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Papoutsis, Konstantinos
Pristijono, Penta
Golding, John Brett
Stathopoulos, Costas E.
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Konstantinos Papoutsis[1]*, Penta Pristijono[1], John B. Golding[1,2], Costas E. Stathopoulos[3], Christopher J. Scarlett[1], Michael C. Bowyer[1] and Quan V. Vuong[1*]

[1]*School of Environmental and Life Sciences, The University of Newcastle PO Box 127 Ourimbah, NSW 2258, Australia*

[2]*NSW Department of Primary Industries, Locked Bag 26 Gosford, NSW 2250, Australia*

[3]*Division of Food and Drink School of Science, Engineering and Technology University of Abertay Dundee DD1 1HG UK*

[*]Corresponding author: PhD Candidate, Konstantinos Papoutsis; email: Konstantinos.Papoutsis@uon.edu.au; Nutrition Food & Health Research Group, School of Environmental and Life Sciences, University of Newcastle, Brush Rd, Ourimbah, NSW 2258, Australia.

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Abstract

The effects of different solvents on the recovery of (i) extractable solids, (ii) total phenolic compounds (TPC), (iii) total flavonoid content (TFC), (iv) vitamin C and (v) antioxidant activity from lemon pomace waste were investigated. The results revealed that solvents significantly affected the recovery of extractable solids, TPC, TFC and antioxidant properties. The combination of organic solvents, ethanol and acetone with water (50%, v/v) had the highest recovery yields for TPC, TFC with increased antioxidant properties compared to their absolute solvents and water. Methanol and 50% acetone resulted in the highest extraction yields of TPC, whereas the methanol resulted in the highest extraction of TFC, whilst the water had the highest recovery of vitamin C. TPC and TFC were shown to be the major components contributing to the antioxidant activity in the lemon pomace.

Keywords: lemon peels, total flavonoids, ascorbic acid, extractable solids, antioxidants

Introduction

Citrus fruits in the family *Rutaceae* include oranges, lemons, limes, grapefruits and tangerines which are well known for their nutritional value as they are good sources of dietary fiber, vitamin C, vitamin B group, carotenoids, flavonoids and limonoids (1). Several recent studies have demonstrated anti-inflammatory activity (2) and have linked the citrus extracts with the prevention of colon cancer (3).

Worldwide citrus production has exceeded 88×10^6 tons (2) and approximately 34 % of this production has been used by the juice industry, resulting in high amounts of waste (4). Citrus pomace includes peel, composed from flavedo, albedo and seed. These have been found to be good sources of phenolic acids, flavonoids, vitamin C (ascorbic acid), molasses, essences, seed oil and pectins (4,5).

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 representing the main component of waste, represents between 50 and 65% of the whole fruit weight (6). Lemon peel contains bioactive compounds, such as vitamin C (ascorbic acid), flavonoids (flavanones, flavonols, flavones) and phenolic acids (ferulic, p-coumaric and sinapic acids) (6,7), which have been linked to antimicrobial (8) and antioxidant activities (9).

Several studies have examined the recovery of bioactive compounds from lemon peel for valorization by the food and pharmaceutical industries (6,8). Solvent type has been shown to play an important role for the optimum recovery of these compounds (10). Several solvents have been used for the recovery of bioactive compounds from citrus, with methanol known as a solvent commonly used for the recovery of phenolic compounds from citrus (11). To the best of our knowledge there is no study investigating the effect of different solvents on the recovery of phenolic compounds, flavonoids, vitamin C and extractable solids from lemon

pomace waste. Therefore the aim of this study was to investigate the effect of different solvents including water, methanol, ethanol and acetone and the combination between these organic solvents with water at a ratio of 50:50 (v/v) on the recovery of total phenolic compounds, total flavonoids, vitamin C and antioxidant activity of the lemon pomace.

Materials and Methods

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. The frozen lemon waste was dipped 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 and labeled container at -18 °C for further analysis.

