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LC-ESI-MS/MS DETERMINATION OF POLYPHENOLS IN KRATOŠIJA WINE



VIOLETA IVANOVA-PETROPULOS¹

*¹Goce Delcev University, Faculty of Agriculture, Stip,
Republic of N. Macedonia*



Elena Bogeveva¹, Aleksandra Sentkowska²,
Magdalena Biesaga³, Ewa Pobozy³

²Heavy Ion Laboratory, University of Warsaw, Warsaw, Poland

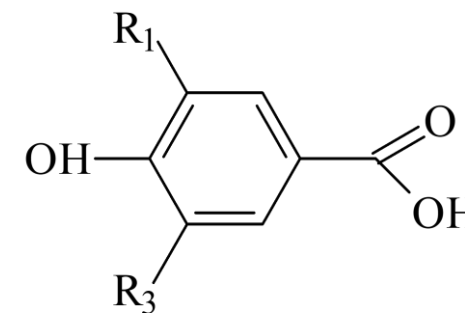
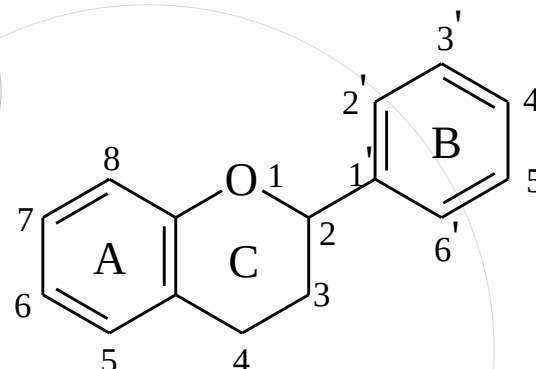
³Faculty of Chemistry, University of Warsaw, Warsaw, Poland





PRESENTATION OVERVIEW

- ✓ **Phenolic** compounds in red wine
- ✓ Liquid **Chromatography**
- ✓ Macedonian **viticulture** and **winemaking**
- ✓ **Kratošija – important Balkan variety**
- ✓ **Results for flavonoids and non-flavonoids in Kratošija wine**



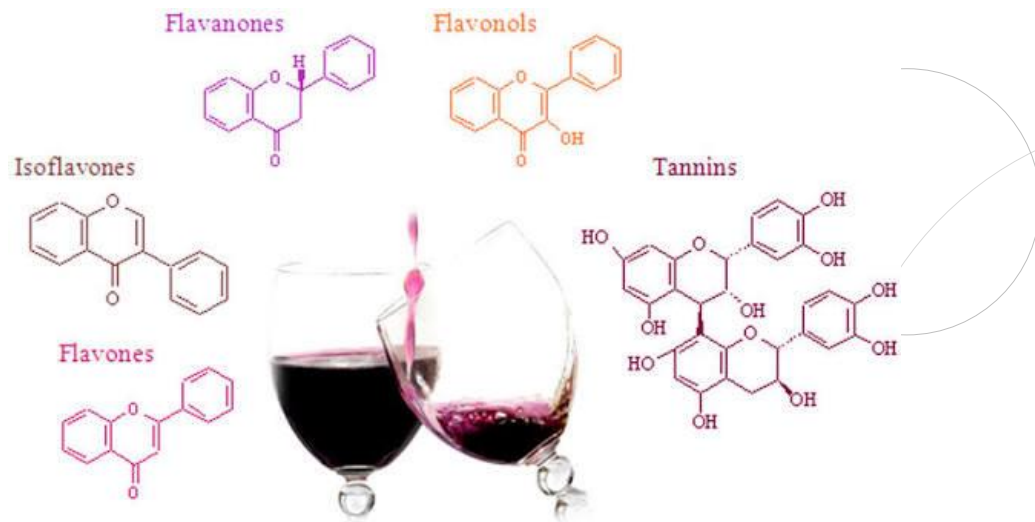


Phenolic compounds in red wine



Very important components in wine and grapes responsible for the quality of wine,

- Determine the color, taste, astringency and bitterness of the wine.
- They are responsible for the differences between red and white wines, especially the color and taste of reds.
- These substances are present in different parts of the grapes and are extracted during vinification.
- Beneficial effects on health: antioxidant, antimicrobial, anticancer ...



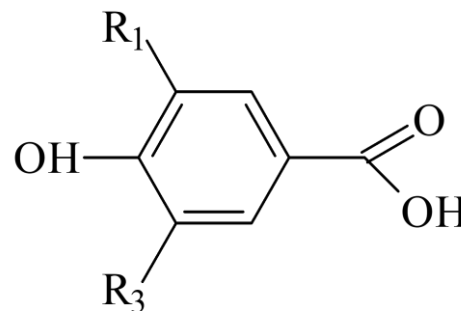


Phenolic composition of wine



NON-FLAVONOIDS

Phenolic acids

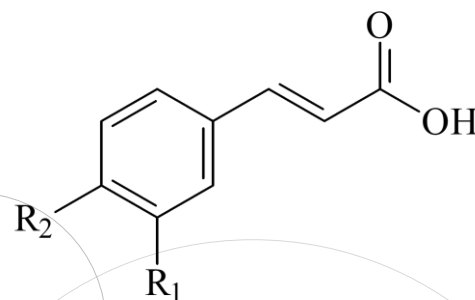


Hydroxybenzoic acids

Gallic acid
Protocatechuic acid

R_1	R_2
OH	OH
OH	H

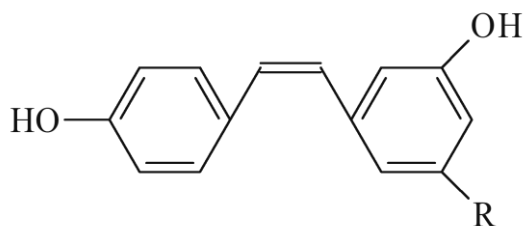
Stilbens



Hydroxycinnamic acids

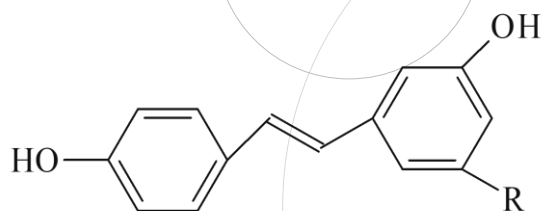
Cinnamic acid
p-Coumaric acid
Caffeic acid
Ferulic acid

R_1	R_2
H	H
H	OH
OH	OH
OH	OCH ₃



cis-

Resveratrol



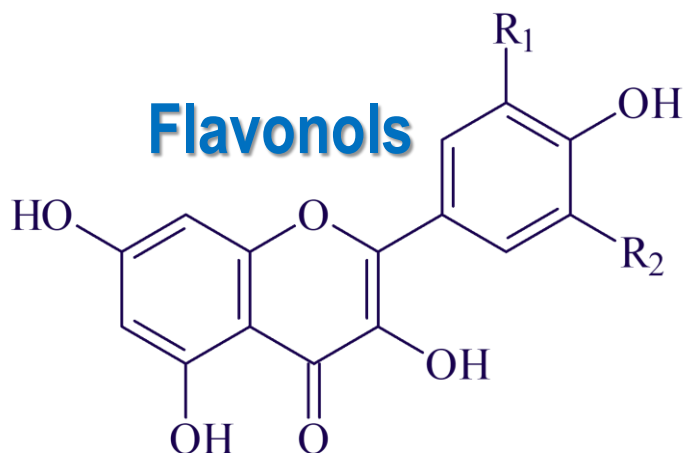
trans-



Flavonoids



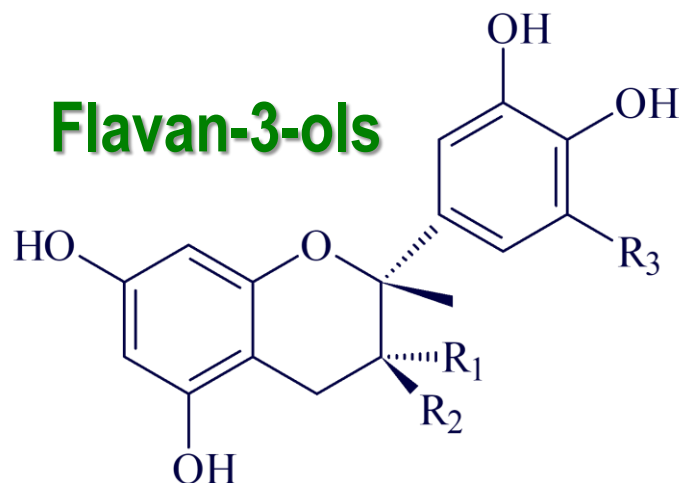
Flavonols



Flavonols

	R ₁	R ₂
Myricetin	OH	OH
Quercetin	OH	H
Laricitrin	OCH ₃	OH
Kaempferol	H	H
Syringetin	OCH ₃	OCH ₃

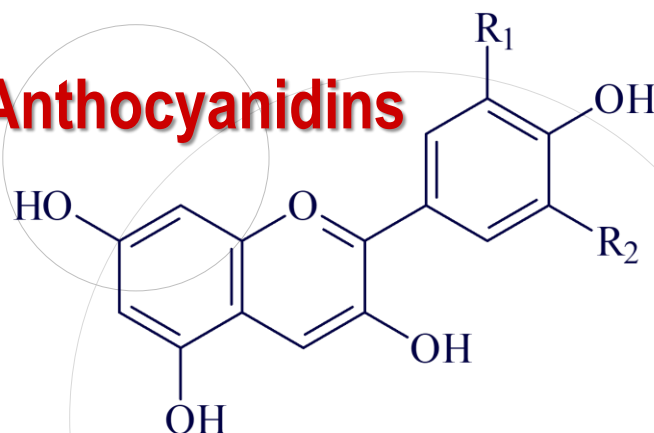
Flavan-3-ols



Flavan-3-ols

	R ₁	R ₂	R ₃
(+)-Catechin	H	OH	H
(-)-Epicatechin	OH	H	H
(-)-Gallocatechin	H	OH	OH
(-)-Epigallocatechin	OH	H	OH
(-)-Epicatechin gallate	OGalloyl	H	H

Anthocyanidins



Anthocyanidins

	R ₁	R ₂
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH ₃	OH
Peonidin	OCH ₃	H
Malvidin	OCH ₃	OCH ₃

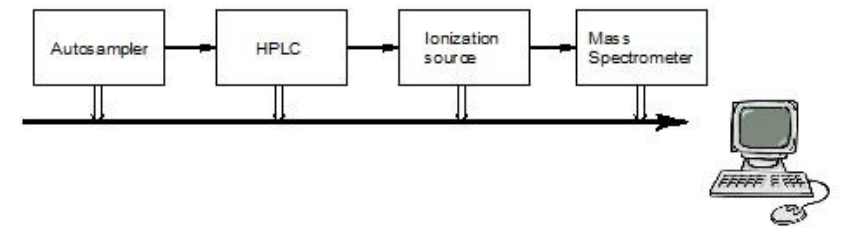
●●● Laboratory Methods for Measuring Phenolic Compounds in Red Wine

- Spectroscopic
- Chromatographic





HPLC- ESI-Q TRAP-MS/MS



High-performance liquid chromatography (HPLC) is a very powerful separation method used to identify and/or quantify one or several components in a mixture simultaneously.

