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ASSESSMENT OF ANTIOXIDANTS BY HPLC-MS IN GRAPEVINE SEEDS

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ABSTRACT

It is well known, that grapevine seeds are rich in significant antioxidants. However, the issue of dealing with the analysis and comparison of antioxidant components in the seeds of *Vitis vinifera* L. in individual cultivars has not yet been sufficiently studied. The experiment was performed with extracts of three varieties (Blaufränkish, Italian Riesling and Cabernet Moravia) and three interspecific cultivars (Nativa, Marlen and Kofranka). Contents of nine major flavonoids (apigenin, astragalin, hyperoside, isorhamnetin, kaempferol, myricetin, quercetin, quercitrin and rutin) and two procyanidins (procyanidin A_2 and procyanidin B_1) were assessed by the HPLC/MS method. The highest contents of antioxidants were found out in interspecific cultivars Marlen and Nativa while the lowest one was assessed in the cultivar Cabernet Moravia. The most represented flavonoid was hyperoside (cultivar Marlen – 15.66 mg·l^{-1}), least represented was kaempferol (cultivar Cabernet Moravia – 1.81 µg·l^{-1}).

Key words: grapevine seeds, flavonoids, procyanidins, HPLC/MS

INTRODUCTION

Nowadays, greater and greater attention is being paid to grapevine seeds because of the fact that they contain a wide spectrum of biologically active components. A high content of proantocyanidins in seeds is rather important; these compounds are studied and evaluated as options for treating various diseases [Charradi et al. 2012, Blanch et al. 2012, Al-Malki et al. 2013, Hassan and Al-Rawi 2013]. Flavonoids contained in grapevine seeds show antibacterial and anxiolytic properties [Harborne and Williams 2000]. Nechita et al. [2012] in their study on extracts originating from grapevine skins, seeds, grape pomace, and sediments mentioned their numerous pharmacological effects. Harris et al. [1999] studied effects of grape seed extracts on patients with leukemia and hemoblastosis and found out that in human leukemic cells these extracts induced apoptic cell death and inhibited their propagation. Raina et al. [2013] studied effects and associated mechanisms of grape seed extracts in two different cell lines of human bladder

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cancer cells and found out that this extract showed a significant inhibiting effect on the viablity of these cells just due to apoptosis. Polyphenols contained in grape products may show a positive effect on the cardiovascular system of subjects with hypertensis [Ras et al. 2013]. Wang et al. [2013] demonstrated a protective effect of oligomeric proanthocyanidins on retinal ganglion cells against oxidative stress-induced apoptosis. Caimari et al. [2013] performed a study on deposition of lipids. They evaluated potential positive effects of low doses of grape seed extracts containing high levels of procyanidins on body mass and fat deposition. In China, Lai at al. [2014] used grape seed oil in an experiment with animals and observed its positive antidiabetic effect. Zhang et al. [2013] published a study on protective effects of grape seed procyanidin B_2 in the development of diabetic nephropathy. Mansouri et al. [2012] demonstrated that grape seed extracts ameliorated albuminuria and renal sclerosis in experimental rats with diabetic nephropathy.

The aim of this study was to characterize flavonoids and procyanidins in grapevine seeds by HPLC-MS method. The emphasis was focused on the comparison among the different varieties.

It is well konwn that grapevine seeds are rich in antioxidants. This fact was corroborated in many scientific studies investigating their antioxidant potential. Grapevine seeds and grape seed products are therefore recommended as preparations prevennting the occurrence of many different diseases. In spite of this, however, problems concerning seed analyses and comparisons of properties of antioxidant components cotained in seeds of different cultivars of *Vitis vinifera* have not yet been adequately studied. That is why the main objective of this study was to assess contents of antioxidants in seed extracts obtained from three (Blaufränkish, Italian Riesling and Cabernet Moravia) and three interspecific (Nativa, Marlen and Kofranka) cultivars of the grapevine species.

MATERIAL AND METHODS

Biological samples. This expeimental study was performed with seeds of three grapevine (*Vitis vinifera* L.) cultivars, namely Blaufränkish, Italian Riesling, and Cabernet Moravia and three interspecific ones – Marlen, Nativa and Kofranka, parental Marlen (Merlan × Fratava), Nativa (Merlan × Fratava) and Kofranka (Merlan × Fratava). The experimental material originated from the Department of Viticulture and Enology, Faculty of Horticulure in Lednice na Moravě, Czech Republic.

Chemicals. Chemicals (Sigma Aldrich, Germany): deionised water, methanol, acetic acid (0.2%), liquid nitrogen, hydrochloric acid, acetate buffer, sodium acetate.

Standard antioxidants (Extrasynthese, France): gallic acid, apigenin, astragalin, hyperoside, isorhamnetin, kaempferol, myricetin, quercetin, quercitrin, rutin, proanthocyanidin A_2, proanthocyanidin B_1.

Method of sample preparation. The experimental material originated from grape pomace. To eliminate undesirable water residues, grapevine seeds were screened and purified. Thereafter the seeds were crushed with liquid nitrogen in a mortar. Subsequently, 10 g of the homogenate were quantitatively transferred into a volumetric flask. The extraction was performed in a dark and cool environment with 100 mL of 75% methanol using the shaker IKA KS 260 Basic (manufaturer Merci, France) for a period of 5 days. Final extracts were centrifuged and transferred into vials and micro test tubes (manufaturer Eppendorf, Germany).

Assessment of antioxidants by the HPLC/MS method. The HPLC/MS method was used to quantify of antioxidants that are interesting above all with regard to their protective properties. Results of performed analyses were expressed as arithmetic means and standard deviations of three measurements. Correlations existing between levels of individual antioxidans were expressed by means of the Pearson's correlation coefficient.