Extraction process

Seven extraction solvents were used for comparison, including: water, absolute methanol, ethanol, acetone, 50% methanol, 50% ethanol and 50% acetone. An ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd., Thebarton, Australia) was used for the extraction. Briefly, 1g of dried lemon pomace was mixed with 100 mL of solvent and exposed to 60 W ultrasonic power, for 20 min at a temperature of 30 °C. Agitation was conducted for 10 s once every five min using a Vortex. After completion of the extraction process, the extracts were centrifuged at 3500 × *g* for 10 min at 14 °C. Then the supernatants were collected using pipet and diluted 10 folds (for the determination of TPC, Vitamin C (ascorbic acid), DPPH, CUPRAC and ABTS assays), while sample without dilution was used

for the determination of TFC and extractable solids. Subsequently, they stored in the dark at -18 °C until used for quantitative analysis and antioxidant determination.

Extractable solids

Extractable solids of the lemon pomace were estimated according to the method reported by Vuong (12) with a minor modification. 2 mL of the supernatant was put into an oven (set at 110 °C) until the solvent being completely evaporated. Extractable solids were expressed as percentage and the following equation: $ES\ (\%) = W\ x\ 100/2$ (ES: Extractable solids, W: Weight of 2 mL after drying in g), was used for the calculation.

Total phenolic content (TPC)

The total phenolic content was measured as described by Vuong (13). 5 mL of 10% (v/v) Folin-Ciocalteu reagent were mixed with 1 mL of diluted sample and 4 mL of 7.5% (w/v) Na_2CO_3 and incubated under dark at room temperature for 1 h. The absorbance was measured at 760 nm using a UV spectrophotometer (Varian Australia Pty. Ltd., Victoria, Australia). The results were expressed as mg of gallic acid equivalents per g of sample dry weight (mg GAE/g dw).

Total flavonoid content (TFC)

The total flavonoid content was measured as described by Zhishen (14). 2 mL of H_2O, 0.15 mL of 5% (w/v) $NaNO_2$ and 0.5 mL of sample were mixed and left for 6 min at room temperature. Then 0.15 mL of 10% (w/v) $AlCl_3$ was added and left for 6 min. Subsequently, 2 mL of 4% (w/v) NaOH and 0.7 mL of H_2O were added and left at room temperature for 15 min before the absorbance was measured at 510 nm. The results were expressed as mg of catechin equivalents per g of sample dry weight (mg CE/g dw).

Total vitamin C

The total vitamin C was measured according to a method described by Vuong (15) with a minor modification. A solution was prepared by mixing 500 ml of 0.6 M sulfuric acid with 5.3218 g of Sodium Phosphate and 2.471 g of ammonium molybdate. 3 mL of the solution were mixed with 0.3 mL of diluted sample and incubated at 95 ^{o}C for 90 min in a water bath. After incubation, they were left at room temperature for 30 min and the absorbance was measured at 695 nm. The results were expressed as mg ascorbic acid equivalents per g of dry weight (mg AAE/g dw).

Assays for the measurement of the antioxidant activity

DPPH assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) was used for the measurement of the antioxidant activity as reported by Thaipong (16), with minor modifications. A stock solution was prepared and stored at −20 °C until used. The working solution was prepared by mixing 10 mL of the stock solution with 45 mL methanol to obtain an absorbance of 1.1±0.02 at 515 nm. 2.85 mL of working solution was mixed with 0.15 mL of diluted sample and left under dark for 3 h before measuring the absorbance at 515 nm. The results were expressed as mg of trolox equivalents per g of dry weight (mg TE/g dw).

CUPRAC assay

CUPRAC (cupric reducing antioxidant capacity) was performed as described by Apak (17) with some modifications. 1 mL of $CuCl_2$, 1 mL of neocuproine, 1 mL of NH_4Ac and 1.1 mL of diluted sample were mixed. The mixture was left at room temperature for 1.5 h before the

absorbance was measured at 450 nm. The results were expressed as mg of trolox equivalents per g of sample (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 (16) with some modifications. A stock solution was prepared and stored at -20 ^{o}C until required. The working solution was prepared by diluting 1 mL of stock solution with 60 mL of methanol to obtain an absorbance value of 1.1 ± 0.02 at 734 nm. 2.85 mL of the working solution was mixed with 0.15 mL of diluted sample and left under dark at room temperature for 2 h before the absorbance was measured at 734 nm. The results were expressed as mg trolox equivalents per g of dry weight (mg TE/g dw).

