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Summary

The aim of this study was to optimize the aqueous extraction conditions for the recovery of phenolic compounds and antioxidant capacity of lemon pomace using response surface methodology. An experiment based on Box–Behnken design was conducted to analyse the effects of temperature, time and sample-to-water ratio on the extraction of total phenolic

compounds, total flavonoids, proanthocyanidins and antioxidant capacity. Sample-to-solvent ratio had a negative effect on all the dependent variables, while extraction temperature and time had a positive effect only on TPC yields and ABTS antioxidant capacity. The optimal extraction conditions were 95 ^{o}C, 15 min, and a sample-to-solvent ratio of 1:100 g/ml. Under these conditions, the aqueous extracts had the same content of TPC and TF as well as antioxidant capacity in comparison with those of methanol extracts obtained by sonication. Therefore these conditions could be applied for further extraction and isolation of phenolic compounds from lemon pomace.

Keywords Antioxidants, phenols, flavonoids, Response Surface Methodology (RSM)

Introduction

Lemon (*Citrus limon* L.) is considered as the third most important citrus species after orange and mandarin, with a strong commercial value, generating a large amount of waste. Lemon peel represents the main component of waste, accounts for 50 to 65% of the whole fruit weight (González-Molina *et al.*, 2010). Lemon peel contains bioactive compounds, such as vitamin C (ascorbic acid), flavonoids (flavanones, flavonols, flavones) and phenolic acids (ferulic, p-coumaric and sinapic acids) (Bocco *et al.*, 1998; González-Molina *et al.*, 2010), which have been linked to antimicrobial (Dhanavade *et al.*, 2011), anticancer (Sun *et al.*, 2002) and antioxidant activities (Wilmsen *et al.*, 2005; Proteggente *et al.*, 2002).

Extraction of bioactive compounds and antioxidants has been achieved by both conventional and non-conventional methods (Khoddami *et al.*, 2013). Several parameters affect the extraction efficiency of bioactive compounds from plant tissues, including extraction time, temperature, sample-to-solvent ratio, and solvent character (Sarwar *et al.*, 2012; Khoddami *et al.*, 2013; Vuong *et al.*, 2013; Candrawinata *et al.*, 2015).

The solubility of bioactive compounds typically increases as a function of longer extraction times and higher temperatures, resulting in higher extraction yields (Davidov-Pardo *et al.*, 2011). However, extending extraction times and temperatures promote the degradation of the bioactive compounds (Joana Gil-Chávez *et al.*, 2013; Vuong *et al.*, 2013). Several solvents have been being used for the extraction of bioactive compounds from citrus; methanol being the most commonly employed solvent (Abad-García *et al.*, 2007). Preliminary work in our lab showed that among different solvents methanol was the most efficient for the extraction of phenolic compounds and antioxidants from lemon peels under ultrasound extraction (Papoutsis *et al.*, under review). Methanol is however classified as a class 2 solvent, which means its use should be limited in pharmaceutical products due to its inherent toxicity (FDA, 2012). Investigations to assess the effectiveness of alternative, less toxic solvents to maximise the recovery of the bioactive compounds from the lemon peel are therefore required. Water could be an effective solvent for the recovery of phenolic compounds and antioxidants (Xu *et al.*, 2008).

Several studies have shown that the extraction yields of phenolic compound from plant sources increase with decreasing sample-to-solvent ratio (Tan *et al.*, 2011; Dailey and Vuong, 2015). However, from an economical standpoint optimisation must also take into consideration solvent and energy costs (Al-Farsi and Chang, 2008; Santana *et al.*, 2009). Citrus waste which is a by-product generated by the citrus juice industry, contains phenolic compounds which present antioxidant activities and could be validated by both pharmaceutical or food industries. Organic solvents are effectively used for the extraction of these compounds, however their use should be limited due to their toxicity and the risk of chemical residues. Water is a safe, cheap and accessible solvent. Therefore, it is necessary to optimize the extraction conditions for the maximum recovery of the bioactive compounds from the lemon pomace waste, in order to minimize the production cost and obtain the safest

extract for further utilization. To the best of our knowledge, no studies have yet been undertaken to optimise the aqueous extraction of phelolics from lemon peel. The aim of this study was to use response surface methodology (RSM) to determine the optimal conditions for the aqueous extraction of phenolic compounds and antioxidants from lemon peel.

