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Waste grape skins: evaluation of safety aspects for the production of functional powders and extracts for the food sector

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ABSTRACT

Skin powders and aqueous alcohol extracts were obtained from waste marcs from different grape varieties (Barbera, Nebbiolo, Pinot Noir, Chardonnay, Moscato and Müller-Thurgau). Both skins and extracts were analysed for the content of chemical contaminants: ochratoxin A (OTA), biogenic amines (BIAs), pesticides and metals. OTA was detected in low concentrations in Barbera, Moscato and Nebbiolo skins, but only in Barbera and Moscato extracts. Cadaverine, putrescine, ethanolamine and ethylamine were found in extracts at very low levels, while potential allergenic amines, tyramine and histamine, were never detected. Different pesticides were present in both skins and extracts. Pb and Cd were found in trace only in the powders, and K, Ca and Mg were the most abundant elements in both skin powders and extracts. Concentrations of the different contaminants were related to fibre content or total phenolics content of powders and extracts, respectively, in order to evaluate their use in the food sector.

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Introduction

Grape marcs are the principal solid byproducts of wine-making process. They are produced in a limited period of the year and their disposal is, in most cases, a problem for wineries since, due to the organic nature, they cannot be directly released in the environment (Spigno et al. 2008). On the other hand, grape marcs, principally composed of seeds and skins, still contain valuable compounds (fibre, phenolic compounds, lipids and sugars) with potential applications in different sectors such as the pharmaceutical, chemical, material, cosmetic and food industries (Arvanitoyannis et al. 2006; Amendola et al. 2010; Basalan et al. 2011; Sri Harsha et al. 2013; Yu & Ahmedna 2013; Denny et al. 2014; Bordiga 2015; Beres et al. 2016; Lingua et al. 2016).

In particular, with a focus on the food sector, grape skins and seeds could be directly used in the form of dried powder as a source of fibre and antioxidant compounds (Sant'Anna et al. 2014a; Lavelli et al. 2015; Marchiani et al. 2015; Marinelli et al. 2015; Seo et al. 2015) or, indirectly, through the production of antioxidant phenolic extracts (Peng et al. 2009; Chouchouli et al. 2013; Spigno et al. 2013; Baiano et al. 2015). However, contaminants present on the grapes may

remain or even accumulate in the residual grape marcs, including mycotoxins (ochratoxin-A, OTA), biogenic amines (BIAs), pesticides and heavy metals.

OTA is a well-known nephrotoxin and it is classified by the IARC as a possible human carcinogen (group 2B) (IARC 1993). It is produced in the vineyard by *Aspergillus* section *Nigri*, the so-called black *Aspergilli* (Battilani & Pietri 2002; Sage et al. 2002). During winemaking, OTA transfers only partially into wine since most of it remains bound to the marc and lees (Grazioli et al. 2006).

BIAs (including also polyamines) are basic nitrogenous compounds formed mainly by the decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. The undesirable toxicological effects in humans associated to some BIAs include rash, oedema, headaches, hypotension, vomiting, palpitations, diarrhoea and heart problems (Kalač 2014; Linares et al. 2016). Histamine, tyramine and putrescine are major amines in wine, but the origin and evolution of the BIAs in wines is still a matter of controversy. The abundance of amines, in fact, is strictly related to the microflora but also to the amino acid composition of the wine after alcoholic fermentation. It was shown that there is a relationship between some amines and important factors such as

variety, sub-region, vintage, safety of the grapes and oenological parameters (Herbert et al. 2005; Marcobal et al. 2006; Del Prete et al. 2009). Grape marcs of white varieties may contain residual BIAs present on the unprocessed grapes, while grape marcs of red varieties may adsorb BIAs also produced during maceration and fermentation steps. Legislation dealing with limits for BIAs contents in wines is still lacking and regulatory limits have not yet been established; however, upper limits for histamine content in wine (2–10 mg l⁻¹) are recommended in some European countries (Manetta et al. 2016).

Pesticides are used in farming on fruit and vegetables, including grapes, to increase yields and product quality. Due to the toxicity of these compounds, their residual content in food products has to be monitored and comply with specific legislation. These contaminants generally accumulate in the outer layers of the vegetable/fruit, so the grape residue after pressing can as a result be enriched in pesticides (Cabras & Angioni 2000; Rose et al. 2009).

Trace elements and heavy metals may be present in high concentrations in food, and excessive dietary intake of these elements can also have adverse effects on health. According to the Environmental Protection Agency (EPA), Pb, Cd, Cu, Zn and Se are some of the most common heavy metals inducing pollution. It is well established that the Cd and Pb have the greatest detrimental effects on the kidneys and nervous system, respectively (Shahbazi et al. 2016). On the other hand, some metals such as Fe, Zn, Cu, Cr, Co and Mn are essential for human metabolism (Daşbaşı et al. 2016). For example, Fe is an integral part of many proteins and enzymes and is a crucial component of proteins involved in oxygen transport, regulation of cell growth and differentiation. Insufficient Fe in humans leads to fatigue, decreased immunity and death. Cr is considered to be an essential trace element for the maintenance of effective glucose, lipid and protein metabolism in mammals. Various clinical disorders such as anaemia, depression of growth, impaired reproductive performance, heart failure and gastrointestinal disturbances have been associated with a dietary insufficient of Cu. Zn is an essential part of more than 200 enzymes involved in digestion, metabolism, reproduction and wound healing. Zn deficiency also leads to cognitive defects and impairs the immune system. The minerals in grapes and grape marcs have two major sources. The first, natural source is the soil. The second, anthropogenic source includes application of organic and mineral fertilisers, pesticides, pollution of the surrounding environment, and use of food additives and plants in the winemaking process (Kment et al. 2005).

