frutescens* extract.

anthocyanins were resistant within the acidic extraction conditions used. Even nitric acid showed the highest ratio for the two major anthocyanins (malonylshisonin + shisonin = 78.4%). This result is in favor of our choice to use nitric acid as the acidifying agent for the extraction of *P. frutescens* polyphenolics.

Extraction−Concentration Processing of *P. frutescens* Extract at Pilot Plant Scale. Nylon cloth prefiltered *P. frutescens* water extract was microfiltrated. The total volume of 59 L of extract was filtered rapidly within 1 h. We recovered 48.5 L of clear reddish permeate, whereas the remaining extract could not be recovered as it constituted the dead volume of the CFM pilot plant unit. The CFM permeate flux stabilized rapidly after the start of the CFM to an average value of 150 L/h/m^2 at a constant transmembrane pressure of 0.6 bar (**Figure 2A**).

The CFM filtrate was concentrated by RO. The flux of the RO permeate (pure water) showed an immediate stabilization at the value of 22 L/h/m^2 and stayed constant at this level for more than 45 min of operation (**Figure 2B**). In the meantime, the VRF of the recycled RO retentate increased slowly from 1 to 5 during the first 40 min. Then, the RO permeate flux started decreasing slowly, while the VRF increased rapidly, until it reached a value of 9.4 at the end of the operation. The RO permeate flux was still at 19 L/h/m^2 near the end of the concentration step. The RO concentration was then stopped because the 48.5 L of feed CFM *P. frutescens* extract was already reduced to nearly the dead volume of the RO pilot plant unit, under which we could not go down. A volume of 5.2 L of RO concentrated extract was collected within 1 h of time operation.

At a higher scale using a larger volume of CFM extract, the reachable VRF could be higher than the one obtained in this work. It seemed that the *P. frutescens* extract did not contain molecules with intrinsically high osmotic pressure, such as sugars. No RO flux limitation, at constant transmembrane pressure, due to increasing osmotic pressure with VRF, seemed to occur in this case. The more feed volume, the higher VRF, because more water could be eliminated as retentate with a RO flux kept at the same high level. Moreover, previous CFM had well-cleaned the extract, avoiding any noticeable RO membrane clogging at this step, helping to maintain the RO flux at a constant level.

The recovered RO concentrate (5.2 L) was submitted to RE until its volume was reduced to 0.96 L. The duration of this operation was about 4 h to remove 4.24 L of water from the RO concentrated extract. The VRF for this operation step was 5.4, raising the global VRF of the whole process up to 50.7.

Analysis of *P. frutescens* Extracts Obtained during the Multistep Process. The total flavone and anthocyanin contents in *P. frutescens* extract were used to optimize the process **(Table 4)**. Evolutions of measured OD_{530} and of total anthocyanin content of the RO retentate showed similar behavior (**Figure 2C**). The curves had both exponential type shapes. OD_{530} readings increased more rapidly than the total anthocyanin content during the RO concentration step. This might be the effect of an extra contribution to the global OD of nonanthocyanin type molecules extracted from *P. frutescens*, giving an absorption background, even at 530 nm. These molecules were also concentrated in the course of the RO concentration step. At $VRF_t = 5.5$ ($t = 50$ min), this absorption contribution represented almost one-half of the OD_{530} of the concentrated extract.

During the RO concentration step, the CFM *P. frutescens* extract was concentrated 9.4 times, as shown by VRF values (**Table 4**). Total anthocyanins and total flavones were concentrated about the same factor (7.6 and 7 times, respectively), calculated from total anthocyanin and flavone HPLC peak area. This indicated that some slight degradation of flavonoids probably occurred during the RO step. In the same time, titrable acidity increased only 6.2 times (from 7.4 to 46.2 mequiv/L) for this step. Concentrations of solutes anthocyanin, flavone, and titrable acidity thus showed less increases than VRF did.

Considering titrable acidity and corresponding pH values of each product obtained at this step, observed pH values seemed not to correspond to the total acidity measured, if we consider that this acidity has to be exclusively attributed to nitric acid, added at the extraction step. Crude *P. frutescens* extract showed a titrable acidity of 7.4 mequiv/L. If nitric acid was the only one present in the extract, this corresponds to 0.007 mol/L nitric acid, which would have given a theoretical pH of 2.15. In fact, the measured pH was different (pH 3.0). The rise of titrable acidity of RO concentrates collected, during RO concentration, did not follow strictly the rise of VRF values calculated for each sample (**Figure 3**). The difference of these two slopes increased regularly within the advance of the concentration process. The RO filtrate did not show any titrable acidity.

During the course of the second concentration step, using a rotating evaporator, data obtained (**Table 4**) showed that the

Table 4. Characterization of Products Obtained along the Concentration Process Steps of *P. frutescens* Extract

product obtained at each process step	volume of product recovered (L)	VRF	pH	acidity (mequiv/L)	relative flavonoid content[a]	
					anthocyanins	flavones
crude *P. frutescens* water extract	59		3.1	7.4	1.00	1.00
CFM permeate	48.5	1	3.1	7.4	1.04	1.00
RO concentrate	5.2	9.4	3.0	46.2	7.64	6.97
CFM + RO + RE concentrate	0.96	50.7	2.7	211	39.30	36.94

[a] Total HPLC peak area of sample/total HPLC peak area of crude extract (standard deviation, ±5%).

