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RESEARCH

Targeted Characterization of Flavonoids and Nonflavonoids in Kratošija Red Wine with HPLC-ESI-MS/MS Technique

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Abstract
In this study, a rapid and sensitive HPLC-mass spectrometric method was applied for identification and semi-quantification of flavonoids and nonflavonoids in Kratošija wines. Wines were produced by inoculation of two commercial yeasts (Zymaflore™ Xpure (Laffort) and Lalvin ICV D80 (Lallemand)) in order to study the effect of the yeast on the phenolics profile of wines. The targeted analyses of phenolic compounds have been performed using HPLC with a tandem mass spectrometer in a single reaction monitoring mode (SRM) or fragmentation spectrum mode to determine each individual compound. A total of 26 phenolic compounds, including 13 nonflavonoids (10 phenolic acids and 2 stilbenes and 1 stilbenoid) and 13 flavonoids (6 flavan-3-ols, 3 flavonols, 2 flavones, 1 flavanone, and 1 flavanonol), have been determined. Flavones chrysin and luteolin, flavanone naringenin, flavanonol taxifolin, and stilbenoid ε-viniferin were reported for the first time in Macedonian red wine. The effect of yeast on the phenolic profile of wines was noticed, as a result of the different fermentation rates in accordance to their specifications. Flavan-3-ols were present in a higher content in the wine fermented with Lalvin ICV D80, while wine fermented with Zymaflore™ Xpure presented higher amounts of all other phenolic compounds.

Keywords Flavonoids · Nonflavonoids · Yeast fermentation · Red wine · Kratošija · HPLC-ESI-MS/MS

Introduction

Polyphenols are considered one of the most important compounds in wines, determining the overall quality of wine, significantly impacting the color, stability, sensory characteristics, astringency, and bitterness and stabilizing the wine during the aging period. In general, polyphenols are a huge group of compounds mainly divided into (a) flavonoids and (b) nonflavonoids (Ivanova et al. 2011a, 2011b; Causon et al. 2019; Ivanova-Petropulos et al. 2015; Wu et al. 2021). According to the molecular structure, phenolic compounds can be classified at four major groups: (a) group with one phenolic ring (benzoic and cinnamic acids), (b) group with two phenolic rings (stilbenes), (c) group with three phenolic rings (anthocyanins, flavan-3-ols and flavonols), and (d) group with more complex ring structures (ellagic acids) (Champ & Kundu-Champ 2019). Flavonoids are the dominant phenolics in wine, consisted of anthocyanins, flavan-3-ols, and flavonols, as well as proanthocyanidins, while nonflavonoids are composed of phenolic acids (hydrozybenzoic and hydroxycinnamic acids) and stilbenes. In regard to the health benefits of wine, phenolic compounds are considered important phytochemicals, possessing antioxidant, anti-microbial, anti-cancer, and anti-inflammatory properties (Pandey and Rizvi 2009; Rashmi and Negi 2020).

The most suitable analytical technique for analysis of individual phenolic compounds is liquid chromatography. High-performance liquid chromatography coupled with DAD is the classical technique which can be successfully applied for identification and quantification of various groups of phenolic compounds due to their different maximal absorbances (Viñas et al. 2000; Rodrígez-Bernaldo de Quirós et al. 2007; Ivanova-Petropulos et al. 2015; Tzanova & Peeva 2018; Pajović Šćepanović et al. 2019). In the last years, mass detectors became the best choice of detection

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technique for analyses of specific compounds, especially secondary compounds present in a low concentration (Sun et al. 2007; Rubilar et al. 2007; Ivanova et al. 2011a; Raičević et al. 2020).

Grape and wine production in Republic of N. Macedonia has a very long tradition. The main red and white varieties are Vranec and Smederevka, respectively, with a total area of 13,002 ha for both varieties (7616 ha for Vranec and 5386 ha for Smederevka) (MAFWE 2023). Overall, among the red wine varieties, Vranec dominates with 7616 ha, followed by Merlot, Cabernet Sauvignon and Kratošija. Kratošija is considered an old ancient variety, grown at the Macedonian vineyards in the period when wine was produced in amphora. In the period of Yugoslavia, Kratošija was replaced with Vranec variety, which from the other hand produces characteristic deep-colored wines (Ivanova et al. 2011a, 2012). Nowadays, Kratošija is found mainly at the Macedonian vineyards in combination with Vranec vines. Since the Macedonian Ministry of Agriculture, Forestry and Water Economy started to support the implementation of Kratošija plants at new vineyards, this variety began to expand and it can be found nowadays at small locations alone, mainly in the Tikveš wine region and used for production of high-quality wines. Expansion of the vine areas is expected in the coming years. Even in some publications, Kratošija is considered an autochthonous Montenegrin variety (Pajović Šćepanović et al. 2019; Pajović-Šćepanović et al. 2019, 2024), and the results of DNA analyses obtained by Calò et al. (2008) showed that Kratošija is a synonym of Zinfandel (USA), Primitivo (Italy), and Crljenak Kaštelanski (Croatia). Moreover, previous investigations of Maletić et al. (2004) indicated that Zinfandel has a Croatian origin because of its microsatellite allelas typical for the Croatian gene pool. Consequently, these studies do not confirm the origin of Kratošija and more additional investigations are necessary to be done. Therefore, it would be the best if Kratošija is considered a Balkan variety for which additional genetic investigations should be performed.