Evaluation of the presence of the above compounds is of great importance to properly evaluate food

applications of residual grape marc and related powders or extracts. To our knowledge, however, this aspect has been little investigated until now (Cabras & Angioni 2000; Rose et al. 2009; Sant'Anna et al. 2014b; Solfrizzo et al. 2015). The principal aim of this study was, therefore, to assess the levels of some contaminants present in grape skins and related extracts. Dried grape skins were obtained from marcs collected from different red and white grape varieties and aqueous alcohol used to produce extracts. The initial skins and the extracts were analysed for OTA, BIAs, pesticides and minerals. Since grape skin powders could be exploited to increase principally the fibre content of foods but also the phenolic content, they were characterised for total fibre content and the contaminants content was related to this parameter. In particular, many literature works have investigated the use of grape skin or grape marc powder for the development of cereal based products (Peng et al. 2009; Mildner-Szkudlarz et al. 2011; Sant'Anna et al. 2014a; Baiano et al. 2015; Marinelli et al. 2015; Lavelli et al. 2016). Therefore, the current legislation on maximum residue levels in unprocessed cereals for the analysed contaminants has been considered in relation to the potential of grape skin powders for addition to mixed cereal flours.

On the other hand, since extraction is aimed to the production of enriched antioxidant extracts, the extracts were also analysed for the total phenolics content and the contaminants content was related to this parameter.

Materials and methods

Chemicals

Methanol was from BDH (Poole, UK). Acetic acid, dichloromethane, sodium hydrogen carbonate, perchloric acid, sodium chloride, hydrogen chloride, sodium hydroxide and sodium tetraborate decahydrate were from Carlo Erba (Milan, Italy). Polyethylene glycol 8'000 (PEG), 2-mercaptoethanol (99%), *o*-phthalaldehyde (≥ 99%), 1,6-diaminohexane (98%) and standards of amines (histamine dihydrochloride 97%, tyramine hydrochloride 98%, serotonin > 98%, putrescine 99%, cadaverine dihydrochloride > 99%, ethanolamine > 99.5%, and ethylamine hydrochloride 98%), pesticides (> 99%) and gallic acid (≥ 98%) were from Sigma-Aldrich (St. Louis, MO, USA). Di-sodium hydrogen phosphate and acetonitrile were from Merck (Darmstadt, Germany). All solvents used for chromatographic analyses were HPLC grade. The OTA standard (98%) was from Biopure (Waterlooville, UK). Mineral standard solutions were

prepared by dilution of multi element standard solutions (100 mg l^{-1} , > 99.9%) from CPI International (Amsterdam, the Netherlands). Ultrapure water was prepared using a Milli-Q system (18 M Ω m resistance, Millipore system, Millipore, Bedford, MA, USA).

Grape marc

Grape marc samples from Barbera (BA), Chardonnay (CH), Moscato (MO), Müller-Thurgau. (MT), Nebbiolo (NE) and Pinot Noir (PN) grape varieties were kindly provided by different wineries located in Northern Italy during the 2012 vintage. Marcs from BA and NE were collected after alcoholic fermentation, while marcs from PN, CH, MO and MT were collected immediately after the pressing operation. The samples were sieved to separate the skins from the seeds, and the skins were dried at 53–54°C for 48–72 h. After drying, skins were milled (final particle size < 2 mm) and stored in plastic bags under vacuum, in the dark, at RT, before performing characterisation (OTA, BIAs, pesticides and metals) and extraction. Moisture content of the skin powders was determined by drying in a vacuum at 105°C for 24 h.

Skin extracts

Phenolic extracts were obtained (in triplicates) by solvent extraction with aqueous ethanol according to the procedure reported in Amendola et al. (2010) slightly modified. Briefly, 125 g of skin powder was extracted with 1 L of 60% aqueous ethanol keeping the mixture stirred at 3500 rpm (mixer Silverson, L5M) for 2 h at 60°C (by means of an electric heating plate). After extraction, the mixture was centrifuged at 2000g for 15 min at RT and the supernatant (extract) recovered, taking note of the exact volume. Extracts were characterised for the total phenolics content, stored at 4°C and analysed within 24 h to detect: OTA, BIAs, residues of pesticides and minerals.

Total fibre content

The total fibre content of skin powders, as the sum of cellulose, hemicellulose and lignin, was evaluated (in triplicate) based on the neutral detergent fibre resulting from the Van Soest method (Spigno et al. 2008). This method cannot account for the pectin content (since this is washed out with the neutral detergent). However, it was selected because it presents a good compromise between the quality of data obtained and the time of analysis.

Total phenolics content

Total phenolics content (TPC) of the skin extracts was assessed (in triplicate) by Folin–Ciocalteu analysis (Folin Index), expressing the results as mg l^{-1} of gallic acid equivalents (GAE) based on a calibration curve with standard gallic acid (Amendola et al. 2010). Briefly, 25 ml of water, 0.5 ml of extract sample (diluted as necessary with water), 2.5 ml of the Folin reagent and 5 ml of 20% sodium carbonate were mixed and brought to 50 ml. The absorbance was measured at 750 nm after 30 min heating at 40°C. A mixture of water and reagents was used as a blank. Based on extract volume and concentration, the total phenolics extraction yield, $g_{\text{GAE}}/100 \text{ g}$ of skin powder (d.w.), was then calculated.

HPLC analyses

The chromatographic system used for OTA and BIAs analysis consisted of a Perkin Elmer (Norwalk, CT, USA) instrument equipped with a 200 Series pump, a Perkin-Elmer 650-10S fluorescence detector, a Jasco LC-Net II/ADC (Oklahoma City, OK, USA) communication module and operated by ChromNAV Control Center software.

The chromatographic system used for evaluation of pesticide residues was a HPLC-MS/MS instrument equipped with a load pump Surveyor LC Pump Plus and Accela UHPLC Pump (Thermo Scientific, Waltham, MA, USA) coupled to a triple-stage quadrupole mass spectrometer TSQ Quantum Series (Thermo Scientific).

