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Residues of grape (*Vitis vinifera* L.) seed oil production as a valuable source of phenolic antioxidants

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ABSTRACT

Phenolic compounds of seven grape seed samples originating from mechanical seed oil extraction were identified and quantified by HPLC–DAD before (intact seeds) and after (press residue) the oil recovery process. Total amounts of all identified compounds ranged from 4.81 ('Cabernet Mitos') to 19.12 g/kg ('Schwarzriesling') of defatted dry matter (DM; 'Schwarzriesling') for integral grape seeds, whereas their content in the press residues ranged from 2.80 ('Cabernet Mitos') to 13.76 g/kg of defatted DM ('Spätburgunder'). This is the first study presenting comprehensive data on the contents of individual phenolic compounds comprising all polyphenolic subclasses of press residues from grape seed oil production also covering the determination of the antioxidant activities of each subclass (Folin–Ciocalteu, FRAP and TEAC assays). Additionally, the effects of different solvents on the yields of phenolic compounds were determined. Maximum yields were obtained using methanol/0.1% HCl (*v:v*), water [75 °C] and a mixture of ethanol and water [3:1; *v:v*], respectively, whereas pure ethanol resulted in poor polyphenol extraction. The results of the present study confirm the press residues of grape seed oil production still to be a rich source of polyphenolics with strong antioxidant activity.

1. Introduction

Grapes (*Vitis vinifera* L.) belong to the world's largest fruit crops with a global production of around 69 million tons in 2006 (FAOSTAT, 2007). Since about 80% of the total amount is used in winemaking, some 10 million tons of grape pomace arise within a few weeks of the harvest campaign. The seeds constitute a considerable proportion of the pomace, amounting to 38–52% on a dry matter basis. Their oil is rich in unsaturated fatty acids, in particular linoleic acid (Schieber, Müller, Röhrig, & Carle, 2002). Grape seed oil is mainly produced in Italy, France and Spain; however, the demand for this oil has also increased in the rest of Europe (Kamel, Dawson, & Kakuda, 1985).

Apart from being a rich source of a high-value fatty oil, grape seeds have also been appreciated because of their content of phenolic compounds such as gallic acid, catechin and epicatechin, and a wide variety of procyanidins. The latter are also referred to as condensed tannins. Grape seed extracts and procyanidins have been a matter of intense investigations with respect to their potentially beneficial effects on human health. Recent reports indicate a wide range of biological activities, e.g. antioxidant properties and radioprotective effects (Castillo et al., 2000), prevention of cataract (Yamakoshi, Saito, Kataoka, & Tokutake, 2002), antihyperglycemic effects (Pinent et al., 2004), enhancement of postprandial lipemia (Del Bas et al., 2005), modulation of the expression of antioxidant enzyme systems (Puiggròs et al., 2005), improvement of insulin sensitivity and prevention of hypertriglyceridemia (Al-Awwadi et al., 2005), inhibition of aromatase and suppression of aromatase expression (Kijima, Phung, Hur, Kwok, & Chen, 2006), inhibition of protein kinase activity of the epidermal growth factor receptor, protective effects against oxidative damage in mouse brain cells (Guo et al., 2007), and anti-inflammatory effects (Terra et al., 2007).

While it is well known that grape seed polyphenolics display antioxidant activities, the fate of individual phenolic compounds in the course of seed oil recovery as well as their contribution to the overall antioxidant properties of seed extracts has not yet been investigated. In the present study, grape seed oil was produced from seven grape cultivars grown in southern Germany. The polyphenols were extracted from the press residues, fractionated into phenolic acids and flavonoids, and their contents and antioxidant activities were determined. Furthermore, the effects of different solvents on the yields and phenolic profile of extracts from the residues of the oil recovery process were assessed.

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2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All reagents and solvents were of analytical or HPLC grade and were purchased from VWR (Darmstadt, Germany). C_{18} reversed-phase cartridges (Chromabond®, 1000 mg) were from Macherey-Nagel (Düren, Germany). The following standards were used for identification and quantification purposes with HPLC–mass spectrometry (MS) and HPLC-diode array detection (DAD): (+)-catechin, *p*-coumaric acid (−)-epicatechin, ferulic acid, gallic acid, caffeic acid, protocatechuic acid, quercetin (Roth, Karlsruhe, Germany); quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, procyanidin B1, procyanidin B2 (Extrasynthèse, Lyon, France); epicatechin gallate, *trans*-resveratrol (Sigma, St. Louis, MO, USA); *trans*-resveratrol 3-*O*-glucoside (*trans*-polydatin) (Sequoia Research Products, Oxford, UK).

ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)], trolox [6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid], ABAP [2,2′-azobis(2-amidinopropane) dihydrochloride] and TPTZ-Fe(II) [2,4,6-tri(2-pyridyl)-s-triazine] were used for the determination of the antioxidant activity and the Folin–Ciocalteu assay, respectively. These reagents were purchased from Sigma (St. Louis, MO, USA).

2.1.2. Plant material

Grape pomace was obtained from a commercial winery (Felsengartenkellerei Besigheim, Hessigheim, Germany). Seeds from the pomace of five different red grape cultivars (vintage 2006) were used for polyphenol analysis and oil production, respectively ('Cabernet Mitos', 'Lemberger', 'Samtrot', 'Spätburgunder'). The red wines were produced using high-temperature short-time treatment of the mash and subsequent enzymatic degradation of grape pectins. Musts were obtained using a screw extrusion press. Additionally, seeds of 'Schwarzriesling' (rosé wine production) and two pomace samples from white wine production ('Kerner', 'Müller-Thurgau'; vintage 2006) originating from the same process but without mash heating were included in the study. Pomace samples were collected after pressing the mash, sealed in polyethylene bags and kept at −20 °C.

2.2. Methods

2.2.1. Grape seed oil production

Frozen grape pomace was manually separated into skins and seeds using a sieve (mesh size 5.6 mm). The seeds were sealed in polyethylene bags *in vacuo* and kept at −20 °C until analysed. Prior to oil production, the seeds were dried in a cabinet drier for 8 h (60 °C) and pressed using a screw extrusion press (KOMET S 87 G, IBG Monforts, Mönchengladbach, Germany). Grape seed pressing was performed without heating, however, temperature increased to 60–68 °C due to dissipation of mechanical energy. The resulting press residues were cooled, sealed in polyethylene bags *in vacuo* and kept at −20 °C until analysed.

