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ENHANCING PRODUCTION THROUGH OPTIMISATION OF DPPH AND RADICAL SCAVENGING ACTIVITY OF GRAPE SEED EXTRACTS

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ABSTRACT

Polyphenols are important for their pharmacological activity and positive contribution to cellular processes within the body. They have the capacity to protect against oxidation of High Density Lipids (HDL) and, thus help the body to retain HDL, while removing the problematic Low Density Lipids (LDL). Polyphenols also possess anti-ulcer, anti-carcinogenic and anti-mutagenic activities. The objective of this study was to evaluate the effect of temperature and grain size of grape seed on the efficiency of extraction of polyphenols from grape seed, using the compressed hot water and solvent extraction techniques. Polyphenols were extracted from milled (<0.5 mm) and whole grape seed, using compressed hot water (high temperature and high pressure) and solvents (Acetone, Methanol and Ethanol). The total polyphenol content and DPPH radical scavenging activity of the extracts were determined using spectrometer and the active compounds identified using HPLC. Total polyphenol content increased with extraction temperature, but decreased at 200 °C. The difference in polyphenol extracts from the milled and whole seed decreased with increase in temperature, but was more evident at 135 °C. The 2 hour extracts showed relatively higher values than those for 1 hour, with the lowest difference occurring at 165 °C and the highest at 180 °C. Solvent extracts from whole seeds were very low compared with the milled seeds, with acetone showing the highest value of 105 mg g^{-1} dry matter for polyphenol content and 110 mg g^{-1} of dry matter for DPPH radical scavenging activity. Methanol had the lowest value (78 mg g^{-1} dry matter) for polyphenol extracts and 80 mg g^{-1} for the DPPH radical scavenging activity. The main extract compounds were gallic acid, catechin and epicatechin.

Key Words: High density lipids, polyphenol, radical scavenging

RÉSUMÉ

Les polyphénols sont importants eu égard à leur activité pharmacologique et leur contribution positive aux processus cellulaires dans le corps. Ils sont doués d'une capacité protective contre l'oxydation des Lipides à Densité Elevée (HDL) et, ainsi aident le corps à maintenir le HDL, tout en éliminant les problématiques Lipides à Basse Densité. Les polyphénols possèdent aussi des activités anti-ulcères, anticarcinogènes et antimutagènes. L'objectif de cette étude était d'évaluer les effets de la température et taille des grains de raisin sur l'efficacité de l'extraction des polyphénols des grains de raisin, utilisant une eau chaude compressée et des techniques d'extraction au solvant. Les polyphenols étaient extraits des grains entiers de raisin et des grains moulus (<0.5 mm) à l'aide d'une eau chaude compressée (température élevée et haute pression) et des solvants (Acétone, Méthanol et Ethanol). La concentration totale des polyphénols et l'activité d'absoption du radical DPPH des extraits étaient déterminés à l'aide du spectrophotomètre et les composés actifs identifiés par HPLC. La concentration totale des polyphénols a augmenté avec la température d'extraction, mais a diminué à 200 °C. La différence dans les extraits de polyphénol des grains moulus et entiers a diminué avec l'augmentation de la temperature, mais était plus

évidente à 135 °C. Les extraits de deux heures ont montré des valeurs relativement plus élevées que ceux d'une heure, avec les différences les plus faibles apparaissant à 165 °C et les plus élevées à 180 °C. Les extraits de graines entières aux solvants étaient de faible quantité en comparaison avec ceux des grains moulus, les valeurs les plus élevées étant de 105 mg g^{-1} de matière sèche de polyphénol et 110 mg g^{-1} de matière sèche d'absorption du radical DPPH obtenues en utilisant l'acétone comme solvant. Les extraits obtenus au méthanol comme solvant étaient encore en plus faible quantité avec 78 mg g^{-1} de la matière sèche pour les extraits du polyphénol et 80 mg g^{-1} pour l'activité d'absorption du DPPH. Les principaux composés de ces extraits étaient des acides galliques, des catéchines et des epicatéchines.

Mots Clés: Lipides à densité élevée, polyphénol, radical de fouille

INTRODUCTION

Plant polyphenols are the most important group of natural antioxidants because of their diversity and extensive distribution. They possess the ability to scavenge both active oxygen species and electrophiles. Recent investigations have shown that many phenolic compounds, including flavonoids, tannins and phenolic acids, exhibit strong antioxidant properties. In some fruits, polyphenols with antioxidant properties such as flavonoids, procyanidins and anthocyanins, have been identified by High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR) or Mass Spectrometer (MS) methods (Sun *et al.,* 2007).

Several methods have been used to approximate the antioxidant efficiency of natural extracts, and one such common method is the diphenylpicrylhydrazyl (DPPH) radical scavenging activity method. Some of the areas of application of antioxidants that are of great concern to human beings include aging and age-related diseases. Attention has, therefore, been focused on plant resources that contain physiologically active phenolic compounds that show chelation and antioxidative effects of the radicals responsible for the aging process (Barclay, 2008).

