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Running Head: Drying method effect on lemon peel polyphenols

Summary

The aim of this study was to investigate the effect of freeze drying, hot air and vacuum drying at 70, 90 and 110 °C, on dried lemon pomace polyphenols and antioxidant capacity. The total phenolic content and antioxidant capacity were higher in lemon pomace dried by hot air or under vacuum than those dried by freeze drying and increased as the temperature increased. The highest total flavonoid content was recorded in the pomace dried under vacuum at 70 and 90 °C. Lemon pomace dried by freeze drying had the highest neohesperidin content, whereas pomace dried under vacuum at 70 °C had the highest rutin and *p*-coumaric acid content. The highest gallic acid content was recorder in the pomace dried by hot air at 110 °C. The results of this study indicate that drying technique should be carefully selected according to the bioactive compounds aimed to be extracted.

Keywords: *Citrus, phenols, flavonoids, antioxidants.*

Introduction

The last decades witnessed an increased interest for the valorisation of phenolic compounds, such as flavonoids and phenolic acids by both pharmaceutical and food industries (Ledesma-Escobar *et al.*, 2016a). Lemon (*Citrus limon*) peels being the main residue generated by lemon juice industry, account for 50 to 65% of the whole fruit weight (González-Molina *et al.*, 2010) and are a good source of phenolic compounds, such as phenolic acids (ferulic, *p*-coumaric and sinapic acids) (Bocco *et al.*, 1998) and flavonoids (flavanones, flavonols, flavones), which have been linked to antimicrobial (Dhanavade *et al.*, 2011), anticancer (Wang *et al.*, 2014) and antioxidant activities (Park *et al.*, 2014).

Drying of material precedes extraction and is an important step in the recovery of phenolic compounds from plant matrixes (Khoddami *et al.*, 2013). Previous studies have indicated that phenolic compound extraction yields might be influenced by the drying method (Chen *et al*., 2011). Ledesma-Escobar *et al.* (2016b) examined the effect of freeze drying and air drying at 45 ˚C on the different compounds of whole lemons using 53% ethanol as a solvent for the extraction and showed that freeze drying was more suitable for the extraction of flavanones or flavones of lemon, whereas air drying facilitated the extraction of flavanols. Sun *et al.* (2015) showed that freeze-drying was an effective drying method for the preservation of phenolic compounds of four *Citrus* species, whereas hot air drying was more efficient for the retention of flavonoids. Lou *et al.* (2015) investigated the effect of different drying temperatures and intervals on the phenolic compounds of immature kumquat (*Citrus japonica* var. margarita) and compared the results with those of the fresh samples.

Phenolic compounds might be degraded either by high temperature or by oxidation (Wojdyło *et al.*, 2014). Therefore, a considerable amount of polyphenols might be lost during drying process. Most of the studies have been applied on *Citrus* waste have investigated the effect of freeze drying and hot air drying on polyphenol content. To the best of our knowledge

there is no study investigating the effect of high temperature under vacuum on the polyphenol content of lemon peels. Therefore, the aim of this study was to examine the effect of different drying techniques, such as freeze drying, hot air drying (at 70, 90 and 110 °C) and drying under vacuum (at 70, 90 and 110 °C) on the phenolic content and antioxidant capacity of lemon pomace, using hot water for the extraction. Optimal drying conditions for enhancing the antioxidant capacity and phenolic content of lemon pomace were proposed.

Materials and methods

Chemicals and reagents

All chemicals used in this study were of analytical grade. Methanol, ethanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu's reagent, anhydrous sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), hydrochloric acid (HCl), formic acid, copper (II) chloride ($CuCl_2$), ammonium acetate (NH_4Ac), 2,2-diphenyl-1-picryl-hydrazil (DPPH), trolox, neocuproine, gallic acid, *p*-coumaric acid, neohesperidin, rutin, quercetin, and catechin were purchased from Sigma-Aldrich Pty Ltd. (Castle Hill, Sydney, Australia). Aluminium chloride ($Al_2Cl_3 \bullet 6H_2O$) was obtained from J. T. Baker Chem. 9 Co. (Belgium, Zedelgem). Sodium hydroxide (NaOH) was purchased from Ajax Chem. (NSW, Australia).