2.2.2. Determination of total lipid contents

Oil contents of the seed samples and press residues were determined after acid hydrolysis of matrix components and Soxhlet extraction with petroleum ether ('Weibull-Stoldt'; Matissek, Schnepel, & Steiner, 1992) using a Soxtherm 2000 automated extraction equipment (Gerhardt, Bonn, Germany; Matthäus & Brühl, 2001).

2.2.3. Extraction of phenolic compounds

For the extraction of phenolic compounds, the integral seeds and the press residues originating from oil production were lyophilised and finely ground using an S 1/2 ball mill (Retsch, Haan, Germany). Aliquots of 5 g of the pulverised samples were extracted and individual phenolic compounds were determined according to a previously published method (Kammerer, Claus, Carle, & Schieber, 2004). Before the identification and quantification of phenolic compounds by HPLC and the determination of total phenolic contents (Folin–Ciocalteu assay) and of the antioxidant activity using TEAC and FRAP assays (see below), the crude extracts were fractionated using RP18 Sep-Pak cartridges. Briefly, 5 mL of the polyphenolic crude extracts were adjusted to pH 7 and applied to the preconditioned cartridges. Phenolic acids were eluted with 10 mL of deionized water and 10 mL of 0.01% HCl (*v*/*v*). Subsequently, flavonoids were recovered with 20 mL of ethyl acetate (Kammerer et al., 2004).

For assessing the effects of the solvent composition on extraction yields and the phenolic profile of the extracts, the press residue of 'Lemberger' seeds was extracted using ethanol, a mixture of ethanol and water (3:1; *v:v*), bidistilled water (75 °C), and methanol/0.1% HCl (*v:v*), respectively. Aliquots of 5 g of the pulverised samples were weighed into Erlenmeyer flasks and extracted with 100 mL of the aforementioned solvents for 2 h under stirring after flushing with nitrogen in order to prevent oxidation. The extracts were centrifuged (10 min, 5366*g*), and solids were re-extracted with 100 mL of the respective solvent (60 min).

2.2.4. Determination of individual phenolic compounds by HPLC

2.2.4.1. HPLC–DAD system. The determination of phenolic compounds was performed using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany), equipped with Chemstation software, a model G1322A degasser, a model G1312A binary gradient pump, a model G1329/G1330A thermoautosampler, a model G1316A column oven, and a model G1315A diode array detector. The separation was carried out with a Phenomenex (Torrance, CA, USA) Aqua C18 column (250 × 4.6 mm i.d.; 5 μm particle size) with a C18 ODS guard column (4.0 × 3.0 mm i.d.) operated at 25 °C. UV–Vis spectra were recorded in the range of 200–600 nm at a spectral acquisition rate of 1.25 scans/s (peak width 0.2 min). A mobile phase consisting of water, acetic acid, and acetonitrile was employed for the determination of phenolic acids and flavonoids as previously described (Kammerer et al., 2004). The injection volume for all samples was 10 μL. Simultaneous monitoring was performed at 280 nm (hydroxybenzoic acids, flavanols), 320 nm (hydroxycinnamic acids) and 370 nm (flavonols), respectively, at a flow rate of 1.0 mL/min.

2.2.4.2. HPLC–MS^n system. For peak assignment, polyphenols were analysed with the HPLC system described above coupled on-line to a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an electrospray ionisation (ESI) source. Data acquisition and processing were performed using Esquire Control software. Mass spectra were recorded in the negative ion mode. Mass spectrometric conditions were applied as previously reported (Kammerer et al., 2004).

2.2.4.3. Quantification of individual phenolic compounds in the seeds and press residues. Individual polyphenols were quantified using a calibration curve of the corresponding standard compound. In case of lacking reference components, the calibration of structurally related substances was used including a molecular weight correction factor (Chandra, Rana, & Li, 2001). The yields of the target compounds were calculated based on total amounts of the respective compounds in grape pomace, which were determined after extraction with methanol/0.1% HCl (*v:v*; Kammerer et al., 2004).

2.2.4.4. Quantification of individual phenolic compounds in grape seed oil. The phenolic content of 'Lemberger' grape seed oil, produced as described under 2.2.1, was determined according to Pour Nikfardjam (2001). Aliquots of 20 g of the grape seed oil were weighed

into Erlenmeyer flasks and extracted with 30 mL of a methanol/water solution (80:20; v/v) in an ultrasonic bath for 30 min after adding 500 μL of Tween® 20. After centrifugation (30 min, 5366*g*), the upper phase was removed and the oil was re-extracted as mentioned above. The residual oil of the combined upper phases was eliminated by freezing overnight at −30 °C and decanting. The resulting solution was evaporated to dryness *in vacuo* at 30 °C, and the residue was dissolved in 20 mL of acetonitrile. The acetonitrile solution was extracted twice with 20 mL of hexane. The acetonitrile phase was evaporated to dryness *in vacuo* at 30 °C and dissolved in 5 mL of methanol/0.1% HCl (*v:v*). The phenolic content of this solution was determined as described above.

2.2.5. Photometric determination of total phenols and of antioxidant activity

2.2.5.1. Total phenolic content (TPC): Folin–Ciocalteu assay. Total phenols were determined using the Folin–Ciocalteu reagent according to Singleton, Orthofer, and Lamuela-Raventós (1999). The absorption was determined after 60 min at 720 nm with a Cary 100 photometer (Varian, Darmstadt, Germany). The results were expressed as gallic acid equivalents (mg GAE/100 g defatted seeds and mg GAE/100 g defatted press residue, respectively).