Statistical analysis

The one-way analysis of variance was conducted using SPSS (version 23). The least significance difference (LSD) was applied for the comparison of the means at p <0.05. Data were reported as means ± standard deviations. The Pearson correlation test was employed to determine the correlation coefficients among bioactive compounds and different antioxidant assays at p <0.01.

Results and Discussion

Effect of solvent on extractable solids

Extractable solids comprise of all soluble compounds such as sugars, proteins, pectins, vitamins, minerals and phytochemicals, which are extracted during the extraction process. The solvents had a significant effect on the extractable solid content (p <0.05) (Fig. 1). Water,

absolute methanol and 50% ethanol gave the highest levels of extractable solids, whereas absolute acetone had the lowest content of extractable solids. Variation in extractable solid content can be explained by the different type of bioactive compounds presented in lemon peels, such as carotenoids, phenolic compounds, ascorbic acid, fibres and pectins, and their different solubilities in various types of solvents. For instance, lipophilic compounds, such as carotenoids can be easily extracted by organic solvents (18), whereas hydrophilic compounds such as ascorbic acid, pectins and sugars can be extracted by water or aqueous alcohols (19,20). The results are supported by the results of a previous study in almond hulls, which reported that extractable solids were significantly affected by the extraction solvents (21).

Effect of solvent on total phenolic content (TPC)

The phenolic compound extraction yields can be influenced by the choice of extraction solvents, ranging from polar to non-polar solvents (10). The type of solvent had a significant effect on the extraction yields of total phenolic compounds from the lemon peel (p <0.05). Results can be seen in Fig. 2 and are in accord with the findings in previous study, which reported that extraction solvents significantly affected the extraction yields of TPC from citrus materials (11). Absolute methanol and 50% acetone had the highest recovery yields with 13.24 and 12.37 mg GAE/g dw, respectively, while water gave less phenolic compound yields compared to 50% acetone but higher compared to absolute acetone. These results are in agreement with Nayak (22) who found that 51% acetone had the highest recovery yield of TPC (12.20 mg GAE/g dw) from orange peels. Park (23) also reported that methanol gave higher extraction yield of TPC from orange peel in comparison with other solvents. These differences in extraction efficiency of TPC can be attributed to the variation in polarity of the tested solvents, which selectively extracted phenolic compounds with different polarities. The highest extraction yield obtained by methanol and 50% acetone can be due to the reduced

polyphenol oxidase (PPO) activity in these extracts, since polar solvents result in reduced PPO activity, which is an enzyme responsible for the oxidation of phenolic compounds (11). In summary, among the different solvents examined in this study, absolute methanol and 50% acetone were found to be the most efficient solvents for the recovery of TPC from the lemon pomace.

Effect of solvent on total flavonoid content (TFC)

The total flavonoid content was significantly affected by solvent (Fig. 3). Absolute methanol extract had the highest extraction yield of TFC (5.03 mg CE/g dw), followed by 50% ethanol and 50% methanol (4.15 and 3.75 mg CE/g dw, respectively). These findings are in agreement with Ma (24) who reported that methanol was the most effective extraction solvent for hesperidin which is a flavonoid compound (flavanone) and are different to previous results reported by Lou (25) who mentioned that hot water was efficient solvent for the extraction of TFC from calamondin (*Citrus mitis* Blanco) compared to absolute methanol, ethanol or their combination with water. The differences in extraction efficiency of TFC can be related to the different polarity of solvents, as well as the different polarity, class (flavanones, flavones and flavonols) and form (glycoside or aglycone) of flavonoids in lemon peels (6). It has been mentioned that flavonoid glycosides and more polar aglycones can be extracted with alcohols or alcohol–water mixtures, while low polarity solvents can be suitable for the extraction of less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols) (26).

