CHEMICAL ENGINEERING TRANSACTIONS
VOL. 44, 2015
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Riccardo Guidetti, Luigi Bodria, Stanley Best

ISBN 978-88-95608-35-8; **ISSN** 2283-9216

Polyphenols from Grape and Apple Skin: a Study on Non-Conventional Extractions and Biological Activity on Endothelial Cell Cultures

Alessandro A. Casazza*[a,b], Bahar Aliakbarian[a,b], Marzia Mura[b,c], Monica Chasseur[d], Marcello Freguglia[d], Sabina Valentini[d], Domenico Palombo[b,c] and Patrizia Perego[a,b]

[a]Department of Civil, Chemical and Environmental Engineering, Genoa University, Via Opera Pia, 15, 16145 Genoa, Italy
[b]Research Center of Biologically Inspired Engineering in Vascular Medicine and Longevity, Genoa, Italy
[c]Department of surgical sciences and integrated diagnostic, Genoa University, Genoa, Italy
[d]Institut Agricole Régional. La Rochère 1/A, 11100 Aosta, Italy

alessandro.casazza@unige.it

Grape and apple skins and seeds are rich in natural antioxidant compounds known as polyphenols. Polyphenols exhibit a wide range of beneficial biological properties acting as antioxidants and anti-inflammatory that can be exploited for vascular diseases. The polyphenol content in grapes and apples depends on cultivar and growing conditions. For this study, four different cultivars of apples (Golden Delicious, Jonagold, Renetta Canada and Raventze) and three of grapes (Fumin, Premetta and Petit Rouge), typical of Aosta Valley (Italy), were harvested at commercial maturity. Skins were collected and dried. Powdered samples were extracted with methanol using microwave assisted extraction (MAE, 110 °C, 60 min) and high pressure and temperature extraction (HPTE, 150 °C, 150 min). The non-conventional methodologies were compared with the classic solid-liquid extraction (25 °C, 19 h). The extracts were characterized in terms of total polyphenols (TP) content and their antiradical power. HPLC analysis was also performed to quantify main single phenolic compounds. For grape and apple skins, the higher TP yields were obtained by HPTE using Jonagold and Premetta cultivars, respectively. In general, extraction yields of HPTE have reached values higher than 30 and 10 mg of Gallic Acid Equivalent per g of Dry Material for grape and apple skins, respectively. Moreover, the biological vaso-protective activity of apple extracts was investigated by evaluating the expression of endothelial activation markers in an in vitro model of endothelial dysfunction induced by the pro-inflammatory cytokine TNFα.

1. Introduction

Grape and apple skins have been considered as important nutritional material because of their content of polyphenols and dietary fibres (Diñeiro García et al., 2009). Several researches have been focused on the extraction, characterization and recovery of high added-value compounds from these products. Among them, extraction using non-conventional techniques such as microwave assisted extraction (MAE) and high pressure and temperature extraction (HPTE) are considered to be efficient for recovery of phenolics with good antioxidant properties (Casazza et al., 2012a).
Extraction under MW has been used for the recovery of bioactive compounds and essential oils from the leaves of rosemary and peppermint (Dai et al., 2010), and the extraction of azadirachtin-related limonoids from neem seed kernel (Young 1995). MAE was successfully applied in laboratory scale because it is fast, ecocompatible (less solvent required), repeatable and very effective (Pérez-Serradilla et al., 2007). It has been reported that MAE is well suited for the extraction of phenolics although performed at relatively high

temperatures (110–150 °C), a critical feature to handle antioxidants without degradation. In a phenomenological study, it emerged that MAE allowed higher polyphenols recoveries compared to conventional technique, without altering the antioxidant potential of the extracts (Spigno and De Faveri, 2009). In HPTE, the purpose of pressurising the extraction is to prevent the solvent boiling at the extraction temperature and to ensure that the solvent remains in intimate contact with the sample during the entire extraction time (Alonso-Salces et al., 2001). In fact, this will grant a higher solubility of the molecules and accelerate the solvent desorption from the matrix. Furthermore, high temperatures decrease the solvent viscosity and result in a better penetration of matrix particles and indeed enhance the extraction efficiency (Oey et al., 2008).
The aim of this work was to study the effect of MAE and HPTE and the comparison with the conventional extraction method on recovery of phenolic compounds from the skins of three grape cultivars and of four apple varieties, cultivated in Aosta Valley (Italy). Dried samples were subjected to microwave assisted extraction (110 °C, 60 min) and high pressure and temperature extraction (150 °C, 150 min). Extracts were characterized in terms of total polyphenols (TP), single phenolic compounds, and antiradical power (ARP). The results were compared with the classic solid-liquid extraction (25 °C, 19 h). The biological vaso-protective activity of the extracts were evaluated using in vitro tests by employing endothelial dysfunction induced by the pro-inflammatory cytokine TNFα.