Samples

Lemon (*Citrus limon*) fruits (at a commercial stage) with an average weight of 125 ± 1.53 g (mean ± standard deviation) were purchased from a local market in Lisarow (Australia, NSW) in April 2016. The fruits were transferred to the laboratory, washed, squeezed and the remaining peels (pomace) were cut in slices and stored at −18 °C in a sealed plastic container, until dried. The initial moisture content of the peels was 78.21 ± 1.12 per 100 g product (mean ± standard deviation).

Preparation of dried samples

Lemon peels (peel thickness of 3.87 mm ± 0.69 (mean ± standard deviation)) were cut in slices (size of 2 × 1 cm) and dried by three different methods: i) freeze drying (for 48 h) in a freeze dryer (FD3 freeze dyer, Thomas Australia Pty. Ltd., Seven Hills, NSW, Australia), ii) air drying (at 70, 90 and 110 °C for 8, 5.5 and 3 h, respectively) in an oven (LABEC, Laboratory Equipment Pty, Ltd., Marrick Ville, NSW, Australia) and iii) vacuum drying (at 70, 90 and 110 °C for 18, 7 and 4 h, respectively) in a vacuum oven (Thermoline, Australian Marketing Group, Marrick Ville, NSW, Australia), until constant weight. Approximately 20 g ± 1 g of sample were weighed before drying and placed on an aluminium tray (single layer). Each drying method was conducted in triplicate. The drying time, energy consumption, residual moisture content (RSC), water activity, color and pH of extracts of dried lemon peels can be seen in Table 1.

The dried peels obtained by the different drying methods were chopped using a stainless knife and the powder passed through a 1.4 mm steel mesh sieve (EFL 2000; Endecotts Ltd., London, England) was stored in a sealed container at −18 °C for further analysis.

Residual moisture content, water activity (a_w), color of the powder and energy consumption

The residual moisture content of the dried peels were determined according to Nguyen *et al.* (2016). The water activity (a_w) of dried samples was measured using an Aqualab Water Activity Meter (Decagon Devices, Inc., Pullman, WA). The color characteristics of the dried peels were measured using a CR-400 Minolta Chroma Meter (Konica Minolta Ltd., North Ryde, NSW, Australia) calibrated using a white tile standard to establish lightness (L*), red/green balance (a*) and yellow blue balance (b*). Hue angle (H°) was determined from a*

and b*, as has been described by Vuong et al. (2015). The energy consumption of the different drying methods was estimated as it has been described by Nguyen et al. (2016) using Eq. (1).

Energy consumption (kwh) = P × t (1)

Where P is the electrical power supplied (kW), and t is the time needed for drying the sample (h).

Extraction procedure

The lemon peel aqueous extracts were prepared according to Papoutsis *et al.* (2016) with minor modifications. Briefly, dried lemon peels (0.1 g) were extracted using hot water (10 mL) at 95 °C ± 1 °C for 15 min in a water bath (Ratek Instruments Pty. Ltd., Boronia, Vic., Australia). After extraction, the samples were filtered through Whatman filter paper number 1 at ambient temperature. Subsequently, the samples were stored at −18 °C until used for analysis.

Phytochemical analysis

Total phenolic content (TPC)

TPC was determined according to Papoutsis *et al.* (2016). The absorbance was measured at 760 nm using UV spectrophotometer (Varian Australia Pty. Ltd., Victoria, Australia) and the results were expressed as mg gallic acid equivalents per g of sample dry weight (mg GAE (g dw)$^{-1}$).

Total flavonoid content (TF)

TF was measured according to Papoutsis *et al.* (2016). The absorbance was measured at 510 nm. The results were expressed as mg catechin equivalents per g of sample dry weight (mg CE (g dw)$^{-1}$).

Total antioxidant capacity

Cupric reducing antioxidant capacity (CUPRAC) assay

CUPRAC assay was determined as described by Papoutsis *et al.* (2016). The absorbance was measured at 450 nm and the results were expressed as mg trolox equivalents per g of sample dry weight (mg TE $(g\ dw)^{-1}$).

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assay was measured according to Papoutsis *et al.* (2016). The absorbance was measured at 515 nm and the results were expressed as mg trolox equivalents per g of sample dry weight (mg TE $(g\ dw)^{-1}$).

