Separation and Identification of Phenolic Acids from Red Grape Seeds by HPLC

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Abstract Red grape seed extract (GSE) was examined for its phenolic acids content. Grape seed powder was defatted twice with hexane for 1h and the defatted residues were kept in methanol for 2h with gentle agitation then centrifuged at 5000rpm for 10 minutes, and the precipitates were extracted using 2M NaOH for 4h with gentle agitation, acidified with HCl to pH 2.2 and centrifuged at 5000 rpm for 10 minutes. The pooled supernatants were extracted using a separating funnel. The pooled extracts were collected, the solvent was evaporated, and the extracted residues were dissolved in methanol: water (4:1 v/v) and subjected to analysis by high-performance liquid chromatography (HPLC). Ferulic and coumaric acids were abundant among other phenolic acids detected in GSE, as their concentrations reached 103.16±6.18 and 93.66±3.42 mg/100g dry weight (DW) respectively. Caffeic, gallic, hydroxybenzoic, and vanillic acids were also identified and quantified in GSE but were at relatively low concentrations of 0.05±0.01; 2.37±0.07; 1.19±0.03; and 1.96±0.04 mg/100g DW respectively. Moreover, chlorogenic and syringic acids were also identified in GSE however; they were not quantified. The results of the present study indicated that red grape seeds contain considerable concentrations of ferulic and coumaric acids. These findings support the postulation that grape seeds have a potential for application in the pharmaceuticals and functional food industry.

Keywords: grape seed extract, Ferulic acid, Coumaric acid, Caffeic acid, Vanillic acid, p-Hydroxybenzoic acid

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1. Introduction

Increasing attention has been focused on the study of substances with protective effects against free radicals, and reactive oxygen and nitrogen species. Free radicals, and reactive oxygen and nitrogen species can cause oxidative stress resulting in damage to several biomolecules, which consequently leads to serious illness and chronic disease [1]. Plants, including cereals, fruits, herbs, and vegetables have various bioactive compounds that have anti-inflammatory, antibacterial, anti-cancer, anti-atherogenic, and anti-diabetic effects [2]. Wongnarat and Srihanam [3] studied the antioxidant activities of white and red grape seed cultivars grown in Thailand, and reported that their total phenolic content was 151.56±23.01 and 481.25±25.41 mg GAE/g DW, respectively, whereas their total flavonoid content was 133.08±2.14 and 330.60±13.43 mg CE/g DW, respectively. Phenolic compounds from the seeds of three wild grapevine species were studied by Weidner et al. [4] using either 80% methanol or 80% acetone, and the authors attributed the antioxidant and anti-radical activities of these extracts to their high tannin and catechin contents. Grape seeds have been reported to have a considerable amount of polyunsaturated fatty acids, including linoleic acid (ω-6) which present at a concentration of about 60% [5].

Both grape seeds and skin are reported to possess a high concentration of tocopherol that reached 402.28 and 231.6 mg/Kg, respectively, and the powerful antioxidant activity of grape seeds extract was attributed to its high tocopherol content [6]. Pinent et al. [7] orally administered procyanidins extracted from grape seeds to streptozotocin - induced diabetic rats and concluded that the anti-hyperglycemic effect of procyanidins from grape seeds may be attributed to its insulin-like effect in insulin sensitive cells. Grape seed polyphenols showed potent protective effects against high glucose- induced oxidative stress and inhibit cytotoxicity in renal cells [8].

Several health benefits of grape seeds have been reported including decreased low-density lipoprotein levels [9,10]. Polyphenols extracted from grape seeds have been reported to enhance endothelial nitric oxide production, thereby improving cardiovascular and endothelial properties in high-risk subjects. [11]. Grape seed extract was reported to reduce the serum levels of total cholesterol, low-density lipoprotein cholesterol, and interleukin-6 in Wistar rats induced with polycystic ovarian syndrome [12].
The antibacterial activity of defatted grape seed extract (GSE) was examined by Jayaprakasha et al. [13] against Gram-positive and negative bacteria. The authors reported that GSE completely inhibited Gram-positive bacteria at a concentration of 850-1000 ppm, whereas Gram-negative bacteria were inhibited at a concentration of 1250-1500 ppm.