2. Materials and Methods

2.1 Raw material

Methanol (analytical and HPLC grade), Folin–Ciocalteu reagent and authentic standards of phenolic compounds, were purchased from Sigma Chemical Co (St. Louis, MO). Standard stock solutions were prepared with methanol and stored at -20 °C in dark conditions. The skins from three grape (Fumin, F; Premetta, P and Petit Rouge, PR) and four apple cultivars (Golden delicious, G; Jonagold, J; Renetta Canada, RC and Raventze, R), cultivated in Aosta Valley (Italy), were collected at commercial maturity and stored at -20 °C. All the samples were oven-dried at 60 °C, to reach a constant moisture of about 4–5 % (D-82152, MMM Medcenter, München, Germany). Samples were grounded with the help of a laboratory mixer to obtain a homogeneous powder for extraction with a particle size of 0.8 mm separated by sieves.

2.2 Extraction processes

Grape and apple skins were extracted with methanol using solid–liquid ratio of 0.20 grams of dried material per millilitres of solvent (g_{DM}/mL_S). The classic solid/liquid extraction was compared with non-conventional extractions (High Pressure and Temperature Extraction and Microwave Assisted Extraction) in order to reduce the long extraction times required by the maceration treatment.

- SLE. Samples were placed in glass test tubes with screw caps on a magnetic shaker (Heidolph Mr. 2002, Kelheim, Germany). Skins were extracted with methanol for 19 h. The extraction was performed in dark conditions at room temperature (25 °C) (Casazza et al., 2012a).
- HPTE. For this method an agitated high pressure and temperature reactor (Parr Instruments, model 350 M – 4650 Series), equipped with a mechanical stirrer, was used. The extraction was performed at 150 °C for 150 min under nitrogen atmosphere (Casazza et al. 2012b).
- MAE. A microwave multimode oven was used. Grape and apple skins were extracted following the methodology described by Casazza et al. (2010).

After the extractions, the methanol fraction was removed from matrix by centrifugation (6000 *x*g for 10 min) (ALC PK131 Centrifuges, Alberta, Canada) and stored at -20 °C until analysis.

2.3 Total polyphenol and Antiradical power analysis

The Total Polyphenols (TP) content was determined using the Folin-Ciocalteu assay (Swain & Hillis, 1959). Briefly, 0.2 mL of sample and 0.5 mL of Folin–Ciocalteu reagent were added to adequate flask. The content was mixed, and 1.0 mL of sodium carbonate solution (20 %) was added, followed by the addition of distilled water to reach the final volume of 10 mL. The solution was mixed and stored for 1 h at room temperature in the dark. Analysis was carried out at 725 nm using an UV-Vis spectrophotometer (Lambda 25, Perkin Elmer, Wellesley, MA, USA). A calibration curve was obtained using gallic acid standard solutions (0.01–1.00

mg/mL). TP yield was expressed as milligrams of Gallic Acid Equivalent per gram of Dried Material (mg_{GAE}/g_{DM}).
The antiradical power (ARP) of the extracts was measured in terms of radical-scavenging ability by means of the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$), using a modified version of the methodology described by Brand-Williams and Berset (1995). ARP was defined as 1/EC50 ($mg_{DPPH^{\cdot}}/mL_{extract}$).