Generally speaking, the quality of the red wine depends on the vinification protocols used, but also on the grape variety, the quality of the grapes, the "terroir," and the climatic conditions, resulting in different flavors and unique characters (Gómez-Plaza et al. 2002; Ivanova et al. 2011a, 2012; Lukić et al. 2017). To the best of our knowledge, there are no published results for Kratošija wines produced in Republic of N. Macedonia in the relevant scientific databases, neither for genetic analyses nor for chemical composition, except one paper which is focused on the determination of chromium and manganese using square-wave voltammetry (Šoptrajanova et al. 1998). Since there are no other available data for this important Balkan grape variety grown in Macedonia and since it is spread in USA, Italy, Croatia, and Montenegro and has international high importance, it is highly valuable obtaining preliminary data for Kratošija cultivar grown at a territory with other/different climate conditions and long tradition for vine growing and winemaking, as Macedonia is. Therefore, the aim of this work was to study flavonoids and nonflavonoids in Macedonian Kratošija wines in order to determine the individual phenolic profile by applying winemaking with two commercial yeasts and to study the effect of yeast on the phenolic profile and phenol's levels. Sophisticated HPLC-ESI-MS/MS technique was applied for characterization and targeted analysis of these compounds important for the color and stability of wine.

Materials and Methods

Chemicals and Reagents

HPLC grade acetonitrile and formic acid were purchased from Merck-Sigma (Darmstadt, Germany). Ultra-pure water was obtained from Milli-Q system (Millipore, Bedford, MA, USA). All the other chemicals were of analytical grade purchased from Merk-Sigma (Darmstadt, Germany).

Vinification

Kratošija grapes (*Vitis vinifera* L.) grown in the Tikveš wine region, near town Kavadarci, were late harvested on October 9, 2023, at adequate technological maturity (26.3°Brix, total acidity 6.32 g/L and pH 3.51) and at an excellent health conditions. In total, 100 kg of harvested grapes was transported to a small private winery for vinification and divided into two lots (50 kg each). The grape berries were carefully separated from the steams, by hands, then crushed and followed by addition of potassium metabisulfate (5 g/100 L). Next day, vinification continued with addition of enzyme (Lafaze HE GRAND CRU, Laffort, Bordeaux, France) in a dose of 3 g/hL. This enzyme is recommended for production of structured red wines, rich in color and polymerized tannins, destined for aging, compatible for the variety and wine that we wanted to produce. Two commercial *Saccharomyces cerevisiae* yeasts were used to initiate controlled alcoholic fermentation: Zymaflore^{TM} Xpure (Laffort, Bordeaux, France) in the first wine lot, marked as Kratošija **1**, and Lalvin ICV D80 (Lallemand, Blagnac, France) in the second wine lot, marked as Kratošija **2**. Both yeasts were rehydrated using 30 g of dry yeast per 100 kg of grapes and applied to the grape mashes. Afterwards, the délestage (fermentation and maceration technique used in red winemaking) lasted for 21 day, applying every day punching down, two times a day during the first 14 days of the fermentation pomace contact period, followed with one punching down a day during the next 7 days. The fermentation rate was followed by determination of the content of reducing sugars, using volumetric

method based on reduction-oxidation (redox) reaction between sugars and Fehling's solution (Ivanova-Petropulos and Mitrev 2014) and when it was below 2.5 g/L, it was considered that the fermentation was finished. The temperature during the fermentation and maceration was between 18 and 22 °C. After finishing the fermentation, the free-run wine from both lots was separated from the pomace and put in glass vessels. The first decantation from the sediment was conducted after 14 days, followed with addition of potassium metabisulfite (10 g/hL) and the second decantation was conducted after 3 weeks of storage, and again followed with addition of potassium metabisulfite (3 g/hL).

The HPLC-ESI-MS/MS analysis of flavonoids and non-flavonoids was performed after 1 year of the wine storage. Wines were stored in a cellar at a constant temperature (between 13 and 14 °C). Before HPLC analysis, wines were diluted with ultra-pure water (ration 1:10) in order to prevent introduction of high concentrations of compounds from the matrix into the mass spectrometer, as this could affect signal suppression, and filtered with cellulose filter (0.45 µm).

HPLC-ESI-MS/MS

The method used to separate the tested compounds was based on the previously optimized method described by Biesaga and Pyrzynska (2009) and slightly modified (Sergiel et al. 2014). Shimadzu LC system (Shimadzu, Kyoto, Japan) consisted of LC20-AD binary pump, a DGU-20A5 degasser, a SIL-20AC autosampler, and a CTO-20AC column oven, connected to a 3200 QTRAP mass spectrometer (Applied Biosystem/MDS SCIEX). The separation was performed on a Phenomenex Luna C18(2) column 100 Å (size: 100 × 2 mm; 3 µm particle size) using the following mobile phase: (A) 8 mM formic acid at pH 2.8 and (B) acetonitrile. Gradient elution was used according to the program: 0.0–2 min, 5% B; 2–30 min, 60% B; 30–40 min, 100% B, 40–50 min, 5% B. The injection volume was 10 µL, flow rate at 0.2 mL/min, and the column temperature was maintained at 30 °C. The wine samples, after minimal sample preparation (dilution and filtration), were directly injected into the HPLC system.

An electrospray ionization (ESI) source was operated in the negative or positive acquisition mode. ESI conditions were as follows: capillary temperature 450 °C, curtain gas at 0.3 MPa, auxiliary gas at 0.3 MPa, negative ionization mode source voltage −4.5 kV, positive ionization mode source voltage 4.0 kV. Nitrogen was used as the curtain and assist gas. Continuous mass spectra were obtained by scanning *m/z* range from 50 to 650. The mass spectrometer operated in SRM mode, detecting characteristic transitions for each compound (e.g., the precursor ion pair and its characteristic product ion produced during fragmentation in the collision cell). Optimal SRM conditions were determined for each compound in the infusion mode. Analyst 1.4.2. software was used to acquire and process the data. All the measurements were performed in duplicate.

Some of the compounds in wines were identified by comparing the retention times and the *m/z* values obtained by the mass spectra and SRM modes, with the mass spectra and SRM modes from a mixture of 21 available standards, listed in alphabetical order in Table 1. All standards were analyzed separately in full scan in positive $[M+H]^+$ and negative $[M-H]^-$ ionization modes to detect the precursor ions characteristic of each compound, observing maximum sensitivity in the negative ion mode. However, most of the phenolic compounds present in Kratošija wines have been identified by applying ESI-MS and MS/MS data compared with data published in the literature and SRM conditions previously optimized (Carando et al. 1999; Le Scanff et al. 2024; Fang et al. 2007; Ivanova et al. 2011a; 2011b; Flamini 2013; Simonetti et al. 2022; Temerdashev et al. 2024).