Effect of solvent on total vitamin C

Solvent had a significant effect on the extraction yield of vitamin C (ascorbic acid) (p <0.05) (Fig. 4). As we expected, the water extract had the highest content of vitamin C (209

228 mg AAE/g dw), followed by absolute methanol and 50% acetone (177 and 165 mg AAE/g
229 dw, respectively), since vitamin C is a cyclic polar molecule and its solubility increases as the
230 solvent polarity increases (27). Higher extraction yields obtained by 50% acetone and 50%
231 ethanol compared to their absolute solvents, can be attributed to the presence of water, which
232 may increase the polarity of the solvent. These results are in accord with the results mentioned
233 by Shalmashi and Eliassi (27), who found that the solubility of vitamin C is in decreasing
234 order as follows: water, methanol, ethanol, acetone, acetonitrile, and ethyl acetate.
235
236 **Effect solvent on antioxidant activity**
237 The effect of solvents on the antioxidant activity of lemon pomace was determined
238 using three antioxidant assays. The results showed that the tested solvents had a significant
239 effect on the antioxidant properties of the extracts (Fig. 5).
240 For the DPPH assay the extracts obtained using 50% acetone proved to have the
241 highest antioxidant properties (0.15 mg TE/g dw), followed by methanol, while water and
242 absolute acetone extracts had the lowest antioxidant properties (0.03 and 0.02 mg TE/g dw,
243 respectively) (Fig. 5A) (p <0.05). The high antioxidant activity of 50% acetone and methanol
244 extracts given by DPPH assay maybe due to the high level of TPC and TFC extracted with
245 these solvents. In addition, the low antioxidant activity of water extracts can be explained by
246 the ability of DPPH assay to mainly measure the antioxidants which are soluble in organic
247 solvents (28). The high value obtained by methanol can be attributed to the very fast electron
248 transfer from phenoxide anion to the radical, due a partial ionization, since DPPH is an
249 electron transfer assay (29).
250 For the CUPRAC assay the extracts obtained using absolute methanol proved to have
251 the highest antioxidant properties (57 mg TE/g dw), while absolute acetone the lowest (10 mg
252 TE/g dw) (Fig. 5B) (p <0.05). The high antioxidant activity of methanol extracts could be

explained by the high level of TPC and TFC, which are compounds with antioxidant activity, as well as the partial ionization of the phenols resulting in a very fast electron transfer, since methanol is an alcohol that enhances ionization (29,30). These findings are in agreement with the Çelik (30), who mentioned higher CUPRAC values in absolute methanol compared to other solvents.

For the ABTS assay, the extracts obtained using absolute methanol and 50% methanol had the highest antioxidant properties (0.46 and 0.43 mg TE/g dw, respectively), followed by 50% ethanol (0.27 mg TE/g dw) (Fig. 5C) (p <0.05), while 50% and absolute acetone extracts had the lowest. These findings are in agreement with Van den Berg (31) who reported different ABTS values among different solvents. These differences can be explained by the limited solubility of some antioxidants at these solvents. The high ABTS value obtained by methanol can be attributed to the very fast electron transfer from phenoxide anion to the radical, due a partial ionization (29). 50% acetone extracts showed a large variation in their antioxidant activities among the different assays. These variations should be attributed to the different reaction mechanisms of the antioxidants extracted by 50% acetone with the different antioxidant assays (30). This is in accord with Çelik (30) who reported that the antioxidant activity of catechin solved in dichloromethane/ethanol, measured by ABTS was the lowest among different solvents, while its antioxidant activity in the same solvent measured by CUPRAC was quite similar with those obtained by the other solvents.