Quantification of individual phenolic compounds

Individual phenolic compounds were determined using high-performance liquid chromatography (HPLC) (Shimadzu LC-20AD, Rydalmere, NSW, Australia) according to Lou *et al.* (2016) with some modifications. Briefly, the standards and samples were filtered through a 0.45 µm nylon filter and 200 µL was individually injected into a C_{18} reversed-phase column (Gemini 110A 5 µm, 150 × 4.6 mm Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia) supplied with a guard column (Gemini C_{18}, 4 × 3.0 mm). The column temperature was maintained at 30 °C using a temperature controller (Phenomenex Therma Sphere TS 130, Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). The mobile phase contained water: acetonitrile: formic acid, 95:4:1 (v:v:v) (Mobile Phase A) and acetonitrile (Mobile Phase B). The flow rate of the solvents was 1 mL/min and the following gradient solution was used: 0 min 5% B; 15 min, 20% B; 35 min, 100% B; 40 min, 5% B; 50 min, 5% B. The analysis was stopped after 60 min. The system was equilibrated between runs for 10 min using the 50% B. Photodiode array (PDA) detection was performed between 210 and 480 nm.

Individual phenolic compounds were identified based on their elution time and quantified from the peak area of 280 nm. The identified phenolic compounds were quantified using external standards (neohesperidin, rutin, gallic acid and *p*-coumaric acid) which were prepared by dissolving standard compounds in methanol at a concentration of 200 µg mL^{-1}. The chromatograms obtained by the different drying methods can be seen in Fig. 1.

Statistical analysis

Analysis of variance was conducted using SPSS statistical software (version 23, IBM, Crop., NY, USA) and *P*-value < 0.05 was considered significant. The comparison of mean averages was performed with the Duncan's post hoc multiple comparison test using SPSS statistical software (version 23, IBM, Crop., NY, USA). The Pearson's correlation (*r*) and *P*-value were used to determine the correlation coefficients among phenols and antioxidant assays. All the experiments were conducted in triplicate.

Results and discussion

Impact of different drying methods on the total phenolic content (TPC)

The TPC of dried lemon pomace aqueous extracts was significantly affected by the different drying methods ($P < 0.05$) and the results are given in Table 2. In general, lemon pomace dried by hot air at 110 °C had the highest TPC (20.71±2.29 mg GAE $(g\ dw)^{-1}$), whereas freeze dried lemon pomace had the lowest (15.76±0.86 mg GAE $(g\ dw)^{-1}$). These results could be attributed to the liberation of some phenolic acids and flavonoids which are mainly found in bound form in plant matrix due to the heat (Hayat *et al.*, 2010), as well as in the reduction of polyphenol oxidase (PPO) activity, which is an enzyme responsible for the selective oxidation of polyphenols, since high temperature tends to reduce PPO activity (Krapfenbauer *et al.*, 2006). These results are in agreement with Lou *et al.* (2014) who found that when air drying

temperature increased from 70 to 150 °C the TPC of immature calamondin (*Citrus mitis* Blanco) peels increased. On the other hand, Ledesma-Escobar *et al.* (2016a) reported that lemons dried by freeze drying had higher content of phenolic compounds compared to those dried by air drying at 45 °C. This difference could be attributed to the oxidation of some phenolic compounds in the samples dried at 45 °C, since samples dried at low temperatures are exposed to oxygen for a long time (Wojdyło *et al.*, 2014).

Impact of different drying methods on the total flavonoid content (TF)

The TF of dried lemon pomace aqueous extracts was significantly affected by the different drying methods ($P < 0.05$) and the results are given in Table 2. Lemon pomace dried under vacuum at 90 and 70 °C had the highest TF, whereas lemon pomace dried by freeze drying, hot air and vacuum drying at 110 °C had the lowest. A dramatic decrease in TF was observed when the temperature (either in vacuum or hot air drying) increased from 90 to 110 °C. The increase of TF could be attributed to the liberation of some flavonoids due to high temperature (Hayat *et al.*, 2010), since flavonoid compounds of *Citrus* peels are mainly present in glycoside forms (González-Molina *et al.*, 2010), as well as to the reduced PPO activity, since previous studies have reported that high temperatures (80-100 °C) destroy its catalytic activity (Krapfenbauer *et al.*, 2006; Queiroz *et al.*, 2008). However, the decrease in the TF observed at higher temperatures (110 °C) could be due to the heat degradation of some released flavonoid compounds. Lou *et al.* (2014) found that high temperature applied for drying resulted in the degradation of some flavonoid compounds of immature calamondin peels, including 30´,50´-di-C-β-glucopyranosylphloretin (DGPP) and hesperidin. To sum up, vacuum drying at 70 or 90 °C could be applied for retaining the total flavonoid content of lemon pomace.