A semi-quantitative analysis of the individual phenolic compounds was performed using extracted ion chromatograms (EICs). The relative amounts were estimated by calculation of the relative peak area of ion intensity extracted

Table 1 Ion pairs of available standards of phenolic compounds in SRM mode (Q1 and Q3)

Compounds	Q1	Q3	DP (V)[3]	CE (V)[4]
Apigenin	269	117	−55	−42
Caffeic acid	179	135	−10	−24
Chlorogenic acid	353	191	−20	−22
Chrysin	255	153	81	37
Ferulic acid	193	134	−30	−20
	193	178	−30	−16
Gallic acid	169	125	−45	−20
Genistein	269	133	−75	−52
Isorhamnetin	315	300	−35	−24
Kempferol	285	151	−45	−25
Luteolin	285	133	−60	−44
Myricetin	317	151	−20	−26
Pelargonin	271	121	141	43
p-Coumaric acid	163	119	−20	−18
p-HBA	137	93	−25	−18
Quercetin	301	151	−40	−30
Rhamnetin	315	165	−35	−30
Rhamnozide quercetin	449	303	31	21
Sophorozide cyanidin	611	287	61	33
Syringic acid	197	182	−20	−18
	197	167	−20	−24
Tectochrysin	269	124	76	49
Vanillic acid	167	152	−30	−16
	167	123	−30	−16

Q1: precursor ion, Q3: product ion, *DP (V)*[3] declustering cone potential, *CE (V)*[4] collision energy, *p-HBA* p-hydroxybenzoic acid

at *m/z* values of the pseudomolecular ([M+H$^+$] or [M−H]$^-$) ions for each phenolic compound from the EICs. Results were average value of three repetitions.

SRM is a mode of tandem mass spectrometry used to detect and quantify specific compounds with high sensitivity. It involves selecting a specific precursor ion (Q1), fragmenting it, and monitoring one specific product ion (Q3). This pair of ions is called transition, and it is characteristic from the analyte. SRM is highly specific and it is used for quantifying known molecules in complex samples, by defining multiple SRM-transitions (Q1/Q3-pairs) in one experiment. Even if some flavonoids have same fragmentation ions, there is no problem in differentiating between them since the ion pair rule must be fulfilled (precursor ion and fragmentation ion). In the case of LC-MS/MS, the confirmation of the presence of analytes is not only the retention time, but also the characteristic SRM pair.

Statistical Analysis

Statistical treatments, including means and standard deviations, were performed on the results for all individual phenolic compounds determined with the chromatographic assays. ANOVA Student–Newman test was applied in order to make the multiple comparison of mean values to ascertain possible significant differences between the studied Kratošija wines, using XLStat software (2024). Significant difference was statistically considered at the level of $p < 0.05$.

Results and Discussion

HPLC-ESI-MS/MS Analysis

Applying HPLC-ESI-MS/MS technique in this study, 26 phenolic compounds were detected and semi-quantified in Kratošija wines produced with two different commercial yeasts for fermentation. The various groups of phenolics were considered, as follows: 10 phenolic acids, 2 stilbenes and 1 stilbenoid, 6 flavan-3-ols, 3 flavonols, 2 flavones, 1 flavanone, and 1 flavanonol. The results are presented in Table 2.

The relative amount of individual phenolic compounds was calculated based on the peak area calculation from the appropriate ion peak chromatogram. The ion intensities have been extracted at the appropriate *m/z* values of the molecular ions of the corresponding detected compounds. The total ion chromatograms (TIC) in SRM modes for all analyzed compounds of both wines are presented at Fig. 1. The extracted ion chromatograms of the individual phenolic compounds divided into groups according to their molecular ions, in both Kratošija wines, are presented in Fig. 2.

Data for the semi-quantified compounds (peak area calculated) separated by appropriate groups are shown in Fig. 3.

Table 2 Phenolic compounds found in the Kratošija wines and identified by their retention time and ion pair monitored in SRM mode (Q1 and Q3)

Compounds	t_R/min	Q1[1]	Q3[2]	DP (V)[3]	CE (V)[4]
Nonflavonoids					
Phenolic acids					
Gallic acid	4.28	169	125	−45	−20
3,4-DHBA	8.02	153	109	−15	−18
Chlorogenic acid	9.48	353	191	−20	−22
Dihydroferulic acid	11.27	195	136	−55	−22
Vanillic acid	12.15	167	152	−30	−16
Caffeic acid	12.41	179	135	−10	−24
Syringic acid	12.54	197	182	−20	−18
p-Coumaric acid	14.86	163	119	−20	−18
Ferulic acid	15.66	193	134	−30	−20
p-HBA	18.4	137	93	−25	−18
Stilbenes and stilbenoids					
Resveratrol-3-glucoside (Piceid)	17.19	389	227	−40	−20
Resveratrol	19.8	227	143	−55	−38
ε-Viniferin	22.54	453	347	−75	−26
Flavonoids					
Flavan-3-ols					
Dimer 1 (B3)**	10.4	577	289		
Catechin	11.31	289	109	−45	−20
Dimer 2 (B1)**	12.15	577	289		
Epicatechin	12.86	289	109	−40	−32
Dimer 3 (B4)**	13.86	577	289		
Dimer 4 (B2)**	15.37	577	289		
Flavonols					
Myricitin	18.48	317	151	−20	−26
Quercitrin	18.67	447	300	−60	−32
Syringetin	23.22	345	315		
Flavones					
Chrysin*	25.40	255	153	81	37
Luteolin	20.82	285	133	−60	−44
Flavanones					
Naringenin	22.75	271	151	−45	−26
Flavanonols					
Taxifolin	16.06	303	125	−50	−30

p-HBA, *p*-hydroxybenzoic acid; *3,4-DHBA*, 3,4-dyhydroxybenzoic acid; *positive ionization mode; **data based on the literature (Absalon et al. 2011); [1]the first quadrupole; [2]the third quadrupole; [3]*DP*, declustering (cone) potential; [4]*CE*, collision energy