2.2.5.2. Antioxidant activity: TEAC assay. The assay is based on the decolorisation of the radical cation 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) [$ABTS^{\circ+}$] after reduction to ABTS. Spectrophotometric analyses were performed as published by Schilling et al. (2007). A phosphate buffer was prepared by mixing 818 mL of a Na_2HPO_4 solution (66 mmol/L) with 182 mL of a KH_2PO_4 solution (66 mmol/L) and 150 mmol sodium chloride. For the daily preparation of the radical solution, 0.5 mL of an ABTS solution (20 mmol/L) in the phosphate buffer was mixed with 100 mL of an ABAP solution (2.5 mmol/L) in the phosphate buffer and heated at 60 °C for 15 min in a water bath. The reaction was initiated by adding 1.96 mL of the $ABTS^{\circ+}$ solution to 40 μL of the sample or trolox (standard) solutions or 40 μL of water as a control (Van den Berg, Hänen, Van den Berg, & Bast, 1999; Van den Berg, Hänen, Van den Berg, Van der Vijgh, & Bast, 2000). The mixture was allowed to stand for 6 min at room temperature before the absorption was measured at 734 nm (Cary 100 photometer; Varian, Darmstadt, Germany). Aqueous solutions of trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) in a range of 50–1000 mmol/L were used for calibration of the TEAC and FRAP assays. The values were expressed as mol of Trolox per 100 g of sample (mol Trolox antioxidant equivalent (TAE)/100 g DM of defatted seeds and mol TAE/100 g DM of defatted press residue, respectively).

2.2.5.3. Antioxidant activity: FRAP assay. This method is based on an increase of the absorbance at 593 nm due to the formation of tripyridyl-*S*-triazine complexes with Fe^{2+} [TPTZ-Fe(II)] in the presence of a reductive agent (Benzie & Strain, 1996; Benzie & Szeto, 1999). The FRAP reagent was prepared from 2.5 mL of a TPTZ solution (10 mmol/L) in hydrochloric acid (40 mmol/L) and 2.5 mL of a $FeCl_3$ solution (20 mmol/L) mixed with 25 mL of an acetate buffer (0.3 mol/L, pH 3.6). For the determination of the antioxidant capacity, the FRAP reagent (1.5 mL) was mixed with 100 μL of water and 100 μL of the appropriately diluted sample. The mixture was allowed to stand for 4 min at room temperature before the absorption was measured at 593 nm (Cary 100 photometer; Varian, Darmstadt, Germany). The calibration was performed with trolox as described above.

2.3. Statistical analysis

The determination of phenolic compounds was performed in duplicate. The antioxidant activity and TPC assays were carried out in quadruplicate. For comparison of the results of TPC, FRAP and TEAC assays, the coefficients of correlation were determined for each combination.

3. Results and discussion

3.1. Lipid contents of grape seeds and of the press residues originating from the oil recovery process

Oil recovery from grape seeds was performed using a screw extrusion press. Even though pressing was conducted without heating, the temperature of the solid material increased to 60–68 °C as a result of frictional forces. Thus, the oil yield was enhanced due to reduced viscosity of the lipids. Total oil contents ranged from 7.6% ('Schwarzriesling') to 16.0% ('Müller-Thurgau') for the seeds and from 2.9% ('Kerner') to 4.3% ('Spätburgunder') for the press residues (data not shown), which is in accordance with literature data (Pour Nikfardjam, 2001; Schieber et al., 2002; So-Young et al., 2006). The contents of phenolic compounds given in the present study are based on the defatted samples.

3.2. Contents of individual phenolic compounds

3.2.1. Polyphenols in grape seeds

For the determination of individual phenolic compounds by HPLC, grape seeds of seven cultivars were selected. The total amounts of all compounds calculated as sum of individually quantified components ranged from 4.81 ('Cabernet Mitos') to 19.12 g/kg dry matter (DM, 'Spätburgunder') for non-extracted grape seeds. The phenolic profile of the seeds was dominated by flavonoids, whereas phenolic acids were detected in lower amounts. Among the cultivars investigated 'Lemberger' and 'Cabernet Mitos' showed the most complex phenolic profile with seven phenolic acids and nine flavonoids identified in the seeds and press residues.

3.2.1.1. Phenolic acids. Phenolic acid contents ranged from 188.7 mg/kg DM ('Müller-Thurgau') to 1165.8 mg/kg DM ('Samtrot'), with gallic acid being the predominant compound in all samples. The phenolic acid contents are presented in Table 1. Great variabilities in the phenolic acid contents were observed between the samples. However, marked differences between seeds from red and white grape pomace were not found. With the exception of the cultivar 'Samtrot', the gallic acid content of the samples ranged from 188.7 mg/kg DM ('Müller-Thurgau') to 332.1 mg/kg DM ('Lemberger') in the seeds. The seeds of 'Samtrot' grapes exhibited higher gallic acid amounts (1116.5 mg/kg DM). These findings can be attributed to the large variabilities in the phenolic composition of samples from different cultivars and vintages (Kammerer et al., 2004).

3.2.1.2. Flavonoids. The flavan-3-ols catechin, epicatechin, epicatechin gallate and the dimeric procyanidins B1 and B2 were the predominant components in all cultivars (Table 2). Quercetin, quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside and quercetin 3-*O*-glucuronide were also detected in some of the samples. However, their contents were negligible. Highest total flavonoid amounts were found in 'Spätburgunder' (Pinot noir) seeds (18.78 g/kg), followed by 'Samtrot' (14.76 g/kg) and 'Schwarzriesling' (8.88 g/kg). The contents of the white grape cultivars were 7.49 ('Müller-Thurgau') and 5.34 g/kg ('Kerner'), respectively.