Correlation between bioactive compounds and antioxidant properties

The antioxidant properties of the lemon pomace can be contributed by bioactive compounds, such as TPC, TFC, as well as vitamins C and E. In this study TPC and TFC had a strong correlation with antioxidant properties of the extracts prepared from the lemon pomace (Table 1). The r values between TPC and DPPH, CUPRAC and ABTS were 0.95, 0.94, and

278 0.59 (p <0.01), respectively, revealing that TPC was a major contributor to antioxidant
279 properties of the lemon pomace extracts. Similarly, the r values between TFC and DPPH,
280 CUPRAC and ABTS were 0.75, 0.91 and 0.80 (p <0.01), respectively, indicating that TFC
281 was also a major antioxidant contributor. The r values between vitamin C and antioxidant
282 properties measured by DPPH, and CUPRAC were 0.45, 0.55 and (p <0.01), respectively,
283 while no correlation observed between vitamin C and ABTS assay, indicating that vitamin C
284 contributed to antioxidant properties of the lemon pomace extracts but not significantly.
285 These findings are supported by previous study which showed that TPC had close correlation
286 with the antioxidant properties and were the major contributor to the antioxidant properties of
287 citrus extracts because of being potential electron donors, due to their hydroxyl groups (32).
288 However, these findings are different to those reported by Ghasemi (33) who found no
289 correlation between phenolic compounds or flavonoids and antioxidant activity of the citrus
290 peel. These findings were also in accord with a previous study, which found that antioxidant
291 power of plant extracts is largely contributed by phenolic compounds rather than ascorbic
292 acid (34). However another study found that vitamin C contributed to antioxidant capacity of
293 citrus fruits more than phenolic compounds (35). The differences can be explained by the
294 potency of each phenolic compound contained in the extracts as well as their levels in the
295 extracts, which could be linked with the correlation with the antioxidant properties (30).

296 To sum up, the type of solvent significantly affected the recovery of extractable solids,
297 TPC. TFC, vitamin C and the antioxidant properties from the lemon pomace. Water, methanol
298 and 50% ethanol resulted in the highest extractable solids. Methanol and 50% acetone
299 resulted in the highest extraction yields of TPC, methanol provided the highest extraction
300 yield of TFC, whereas, water had the highest recovery of vitamin C. Methanol, 50%
301 methanol, 50% ethanol and 50% acetone were found to provide the most potent antioxidant
302 properties. TPC and TFC were strongly correlated with antioxidant properties, whereas

vitamin C had a relatively low correlation with antioxidant properties, revealing that the lemon pomace waste is a great source of TPC and TFC, which are the major source of antioxidants.

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Figure Captions

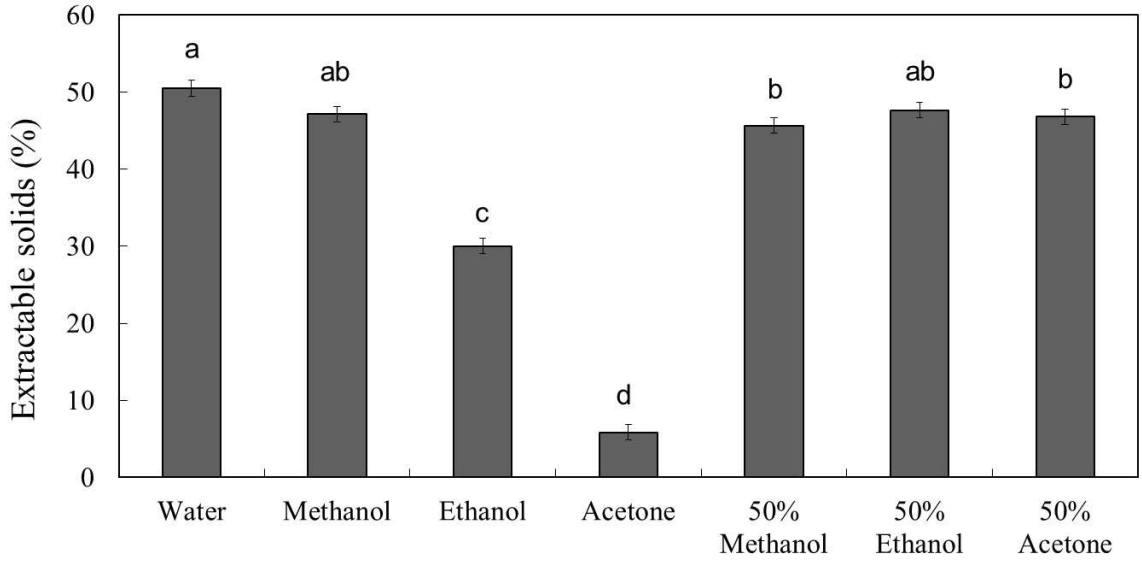

Fig. 1. Effect of solvents on the extractable solids from lemon peels. The values are the mean average of three replications for each solvent ± standard deviation. Columns not sharing the same superscript letter are significantly different $p < 0.05$.