Here, we studied the phenolic acid profile of red grape seed extract and highlighted the possible applications of these extracts in different industries.

2. Materials and Methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Methanol and water were of HPLC grade. Other chemicals were of analytical grade and obtained from BDH (Poole, UK).

2.2. Material

Red grape was purchased from the local market in Riyadh. The seeds were manually collected and rinsed with Milli-Q ultrapure water and kept in an electrical oven at 50°C for 2d until reaching a constant weight. The final weight after moisture evaporation were recorded. Grape seed samples were powdered using a Braun Grinder (Germany) and kept in a plastic container away from light [14].

2.3. Methods

Grape seeds powder, 2g was weighed, and the lipid material was removed by adding 20 ml of hexane for 1 h. After defatting was done twice, the hexane layer was discarded, and 20 ml of 75% methanol was added to the residue and kept at 23°C with stirring for 2h. The suspension was centrifuged at 5000 rpm for 10 min. The precipitates were collected, and phenolic acids were extracted with 2M sodium hydroxide and left at 23°C with stirring for 4h. The suspensions were acidified with 6 M HCl to pH 2.2. Phenolic acids were extracted twice using a diethyl ether and ethyl acetate (1:1 v/v) solution [15]. The organic layers (upper layers) were extracted using a separating funnel. The pooled extracts were collected, and the solvent was evaporated using a rotary evaporator under vacuum. After complete evaporation, the residues were dissolved in methanol: water (4:1 v/v), and the final volumes of the extracts were recorded. The phenolic acids in the extracts were subjected to analysis by HPLC.

2.4. High-performance Liquid Chromatography

Analyses were performed using the Jasco LC-4000 high-performance liquid chromatography system, consisting of AS-4050 auto-sampler, two PU-4180 chromatographic pumps, CO-4061 column oven, and MD-4010 PDA detector. The stationary phase was a 5µm (4.6x150mm) Venusil XBP C18 column (Agela Technologies, Wilmington, USA), and the mobile phase was water: methanol: acetic acid at 90:20:5 (v/v/v). The GSE injection volume varied from 0.1-5.0 µL and isocratic runs were performed. Two sets of standard mixtures were prepared for the calibration curves, the first one consisted of coumaric acid, ferulic acid, and caffeic acid, and the second mixture consisted of gallic acid, chlorogenic acid, hydroxybenzoic acid, vanillic acid, and syringic acid. All phenolic acids standards were in the range of 50-200 ng/µL. UV intensity was monitored at 300 nm for caffeic, coumaric, and ferulic acids, and at 280 nm for gallic, chlorogenic, hydroxybenzoic, vanillic, and syringic acids. Data processing was performed using the Jasco ChromNAV version 2.0 software.

2.5. Statistical Analysis

All experiments were performed in triplicate. The results are presented as mean and standard deviation, which were calculated using the MS Excel 2010 program.

3. Results and Discussion

The chromatographic pattern of a mixture consisted caffeic, coumaric, and ferulic acid standards, with each phenolic acid at a concentration of 50 ng/µL, is shown in Figure 1. Correlation coefficients, retention times, and slopes of the calibration curves are shown in Table 1.
Table 1. Correlation coefficients, retention times, and the slopes of caffeic, coumaric, and ferulic calibration curves

| Phenolic acid | Slope | Correlation coefficient | Retention time (min.) |
|---------------|-------|------------------------|-----------------------|
| Caffeic (CF)  | $y = 22408.2 + 1543.78x$ | 0.986243 | 4.513 |
| Coumaric (CO) | $y = 30745.5 + 2175.72x$ | 0.986947 | 8.193 |
| Ferulic (FA)  | $y = 16057.2 + 1264.25x$ | 0.989416 | 10.163 |

Figure 2 shows the chromatographic pattern of GSE in terms of caffeic, coumaric, and ferulic acids. Ferulic acid concentration was found to be 103.16mg/100g DW in GSE, whereas, coumaric acid was present at a concentration of 93.66mg/100g DW.

