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Offline combination of pressurized fluid extraction and electron paramagnetic resonance spectroscopy for antioxidant activity of grape skin extracts assessment☆

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ABSTRACT

A comprehensive characterization of grape skin methanolic and ethanolic extracts prepared by pressurized fluid extraction (PFE) at various temperatures within 40 to 120 °C from two wine grape varieties, St. Laurent and Alibernet was performed. For the first time, an offline combination of PFE and electron paramagnetic resonance (EPR) spectroscopy together with other experimental methods was employed to assess the effect of extraction conditions on numerous extract characteristics including antioxidant or radical-scavenging ability, HPLC profile of anthocyanins, total phenolic compounds content (TPC), tristimulus color values (CIE Lab), and pH values. The properties of extracts depend on the solvent used, the mass of grape skins as well as on the extraction conditions among which the temperature plays a crucial role. In spite of wide interval of extraction temperatures, all extracts still retain their antioxidant and/or radical-scavenging properties, indicating that the extracts prepared by PFE can serve as potential source of functional food supplements or color enhancers.

1. Introduction

Grape skins contain a large number of polyphenolic compounds the concentration of which varies greatly according to the variety of grapevine, cultivar, season and environmental factors. Up to date, more than 8000 different phenolic structures have been recognized. They include both flavonoids (e.g., flavonols, flavan-3-ols as well as polymers of the latter, defined as procyanidins and anthocyanins) and non-flavonoids (e.g., hydroxycinnamates and hydroxybenzoates). Anthocyanins represent the most abundant group of polyphenols and are associated with the color of several aerial and subterranean organs in many plants [1–6].

In grapevines, anthocyanins are accumulated in leaves during ripening and are responsible for the coloration of grape skins in red and rosé cultivars, and in the grape pulp, respectively [1–6]. The most abundant anthocyanins identified in Vitis vinifera grapes and wines are the 3-O-glucosides, 3-O-acetyl glucosides and 3-O-p-coumaroyl glucosides of delphinidin (De), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv) as the dominant form. Besides them, tartaric esters of hydroxycinnamic acids, monomeric and dimeric flavanols, flavonols and stilbenes were also identified [4–6].

These pigments are water soluble, revealing also the beneficial effects on human health, including the enhancement of visual acuity, but evinced also anticarcinogenic, antimutagenic or anti-inflammatory action [7–10].

Although grape skins represent the best source for their isolation, only about 30–40% of polyphenols (mainly anthocyanins) are extracted from grapes during the winemaking process [5]. Thus, in order for polyphenols to be used as food supplements, efforts have recently been made to find a suitable extraction system for their isolation from grapes in larger quantities. One also has to respect the limited stability of polyphenols under specific conditions [2,11].

Grape anthocyanins are frequently extracted by conventional extraction techniques using acidified methanol, ethanol, acetone or their aqueous mixtures. However, the use of acidic solvents may lead to the denaturation of cellular membranes, thus facilitating, besides other processes, the solvolysis of anthocyanins. In addition, the extraction process is time consuming and laborious [12,13].

Pressurized fluid extraction (PFE) [14–17] operating at elevated temperatures and pressures was previously used for the extraction of different phenolic compounds from grapes and wines [18–20]. The advantages of PFE over conventional extraction techniques can briefly be summarized as follows: higher temperature increases

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the solubility, diffusion rate and mass transfer of the compounds and decreases the viscosity and surface tension of the extraction solvent. These changes improve the contact of analytes with the solvent and enhance extraction which can be achieved more rapidly with less solvent consumption. Moreover, the elevated pressure improves the contact of solvent with the analytes trapped in the matrix pores [21].

For ecological and economical reasons, ethanol, methanol and water are the most frequently employed solvents in PFE. Ju and Howard [7] investigated the effect of different solvents and temperature on the efficiency of PFE of anthocyanins from the skin of highly pigmented red wine grapes. They concluded that 60% acidified methanol at 60 °C extracted the highest level of anthocyanins [7,22]. The type and polarity of the extracting solvent influence not only the yield and the composition of isolated polyphenols, but also the antioxidant activity of the final extract. As follows from available data, maximum total phenolic extraction yields were obtained with methanol but optimal solvent providing maximum antioxidant activity is required for each substrate [23]. In this context, it should be noted here that different relationships between phenolic content and antioxidant activity have been reported, some authors found a positive correlation while the others were not able to find any relationship [1,2,23–28]. Moreover, as follows from previously published papers, polyphenols can act as either anti- or pro-oxidants depending on the reaction conditions [26,29–31].

Various methods have been used to monitor and to compare the radical-scavenging or antioxidant activity of foods and biosystems, among which electron paramagnetic resonance (EPR) spectroscopy, due to its high sensitivity and selectivity, is considered to be one of the most efficient [32–36]. Most frequently, the ability of the studied system to scavenge different free radicals, e.g., 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhydrazyl (•DPPH), or hydroxyl radical (•OH) added or generated directly in the experimental system is monitored [8,26,37–39]. As the redox reaction occurring in the experimental system is frequently accompanied by the development or disappearance of color at specific wavelength, UV–vis spectroscopy is frequently effectively involved as well [1,2,11,15–17,24–27,40].

Methods of multivariate statistics, notably principal component analysis (PCA) and canonical discriminant analysis (CDA) [41], represent valuable tools, making possible the categorization of different food samples via the consideration of many variables that can be measured, often in a single analytical level. The analysis of chromatographic, electrophoretic or elemental data was previously effectively used for the discrimination of many kinds of foods, e.g., of cheeses and cheese-derived products or wines, based on the different geographical origin, varieties or quality, but also for the prediction of spices γ-irradiation [42–44].

In this contribution, a comprehensive study of grape skin methanolic and ethanolic extracts prepared by PFE at various temperatures from 40 up to 120 °C from two wine grape varieties, St. Laurent and Alibernet from Velké Pavlovice and Mikulov subregions (South Moravia region, Czech Republic) is presented. To the best of our knowledge, this is the first application of the offline combination of PFE and EPR. The latter was used to assess the effect of the extract preparation procedure on the radical-scavenging ability of the extracts. Other basic characteristics of extracts prepared by PFE, i.e. the HPLC profile of polyphenols as well as total phenolic compounds' content (TPC), tristimulus color values (CIE Lab color space) and pH values were simultaneously monitored as well, in order to obtain as comprehensive information on extracts quality as even feasible.

All the experimental data were processed with PCA and CDA to specify the optimum extraction conditions for extract preparation from the perspective of the potential further application of the extracts as food supplements.

2. Materials and methods

2.1. Sample characterization

Grape skins from two wine grape varieties, St. Laurent and Alibernet from Velké Pavlovice and Mikulov sub-regions (South Moravia region, Czech Republic), collected in 2007 vintage were used in experiments. Harvested grapes were placed into polystyrene boxes filled with dry ice and transported to the laboratory. Grape skins were then manually separated from the pulps at inert atmosphere and lyophilized. The dried skins were ground to a fine powder under liquid nitrogen and stored at −20 °C in dark glass vials.