Antioxidant compounds are chemical substances that donate an electron to the free radical and convert it to a harmless molecule. They are able to intercept free radicals and protect cells from the oxidative damage that cause aging and age related diseases. They also prevent injury to blood vessel membranes, help to optimise blood flow to the heart and brain, provide defence against cancer-causing DNA damage, and help to lower the risk of cardiovascular disease and dementia including Alzheimer's disease (Duthie, 1999; Barclay, 2008).

There is increasing consciousness among consumers about the role of food in the management of lifestyle diseases, and more people are now adopting natural and healthy body care practices. Thus, more healthy products are being developed and recognised as useful commercial products. One of the well known sources of such healthy products is the grape seed. Effective and efficient extraction of polyphenols from grape seeds has, however, remained one of the biggest challenges. Use of water as a solvent has been recommended by many researchers (Murga *et al.*, 2002; García-Marino, 2006; Nawaz *et al.*, 2006; Yilmaz and Toledo, 2006) since it has been found to be safe and efficient. This method is classified as a hydrothermal reaction (reaction with hot water at high pressure). During hydrothermal reaction, the rise in temperature decreases permittivity of the water resulting in change of the ionic compounds. It is, therefore, a simple process through which extractions can be done at about 180 °C. Such high temperature of extraction falls in the super-criticality and sub-critical regions (Amano, 2006). Although much work has been done on the properties of grape seed extracts, optimisation of effects of temperature and grain size of grape seed on the efficiency of extraction of polyphenols from grape seed using the compressed hot water and solvent extraction techniques has not been done.

The objective of this study was to evaluate the effect of temperature and grain size of grape seed on the efficiency of extraction of polyphenols from grape seed using the compressed hot water and solvent extraction techniques.

MATERIALS AND METHODS

Seed preparation. The test material used was grape (*Vitis vinifera*) seed (cv. Campbell Early) from a wine factory in Iwate Prefecture, Japan. The grape seed was washed sufficiently in a laboratory with tap water, allowing it to attain 20% moisture content. The seeds were then dried in a constant temperature oven (SANYO, MUV-212) to 13% moisture content and stored under refrigeration at 4 °C.

Variation of grain size was attained through pulverisation using a rice mill (National, MK-51M, Japan). Pulverisation was done intermittently using a rice mill at 10 seconds interval to minimise heat generation. The seeds were milled using a rice mill and sieved using a 0.5 mm screen sieve to obtain samples of <0.5mm. Before extraction, about 5 g of the seed and milled sample were weighed and used to determine the moisture content using the oven method at 105 °C for 24 hours. The final dry weight of the sample was obtained at equilibrium and used in the computation of the wet and dry basis moisture content.

Sample extraction

Hydrothermal extraction. Three samples were extracted each at 80, 105, 120, 135, 150, 165, 180 and 200 °C. A sample of 0.4 g was taken and put in a batch type titanium autoclave container of 80 ml capacity (40 mm diameter, 125 mm length and 4 mm thickness) (Figs. 1 and 2). A volume of 40 ml of distilled water was then added before tightly closing the container and putting it in a hot air oven for 1 and 2 hours for each sample.

After extraction, the titanium autoclave container was removed from the oven and cooled suddenly with cold tap water. Samples from the container were filtered using a 0.2 µm micro-filter (ADVANTEC, DISMIC-25cs) as described by Xu *et al.* (2003), Buciæ-Kojiæ *et al.* (2007) and Wiboonsirikul *et al.* (2008). The filtered sample (Fig. 3) was then subdivided into 1.5 ml tubes and frozen in liquid nitrogen before being stored in a freezer. The frozen sample was only removed and thawed at room temperature for analysis.

Organic solvent extraction. Solvent extraction was carried out using 50% proportion of acetone, methanol and ethanol solutions separately at 25 °C. A total of 0.4 g of whole seed and milled samples (<0.5 mm) were put in a conical flasks

Figure 2. Titanium extractor.

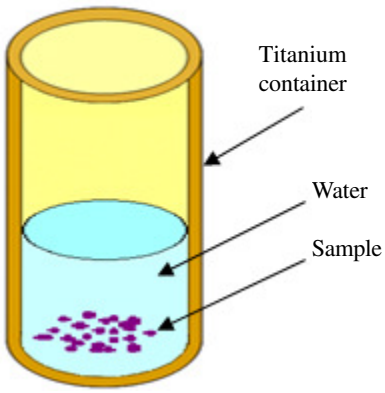

Figure 1. Sketch of Titanium extractor.

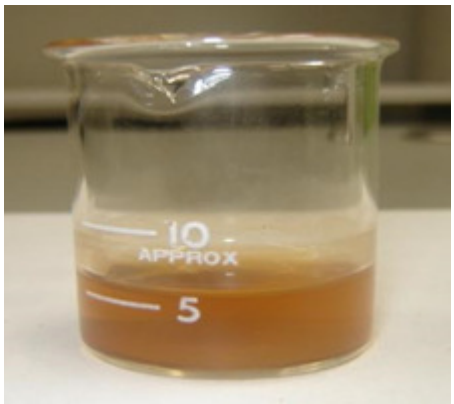

Figure 3. Grape seed extract at 200 °C (2 hrs).

and 40 ml of the solvent added. The mixtures were covered with aluminium foil and allowed to settle, without shaking, for 2 hours. The extract was then filtered using a 0.2 µm micro-filter (ADVANTEC, DISMIC-25cs). The filtrate was directly used for analysis.

