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Determination of organic acids in wines using capillary zone electrophoresis-electrospray ionization /qudrupole-time-of-flight-mass spectrometry (CZE-ESI/QTOF-MS)

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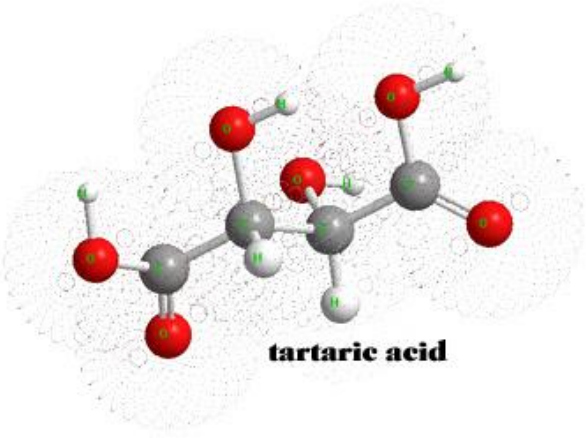
INTRODUCTION

Wine components

- Water
- (Ethyl) alcohol
- Organic acids

  - ✓ tartaric acid
  - ✓ malic acid
  - ✓ succinic acid
  - ✓ lactic acid
  - ✓ citric acid
  - ✓ acetic acid



INTRODUCTION

Organic acids

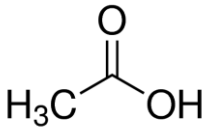
- ✓ Significantly influence the quality of wine
- ✓ The sensory perception, such as flavor, aroma and colour
- ✓ Have effect on the pH
- ✓ Effect on chemical and microbiological stability of wines

➢ Monitoring during the whole vinification process: starting from the grapes juices, continuing to the alcoholic fermentation and wine stabilization processes.

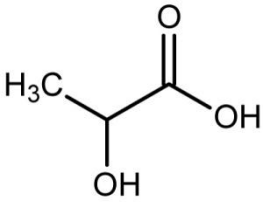
Organic acids in wine

Monoprotic acids:

- Acetic acid

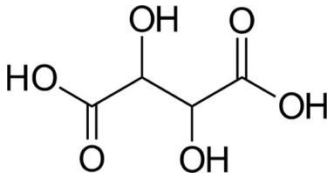


- Lactic acid

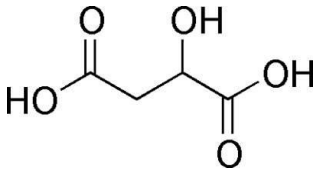


Diprotic acids:

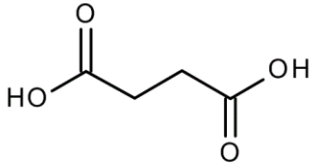
- Tartaric acid



- Malic acid

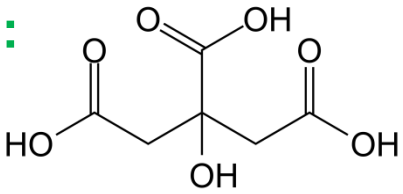


- Succinic acid



Triprotic acids:

- Citric acid



❖ ***In grape juices, tartaric, malic and citric acids are the main organic acids.***

❖ ***Acetic, Lactic and Succinic acids are products of fermentation.***

Organic acids in wine

The content of acids in:

- **grapes: 8-13 g/L**
- **wines: 5.5 to 8.5 g/L**

Principal organic acids are tartaric acid and malic acid.

- Tartaric acid (most abundant) - Stereochemistry was elucidated by Louis Pasteur in 1849.
- Stable to microbial fermentation but forms insoluble salts with potassium (K2Tar found on the bottom of the cork or bottle in aged wines, KHTar is cream of tartar).
- Total acidity is usually expressed as tartaric acid equivalents.
- The content of tartaric acid decreases during the fermentation as a result of precipitation in a form of tartaric crystals.

- Malic acid (second abundant) can be metabolized by yeast and bacteria.
- During the malolactic fermentation, the content of malic acid decreases due to the conversion to lactic acid, resulting an increasing content of that.
- Citric acid influences the acidity of wines.
- Shikimic acid - present in a concentration range of 10-150 mg/L in the wines.