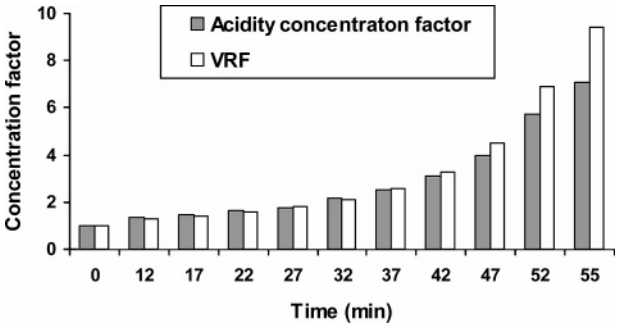

Figure 3. Titrable acidity and VRF of *P. frutescens* extract during RO concentration.

VRF obtained was 50.7. This concentration step led to an extra concentration of 5.4 times that took nearly 4 h of evaporation time in the experimental conditions used. For the final concentrated extract, the rise in concentration was 5.1 times for anthocyanins and 5.3 for flavones, while VRF was 5.4 (9.4−50.7) for this step. If the slight differences observed between of these concentration factors might be attributed to experimental uncertainty, it could be concluded that not many anthocyanins and flavones degraded during this second concentration step using RE. Titrable acidity showed a 4.6 times rise, from 46.2 to 211 mequiv/L, which was about the same increase as for VRF (5.4). However, the final pH did not change so much, from pH 3.1 to 2.7 (about 10% decrease), as compared to VRF, from 1 to 50.7 (about 500% increase).

If we compared the variation of the total acidity that occurred within the whole concentration step, including RO and RE, the total amount of nitric acid of the CFM permeate submitted to RO accounted for 358.9 mequiv (48.5 mequiv/L × 7.4 L) and the residual titrable acidity found in the RE concentrate was only 202.6 mequiv (211 mequiv/L × 0.96 L). This indicated that 156.3 mequiv of free acidity, brought by the added nitric acid for *P. frutescens* extraction, was not titrable because it probably combined into salt formation, buffering the final concentrate to the acceptable pH of 2.7 found. These results lead us to conclude that the extracted medium was strongly buffered by some endogenous organic acids and salts extracted from the *P. frutescens* plant.

The final *P. frutescens* concentrate contained 422 mg/L of total anthocyanins (as cyanidin equivalents), measured by HPLC-DAD. The two major anthocyanins found in this concentrated extract were cyanidin malonylshisonin and shisonin, and the two major flavones were apigenin-7-*O*-diglucuronide and luteolin-7-*O*-diglucuronide. The balances of both anthocyanins and flavones in the concentrate were the same as the one found in the crude acidified water extract of *P. frutescens*.

The concentration was in fact stopped at this level (VRF = 50.7) because some precipitate appeared at this concentration level. After settling overnight, the precipitate was separated from the deep red concentrated *P. frutescens* extract. It was washed with deionized water, and after it dried, the precipitate showed white crystals. This precipitate was also formed for a high-level concentration of *P. frutescens* extracts obtained by direct RE of the crude extract, during trials conducted at the lab scale. This precipitate appeared more or less with other organic or mineral acids used for acidifying water at the extraction step.

Identification of the Crystallized Precipitate. The precipitate was submitted to microanalysis and NMR analysis. For the lab scale trials, conducted with acidified tap water relatively loaded in Ca^{2+} ions (water hardness = 128 mg/L), the ICP spectrophotometer revealed that the major mineral element found was Ca (15.8% w/w). The other major elements were K (0.65%), P (0.051%), and Mg (0.061%), while the minor elements showed the following contents: Na (739 ppm), Zn (92.8 ppm), Cu (19.2 ppm), Al (10.7 ppm), Fe, and Mn (<10 ppm). For the deposit collected from multistep concentration process conducted at the pilot plant scale, the results were as follows: K (16.67%), P (0.017%), Ca (1.27%), Mg (traces), Na (52 ppm), and Al (28 ppm). The Ca content was in this case lower than in the previous extraction trial made with tap water. During the course of tap water extraction, water Ca^{2+} ions have replaced K^+ ions in the extracted endogenous salts from *P. frutescens* that seemed to contain a very few amount of Ca^{2+} ions.

The ^{1}H NMR analysis in DMSO-d_6 + CF_3COOD at 300 MHz showed only two singlet signals at 8.7 ppm for −O*H* and 4.3 ppm for −C*H*. The ^{13}C NMR showed typical signals corresponding to the functional groups −*C*OO$^-$ (173.7 ppm) and >*C*HOH (72.6 ppm), which were characteristic signals for tartaric acid in agreement with the fact that crystals (Ca tartrate) were not soluble in either pure water or organic solvents such as methanol, $CHCl_3$, or DMSO, showing therefore an ionic origin only soluble in concentrated acids. Such a structure was in good agreement with the results of the elemental analysis that showed that tap water-extracted crystals contained 18.9% C, 4.7% H, and 15.4% Ca. Because of the absence of Ca^{2+} ions when using deionized water for *P. frutescens* extraction, only K^+ ions were found, accounting for the same mass percentage in the composition of potassium tartarate molecule. Further analysis confirmed the structure of this molecule in the deposit. The optical rotation was found to be +20°, in agreement with the optical rotation of L-(+)-Ca tartrate.

The results of this investigation indicated that this membrane technology is a good alternative for extraction−concentration of polyphenolics from plants containing functional, active, but sensitive molecules such as anthocyanins and flavones. The concentrated extract was purified and contained only soluble compounds. The coupled membrane technology avoided pollution of the extract by other undesirable macromolecules, cell walls, or endogenous flora, leading to a relative pure concentrate of polyphenolics. This anthocyanin and flavonoid extract can be used as a natural coloring ingredient with active properties; among them, the antioxidant property is a well-known intrinsic characteristic of these molecules. Moreover, the multistep process lead to a high level of concentrated *P. frutescens* extract,

minimizing time and energy consumption, as compared to the traditional single evaporation process. It offers the opportunity to operate in low-demanding technical environment, such as in developing countries where such herbs are grown and used in traditional ways by local people.

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