Ochratoxin A

OTA in skin powders was evaluated (in triplicate) according to the method described by Serra et al. (2004). To 10 g powder, 150 ml of solution A (5% NaHCO_3 , 1% PEG 8000) were added. The resulting mixture was poured into a 250 ml centrifuge bottle and stirred magnetically for 30 min and then centrifuged at 4°C, 8800g for 20 min. The supernatant was filtered through a glass microfiber filter (1.5 μm pore size) and the filtrate collected in a graduated cylinder. An aliquot of the filtrate (20 ml) was extracted through 3 ml immunoaffinity columns (Ochraprep, R-Biopharm Rhône, Lanarkshire, UK) using 5 ml of washing solution containing NaCl (2.5%) and NaHCO_3 (0.5%) and then 2 ml of distilled water. OTA was eluted with 2 ml methanol, evaporated to dryness under dry nitrogen gas stream and reconstituted with the mobile phase. For evaluation of OTA content in skin extracts, the samples were two-fold diluted with water, mixed with 10 ml of solution A and filtered. An aliquot (20 ml) of the filtered solution

was cleaned up by immunoaffinity columns as described above. The final reconstituted samples were filtered again and analysed according to Visconti et al. (1999). Chromatographic separation was performed on a Zorbax column (Agilent Technologies, Santa Clara, CA, USA) SB-C18, 4.6 × 150 mm, fitted with 3.5 µm. The mobile phase consisted of a mixture of acetonitrile/water/acetic acid (49:49:2 v/v) eluted at a flow rate of 1.0 ml min⁻¹. Quantification of OTA was performed by measuring peak area at OTA retention time and comparing it with a calibration curve constructed with standard OTA solutions in the 0.05–10 µg l⁻¹ concentration range.

Based on volume extract and its OTA concentration, the OTA extraction yield, $g_{OTA}/100\text{ g}$ of skin powder (d.w.), was calculated and divided by the OTA content in skin powders to give the contaminant migration during extraction.

Biogenic amines

BIAs in skin powders were determined (in duplicate) according to the method described by Bauza et al. (1995) and also to the procedure of Method OIV-MA-AS315-18 (OIV 2016). The powder (2.5 g) was extracted by adding a 5% (v/v) perchloric acid solution (50 ml). After 12 h soaking at ambient temperature with intermittent stirring, the residual solid was separated by centrifugation (20 min at 4°C, 8800g) and the supernatant filtered through a 0.45 µm filter and twofold diluted with methanol. Standard and samples were submitted to derivatisation with *o*-phthalaldehyde with a solution composed of 2 ml of tetraborate buffer (0.1 M, adjusted at pH 10.5 with NaOH 10 N), 2 ml of *o*-phthalaldehyde (0.4% in methanol), 0.6 ml of 2-mercaptoethanol and 0.4 ml of sample (or standard). Derivatised samples were injected immediately (20 µl) into a Gemini NX-C18 column, 250 mm × 4.6 mm, 5 µm (Phenomenex, Torrance, CA, USA) maintained at 35°C. The mobile phases were phase A phosphate buffer 0.03 M, and mobile phase B acetonitrile. The stepwise gradient was: 0–15 min 20–30% B, 15–23 min 30–40% B, 23–42 min 40–50% B, 42–55 min 50–65% B, 55–60 min 65% B, 60–70 min 65–20% B, 70–95 min 20% B. The fluorescence detector was set at 356 nm excitation and 445 nm emission wavelengths. Quantification of BIAs was performed by measuring peak areas at the appropriated retention times using 1,6-diaminohexane as internal standard, and calibration curves with standard amines (histamine, tyramine, serotonin, putrescine, cadaverine, ethanolamine, ethylamine).

For evaluation of BIAs in the skin extracts, these were filtered through 0.45 µm filters and subject to

derivatisation as reported above. The extracted contaminants were calculated as reported for OTA.

Pesticides

Evaluation of the pesticide residues was carried out in triplicate based on the BS EN 15662:2008 (2008) method. The skin extracts, and the extracts obtained from the skin powders according the BS EN 15662:2008 method, were concentrated using an EQuan system consisting of two HPLC pumps with a preconcentration column (Hypersil GOLD, Thermo Scientific, 20 × 2 mm, 12 µm particle fitted) and an analytical column (Hypersil GOLD, Thermo Scientific, 50 × 3 mm, 1.8 µm particle fitted) operated at a flow rate of 0.4 ml min⁻¹. The following elution program was used: 95% solvent A (water containing 0.1% formic acid and 0.1% ammonium formate) and 5% solvent B (methanol containing 0.1% formic acid and 0.1% ammonium formate), injection volume 2 µl. The extracted contaminants were calculated as reported for OTA.

Mineral content

The mineral content (Ca, Cu, Fe, K, Mg, Mn, Na and Zn) was assessed (in duplicate) by atomic absorption spectroscopy (Perkin Elmer AAnalyst 300 equipped with HGA 850 Graphite furnace and AS 800 Autosampler). In the case of skin powders, samples (10 g) were placed in platinum capsules and heated at 550°C until a white residue was obtained. Then, 2 × 1 ml of 0.001 N nitric acid were added and the samples were dried under infrared lamps, suspended in 5 ml of 0.001 N nitric acid and analysed. In the case of skin extracts, the samples were filtered and diluted with Milli-Q water and directly analysed.

The Pb, Cd and Cr contents were evaluated by digestion of 0.5 g of skin powder with 6 ml 65% nitric acid and 1.5 ml hydrogen peroxide 30% w/w. The digested samples were then analysed by Inductively Coupled Plasma Mass Spectrometer (Agilent 7700x) with Octapole Reaction System (ORS System). The skin extracts were directly analysed after filtration and appropriate dilution with Milli-Q water.

The minerals extracted were calculated as reported for OTA.

Statistical analysis of the data

Results are presented as mean values of the carried out replicates with their corresponding standard deviations. Influence of the grape variety on the different evaluated parameters was assessed by one-way analysis

of variance (ANOVA) using statistical software SPSS® (version 19.0, SPSS Inc., Chicago, IL, USA). Differences at $p < 0.01$ were considered significant. In case of significant difference, the means were assessed applying the post-hoc Tukey test.