Materials and methods

Chemicals and reagents

All chemicals used in this study were analytical grade. Methanol, ethanol, vanillin, and potassium persulfate were purchased from Merck (Germany). Folin–Ciocalteu phenol regent, anhydrous sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), hydrochloric acid (HCl), ferric chloride ($FeCl_3$), gallic acid, catechin, copper (II) chloride ($CuCl_2$), ammonium acetate (NH_4Ac), neocuproine, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (New South Wales, Australia). Sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) was purchased from Government Stores Department (Australia). Aluminium chloride ($Al_2Cl_3 \cdot 6H_2O$) was obtained from J. T. Baker Chem. Co. (Belgium). Acetic acid was obtained from BDH Laboratory Supplies (Poole, England). Sodium hydroxide (NaOH) was purchased from Ajax Chem. (Australia).

Materials

Lemon (*Citrus limon* L.) waste including peel and seeds (flavedo and albedo) was obtained from a commercial juicing factory at Kulnura, NSW Australia. After collection, the seeds were removed and the remaining peel and pomace flesh were stored immediately at -18 °C to minimize the oxidation of the bioactive compounds. The frozen lemon waste was immersed

in liquid nitrogen and freeze dried (FD3 freeze dyer, Thomas Australia Pty. Ltd., Seven Hills, NSW, Australia). The dried waste was ground using a commercial blender (John Morris Scientific, Chatswood, NSW, Australia) and sieved using a steel mesh sieve (1.4 mm EFL 2000; Endecotts Ltd., London, England). The ground lemon waste was kept in a sealed container at -18 °C for further analysis.

Water Extraction process

Hot water extraction of bioactive compounds was carried out by using water bath (Ratek Instruments Pty. Ltd., Victoria, Australia). Specifically, a required amount of lemon dry powder was mixed with distilled water according to the experimental design shown in Table 1. During the extraction the rest tubes were wrapped with parafilm to minimize the evaporation. After extraction was completed, the extracts were maintained in ice, and then centrifuged at 3500 x g for 10 min at 14 °C. The samples were then stored in the dark at -18 °C until used for quantitative analysis and antioxidant determination.

Ultrasound extraction

Ultrasound-assisted extraction was applied with methanol as a solvent at a sample-to-solvent ratio of 1:100 g/mL of dried sample. The extraction process was conducted in an ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd., Thebarton, Australia) with pre-set conditions at temperature of 30 °C, time of 20 min and power of 60W. After completion of the extraction, the extracts were immediately cooled on ice to room temperature, and then centrifuged at 3500 x g for 10 min at 14 °C. The sample supernatant was then collected at storage conditions.

Experimental design

Response Surface Methodology (RSM) of JMP (Version 11) was applied to design and optimise the conditions for the aqueous extraction of bioactive compounds and antioxidant capacity from lemon pomace. Three parameters including temperature (X_1: 75-95 ^oC), time (X_2: 5-15 min) and sample-to-solvent ratio (X_3: 1-10 g / 100 mL) were tested. The optimal ranges of these parameters were determined in preliminary experiments. A three-factor and a three-level Box–Behnken design consisting of fifteen experimental runs was employed including three central points replicated in the design of the experimental conditions, since Box–Behnken design is considered as an efficient design for response surface methodology (Ferreira *et al.*, 2007).

A second-order polynomial equation was used to express the amount of total phenolic compounds, total flavonoids, proanthocyanidins, and antioxidant capacity. The generalised second-order polynomial model used in the response surface analysis is shown in Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{\substack{i=1 \\ i<j}}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 \qquad \text{Equation (1)}$$

Y is the predicted response, b_0, b_i, b_{ii} and b_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables (Vuong *et al.*, 2011).

For the validation of the model, the optimised conditions of the independent variables were further applied in triplicate, using the same experimental procedure as mentioned previously.