2.2. Chemicals

The substances used in experiments included 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), freshly distilled before use and stored at −18 °C under argon (Sigma Aldrich Ltd, Milwaukee, WI), stable free radical 1,1-diphenyl-2-picrylhydrazyl (•DPPH) (Fluka, Buchs, Switzerland), 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) salt (ABTS), (Polysciences, Inc., Warrington, PA), $K_2S_2O_8$ (Merck GmbH, Darmstadt, Germany), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma Aldrich Ltd, Milwaukee, WI, USA), H_2O_2, NaOH, $Fe(NH_4)_2(SO_4)_2{\cdot}9H_2O$ (Lachema Brno, Czech Republic), methanol and ethanol of spectroscopic grade purity (AFT, Bratislava, Slovak Republic), and deionized water. The standards for HPLC were Brillant blue FCF (Lachema, Brno, Czech Republic) and 3-O-monoglucosides of malvidin (Mv-3-glc), delphidin (De-3-glc), peonidin (Pn-3-glc), cyanidin (Cy-3-glc), petunidin (Pt-3-glc), and pelargonidin (Pg-3-glc) of analytical grade purity (Polyphenols Laboratories AS, Sandnes, Norway). These 3-O-monoglucosides constitute the principal portion of grape anthocyanins [1–6] and were selected for the present study as a result of trade-off between the need for a representative selection of the antioxidants present in grapes and the resources available. We believe that the selection introduces a tolerable uncertainty into the results described below.

2.3. Pressurized fluid extraction

Static PFE of grape skins was performed using a *one*PSE extractor (Applied Separations, Allentown, PA). A respective portion of grape skin powder (0.5 and 1.0 g, respectively) was placed into 11 ml extraction cell containing inert material (glass beads, 570–700 μm) at the bottom. The extraction was performed using methanol and ethanol as extraction solvents, respectively, under the following conditions: extraction temperature, 40–120 °C (20 °C step); pressure, 15 MPa; extraction time, 3 × 5 min; rinsing time, 20 s; nitrogen purge time, 20 s after each cycle and 120 s after the extraction run. After the PFE run, the extract was cooled to 5 °C and stored in a fridge until further analysis. Two extracts were prepared in parallel under the same conditions for each amount of grape skins. The extracts were diluted with the corresponding solvents, if appropriate. Further details on extracts composition, conditions of preparation as well as their basic characteristics are summarized in Table 1 and Table 2 for methanolic and ethanolic extracts, respectively.

Just for comparison of the extraction methods efficiency from the viewpoint of polyphenols composition and concentrations, conventional Soxhlet extraction was also employed for methanolic and

Table 1
Basic characterization and abbreviations of Alibernet (A) and St. Laurent (L) grape skin extracts in methanol (M), along with the extraction conditions, the pH value, and TPC.

Sample ID	Skin quantity[a] (g)	Extraction temperature (°C)	pH (25 °C)		TPC content (GAE)[b]	
			L	A	L	A
L_{M1}/A_{M1}	0.5	40	5.9	5.5	40.72	123.82
L_{M3}/A_{M3}	1.0		5.8	5.6	68.74	214.30
L_{M7}/A_{M7}	0.5	60	6.0	5.8	47.51	82.30
L_{M9}/A_{M9}	1.0		5.7	5.6	61.45	151.68
L_{M13}/A_{M13}	0.5	80	5.9	5.9	28.74	66.58
L_{M15}/A_{M15}	1.0		5.7	5.8	71.12	133.77
L_{M19}/A_{M19}	0.5	100	5.8	5.9	26.29	70.96
L_{M21}/A_{M21}	1.0		5.8	5.8	76.50	140.34
L_{M25}/A_{M25}	0.5	120	5.9	6.0	31.07	71.91
L_{M27}/A_{M27}	1.0		5.7	5.8	47.80	150.58

[a] Amount of lyophilized grape skins used for extract preparation.
[b] GAE—gallic acid equivalent, mg/100 g.

ethanolic extracts preparation in parallel, as described by other authors [7].

2.4. HPLC analysis

A Spectra SYSTEM HPLC apparatus (Spectra SYSTEM, Thermo Fisher Scientific, Waltham, MA) equipped with gradient pump autosampler, and DAD detector set at 520 nm was used for anthocyanin analysis. All extracts were filtered through a 0.45 μm syringe filter prior to the analysis. The separation was performed on 4.6 mm i.d., Synergi C12 Max-RP column (length, 250 mm, stationary phase, 4 μm) (Phenomenex, Torrance, CA). A mixture of water/acetonitrile, 97:3 (solvent A) and 40:60 (solvent B) adjusted at pH 1.8 by formic acid was used as the mobile phase. The 0.5 ml/min flow rate was kept constant during the measurement (run time, 50 min) under the following linear gradient program: 0 min 6% B; 20 min 20% B; 35 min 40% B; 40 min 60% B; 45 min 90% B; 47–55 min 6% B. Identification of the target analytes was performed by comparing their retention times with those of the respective standards. Quantification was performed by the internal standard method involving Brillant blue FCF as the internal standard. All analyses were carried out in triplicate and the results are presented as averages of three measurements [45].

2.5. EPR experiments

All experiments were performed using a portable X-band EPR spectrometer e-scan (Bruker BioSpin, GmbH, Karlsruhe, Germany) with accessory. Reactive radical species for testing of antioxidant activity of extracts were generated immediately before EPR measurements by mixing the extracts with Fenton reagents (Fe^{2+}, H_2O_2). The formation of short-lived radical species (primarily •OH) was evidenced by addition of DMPO spin trapping agent. In another series of experiments, the decay of •DPPH and $ABTS^{\bullet+}$ radicals after their addition into the experimental system was monitored to assess the radical-scavenging ability of extracts. Reference samples were prepared by replacing the extracts with the respective pure solvent.

Experimental systems used in tests of antioxidant and radical-scavenging activity (RSA) of grape skin extracts are summarized in Table 3. All samples were mixed in an Eppendorf tube and the reaction time was measured from the moment of addition of reactive agents (i.e. hydrogen peroxide, •DPPH, or $ABTS^{\bullet+}$). The samples were transferred into a flat quartz EPR cell, tightened with a stopper and inserted into the cavity of spectrometer. Measurements were performed at ambient temperature. All measurements were started precisely 3 min after the last reagent's addition and 10 accumulated EPR spectra were recorded in the time domain during 30 min (Fenton system) and 15 min (•DPPH and $ABTS^{\bullet+}$), respectively. Each experimental spectrum represented an average of 30 individual scans. The experiments were performed in triplicate mode. The relative standard deviation among the individual measurements was less than 5%. The response and settings of EPR spectrometer was checked by means of Strong pitch standards (Bruker) daily before the experiments. The experimental EPR spectra processing, evaluation and simulation was carried out using WIN EPR and SimFonia programs (Bruker) as described e.g., in Refs. [42,46]. Numerical values obtained were expressed as mean ± SD ($n = 3$).

2.6. UV–vis experiments

A double-beam UV–vis spectrometer Specord M40 (Carl Zeiss, Jena, Germany) with an appropriate equipment was used to monitor the grape skin extract characteristics. All experiments were carried out in the same square quartz UV–vis transparent cells (1 cm path length). Total phenolic compounds' content (TPC) of individ-

Table 2
Basic characterization and abbreviations of Alibernet (A) and St. Laurent (L) grape skin extracts in ethanol (E), along with the extraction conditions, the pH value, and TPC.