3.2.2. Polyphenols in the seed oil press residues

3.2.2.1. Phenolic acids. The phenolic acid contents of the press residues ranged from 147.4 ('Müller-Thurgau') to 492.7 mg/kg DM ('Samtrot'). In accordance with the aforementioned results, gallic acid was the predominant phenolic compound ranging from

Table 1
Phenolic acid contents (mg/kg DM) of seeds and seed oil press residues from seven grape (*Vitis vinifera* L.) cultivars grown in southern Germany (mean ± standard deviation of analyses performed in duplicate)

	'Cabernet Mitos'		'Lemberger'		'Spätburgunder'		'Samtrot'		'Müller-Thurgau'		'Kerner'		'Schwarzriesling'	
	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue
Caftaric acid	91.1 ± 1.6	53.5 ± 6.6	112.5 ± 1.4	96.6 ± 0.8	18.9 ± 0.9	21.5 ± 0.5	49.2 ± 7.2	139.0 ± 16.5	n.d.	13.7 ± 1.6	15.6 ± 3.6	23.2 ± 2.3	127.9 ± 10.7	38.8 ± 2.7
Coutaric acid	28.3 ± 0	22.1 ± 2.3	31.7 ± 1.9	19.5 ± 1.7	16.9 ± 1.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.7±0.5	n.d.	20.9 ± 1.5
Fertaric acid	21.1 ± 0.3	n.d.	21.8 ± 1.2	2.9 ± 0.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gallic acid	278.8 ± 3.4	152.9 ± 7.8	332.1 ± 20.3	127.0 ± 8.3	244.3 ± 24.9	241.7 ± 10.1	1116.5 ± 60.0	353.7 ± 22.0	188.7 ± 12.8	133.7 ± 12.4	248.6 ± 14.2	133.2 ± 19.7	307.1 ± 5.5	93.1 ± 14.8
Caffeic acid	n.d.	16.9 ± 0.3	n.d.	12.3 ± 2.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.2 ± 0.4
p-Coumaric acid	n.d.	17.4 ± 0.1	n.d.	9.0 ± 1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.7 ± 0.2
Ferulic acid	n.d.	n.d.	n.d.	7.9 ± 1.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total content	419.3 ± 3.8	262.8 ± 12.5	498.2 ± 20.5	275.2 ± 10.0	280.0 ± 25.0	263.2 ± 10.1	1165.8 ± 60.5	492.7 ± 27.5	188.7 ± 12.8	147.4 ± 12.5	264.2 ± 14.6	164.0 ± 19.8	435.0 ± 12.1	168.7 ± 15.1

Abbreviation: n.d., not detected.

Table 2
Flavonoid contents (g/kg DM) of seeds and seed oil press residues from seven grape (*Vitis vinifera* L.) cultivars grown in southern Germany (mean ± standard deviation of analyses performed in duplicate)

	'Cabernet Mitos'		'Lemberger'		'Spätburgunder'		'Samtrot'		'Müller-Thurgau'		'Kerner'		'Schwarzriesling'	
	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue	Seeds	Press residue
Proc B1	0.77 ± 0.20	0.34 ± 0.00	0.71 ± 0.24	0.69 ± 0.00	4.99 ± 0.55	1.84 ± 0.11	2.07 ± 0.1	1.49 ± 0.03	1.21 ± 0.02	1.06 ± 0.05	0.61 ± 0.04	0.55 ± 0.09	0.91 ± 0.03	1.08 ± 0.13
Cat	1.22 ± 0.13	0.74 ± 0.06	1.43 ± 0.03	1.00 ± 0.00	3.76 ± 0.28	6.18 ± 0.05	4.64 ± 0.78	4.56 ± 0.08	2.17 ± 0.04	2.20 ± 0.04	0.88 ± 0.12	1.01 ± 0.10	2.38 ± 0.09	2.38 ± 0.00
Proc B2	0.97 ± 0.15	0.47 ± 0.07	1.29 ± 0.40	0.86 ± 0.02	2.98 ± 0.12	1.39 ± 0.11	2.73 ± 0.27	1.02 ± 0.02	1.21 ± 0.00	1.07 ± 0.06	1.16 ± 0.15	0.61 ± 0.07	1.22 ± 0.11	0.97 ± 0.11
Epicat	1.23 ± 0.15	0.79 ± 0.06	1.64 ± 0.07	1.24 ± 0.00	6.12 ± 0.38	3.92 ± 0.33	3.31 ± 0.37	2.42 ± 0.06	2.06 ± 0.03	1.10 ± 0.02	2.23 ± 0.43	1.06 ± 0.01	2.66 ± 0.27	1.73 ± 0.10
Epicat gall	0.18 ± 0.04	0.14 ± 0.01	0.83 ± 0.23	0.60 ± 0,03	0.92 ± 0.08	0.17 ± 0.01	1.98 ± 0.04	0.19 ± 0.00	0.80 ± 0.02	0.02 ± 0.00	0.41 ± 0.05	0.44 ± 0.03	1.69 ± 0.07	0.76 ± 0.05
q-3-*O*-gal	0.02 ± 0.00	0.03 ± 0.00	<0.01 ± 0.00	0.06 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<0.01 ± 0.00	n.d.	0.01 ± 0.00
q-3-*O*-glc	n.d.	<0.01 ± 0.00	<0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.00	n.d.	0.04 ± 0.00
q-3-*O*-gluc	n.d.	n.d	n.d.	0.02. ± 0.00	<0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	n.d.	0.03 ± 0.00	n.d.
q-3-*O*-rha	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 ± 0.00	n.d.	n.d.
q	n.d.	0.01 ± 0.00	<0.01 ± 0.00	0.01 ± 0.00	<0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 ± 0.00	n.d.	<0.01 ± 0.00
Total content	4.39 ± 0.29	2.52 ± 0.1	5.91 ± 0.52	4.48 ± 0.03	18.78 ± 1.01	13.50 ± 0.39	14.76 ± 0.91	9.69 ± 0.08	7.49 ± 0.06	5.65 ± 0.09	5.34 ± 0.54	3.71 ± 0.22	8.88 ± 0.31	6.98 ± 0.16

Abbreviations: proc, procyanidin; cat, catechin; epicat, epicatechin; epicat gall, epicatechin gallate; q, quercetin; glc, glucose; gal, galactoside; gluc, glucuronide; rha, rhamnoside; n.d., not detected.