Organic Acid Measurement

- **Measured by titrating with a base of known concentration (NaOH) in the presence of a chemical indicator with a known pH end point.**
- **This measurement called titratable acidity (TA)**
- Concentrations range from 8.0 g/L to 5.5 g/L
- pH ranges from 2.8 to 4.0.
  - **White wine 3.0-3.3; Red wine 3.2-3.4**

ANALYTICAL TECHNIQUES FOR DETERMINATION OF ORGANIC ACIDS

- ✓ **Chromatographic techniques – HPLC**, **GC –** Sample preparation necessary!!
- ✓ **Capillary electrophoresis coupled to UV detection -** fast analyses and efficient resolution of the analytes.
- ✓ **Capillary electrophoresis directly coupled to a mass spectrometer (CE-MS) -** higher separation sensitivity, selective mass detection in a single run analysis
- ✓ **Capillary electrophoresis coupled to electrospray ionization mass spectrometer (CE-ESI-MS)**
- ✓ **Capillary electrophoresis coupled to an accurate-mass quadrupole time-of-flight mass spectrometer (QTOF-MS) -** increased sensitivity, provides a high mass accuracy and resolution at high acquisition rates.

No publications where CZE-ESI/QTOF-MS was used for analysis of organic acids in wine samples.

The Advantages of CE

- The number of theoretical plates is typically in the hundreds of thousands.
- There is no mass transfer between mobile and stationary phases as with HPLC and GC, therefore the analytes remain in a “plug” instead of spreading as a result of laminar flow. (Peaks can still broaden however.)
- Altering column conditions allows focusing or concentration of samples.

Republic of Macedonia - long tradition for wine production.

- **wine is the first and most important exported product** in the class of alcoholic beverages and the second most important agro-product after the tobacco

- Increased production and export of wine
  in 2008, **70.3** million liters exported - **39** million euros
  in 2013, **88.5** million liters exported - **50** million euros

The aims of the work:

(1) To optimize and validate capillary CZE-ESI/QTOF-MS method for the determination of organic acids in red wines,

(2) To apply the method on Vranec wines analysis from different regions.

EXPERIMENTAL PART

Wine samples: Vranec wines from different wine regions produced in experimental winery

Vranec grapes:100 kg, traditional winemaking

Vranec wines	Locality	Wine region
V1	Bistrenci	Tikveš
V2	Barovo	Tikveš
V3	Demir Kapija	Tikveš
V4	Disan	Tikveš
V5	Drenovo	Tikveš
V6	Gradsko	Tikveš
V7	Krivolak	Tikveš
V8	Kurija	Tikveš
V9	Lepovo	Tikveš
V10	Manastirec	Tikveš
V11	Veles	Tikveš
V12	Vilarov	Tikveš
V13	Ridiste	Tikveš
V14	Štip	Tikveš
V15	Bitola	Bitola
V16	Gevgelija	Gevgelija-Valandovo
V17	Radoviš	Strumica-Radoviš

Sample preparation: Wine samples were diluted with deionized water (ratio 1:5), filtered with a 0.22 μm membrane filter (PVDF syringe filter, Nantong FilterBio Membrane Col, Ltd, China) and injected into the capillary electrophoresis system.

CE-ESI/QTOF-MS instrumentation

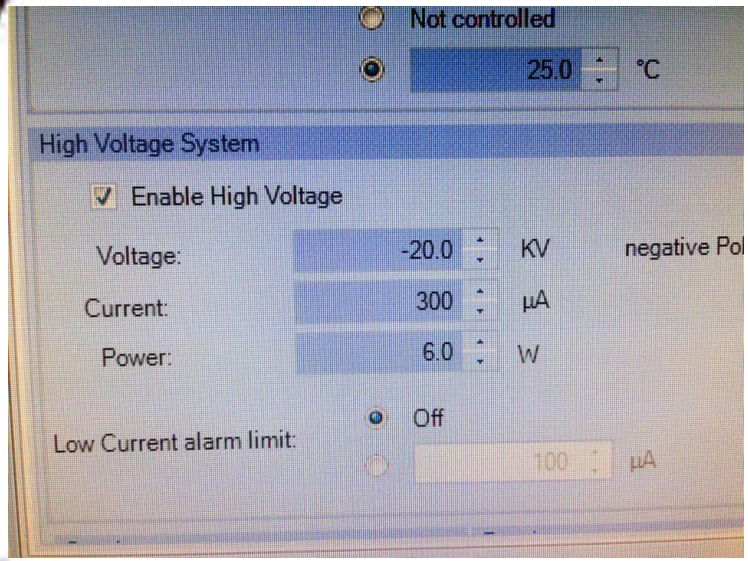
- **7100 Capillary Electrophoresis (CE) system (Agilent Technologies, Waldbronn, Germany).**
- Detection: 6530 Accurate-Mass Quadrupole Time-of-flight Mass Spectrometer (**QTOF-MS**) coupled to the CE instrument.
- Separation – **Capilary**: 80 cm x 50 µm internal diameter, fused-silica capillary (Polymicro Technologies, Phoenix, USA).
- 1% (*v/v*) solution of formic acid, sheath liquid