Sample analysis

Polyphenol content. Total polyphenol content was determined using the Folin-Ciocalteu method, outlined by the Journal of the Japanese Society for Food Science and Technology (2006). The samples from the fridge were warmed to melt and diluted five times for those extracted between (80-165 °C); and ten times for those extracted between 180-200 °C, using distilled water as per equipment specification. A volume of 0.1 ml of the diluted sample was added to 1.6 ml of distilled water before adding 0.1 ml of Folin-Ciocalteu liquid and 0.2 ml of 20% $NaCO_3$. The mixture was homogenised using an electric stirrer and allowed to react under darkness for 30 minutes. The absorbance of the mixture was measured at 760 nm, three times using a UV/VIS spectrometer (JASSO, V-530). The spectrometer absorbance was calibrated using gallic acid solution of 10 mM L^{-1} obtained by dissolving 170.1 mg of gallic acid in 100 ml of distilled water. The solution was diluted to give 5, 2, 1 and 0.5 mM L^{-1} solutions, which were used in place of the sample. The spectrometer reading of the diluted solutions was plotted against their concentrations to derive the calibration curve to standardise the sample spectral readings. The total phenol content was, therefore, calculated as the weight of gallic acid equivalent per dry matter content of the sample. The test was repeated twice for the temperature range of 150-180 °C, in order to improve accuracy of results.

DPPH Radical scavenging activity. The DPPH radical scavenging activity was determined according to the methods by Ye *et al.* (2009) and Leong and Shui (2002), with minimal modification. DPPH (1-1Diphenyl-2Picrylhydrazyl) solution was prepared by dissolving 8 mg of DPPH in ethanol and then diluting it with distilled water to make 50 ml of DPPH solution. A total of 0.2 ml of water was mixed with 1.8 ml of DPPH solution and its absorbance measured immediately using the spectrometer set at 517 nm to give the reading C_0. A similar sample was kept in a dark cabin at 25 °C for 30 minutes and its absorbance then measured to give the reading C_{30}. A measure of 0.2 ml of the diluted sample was added to 1.8 ml of DPPH solution and 1.8 ml of distilled water. The samples were shaken and kept at 25 °C in a dark cabin for 30 minutes. The absorbance of the samples was then measured by the spectrometry to give the readings C_D for DPPH mixture and C_W for water mixture. The DPPH radical scavenging activity of the sample (SA) was computed as:

$$SA = \frac{\{C_0 - (C_D - C_W)\} \times 100}{C_{30}} \quad \text{..... (1)}$$

Calibration of the spectrometer was done using Trolox (97%) solution of 1 mM L^{-1} obtained by dissolving 25 mg of the organic compound in 100 ml of water. The solution was diluted into four different portions of 0.5, 0.4, 0.2 and 0.1 mM L^{-1}. The four portions were then used in place of the sample during the spectrometer analysis. A calibration curve of SA value for Trolox against Trolox concentration was plotted and its gradient used to approximate the actual SA value of the sample reported as mg of Trolox equivalent per g of dry matter.

HPLC analysis. The High Performance Liquid Chromatography analysis was carried out using the LC-20A type (Shimazu-Japan) analyser using 23% Methanol and 77% phosphoric acid mixture as column fluid flowing at 1.0 ml min^{-1}. The test column temperature, pump pressure and wavelength were set at 40 °C, 6.0 MP and 280 nm, respectively. The samples were diluted 5, 10 or 20 times based on their concentration. Measurement was done by injecting 20 µl of the diluted sample into the analyser resulting in the print out of the sample chromatogram. The calibration of the analyser was done using gallic acid, catechin, epicatechin, epigallocatechin and epigallocatechin gallate. Using the sample chromatogram area and that of the calibration compound with the same retention time, the compounds in the sample were identified and quantified in mg 100 ml^{-1}. The tests were repeated twice and the average computed. Statistical

analysis was done using the Tukey method, at five percent confidence level ($P < 0.05$).

RESULTS AND DISCUSSION

Compressed hot water extraction

Polyphenol content. The polyphenol content in the extracted samples generally increased with increase in temperature, with the fine milled sample showing the highest value (Fig. 4). The extractable value for seeds more than doubled between 80 °C (hot water extraction) and the 120 °C (compressed hot water extraction) with a similar trend occurring between 120 and 135 °C. The extractable values for milled seeds increased gradually with temperature, increasing sharply at 180 °C and then decreasing at 200 °C. The decrease could be attributed to denaturing of polyphenols due to high temperatures. There was a significant difference in the extractable values from whole and milled seeds between 80 and 120 °C and at 180 °C (Fig. 4). However, between 135 and 165 °C and at 200 °C, no significant difference ($P>0.05$) was observed, indicating that whole seed would be suitable for polyphenol extraction.

Extraction time between one and two hours, showed no significant variation in the extractable values except at 180 °C (Fig. 5). This implies that extraction time could significantly be reduced with minimal effect on the efficiency of polyphenol extraction.