- used in food science, environmental science, pharmaceutical industry, biological and chemical research, etc.

Mass spectrometry (MS) is a detection technique by measuring mass-to-charge ratio of ionic species.

(1) - a sample is injected in the instrument and then evaporated.

(2) - species in the sample are charged by certain ionized methods, such as electron ionization (EI), electrospray ionization (ESI), chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI).

(3) - the ionic species will be analyzed depending on their mass-to-charge ratio (m/z) in the analyzer, such as quadrupole, time-of-flight (TOF), ion trap and fourier transform ion cyclotron resonance.

➤ Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has the advantages of **high sensitivity, good selectivity, high throughput, good reproducibility, and wide coverage of metabolites.**

➤ In targeted metabolomics, a triple quad mass spectrometer can be used with **high sensitivity, good reproducibility, and a wide linear dynamic range in MRM mode.**



MACEDONIAN VITICULTURE AND WINEMAKING

- Grape and wine production in Republic of N. Macedonia has a very long tradition.
- Viticulture and winemaking are one of the key agricultural-related economic activities in the Macedonia. With its terroir, climate, soil, terrain and tradition, the country has all the conditions for the production of high-quality wine.



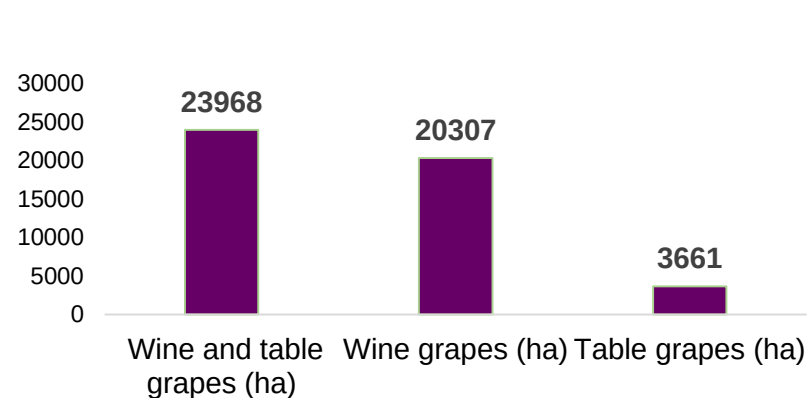


MACEDONIAN VITICULTURE AND WINEMAKING

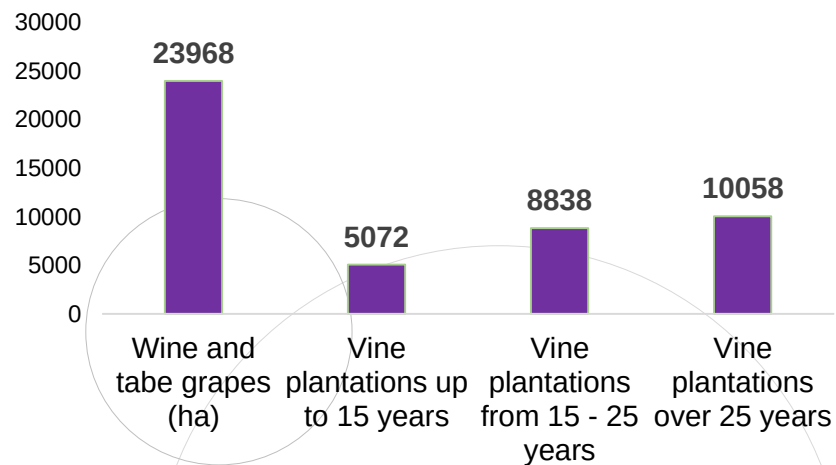


- **Wine grape** varieties account for about: **85%** (60 % red and 40 % white)
- **Table grape** varieties account for about: **15%.**

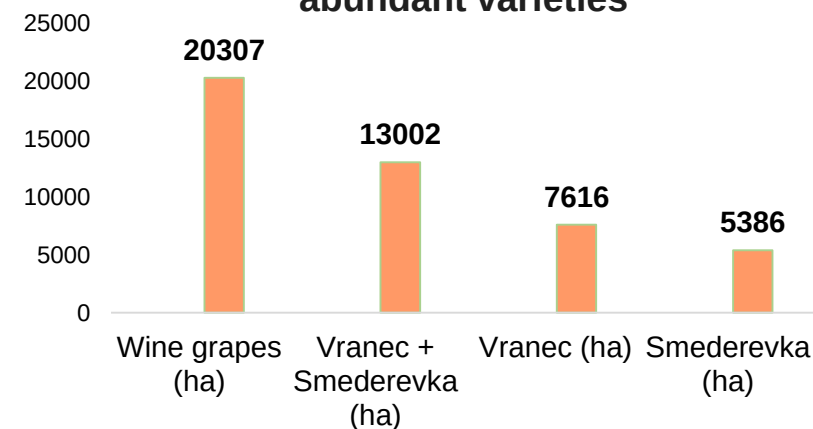
Total area under wine and table grapes



Total area under vineyards and age structure



Total area under wine grapes and most abundant varieties



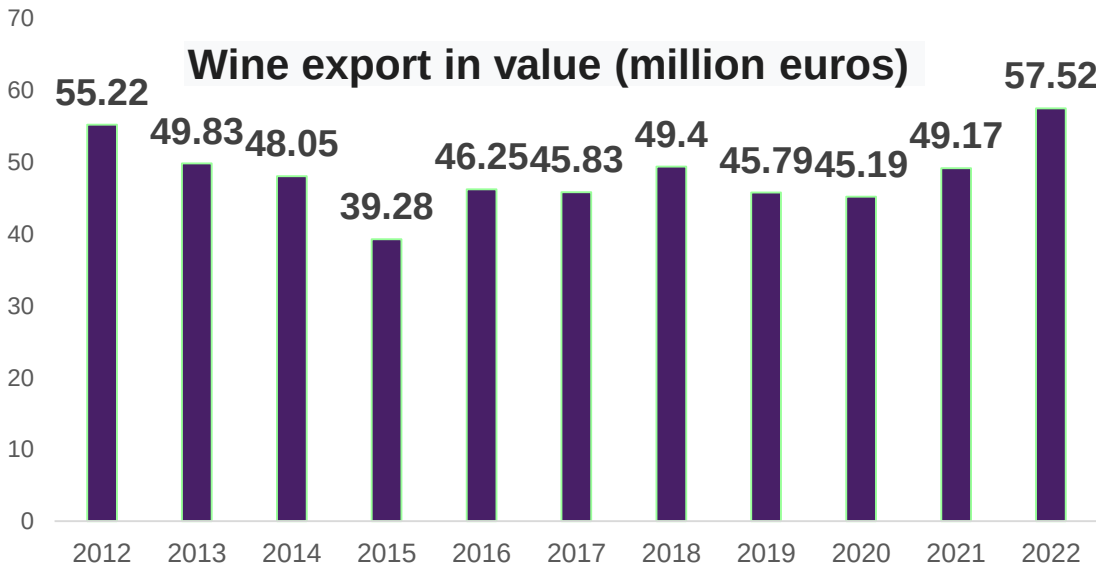
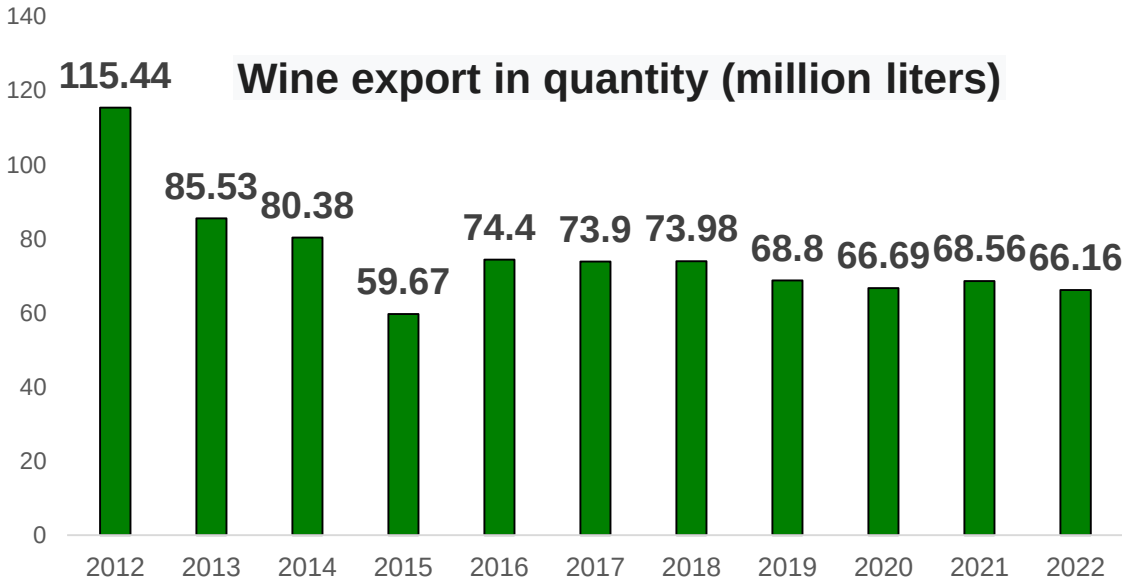


MACEDONIAN VITICULTURE AND WINEMAKING



Capacity of wineries

Capacity of wineries	Number of wineries	% of total number of wineries	Total wine capacity (hl)	% of total wine capacity
from 151.000 hl to 500.000 hl	4	2.45	896.000	51.83
from 51.000 hl to 150.000 hl	5	3.07	408.930	23.65
from 11.000 hl to 50.000 hl	12	7.36	359.400	20.79
from 1.000 hl to 10.000 hl	19	11.66	47.450	2.74
~ 500 hl	123	75.46	16.975	0.98
TOTAL	163		1.728.755	