Sample ID	Skin quantity[a] (g)	Extraction temperature (°C)	pH (25 °C)		TPC content (GAE)[b]	
			L	A		
L_{E1}/A_{E1}	0.5	40	5.4	5.4	21.46	33.92
L_{E3}/A_{E3}	1.0		5.4	5.4	40.37	72.18
L_{E7}/A_{E7}	0.5	60	5.4	5.4	23.96	43.45
L_{E9}/A_{E9}	1.0		5.4	5.4	53.25	88.22
L_{E13}/A_{E13}	0.5	80	5.4	5.4	27.76	48.08
L_{E15}/A_{E15}	1.0		5.4	5.4	58.67	95.13
L_{E19}/A_{E19}	0.5	100	5.4	5.4	29.31	51.27
L_{E21}/A_{E21}	1.0		5.4	5.4	59.84	102.30
L_{E25}/A_{E25}	0.5	120	5.4	5.4	32.79	57.67
L_{E27}/A_{E27}	1.0		5.4	5.4	63.27	123.05

[a] Amount of lyophilized grape skins used for extract preparation.
[b] GAE—gallic acid equivalent, mg/100 g.

Table 3
The composition of experimental systems used in EPR experiments.

System	Variety	Methanolic extract		Ethanolic extract		Other components of experimental system
		Dilution[a]	Addition	Dilution[a]	Addition	
Fe^{2+}/H_2O_2/DMPO	Alibernet	1:0	300 μl	1:0	300 μl	DMPO in H_2O (0.2 M, 100 μl); $(NH_4)_2Fe(SO_4)_2{\cdot}9H_2O$ in H_2O (0.01 M, 100 μl); H_2O_2 (0.1 M, 100 μl)
	St. Laurent	1:0	300 μl	1:0	300 μl	
•DPPH	Alibernet	1:10	300 μl	1:3	300 μl	•DPPH in methanol or ethanol ($c_{DPPH} = 5 \times 10^{-4}$ mol dm^{-3})[c]
	St. Laurent	1:5	300 μl	1:3	150 μl	
$ABTS^{\bullet+}$	Alibernet	1:50	150 μl	1:25	300 μl[b]	$ABTS^{\bullet+}$ in H_2O ($c_{(ABTS^{\bullet+})} = 8–10 \times 10^{-5}$ mol dm^{-3})[c]
	St. Laurent	1:50	300 μl	1:25	300 μl	

[a] Dilution in respective solvent prior to the addition of extract into experimental system.
[b] For extracts from 1.0 g, 150 μl was used.
[c] Final volume of experimental system after mixing, 1000 μl.

ual extracts was determined using the Folin-Ciocalteu assay and their tristimulus color values (CIE Lab) were estimated as previously described e.g., in Ref. [47]. In addition, pH values of all extracts were measured using the combined glass electrode.

2.7. Statistical correlation

To get a complex insight in the effects of extract preparation conditions on the antioxidant status and other important characteristics, all data obtained from HPLC, EPR and UV–vis experiments as well as the extracts' preparation conditions (amount of solid sample, extraction temperature) were taken into account and processed using multivariate statistics involving CDA and PCA. The calculations were performed by means of Unistat® (Unistat, London, United Kingdom) statistical software.

3. Results and discussion

To obtain comprehensive information on the PFE efficiency, its influence on the properties of extracts and to specify the optimal extraction conditions, a series of parallel analyses was performed. First of all, the effects of extraction conditions on the pH value, TPC, and CIE Lab color coordinates were investigated.

3.1. Basic characteristics of the extracts

As follows from the data presented in Tables 1 and 2, pH values of the extracts reached 5.4–6.0 (methanolic extracts) and ~5.4 (ethanolic extracts) regardless of the grape skin variety. Compared to pH of pure methanol (pH = 6.9) and ethanol (pH = 6.4) measured under the same conditions, there is an obvious slight shift of pH of all extracts to the acidic region, probably due to the presence of polyphenols, organic acids and/or other compounds. On the other hand, there is no direct correlation between the extraction conditions and pH or it is only negligible in the case of Alibernet extracts in methanol (Pearson's correlation coefficients PL < 0.2 for St. Laurent and PA < 0.4 for Alibernet). We conclude that the pH values of extracts are virtually unaffected by the extraction conditions, i.e., the solvent and the amount of grape skins, in the whole temperature range.

In accord with expectations, the average TPC in the individual extracts depends on the amount of grape skins used for extract preparation. Good correlation was also confirmed by the Pearson's coefficients for both varieties and solvents, $P > 0.8$. When TPCs of the extracts prepared from the two grape skin varieties at the same extraction conditions are compared, the TPC in Alibernet extracts is approximately three times and two times higher than that in St. Laurent methanolic and ethanolic extracts, respectively. We suppose that the differences come mostly from biological/botanical diversity as the climatic conditions are not so different in this case—both wine varieties originate from small wine making regions close to each other. Some other aspects should also be considered, e.g., the polarity of extraction solvent and extrability of individual components as will be discussed later.

The effect of extraction conditions on TPC was considered to be one of the most important characteristics. In methanolic extracts of St. Laurent skins, increasing extraction temperature up to 60 °C resulted in a slight increase of TPC in extracts prepared from 0.5 and 1.0 g whereas the additional increase of extraction temperature resulted in ~10% reduction of polyphenol concentrations in all samples. The latter trend was more significant in Alibernet grape skins methanolic extracts for which the decrease of TPC in the whole temperature range was observed, reaching ~30% in extracts prepared at 120 °C. In ethanolic extracts, the trends in TPC content were completely different, as in the whole extraction temperature range and for both grape skin varieties TPC increased with extraction temperature. The above-discussed trends were fully confirmed by statistical analysis of the results. The Pearson's correlation coefficients for methanolic extracts were PL,M = 0.11 and PA,M = 0.66 whereas for ethanolic extracts PL,E = 0.37 and PA,E = 0.43.

The results of HPLC analysis of anthocyanins performed using several monoglucosides as standards (Fig. 1) are in good agreement with the above-described trends in TPC although they cannot be directly compared on the absolute basis as the former are expressed as gallic acid equivalents and the latter represent absolute concentrations of individual compounds in the extracts. However, as presented in Table 4, a decrease of anthocyanin concentrations in methanolic extracts prepared from 0.5 g of grape skin was observed in the whole range of extraction temperatures regardless of the grape skin variety and the type of anthocyanin identified. On the contrary, in ethanolic extracts prepared at temperatures up to 80 °C a gradual increase of anthocyanin concentrations was revealed. Further increase of extraction temperature resulted in a slight decrease of anthocyanins content except for De-3-glc (Alibernet, St. Laurent), Cy-3-glc and Pt-3-glc (Alibernet) the content of which in extracts prepared at 120 °C was even higher than in those prepared at 40 °C. The behavior of anthocyanins prepared from higher amounts of grape skins was practically identical. The limits of detection (LOD, S/N = 3) were 488 ng/l for Mv-3-glc and De-3-glc, and 244 ng/l for Pt-3-glc, Cy-3-glc and Pn-3-glc.

The observed phenomena may have resulted from biodiversity but rather from the different extrability/solubility of individual components into the respective solvents. The equilibrium solubility depends on the properties of individual components, on the properties of methanol and ethanol, and on the component–solvent

Table 4
Influence of extraction conditions on the content of anthocyanins in St. Laurent and Alibernet grape skin extracts prepared from 0.5 g of dry matter, as determined by HPLC.