DPPH radical scavenging activity. The DPPH radical scavenging activity of grape seed generally increased with temperature and reduction in grain size, with a sharp increase for seed between 80 and 105 °C and between 120 and 135 °C. The extractable values for milled seed, however, increased gradually over the whole temperature range, showing a decrease at 200 °C (Fig. 6). The extractable values after one hour of treatment were generally lower than those after two hours. The difference between the two, however, decreased with increase in temperature (Fig. 7).

There was a strong linear correlation between polyphenol content of whole seed and the DPPH radical scavenging activity ($r^2 = 0.974$, Fig. 8). The relationship showed that the DPPH radical scavenging activity of the seed extract was higher than the polyphenol content by about 20%, irrespective of temperature and form of seed used to extract the compounds. The relationship for milled seed was, however, exponential, with a correlation coefficient of $r^2 = 0.953$ (Fig. 9). In this case, the DPPH radical scavenging activity was still high.

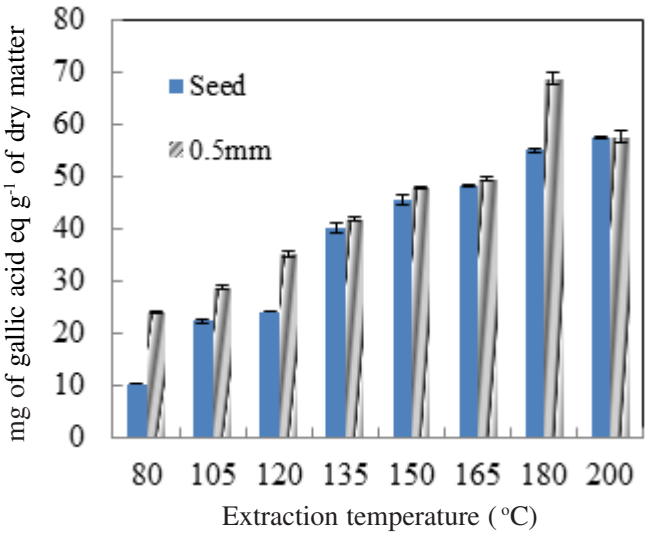

Figure 4. Effect of temperature and seed size on polyphenol content of whole and milled grape seed.

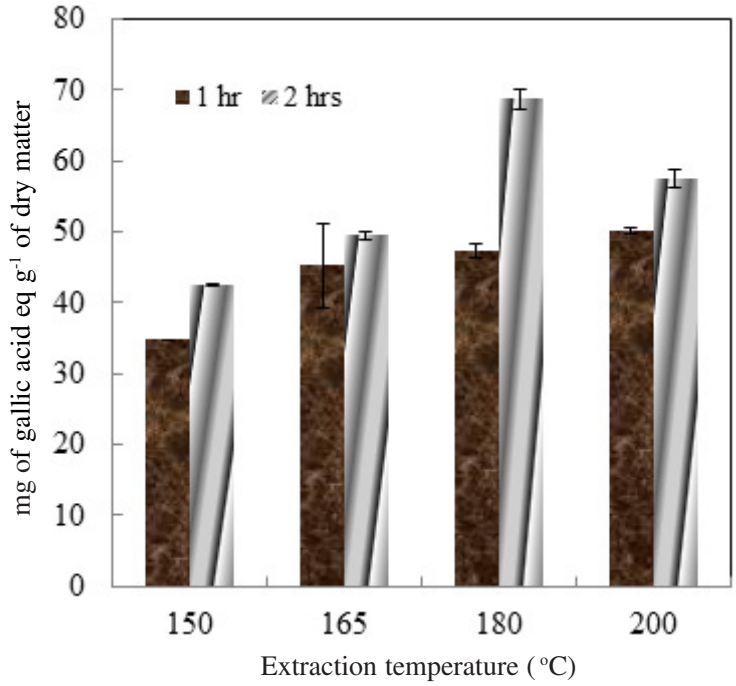

Figure 5. Effect of time and temperature on polyphenol content of milled grape seed.

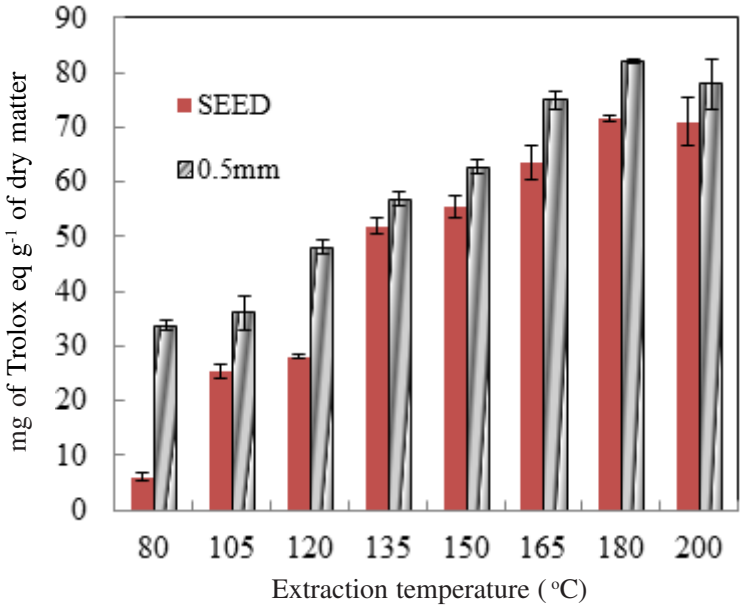

Figure 6. Effect of temperature in DPPH radical scavenging activity of whole and milled grape seed.