Wine export by year (2012-2022) in quantity and value

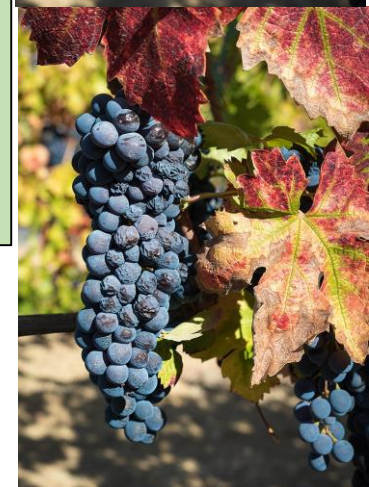
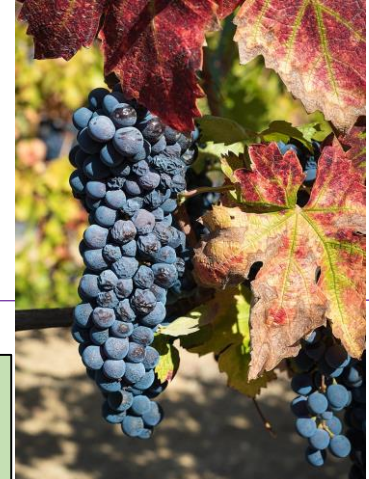


KRATOŠIJA – IMPORTANT BALKAN VARIETY

- ✓ Kratošija is considered as 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.
- ✓ Nowadays, Kratošija is found mainly in combination with Vranec vines and at small locations alone, mainly in the Tikveš wine region.
- ✓ **Expansion of the vine areas is expecting in the coming years.**

ORIGIN of Kratošija

- ✓ In some publications, **Kratošija is considered as autochthonous Montenegrin variety** (Pajović Šćepanović et al. 2019a; 2019b; 2024)
- ✓ 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 alleles typical for the Croatian gene pool.
- ✓ Consequently, more additional genetic investigations are necessary to be done.
- ✓ **Therefore, it would be the best if Kratošija is considered as a Balkan variety.**





Winemaking

Kratošija grapes, harvest at 260 °Brix

**Kratošija 1 with yeast: Zymaflore™
Xpure (Laffort, Bordeaux, France)**

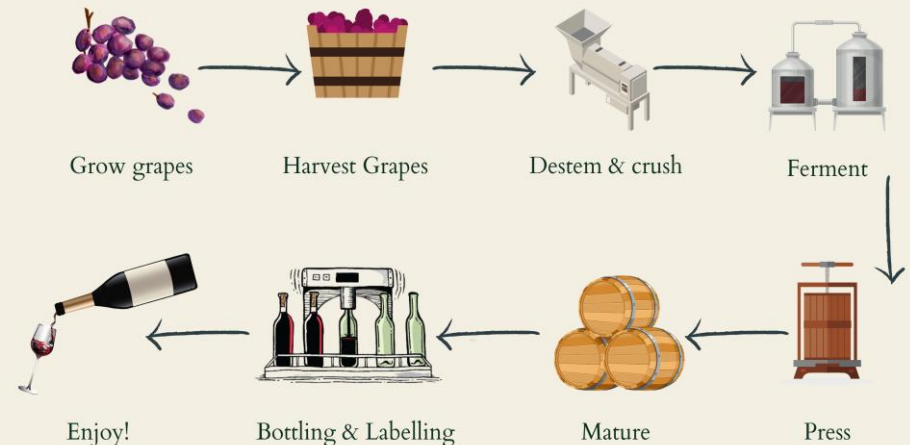
Yeast recommended for wines with **high aromatic purity, freshness**, expression of **black fruit notes** and great smoothness on mouthfeel.

**Kratošija 2 with yeast: Lalvin ICV D80
(Lallemmand, Blagnac, France)**

Yeast brings high foremouth volume, big mid-palate mouthfeel and intense fine grain tannin to reds. **It is one of the best strains for contributing big tannin volume** and is characterized by ripe fruit, smoke and a licorice finish.

Added:

**Potassium metabisulfite, $K_2S_2O_5$
Enzyme Lafaze HE GRAND CRU**



Note: For white wines, reverse order of pressing & fermenting wine

21 day alcoholic fermentation (18-20 °C)

Punching down:

Two times a day - 14 days

Once a day – 7 days

HPLC-ESI-MS/MS analysis of flavonoids and non-flavonoids

LC 20 Shimadzu, ESI-MS 3200

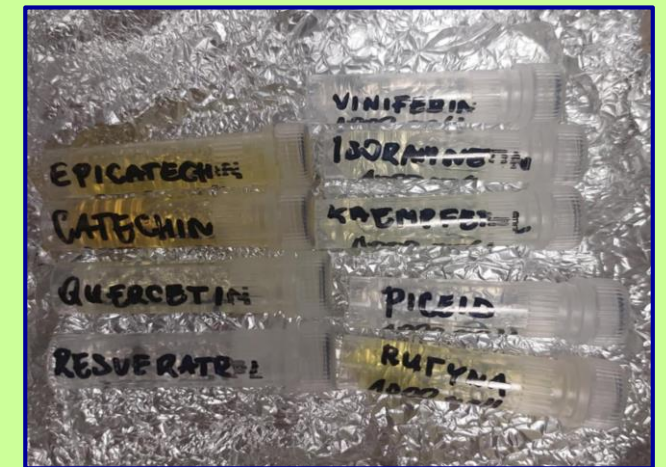
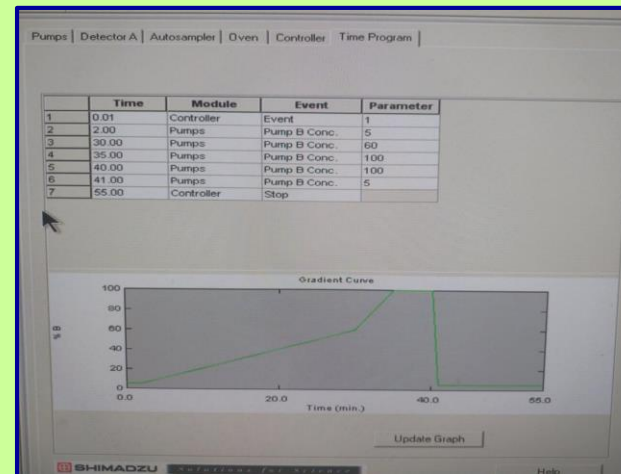
- ✓ Negative/positive mode, ESI
- ✓ Sample volume: 10 mL injection
- ✓ Column: Luna, 3 μ m C18 (100x2mm) (Phenomenex)
- ✓ **Eluent A: HCOOH pH 2.8, eluent B: ACN**

Time program for gradient elution:

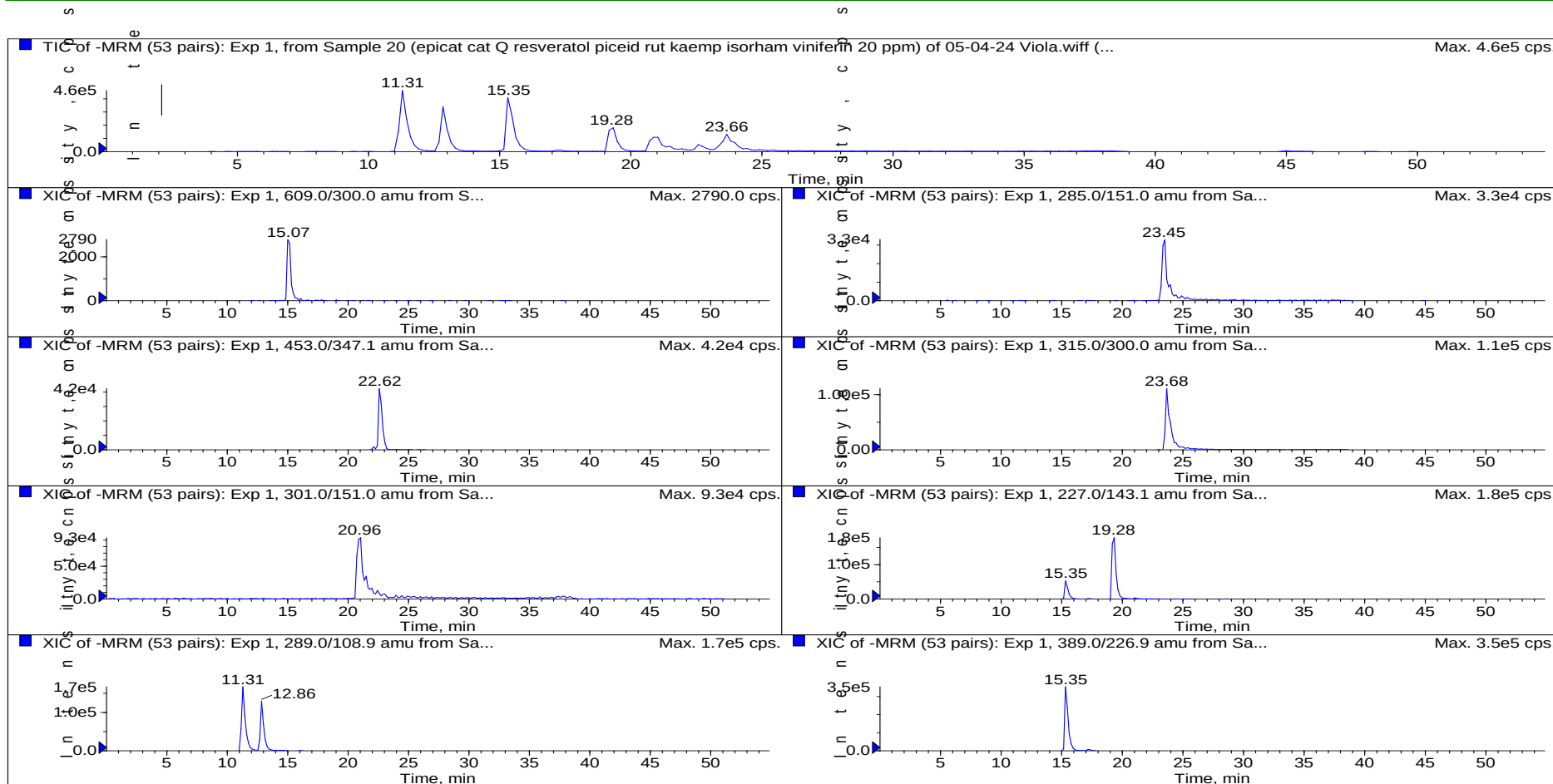
0-2 min 5% ACN
30min 60 % ACN
35 min 100% ACN
40 min 100% ACN
41 min 5% ACN
55 stop