Analyses of samples used for the estimation of flavonoids and proanthocyans were performed in a chromatographic system Agilent Technologies 1200 (manufacturer Waldbron, Germany) consisting of the following devices: mobile phase reservoirs, degasser, binary pump, sample reservoir with an autosampler, mass detector with an Agilent 6460 Triple Quadrupole LC/MS (manufacturer Jet Stream Technologies, USA). Separation of samples was performed in chromatographic columns Zorbax SB C18 with dimensions 50 × 2.1 mm and the size of particles 2.7 µm.

The period of analysis was always 6 minutes. The flow rate of the carrier gas was 0.6 mL·min^{-1}. The organic component of the mobile phase was methanol gradient grade (solvent B) and the aqueous constituent contained 0.05 M of ammonium formate (solvent A). The gradient of the moving phase was as follows: time 0 minute: 50% of solvent A and 50% of solvent B; time 2.5 minutes: 100% of solvent A and 0% of solvent B; time 5 minutes: 50% of solvent A and 50% of solvent B. The temperature of the column thermostat was adjusted to 45°C.

Parameters for the assessment of flavonoids. The analysis lasted 6 minutes. The flow rate of the carrier gas was 0.6 ml·min^{-1}. The organic component of mobile phase was methanol gradient grade (solvent B) and the aqueous constituent contained 0.2% of ammonium formate (solvent A). The gradient of the mobile phase was as follows: time 0 minute, 50% of solvent B; time 2.5 minutes, 0% of solvent B; time 5 minutes, 50% of solvent A and 50% of solvent B. The temperature of the column thermostat was adjusted to 45°C.

Parameters for the assessment of proanthocyans. The analysis lasted also 6 minutes. The flow rate of the carrier gas was 0.7 mL·min^{-1}. The organic component of mobile phase was methanol gradient grade (solvent B) and the aqueous constituent contained 0.2% of ammoniumformate (solvent A). The gradient of the moving phase was as follows: time 0 minutes: 40% of solvent B; time 3.0 minutes: 0% of solvent B; time 4.5 minutes: 50% of solvent B; time 5 minutes: 60% of solvent A and 40% of solvent B. The temperature of the column thermostat was adjusted to 55°C.

When performing the quantitative analysis the method of calibration curve with external standard was used. The chromatograhic software was used to integrate estimated values and to determine exactly areas below given peaks and the calibration curves characterising the dependence of peaks on concentrations. This calibration of the dependence of peaks on concentrations was performed for each standard compound, i.e. for apigenin, astragalin, hyperoside, isorhamnetin, kaempferol, myricetin, quercetin, quercitrin, rutin, proanthocyanidin A_2 and proanthocyanidin B_1. At the same time also the retention time was determined as well as the regression line equations and correlation coefficients R for each of the aforementioned substances.

RESULTS AND DISCUSSION

The flows of ions contained in the standard sample (A) is and in the virtual sample of grapeseed extract (B) are presented in Figure 1.

Assessment of rutin. Rutin is quercetin-3-0-rutinoside. Its synthesis runs via a rutin synthase activity. Health effects of rutin were studied from many points of view. Rutin is abundant not only in grapevine (both in wine and grapes) a but also in tomatoes, cranberries, mulberries, elder, asparagus, buckwheat, and citrus fruit [Krvavac et al. 2009, Capanoglu et al. 2012, Lee et al. 2012, 2013]. It functions above all as a compound that protects plants against harmfull effects of UV-radiation [Gaberscik et al. 2002].

A record of the standard rutin sample in the retention time of 1.5 minutes is presented in Figure 2A; a record of virtual rutin sample exctracted from seeds of the Kofranka cultivar in Figure 2B. The highest and the lowest concentrations of rutin were found out in cultivars Italian Riesling (1957 μg·L^{-1} of extract) and Blaufränkish (676 μg·L^{-1} of extract), respectively.

Assessment of hyperoside. Hyperoside is quercetin-3-O-galactoside. It was found out in strawberries [Bagdonaite et al. 2013], blueberries [Hicks et al. 2012], whitethorn [Belkhir et al. 2013] and rowan [Gaivelyte et al. 2013]. Among herbs, it is abundant above all in St John's wort [Cirak et al. 2013]. According to Web of Science (WOS), the occurrence of this compound has not been studied yet. A record of the standard hyperoside sample in the retention time of 1.5 minutes is presented in Figure 3A and that of the virtual rutin sample exctracted from seeds of the Kofranka cultivar is illustrated in Figure 3B. The highest and the lowest concentrations of hyperoside were found out in cultivars Marlen (15.6 mg·L^{-1}) and Cabernet Moravia (2.4 mg·L^{-1}), respectively.