Determination of chemical properties

Total phenolic content (TPC): TPC was determined as described by Vuong *et al.* (2013). Briefly, 5 mL of 10% (v/v) Folin-Ciocalteu reagent was added to 1 mL of sample, followed by the addition of 4 mL of 7.5% (w/v) Na_2CO_3, then mixed well on a vortex for 2 min and incubated in the dark at room temperature for 1 h prior to measurement of solution

absorbance (λ= 760 nm) by UV spectrophotometer (Varian Australia Pty. Ltd., Victoria, Australia). Gallic acid was used as the standard for a calibration curve, with the results expressed as mg of gallic acid equivalents per g of sample dry weight (mg GAE/g dw).

Total flavonoid content (TFC): Total flavonoid content was measured as described by Zhishen *et al.* (1999). Briefly 2 mL of H_2O and 0.15 mL of 5% (w/v) $NaNO_2$ were added to 0.5 mL of sample and left at room temperature for 6 min. Then 0.15 mL of 10% (w/v) $AlCl_3$ was added and left at room temperature for 6 min. Subsequently, 2 mL of 4% (w/v) NaOH and 0.7 mL of H_2O were added and the final volume of the mixture left at room temperature for a further 15 min before the absorbance was measured (λ= 510 nm) using a UV spectrophotometer. Catechin was used as the standard for a calibration curve and the results were expressed as mg of catechin equivalents per g of sample dry weight (mg CE/g dw).

Proanthocyanidins: Proanthocyanidins were determined according to a method described by Li *et al.* (2006). Vanillin (3 mL, of 4% w/v) was added to 0.5 mL of sample. Subsequently, 1.5 mL of concentrated HCl was added to the mixture and left for 15 min at room temperature before the absorbance was measured at 500 nm using a UV spectrophotometer. Catechin was used as the standard for a calibration curve and the results were expressed as milligrams of catechin equivalents per g of sample dry weight (mg CE/g dw).

Methods for the determination of antioxidant properties

DPPH assay: DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used for the determination of the antioxidant activity as described by Thaipong *et al.* (2006), with some modifications. A stock solution was prepared by dissolving 24 mg DPPH in 100 mL methanol and then stored at −20 °C until required. The working solution was then prepared fresh by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 ± 0.02 (λ= 515 nm). Subsequently, 2.85 mL of working solution was added to 0.15 mL of sample and left under

darkness at room temperature for 30 min before measuring the absorbance (λ= 515 nm) using a UV spectrophotometer. Trolox was used as the standard for a calibration curve and the results were expressed as mg of trolox equivalents per g of sample dry weight (mg TE/g dw).

ABTS assay: ABTS (2,2′- azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) assay was used for the determination of the antioxidant activity as described by Thaipong *et al.* (2006) with some modifications. A stock solution was prepared by adding 10 mL of 7.4 mM ABTS solution to 10 mL of 2.6 mM K_2S_2O8 and left at room temperature in the dark for 15 h, and then stored at -20 ^oC until required. The working solution was freshly prepared by diluting 1 mL of stock solution with 60 mL of methanol to obtain an absorbance value of 1.1 ± 0.02 (λ= 734 nm). Subsequently, 2.85 mL of the working solution was then added to 0.15 mL of sample and left in the dark at room temperature for 2 h before its absorbance was recorded (λ= 734 nm) using a UV spectrophotometer. Trolox was used as a standard and the results were expressed as mg trolox equivalents per g of sample dry weight (mg TE/g dw).

FRAP assay: FRAP (Ferric reducing antioxidant power) was measured as described by Thaipong *et al.* (2006) with some modifications. A working FRAP solution was prepared by mixing 300 mM acetate buffer, 10 mM Tripyridil-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$ in the ratio of 10:1:1 and warmed to 37 ^oC in a water bath (Ratek Instruments Pty. Ltd., Victoria, Australia). Working FRAP solution (2.85 mL) was added to 0.15 mL of sample and incubated at room temperature in the dark for 30 min before its absorbance was read (λ= 593 nm) using a UV spectrophotometer. Trolox was used as a standard and the results were expressed as mg trolox equivalents per g of sample dry weight (mg TE/g dw).