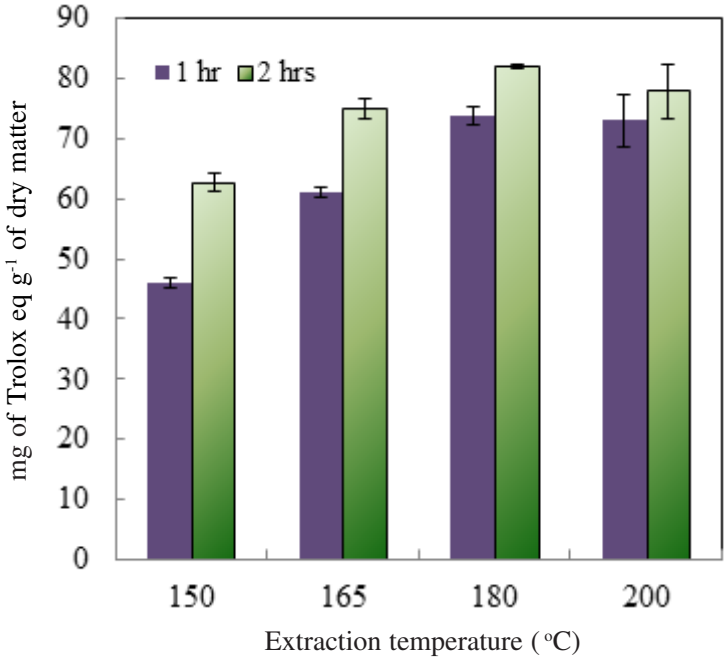

Figure 7. Effect of time and temperature on milled grape seed (<0.5 mm).

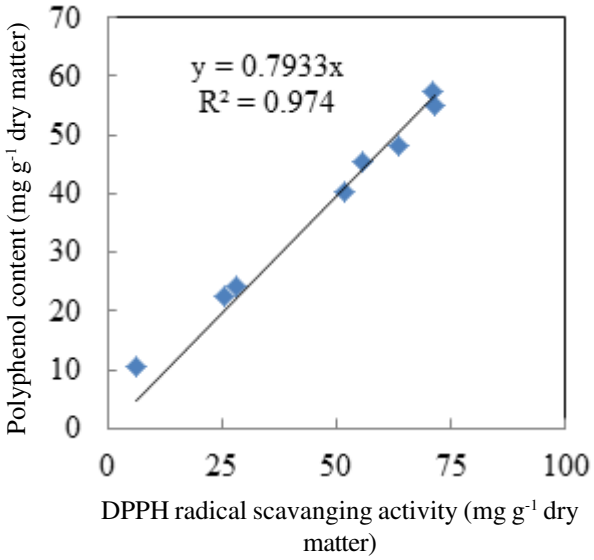

Figure 8. Correlation of polyphenol and DPPH for whole grape seed.

HPLC analysis. Based on HPLC analysis, four types of organic compounds were identified in the compressed hot water extracts at all temperatures (Fig. 10). The identified compounds were mainly Gallic acid (GA), Catechin (CT), Epicatechin (ECT), Epigallocatechin (EGCT) and Epigallocatechin gallate (EGCTG). There were, however, many other compounds which were not identified.

The content of organic compounds increased slightly with increase in temperature, with an extract content of less than 10 mg/100 g^{-1} of dry

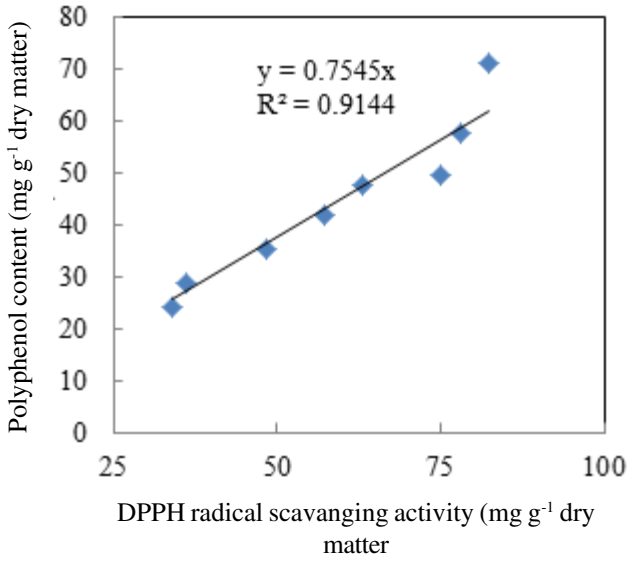

Figure 9. Correlation of polyphenol and DPPH for milled grape seed.