Solvent	Extraction temperature (°C)	Anthocyanins content (mg/g)				
		De-3-glc[a]	Cy-3-glc[a]	Pt-3-glc[a]	Pn-3-glc[a]	Mv-3-glc[a]
Alibernet						
Methanol	40	53.74	4.66	50.46	108.52	267.84
	60	33.27	2.28	24.93	53.87	98.39
	80	26.21	0.89	20.57	44.38	87.62
	100	31.59	1.83	24.10	48.31	90.87
	120	25.32	0.73	20.43	45.47	84.86
Ethanol	40	9.11	0.44	10.43	23.04	46.35
	60	13.21	0.71	12.35	27.18	60.14
	80	15.44	0.78	13.82	28.28	63.32
	100	11.90	0.48	10.82	19.29	48.58
	120	10.17	0.52	11.31	19.99	49.10
St. Laurent						
Methanol	40	40.83	3.74	30.66	56.72	178.27
	60	37.15	2.98	28.56	53.95	169.52
	80	23.78	0.78	18.34	25.52	72.04
	100	13.20	0.68	11.43	12.51	42.20
	120	6.95	0.28	7.89	5.64	32.35
Ethanol	40	6.33	0.36	6.05	6.69	28.20
	60	9.72	0.39	7.39	7.89	34.65
	80	10.92	0.45	9.49	8.55	37.80
	100	9.56	0.39	8.21	6.65	32.63
	120	6.72	0.21	5.62	4.86	24.16

[a] De-3-glc, delphinidin-3-glucoside; Cy-3-glc, cyanidin-3-glucoside; Pt-3-glc, petunidin-3-glucoside; Pn-3-glc, peonidin-3-glucoside; Mv-3-glc, malvidin-3glucoside.

interactions. The most important properties of the solvents include the relative permittivity ε (at 298 K, relative permittivity $\varepsilon_{MeOH} = 32.63$ and $\varepsilon_{EtOH} = 24.30$) [48] and the solubility parameter δ (=square root of the cohesive energy density). Employing the equations of state for methanol and ethanol [49], one can show that, at a constant pressure of 15 MPa, δ_{MeOH} drops from 29.38 $(J\,cm^{-3})^{1/2}$ at 40 °C to 26.18 $(J\,cm^{-3})^{1/2}$ at 120 °C whereas δ_{EtOH} drops from 25.95 $(J\,cm^{-3})^{1/2}$ at 40 °C to 22.90 $(J\,cm^{-3})^{1/2}$ at 120 °C. Hence, the polarities of both solvents decrease somewhat with the rising temperature, and the effect on the solubilities depends on the respective polarities of the individual polyphenols. Therefore, there appears to be no clear-cut, universal explanation for the difference between the solubilities of polyphenols in methanol and ethanol at a particular temperature.

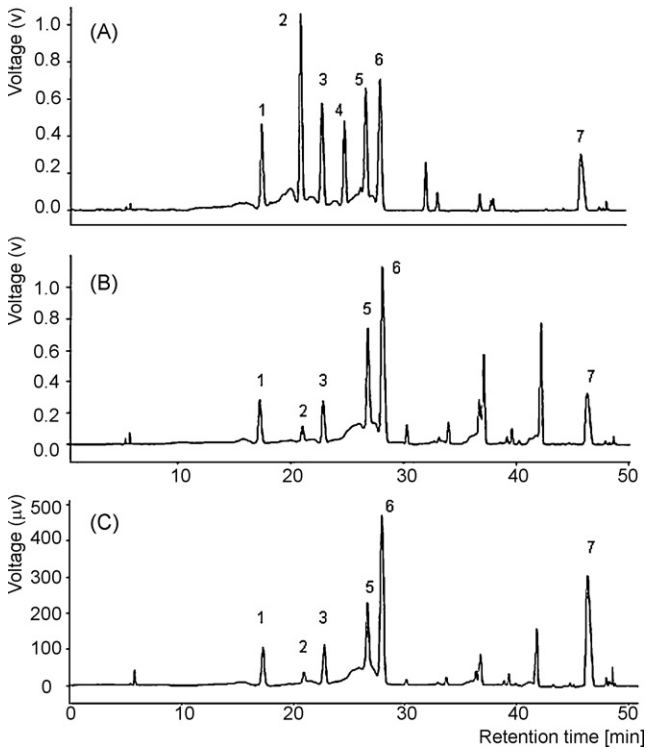

Fig. 1. HPLC chromatograms of anthocyanins—standard of 3-monoglucosides (A), methanolic extract from Alibernet (B) and from St. Laurent (C) grape skins. Extracts were prepared from 0.5 g of dry matter by PFE at 40 °C. For experimental conditions, refer to Section 2. Peak assignment: (1) De-3-glc, (2) Cy-3-glc, (3) Pt-3-glc, (4) Pg-3-glc, (5) Pn-3-glc, (6) Mv-3-glc, and (7) Brillant blue FCF–internal standard.

Apart from depending on the solubility, the extrability of a component depends also on the character of the sample matrix, notably on accessibility of the matrix pores by the solvent. An important parameter in this respect is the dynamic viscosity (η) of the solvent, and there is a distinct difference between the viscosities of the two solvents. At the pressure of 15 MPa, η_{MeOH} drops from 488 μPa s at 40 °C to 199 μPa s at 120 °C whereas η_{EtOH} drops from 869 μPa s at 40 °C to 288 μPa s at 120 °C [50]. At any particular temperature, therefore, the dynamic viscosity of methanol is markedly lower than that of ethanol, and so methanol should be able to penetrate the matrix more easily than ethanol. Further, the matrix morphology may differ between the two grape varieties, adding to the complexity of the problem and making it very difficult to identify a single cause of the different temperature courses of the TPC in methanolic and ethanolic extracts. Besides, there is the problem of thermal stability of polyphenols in individual foods which is currently only poorly discussed and available data are frequently contradictious [51–53].

Valuable information on PFE efficiency regarding the anthocyanins content and retention under different extraction conditions follows also from comparison of PFE to Soxhlet extraction. As confirmed by HPLC analysis, in methanolic extracts prepared from 0.5 g of skins at 64.7 °C by Soxhlet extraction, the content of all identified anthocyanins reached less than 50% of their concentration in extracts prepared by PFE at 60 °C for both grape skin varieties. In case of Cy-3-glc, even an 80% and 90% decrease for Alibernet and St. Laurent varieties was observed, respectively. The results obtained with ethanolic extracts or extracts prepared from higher amounts of grape skins were virtually identical. Based on this comparison, we can conclude that PFE represents a valuable and effective method of extract preparation, sensitive enough to protect and sus-