CUPRAC assay: CUPRAC (cupric reducing antioxidant capacity) was determined as described by Apak *et al.* (2004) with some modifications. $CuCl_2$ (1 mL) were mixed with 1 mL of neocuproine and 1 mL of NH_4Ac. Subsequently 1.1 mL of sample were added to this mixture. The mixture was incubated at room temperature for 1.5 h before measuring the

absorbance (λ= 450 nm) using a UV spectrophotometer. Trolox was used as the standard for a calibration curve and the results were expressed as mg of trolox equivalents per g of sample dry weight (mg TE/g dw).

Statistical analysis

The Pearson correlation test was used to evaluate the correlation between the bioactive compounds and different antioxidant assays. The correlation analysis was performed using SPSS statistical software (version 23), with a *p*-value < 0.05 considered significant. The predicted and observed values were compared with the least significant difference at $p < 0.05$ and expressed as means ± standard deviations. For the comparison of hot water with methanol extracts, a one-way ANOVA and the Least Significance Difference (LSD) at $p < 0.05$ were conducted using SPSS statistical software (version 23). All the experiments were conducted in triplicate.

Results and discussion

Fitting of the models and validation

Fitting of model for the TPC, TF, and proanthocyanidins, was analysed, the results displayed in Table 2 and Figure 1. The RSM application of TPC values showed that the model was significant ($p < 0.003$), did not present lack of fit ($p = 0.066$), the adjusted $R^2 = 0.90$ and could explain 97% of all variance in the data.

The RSM application of TF values showed that the model was significant ($p < 0.026$), presented lack of fit ($p = 0.004$), the adjusted $R^2 = 0.78$ and could explain 92% of all variance in data. For the proanthocyanidins values the RSM application showed that model was not significant ($p < 0.11$), presented lack of fit ($p = 0.001$), the adjusted $R^2 = 0.58$ and could

explain 85% of all variance in data. That means that the model is not reliable for the prediction of proanthocyanidin yields.

Fitting of model for the antioxidant capacity measured by DPPH, ABTS, FRAP, and CUPRAC was analysed and the results can be seen in Table 2 and Figure 2. The RSM application of DPPH showed that the model was significant ($p < 0.022$), presented lack of fit ($p = 0.002$), the adjusted $R^2 = 0.80$ and could explain 93% of all variance in data. For the ABTS the RSM application showed that the model was significant ($p < 0.002$), did not present lack of fit ($p = 0.065$), the adjusted $R^2 = 0.93$ and could explain 98% of all variance in data. For the FRAP assay, the model was significant ($p < 0.036$), presented lack of fit ($p = 0.001$), the adjusted $R^2 = 0.75$ and could explain 91% of all variance in data. Finally, for CUPRAC assay the model was significant ($p < 0.0001$), did not present lack of fit ($p = 0.129$), the adjusted $R^2 = 0.98$ and could explain 99% of all variance in data. In general, analysis of results revealed that the mathematical models are reliable for the prediction of bioactive compounds and antioxidant properties and are expressed and fitted in the following equations.

$$Y_{TPC} = 5.27333 + 0.077875 \times (X_1-85) + 0.1455 \times (X_2-10) - 0.6222 \times (X_3-5.5) - 0.00035 \times (X_1 \times X_2-10 \times X_1-85 \times X_2+850) - 0.02944 \times (X_1 \times X_3-5.5 \times X_1-85 \times X_3+467.5) + 0.03055 \times (X_2 \times X_3-5.5 \times X_2-10 \times X_3+55) + 0.0055333 \times (X_1-85)^2 + 0.0636 \times (X_2-10)^2 + 0.094485 \times (X_3-5.5)^2$$

Equation (2)

$$Y_{TF} = 1.75 + 0.01025 \times (X_1-85) - 0.01375 \times (X_2-10) - 0.24861 \times (X_3-5.5) - 0.0047 \times (X_1 \times X_2-10 \times X_1-85 \times X_2+850) - 0.0017 \times (X_1 \times X_3-5.5 \times X_1-85 \times X3+467.5) - 0.0057 \times (X_2 \times X_3-5.5 \times X_2-10 \times X_3+55) - 0.0031875 \times (X_1-85)^2 + 0.00315 \times (X_2-10)^2 + 0.03648 \times (X_3-5.5)^2$$ (Equation 3)