Wines were produced using two commercial *Saccharomyces cerevisiae* yeasts for alcoholic fermentation: Zymaflore^{TM} Xpure (Laffort, Bordeaux, France) (wine marked as Kratošija **1**) and Lalvin ICV D80 (Lallemand, Blagnac, France) (wine marked as Kratošija **2**). According to the yeast characteristics given by the producer, Zymaflore^{TM} Xpure is adopted for red wines with a great aromatic purity,

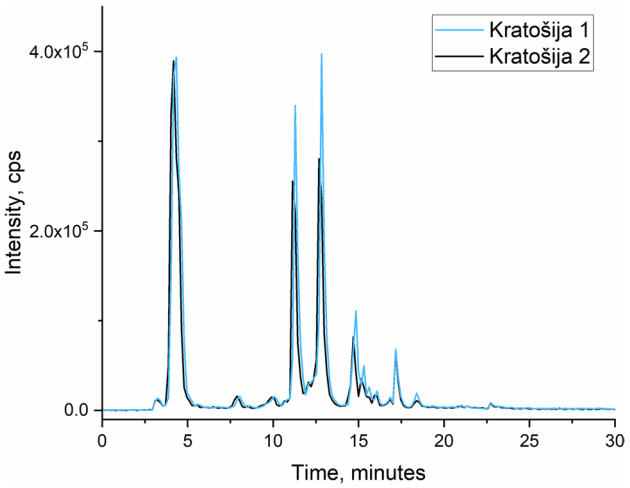


Fig. 1 Total ion chromatograms in SRM modes of Kratošija 1 and Kratošija 2 wines obtained with HPLC-ESI-MS/MS under single reaction monitoring (SRM). Blue line corresponds to Kratošija 1 (fermented with Zymaflore^{TM} Xpure yeast) and black line corresponds to Kratošija 2 (fermented with Lalvin ICV DB80 yeast)

enhances the aromatic freshness and provides great smoothness of mouthfeel. The second yeast, Lalvin ICV D80, has the ability to intensify fine grain tannins and to bring high volume and big mid-palate mouthfeel, effecting on the structure and good color stability. We chose these yeast strains in order to study their effect on the phenolic profile, as well as to compare the phenolic profile of two wines produced from same grapes inoculated with different yeasts for fermentation, since both yeasts influence differently on the wine characteristics. As we expected, differences in the flavonoids and nonflavonoids profile and content in both wines have been noticed, as described below by each phenolic group separately.

Nonflavonoids

Phenolic Acids

Phenolic acids are nonflavonoids, divided into two groups: hydroxybenzoic and hydroxycinnamic acids (Ivanova et al. 2011a; 2011b). Gallic acid is the dominant hydroxybenzoic acid in grapes and wine, while the main hydroxycinnamic acids in wine are caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid, mainly present as esters of tartaric acid.

In this study, 10 phenolic acids have been identified and semi-quantified in the investigated Kratošija wines. Their molecular ions (Q1) and fragment ions (Q3) are shown in Table 2. Gallic acid that mainly originates from the grapes, but also from the breakdown of condensed tannins (Ivanova-Petropulos et al. 2015), was the dominant compound in both wines, followed by *p*-coumaric, syringic, caffeic, vanillic, and ferulic acids. Gallic acid was detected as a deprotonated $[M-H]^-$ ion at *m/z* 169, producing fragment ion at *m/z* 125 which corresponds to the elimination of CO_2 from the carboxylic acid (Ivanova et al. 2011a; 2011b).

Investigating the effect of yeast, it was noticed that the sum of relative amounts of phenolic acids was higher in Kratošija 1, wine fermented with Zymaflore^{TM} Xpure yeast ($p < 0.05$). Consequently, the relative amounts of all individual phenolic acids was higher in Kratošija 1, wine fermented with Zymaflore^{TM} Xpure yeast, except of gallic and chlorogenic acids, which relative contents were slightly higher, but significantly not different ($p > 0.05$) in the wine 2, fermented with Lalvin ICV D80 yeast. In fact, it is already known that the levels of chlorogenic acid can be influenced by the yeast enzymatic activity, so probably Zymaflore^{TM} Xpure yeast influenced slightly lower amount of this compound in the wine (Zhang et al. 2021; Boban et al. 2024).

Stilbenes and Stilbenoids

Stilbenes are nonflavonoids considered as phytoalexins, synthesized in the grape vine as a defense system against fungal infection, mostly *Botrytis cinerea*, and from UV radiation (Flamini 2013). The most significant and commonly studied stilbene is resveratrol, which exists in two isomeric forms, *cis*- and *trans*-forms. Moreover, stilbenes exist in glucoside, dimers (pallidol), trimers (grandiphenol C), *α*-viniferin, and polymers (Pandey et al. 2014).

In the analysis of Kratošija wines, two stilbenes and derivatives were identified and semi-quantified, among them *trans*-resveratrol and *trans*-resveratrol-3-glucoside (piceid) and one stilbenoid, *ε*-viniferin (detected for the first time in Macedonian wines). The deprotonated molecular ion of resveratrol-3-glucoside $[M-H]^-$ detected at retention time of 17.10 min, at *m/z* 389, gave a fragment ion at *m/z* 227 corresponding to the resveratrol moiety by loss of the glucoside group (162 Da). The monomer resveratrol (t_R at 19.8 min) and *ε*-viniferin (resveratrol dehydrodimer) (t_R at 22.54 min) presented molecular ions at *m/z* 227 and 453, respectively (Ivanova et al. 2011a; Flamini 2013). The molecular (Q1) and fragment ions (Q3) of stilbenes and stilbenoids are shown in Table 2.