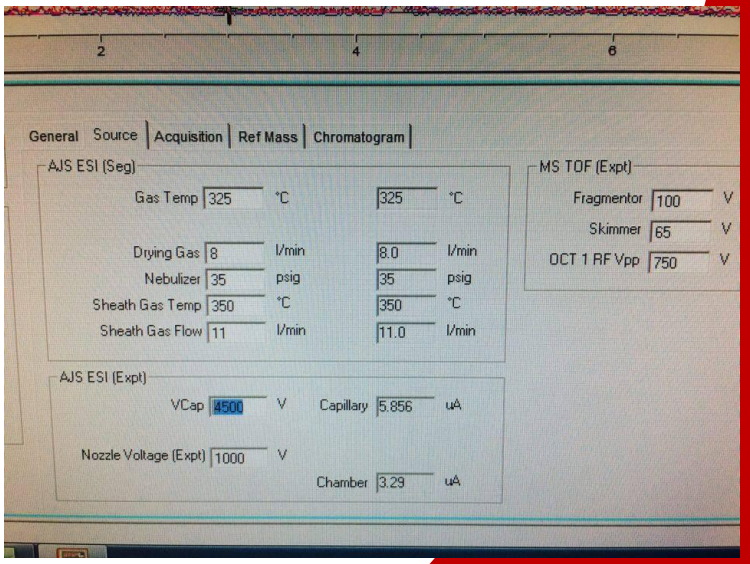
CE-ESI/QTOF-MS instrumentation

- ESI/QTOF-MS operated in **negative** ionization mode

-The data processing was performed on ChemStation B. 04.03. version and MassHunter B. 04 version software.

Working conditions:





Capillary preconditioning

- 1 % hexadimethrine bromide (polybrene, PB) for coating the capillary inner surface
- 50 mM ammonium acetate buffer, at pH 6 - as background electrolyte
- new capillary flushed with: aceton (2 min), water (2 min), 1 M NaOH (20 min), water (5 min), PB coating solution (15 min) and BGE (5 min).
- short preconditioning: pressure flush of PB solution (2 min), water (2 min) and BGE (4 min).

Validation parameters

Calibration curves:

- Six concentration levels: **0.025, 0.05, 0.1, 0.25, 0.5** and **0.8** g/L for each organic acid (lactic, succinic, malic, tartaric, shikimic and citric).

Linearity

Limit of quantification (LOQ)

Recovery

Repeatability and reproducibility

RESULTS AND DISCUSSION

The effect of buffer on compounds separation

- A volatile buffer system is necessary to be used.
- Two buffers tested: **ammonium acetate** and **ammonium formate** - founding that ammonium acetate presented better effect on separation instead of ammonium formate

- **Ammonium acetate** tested at different concentrations: ***10, 20, 25, 50 and 75 mM***