Results and discussion

Fibre and total phenolics content

All the skin powders showed a fibre content higher than 43%, confirming they could be used to fortify different food products. MT skins had the highest content ($55.01 \pm 0.07\%$), followed by NE and PN ($48.08 \pm 0.28\%$ and $46.52 \pm 0.77\%$ respectively and not statistically different), BA, CH and MO ($43.48 \pm 1.31\%$, $43.33 \pm 0.15\%$ and $43.07 \pm 0.99\%$ respectively and not statistically different).

The applied extraction procedure allows recovery only of free phenolic compounds, therefore the results can be used to compare the different grape variety marcs in terms of content of extractable free phenolics. The highest TPC was obtained for PN skins ($6.45\% \pm 0.67$) which was expected since they came from marc which had not been fermented. BA and CH skins gave comparable intermediate yields ($3.55 \pm 0.33\%$ and $3.18 \pm 0.35\%$, respectively), while MT, MO and NE gave the lowest (not statistically different) values ($2.33 \pm 0.33\%$, $1.80 \pm 0.024\%$ and $1.51 \pm 0.26\%$, respectively).

Ochratoxin A

OTA was detected only in the BA, NE and MO skin powders, at very low levels: 0.32 ± 0.03 , 0.20 ± 0.00 and $0.10 \pm 0.01 \mu\text{g kg}^{-1}_{\text{dw}}$, respectively. EU legislation has set OTA maximum residue levels of $10 \mu\text{g kg}^{-1}_{\text{fw}}$ for dry grapes, and $5 \mu\text{g kg}^{-1}_{\text{fw}}$ for unprocessed cereals, therefore addition of these skin powders to cereal

flours would not cause the MRL for this mycotoxin to be exceeded.

The presence of OTA was monitored in various grape-marc-derived food supplements. In most samples, this contaminant was found either to be absent or lower than the LOQ, but it was present in detectable amounts in some products. Indeed, OTA contamination is particularly critical in some climatic regions, such as South Italy, where levels up to $849.1 \mu\text{g kg}^{-1}$ of OTA were reported for samples of grape marcs (Solfrizzo et al. 2015). The sampling region considered in this study is Northern Italy and the different climate could account for the lower levels of OTA found in the skin samples.

OTA was detected only in BA and MO extracts at a concentration of 0.044 ± 0.004 and $0.005 \pm 0.001 \mu\text{g l}^{-1}$, respectively, corresponding to an average 87.5% and 35.8% migration of this contaminant during extraction. It was suggested that this toxin does not pass into ethyl alcohol during distillation of grape marc. On the other hand, OTA was found in some food supplements containing grape marc extract (Solfrizzo et al. 2015). Thus the factors affecting OTA migration rate are still unknown.

Biogenic amines

Table 1 reports the results obtained for the BIAs content of both skin powders and related extracts.

Ethanolamine was the major amine found in all the skin powder samples and this compound does not present any particular risk to health or food quality. Ethanolamine extracted by aqueous alcohol was recovered at levels ranging from 6% (from NE skins) up to 52% (from PN) depending on the grape variety and without any correlation with the type of grape (red or white), the vinification process or the initial concentration in the skins. No correlation

Table 1. Biogenic amines detected in waste grape skin powders and extracts and corresponding percentage of recovery.

Grape variety	Ethanolamine			Ethylamine		
	Skin powder ($\mu\text{g g}^{-1}$)	Extract (mg l^{-1})	Recovery (%)	Skin powder ($\mu\text{g g}^{-1}$)	Extract (mg l^{-1})	Recovery (%)
Chardonnay	53.84 ± 3.76^b	3.69 ± 0.25^b	38.58 ± 3.86^b	4.16 ± 0.06^b	0.46 ± 0.03^c	61.64 ± 6.27^a
Moscato	24.29 ± 7.07^c	1.93 ± 0.16^c	50.70 ± 3.61^a	8.67 ± 1.13^a	0.64 ± 0.05^b	46.87 ± 3.19^b
Müller T.	85.37 ± 2.75^a	4.58 ± 0.12^a	34.71 ± 0.29^b	4.54 ± 0.14^b	0.16 ± 0.01^e	23.47 ± 1.60^c
Barbera	84.93 ± 4.95^a	4.49 ± 0.28^a	33.71 ± 1.43^b	4.32 ± 0.01^b	0.38 ± 0.01^d	56.19 ± 1.92^{ab}
Nebbiolo	49.07 ± 4.58^b	0.44 ± 0.12^d	6.40 ± 1.62^c	0.51 ± 0.01^c	n.d.	n.d.
Pinot N.	46.72 ± 14.94^b	3.54 ± 0.13^b	52.25 ± 6.86^a	7.99 ± 2.19^a	0.75 ± 0.03^a	64.94 ± 8.52^a
Putrescine						
Chardonnay	7.31 ± 0.67^d	0.32 ± 0.01^b	24.54 ± 1.25^a	2.80 ± 0.02^{cd}	0.19 ± 0.01^b	37.79 ± 2.90^b
Moscato	4.31 ± 0.76^e	0.08 ± 0.01^d	11.26 ± 0.79^b	0.60 ± 0.07^e	n.d.	n.d.
Müller T.	13.43 ± 0.30^b	0.50 ± 0.05^a	24.02 ± 3.11^a	2.31 ± 0.16^d	0.20 ± 0.01^a	57.29 ± 2.95^a
Barbera	10.35 ± 0.27^c	0.26 ± 0.01^c	15.81 ± 0.06^b	8.40 ± 0.90^a	n.d.	n.d.
Nebbiolo	15.93 ± 1.56^a	n.d.	n.d.	4.03 ± 0.14^b	n.d.	n.d.
Pinot N.	5.41 ± 0.84^{de}	0.23 ± 0.02^c	29.39 ± 4.83^a	3.36 ± 0.04^{bc}	0.14 ± 0.01^c	28.80 ± 1.61^c
Cadaverine						

Note: Values are means $\pm$ SD. The same superscript letter under each parameter indicates the means are not statistically different according to analysis of variance (ANOVA) and Tukey's post-hoc test ($p < 0.01$).

between percent recovery and initial content in the skins or skin fibre content could be found (no shown data).