Concerning the yeast effect, relative amount of the individual stilbenes (*trans*-resveratrol and *trans*-resveratrol-3-glucoside) was higher in Kratošija 1, wine fermented with Zymaflore^{TM} Xpure yeast, similarly as the content of phenolic acids, while the content of *ε*-viniferin was only slightly higher in Kratošija 1, without significant difference between the wines ($p > 0.05$). In fact, higher content of nonflavnoids (phenolic acids and stilbenes) in the wine fermented with Zymaflore^{TM} Xpure yeast can be attributed to a higher extraction of these

compounds during the alcoholic fermentation, as well as interaction with the yeast cell walls during the fermentation, followed by adsorption of nonflavonoids, which decreased their amount in the wine fermented with Lalvin ICV DB80 yeast.

Fig. 2 Ion-extracted peak chromatograms of phenolic compounds in Kratošija wine in SRM mode. **a** Ion-extracted peak chromatograms of phenolic acids. **b** Ion-extracted peak chromatograms of stilbenes and stilbenoids. **c** Ion-extracted peak chromatograms of flavan-3-ols. **d** Ion-extracted peak chromatograms of flavonols, flavones, flavanone, and flavanonol

Flavonoids

Flavan-3-ols

Flavan-3-ols are compounds responsible for the astringency, bitterness, and structure of the wine. These compounds can be present as monomers, oligomers, and polymers (Monagas et al. 2005; Zhang et al. 2021). The main flavan-3-ol monomers in grapes and wine are (+)-catechin and (−)-epicatechin, followed by (+)-gallocatechin, (−)-epigallocatechin and (−)-epicatechin-3-*O*-gallate (Ivanova et al. 2011a; 2011b). The monomeric flavan-3-ols, (+)-catechin and (−)-epicatechin with $[M-H]^-$ pseudomolecular ions at *m/z* 289 and retention times at 11.31 and 12.86 min, respectively, were detected in both Kratošija wines. The four flavan-3-ol dimers were identified in wines, presenting molecular ions at *m/z* 577, and producing fragment ion at *m/z* 289. Dimers were detected at four retention times at 10.4, 12.15, 13.86, and 15.37 min and identified as procyanidin B3, B1, B4, and B2, respectively, in accordance to the literature (Carando et al. 1999; Ivanova et al. 2011b). Procyanidins B1 and B3 dominated in both wines. In terms of the influence of yeast, Kratošija 2, wine fermented with Lalvin ICV DB80 yeast, contained higher relative amounts of flavan-3-ol dimers, probably due to the yeast ability to increase the tannin's content and better extract flavan-3-ols from the grape skins, while the other used yeast (Zymaflore^{TM} Xpure) could be less efficient, resulting with lower content of flavan-3-ol monomers, even there was no statistical difference between the levels of monomers in both wines. Concerning the individual procyanidins, the most abundant dimers were B3, B1, and B4, present in significantly higher levels ($p < 0.05$) in Kratošija 2, while the content of dimer B2 was not significantly higher in this wine.

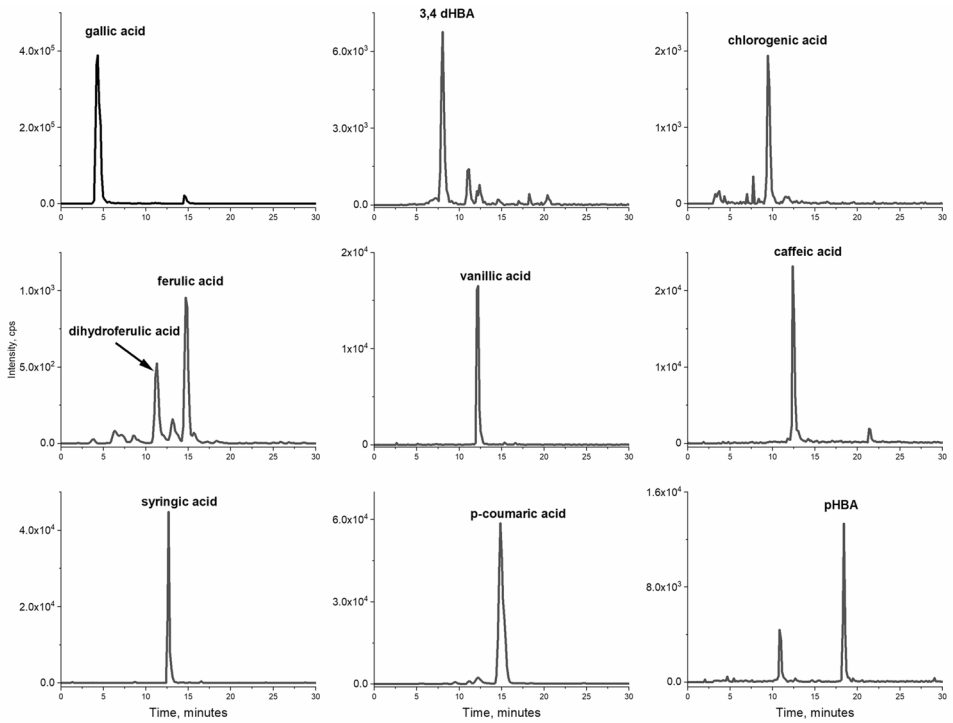
Flavonols

Regarding the flavonols, 3 compounds were detected as follows: myricitin and syrengetin as aglycones with molecular ions at *m/z* 317 and 345, at retention times of 18.48 and 23.22 min, respectively, and quercitrin (quercetin-rhamnozide) as a glucose, giving molecular ion at *m/z* 447, at retention time of 18.67 min, in accordance to the literature (Simonetti et al. 2022). Myricitin dominated in both wines, present in a significantly higher amount in Kratošija 1 ($p < 0.05$), while relative contents of syrengetin and quercitrin were similar in the wines ($p > 0.05$), slightly higher in Kratošija 1 wine. Concerning the yeast influence, Kratošija wine fermented with Zymaflore^{TM} XPure yeast presented higher relative amounts of all flavonols, probably as a result of the higher fermentation rate of this yeast, compared to Lalvin ICV DB80.