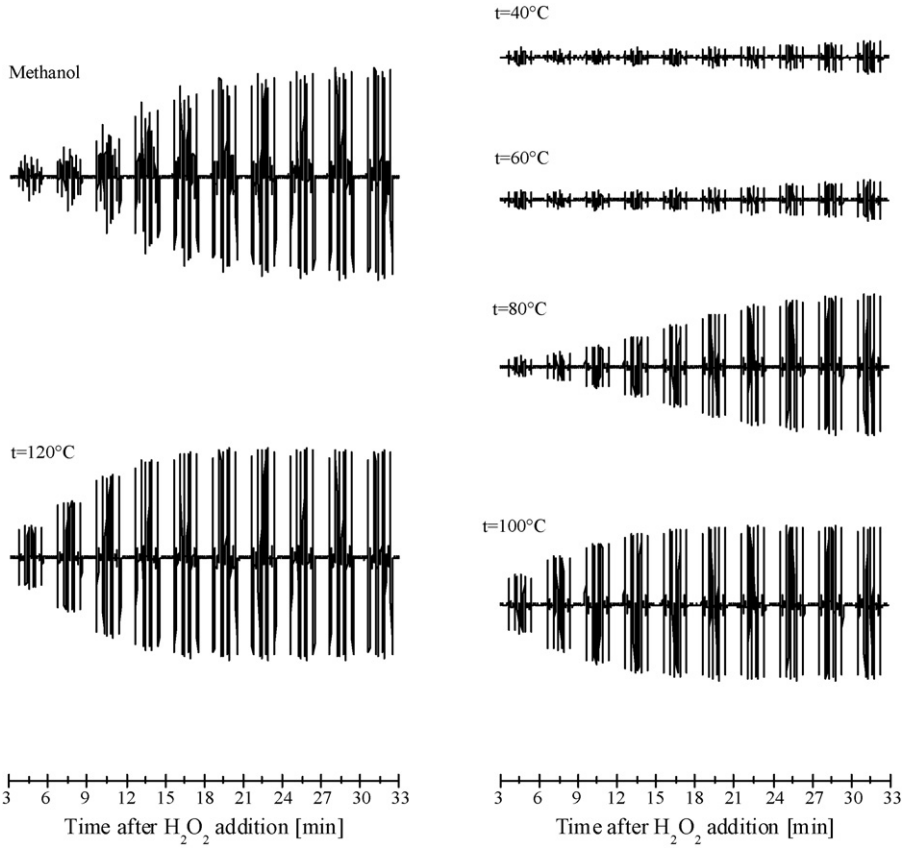

Fig. 2. Time evolution of EPR spectra recorded in system containing methanol (reference) and methanolic extracts of St. Laurent variety prepared from 1.0 g of crude grape skins at 40–120 °C, respectively; Fenton's reagents and DMPO spin trap. Spectra were recorded at 298 K using magnetic field sweep width, SW = 8 mT.

tain the maximum of components of potential interest, taking into account both their quantity and quality.

3.2. Antioxidant activity assays

The effects of extraction conditions on antioxidant properties of grape skin extracts were tested in experimental systems in which reactive radicals were generated in Fenton reaction or added into the system in the form of stable free radical (•DPPH, $ABTS^{\bullet+}$), and the response of the system to oxidation stress conditions was evaluated.

Fig. 2 shows typical time evolution of EPR spectra recorded in system containing grape skin extracts of St. Laurent wine variety in methanol and Fenton's reagents, in the presence of DMPO spin trap. As some of the radicals formed in the experimental system are not stable enough to be detected directly by EPR, the DMPO spin trap was effectively employed to stabilize them in the form of respective spin adducts [37,39].

As follows from the simulation analysis, in the presence of pure water, dominantly the formation of •OH radicals (in the form of DMPO–OH• adducts) is observed (a_N = 1.44 mT, a_H = 1.48 mT; g = 2.0059) whereas in pure methanol or ethanol, the formation of different types of carbon-centred radicals (in methanol, dominantly •DMPO–CH_2–OH, •DMPO–R and •DMPO–CX and in ethanol •DMPO–$CHOHCH_3$, •DMPO–R and •DMPO–CX) is to be found, in accord with our previous experiments and already published data [45,46,54]. It should be noted here, that the low-intensive three-line EPR signal (a_N = 1.71 mT; g = 2.0059) which was observed in the presence of both, ethanol and methanol, correspond well to •DMPO-CX adducts formation that were previously attributed to DMPO spin trap decomposition products [46].

As an illustration of the complexity of the reaction routes of radicals intermediates formation induced by the Fenton reaction, the following mechanism is suggested in case of ethanolic extracts:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (1)$$

$$RH + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O \quad (2)$$

$$CH_3CH_2OH + {}^{\bullet}OH \rightarrow CH_3({}^{\bullet}CH)OH + H_2O \quad (3)$$

$$CH_3({}^{\bullet}CH)OH + RH \rightarrow {}^{\bullet}R + CH_3{-}CH_2{-}OH \quad (4)$$

$$R^{\bullet},\ OH^{\bullet},\ ({}^{\bullet}CH)OHCH_3 + DMPO \rightarrow {}^{\bullet}DMPO(-R,\ -OH,\ -(CH)OHCH_3) \quad (5)$$

The addition of grape skin extracts prepared at 40 °C into the system resulted in significant decrease or nearly total elimination of spin adduct concentration as a result of competitive reactions between the antioxidants, the generated free radicals and the spin trap used [39,46].

As presented in Fig. 2, the increase of extraction temperature from 40 to 120 °C resulted in gradual worsening of RSA of methanolic and ethanolic extracts of both varieties. As expected, this effect is proportional to the amounts of grape skins used to prepare the respective extracts, and it is more significant for methanolic extracts. Staško et al. [39] suggested previously a way to quantify the antioxidant effects of Tokay wines via the calculation of relative amounts of the radicals scavenged (RS parameter). Using this approach (Fig. 3), we can conclude that antioxidant properties of the St. Laurent variety extracts are significantly lower than those of Alibernet, in both solvents. In methanolic extracts prepared from 1.0 g at 40 °C, RS ≈ 66% and RS ≈ 89% were estimated,

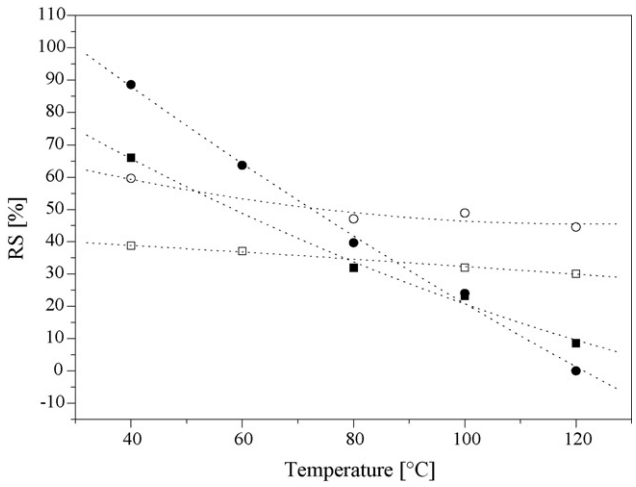

Fig. 3. Thermal dependence of antioxidant activity of St. Laurent (■, methanol; □, ethanol) and Alibernet (●, methanol; ○, ethanol) extracts. Extracts were tested in Fenton's system. Results are expressed as the relative amounts of radicals scavenged (RS, %).

respectively, while at 120 °C the RS parameter fell down to 9% (St. Laurent) or descended virtually to zero (Alibernet). In ethanolic extracts, the situation is very similar but not so pronounced. Generally, the RS values of extracts of both varieties are lower than those estimated in methanol. As regards the effect of extraction temperature, the gradual decrease of RS values from 38.7% to 30.1% (St. Laurent) and from 59.6% to 44.5% (Alibernet) were noticed as a result of extraction temperature increase from 40 to 120 °C. The observed differences between the solvents can satisfactorily be explained by the differences in the solvent polarities influencing the extrabilities of grape skin components and, consequently, the composition of polyphenols and other antioxidants in the extracts [48].