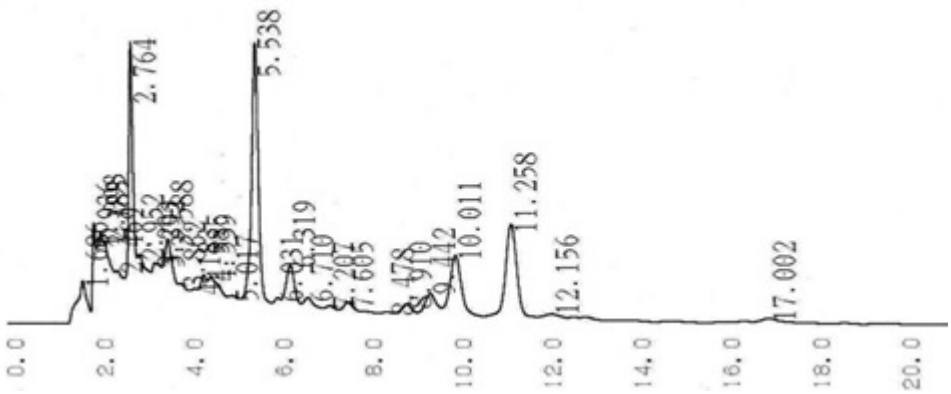

Figure 10. HPLC Chromatograph of milled grape seed extracts at 120 °C.

matter for both whole and milled grape seeds. The trend was, however, different for the catechin which increased significantly above 150 °C and sharply at 200 °C to 113 mg 100 g^{-1} of dry matter (Figs. 11 and 12, respectively). This trend agreed with that shown for tea extracts (Xu, 2003), which is indicative of lack of epimerisation of catechin at high temperatures. The whole seed extracts had generally low contents, particularly at temperatures below 165 °C, contrasting with the data for the milled seeds extracts. The trend, however, changed at higher temperatures, which shows increased ease of release of organic compounds from the seeds. This finding suggests that whole seeds could effectively be used for organic compounds extraction at high temperatures without milling, thus saving the milling time energy and cost.

Organic solvent extraction

Polyphenol content. Polyphenol content for solvent extracts is shown in Figure 13. Acetone showed the highest extractable polyphenol content for milled grape seeds (79.3 mg g^{-1}); whereas ethanol had the lowest value (36.3 mg g^{-1}). The acetone extract compared very well with that of compressed hot water at 180 °C, which

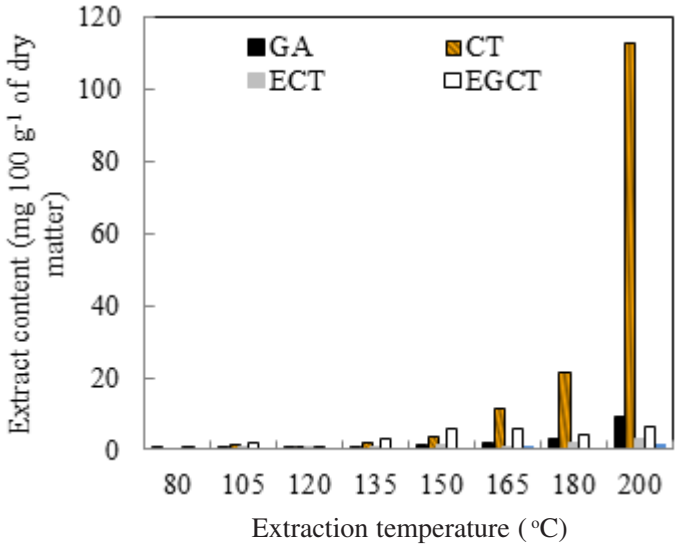

Figure 11. Organic compounds in whole grape seed extract (GA = Gallic acid, CT = Catechin, ECT = Epicatechin, EGCT = Epigallocatechin).

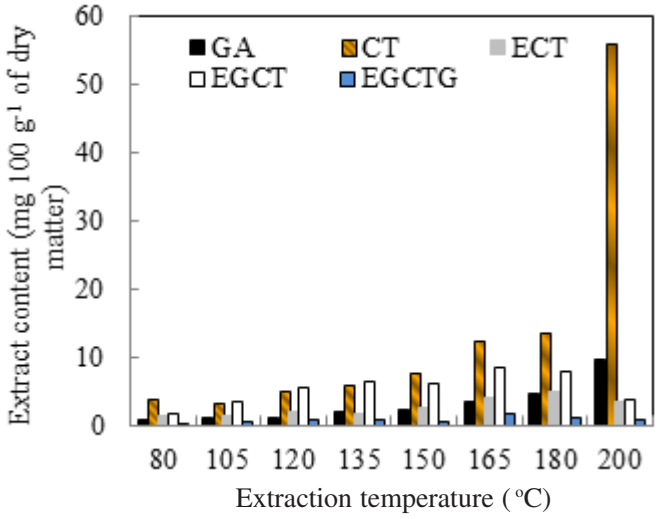

Figure 12. Organic compounds in milled grape seed extract (GA = Gallic acid, CT = Catechin, ECT = Epicatechin, EGCT = Epigallocatechin).

stood at 74.0 mg g^{-1}. The whole seed solvent extracts, however, showed very low polyphenol yields, (<12 mg g^{-1}) compared with 55 mg g^{-1} for compressed hot water. Thus, the solvent can only effectively be used on milled grape seeds.