Y_{DPPH} = 0.078033 + 0.0007625 × (X_1-85) - 0.000815 × (X_2-10) - 0.0088833 × (X_3-5.5) - 0.0000335 × (X_1×X_2-10×X_1-85×X_2+850) - 0.00014 × (X_1×X_3-5.5×X_1-85×X_3+467.5) + 0.00068 × (X_2×X_3-5.5×X_2-10×X_3+55) + 0.000153 × (X_1-85)^2 - 0.000156 × (X_2-10)^2 + 0.00074 × (X_3-5.5)^2 (Equation 4)

Y_{ABTS} = 0.086367 + 0.0035975 × (X_1-85) + 0.006585 × (X_2-10) - 0.0148 × (X_3-5.5) + 0.000326 × (X_1×X_2-10×X_1-85×X_2+850) - 0.0011 × (X_1×X_3-5.5×X_1-85×X_3+467.5) - 0.001462 × (X_2×X_3-5.5×X_2-10×X_3+55) + 0.000192 × (X_1-85)^2 + 0.001036 × (X_2-10)^2 + 0.0022 × (X_3-5.5)^2 (Equation 5)

Y_{FRAP} = 4.3329 + 0.064045 × (X_1-85) + 0.0025275 × (X_2-10) - 0.413808 × (X_3-5.5) - 0.0058105 × (X_1×X_2-10×X_1-85×X_2+850) - 0.019195 × (X_1×X_3-5.5×X_1-85×X_3+467.5) + 0.007098 × (X_2×X_3-5.5×X_2-10×X_3+55) + 0.003956 × (X_1-85)^2 - 0.034303 × (X_2-10)^2 + 0.015504 × (X_3-5.5)^2 (Equation 6)

Y_{CUPRAC} = 14.50793 + 0.2775875 × (X_1-85) + 0.1329075 × (X_2-10) - 2.0336194 × (X_3-5.5) - 0.033289 × (X_1×X_2-10×X_1-85×X_2+850) - 0.0617511 × (X_1×X_3-5.5×X_1-85×X_3+467.5) - 0.0647056 × (X_2×X_3-5.5×X_2-10×X_3+55) + 0.01823845 × (X_1-85)^2 + 0.1032048 × (X_2-10)^2 + 0.1673763372 × (X_3-5.5)^2 (Equation 7)

Effect of Extraction parameters on Total Phenolic Compounds (TPC), Total Flavonoids (TF), and Proanthocyanidins

The effect of the extraction variables on TPC, TF, and proanthocyanidins can been seen in Table 3. Extraction temperature, and time had a positive significant linear effect on TPC, while sample-to-solvent ratio had a negative significant linear effect on the TPC, TF, and

proanthocyanidins. Ratio had a positive significant quadratic effect on TPC and TF, while extraction time had a positive significant quadratic effect only on TPC. A significant interaction effect between extraction temperature and ratio on TPC was observed. The impact of temperature (75–95 °C), time (5–15 min), and sample-to-solvent ratio (1–10 g / 100 mL) on TPC can be seen on Figure 3. The extraction yields of TPC, TF, and proanthocyanidins varied from 3.77 to 12.41 mg GAE/g dw, 0.81 to 4.07 mg CE/g dw, and 0.57 to 1.86 mg CE/g dw, respectively. Overall, an increase in sample-to-solvent ratio tends to decrease the extraction yields of TPC, TF, and proanthocyanidins, while an increase of extraction temperature and time tends to increase the extraction yields of TPC. These results are in agreement with Xu *et al.* (2008) who have reported that hot water led to increased extraction yields of phenolic compounds in two citrus varieties and Hayat *et al.* (2009) who mentioned that the extraction yield of phenolic acids in citrus mandarin increased when the liquid-to-solid ratio increased. The high temperature may affect positively the extraction yields i) by enhancing the solubility of phenolic compounds and the mass transfer rate (Dai and Mumper, 2010), as well as ii) by weakening the plant tissues (Al-Farsi and Chang, 2008), while an decrease sample-to-solvent may promote the extraction of bioactive compounds from plant materials, since a larger solvent volume can generally dissolve the bioactive ingredients contained in the sample more effectively (Zhang *et al.*, 2011).