Standards



Characterization of standards

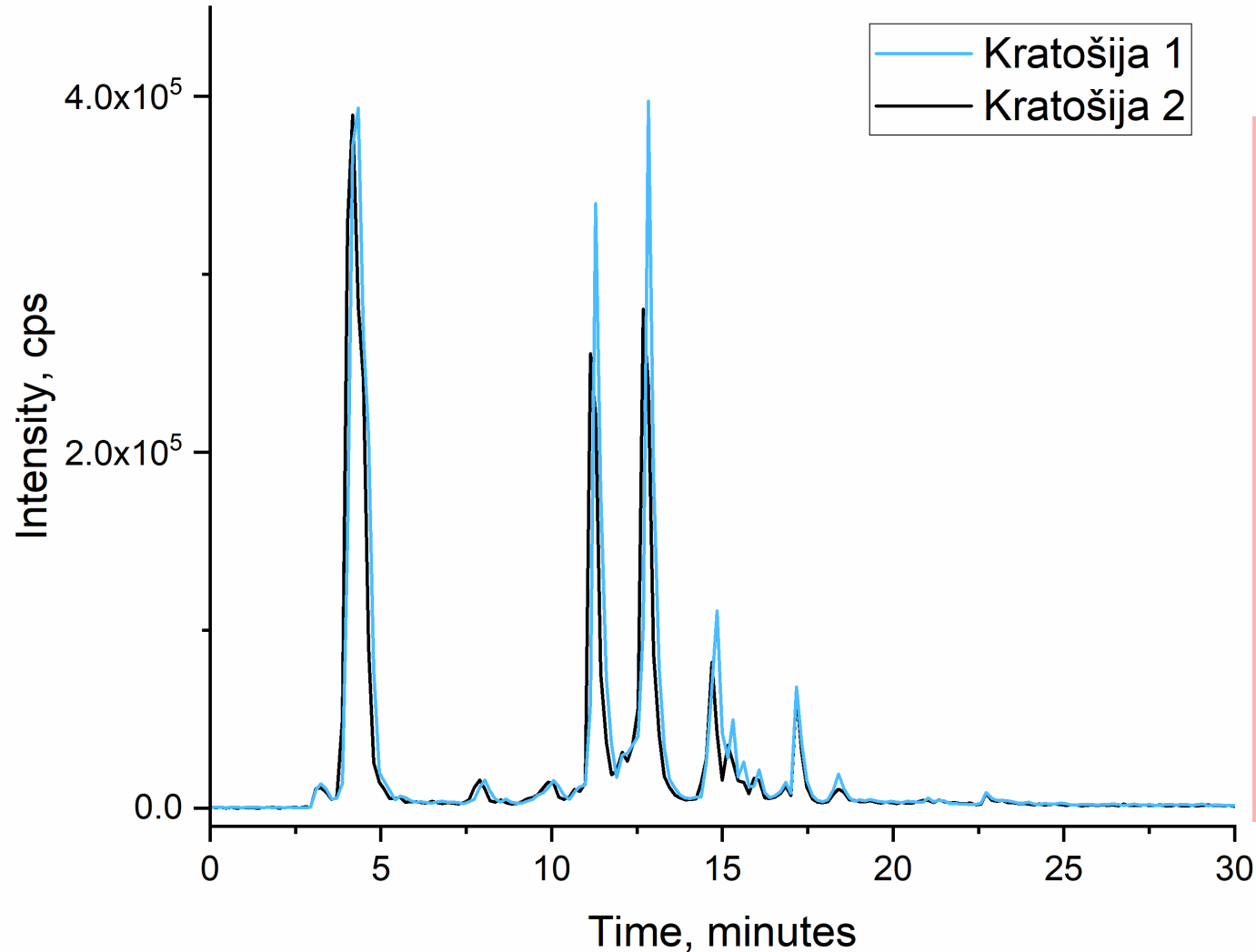


Standards (20 ppm)

1. Resveratrol
2. Piceid
3. Catechin
4. Epicatechin
5. Quercetin
6. Viniferin
7. Isorhamnetin
8. Kaempferol
9. Rutin

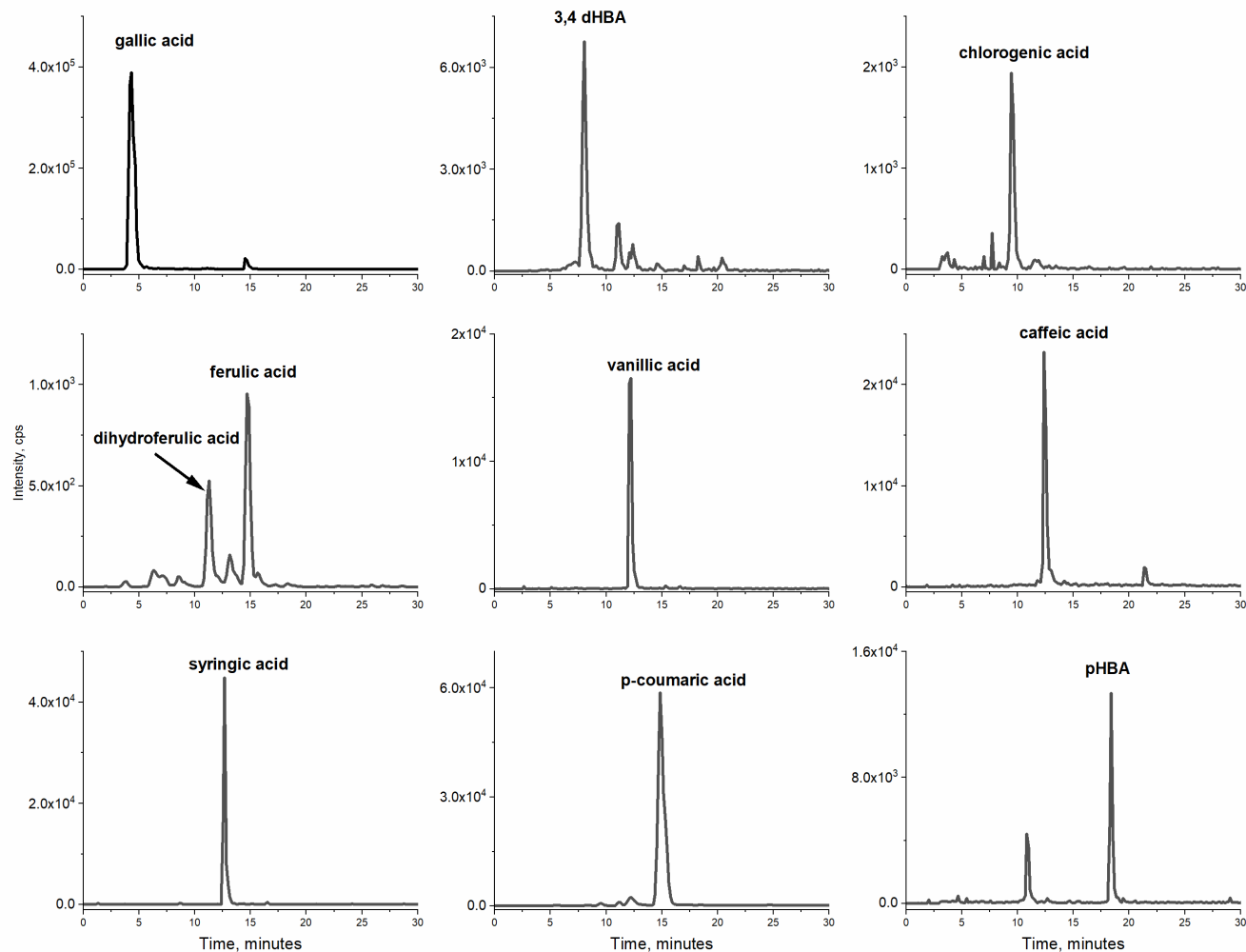
- All standards were analyzed separately in full scan in positive $[M+H]^+$ and negative $[M-H]^-$ ionization modes to detect the precursor ions characteristic of each compound, observing maximum sensitivity in the **negative** ion mode.

Total ion chromatogram (TIC) in SRM mode of two wine samples



- **SRM** (single reaction monitoring mode) is a mode of tandem mass spectrometry used to detect and quantify specific compounds with high sensitivity.
- Involves selecting a specific precursor ion (Q1), fragmenting it, and monitoring one specific product ion (Q3).
- This **Q1/Q3 pair** is called **transition**, and it is characteristic from the analyte.
- Even some flavonoids have same fragmentation ions, there is no problem in differentiating between them since they have characteristic SRM pair.

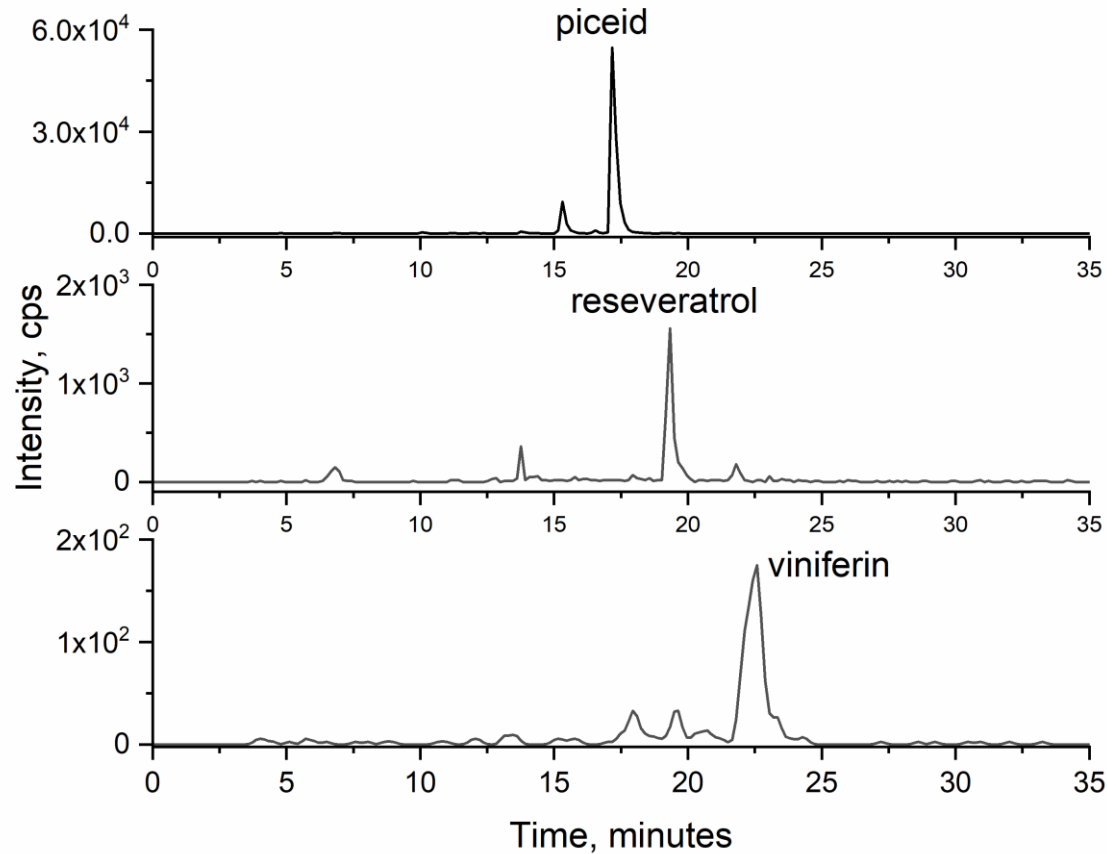
Extracted ion chromatogram (EIC) of phenolic acids in Kratošija wines in SRM mode