DPPH radical scavenging activity. The DPPH radical scavenging activity of the solvent extracts, for whole and milled seeds showed a similar trend as that of the polyphenol content (Fig. 14). Milled seed acetone extract had the highest value (104.4 mg g^{-1}), whereas ethanol had the lowest (60.6 mg g^{-1}. The acetone extract compared very well with that of compressed hot water at 180 °C, which yielded 82.3 mg g^{-1}. However, the solvent extracts from whole seeds yielded very low values of less than 7 mg g^{-1} compared with compressed water yield of 71.4 mg g^{-1}. This showed that the

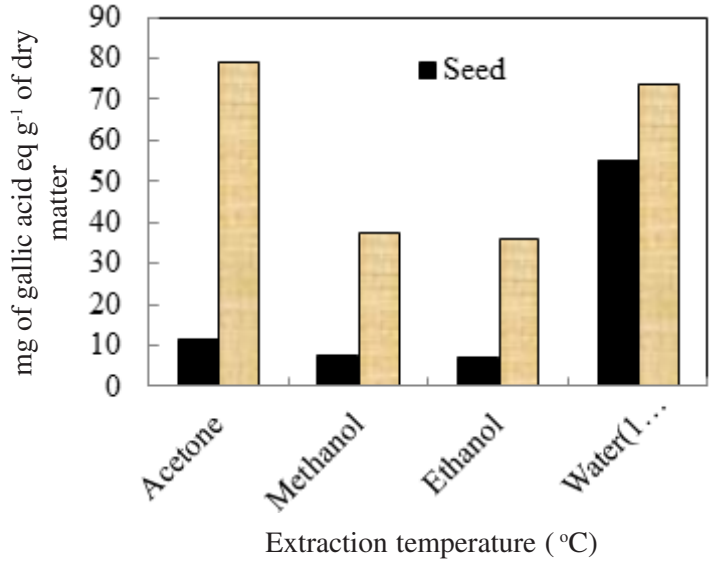

Figure 13. Polyphenol content of solvent extracts of whole and milled seeds.

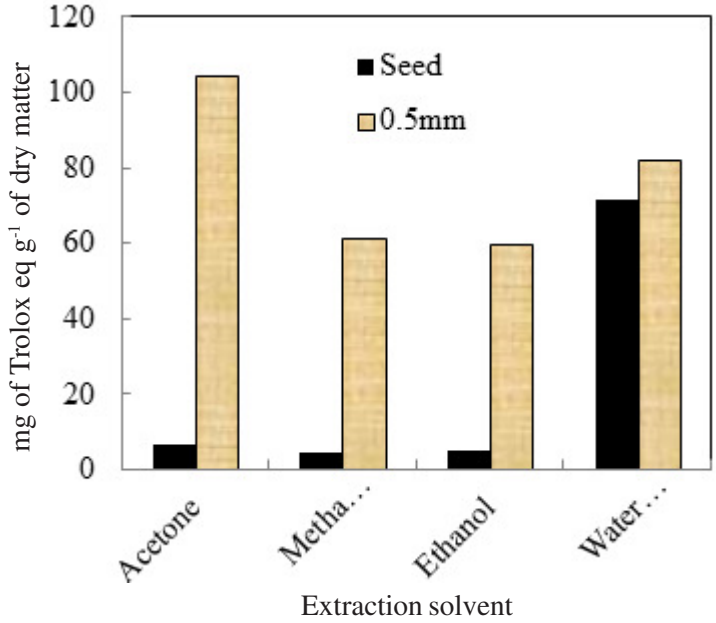

Figure 14. DPPH Radical scavenging activity for solvent extracts of whole and milled seeds.

solvents were unsuitable for polyphenol extract from whole grape seeds.

There was a strong correlation ($r^2 = 1$) between the polyphenol content and DPPH radical scavenging activity of the solvent extracts for milled seeds compared to the whole seed (r^2 = 0.8790, Figs. 15 and 16). In both cases, however, the DPPH radical scavenging activity was generally higher than the polyphenol content in the milled seed extracts, showing the highest difference of over 20 mg g^{-1} of dry matter. This correlation was the first of its kind, showing that an empirical relation can be developed between the tested parameters. It also clearly indicated

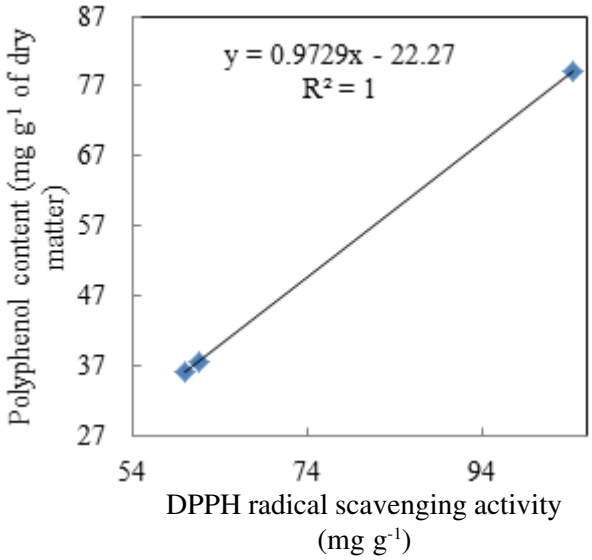

Figure 15. Polyphenol and DPPH comparison for solvent extracts of milled grape seeds.