Ethylamine, another volatile amine like ethanolamine, was found in lower concentrations in all the skin powder samples, but its recovery during the extraction process was generally higher compared to ethanolamine.

Again, as for ethanolamine, it was not possible to find any correlation between the recovery percentage and other factors. Furthermore, the correlation of migration with variety was not the same for the two amines.

The polyamines, putrescine and cadaverine were identified in all the skin powder samples at lower levels compared to ethanolamine, but comparable to ethylamine. Extract concentrations never exceeded 0.50 mg l^{-1} and migration of these two polyamines into aqueous ethanol was below 30% for most of the samples, except

for cadaverine which migrated by 38% and by 57% from CH and MT, respectively.

None of the samples (neither skin powders nor extracts) contained the most toxic BIAs: histamine and tyramine. Also serotonin was never detected.

Pesticides

Tables 2 and 3 show the pesticide residue levels detected in the analysed samples. Many differences were found among the varieties regarding both total pesticides concentration and active principles. The grape marcs used in the present study were all derived from conventionally cultivated grapes. CH, MO, MT and NE were the most contaminated skins. In particular, NE together with MT presented the highest number of identified active principles and the highest level of total pesticide

Table 2. Pesticide residues detected in waste grape skin powders.

Active principle	Content in skin powder ($\mu\text{g g}^{-1}_{\text{dw}}$)						European Union MRL ($\mu\text{g g}^{-1}_{\text{dw}}$) [†]	
	Chardonnay	Moscato	Müller T.	Barbera	Nebbiolo	Pinot N.	Wine grapes	Wheat
Boscalid	n.d.	n.d.	n.d.	n.d.	0.54 ± 0.08	n.d.	25	0.58
Cyprodinil	1.07 ± 0.26	1.94 ± 0.44	n.d.	n.d.	1.50 ± 0.14	n.d.	15	0.58
Dimethomorph	0.56 ± 0.11	0.88 ± 0.17	2.73 ± 0.24	n.d.	1.53 ± 0.14	0.18 ± 0.02	15	$0.01^{\ddagger}$
Etofenprox	0.27 ± 0.00	0.39 ± 0.19	0.24 ± 0.02	0.26 ± 0.202	0.27 ± 0.13	0.21 ± 0.03	25	0.58
Famoxadone	n.d.	n.d.	n.d.	n.d.	1.55 ± 0.02	n.d.	10	0.12
Fenazaquin	n.d.	n.d.	n.d.	n.d.	0.07 ± 0.08	n.d.	1	$0.01^{\ddagger}$
Fenhexamid	n.d.	n.d.	4.91 ± 1.52	n.d.	n.d.	n.d.	25	$0.06^{\ddagger}$
Fludioxonil	0.72 ± 0.26	1.88 ± 0.49	n.d.	n.d.	1.58 ± 0.16	n.d.	20	$0.01^{\ddagger}$
Flufenoxuron	0.11 ± 0.01	0.14 ± 0.08	0.11 ± 0.03	0.12 ± 0.04	0.20 ± 0.16	n.d.	10	$0.06^{\ddagger}$
Metaxyl	n.d.	0.20 ± 0.11	n.d.	n.d.	n.d.	n.d.	5	0.06
Quinoxifen	n.d.	n.d.	n.d.	n.d.	0.11 ± 0.02	n.d.	5	$0.02^{\ddagger}$
Spiroxamine	n.d.	n.d.	1.17 ± 0.28	n.d.	n.d.	n.d.	5	$0.06^{\ddagger}$
Thiamethoxam	0.33 ± 0.09	0.44 ± 0.16	n.d.	n.d.	0.15 ± 0.03	0.11 ± 0.01	5	$0.06^{\ddagger}$
Trifloxystrobin	0.07 ± 0.08	n.d.	0.49 ± 0.08	n.d.	0.27 ± 0.04	n.d.	25	0.06
Total	$3.11^{\text{cd}} \pm 0.81$	$5.87^{\text{bc}} \pm 1.64$	$9.66^{\text{a}} \pm 2.16$	$0.38^{\text{d}} \pm 0.24$	$7.75^{\text{ab}} \pm 0.69$	$0.49^{\text{d}} \pm 0.02$		

Notes: Values are means $\pm$ SD. The same superscript letter in the last row refers to means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

n.d., Not detected; MRL: maximum residue level from the EU pesticide database; †values calculated assuming an average water content of 80% in grape and 14% in wheat; ‡lower limit of analytical determination.

Table 3. Pesticide residues detected in waste grape skin extracts.