93.1 mg/kg DM ('Schwarzriesling') to 353.7 mg/kg DM ('Samtrot'). Interestingly, the gallic acid content of 'Samtrot' press residue was only slightly higher compared to the other cultivars, which may be ascribed to condensation reactions with further grape seed phenolics as a consequence of the thermal impact. As can be seen from Table 1, the phenolic acid profile of the press residues was more complex than that of the intact seeds. As an example, caffeic, *p*-coumaric and ferulic acids were detected in 'Lemberger' press residue, whereas the respective seed sample was devoid of these compounds. These findings are in accordance with a previously published study showing that heating of grape seed extracts may change their polyphenol profile and contents (So-Young et al., 2006). These components may originate from the degradation of higher molecular phenolic components which were not determined by HPLC.

3.2.2.2. Flavonoids. Total amounts of individually quantified flavonoids ranged from 2.52 ('Cabernet Mitos') to 13.50 g/kg DM ('Spätburgunder'). As observed for flavonoids in grape seeds, highest amounts were determined in 'Spätburgunder' followed by 'Samtrot' (9.69 g/kg DM) and 'Schwarzriesling' (6.98 g/kg DM; Table 2). The flavonoid contents of the press residues were below those of the integral grape seeds. Thus, phenolic contents of the press residues were lowered through oil recovery, even though polyphenols are hardly soluble in fatty oil (Fig. 1A). The average relative flavonoid loss amounted to 29.4%, without significantly changing the phenolic profile. Decreasing the thermal impact during pressing might even enhance flavonoid retention, thus supporting the high potential of the seed oil press residues as a source of phenolic compounds.

3.2.3. Polyphenols in grape seed oil

The loss of phenolic compounds in the solid matrix during pressing might be ascribed both to the thermal impact and to a partial transfer into the oil. Even though the solubility of phenolic compounds in the oil is poor, small amounts might be carried over into the oil through processing. Therefore, phenolic contents in the fatty oil were determined. Since the crude oil contained

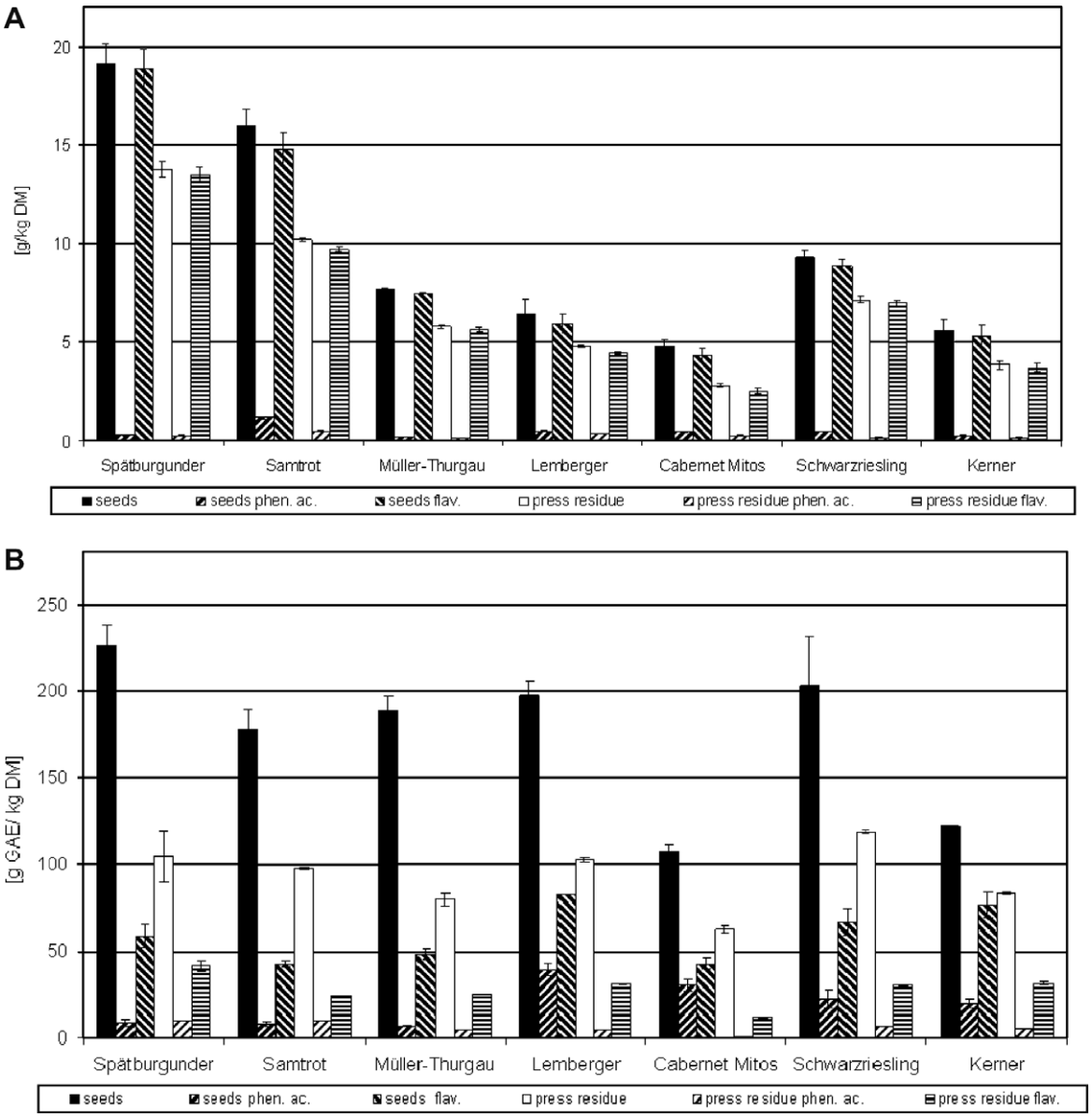

Fig. 1. Phenolic contents of grape seeds of different cultivars and seed oil press residues derived thereof as determined by HPLC–DAD (A) and the Folin–Ciocalteu assay (B; mean ± standard deviation of analyses performed in duplicate (A) and quadruplicate (B), respectively).

sediments, the phenolic content of the oil was determined both with and without removal of the sediments. The total polyphenol amount of the oil after centrifugation was 2.9 mg/kg, whereas total phenolics of the sediment containing oil amounted to 359.3 mg/kg (data not shown). Only minor amounts of catechin, epicatechin (1.3 mg/kg each) and *trans*-resveratrol (0.3 mg/kg) were detected in the clarified oil. Since the oil has not been refined, its phenolic content after refinement is assumed to be negligible. These findings are in agreement with literature data showing that procyanidins were not detectable in two different grape seed oils using the vanillin–HCl assay (Nakamura, Tsuji, & Tonogai, 2003). In another study, only negligible amounts of procyanidins were determined in commercially available grape seed oils (Pour Nikfardjam, 2001). In contrast, the turbid oil with sediment exhibited significantly higher amounts of polyphenols, the profile of which was comparable to that of grape seeds, thus explaining in part the lower phenolic amounts of the press residues as compared to the integral grape seeds. Therefore, the sediments of the grape seed oil production might also serve as a rich source of phenolic antioxidants.