2.4 High Performance Liquid Chromatography (HPLC) analysis

The single phenolic compound analysis was carried out using a HPLC (1100 Series, Hewlett Packard, Palo Alto, CA,USA) equipped with a C18 reverse-phase column (Model 201TP54, Vydac, Hesperia, CA) coupled with a diode-array detector (Agilent 1200), following the methodology described by Ferrari et al. (In Press). The mobile phase A was water/acetic acid (99:1 %, v/v) and the B was methanol/acetonitrile (50:50 %, v/v). The mobile phase gradient changed as follow: from 0 to 5 % B in 5 min, from 5 to 30 % B in 25 min, from 30 to 40 % B in 10 min, from 40 to 48 % B in 5 min, from 48 to 70 % B in 10 min, from 70 to 100 % B in 5 min and isocratic at 100 % B for 5 min. Colum temperature was 30 °C. Before each analysis, the extracts were filtered through a cellulose acetate filter (20 µm).

2.5 Biological vaso-protective activity

The efficiency of polyphenols from Jonagold microwave extract to counteract endothelial dysfunction was tested on human endothelial cell line EAhy926. The extract from Jonagold apple skin was administered to cells and the potential vaso-protective activity evaluated by measuring the levels of two endothelial function markers: Intracellular Adhesion Molecules-1 (ICAM-1) and Nitric Oxide Synthase (e-NOS). Cells were cultured in DMEM with 10% foetal bovine serum. For the treatment experiments, confluent cells were put in serum free medium for 1 h, pre-treated for 1 h with the MAE (polyphenols concentration 0.05 and 0.1 mg/ml) and then subjected to TNFα (100 ng/ml, Sigma Aldrich, T6674). After 24 h, cells were lysed with RIPA buffer and Western blot analysis was performed as previously described (Palmieri et al., 2012). Primary antibodies used were: polyclonal anti-ICAM-1 (Santa Cruz, sc-7891, 1:800 dilution), and polyclonal anti-eNOS (Millipore, 07-520, 1:1000).

2.6 Statistical analysis

Influence of the various parameters was assessed by analysis of variance (ANOVA) and Tukey's post hoc test ($p < 0.05$), using ''Statistica'' software version 8.0 (StatSoft, Tulsa, USA). The statistically significant differences were illustrated in tables and figures by different letters.

3. Results and Discussions

3.1 Total polyphenols and HPLC analysis

As can be seen in Figure 1, TP extraction yield from apple skins slightly varied using SLE (from 8.6 to 14.9 mg_{GAE}/g_{DM}). Using MAE, the higher extraction yield was observed for Raventze skins (20 mg_{GAE}/g_{DM}). Even if the SLE and MAE extraction yields were statistically similar, however the extraction times were different: 1 and 19 hours for MAE and SLE, respectively.
The HPTE reported the highest TP recoveries, with values of more than 460, 355, 267 and 63 % if compared with SLE for GD, J, RC and R skins, respectively.
Different results were observed with the recovery of phenolic compounds from grape skins. In general, for SLE, the Petit Rouge and Premetta cultivars resulted in TP concentration similar to apple skins, while Fumin skins showed higher levels of phenolic compounds, up to 50 mg_{GAE}/g_{DM}. The MAE behaved differently depending on the cultivar. A statistically significant TP yield increase, respect to SLE, was observed only for Petit rouge skins (153 %).
In general, HPTE gave a TP yield increase if compared with SLE (434 and 76 % for Premetta and Petit Rouge, respectively), and only with Fumin skins a yield reduction was observed (13 %), this reduction could be explained by the presence of more active phenolic compounds.
In Table 1 are reported the main phenolic compounds analysed by HPLC, from these results we can conclude that the high concentration of single phenolic compounds was belong to catechin-derivatives from apple and grape skins. The highest concentration of catechin-derivatives was obtained using SLE for Raventze and using SLE and HPTE for Fumin and Premetta, respectively.