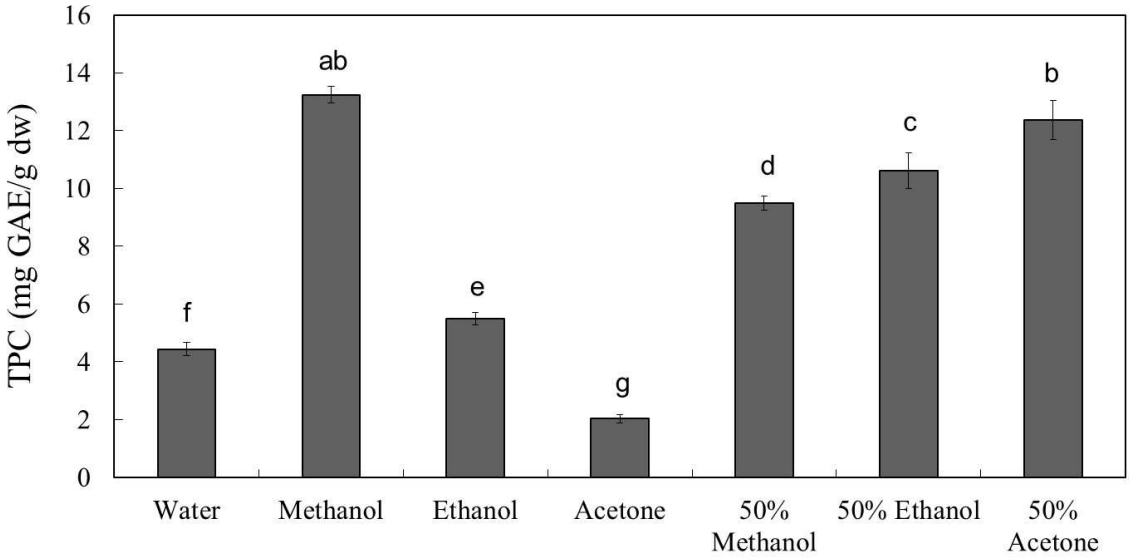

Fig. 2 Effect of solvents on the recovery of total phenolic compounds (TPC) from lemon peels. The values are the mean of three replications for each solvent ± standard deviation. Columns not sharing the same superscript letter are significantly different at $p < 0.05$.