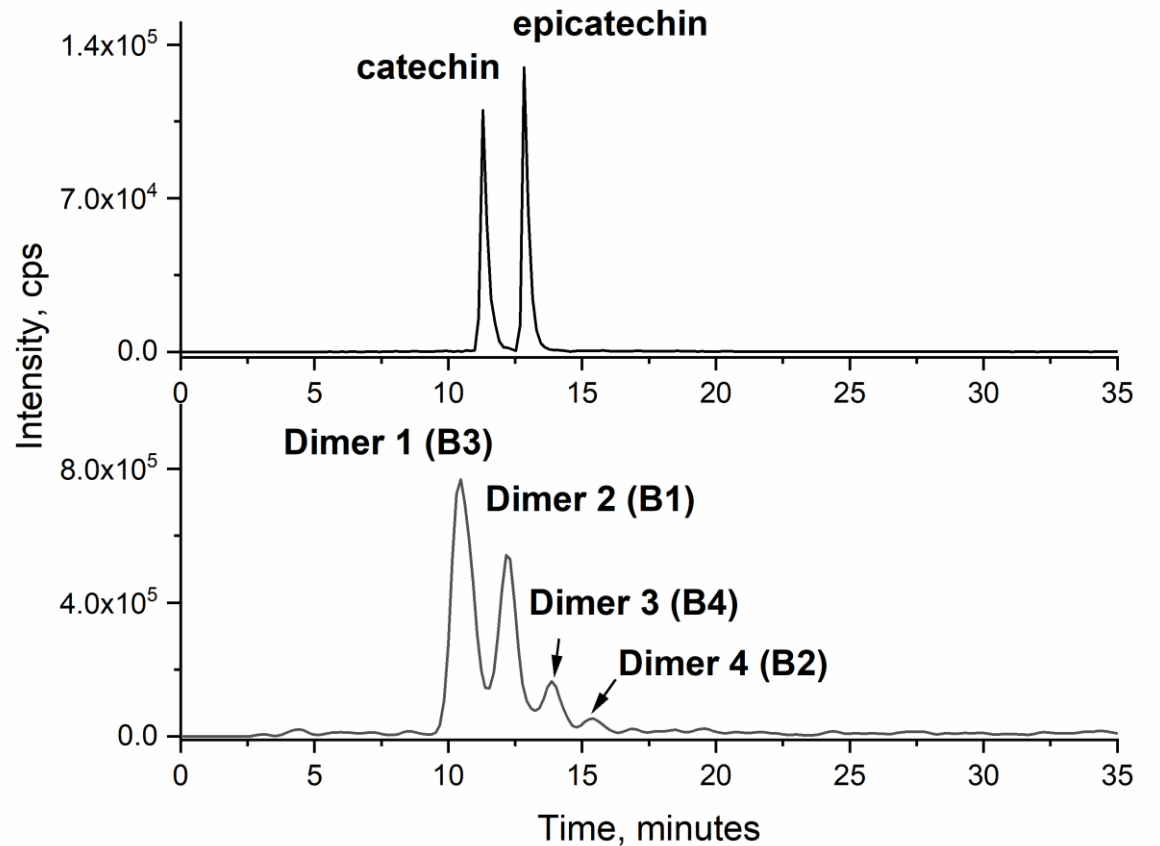
	Phenolic acids	Q1	Q3	t _R /min
1	Gallic acid	169	125	4.28
2	3,4 DHBA	153	109	8.02
3	Chlorogenic acid	353	191	9.48
4	Dihydroferulic acid	195	136	11.27
5	Vanillic	167	152	12.15
6	Caffeic	179	135	12.41
7	Syringic	197	182	12.54
8	<i>p</i> -Coumaric	163	119	14.86
9	Ferulic	193	134	15.66
10	<i>p</i> -HBA	137	93	18.4

EIC of phenolic acids

Extracted ion chromatogram (EIC) of stilbenes and flavan-3-ols in Kratosija wines in SRM mode

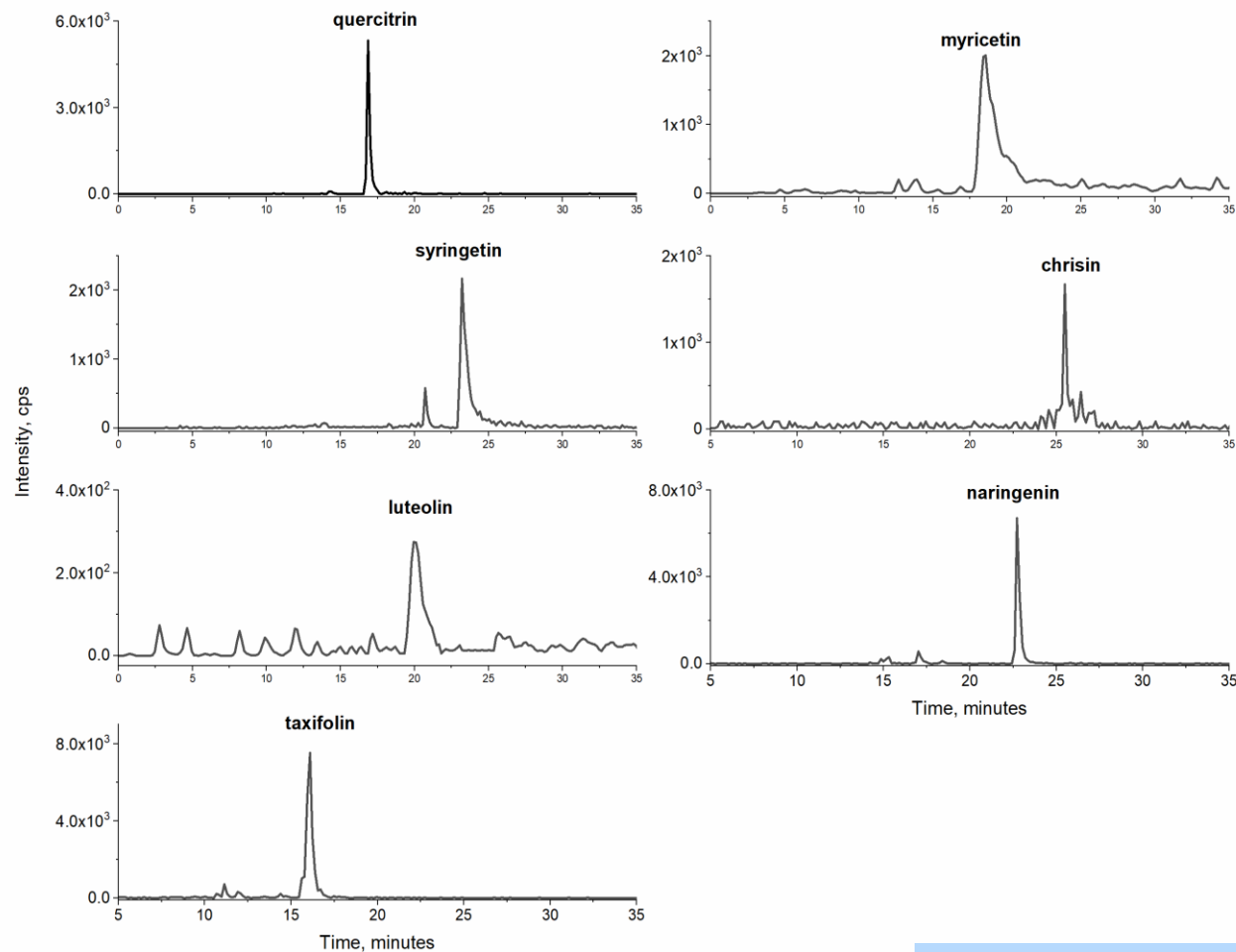


EIC of stilbenes



EIC of flavan-3-ols

Extracted ion chromatogram (EIC) of flavonols, flavones, flavanone, and flavanonol