Caffeic acid has also been identified and quantified in GSE, however, it was found at a low concentration of 0.05mg/100g DW (Table 2). Tang et al. [16] reported the presence of ferulic acid in black grape seeds at a concentration of 24.1mg/100g, however, our findings regarding ferulic acid content in red grape seeds was five times higher (103.16mg/100g) than what was previously reported. Ferulic acid has been reported to improve the lipid profile in diabetic rats, thereby preventing the progression of diabetic complications [17]. Ferulic acid has been reported as an additive in the food industry [18]. The inclusion of ferulic acid in the manufacture of cosmetic and skin lotions has also been reported [19]. The hepatoprotective effect of ferulic acid against rats that were administered with ethanol has also been reported [20]. Our results showed that grape seed extract has a considerable concentration of coumaric acid (93.66mg/100g). Amalan et al. [21] reported that coumaric acid has a significant effect on glucose and lipid metabolism, and improved metabolic disorders through GLUT2 activation. Based on Amalan et al. [21].

Table 2. Caffeic, coumaric, and ferulic acid content in red grape seed extracts

| Phenolic Acid | Red grape seed extract (mg/100g DW) |
|---------------|-----------------------------------|
| Caffeic (CF)  | 0.05± 0.01                        |
| Coumaric (CO) | 93.66± 3.42                       |
| Ferulic (FA)  | 103.16± 6.18                      |

Data are expressed as mean ±SD for three samples.

Our findings support that coumaric acid from GSE can have pharmaceutical applications. The phenolic content in seed, cane, leaf, skin, and pulp of five native grape cultivars in West Azerbaijan province, Iran was investigated by Farhadi et al. [22], who reported the presence of caffeic acid in grape seeds at a mean concentration of 10.3µg/g (1.03mg/100g) which is comparable to our finding regarding the same parameter (0.05mg/100g).

The chromatographic pattern of a mixture consisted gallic, chlorogenic, hydroxybenzoic, vanillic, and syringic acid standards, with each phenolic acid at a concentration of 100 ng/µl is shown in Figure 3. Correlation coefficients, retention times, and the slopes of the calibration curves are shown in Table 3.

Table 3. Correlation coefficients, retention times, and the slopes of gallic, chlorogenic, hydroxybenzoic, vanillic, and syringic acid calibration curves

| Phenolic acid | Slope                  | Correlation coefficient | Retention time (min.) |
|---------------|------------------------|-------------------------|-----------------------|
| Gallic (GA)   | $y = 24487.2 + 1385.49x$ | 0.972618 | 1.707 |
| Chlorogenic (CG) | $y = 11653.9 + 639.014x$ | 0.971021 | 3.463 |
| Hydroxybenzoic (HBA) | $y = 13798.3 + 707.183x$ | 0.971111 | 4.177 |
| Vanillic (V)  | $y = 11059.2 + 622.671x$ | 0.972489 | 5.323 |
| Syringic (SG) | $y = 28538.7 + 1611.78x$ | 0.973834 | 6.217 |
Gallic acid, hydroxybenzoic acid, and vanillic acid were detected and quantified in GSE at a concentration of 2.37±0.07, 1.19±0.03, and 1.96±0.04 mg/100g DW, respectively (Figure 4, Table 4). Although chlorogenic and syringic acids were identified in GSE, the HPLC system failed to quantify them. Stanciu et al. [23] reported the presence of gallic, chlorogenic, caffeic, vanillic, and ferulic acids in black magic grape seeds at a concentration of 294.437, 20.062, 5.868, 7.027, and 16.334 mg/100g respectively. Our finding that the GSE content of gallic acid is 2.37±0.07 was comparable with the reports of Mattos et al. [24] and Farhadi et al. [22], both of which reported that gallic acid content in GSE was 0.98 and 0.81 mg/100g, respectively. Mattos et al., [24] also reported that the caffeic acid in GSE was 0.106 mg/100g which coincides with the result obtained in the present study (0.05 mg/100g). A report conducted by Liu et al. [25], who studied the phenolic content in the pulps of 30 grape varieties, stated that the content of gallic and caffeic acids ranged between 0.219-2.262; and 0.301-2.115 µg/g fresh weight (FW), respectively. It can be concluded that the content of gallic acid is 10 times higher in grape seeds compared with grape pulps, whereas grape pulps contained a caffeic acid level that is four times higher than that in grape seeds.