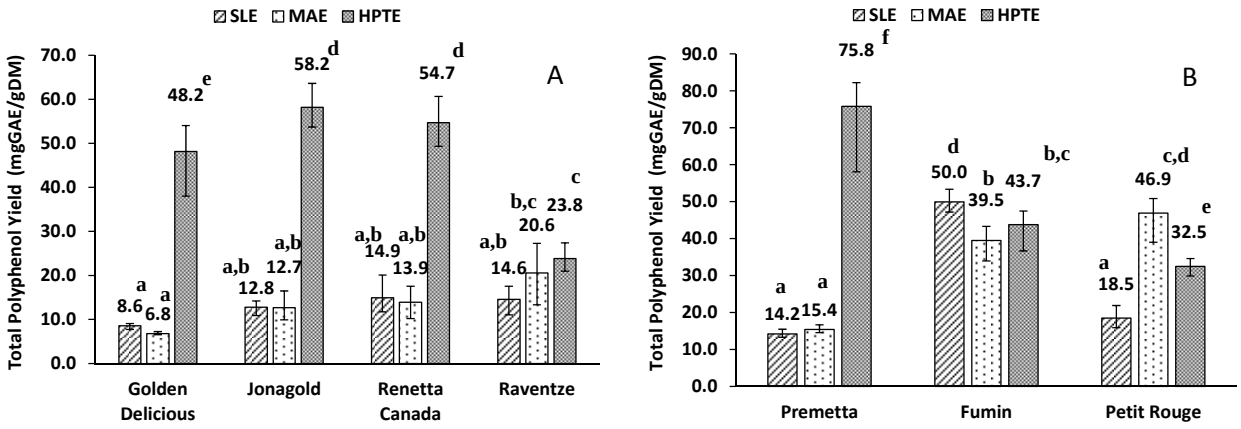

Figure 1. Total polyphenols (mg_{GAE}/g_{DM}) extracted from apple (A) and grape (B) skins using three different extraction methodologies. Different letters within each panel show significant differences at $p < 0.05$

Table 1. Main single phenolic compounds detected by HPLC

Matrix	Extraction method	Variety	Yield (mg/100gDM)				
			GA	Ca	Ca-D	Qe	Res
Apple skins	SLE	Golden Delicious	$0.17^{a,b,c}$	0.46^{a}	$8.71^{a,b,c}$	0.18^{a}	-
		Jonagold	$0.13^{b,c}$	$0.59^{a,c}$	$18.05^{d,e,f}$	$0.22^{a,d}$	-
		Renetta Canada	0.27^{d}	1.05^{e}	46.62^{g}	$0.29^{d,e}$	-
		Raventze	0.21^{a}	$0.68^{a,b,c,d}$	59.64^{h}	$0.35^{b,e}$	-
	MAE	Golden Delicious	$0.18^{a,c}$	$0.63^{a,c,d}$	6.23^{a}	0.36^{b}	-
		Jonagold	0.12^{b}	0.44^{a}	$6.75^{a,b}$	0.11^{c}	-
		Renetta Canada	$0.18^{d,e}$	$0.91^{b,e}$	$12.76^{b,c,d}$	0.11^{c}	-
		Raventze	$0.16^{a,b,c}$	$0.87^{b,d,e}$	$14.74^{c,d,e}$	$0.17^{a,c}$	-
	HPTE	Golden Delicious	0.08^{f}	$0.73^{b,c,d}$	$11.58^{a,b,c}$	0.38^{b}	-
		Jonagold	0.18^{a}	$0.89^{b,e}$	$9.20^{a,b,c}$	$0.24^{a,d}$	-
		Renetta Canada	0.27^{a}	1.51^{f}	21.07^{ef}	0.61^{f}	-
		Raventze	0.32^{e}	1.56^{f}	24.54^{f}	$0.34^{b,e}$	-
Grape skins	SLE	Premetta	0.25^{a}	3.88^{e}	150.98^{e}	-	0.44^{c}
		Fumin	0.23^{a}	$7.03^{c,d}$	$606.51^{c,d}$	-	0.73^{b}
		Petit Rouge	0.06^{b}	1.40^{a}	14.09^{a}	-	0.13^{a}
	MAE	Premetta	0.25^{a}	2.51^{b}	141.82^{b}	-	0.55^{c}
		Fumin	$0.20^{a,c}$	$2.26^{a,b}$	$384.13^{a,b}$	-	0.58^{b}
		Petit Rouge	$0.09^{c,d}$	$2.23^{a,b}$	$24.87^{a,b}$	-	0.11^{a}
	HPTE	Premetta	1.00^{e}	7.71^{d}	606.50^{d}	-	0.73^{b}
		Fumin	0.64^{d}	6.17^{c}	584.28^{c}	-	0.58^{b}
		Petit Rouge	0.70^{d}	$6.87^{c,d}$	$30.33^{c,d}$	-	0.00^{a}

GA, gallici acid; Ca, catechin; Ca-D, catechin-derivatives; Qe, quercetin; Res, resveratrol.
Means (n=3) ± standard deviation with different letters (a - h) in the same column, for grape and apple skins separately, are significantly different ($p < 0.05$).