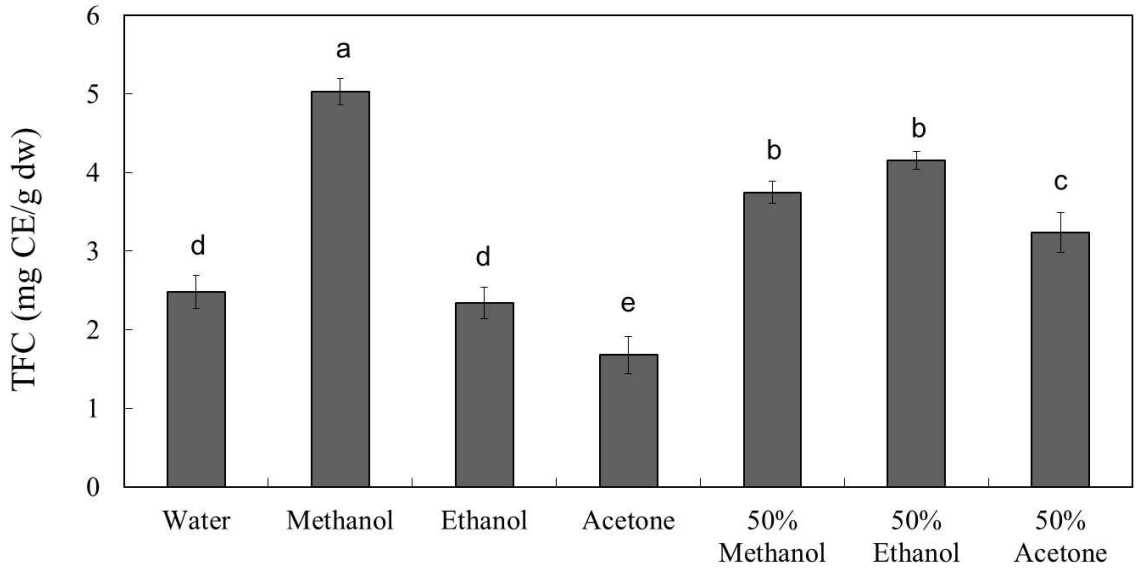

Fig. 3. Effect of solvents on the recovery of total flavonoid content (TFC) from lemon peels. The values are the mean of three replications for each solvent ± standard deviation. Columns not sharing the same superscript letter are significantly different at $p < 0.05$.

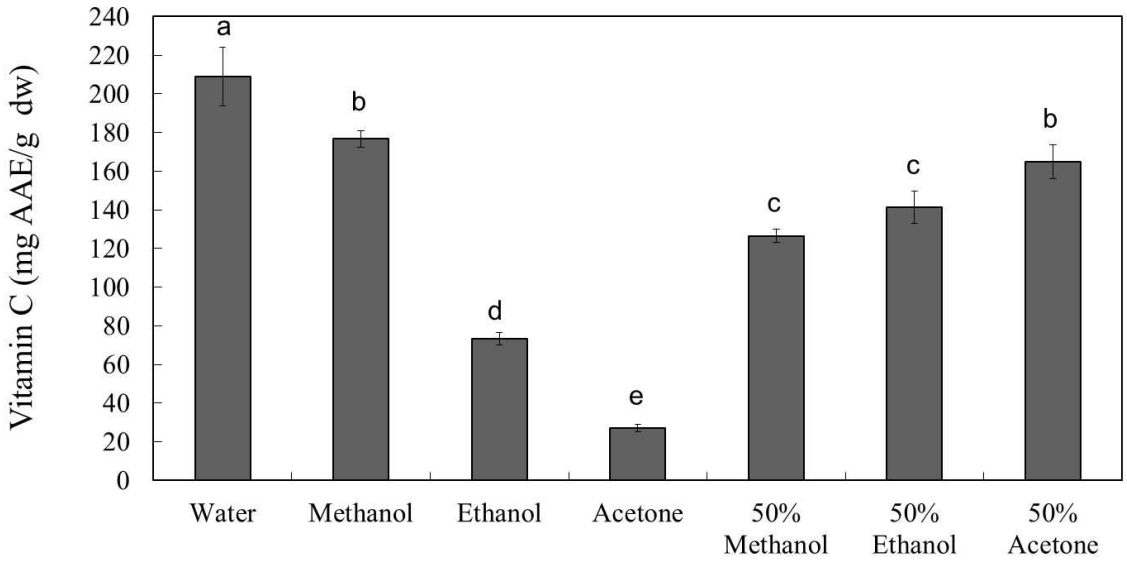

Fig. 4. Effect of solvents on the recovery of vitamin C from lemon peels. The values are the mean of three replications for each solvent ± standard deviation. Columns not sharing the same superscript letter are significantly different at $p < 0.05$.