Assessment of quercitrin. Quercitrin is quercetin-3-O-rhamnoside. It is contained in apricots, apples, carrots, potatoes and summer squash courgette (zucchini) [Andlauer et al. 2003]. A record of the standard rutin sample in the retention time of 2.0 minutes is presented in Figure 4A while that of a virtual rutin sample exctracted from seeds of the Kofranka cultivar

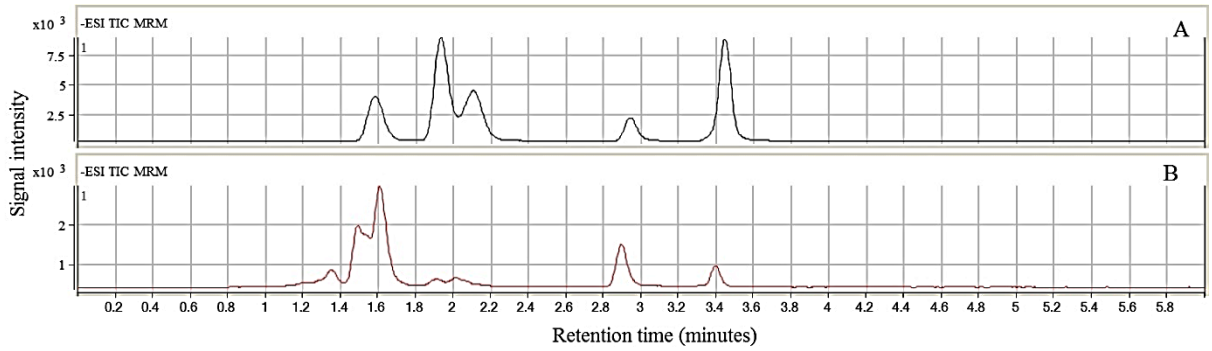

Fig. 1. The flows of ions contained in the standard sample (A) and in the virtual sample of grapeseed extract (B)

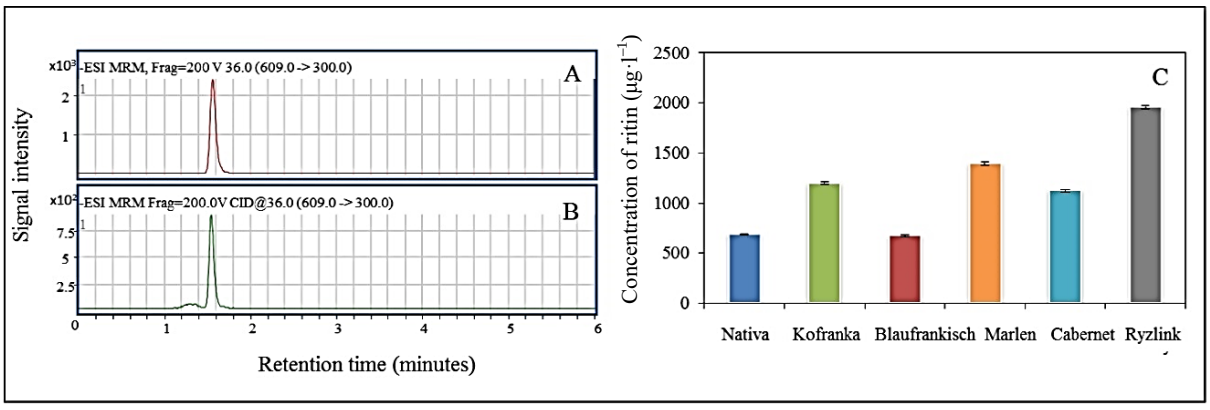

Fig. 2. A. Record of the standard rutin sample in the retention time of 1.5 minutes. B. Record of virtual rutin sample exctracted from seeds of the Kofranka cultivar. C. Concentrations of rutin in seeds of cultivars

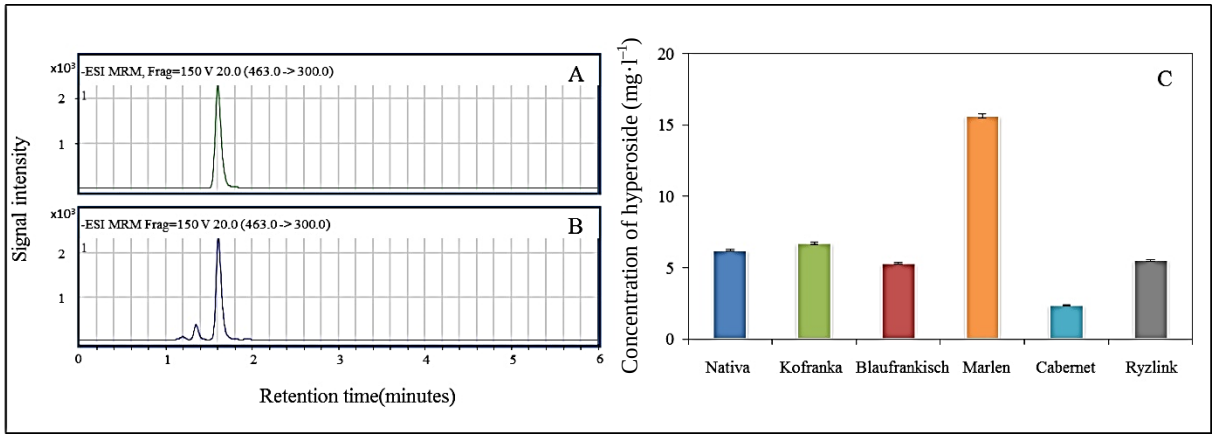

Fig. 3. A. Record of the standard hyperoside sample in the retention time of 1.5 minutes. B. Virtual hyperoside sample exctracted from seeds of the Kofranka cultivar. C. Concentrations of hyperoside in seeds of cultivars