3.2 Antiradical power and biological evaluation

Because all single phenolic compound do not show the same antiradical power, the correlation between polyphenols of skins and their antioxidant capacity is a great concern to be studied (Lataoui et al., 2014). In

this study, ARP augmented by increasing TP concentrations (data not reported), but not in a linear way (R^2 = 0.4129). Likely due to different phenolic compounds profiles (Table 1) in the extracts or to the release of additional non-phenolic compounds (proteins, sugars) that interact with Folin-Ciocalteu method and not with DPPH assay.
The ARP values of the extracts, obtained using the DPPH assay, fall in the range from 5.31 to 89.75 $mg_{DPPH}/mL_{extract}$ (Table 2). For SLE, no statistical differences were observed within apple skin extracts. For grape skin extracts, the ARP value of Fumin SLE extract was statistically similar with the ARPs obtained with HPTE of Premetta, Fumin and Petit Rouge skins. In general, both for apples and grapes, ARP increased using SLE, MAE and HPTE. Like for total polyphenol yield, only for Fumin samples, extracts from MAE and HPTE resulted in lower ARP values respect to SLE, even if the values do not differ statistically.

Table 2. Antiradical power of the extracts obtained by SLE, MAE and HPTE

Matrix	Variety	ARP ($mg_{DPPH}/mL_{extract}$)		
		SLE	MAE	HPTE
Apple skins	Golden Delicious	5.98 ± 0.01^{a}	5.96 ± 0.56^{a}	47.75 ± 0.05^{b}
	Jonagold	$8.87\pm0.07^{a,c}$	$22.64\pm2.01^{c,d}$	42.35 ± 1.58^{b}
	Renetta Canada	$8.41\pm0.14^{a,c}$	$33.69\pm6.90^{b,d}$	62.79 ± 7.25^{e}
	Raventze	5.31 ± 1.17^{a}	7.76 ± 0.56^{a}	$36.41\pm7.49^{b,d}$
Grape skins	Premetta	22.23 ± 1.92^{b}	70.69 ± 3.97^{a}	86.01 ± 2.06^{a}
	Fumin	89.75 ± 12.36^{a}	78.95 ± 3.16^{a}	77.09 ± 8.88^{a}
	Petit Rouge	31.38 ± 1.87^{b}	25.03 ± 0.47^{b}	77.74 ± 1.82^{a}

Means (n=3) ± standard deviation with different letters (a - d), for grape and apple skins separately, are significantly different (p < 0.05).

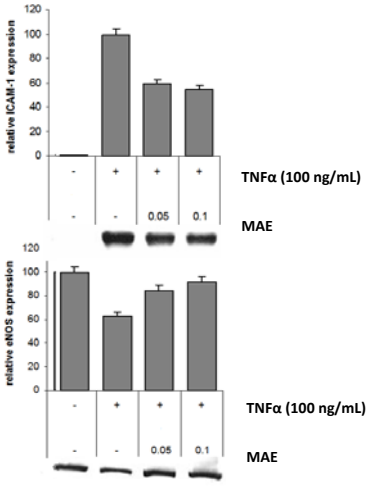

Figure 2. Western blotting of cell lysates and densitometric analysis for ICAM-1 and eNOS expression. Data shown are mean ± SD of three experiments

Western blot analysis showed that polyphenol extracts from Jonagold apple skin counteract the TNFα-induced endothelial dysfunction: Figure 2 shows that MAE restored the expression of eNOS, a vaso-protective molecules which is down-regulated by TNFα. Similarly, extracts decreased the TNFα-induced level of ICAM-1.

4. Conclusions

The extraction of apple and grape skins using MAE and HPTE gave samples rich in total polyphenols and with a high antiradical power. The HPLC analysis reported significant concentration of catechin-derivatives in both

of them. In this work, we demonstrated that polyphenols extracted by MAE have good activities in counteracting the endothelial dysfunction induced by TNFα. The extracts after the solvent recycle could be used for food, cosmetic and pharmaceutical purposes**.**

Acknowledgments

We would like to thank Les Crêtes ss, Maley srl and Ottin ss for providing many of the samples analyzed in this study.