Table 4. The contents of gallic, chlorogenic, hydroxybenzoic, vanillic, and syringic acids in red grape seed extracts

| Phenolic Acid                  | Red grape seed extracts (mg/100g DW) |
|-------------------------------|--------------------------------------|
| Gallic acid (GA)              | 2.37±0.07                            |
| Chlorogenic acid (CG)         | N.D.*                                |
| Hydroxybenzoic acid (HBA)    | 1.19±0.03                            |
| Vanillic acid (V)             | 1.96±0.04                            |
| Syringic acid (SG)            | N.D.*                                |

Data are expressed as mean ±SD for three samples; N.D.*: Not determined.
4. Conclusion

The results of the present study indicated that red grape seeds contain considerable concentrations of ferulic and coumaric acids, whereas gallic, caffeic, hydroxybenzoic, and vanillic acids were present at a relatively low concentrations. These findings support the postulation that grape seeds have a potential for application in the pharmaceuticals and functional food industry.

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Conflicts of Interest

The author declares no conflict of interest.

References

[1] Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., Milzani, A., "Biomarkers of oxidative damage in human disease", Clinical Chemistry, 52(4). 601 - 623. April 2006.

[2] Saldana, M., Gamarra, F., Siloto R. In Emerging Technologies Used for the Extraction of Phytochemicals from Fruits, Vegetables, and Other Natural Sources, de la Rosa, L.A., Alvarez-Parrilla, E., Gonzalez-Aguilar, G.A., Eds., Blackwell Publishing, John Wiley and Sons, USA, 2010, 246.

[3] Wongnarat, C., Srihanam, P., "Phytochemical and antioxidant activity in seeds and pulp of grape cultivated in Thailand", Oriental Journal of Chemistry, 33(1). 113-121. January 2017.

[4] Weidner, S., Powlaka, A., Karamac, M., Amaroowicz, R., "Extracts of phenolic compounds from seeds of three wild grapevines: Comparison of their antioxidant activities and the content of phenolic compounds", International Journal of Molecular Sciences, 13(3). 3444-3457. March 2012.

[5] Lucarini, M., Durazzo, A., Kiefer, J., Santini, A., et al., "Grape seeds: chromatographic profile of fatty acids and phenolic compounds and qualitative analysis by FTIR-ATR spectroscopy", Foods, 9(1). 10. Dec. 2019.

[6] Burcova, Z., Kreps, F., Schmidt, S., Strizincova, P., Jablonsky, M., Kyselka, J., Haz, A., Surina, I., "Antioxidant activity and the tocopherol and phenol contents of grape residues", BioResources, 14(2). 4146-4156. April 2019.

[7] Piment, M., Blay, M., Blade, M.C., Salvado, M.J., Arola, L., Ardevol, A., "Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines", Endocrinology, 145(11). 4985-4990. Nov.2004.

[8] Fujii, H., Yokozawa, T., Kim, Y.A., Tohda, C., Nonaka, G., "Protective effect of grape seed polyphenols against high glucose-induced oxidative stress", Bioscience, Biotechnology, and Biochemistry, 70(9). 2104-2111. Sep.2006.

[9] Teissedre, P.L., Frankel, E.N., Waterhouse, A.L., Pele, G.H., German, J.B., “Inhibition of human LDL oxidation by phenolic antioxidants in-vitro from grapes and wines”, Journal of the Science of Food and Agriculture, 70(1). 55-61. January 1996.

[10] Vigna, G.B., Constantini, F., Aldini, G., Carini, M., Catapano, A., Schena, F., Tangerini, A., Zanca, R., Bombardelli, E., Morazzone, P., Mezzetti, A., Fellin, R., Maffei-Facino, R., “Effect of a standardized grape seed extract on low-density lipoprotein susceptibility to oxidation in heavy smokers", Metabolism, 52(10).1250-1257. Oct. 2003.