•DPPH and ABTS•+ radical assays were previously successfully employed to characterize, e.g., tea, wine, or spices extracts [39,42,46]. Here we have confirmed that the extracts of both varieties demonstrated significant abilities to terminate •DPPH and ABTS•+. Fig. 4 shows the time evolution of EPR spectra of methanolic extracts prepared from 1.0 g of St. Laurent grape skins in the presence of ABTS•+ cation radical. While the EPR signal intensity remained practically constant in the reference sample (pure methanol), a decay of radicals occurred as a result of the respective extract addition and the spectra intensity fell down. The ethanolic extracts and the experiments with •DPPH also revealed identical trends.

Pellegrini et al. [55] suggested an effective comparison of RSA for different food products, based on Trolox-equivalent antioxidant capacity (TEAC) calculation. Following this approach, the TEAC values of each extract were calculated for the reactions with both •DPPH and ABTS•+, taking the double-integrated EPR spectra intensities measured exactly 10.5 min after the respective radical addition into the experimental system. The TEAC value is related to the experimental parameters by the following equation:

$$TEAC_{\bullet\,DPPH/ABTS^{\bullet+}} = \frac{(c_{0(\bullet\,DPPH/ABTS^{\bullet+})} - c_{t(\bullet\,DPPH/ABTS^{\bullet+})}) * V_{(\bullet\,DPPH/ABTS^{\bullet+})}}{V_{(Sample)}} * \nu * Z \quad (6)$$

where $c_{0(\bullet\,DPPH/ABTS^{\bullet+})}$ is the initial concentration of •DPPH (ABTS•+) solution; $c_{t\,(Sample)}$ is the concentration after the addition into sample extract determined in chosen time t; $V_{(\bullet\,DPPH/ABTS^{\bullet+})}$ is the volume of •DPPH (ABTS•+) solution added to the system; $V_{(Sample)}$ is the volume of sample added to the system; ν is the stoichiometric coefficient of the reaction between •DPPH (ABTS•+) and TROLOX (in both cases, $\nu = 1/2$); Z is the dilution factor.

As follows from the TEAC values depicted in Fig. 5, the methanolic extracts showed better RSAs than the ethanolic ones, and Alibernet extracts were more efficient than those of St. Laurent variety, similarly as described above for Fenton's system, probably in close relationship with TPC. In addition, $TEAC_{ABTS^{\bullet+}}$ values are several times higher in comparison to $TEAC_{\bullet DPPH}$ values of the corresponding extracts.

As regards the effect of extraction conditions on TEAC values, it is clear that the methanolic extracts of Alibernet are more resistant to extraction temperature than the extracts of St. Laurent. Only small variations with the increasing temperature were observed. In the case of Alibernet ethanolic extracts, a gradual increase of both $TEAC_{\bullet DPPH}$ and $TEAC_{ABTS^{\bullet+}}$ values in the whole temperature range was found, indicating a positive effect of increased extraction temperature on RSA of Alibernet extracts. A similar effect was also found for $TEAC_{\bullet DPPH}$ values of methanolic extracts of St. Laurent variety. On the other hand, $TEAC_{ABTS^{\bullet+}}$ values of St. Laurent methanolic extracts prepared at temperatures from 40 up to 100 °C remained practically unchanged. As a result of additional growth of extraction temperature to 120 °C, both, $TEAC_{\bullet DPPH}$ and $TEAC_{ABTS^{\bullet+}}$ values of St. Laurent methanolic extracts decreased significantly.

In the case of St. Laurent ethanolic extracts, the slightly positive effects of extraction temperature on $TEAC_{\bullet DPPH}$ values within the whole temperature interval was noticed, whereas for $TEAC_{ABTS^{\bullet+}}$ values, their increase in the extracts prepared at temperatures up to 80 °C was found. A further increase of the extraction temperature resulted in the gradual decay of $TEAC_{ABTS^{\bullet+}}$ values. It should be noted here that in spite of the observed decrease, the $TEAC_{ABTS^{\bullet+}}$ values determined for extracts prepared at 120 °C are still higher than those of the extracts prepared at 40 °C, indicating a slight improvement of their antioxidant activity with rising extraction temperature.

The observed phenomena can be effectively explained by consideration of the following four factors:

(i) different polarity of solvents used for extracts preparation,
(ii) changes in partition coefficients with increasing extraction temperature,
(iii) redox potentials of ABTS•+ and •DPPH influencing their reactivity and
(iv) thermal stability of individual extracts' components.

In this context, it is widely accepted that TPC and composition of individual polyphenols have the most significant influence on the overall antioxidant status of biological systems. Thus, the correlation between the TPC and TEACs or RS values obtained from individual antioxidant testing assays was evaluated. Pearson's correlation coefficients for the extracts of both varieties with methanol and ethanol are listed in Table 5. The data presented clearly indicated a significant correlation between TPC and TEACs for both solvents and varieties. However, the slightly lower correlation between the TPC and $TEAC_{\bullet DPPH}$ was found for St. Laurent extracts in ethanol ($P \approx 0.73$), explainable by the same factors as described above as well as by the composition of individual polyphenols in these extracts influencing the reactivity of •DPPH. Regarding the correlation between the TPC and RS in methanolic extracts, it is only average, reaching $P \approx 0.39$ (Alibernet) and $P \approx 0.44$ (St. Laurent) whereas for ethanolic extracts, a better correlation ($P \approx 0.54$ (Alibernet) and $P \approx 0.81$ (St. Laurent)) was revealed. These differences most probably follow from the dissimilarities in proton donating/accepting ability of individual polyphenols, influencing their reactivity with the •OH radicals generated directly in the experimental system via Fenton reaction [56]. When considering the correlation matrix, one additional phenomenon is obvious, i.e.,

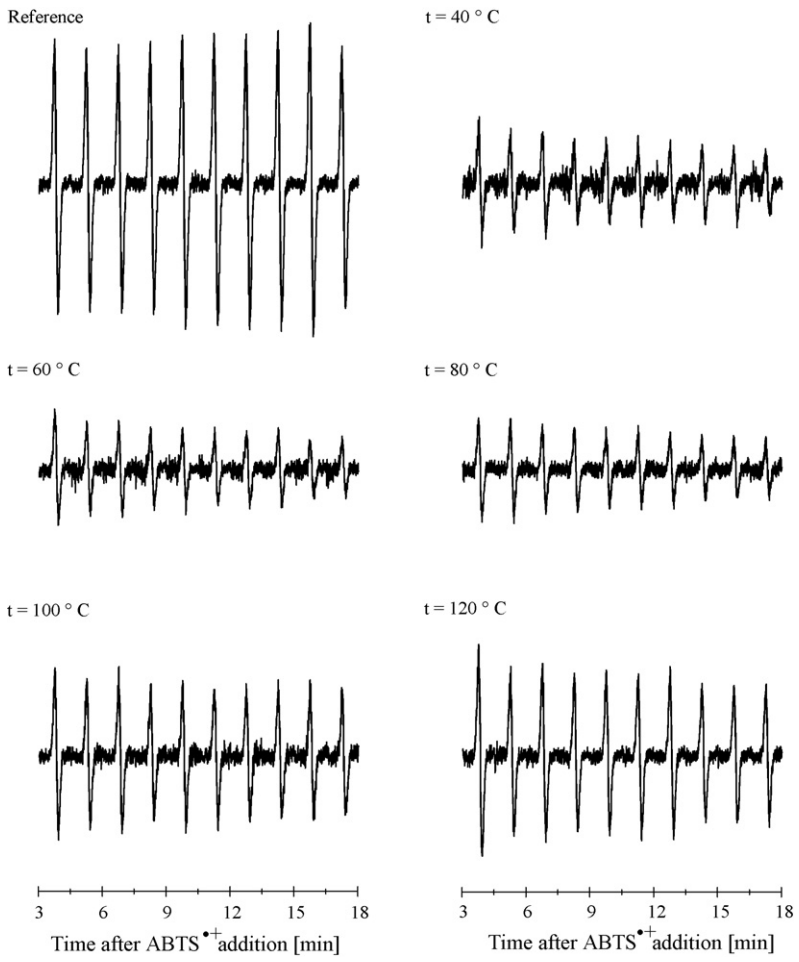

Fig. 4. Time evolution of EPR spectra recorded in system containing methanol (reference) or methanolic extracts of St. Laurent variety prepared from 1.0 g of crude grape skins at 40–120 °C and water solution of ABTS•+ (c_0 (ABTS•+) = 100 μM).