Effect of Extraction parameters on antioxidant capacity measured by DPPH, ABTS, FRAP, and CUPRAC

The antioxidant activity of lemon pomace extracts was determined using four antioxidant assays including DPPH, ABTS, FRAP and CUPRAC, since each antioxidant assay has its own advantages and limitations (Vuong *et al.*, 2015).

The effect of extraction variables on antioxidant capacity of lemon pomace extracts can be seen in Table 4. Sample-to-solvent ratio had a significant negative linear effect on the antioxidants measured by all assays ($p < 0.001$). These results are in accord with Candrawinata *et al.* (2015) who mentioned that an increase in apple pomace-to-water ratio led to a decrease in the antioxidant activity of the extracts. Extraction temperature had a linear effect on the antioxidants measured by ABTS and CUPRAC (positive effect, $p < 0.01$), while time had a positive linear effect only on the antioxidants measured by ABTS at a level of significance of $p < 0.01$. Both extraction temperature and time had no effect on the antioxidants measured by DPPH and FRAP which is in accord with Xu *et al.* (2008) who mentioned that extraction temperature and time had very little impact on the antioxidant capacity of hot water citrus peel extracts. A positive quadratic effect of the extraction time and ratio on the antioxidants measured by CUPRAC ($p < 0.01$) and ABTS ($p < 0.05$ and $p < 0.01$, respectively) was observed. The interaction between temperature and ratio had a negative effect on both ABTS and CUPRAC values ($p < 0.01$ and $p < 0.05$, respectively), while the interaction between time and ratio had a negative effect only on the antioxidants measured by ABTS assay at a level of significance of $p < 0.05$. The antioxidant activity varied from 0.1697 to 1.3806 mg TE/g dw (DPPH), 0.3014 to 2.0067 mg TE/g dw (ABTS), 2.1243 to 9.2256 mg TE/g dw (FRAP), and 10.1438 to 34.8561 mg TE/g dw (CUPRAC). Overall, an increase in sample-to-solvent ratio tends to decrease the antioxidant activity of the extracts, which is consistent with the mass transfer principle where the driving force for mass transfer is considered to be the concentration gradient between the solid and the solvent (Al-Farsi and Chang, 2008; Tan *et al.*, 2011).

Correlation between TPC, TF, proanthocyanidins and antioxidant capacity.

The contribution of TPC, TF, and proanthocyanidins to the total antioxidant activity can be evaluated by the correlation between them and the antioxidant assays were used (Candrawinata *et al.*, 2015). The *r* values between TPC and DPPH, ABTS, FRAP, and CUPRAC were 0.75, 0.86, 0.70, and 0.92 (p<0.01), respectively, revealing that TPC was a major contributor to the antioxidant properties of the lemon pomace extracts. Similarly, the *r* values between TF and DPPH, ABTS, FRAP, and CUPRAC were 0.76, 0.66, 0.67 and 0.86 (p<0.01), respectively, indicating that TF contribute to the antioxidant activity of lemon pomace, as well. The *r* values between proanthocyanidins and DPPH, ABTS, FRAP, and CUPRAC were 0.75, 0.52, 0.51 and 0.61 (p<0.01), respectively, indicating that proanthocyanidins have a lower contribution to the antioxidant activity of lemon pomace compared to the TPC and TF (Table 5). These results are supported by previous studies which indicated that TPC had close correlation with antioxidant character and was the major contributor to the antioxidant properties of citrus extracts, because of their potential electron donor capacity, due to the usual presence of multiple hydroxyl groups (Lagha-Benamrouche and Madani, 2013; Shofinita *et al.*, 2015). In contrast, Ghasemi *et al.* (2009) reported no correlation between phenolic compounds or flavonoids and antioxidant activity of citrus peels. This difference could be attributed to the different extraction method and solvent used by this study.