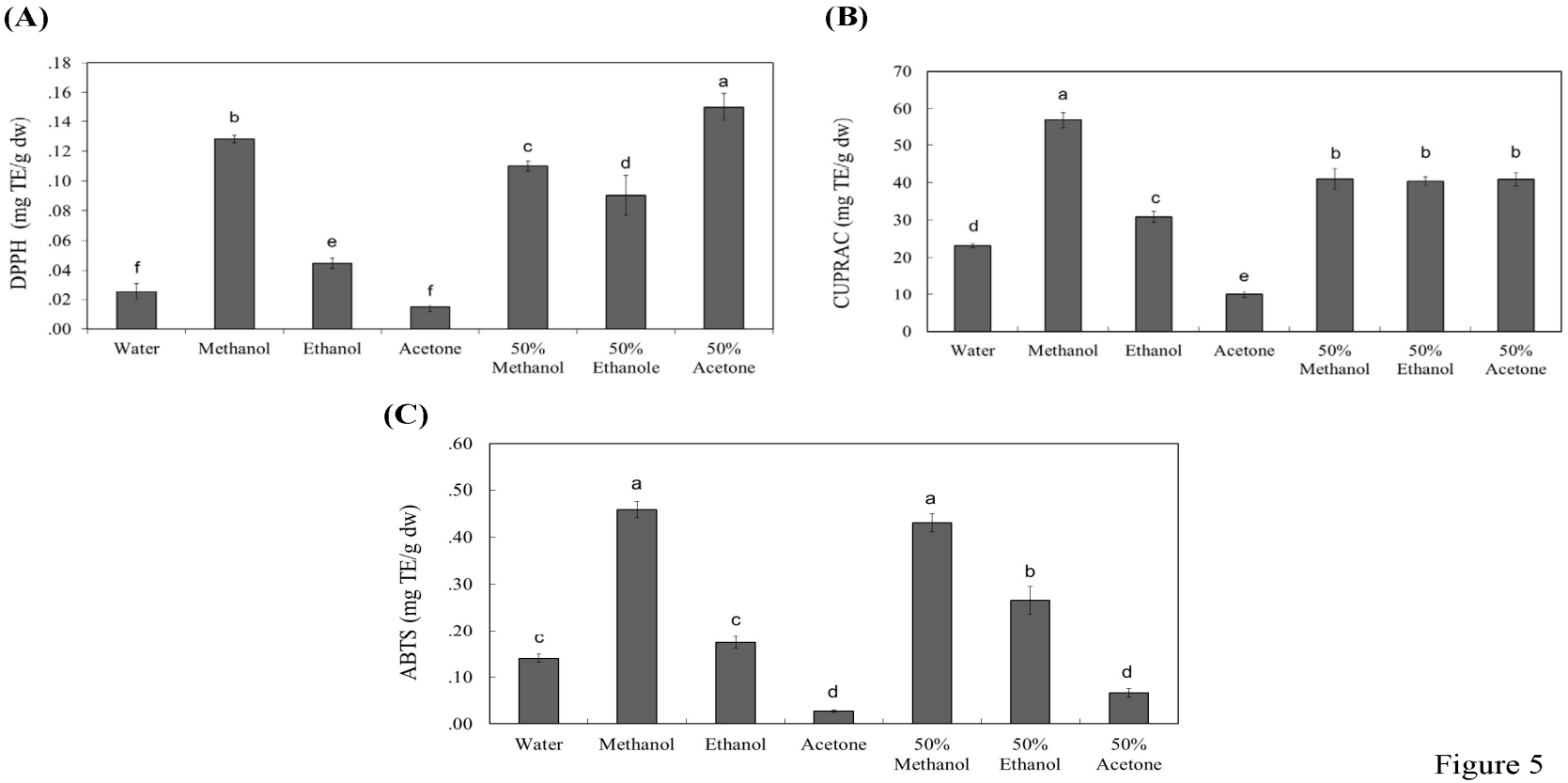

Figure 5

Fig. 5. Effect of solvents on the recovery of antioxidant properties from lemon peels using various antioxidant assays such as DPPH (A), CUPRAC (B) and ABTS (C). The values are the mean of three replications for each solvent ± standard deviation. Columns not sharing the same superscript letter are significantly different at $p < 0.05$.

Table 1 Correlation between bioactive compounds and antioxidant properties of lemon peels (p <0.01).

	Total Phenolic Compounds		**Total Flavonoids**		**Vitamin C**	
	r	***p value***	***r***	***p value***	***r***	***p value***
DPPH	**0.95**	***	**0.75**	***	**0.45**	*
CUPRAC	**0.94**	***	**0.91**	***	**0.55**	*
ABTS	**0.59**	**	**0.80**	***		