50 mM buffer solution, pH 6

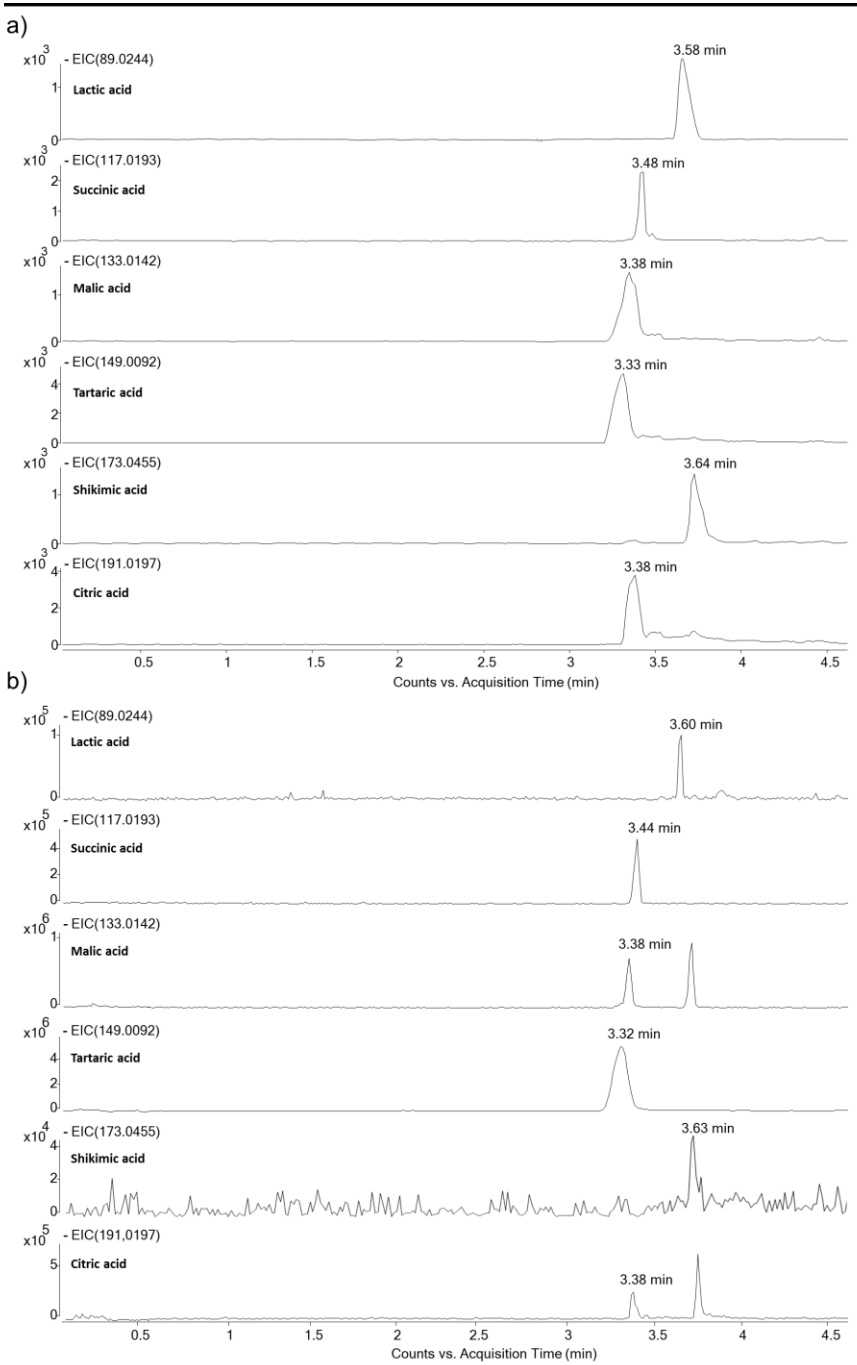
RESULTS AND DISCUSSION

The effect of capillary length

- Two capillary lengths tested: **80 cm** (5 min rung time) and **120 cm** (14 min run tme).

80 cm long *capillary*

- In the total ion electropherogram, no separation was achieved with both columns.
- Baseline separation of the compounds was not necessary – QTOF-MS
- **EIEs used for quantification**
- 1 % (*m/v*, in water) solution of hexadimethrine bromide (polybrene, PB) for coating the capillary inner surface
- 1% (*v/v*) solution of formic acid, sheath liquid



Extracted ion electropherograms of organic acids in:
(a) standard solution
(b) Vranec wine, V13

RESULTS AND DISCUSSION

The effect of separation voltage

- The separation voltages of **-25 kV** and **-20 kV** were tested.
  Lower separation voltage (-20 kV) chosen for the analyses

Final CE conditions:

- CE capillary: **80 cm long x 50 µm internal diameter** coated with a solution of **polybrene (1 %, *m/v*)**.
- Background electrolyte: **50 mM ammonium acetate, pH 6**
- Applied voltage: **-20 kV.**

VALIDATION PARAMETERS

Table 2
Linearity data

Organic acid	Migration time (min)	MS (*m/z*) $[M-H]^-$	Concentration range (mg/L)	Intercept	Slope	R^2	LOQ (mg/L)
Lactic	3.5	89	7-150	425	28790	0.9918	7.17
Succinic	3.3	117	4-70	2673	140997	0.9935	4.68
Malic	3.2	133	0.004-200	2143	118261	0.9905	0.05
Tartaric	3.1	149	5-800	-230	115674	0.9990	5.70
Shikimic	3.6	173	0.5-60	1328	171801	0.9902	0.59
Citric	3.3	191	20-650	-146	58946	0.9982	20.5