Optimisation of aqueous extraction conditions for bioactive compounds and antioxidant properties from lemon pomace.

Based on the RSM predictive models and expected values, the optimal conditions for the total phenolic compounds, flavonoids, proanthocyanidins, and all four different antioxidant assays were selected at temperature = 95 °C, time = 15 min, and sample-to-solvent ratio = 1:100 g / mL. To validate the model, three individual experiments were performed under the optimal

conditions. The results presented in Table 6, show no significant difference between the predicted and observed values, therefore, these conditions were recommended for optimal extraction of lemon pomace for analysis of bioactive compounds and antioxidant properties.

Comparison with methanol extracts obtained by ultrasound assisted extraction

The TPC, TF, proanthocyanidins, DPPH, ABTS, and CUPRAC values obtained by the optimal conditions from the hot water extraction were compared with methanol extract values. The same lemon pomace was used for both hot water and methanol extractions. Analysis of variance showed that there was no significant difference between the values obtained by hot water and methanol for the TPC, TF, and the antioxidant capacity determined by DPPH and ABTS at $p < 0.05$. However, methanol extracts contained 48.27% more proanthocyanidins than water extracts and CUPRAC values were 39.82% greater for the methanol compared to the hot water extracts (Table 7). Dahmoune *et al.* (2013) reported that the optimal microwave extraction conditions using ethanol as a solvent in lemon pomace resulted in TPC yields of 15.78 ± 0.80 mg GAE/g which are close to the yields obtained at the optimal conditions by hot water in this experiment. The efficiency of hot water could be attributed to the high temperature enhancing the solubility of bioactive compounds leading in higher extraction yields (Davidov-Pardo *et al.*, 2011). These results are in agreement with Xu *et al.* (2008) who supported that hot water extracts from citrus peels had the same antioxidant capacity with methanol extracts.

Conclusion

Box-Behnken experimental design was effective in evaluating the effect of extraction temperature, time, and sample-to-solvent ratio on the total phenolic compounds, flavonoids and antioxidant capacity measured by DPPH, ABTS, FRAP, and CUPRAC. Sample-to-solvent ratio was the factor that influenced negatively all the dependent variables, revealing that when the sample-to-solvent ratio increased the dependent variables decreased. Lemon pomace is a good source of TPC and TF since high correlation was observed between them and the antioxidant capacity assays. The optimum conditions for increasing the yield of total phenolic content, total flavonoid compounds and antioxidant capacity were obtained at 95 ^{o}C temperature, 15 min extraction time, and 1 g / 100 ml sample-to-water. Hot water extracts at the optimal conditions had the same content of TPC and TF as methanol extracts obtained by ultrasound extraction. Overall, hot water could be an effective solvent for the recovery of TPC, TF from lemon pomace.

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Conflict of interest statement

The authors declare no conflict of interest.

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Tables and Figures

Table 1 Box-Behnken design and the dependent responses.

Table 2. Analysis of variance for the determination of model fitting.

Table 3. Analysis of variance for the experimental results of total phenolic compounds (TPC), total flavonoids (TF), and proanthocyanidins.

Table 4. Analysis of variance for the experimental results of antioxidant activity measured by DPPH, ABTS, FRAP, and CUPRAC.

Table 5. Correlation between bioactive compounds and antioxidant properties of lemon peels.

Table 6. Validation of the predicted values for TPC, flavonoids, proanthocyanidins, and antioxidant capacity.

513 **Table 7.** Comparison of hot water (water bath) with methanol extracts (ultrasound
514 extraction).

515 **Fig. 1** Correlation between the predicted and experimental values for TPC, flavonoids, and
516 proanthocyanidins.

517 **Fig. 2** Correlation between the predicted and experimental values for DPPH, ABTS, FRAP,
518 and CUPRAC.

519 **Fig. 3** Impact of temperature (75–95 °C), time (5–15 min), and sample-to-solvent ratio (1–10
520 g / 100 mL) on TPC (mg GAE/g dw).

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