the mutual correlation between TEACs is very high or at least very significant ($P \geq 0.69$) but the correlation between RS and TEAC is significantly lower or only average as the *P*-values vary from $P \approx 0.24$ (Alibernet, methanol) up to 0.6804 (Alibernet, ethanol), with the same explanation as mentioned above.

However, as the analysis of anthocyanins was performed in the current study, a basic correlation of their content with the results of antioxidant testing assays was performed on the same basis as described above (data not presented). Regardless of the grape skin variety, very high positive correlation was found for each of the analyzed anthocyanins' 3-glucosides (see Table 4) and $TEAC_{ABTS^{\bullet+}}$, reaching in ethanolic extracts the Pearson's correlation coefficients $P > 0.84$ (De-3-glc, Cy-3-glc, Pn-3-glc) or even $P > 0.90$ (Mv-3-glc, Pt-3-glc). In the same extracts, the correlation of anthocyanins content with $TEAC_{\bullet DPPH}$ was somewhat lower. Although the concentration of anthocyanins in methanolic extracts was higher than in ethanolic ones (Table 4), its correlation with TEAC values was weaker, reaching the maximum value of $P \approx 0.74$ for the Pn-3-glc and $TEAC_{ABTS^{\bullet+}}$. When considering the relationship between the RS and anthocyanins, it is obvious that while the correlation is weak or non-significant ($P \leq 0.44$) in ethanolic extracts, the situation is strongly different in methanolic extracts as the *P*-values range from $P \approx 0.61$ (De-3-glc) to $P \approx 0.72$ (Cy-3-glc). These results are quite contradictious to those obtained for the correlation of TPC.

Table 5
Correlation matrices between the antioxidant activities of grape skin extracts in methanol and ethanol and total polyphenols content without respect to the amount of dry matter used for extract preparation.

		St. Laurent				Alibernet			
		TPC	$TEAC_{ABTS^{\bullet+}}$	$TEAC_{\bullet DPPH}$	% RS	TPC	$TEAC_{ABTS^{\bullet+}}$	$TEAC_{\bullet DPPH}$	% RS
Methanol	TPC	1	0.9778	0.9518	0.4447	1	0.8650	0.9220	0.3923
	$TEAC_{ABTS^{\bullet+}}$	0.9778	1	0.9060	0.5895	0.8650	1	0.6909	0.2503
	$TEAC_{\bullet DPPH}$	0.9518	0.9060	1	0.2810	0.9220	0.6909	1	0.2370
	% RS	0.4447	0.5895	0.2810	1	0.3923	0.2503	0.2370	1
		TPC	$TEAC_{ABTS^{\bullet+}}$	$TEAC_{\bullet DPPH}$	% RS	TPC	$TEAC_{ABTS^{\bullet+}}$	$TEAC_{\bullet DPPH}$	% RS
Ethanol	TPC	1	0.9012	0.7349	0.8147	1	0.9734	0.9750	0.5358
	$TEAC_{ABTS^{\bullet+}}$	0.9012	1	0.7208	0.6282	0.9734	1	0.9654	0.5259
	$TEAC_{\bullet DPPH}$	0.7349	0.7208	1	0.3543	0.9750	0.9654	1	0.6804
	% RS	0.8147	0.6282	0.3543	1	0.5358	0.5259	0.6804	1

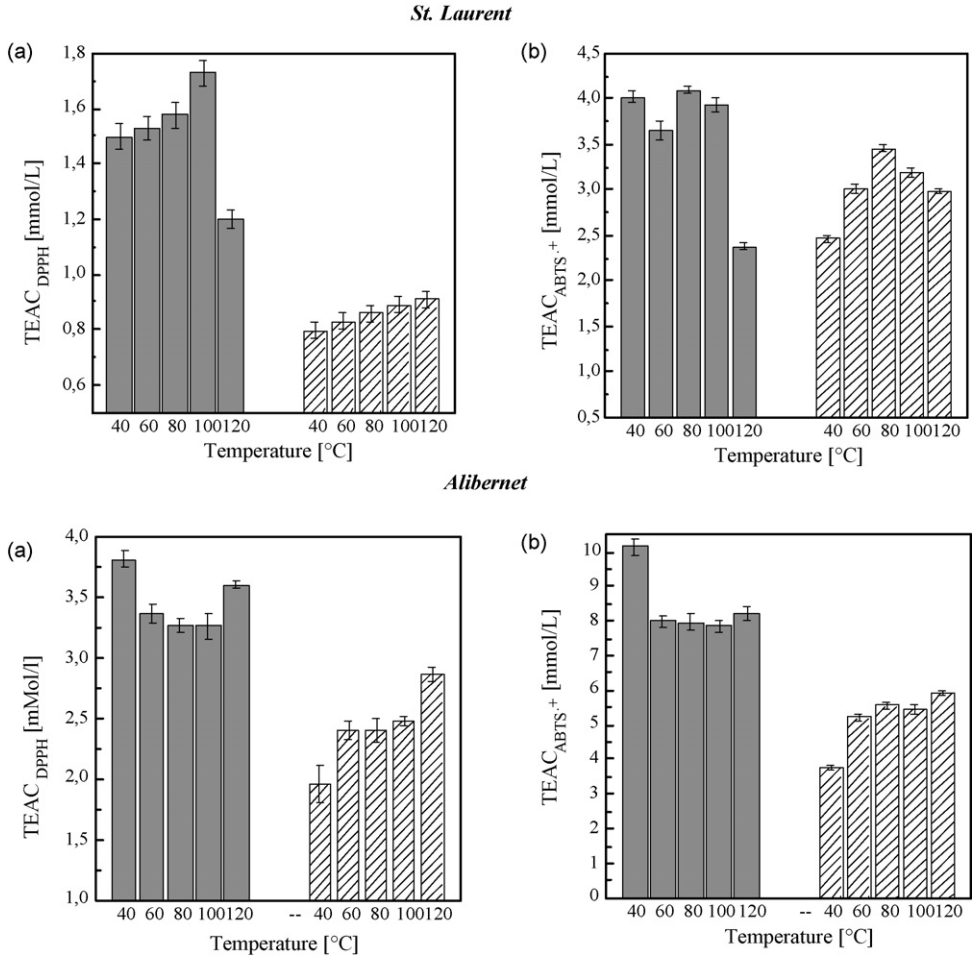

Fig. 5. Trolox-equivalent antioxidant capacity (TEAC, mmol Trolox/L of extract) of St. Laurent and Alibernet extracts in methanol (■) and ethanol (▨), prepared at various temperatures from 1.0 g of crude grape skins in the reaction with (a) •DPPH and (b) ABTS•+. Each value is expressed as mean ± SD ($n = 3$).