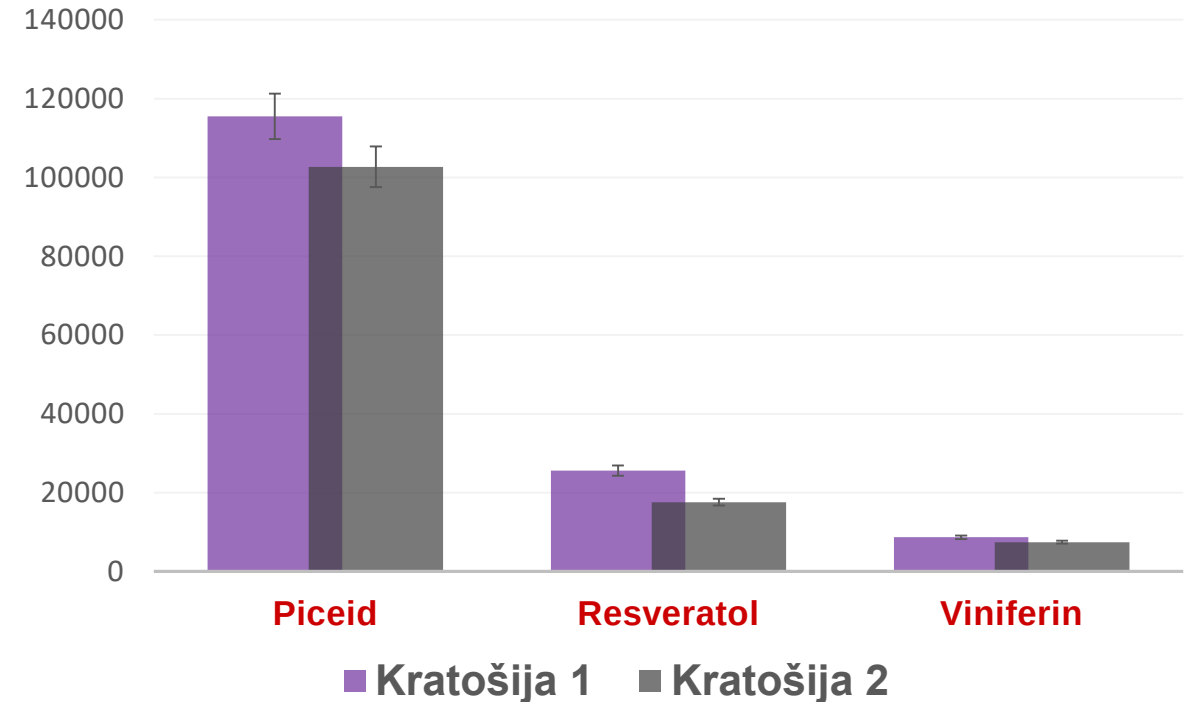
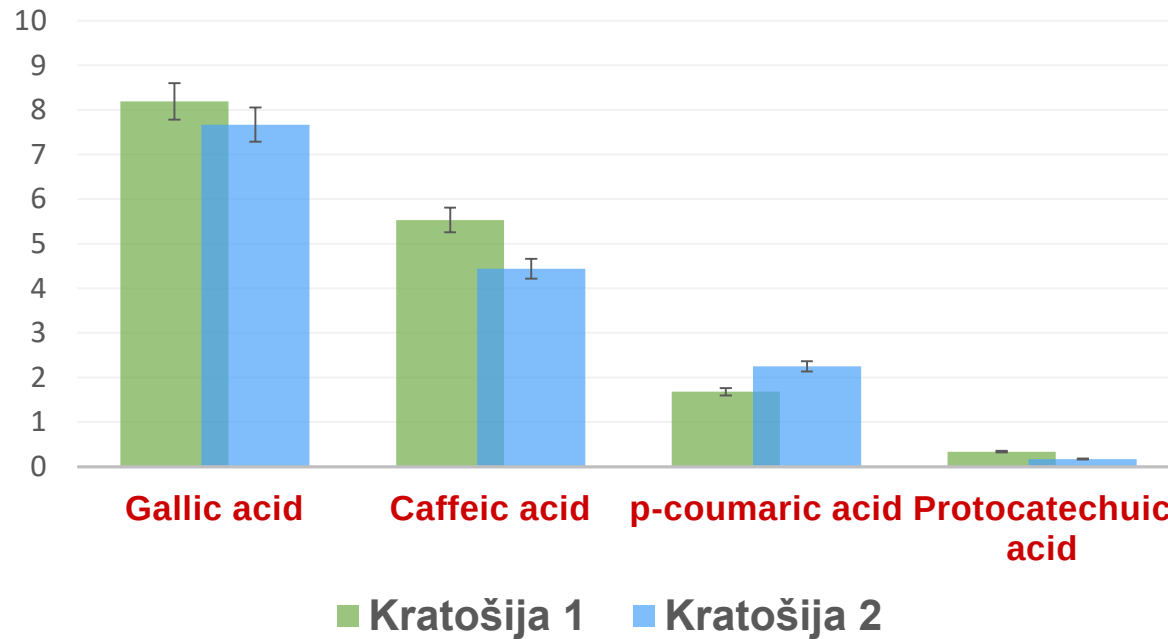
	Flavonols	Q1	Q3	t _R /min
1	Quercetin	447	301	16.85
2	Myricetin	317	151	18.48
3	Syringetin	345	315	23.22

	Flavones	Q1	Q3	t _R /min
1	Chrysin	255	153	?
2	Luteolin	285	133	20.82

	Flavanones	Q1	Q3	t _R /min
1	Naringenin	271	151	22.75

	Flavanonols	Q1	Q3	t _R /min
1	Taxifolin	303	125	16.06

Quantification of non-flavonoids

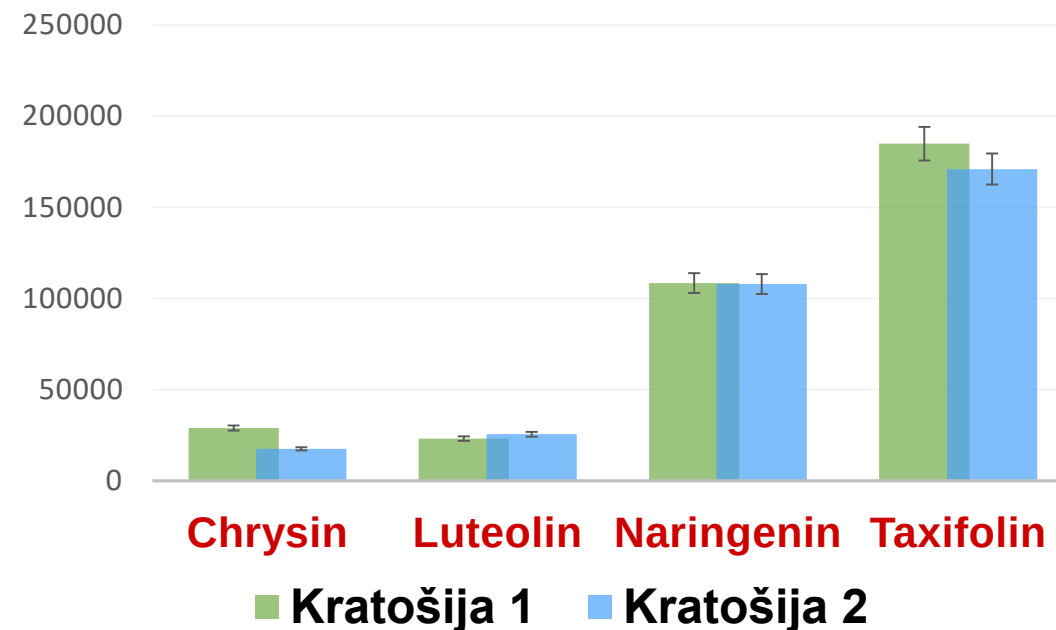
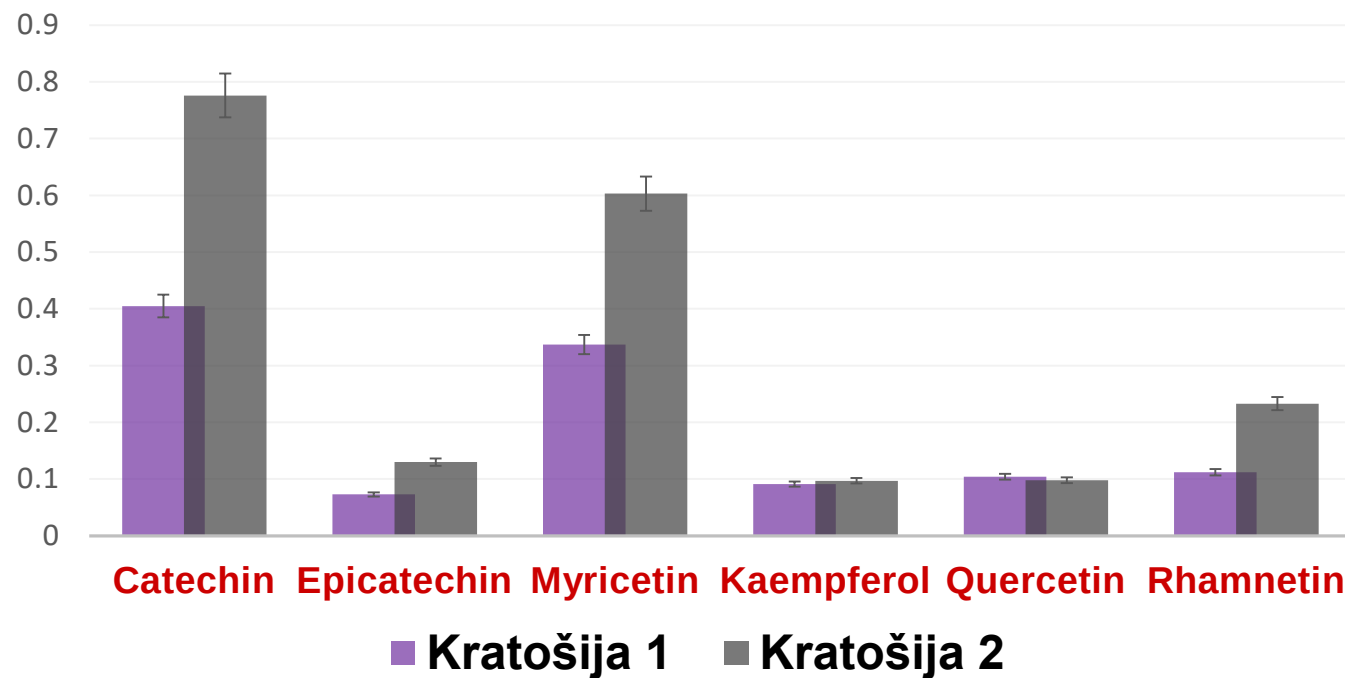


Kratošija 1 with yeast: Zymaflore™ Xpure (Laffort, Bordeaux, France)

Kratošija 2 with yeast: Lalvin ICV D80 (Lallemand, Blagnac, France)

- ✓ Higher content of nonflavonoids (phenolic acids and stilbenes) in **Kratošija 1** (wine fermented with Zymaflore™ XPure yeast) can be attributed to a **higher extraction** of these compounds during the alcoholic fermentation
- ✓ Interaction with the yeast cell walls during the fermentation, followed by **adsorption** of **nonflavonoids**, which decreased their amount in **Kratošija 2** (wine fermented with Lalvin ICV DB80 yeast).