3.3. Total phenolic content (TPC; Folin–Ciocalteu assay)

In order to assess the contribution of various polyphenol classes to the total polyphenol amount, the TPC of the crude seed extracts was measured first. Subsequently, the crude extracts were fractionated into phenolic acids and flavonoids using RP-18 cartridges, and the TPC of both fractions was determined by the Folin–Ciocalteu assay. The TPC of the seeds ranged from 107.4 ('Cabernet Mitos') to 226.0 g gallic acid equivalents (GAE)/kg seeds ('Spätburgunder'; Fig. 1B). The TPC of 'Schwarzriesling' (203.4 g GAE/kg DM), 'Lemberger' (197.1 g GAE/kg DM), 'Müller-Thurgau' (189.1 g GAE/kg DM), Samtrot (177.8 g GAE/kg DM) and Kerner (122.4 g GAE/kg DM) ranged between these extremal values. Interestingly, the sequence of decreasing amounts as determined by HPLC (Fig. 1A) and the Folin–Ciocalteu method (Fig. 1B) differed slightly. Additionally, major differences in the phenolic amounts of the different cultivars can be observed in Fig. 1A illustrating the sum of individually quantified compounds. In contrast, these differences were less pronounced when the Folin–Ciocalteu assay was used (Fig. 1B). This observation is probably due to the fact that both monomeric, oligomeric and polymeric polyphenols are determined by the Folin–Ciocalteu assay, whereas only the low-molecular compounds listed in Tables 1 and 2 were covered by HPLC. Accordingly, the grape seeds studied here are less variable in terms of their contents of oligomeric and polymeric compounds as compared to the low-molecular phenolics.

A similar phenomenon has been described for the determination of the antioxidant activity in shelf life experiments with processed blackberries (Hager, Luke, & Prior, 2008). In this study the antioxidative activity remained constant throughout storage, whereas the samples showed a concomitant decrease of the contents of monomeric anthocyanins. This observation was explained by the high antioxidant activity of the polymeric compounds formed during storage. Since the Folin–Ciocalteu determination is also based on the antioxidant potential of the phenolics, these findings might explain the differences between the determination of individual and total phenolic compounds.

Major differences between the TPC of the crude extracts and the sum of the phenolic acid and flavonoid amounts were observed (Fig. 1B). This might be explained by the fractionation of the crude extracts. Grape seeds are rich in oligomeric and polymeric polyphenols (particularly procyanidins) which were determined in the crude extract by the Folin–Ciocalteu assay. The fractionation was performed using a RP-18 cartridge. High molecular compounds were probably not eluted quantitatively from the SEP-Pak cartridges. Thus, lower amounts of phenolics were determined in the fractions as compared to the crude extracts using the Folin–Ciocalteu assay. In a previous study of Sun, Belchior, Ricardo-da-Silva, and Spranger (1999), a RP-18 cartridge was used for the pre-fractionation of procyanidins. After eluting monomeric and oligomeric flavan-3-ols with ethyl acetate, polymeric procyanidins were still adsorbed onto the C18 material. These findings corroborate our assumption that the marked differences in the results obtained for the raw extract and fractions were due to irreversible binding of polymeric compounds to the adsorbent.

Significantly lower polyphenol contents of the solid residue after oil recovery as deduced from the HPLC results were confirmed by photometric polyphenol determinations (loss of 35.8% on average). Highest TPC values were found in the samples of 'Schwarzriesling' (119.3 g GAE/kg DM), followed by 'Spätburgunder' (105.0 g GAE/kg DM), 'Lemberger' (102.9 g GAE/kg DM) and 'Samtrot' (97.5 g GAE/kg DM; Fig. 1B). In contrast to the HPLC data, 'Lemberger' seeds after oil recovery were still rich in phenolic compounds, which is probably due to high amounts of oligomeric and polymeric compounds which were not detected by HPLC. For this reason, the phenolic contents of the seeds and the press residues as determined by the Folin–Ciocalteu assay exceeded the total amount of individual phenolics as quantified by HPLC.

3.4. Antioxidant activity of phenolic extracts from grape seeds and press residues originating from grape seed oil production

3.4.1. TEAC assay

The results of the TEAC assay are illustrated in Fig. 2A. The values of the crude extracts from the seeds ranged from 48.49 to 104.80 mol TAE/100 g DM. 'Spätburgunder' yielded highest amounts, followed by 'Müller-Thurgau' (81.14 mol TAE/100 g DM), 'Samtrot' (78.59 mol/100 g DM), 'Lemberger' (76.21 mol TAE/100 g DM), 'Schwarzriesling' (72.10 mol TAE/100 g DM), 'Kerner' and 'Cabernet Mitos'. Concerning the flavonoid fraction of the press residues, highest antioxidant activities were observed for 'Samtrot', followed by 'Kerner', 'Spätburgunder', 'Müller-Thurgau', 'Schwarzriesling', 'Lemberger' and 'Cabernet Mitos'. The differing order between TEAC values of the crude seed extracts and of the flavonoid fraction can probably be attributed to irreversible binding of oligomeric and polymeric compounds to the SEP-PAK cartridge as described under Section 3.3.

Furthermore, the TEAC values might also be affected by non-phenolic compounds, explaining the large differences between the results for the crude extracts and the sum of those for the flavonoid and phenolic acid fraction. The seed oil press residues exhibited lower TEAC values, ranging from 26.14 ('Cabernet Mitos') to 46.58 ('Schwarzriesling') mol TAE/100 g DM, again demonstrating the degradation of phenolic antioxidants during processing.