Active principle	Content in skin extract (mg l^{-1})					
	Chardonnay	Moscato	Müller Thurgau	Barbera	Nebbiolo	Pinot Noir
Boscalid	n.d.	n.d.	n.d.	n.d.	0.06 ± 0.00	n.d.
Cyprodinil	0.13 ± 0.00	0.215 ± 0.05	n.d.	n.d.	0.10 ± 0.02	n.d.
Dimethomorph	0.06 ± 0.01	0.11 ± 0.01	0.38 ± 0.03	n.d.	0.17 ± 0.01	0.04 ± 0.00
Etofenprox	0.03 ± 0.01	0.01 ± 0.00	0.021 ± 0.00	0.016 ± 0.001	0.01 ± 0.00	0.03 ± 0.02
Famoxadone	n.d.	n.d.	n.d.	n.d.	0.14 ± 0.05	n.d.
Fenazaquin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fenhexamid	n.d.	n.d.	0.63 ± 0.11	n.d.	n.d.	n.d.
Fludioxonil	0.08 ± 0.04	0.17 ± 0.01	n.d.	n.d.	0.17 ± 0.00	n.d.
Flufenoxuron	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.
Metaxyl	n.d.	0.02 ± 0.00	n.d.	n.d.	n.d.	n.d.
Quinoxifen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Spiroxamine	n.d.	n.d.	0.13 ± 0.02	n.d.	n.d.	n.d.
Thiamethoxam	0.01 ± 0.00	0.03 ± 0.00	n.d.	n.d.	0.01 ± 0.00	0.01 ± 0.00
Trifloxystrobin	0.02 ± 0.01	0.01 ± 0.00	0.06 ± 0.01	n.d.	0.02 ± 0.00	n.d.
Total	$0.34^{\text{c}} \pm 0.059$	$0.57^{\text{b}} \pm 0.08$	$1.23^{\text{a}} \pm 0.17$	$0.02^{\text{d}} \pm 0.00$	$0.68^{\text{b}} \pm 0.06$	$0.11^{\text{d}} \pm 0.02$

Note: Values are means $\pm$ SD. n.d., Not detected. The same superscript letter in the last row refers to means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

residues, followed by MO and CH. Skins from PN and BA marc showed the lowest total contamination, 20–25 times lower than MT. White grapes were not fermented and this might partly explain the higher pesticides content in the residual marc and skins. However, also PN skins were obtained from unfermented marc, while the highly contaminated NE skins had been fermented. One possible explanation is that NE grapes are harvested later than BA and PN and so more in-field treatments may have been carried out during the summer. However, it was not possible to get the list of the pesticide treatment for the different collected grape byproducts in order to test this hypothesis.

The identified compounds and concentrations in the extracts were similar to the skin powder pesticide profiles (Table 3), with a few exceptions, as some active principles present in the skins were not always detected in the extracts (Flufenoxuron, Fenazaquin, Quinoxifen). There was one instance, for the MO variety, in which a pesticide (Trifloxystrobin) was detected in the extract but not in the skin powder. This might be due to inhomogeneity in the sample or, possibly to the very low concentration of the residue in the initial skins.

The percentage of migration of the pesticide residues into the aqueous alcohol extraction was calculated in relation to the total amount of pesticides. Migration was very high for PN skins (almost 100%) and MT skins (77%), followed by MO, CH and NE (52–59%), and BA (only 25%). As mentioned for BIAs, the percent recovery due to migration could not be correlated either to the type of grape, vinification process, fibre content of skins, or to the initial concentration in the skin powders. The only work found in literature reporting pesticide loads of grape skin extracts is that by Corrales et al. (2010) who analysed extracts obtained from white grape skins (Riesling variety) from organic and conventional cultivars, finding a higher content in conventional extracts. Interestingly, for the pesticides identified both in our study and in Corrales et al. (2010) (Boscalid, Cyprodinil, Dimethomorph, Famoxadone, Fenhexamid, Fludioxonil and Quinoxifen), all our extracts showed lower levels than the conventional Riesling extract, with BA and PN extracts also lower than the organic Riesling extract. Indeed, organic cultivated grapes can have some pesticide residues due to the proximity of conventional farming cultivars and possible physical or mechanical transport. Nevertheless, pesticide levels have to be below to the values established by the EEC Regulation (1991) (cited in Corrales et al. 2010).

Regarding the different identified active principles, Etofenprox was the only one present in all the samples. It is an insecticide commonly used in viticulture

against *Scaphoideus titanus*, the natural vector of grapevine flavescence dorée. The low assessed concentrations could be due to its chemical characteristic and the typical application period (June–July). Some varieties showed high values of anti-botrytis products (Cyprodinil, Fenhexamid, Fludioxonil), probably due to the fact that, if necessary, these compounds are normally applied three or four weeks before harvest. Furthermore, considering the identified compounds and the commercially available products, two different anti-botrytis treatments may be applied in the vineyard: in the case of CH, MO and NE, commercial products containing the two active principles Cyprodinil and Fludioxonil were probably used, while for MT, the treatment was made with a product that contains just one active principle, Fenhexamid. However, in some samples the antiperonosporic and antioidium products (Dimethomorph, Famoxadone and Spiroxamine) were present at high levels.

Our results confirmed the few literature studies on pesticide residues in grape byproducts. Rose et al. (2009) found residues of fungicides and insecticides in most of the analysed grape marc, reporting a higher content in the seeds than in the marc.

Maximum residue levels (MRL) for different pesticides in wine grapes are defined at EU level and can be consulted on the EU pesticides database. The values are referred to fresh fruit weight, so they have been recalculated here based on dry fruit weight assuming an average 80% water content in grape (Table 3). If the whole pesticide residue remained in the grape marc and then grape skin, the detected values are well below the legal limit. Assessing the hypothetical use of grape skin powder to enrich common wheat flours in fibre, minerals and antioxidants, the MRLs for the same active principles in wheat are available (Table 3). In this case, the values found for some principles and varieties appeared well above the set limits. It is worth considering that the proposed levels of grape skin addition to wheat flour is dependent on the final product required due to the interactions between grape skin fibre and gluten and the resulting effects on protein networks. In pasta products addition is in the range 2.5–7.5% (Sant’Anna et al. 2014a); for bread production it is in the range 4–10% (Mildner-Szkudlarz et al. 2011; Lavelli et al. 2016); while for biscuits production addition can be even higher, at 10–30% (Mildner-Szkudlarz et al. 2013; Walker et al. 2014). Based on the fibre content reported in Table 1, a 10% addition of BA skins would be enough to produce a ‘source of fibre’ wheat flour bread according to the permitted European Commission nutritional claims (at least 3 g of fibre per 100 g for the ‘source of fibre’ claim). The

BA variety had the less contaminated skins but one of the two identified compounds (flufenoxuron) was higher than the allowed residue in wheat and this could prevent its use in some cereal products.