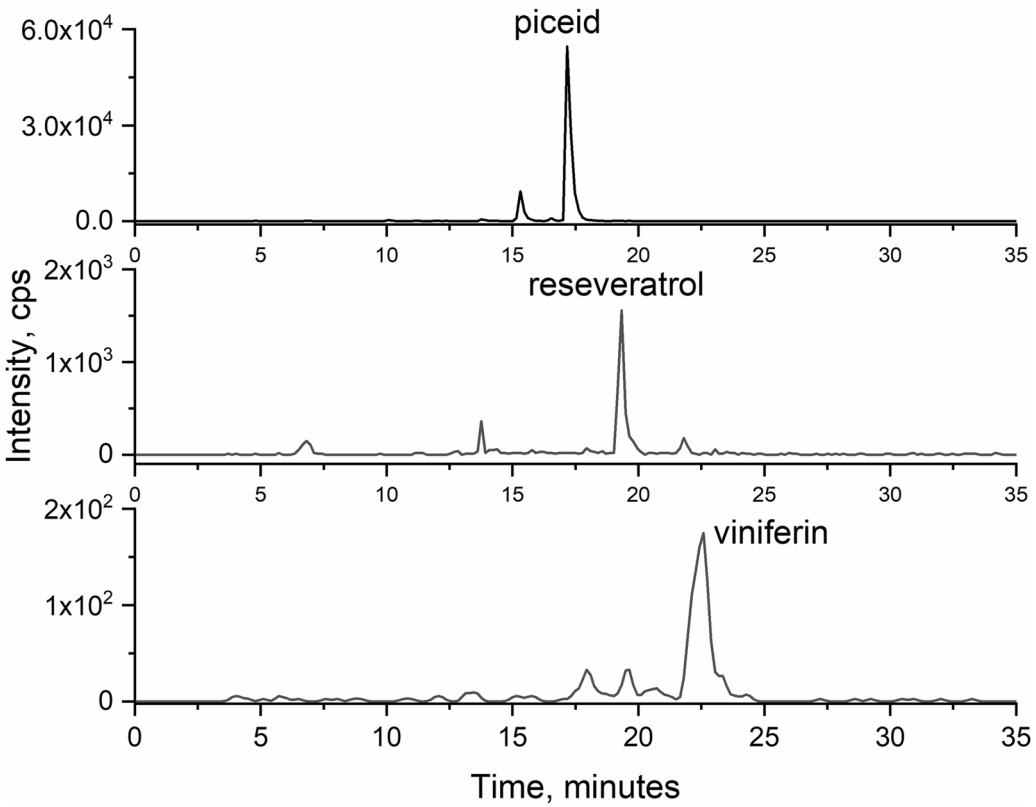
Flavones, Flavanones, and Flavanonols

Chrysin, as well as luteolin which is established as an antioxidant that prevent oxidation of low-density lipoprotein and inhibit lipid peroxidation, has been detected in both wines, in accordance to the literature (Fang et al. 2007; Temerdashev et al. 2024). Chrysin showed molecular and fragment ions at *m/z* 255 and 153, respectively, while luteolin showed molecular ion at *m/z* 285 and fragment ion at *m/z* 133 at retention time of 20.82 min. Slightly higher content of luteolin was found in Kratošija 2 wine ($p > 0.05$), fermented with Lalvin ICV DB80 yeast. In addition, taxifolin and naringenin were detected in both wines at retention time of 16.06 and 22.75 min, presenting molecular ions at *m/z* 303 and 271, and producing fragments ions at *m/z* 125 and 151, respectively (Le Scanff et al. 2024; Temerdashev et al. 2024). The relative content of taxifolin was higher in Kratošija 1, fermented with Zymaflore^{TM} Xpure yeast, while the content of naringenin was slightly higher in Kratošija 1 ($p > 0.05$). In addition, chrysinm, luteolin, taxifolin, and naringenin are detected for the first time in Macedonian red wines. All these flavonoids possess various health benefits (Bai et al. 2025). Thus, chrysin, luteolin, and naringenin possess anti-oxidant, anti-inflammatory, and anti-cancer properties (Neuhouser 2004; Gates et al. 2007; Khoo et al. 2010), and taxifolin is effective for cardiovascular and cancer diseases, inflammation, Alzheimer, diabetes, allergic reaction, etc. (Jain and Vaidya 2023).

Since this is a first study focused on phenolic profile of Kratošija wines produced from the Macedonian vineyards, comparative studies with other wine varieties have not been performed yet. Moreover, there are limited number of studies focused on this grape variety grown at the Balkan region. One of them, comparing Kratošija with Vranac and Cabernet Sauvignon in a Montenegrin study (Pajović Šćepanović et al. 2019a), showed that Kratošija


gallic acid
3,4 dHBA
chlorogenic acid
ferulic acid
dihydroferulic acid
vanillic acid
caffeic acid
syringic acid
p-coumaric acid
pHBA
Intensity, cps
Time, minutes
Time, minutes
Time, minutes