Quantification of flavonoids



- **Chrysin, luteolin, taxifolin and naringenin**
 - **detected** for the **first** time in Macedonian red wines.
 - **possess anti-oxidant, anti-inflammatory and anti-cancer properties.**
- **Taxifolin is effective for cardiovascular and cancer diseases, inflammation, Alzheimer, diabetes, allergic reaction.**

HPLC-ESI-TQMS analysis of anthocyanins and pyranoanthocyanins

LC 20 Shimadzu, ESI-TQMS 8030

- ✓ Negative/positive mode, ESI-TQMS
- ✓ Sample volume: 5 mL injection
- ✓ Column: Kinetex C18 column (100 x 2.1 mm, 2.6 μm)
- ✓ **Eluent A: HCOOH pH 2.8, eluent B: ACN**

Time program for gradient elution:

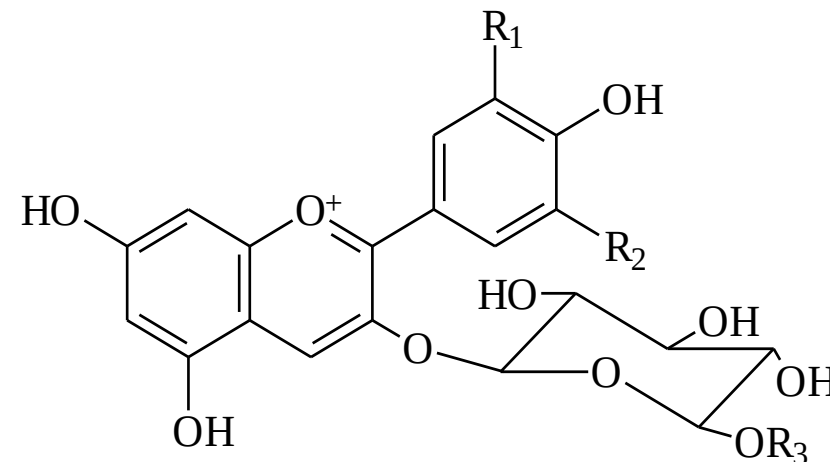
0-3 min 10% ACN

4-10 min 20 % ACN

10-15 min 25% ACN

15-31 min 90% ACN

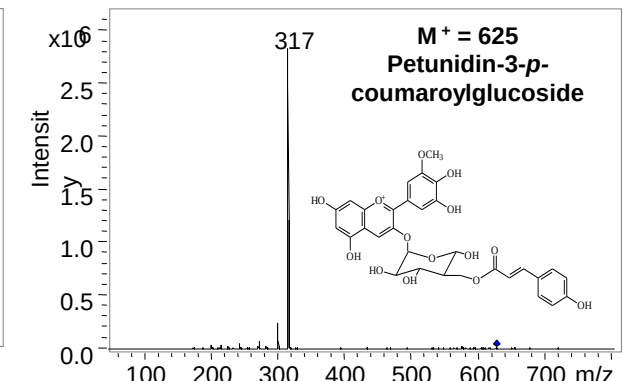
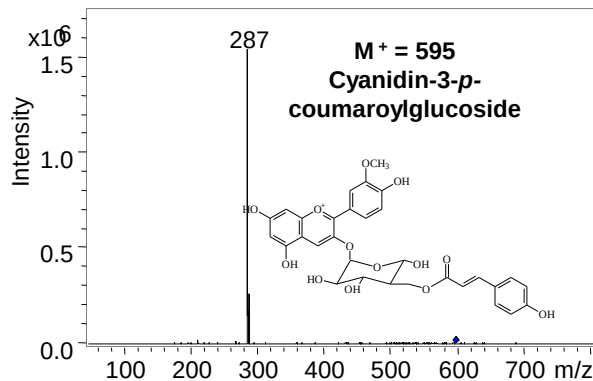
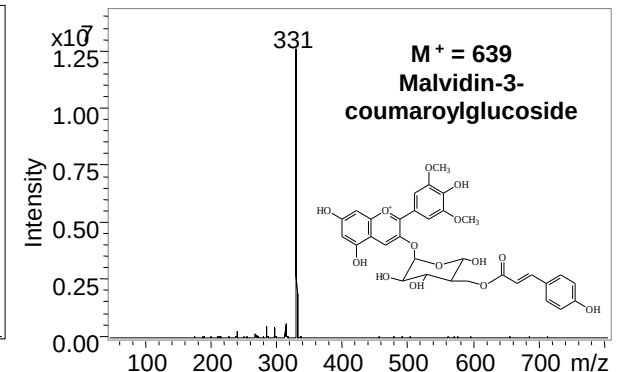
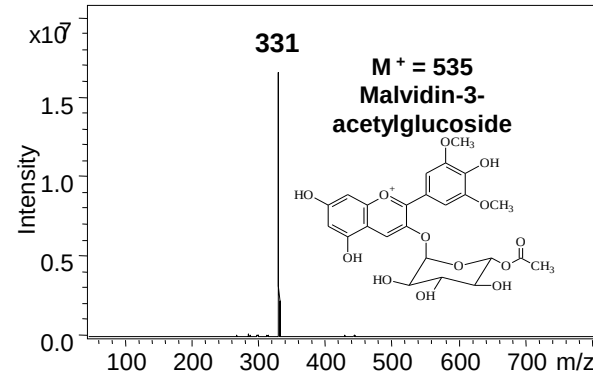
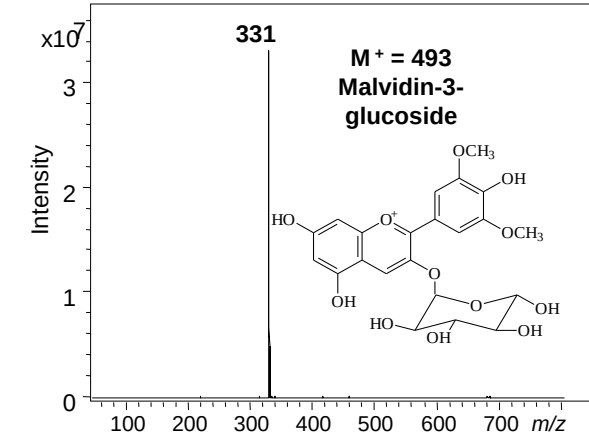
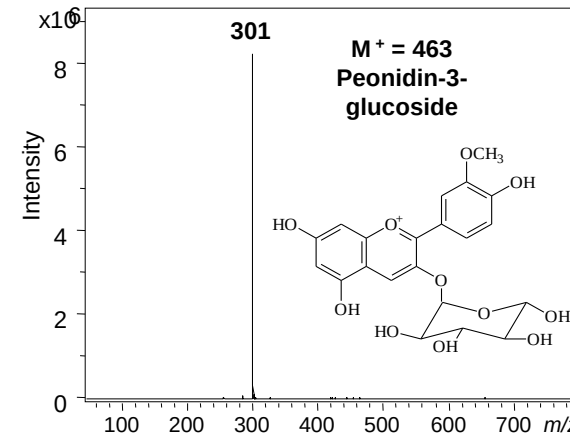
32-50 min 10% ACN



Anthocyanins	R ₁	R ₂
Dp-3-glc	OH	OH
Cy-3-glc	OH	H
Pt-3-glc	OCH ₃	OH
Pn-3-glc	OCH ₃	H
My-3-glc	OCH ₃	OCH ₃

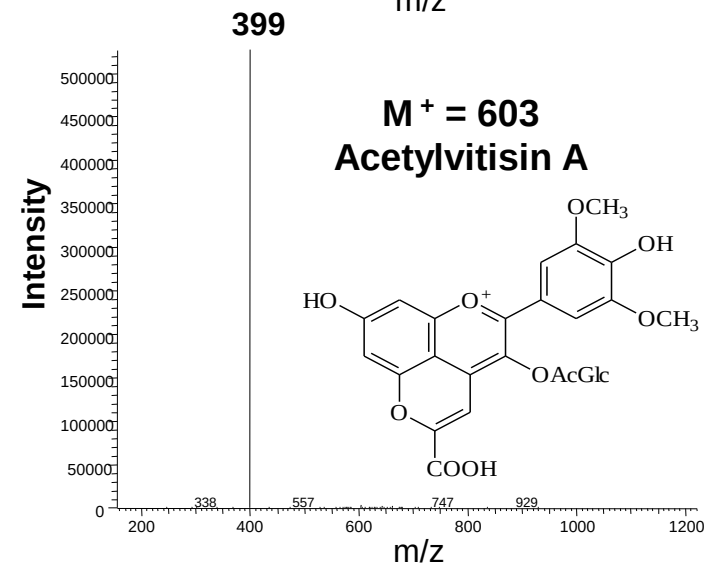
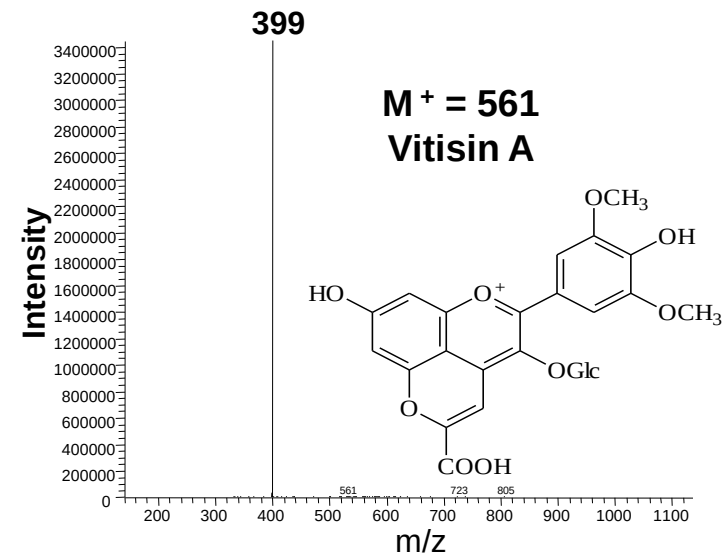
MS identification of anthocyanins

Anthocyanins	t _r (min)	MS (m/z) M ⁺	MS/MS (m/z)
Monoglucosides			
Dp-3-glc	5.1	465	303
Cy-3-glc	6.0	449	287
Pt-3-glc	6.9	479	317
Pn-3-glc	7.4	463	301
My-3-glc	8.3	493	331
Acetylglucosides			
Dp-3-acetylglc	11.4	507	303
Pt-3-acetylglc	13.6	521	317
Pn-3-acetylglc	16.2	505	301
Mv-3-acetylglc	18.1	535	331
p-Coumaroylglucosides			
Dp-3-p-coumglc	13.6	611	303
Cy-3-p-coumglc	17.3	595	287
Pt-3-p-coumglc	20.3	625	317