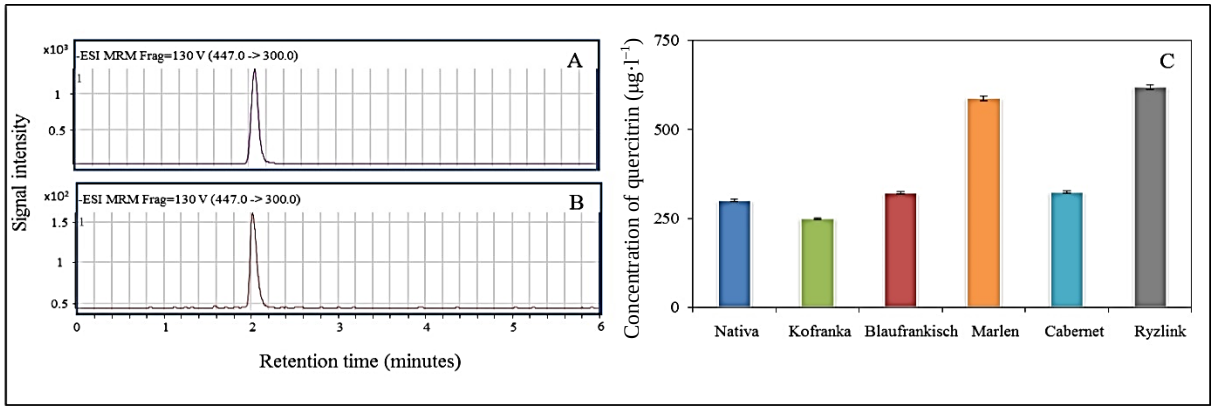

Fig. 4. A. Record of the standard quercitin sample in th e retention time of 2.0 minutes. B. Virtul quercitin sample exctracted from seeds of the Kofranka cultivar. C. Concentrations of quercitrin in seeds of cultivars

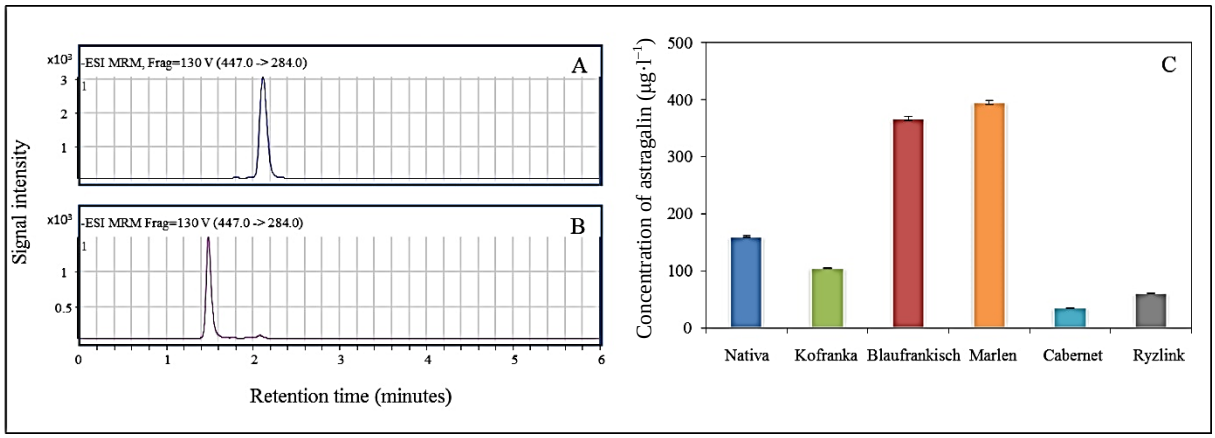

Fig. 5. A. Record of the astragalin standard in the retention time of 2.1 minutes. B. Virtual astragalin sample exctracted from seeds of the Kofranka cultivar. C. Concentrations of astragalin in seeds of cultivars

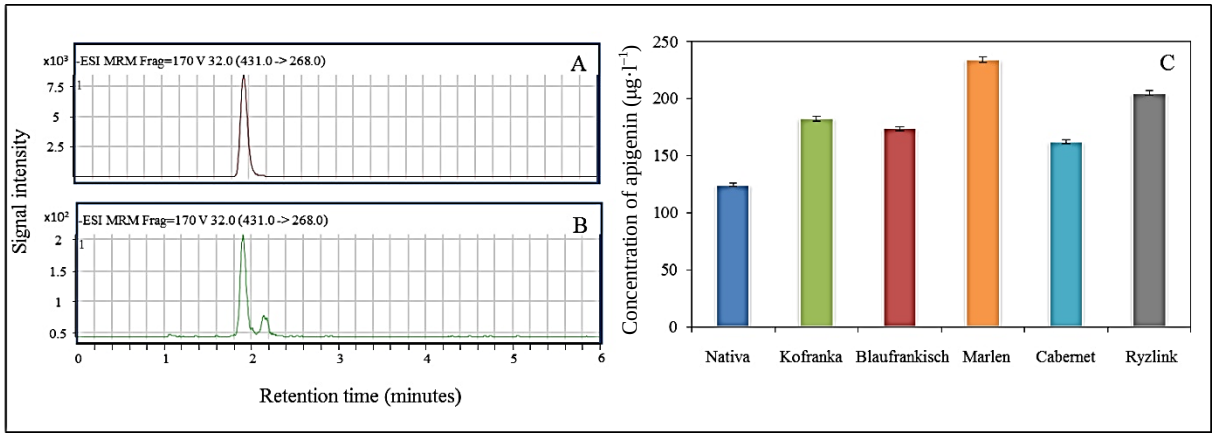

Fig. 6. A. The content of apigenin standard in time 2.1 min. B. Real sample extracted from seeds of Kofranka. C. Concentrations of apigenin in seeds of cultivars