a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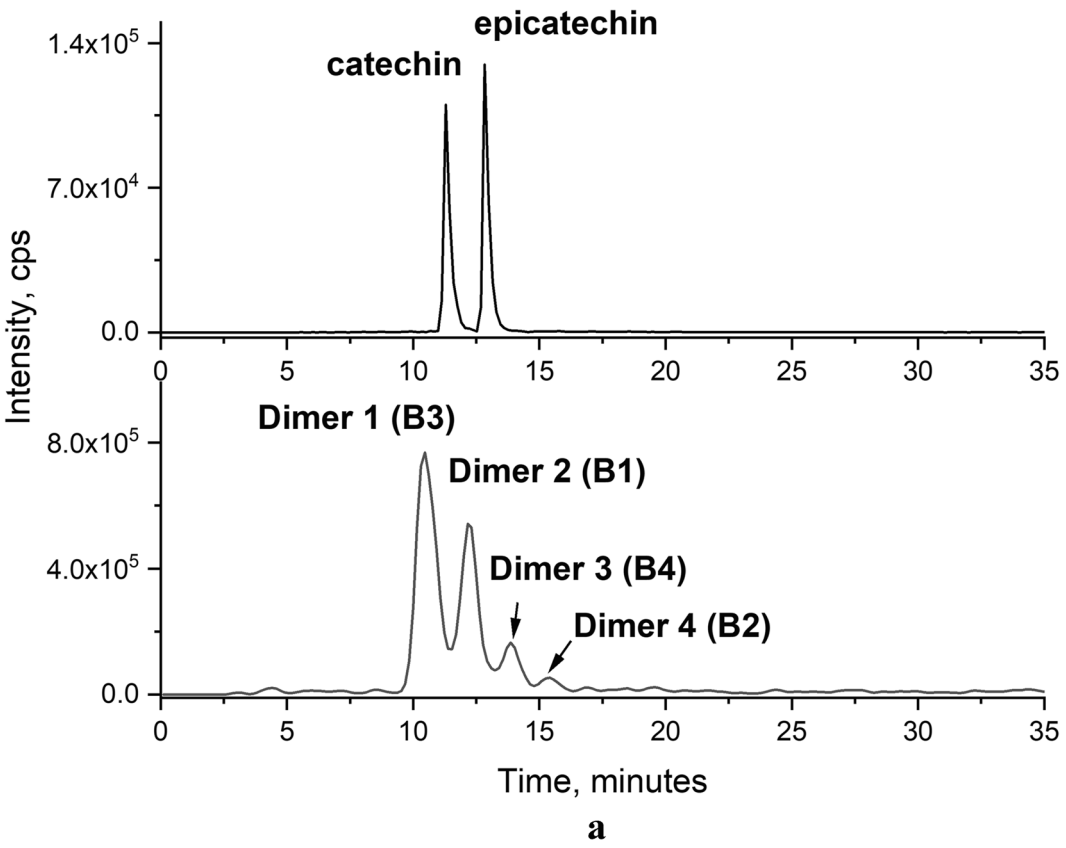
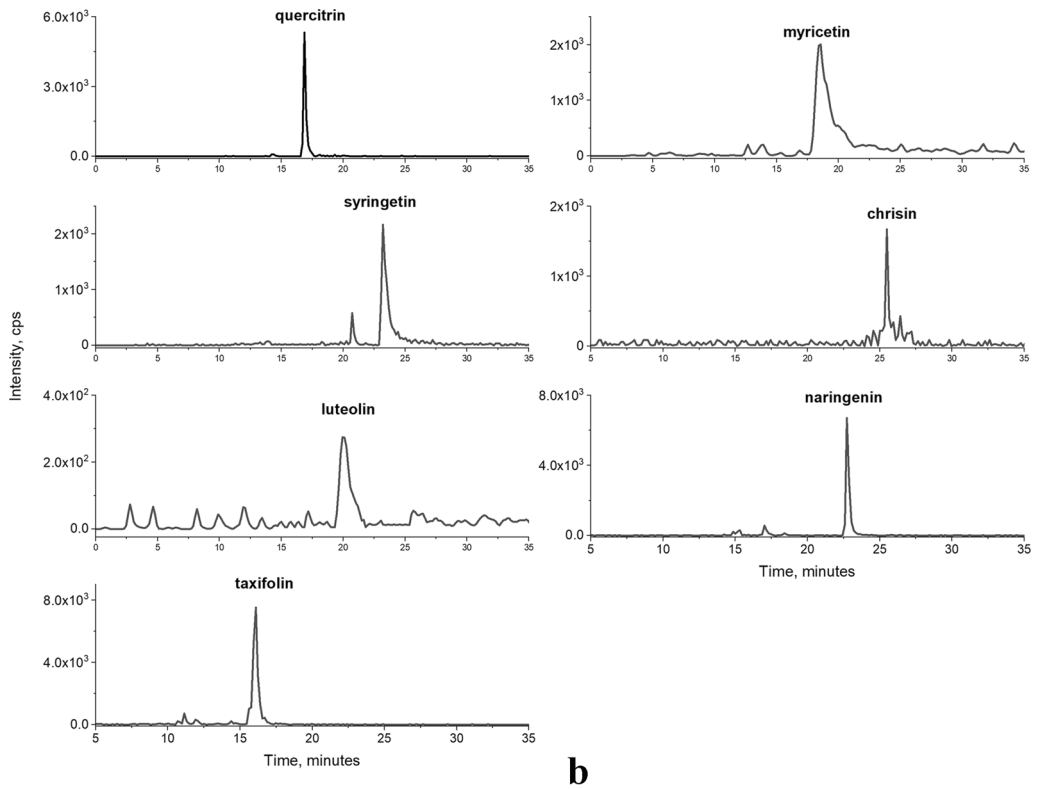
b



a



b

Fig. 2 (continued)