It appears that although anthocyanins 3-glucosides are present at a significant concentration level, they are only one group of polyphenols, and so their behavior can differ from that of other polyphenolic compounds that may also be present in the extracts. The potential synergism of individual polyphenolic compounds must also been taken into account.

3.3. Multivariate statistics

In view of numerous experimental characteristics obtained by different methods and assays on extracts prepared by PFE, interesting information can be obtained by their mutual comparison all-in-one using multivariate statistics. All the experimental data were statistically evaluated separately for methanolic and ethanolic extracts by PCA and CDA.

Using the PCA approach, all the samples, with respect to the solvent used, were clearly differentiated from one another according to the grape skin variety. All the experimental data except for the mass of grape skins and the extraction temperature were used as variables for principal components construction. As follows from the results of PCA performed for methanolic extracts, the first three principal components cumulatively explained more than 82% of the whole system variability. Just as an illustration, to describe 99% of the whole system variability, the construction of seven principal components would be necessary. According to the respective eigenvectors, the total variability in the first component is dominantly influenced by the TPC and both $TEAC_{ABTS^{\bullet+}}$ and $TEAC_{\bullet DPPH}$ values while in the second and third component by the concentration of anthocyanins and the L* (CIE Illuminant A) values, respectively. In ethanolic extracts, the results are very similar. According to the variance table, the first three principal components cumulatively explain more than 88% of the whole data set variability. The experimental parameters with the most significant weights include the $TEAC_{ABTS^{\bullet+}}$ and $TEAC_{\bullet DPPH}$ values in the first component, the CIE b* values (Illuminant C and D65) representing the yellow-blue color axis in the second component, and the content of anthocyanins in the third component, respectively.

To consider the effects of extraction conditions on the properties of grape skin extracts, CDA was performed using all experimental data as variables for discrimination functions construction. The samples were discriminated taking the mass of grape skins and the extraction temperature as the main discriminators, separately for methanolic and ethanolic extracts, and without respect to the solvent used.

In accord with our expectations, absolutely correct differentiation and classification of the samples according to the mass of grape skins used for extract preparation was achieved in both solvents, and also when the samples were differentiated and classified without respect to the solvent. These results unambiguously confirm the importance of the mass of crude sample for the properties of extracts regardless of which method was used for the extraction.

In case the extraction temperature was used as the discriminator, 100% correct differentiation of samples was reached in both solvents. The three most important discriminant functions explain-

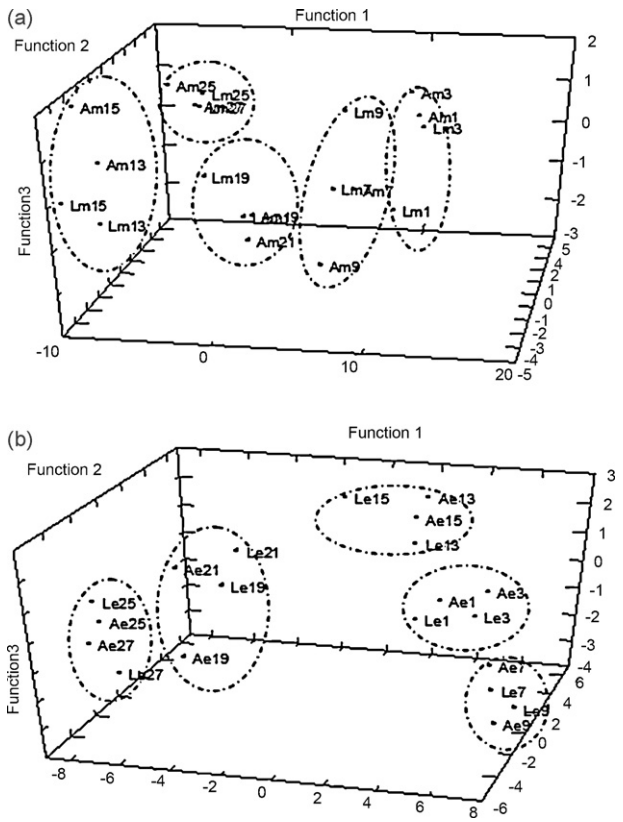

Fig. 6. Classification—recognition of St. Laurent (L) and Alibernet (A) grape skin extracts in (a) methanol and (b) ethanol using the canonical discriminant analysis according to the extraction temperature. Labeling of samples is identical with that used in Tables 1 and 2.

ing cumulatively about 99% of the whole system variability were used for the visualization of statistical results, as clearly depicted in Fig. 6a and b, for St. Laurent and Alibernet extracts, respectively. Here, extracts of both grape skin varieties are nicely divided into five different groups (bordered by dash-dotted oval lines) according to the extraction temperature.

According to the values of CDA standardized coefficients, the most important role in the first discriminant function in methanolic extracts pertained to both $TEAC_{\bullet DPPH}$ and $TEAC_{ABTS^{\bullet+}}$ whereas in the second and the third function the concentrations of Pn-3-glc and Mv-3-glc anthocyanins were the most important. In ethanolic extracts, TPC and specifically, the concentration of Mv-3-glc anthocyanin were found as dominant for construction of the first three discriminant functions. To verify the correctness of discriminant analysis, the prediction ability of the statistical model employed was tested in a standard recognizability tests (data not presented). The results confirmed an absolutely correct classification of the samples into groups according to the extraction temperature for both solvents.

4. Conclusion

The offline combination of PFE with other experimental methods, notably EPR spectroscopy, was presented in this contribution for the first time. Comprehensive multi-experimental analysis confirmed unambiguously that PFE is a method of choice with great potential to be applied in production of natural food supplements and isolates. Compared with conventional extraction methods, e.g., Soxhlet extraction, the efficiency of PFE is much better, reaching approximately two times higher yields of the extracted anthocyanins.

In accord with expectations, the properties of extracts depend on the solvent used, the mass of grape skins, as well as on extraction conditions among which the temperature plays a crucial role.

The influence of extraction temperature on the other characteristics investigated, i.e., pH, content of polyphenols and specifically anthocyanins as well as on color characteristic and antioxidant or radical-scavenging ability of extracts was also monitored and mutual correlations of variables were estimated by the methods of multivariate statistics.

With respect to the complexity of the system studied, an exact specification of the best extraction conditions cannot be performed, as it strongly depends not only on the character of matrix from which the component of interest should be isolated but also on the way of subsequent utilization of the extract/isolates, redox potentials, polarity, extrability, and on other factors.

However, some general conclusions can be done:

(i) the effect of extraction conditions on pH and tristimulus CIE $L^*a^*b^*$ values content is non-significant or negligible.
(ii) HPLC analysis of anthocyanins content revealed their presence at significant levels but their presence contributes only partly to the properties of extracts conventionally related to TPC.
(iii) Antioxidant and radical-scavenging abilities of extracts, as determined by EPR, are in significantly positive correlation with the TPC markedly influenced by the extraction solvent polarity.

In spite of wide interval of extraction temperatures, all the extracts still retain their antioxidant and/or radical-scavenging properties even when prepared at temperatures of 120 °C. This finding indicates that extracts prepared by PFE can serve as potential source of functional food supplements.

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