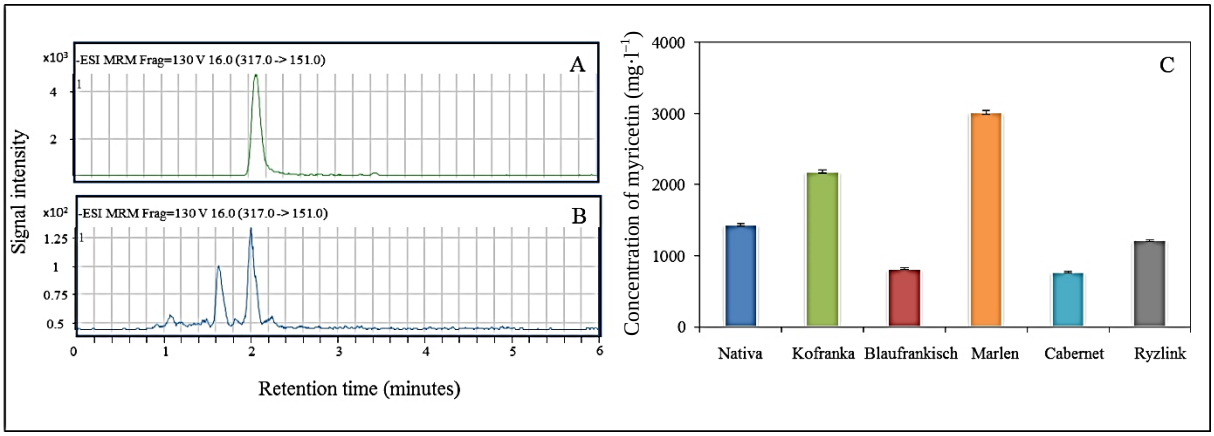

Fig. 7. A. The contents of myricetin standard in time 2.0. B. Recorded on virtual sample of myricetin extracted from seeds of Kofranka. C. Concentrations of myricetin in seeds of cultivars

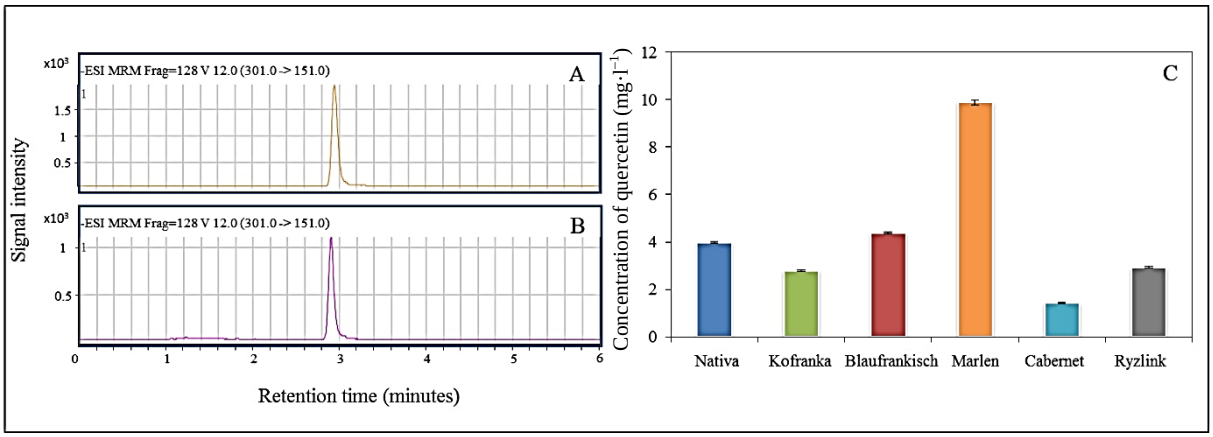

Fig. 8. A. The content of quercetin standard in time 2.9. B. Recorded on virtual sample of quercetin extracted from seeds of Kofranka. C. Concentrations of quercetin in seeds of cultivars

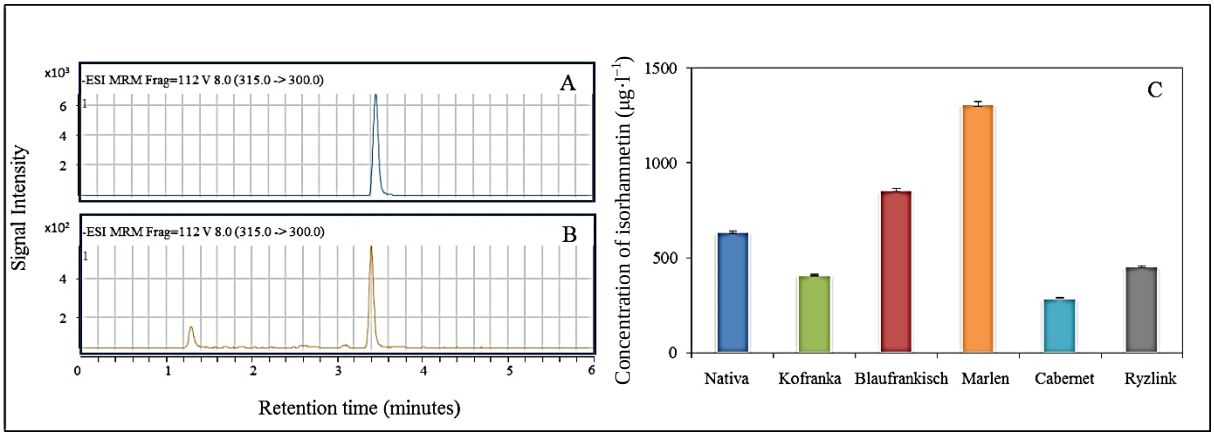

Fig. 9. A. The content of isorhamnetin standard. B. Recorded in the virtual sample of isorhamnetin extracted from seeds of Kofranka. C. Concentrations of isorhamnetin in seeds of cultivars