Mineral content

The results found for the mineral content of skin powders are reported in Table 4.

The metal profile reflects the typical grape composition reported in literature, with K the principal element, much more abundant than Ca, Mg and Na (Ribéreau-Gayon et al. 2000). The heavy metals Cd and Pb were also present. Cd was always far below the allowed maximum level in wheat according to Commission Regulation 1881/2006, while Pb was above the EU limit for CH, NE and PN skins. This means that mixing wheat flour with these skin powders could result, depending on the Cd load of the wheat flour and the mixing ratio (as detailed above in the discussion of pesticide residues), in a final mixed flour above the EU limit for Cd.

The extract composition (Table 5) partly reflected the skin powder profile, since Mg content was higher than Ca, while Cr, Cd and Pb were never detected in the extracts.

The percentage of element extraction into aqueous alcohol was very variable for both variety and element. For example, Na migrated more than 60% from BA, CH and NE skins, but less than 10% from the other varieties. Zn was recovered at 90% from BA, MO and MT, but only at 45% in the other varieties. Mn showed 20–50% migration.

Conclusions

Grape skin powders could be used in cereal based products and in other food products, as a source of functional components, *in primis* fibre but also minerals and polyphenols. From this point of view (Figure 1(a)), powders from fermented BA and PN skins appear the most promising. However, a number of contaminants have been identified and their content has been related to the fibre content (Figure 1(b)). In this case, the highest OTA content was found in BA skins, even though it was far below the legal limits for cereals. The heavy metals Pb and Cd were identified in all the samples. Cd was present at very low levels, but Pb content could make some varieties (CH, NE and PN) not appropriate to be mixed with wheat flour. MO and MT skins gave rise to a puzzlingly high Na content. Some biogenic amines were detected, but not histamine and tyramine, the most toxic for humans.

Table 4. Mineral content in waste grape skin powders.

	Chardonnay	Moscato	Müller Thurgau	Barbera	Nebbiolo	Pinot Noir	Wheat, European Union MRL ^a
K (mg g ⁻¹ _{dw})	29.31 ± 0.94 ^b	31.00 ± 1.42 ^b	28.50 ± 1.61 ^b	69.25 ± 13.90 ^a	49.87 ± 16.70 ^{ab}	23.44 ± 2.43 ^b	
Ca (mg g ⁻¹ _{dw})	1.78 ± 0.60 ^a	1.27 ± 0.18 ^a	1.09 ± 0.17 ^a	2.10 ± 0.37 ^a	2.08 ± 0.99 ^a	1.65 ± 0.15 ^a	
Mg (mg g ⁻¹ _{dw})	0.65 ± 0.25 ^a	0.39 ± 0.05 ^a	0.34 ± 0.02 ^a	0.41 ± 0.10 ^a	0.40 ± 0.07 ^a	0.67 ± 0.03 ^a	
Fe (µg g ⁻¹ _{dw})	96.10 ± 11.86 ^b	69.71 ± 7.93 ^b	55.64 ± 3.97 ^b	76.94 ± 5.61 ^b	192.65 ± 45.33 ^a	110.47 ± 6.27 ^b	
Na (µg g ⁻¹ _{dw})	35.40 ± 6.07 ^b	682.13 ± 9.31 ^a	937.96 ± 239.49 ^a	51.60 ± 7.43 ^b	92.35 ± 23.80 ^b	567.20 ± 115.09 ^a	
Cu (µg g ⁻¹ _{dw})	22.29 ± 1.18 ^a	58.00 ± 0.40 ^b	167.39 ± 3.42 ^a	70.57 ± 0.88 ^{ab}	164.95 ± 26.37 ^a	21.56 ± 1.59 ^a	
Zn (µg g ⁻¹ _{dw})	31.51 ± 0.87 ^a	12.22 ± 0.50 ^d	12.08 ± 0.66 ^d	11.06 ± 1.27 ^d	17.86 ± 2.91 ^c	23.01 ± 0.19 ^b	
Mn (µg g ⁻¹ _{dw})	9.69 ± 0.20 ^b	6.91 ± 0.09 ^c	10.24 ± 0.43 ^{ab}	12.76 ± 0.82 ^{ab}	10.42 ± 2.02 ^{ab}	16.71 ± 0.15 ^a	
Cr (µg g ⁻¹ _{dw})	0.35 ± 0.03 ^b	0.18 ± 0.01 ^b	0.39 ± 0.17 ^b	0.39 ± 0.15 ^b	0.88 ± 0.19 ^a	0.37 ± 0.07 ^b	
Pb (µg g ⁻¹ _{dw})	0.26 ± 0.01 ^{ab}	0.23 ± 0.10 ^{ab}	0.22 ± 0.02 ^{ab}	0.15 ± 0.04 ^b	0.35 ± 0.02 ^a	0.37 ± 0.07 ^a	0.23
Cd (ng g ⁻¹ _{dw})	3.31 ± 0.47 ^a	0.97 ± 0.12 ^a	3.86 ± 0.60 ^a	5.42 ± 3.32 ^a	10.77 ± 6.30 ^a	7.79 ± 1.38 ^a	230

Note: Values are means ± SD. The same superscript letter in the same line indicates means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$). ^aValues calculated assuming an average water content of 14% in wheat.

Table 5. Mineral content in waste grape skin extracts.