MS identification of pyranoanthocyanins

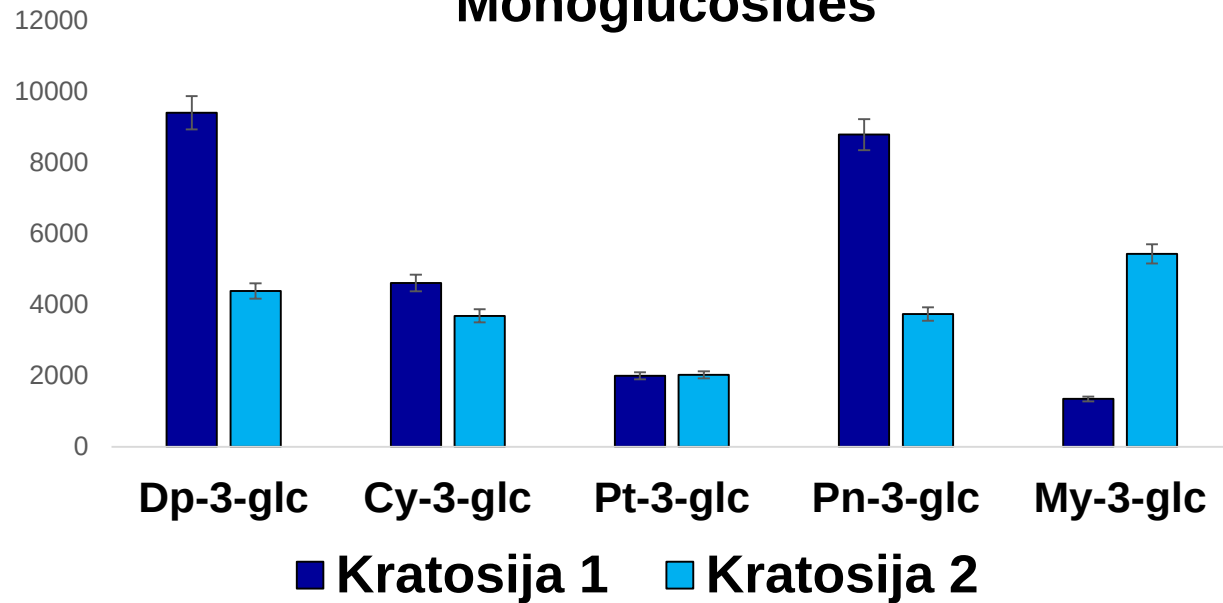
Pyranoanthocyanins	t _r (min)	MS (m/z) M ⁺	MS/MS (m/z)
Vitisin-A	15.2	561	399
Ac-vitisin-A	17.7	603	399
p-Cm--vitisin-A	19.1	707	399
Vitisin-B	16.2	517	355
Ac-vitisin-B	20.2	559	355
p-Cm-vitisin-B	21.7	663	355
10-carboxy-pyranoPn-3-glc		531	369
10-catechin-pyranoMv-3-glc		805	643, 491
10-epicatechin-pyranoMv-3-glc		805	643,491
10-DHP-pymv-3-glc-(pinotin A)	28.0	625	463
10-DHP-pymv-3-acglc	30.4	667	463
10-DHP-pymv-3-cmglc	N.D.	N.D.	N.D.
10-MHP-pymv-3-glc	31.8	609	447
10-MHP-pymv-3-acglc	32.4	651	447
10-MHP-pymv-3-cmglc	35.2	755	447



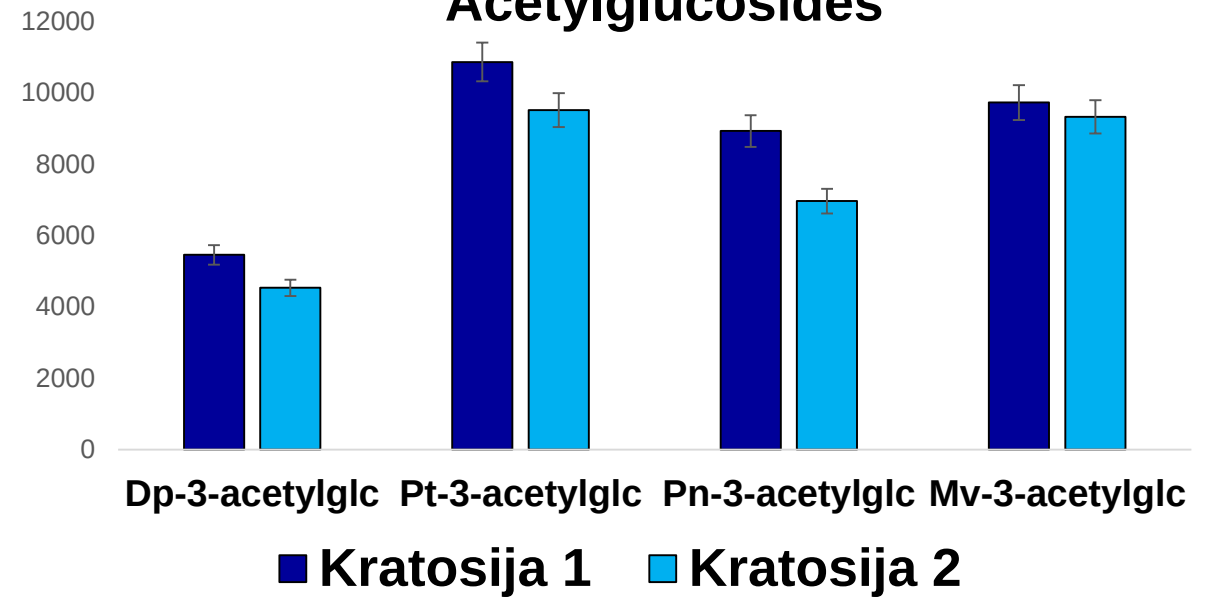
Semi-quantification of anthocyanins



Monoglucosides

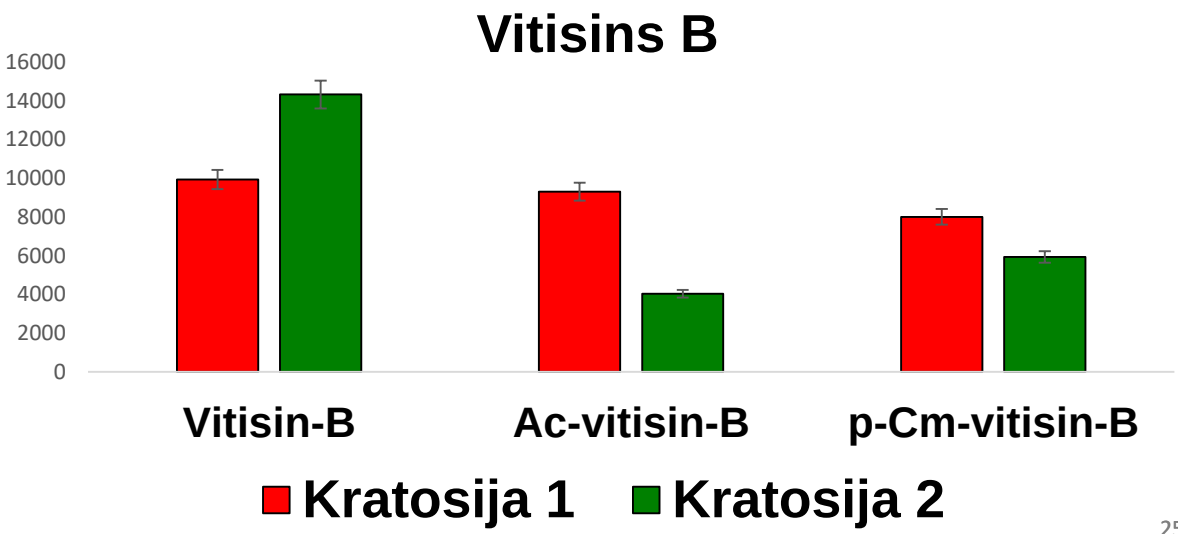
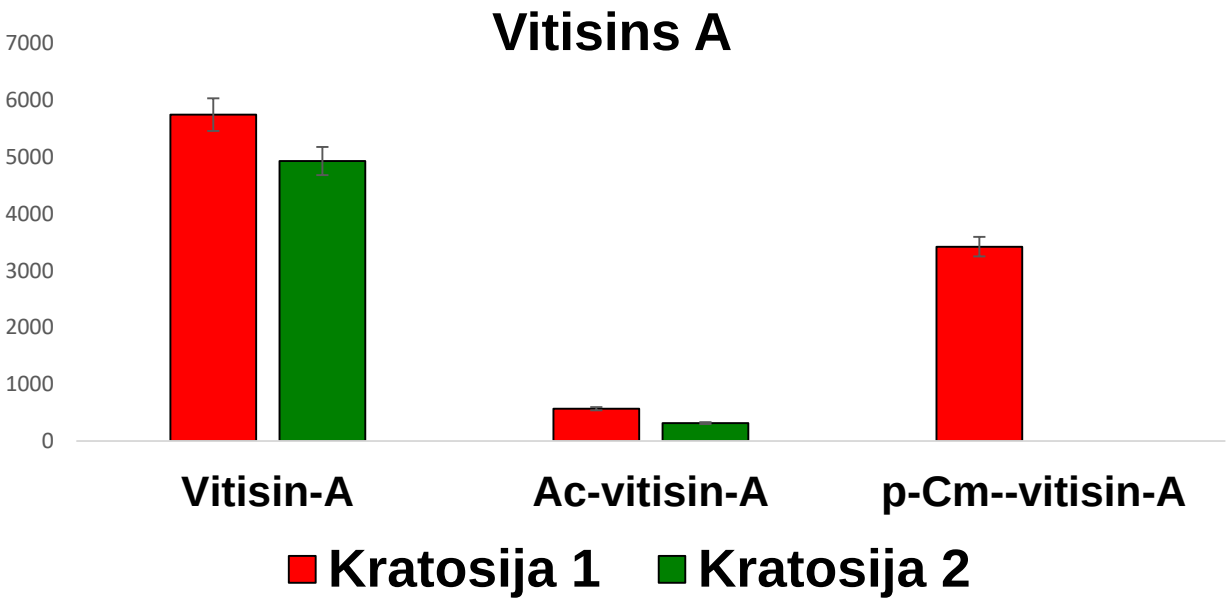


Acetylglucosides





Semi-quantification of pyranoanthocyanins



CONCLUSION



1.

- ✓ HPLC coupled to tandem mass spectroscopy (MS/MS) operated in electrospray ionization (ESI) and quadrupole linear ion trap (Q TRAP) negative/positive mode is sensitive technique for determination of non-flavonoids and flavonoids, as well as small amounts of compounds.

2.

- ✓ **Complex polyphenolic profile of Kratošija wine**
- ✓ **Gallic acid was the dominant phenolic acid, followed by caffeic acid.**
- ✓ Flavan-3-ols procyanidins **B1** and **B3** dominated in wines.
- ✓ **Anthocyanins monoglucosides** dominated in wine.

3.

- **Flavones chrysin and luteolin, flavanone naringenin, flavanone taxifolin and stilbenoid viniferin were reported for the first time in Macedonian red wine.**
- **Influence of yeast was noticed. Wine fermented with Xpure yeast (Kratošija 1) presented higher content of almost all groups of compounds, except for flavan-ols which were higher in wine fermented with D80 yeast (Kratošija 2).**



ACKNOWLEDGEMENT



CEEPUS Network
TEACHING AND LEARNING BIOANALYSIS

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Thank you for your kind attention

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