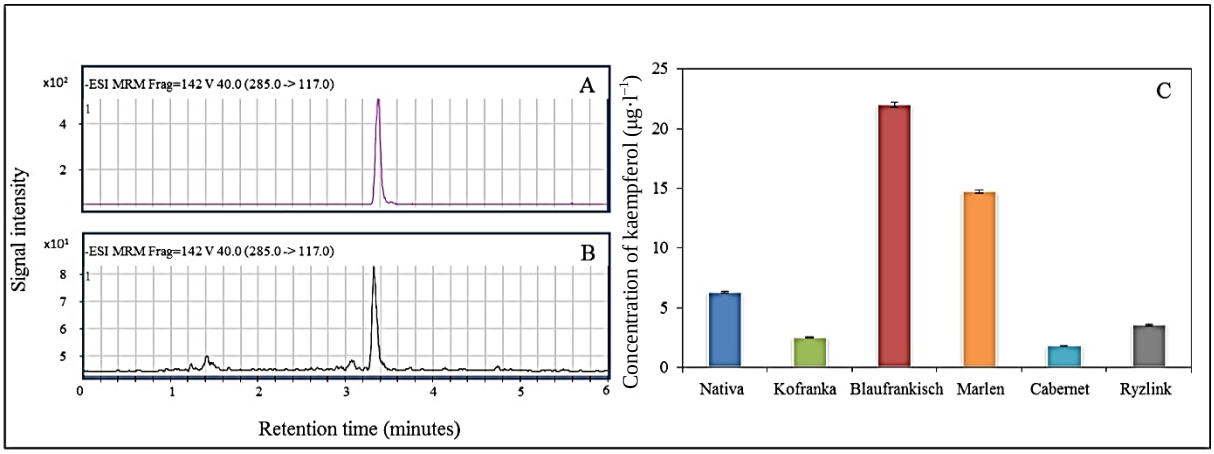

Fig. 10. A. The content of kaempferol standard recorded in time 3.3 minutes. B. Kaempferol extracted from seeds of Kofranka. C. Concentrations of kaempferol in seeds of cultivars

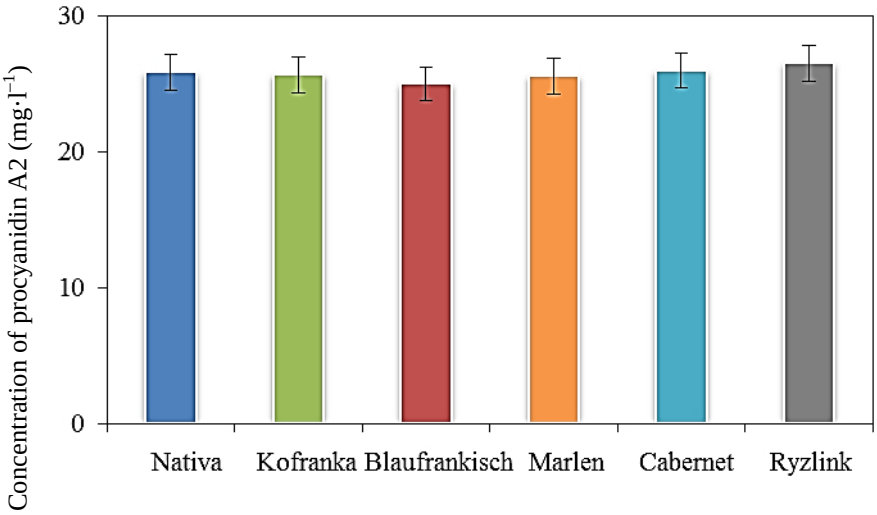

Fig. 11. Contents of procyanidin A_2 in seed extracts of grapevine cultivars

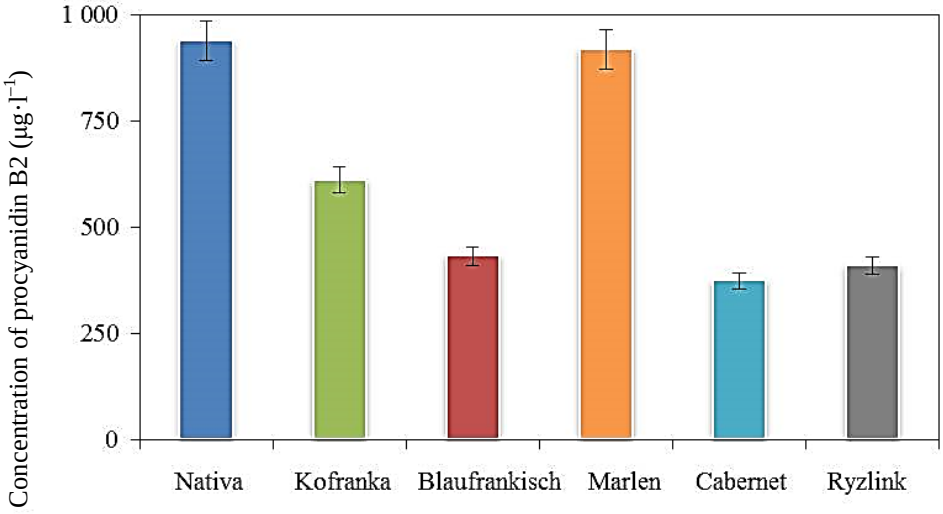

Fig. 12. Contents of procyanidin B_1 in seed extracts of grapevine cultivars

is illustrated in Figure 4B. The highest and the lowest concentrations of rutin were found out in cultivars Italian Riesling, (620 $\mu g \cdot L^{-1}$) and Kofranka (249 $\mu g \cdot L^{-1}$), respectively.