3.4.2. FRAP assay

The FRAP assay showed highest values for both the crude extract and flavonoid fraction of 'Spätburgunder' seeds (58.04 mol TAE/100 g DM and 21.83 mol TAE/100 g DM, respectively). FRAP values of the press residues ranged from 16.71 to 26.50 mol TAE/100 g DM. 'Lemberger' yielded highest amounts followed by 'Schwarzriesling', 'Müller-Thurgau', 'Spätburgunder', 'Samtrot', 'Cabernet Mitos' and 'Kerner' (Fig. 2B).

3.4.3. Correlation of antioxidant activity, contents of individual phenolic compounds and TPC values

Phenolic compounds are known to act as antioxidants not only because they are hydrogen or electron donators but also because they stabilize radical intermediates, thus preventing oxidation of various food ingredients (Ricardo da Silva, Darmon, Fenandez, & Mitjavila, 1991; Sun et al., 1999). To evaluate the antioxidant

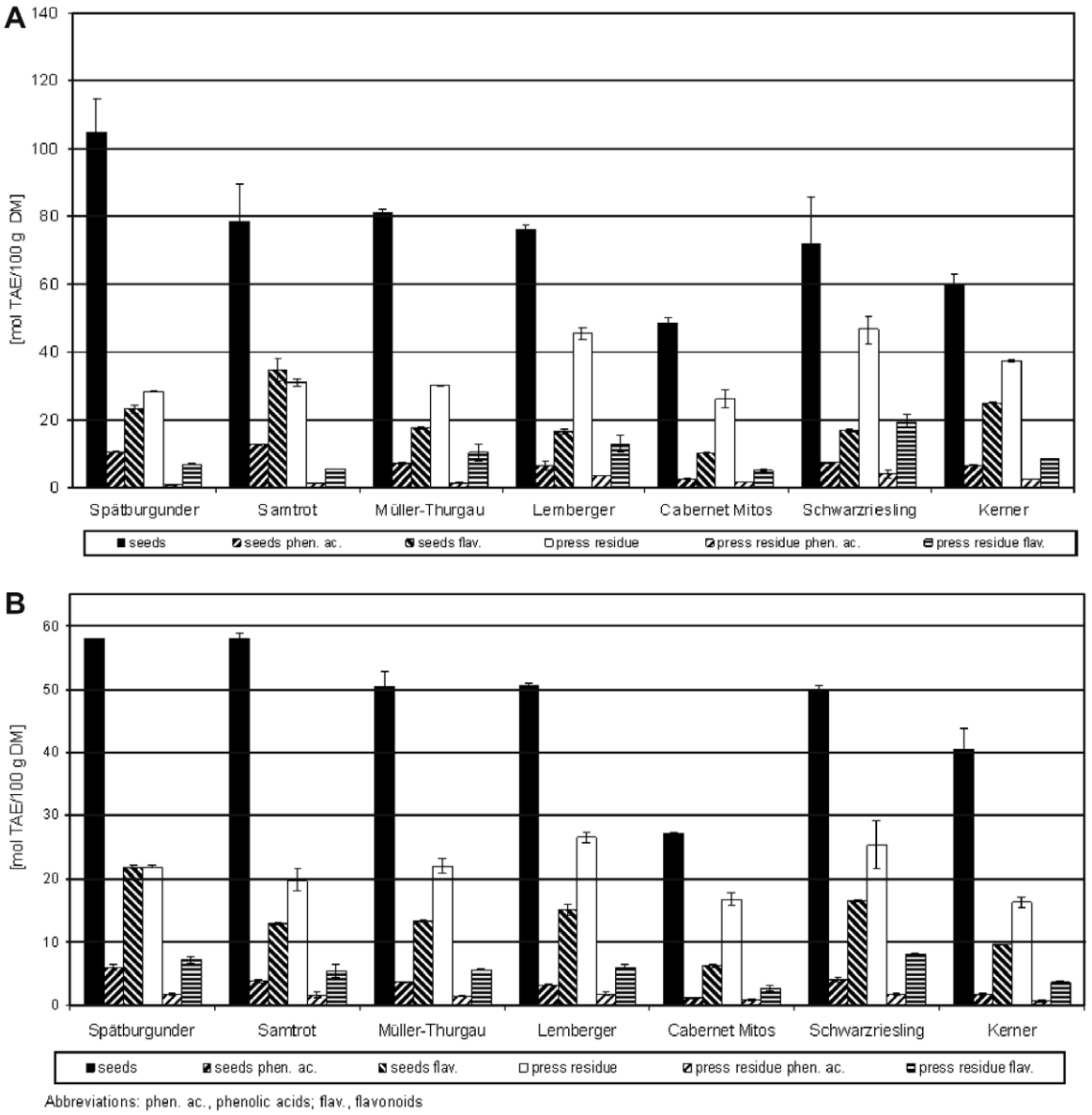

Fig. 2. Antioxidant activity of phenolic compounds extracted from grape seeds of different cultivars and the press residues originating from the oil recovery process: TEAC (A) and FRAP (B) assay (mean ± standard deviation of analyses performed in quadruplicate (B)).

properties of the crude extracts based on their phenolic contents and profile, coefficients of correlation between TEAC, FRAP and the total phenolic contents (Folin–Ciocalteu assay) were determined (Table 3). Each combination of the results showed a high correlation. As can be seen from Fig. 2, the flavonoid fraction always exhibited much higher antioxidant activities compared to the phenolic acid fraction, which is in accordance with the TPC of each fraction (Fig. 1B). Procyanidin B1 has been assumed to be one of the most important radical scavengers in grape seed extracts (Guendez, Kallithraka, Makris, & Kefalas, 2005), which may explain the high TEAC values of 'Spätburgunder' seeds (Fig. 2A and Table 2). However, this sample was also characterised by high catechin and epicatechin contents. In a recent study evaluating the *in vitro* antioxidant activity of dietary grape seed products, the antioxidant activity was shown to depend on several variables and not on single compounds (Monagas et al., 2005). Thus, the more complex phenolic profile of 'Spätburgunder' seeds and synergistic effects might explain the high antioxidant activity of the corresponding extracts.