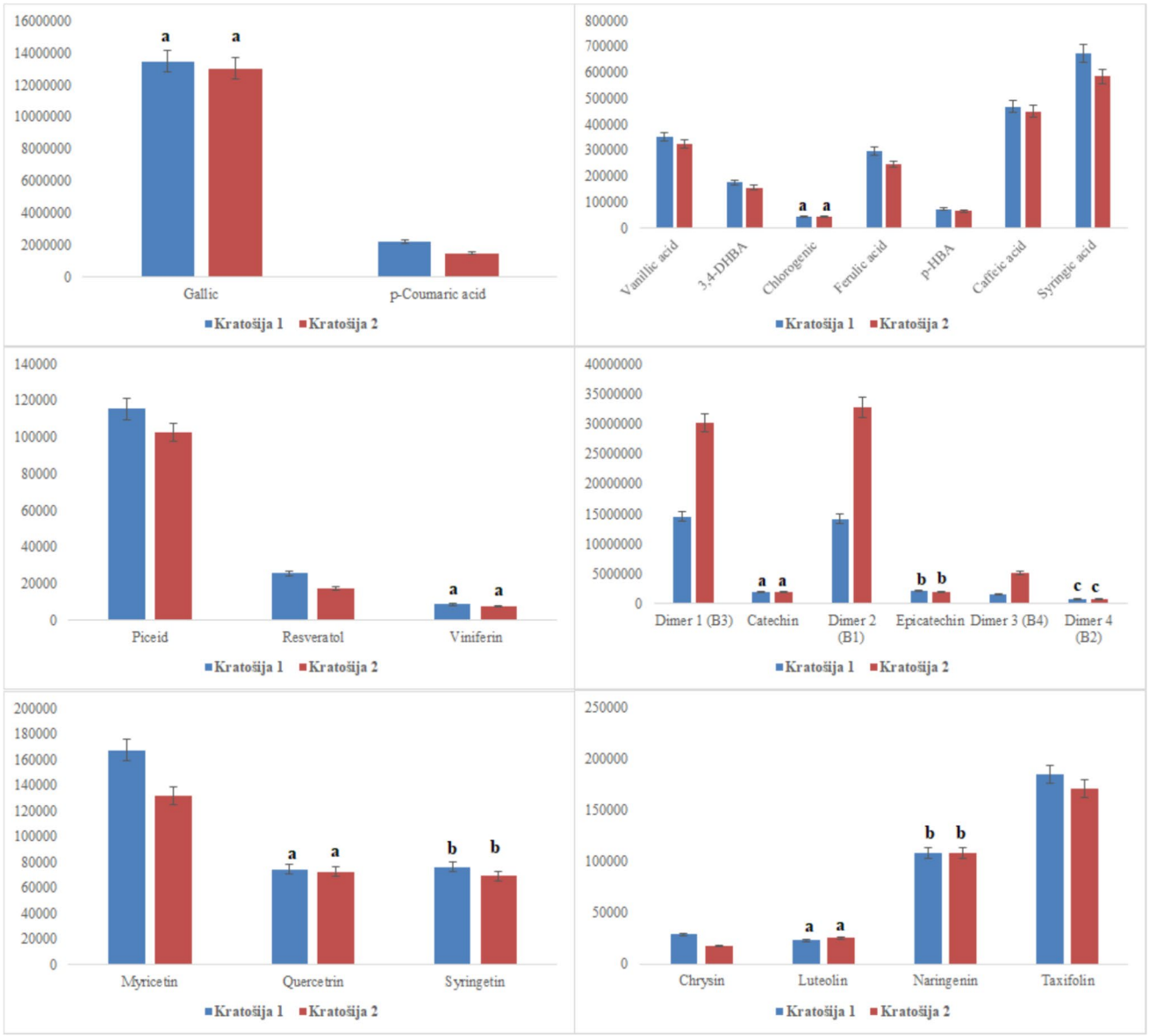


Fig. 3 Relative amounts (±standard deviation of three repetitions) of phenolic compounds determined in Kratošija wines fermented with different commercial yeasts expressed as average peak areas ($n=3$). Same letters in the column indicate values that are not significantly different ($p>0.05$) determined by ANOVA. *p*-HBA, *p*-hydroxybenzoic acid; 3,4-DHBA, 3,4-dyhydroxybenzoic acid. Results for wines Kratošija 1 (fermented with Zymaflore^{TM} Xpure yeast) and Kratošija 2 (fermented with Lalvin ICV DB80 yeast) are presented as average value of the relative peak area of each compound±RSD (relative standard deviation)

wines presented highest content of hydroxycinnamic acids, hydroxybenzoic acids, and flavan-3-ols, but lower amount of antohocyanins. Another study (Pajović-Šćepanović et al. 2024) confirmed that Kratošija wines contained lower amount of anthocyanins and total phenolic content compared to Vranac wines. However, additional studies are necessary to be performed, focusing on Kratošija wines from various locations and vinification protocols, including systematic analyses of increased number of phenolic compounds, as well as other groups of important compounds.

Conclusion

In this study, the flavonoids and nonflavonoids profile of Macedonian Kratošija wines has been evaluated for the first time in this study. Wines were produced by fermentation of two commercial yeasts (Zymaflore^{TM} Xpure (Laffort) and Lalvin ICV D80 (Lallemand)) in order to study their effect on phenolics profile and content. A total of 26 phenolic compounds, including 10 phenolic acids, 2 stilbenes, 1 stilbenoid, 6 flavan-3-ols, 3 flavonols, 2 flavones,

1 flavanone, and 1 flavanonol, have been detected and semi-quantified using HPLC-ESI-MS/MS for analysis. Chrysin, luteolin, taxifolin, naringenin, and ϵ-viniferin are reported here for the first time in Macedonian red wine confirming the anti-oxidant capacity of Kratošija wines. In addition, both wines presented same phenolic profile, just differences in the content of individual phenolic compounds were noticed as a result of the influence of the yeast due to the different fermentation rates in accordance to their specifications. Wine fermented with Lalvin ICV D80 presented higher content of flavan-3-ols since this yeast influences on intense fine grain tannins and extraction, while Zymaflore^{TM} Xpure presented higher amounts of all other phenolic compounds.

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Author Contribution Conceptualization, V.I.P.; writing – original draft, V.I.P., M.B., E.B., E.P. and S.A.; visualization, V.I.P.; corresponding author, review and editing, V.I.P., experimental, V.I.P., M.B., E.B., E.B. and S.A. All authors have read and agreed to the published version of the manuscript.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval This article does not contain any studies with animals or human participants.

Consent to Participate The authors agreed to participate in this work.

Consent for Publication The authors agreed to publish this work.

Conflict of Interest The authors declare no competing interests.

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