Assessment of astragalin. Astragalin is kaempferol-3-O-glucoside. Its presence was demonstrated in mulberries [Choi et al. 2013], rhododendrons [Mok et al. 2013] and larch [Medvedev et al. 1972]. A record of the astragalin standard in the retention time of 2.1 minutes is presented in Figure 5A while that of a virtual rutin sample exctracted from seeds of the Kofranka cultivar is illustrated in Figure 5B. The highest and the lowest concentrations of astragalin were found out in cultivars Marlen (395 $\mu g \cdot L^{-1}$) and Cabernet Moravia (35 $\mu g \cdot L^{-1}$), respectively.

Assessment of apigenin. Apigenin is known also as 4',5,7-trihydroxyflavone. Apigenin occurs in many vegetable species and its highest concentrations can be found out in parsley, celery and chamomile [Guo et al. 2013]. The content of apigenin standard in time 2.1 min. and that found out in the real sample extracted from seeds of Kofranka are presented in Figures 6A and B, respectively. The highest and the lowest concentrations of apigenin were found out in cultivar Marlen (234 $\mu g \cdot L^{-1}$) and Nativa (124 $\mu g \cdot L^{-1}$), respectively.

Assessment of myricetin. Myricetin occurs above all in leaf vegetables, aples, aloe vera, spinach, garlic, and blueberries its content in grapevine bunches was studied as well [Kumar et al. 2009]. The contents of myricetin standard in time 2.0 and that recorded in the virtual sample of myricetin extracted from seeds of Kofranka are presented in Figures 7A and 7B, respectively. The highest and the lowest concentrations of myricetin were found out in cultivars Marlen (3014 $\mu g \cdot L^{-1}$) and Cabernet Moravia (775 $\mu g \cdot L^{-1}$), respectively.

Assessment of quercetin. Quercetin is a plant polyphenol from the flavonoid group, found in many fruits, grapevine berries, vegetables, leaves, and grains [Eftekhari et al. 2012]. The content of quercetin standard in time 2.9 and that recorded in the virtual sample of quercetin extracted from seeds of Kofranka are presented in Figures 8A and 8 B, respectively. The highest and the lowest concentrations of quercetin were found out in cultivars Marlen (9.89 $mg \cdot L^{-1}$) and Cabernet Moravia (1.43 $mg \cdot L^{-1}$), respectively.

Assessment of isorhamnetin. Isorhamnetin is an O-methylated flavonol. This compound protects living organisms against the oxidative stress [Yang et al. 2012]. It was identified in marigold, onions, common sea-buckthorn, red beet, gingko, grapevine and several other plants [Dragoni et al. 2012]. The content of isorhamnetin standard and that recorded in the virtual sample of isorhamnetin extracted from seeds of Kofranka are presented in Figures 9A and 9B, respectively. The highest and the lowest concentrations of isorhamnetin were found out in cultivars Marlen (1312 $\mu g \cdot L^{-1}$) and Cabernet Moravia (287 $\mu g \cdot L^{-1}$), respectively.

Assessment of kaempferol. Kaempferol is a natural flavonoid that occurs in tea, broccoli, cabbage, beans, tomatoes, strawberries, common sea-buckthorn, apples, common nettle and also in grapevine bunches [Xie et al. 2012, Ozcan and Yaman 2013]. The content of kaempferol: standard and that recorded in the virtual sample in time 3.3 minutes is presented in Figure 10A and that of kaempferol extracted from seeds of Kofranka is illustrated in Figure 10B. The highest and the lowest concentrations of kaempferol were found out in cultivars Blaufränkish (23.12 $\mu g \cdot L^{-1}$) and Cabernet Moravia (2.87 $\mu g \cdot L^{-1}$), respectively.

Assessment of procyanidins. Condensed tannins are oligomeric or polymeric compounds that are derived from flavan-3-ols. When heated, ethanol solutions of procyanidins are decomposed to anthocyanidins. For that reason they are also called proanthocyanidins or procyanidins. Basic components of these condensates are derivatives flavan-3-ol (+)-catechin and (-) epicatechin. These substances were isolated from many plant products and species, e.g. tea and cacao [Robbins et al. 2012], cranberries [White et al. 2012] and blueberries [Rodriguez-Mateos et al. 2012]. However, wine is probably their most famous source [Khanal et al. 2012]. In recent years, their content was detected also in grapevine seeds [Sun et al. 2012]. Anti-inflammatory [Pallares et al. 2012] and anti-carcinogenous [Kin et al. 2013].

Assessment of A2 procyanidins. Procyanidin A_2 Procyanidin A is a procyanidin dimer. Procyanidin B2 may be transformed to procyanidin A_2 by a radical oxidation DPPH radical [Kondo 2012]. Contents of procyanidin A_2 in seed extracts of grapevine cultivar under study are presented in Figure 11. The average value was 25.8 $mg \cdot L^{-1}$. As one can see in this

picture, contents of procyanidin A2 in all cultivars were very similar and the differences were only very slight (3%).

Assessment of B1 procyanidins. Procyanidin B_1 is a procyanidin dimer. From the chemical point of view, it is epicatechin-(4β→8)-catechin. Procyanidin B_1 can be transformed to procyanidin A_1 by a radical oxidation DPPH [Kondo et al. 2012].Contents of procyanidin B_1 in seed extracs of grapevine cultivars under study are presented in Figure 12. The highest and the lowest concntrations of this procyanidin were found out in Nativa (941 $mg \cdot L^{-1}$) and in Cabernet Moravia (375 $mg \cdot L^{1}$), respectively.

Correlations existing between contents of individual flavonols were tested by means of Pearson's correlation coefficient. Correlation coefficients of contents individual flavonols, as assessed by the HPLC/MS method, are presented in Table 1.