	Chardonnay	Moscato	Müller Thurgau	Barbera	Nebbiolo	Pinot Noir
K mg l ⁻¹	1196.75 ± 1.25 ^a	451.25 ± 0.25 ^d	958.50 ± 49.00 ^b	109.25 ± 0.25 ^f	302.25 ± 55.75 ^e	783.75 ± 7.75 ^c
Ca mg l ⁻¹	27.60 ± 0.05 ^a	8.48 ± 0.03 ^c	9.63 ± 0.18 ^c	6.85 ± 0.05 ^c	10.60 ± 2.3 ^c	16.28 ± 1.78 ^b
Mg mg l ⁻¹	48.40 ± 0.05 ^a	22.28 ± 0.03 ^c	18.20 ± 0.60 ^{cd}	16.23 ± 0.03 ^d	17.38 ± 2.18 ^{cd}	33.03 ± 3.13 ^b
Fe mg l ⁻¹	2.10 ± 0.10 ^a	1.88 ± 0.13 ^{ab}	1.95 ± 0.01 ^{ab}	1.80 ± 0.10 ^{bc}	1.68 ± 0.03 ^{bc}	1.58 ± 0.03 ^c
Na mg l ⁻¹	6.31 ± 4.29 ^a	7.63 ± 0.48 ^a	9.73 ± 1.28 ^a	6.65 ± 0.10 ^a	9.33 ± 0.23 ^a	8.50 ± 0.01 ^a
Cu mg l ⁻¹	1.90 ± 0.55 ^{bc}	1.58 ± 0.03 ^{bc}	3.80 ± 0.10 ^a	1.65 ± 0.15 ^{bc}	2.10 ± 0.01 ^b	1.08 ± 0.03 ^c
Zn mg l ⁻¹	2.03 ± 0.08 ^a	1.70 ± 0.15 ^b	1.73 ± 0.03 ^b	1.58 ± 0.08 ^{bc}	1.38 ± 0.03 ^c	1.40 ± 0.01 ^c
Mn mg l ⁻¹	0.65 ± 0.01 ^a	0.52 ± 0.03 ^b	0.69 ± 0.03 ^a	0.53 ± 0.01 ^b	0.42 ± 0.01 ^c	0.52 ± 0.01 ^b

Note: Values are means ± SD. The same superscript letter in the same line indicates means not statistically different according to ANOVA and Tukey's post-hoc test ($p < 0.01$).

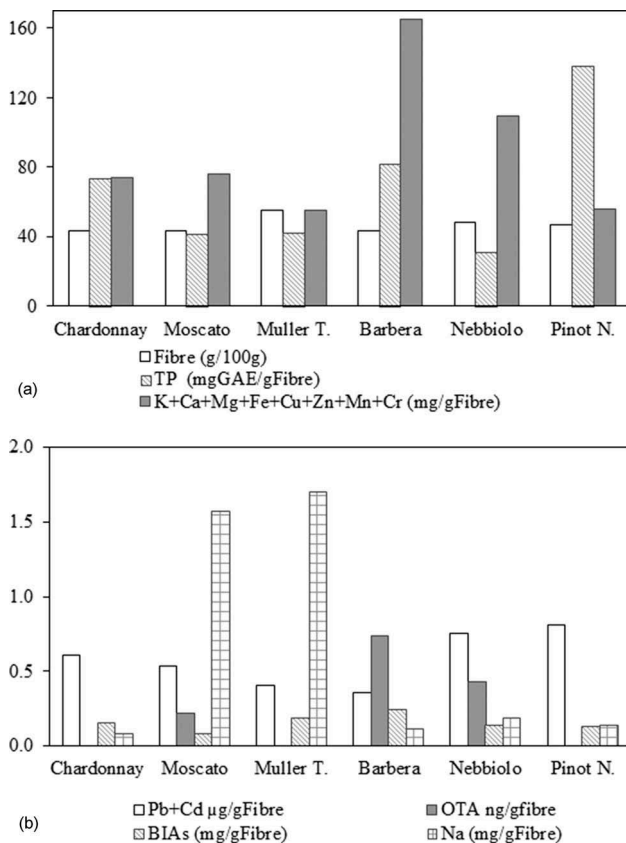


Figure 1. Comparison of skin powders from different grape varieties for fibre and content relative to fibre of (a) functional and (b) undesirable components. TP, total phenolics; GAE, gallic acid equivalents; OTA, ochratoxin A; BIAAs, biogenic amines.

Polyphenolic extracts obtained from waste grape skins could be used as functional ingredients in different foods. The highest amount of free phenolic compounds were extracted from unfermented PN skins which was double that from BA and CH skins. This work showed that both other functional and contaminant compounds can be co-extracted with phenolics at different levels. Considering the TPC as the reference parameter for addition of such extracts in foods, the concentration of all the other assessed compounds has been referred to the TPC (Figure 2). BA extract was contaminated with OTA, since 85% of the initial skin content passed in the extract, but the level was far below the legal limit. White grape extracts and BA extracts were the richest in BIAAs, which however, as mentioned for the skin powders, were not the most toxic amines. BA extract contained low levels of minerals and trace contents of pesticides (comparable to the levels accepted for organic cultivation). Pb and Cd were never extracted.

The obtained results suggest that the initial grape marc has to be carefully selected to obtain flours and extracts to be used in the food sector, on the basis not only of the fibre and or polyphenols content, but also

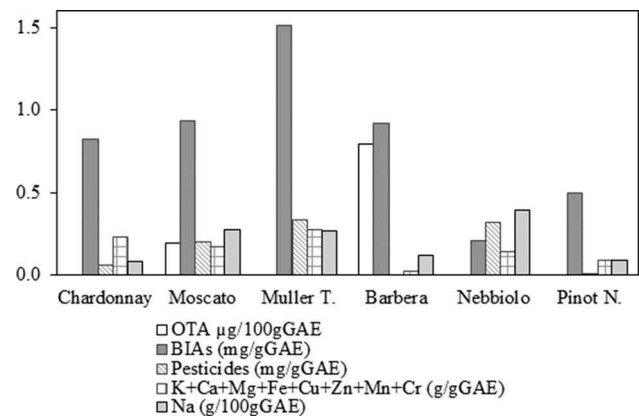


Figure 2. Comparison of skin extracts from different grape varieties for their contents relative to total phenolics of functional and undesirable components. GAE, gallic acid equivalents; OTA, ochratoxin A; BIAAs, biogenic amines.

of the presence of contaminants that can be co-extracted with the target compounds.

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