References

Alonso-Salces R.M., Korta E., Barranco A., Berrueta, L.A., Gallo B., Vicente, F., 2001, Pressurized liquid extraction for the determination of polyphenols in apple, Journal of Chromatography A, 933, 37-43, DOI:10.1016/S0021-9673(01)01212-2.

Brand-Williams W., Cuvelier M.E., Berset C., 1995, Use of free radical method to evaluate antioxidant activity, Lebensmittel-Wissenschaft und –Technologie, 28, 25–30, DOI:10.1016/S0023-6438(95)80008-5.

Casazza A.A., Aliakbarian B., De Faveri D., Fiori L., Perego P., 2012a, Antioxidants from winemaking wastes: a study on extraction parameters using Response Surface Methodology, Journal of Food Biochemistry, 36, 28-37, DOI: 10.1111/j.1745-4514.2010.00511.x.

Casazza A.A., Aliakbarian B., Mantegna S., Cravotto G., Perego P., 2010, Extraction of phenolics from *Vitis vinifera* wastes using non-conventional techniques, Journal of Food Engineering, 100, 50-55, DOI: 10.1016/j.jfoodeng.2010.03.026.

Casazza A.A., Aliakbarian B., Sannita E., Perego P., 2012b, High-pressure high-temperature extraction of phenolic compounds from grape skins, International Journal of Food Science and Technology, 47, 399-405, DOI: 10.1111/j.1365-2621.2011.02853.x.

Dai J., Orsat V., Raghavan G.S.V., Yaylayan V., 2010, Investigation of various factors for the extraction of peppermint (*Mentha piperita* L.) leaves, Journal of Food Engineering, 96 (4), 540-543, DOI: 10.1016/j.jfoodeng.2009.08.037.

Diñeiro García Y., Suárez Valles B., Picinelli Lobo A., 2009, Phenolic and antioxidant composition of by-products from the cider industry: Apple pomace, Food Chemistry, 117, 731-738, DOI: 10.1016/j.foodchem.2009.04.049.

Ferrari P.F., Palmieri D., Casazza A.A., Aliakbarian B., Perego P., Palombo D., In Press, TNFα-induced endothelial activation is counteracted by polyphenol extract from UV-stressed cyanobacterium *Arthrospira platensis*, Medicinal Chemistry Research, DOI: 10.1007/s00044-014-1126-6.

Lataoui M., Seffen M., Aliakbarian B., Casazza A.A., Converti A., Perego P., 2014, Optimisation of phenolics recovery from *Vitex agnus-castus* Linn. leaves by high-pressure and temperature extraction, Natural Product Research, 28, 67-69, DOI: 10.1080/14786419.2013.832678.

Oey I., Lille M., Loey A.V., Hendrickx M., 2008, Effect of high pressure processing on colour, texture and flavour of fruit and vegetable-based food products: a review, Trends in Food Science and Technology, 19, 320-328, DOI: 10.1016/j.tifs.2008.04.001.

Palmieri, D, Perego, P, Palombo, D., 2012, Apigenin inhibits the TNFα-induced expression of eNOS and MMP-9 via modulating Akt signalling through oestrogen receptor engagement, Molecular and Cellular Biochemistry, 371, 129-136, DOI: 10.1007/s11010-012-1429-1.

Pérez-Serradilla J.A., Japon-Lujan R., Luque de Castro M.D., 2007, Simultaneous microwave-assisted solid–liquid extraction of polar and nonpolar compounds from alperujo. Analytica Chimica Acta, 602, 82-88, doi:10.1016/j.aca.2007.09.008.

Spigno G., De Faveri M., 2009, Microwave-assisted extraction of tea phenols: a phenomenological study. Journal of Food Engineering, 93, 210-217, DOI: 10.1016/j.jfoodeng.2009.01.006.

Swain T., Hillis W.E., 1959, The phenolic constituents of *Prunus domestica*. The quantitative analysis of phenolic constituents, Journal of the Science of Food and Agriculture, 10, 63–68, DOI: 10.1002/jsfa.2740100110.

Young J.C., 1995. Microwave-assisted extraction of the fungal metabolite ergosterol and total fatty acids, Journal of Agricultural and Food Chemistry, 43, 2904-2910.