The highest values of correlation coefficients existed between contents of quercetin and isorhamnetin (r = 0.970), quercetin and hyperoside (r = 0.961), and isorhamnetin and astragalin (r = 0.935). The lowest correlation coefficients existed between contents of rutin and kaempferol (r = −0.408) and rutin and astragalin (r = −0330). Thanks to this simple statistical evaluation it was possible to learn more about relationships and dependences existing between individual compounds and/or groups of components under study.

There are many studies dealing with contents of antioxidant components in grapevine seeds. Authors of this study investigated antioxidant activities and contents of polyphenolic compounds not only by spectrophotometry but also by HPLC and HPLC/MS. Positive health effects of grapevine seeds were highlighted in numerous scientific studies.

Chamorro et al. [2012] studied the effect of thermal processing on the content of polypyhenols and the antioxidant activity of grapevine pomace and seed extracts. The thermal treatment of these materials was performed either in laboratory oven or in an autoclave at the temperture of 100°C for periods of 15; 30 and 60 minutes. The HPLC/MS method was used to estimate contents of some selected antioxidant components. The thermal processing in the laboratory oven influenced neither contents of total polyphenols, tannins, and procyanidin components nor the antioxidant activity of tested materials. Autoclaving, however, caused an extensive hydrolysis of gallocatechin (70%), catechin (61%), epicatechin (65%), procyanid B1 (75%) and procyanid B2 (73%); besides, it also resulted in an increase in contents of gallic acid (71%), gallocatechin (100%) and epicatechin gallate (129%). This study referred to the heat resistance of antioxidants contained in grapevine seeds and pomace [Chamorro et al. 2012].

In Brazil, Rockenbach et al. [2011] studied antioxidant activities of and contents phenolic compounds in seeds and skins of red grapes of species *V. vinifera* and *V. labrusca*. In 100 g of seeds, concentrations of phenolic compounds ranged from 2.128 to as much as 16.518 mg of catechin equivalents. In 100 g of skins, however, these contents were lower and ranged only from 660 to 1.839 mg of catechin equivalents. Values of antioxidant activity were assessed by DPPH and

Table 1. The correlation coefficients between contents of individual flavonoids

	Apigenin	Hyperosid	Isorhamnet	Kaempferol	Myricetin	Quercetin	Quercitrin	Rutin
Astragalin	0.378	0.690	0.935	0.931	0.415	0.831	0.191	–0.330
Apigenin	×	0.672	0.517	0.237	0.596	0.605	0.754	0.694
Hyperosid	×	×	0.873	0.398	0.908	0.961	0.525	0.202
Isorhamnet	×	×	×	0.771	0.614	0.970	0.432	–0.102
Kaempferol	×	×	×	×	0.071	0.597	0.101	–0.409
Myricetin	×	×	×	×	×	0.766	0.333	0.258
Quercetin	×	×	×	×	×	×	0.516	0.055
Quercitrin	×	×	×	×	×	×	×	0.785

FRAP with Trolox as a standard antioxidant. In 100 g, seeds, the cultivar Pinot Noir contained 16.925 mmol of Trolox equvivalent (DPPH), and 21.492 mmol Fe^{2+} (FRAP) while skins of the cultivar Isabel contained only 3.640 µmol TE·100 g^{-1} and 4.362 µmol Fe^{2}·100 g^{-1}. In skins of cultivars Cabernet Sauvignon and Primitivo the highest contents of anthocyans were 935 and 832 mg·100 g^{-1}, respectively. Previous studies indicate cultivar differences among horticulture crops on bioactive content [Alibabic et al. 2018, Galiana-Belaguer et al. 2018, Ozdemir et al. 2018]. The grapevine seed extract was rich both in oligomeric and polymeric flavanols. These data indicated that extracts from grapevine seeds and skins contained great amounts of antioxidantas. Weidner et al. [2013] studied phenolic compounds isolated from seeds of European and Japanese species of grapevine (*Vitis vinifera* and *Vitis coignetiae*) using either 80% methanol or 80% aceton. Phenolic (i.e. phenolic acids and catechins) were assessed by the HPLC method. Seeds contained great amounts of tannins and detectable levels of catechins and *p*-coumaric, ferulic and caffeic acids. In the European grapes, the content of total phenolic substances was higher than in Japanese ones.

CONCLUSION

Contents of antioxidants were assesed by the HPLC/MS method. The methodology enabling a very exact quantification and identification of nine flavonoids and two procyanidins was optimised.

Results of studies on antioxidant components contained in grapevine seeds and on their potential benefits for human health represent the greatest contribution in this sphere of research. Our results enable to draw a conclusion that grapevine seeds contain very high levels of antioxidants that are beneficial for human health. Studies on functional components of grapevine seeds are interesting because of several reasons.

So, for example, they are important with regard to organoleptic properties of wine and such products as grapevine seed or grapevine seed flour. However, they are interesting at most from the viewpoint of protection of human health. This fact was demonstrated in many and many studies. Grapevine seeds are available as a part of agricultural and industrial wastes so that they enable a cheap manufacturing of final products. Their potential to be processed to natural dietary supplements and/or other products designed for humans is really great.

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AUTHOR CONTRIBUTIONS

Lenka Sochorova − HPLC-MS analysis of samples, writing an article, Borivoj Klejdus − HPLC-MS analysis of samples, Jiri Mlcek − sample preparation, Tunde Jurikova − writing an article, Mojmir Baron-writing an article, Jiri Sochor − leader of experiments, supervisor, Sezai Ercisli − writing an article, Muhammed Kupe − writing an article.

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