Impact of different drying methods on individual phenolic compounds

The content of individual flavonoids was significantly influenced by the different drying methods ($P < 0.05$) and the results are given in Table 3. Lemon pomace dried by freeze drying had the highest neohesperidin content (64.23 μg mL^{-1}) compared to those dried by hot air or under vacuum at different temperatures, indicating that neohesperidin loss occurs when lemon pomace is dried at high temperatures. These results are in accord to Ledesma-Escobar *et al.* (2016a) who showed that the content of neohesperidin was higher in lemons dried by freeze drying compared to those dried by air. Neohesperidin content of peels dried by hot air was significantly affected by temperature and dramatically decreased when the temperature increased from 90 to 110 °C (from 31.04 to 21.55 μg mL^{-1}, respectively), whereas no significant reduction in neohesperidin content was observed in the peels dried under vacuum at different temperatures (no oxygen environment).

Rutin being a flavonol glycoside, was the major compound identified in this study and was higher in the pomace dried under vacuum at 70 and 90 °C (137.04 and 121.89 μg mL^{-1}, respectively). The rutin content of peels dried at 110 °C either by air or under vacuum was significantly lower (55.79 and 92.90 μg mL^{-1}, respectively) than those dried at lower temperatures and by freeze drying. These results are different to those reported by Ledesma-Escobar *et al.* (2016a), who showed that rutin was higher in lemons dried by air compared to those dried by freeze drying. These differences could be attributed to the lower air drying temperatures applied in this study (45 °C), since high temperature may promote the degradation of rutin (Buchner *et al.*, 2006).

The content of the identified individual phenolic acids was significantly influenced by the drying methods ($P < 0.05$) (Table 3). Lemon pomace dried under vacuum at 70 °C had the highest *p*-coumaric acid content (1,69 μg mL^{-1}). In general, lemon peels dried under vacuum had higher *p*-coumaric content than those dried by hot air, whereas as the temperature increased

from 90 to 110 °C (hot air drying) and from 70 to 110 °C (vacuum drying) the *p*-coumaric acid content sharply decreased (31 and 44%, respectively). These results were expected since hydroxycinnamic acids (*p*-coumaric acid) are heat sensitive (King & Young, 1999) and indicate that both high temperature and oxygen might lead to the degradation of *p*-coumaric acid. These results are different to those reported by Sun *et al.* (2015) who mentioned that the *p*-coumaric acid content of immature *Citrus* fruits was higher in those dried by freeze drying compared to those dried by hot air (at 60 °C for 10 h). These differences could be due to the oxidation of *p*-coumaric acid in the samples dried at 60 °C, since samples dried at this temperature were exposed to the oxygen for longer time compared to those of our study which were dried at higher temperatures (Wojdyło *et al.*, 2014), as well as to the different species used in these studies (Gorinstein *et al.*, 2001).

The gallic acid content was higher in the peels dried by hot air compared to those dried under vacuum, whereas it was not detected in the peels dried by freeze drying. The gallic acid content of peels dried by both air and vacuum drying increased as the temperature increased. These results are in accord to Hayat *et al.* (2010) who showed that as the temperature increased, the gallic acid content of *Citrus mandarin* peels increased, since high temperature promotes the liberation of bound phenolic acids. The gallic acid content of the peels dried by hot air was higher than those dried under vacuum. This difference could be attributed to the presence of oxygen in hot air drying, which might be implicated in the synthesis of gallic acid. Little information is available on the biosynthetic pathway of gallic acid (Vogt, 2010). However, Ossipov *et al.* (2003) suggested that shikimate dehydrogenase might oxidize shikimic acid to dehydroshikimic acid and further to gallic acid.