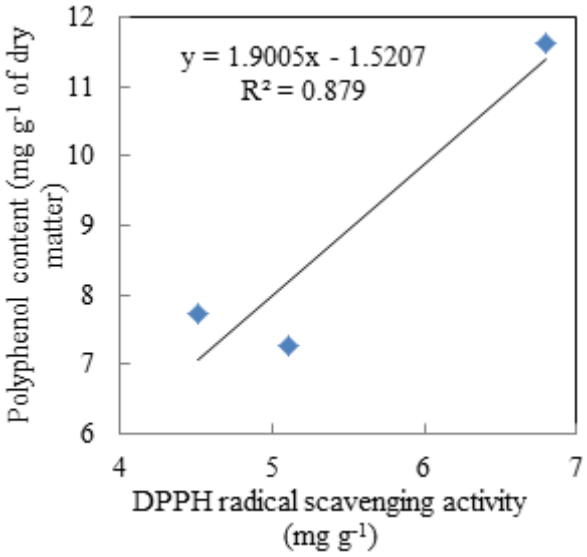

Figure 16. Polyphenol and DPPH comparison for solvent extracts of whole grape seeds.

the effect of the grain size when carrying out extraction of specific compounds from the seeds thus establishing the base for effective and efficient compounds extraction.

HPLC analysis. HPLC analysis chromatogram for whole grape seed acetone extracts is shown in Figure 17. The main peak was located at 2.712 minutes for gallic acid; whereas the other compounds were significantly low. Based on the calibration chromatograms, the other minor organic compounds in the solvent extracts were catechin, epicatechin, epigallocatechin and epigallocatechin gallate.

Gallic seemed to be the only main compound extracted from the seeds (S) by acetone (Fig. 18). The acetone extract from seeds had over 129 mg 100 g^{-1} of dry matter compared to the next highest

Figure 17. HPLC Chromatogram for grape seed acetone extract.

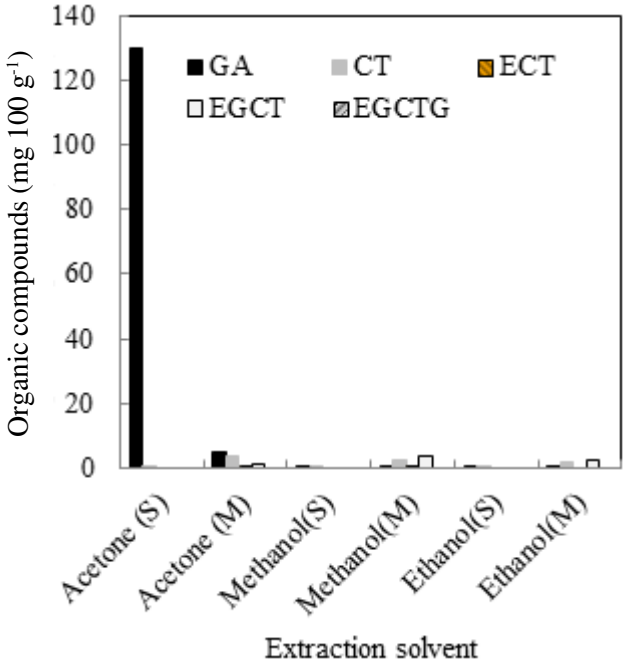

Figure 18. Identified organic compounds in grape seed solvent extracts (GA = Gallic acid, CT = Catechin, ECT = Epicatechin, EGCT = Epigallocatechin).

extract of catechin (4 mg 100 g^{-1} dry matter) by acetone from milled grape seeds (M). It is not clear why acetone extract from milled grape seed showed low gallic acid concentration; but this may be attributed to the epimerisation of catechin during milling. Thus, based on the solvent extraction, acetone was an effective solvent for gallic acid extraction from grape seed without milling. Such similar results for grape seed have not been documented before.

CONCLUSION

The results confirm the presence of anti- oxidising compounds in grape seed, with compressed hot water extraction effectively being used for organic compounds extraction. Higher temperatures above 135 °C for two hours are effective for whole grape seed, thus reducing the need for milling. Reduction in extraction time and temperature above 200 °C; however, shows

decrease in extraction efficiency. Temperature and time are major factors contributing to the compressed hot water extracts content. The main organic compounds identified are catechin, epicatechin, epigallocatechin and gallic acid in decreasing order. Epigallocatechin gallate only occurs in trace levels. Although compressed hot water extraction is the common method for polyphenol extraction, acetone shows very high effectiveness in the extraction of gallic acid from whole seeds. There is, however, need for further research to perfect the extraction of polyphenols from grape seed. This will include evaluation of high temperature effects above 200 °C and effectiveness of other commonly used solvents. A model can then be developed to simulate the extraction process and the extraction condition optimised for economic operation.

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