Table 3
The coefficients of correlation between TEAC, FRAP and Folin–Ciocalteu assays of seed and seed oil press residue samples

	Seeds	Seed oil press residue
FRAP/TEAC	0.9482	0.9283
FRAP/Folin–Ciocalteu	0.9345	0.9559
FRAP/Folin–Ciocalteu	0.9193	0.9078

The large variations in the contents of individual phenolic compounds precluded the establishment of correlations of individual compounds with the antioxidant activity. Therefore, the antioxidant activity is more likely determined by the combination of several phenolic compounds. As an example of these complex findings the TEAC values of the flavonoid fractions of the seeds and press residues of 'Samtrot' showed contrasting results. Using identical *in vitro* tests, the cultivar 'Samtrot' showed the highest TEAC values for the seeds but lowest antioxidant activity among all press

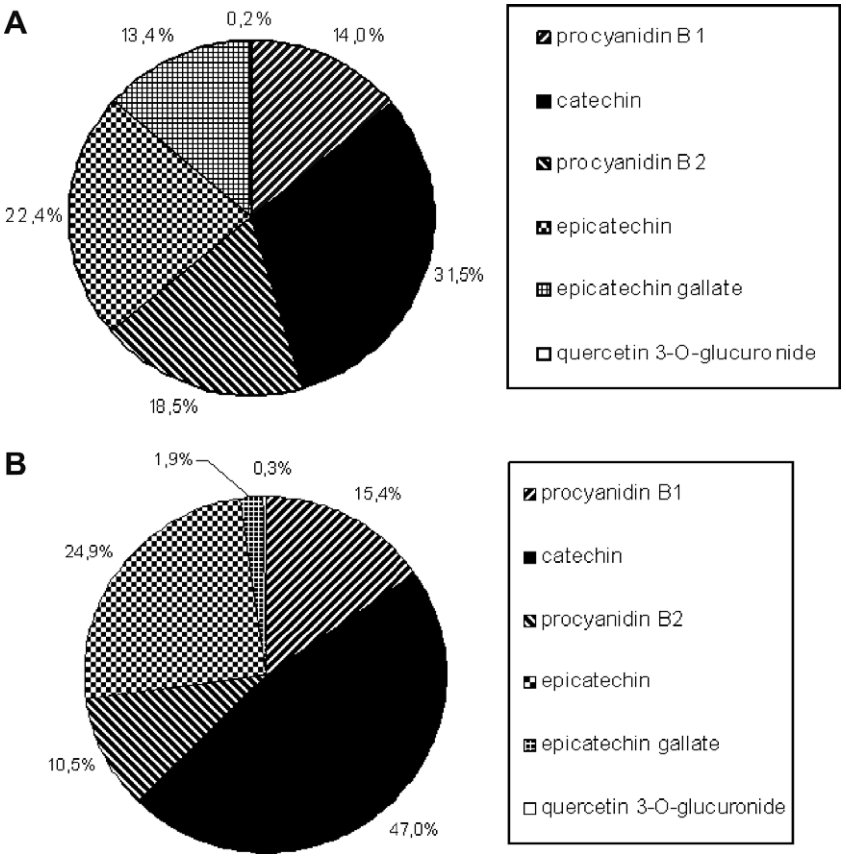

Fig. 3. Flavonoid composition of extracts from *Vitis vinifera* L. *cv*. 'Samtrot' grape seeds (A) and seed oil press residues (B). Values are expressed as percentage of the total amount of flavonoids.

residues. However, these two samples markedly differed in their relative percentages of phenolic compounds. While the seeds were characterised by an evenly distributed proportion of the five phenolic compounds (Fig. 3A), the flavonoid fraction of the seed oil press residue was clearly dominated by catechin (47%) and showed a very low content of epicatechin gallate (1.9%; Fig. 3B). Therefore, a "balanced" mixture of various phenolic compounds seems to enhance antioxidant activity.

3.5. Effects of solvent composition on the yields of phenolic compounds

The aforementioned results demonstrate that grape seeds and the press residues from grape seed oil recovery are a rich source of phenolic compounds with high antioxidant activities. The extraction of these compounds was performed on laboratory scale using methanol/0.1% HCl (v:v), which is unfavourable for polyphenol extraction on a large scale. Therefore, further investigations were performed to optimise solvent extractions under conditions which might also be applied to recover food ingredients on an industrial scale. Using ethanol/water (3:1; *v:v*) and hot water (75 °C) showed optimal extraction yields of 99.8% and 98.2%, respectively. In contrast, recoveries were poor (49.4%) when pure ethanol was used.

The use of organic solvents would require rigorous safety precautions. Furthermore, attention must be paid to ensure maximum trace level amounts of the organic solvents in the final product. Therefore, water as a solvent is an excellent alternative for the recovery of food ingredients. However, Shi et al. (2003) described that when using water, proteins and polysaccharides are also extracted under high pressure and at high temperatures. These findings could not be confirmed in the present study. Hot water extracts did not contain high amounts of undesired compounds. Consequently, concentration of the aqueous extract by evaporation to dryness *in vacuo* did not cause any problems.

The results presented in this study demonstrate the press residues of grape seed oil production still to be rich in phenolic compounds and their extracts to be high in antioxidant activity, making their utilisation worthwhile and thus supporting sustainable agricultural production. Finally, these nutraceuticals can be efficiently extracted using hot water instead of organic solvents.

4. Concluding remarks

This study clearly demonstrates the press residues of grape seed oil production to be a polyphenol-rich by-product with high antioxidant activity. However, the oil extraction process applied in this study needs to be optimised. Since the temperature rose above 60 °C during pressing, losses of target compounds could not be avoided. Thus, a cold pressing process might increase the phenolic content and the antioxidant capacity of the seed oil press residues. Polyphenols can easily be extracted from these by-products in high amounts, enabling their application as ingredients of functional or enriched foods.

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