Impact of different drying methods on the antioxidant capacity

The antioxidant capacity of dried lemon pomace aqueous extracts measured by CUPRAC and DPPH was significantly affected by the different drying methods ($P < 0.05$) (Fig. 2A, B). In general samples dried by hot air or under vacuum had higher antioxidant capacity compared to those dried by freeze drying. As the drying temperature increased the antioxidant capacity of aqueous extracts of samples dried either by hot air or vacuum drying increased. These results are different to those reported by Sun *et al.* (2015) who found that the antioxidant capacity of different *Citrus* species dried by freeze drying was higher compared to those dried by hot air (at 60 °C, for 10 h) or sun drying (at 20-25 °C, for 3 days). These differences could be attributed to the different drying times and temperatures applied, as well as to the different *Citrus* species. The variation of antioxidant capacity measured by both CUPRAC and DPPH was similar to TPC variation, indicating that TPC of lemon pomace is implicated in the antioxidant capacity. This is further supported by the correlation coefficients between TPC and antioxidant assays (CUPRAC and DPPH) (r=0.80 and 0.49, P = 0.000 and 0.024 respectively) (Fig. 3A, B). As the drying temperature increased, the antioxidant capacity of samples dried by hot air or under vacuum increased. Neohesperidin, rutin and *p*-coumaric acid seems to have negative effect on the antioxidant capacity of dried lemon pomace since the correlation coefficients between neohesperidin, rutin and *p*-coumaric acid and CUPRAC were (r = -0.626 P =0.002, r = -0.859 P = 0.000 and r =-0.621 P = 0.003, respectively) and with DPPH were (r = -0.492 P =0.045, r = -0.520 P = 0.016 and r =-0.474 P = 0.030, respectively). On the other hand the correlation coefficients between gallic acid and antioxidant assays (CUPRAC and DPPH) was (r = 0.935 and 0.646, P = 0.000 and 0.002, respectively), indicating that gallic acid contributes to the antioxidant capacity of lemon pomace. Therefore, it could be also mentioned that heat might promote the synthesis of new compounds which contribute to the antioxidant capacity of dried lemon pomace aqueous extracts (Tamanna & Mahmood, 2015).

Conclusion

Drying method significantly affected the TPC, TF, neohesperidin, rutin, p-coumaric acid, gallic acid content and antioxidant capacity of dried lemon pomace aqueous extracts. Vacuum drying at 90 and 70 °C for 7 and 18 h, respectively, is a good method for the preservation of TF, whereas hot air drying at 110 °C for 3 h facilitates high TPC recovery. Drying under vacuum can be effectively used for high recovery of *p*-coumaric acid and rutin content from lemon peels, whereas high neohesperidin content can be achieved by freeze drying, since drying at temperatures more than 70 °C resulted in the decrease of its content. Gallic acid was detected only in the peels dried by hot air or vacuum drying at different temperatures. Since drying at high temperatures either by hot air or under vacuum resulted in the formation of some peaks which were not detected in the samples dried by freeze drying, further studies should be conducted in order to identify the compounds (phenolics or not) which are synthesised during thermal treatment and their contribution in antioxidant capacity.

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Conflict of interest statement

The authors declare no conflict of interest.

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Legends to Figures

Figure 1. Chromatograms at 280 nm of the aqueous extracts of dried lemon (*Citrus limon*) pomace, dried by the different methods: (a) hot air drying at 110 °C, (b) hot air drying at 90 °C, (c) hot air drying at 70 °C, (d) vacuum drying at 110 °C, (e) vacuum drying at 90 °C, (f) vacuum drying at 70 °C and (g) freeze drying.

Figure 2. Antioxidant capacity (CUPRAC (a) and DPPH (b)) of aqueous extracts of dried lemon (*Citrus limon*) pomace, dried by different methods. Data (mean ± standard deviation, n = 3) with different superscripts are significantly different at $P < 0.05$.

Figure 3. Correlation between antioxidant capacity determined by CUPRAC (a) and DPPH (b) and total phenolic content (TPC) of aqueous extracts of dried lemon (*Citrus limon*) pomace.