[11] Clifton P.M., “Effect of grape seed extract and querceitin on cardiovascular and endothelial parameters in high-risk subjects”, Journal of Biomedicine and Biotechnology, 2004(5). 272-278. 2004.

[12] Salmabadi, Z., Mohseni Kouchesfahani, H., Parivar, K., Karimzadeh, L., “Effect of grape seed extract on lipid profile and expression of interleukin-6 in polycystic ovarian syndrome Wistar rat model”, International Journal of Fertility & Sterility, 11(3). 176-183. Aug. 2017.

[13] Jayaprakasala, G.K., Solvi,T., Sakariah, K.K., “Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extracts", Food Research International, 36(2). 117-122. April 2003.

[14] Al-Farsi, M.A. and Lee C.Y., “Optimization of phenolics and dietary fibre extraction from date seeds” Food Chemistry, 108(3). 977-985. June 2008.

[15] Gurukar, M.S.A., and Chilkunda, N.D., “ Morus alba leaf bioactives modulate peroxisome proliferator activated receptor γ in the kidney of diabetic rat and impart beneficial effect”, Journal of Agricultural and Food Chemistry, 66(30). 7923-7934. Aug. 2018.

[16] Tang, G.-Y., Zhao, C.-N., Liu, Q., Feng, X.-L., Xu, Y.-X., Cao, S.-Y., Meng, X., Li, S., Gan, R.-Y., Li, H.-B., “Potential of grape wastes as a natural source of bioactive compounds”, Molecules, 23(10). 2598. Oct. 2018.

[17] Balasubashmini, S.M., Rukkumani, R., Menon, V.P., “Protective effects of ferulic acid on hyperlipidemic diabetic rats”, Acta Diabetologica, 40(3). 118-122. Sep. 2003.

[18] Pei, K., Ou, J., Huang, J., Ou, S., “ p-Coumaric acid and its conjugates. Dietary sources, pharmokinetic properties and biological activities", Journal of the Science of Food and Agriculture, 96 (9). 2952-2962. July 2016.

[19] Ou, S., and Kwok, K., “Ferulic acid: Pharmaceutical functions, preparation and applications in foods”, Journal of the Science of Food and Agriculture, 84(11).1261-1269. July 2004.

[20] Rukkumani, R., Aruna, K., Suresh, V.P., Padmanabhan, M.V., “Hepatoprotective role of ferulic acid: A dose-dependent study”, Journal of Medicinal Food, 7(4). 456-461. Dec. 2004.

[21] Amalan, V., Vijayakumar, N., Indumathi, D., Ramakrishnan, A., “Antidiabetic and antihyperlipidemic activity of p-coumaric acid in diabetic rats, role of pancreatic GLUT 2: In vivo approach”, Biomedicine & Pharmacotherapy, 84. 230-236. Dec. 2016.

[22] Farhadi, K., Esmaeizadeh, F., Hatami, M., Forough, M., Molaie, R., “Determination of phenolic compounds content and antioxidant activity in skin, pulp, seed, cane and leaf of five native grape cultivars in West Azerbaijan province, Iran”, Food Chemistry,199. 847-855. May 2016.

[23] Stanciu, G., Luposor, S., Popescu, A., Oancea, I.A., “Polyphenols isolation and determination in grape seeds by HPLC/DAD”, Journal of Science and Arts, 1(38). 107-112. March 2017.

[24] Mattos, G.N., Tonon, R.V., Furtado, A.A.,Cabral, L.M., “Grape by-product extracts against microbial proliferation and lipid oxidation: A review”, Journal of the Science of Food and Agriculture, 97(4). 1055-1064. March 2017.

[25] Liu, Q., Tang, G.-Y., Zhao, C.-N., Feng, X.-L., Xu, X.-Y., Cao, S.-Y., Meng, X., Li, S., Gan, R.-Y., Li, H.-B., “Comparison of antioxidant activities of different grape varieties”, Molecules, 23(10). 2432. Sep. 2018.