Table 3
Standard additions

Organic acid	Calculated (g/L)*	Experimentally found (g/L)*	SD	Recovery (%)
Lactic	1.25	0.75	0.14	104
Succinic	1.33	0.83	0.20	111
Malic	0.79	0.29	0.05	91.2
Tartaric	3.83	3.33	0.55	101
Shikimic	0.53	31.2	2.41	104
Citric	0.78	0.28	0.02	103

Table 4
Repeatability and reproducibility

Organic acid	Repeatability (5 replicates x 1 day)		Reproducibility (3 replicates x 3 injections x 3 days)	
	Mean concentration (g/L)*	*RSD* (%)	Mean concentration (g/L)*	*RSD* (%)
Lactic	0.35	16.9	0.33	15.8
Succinic	0.54	11.2	0.52	16.8
Malic	1.05	3.44	1.05	1.75
Tartaric	4.69	4.20	4.70	5.90
Shikimic	0.054	8.23	0.053	7.74
Citric	0.33	9.45	0.31	8.29

RESULTS AND DISCUSSION

Application of the method on organic acids determination in Vranec wines from different regions

The quantitative determination of the organic acids was made by the extracted ion electropherograms for each organic acid. The calculated *m/z* values of the quasi-molecular $[M–H]^-$ ions:

***m/z* 89.0244 for lactic acid,**
***m/z* 117.0193 for succinic acid,**
***m/z* 133.0142 for malic acid,**
***m/z* 149.0092 for tartaric acid,**
***m/z* 173.0455 for shikimic acid and**
***m/z* 191.0197 for citric acid**

RESULTS AND DISCUSSION

Results for organic acids in Vranec wines

Wines	Tartaric (g/L)	Malic (g/L)	Lactic (g/L)	Citric (g/L)	Succinic (g/L)	Shikimic (mg/L)	Total acids (g/L)
V1	3.33±0.55a	0.29±0.05	0.75±0.14	0.28±0.02a	0.83±0.20a	31.2±2.41	5.51±0.65a
V2	2.51±0.44b	0.06±0.003	1.46±0.23a	0.71±0.034b	1.10±0.42b	22.8±1.65a	5.87±0.46a
V3	4.26±0.96	1.52±0.23b	0.60±0.16	0.29±0.003a	0.67±0.11b,c	4.15±0.76c	7.34±0.37
V4	2.95±0.60c	1.11±0.20c	0.40±0.10b	0.81±0.05e	0.62±0.05c	15.2±0.93e	5.91±0.32a
V5	3.28±0.44a	1.81±0.07	0.34±0.08b	0.40±0.003d	0.63±0.04c	7.44±0.88	6.46±0.25d
V6	2.80±0.27c	2.05±0.37	0.24±0.08a	0.64±0.05	0.78±0.05a	58.4±8.45b	6.57±1.55
V7	2.09±0.45	0.61±0.07	0.11±0.04a	0.51±0.04c	0.21±0.04d	5.73±0.15d	3.53±0.13e
V8	3.92±0.58	1.66±0.07b	0.35±0.11b	0.26±0.01a	0.71±0.07a	15.9±1.13e	6.93±0.33
V9	3.66±0.41	1.44±0.08b	0.43±0.12b	0.52±0.02c	0.62±0.05c	6.05±.0.85d	6.67±0.26d
V10	4.96±0.82d	0.85±0.05d	0.36±0.05b	0.42±0.02d	0.73±0.07a	<LOQ	7.32±0.20c
V11	2.29±0.11b	0.83±0.11d	0.21±0.05a	0.44±0.03d	0.17±0.006d	<LOQ	3.92±0.06e
V12	3.72±0.36	2.69±0.14a	0.20±0.08a	0.36±0.01	0.56±0.07c	21.2±3.04a	7.54±0.62c
V13	4.87±0.75d	1.04±0.14c	0.34±0.02b	0.32±0.01a	0.50±0.07c	55.9±4.52b	7.12±0.92b
V14	2.61±0.22b	4.03±0.62	0.37±0.09b	0.76±0.04b	0.73±0.08a	3.98±0.32c	8.50±0.23
V15	3.14±0.52a	2.45±0.55a	0.19±0.08a	0.29±0.01a	1.00±0.06b	41.7±4.67	7.11±0.98b
V16	2.91±0.57c	1.40±0.16b	0.22±0.06a	0.89±0.06e	0.43±0.08	<LOQ	5.84±0.19a
V17	3.95±0.65	2.37±0.66	0.39±0.11b	0.55±0.04c	1.19±0.24b	13.7±1.84	8.47±0.59

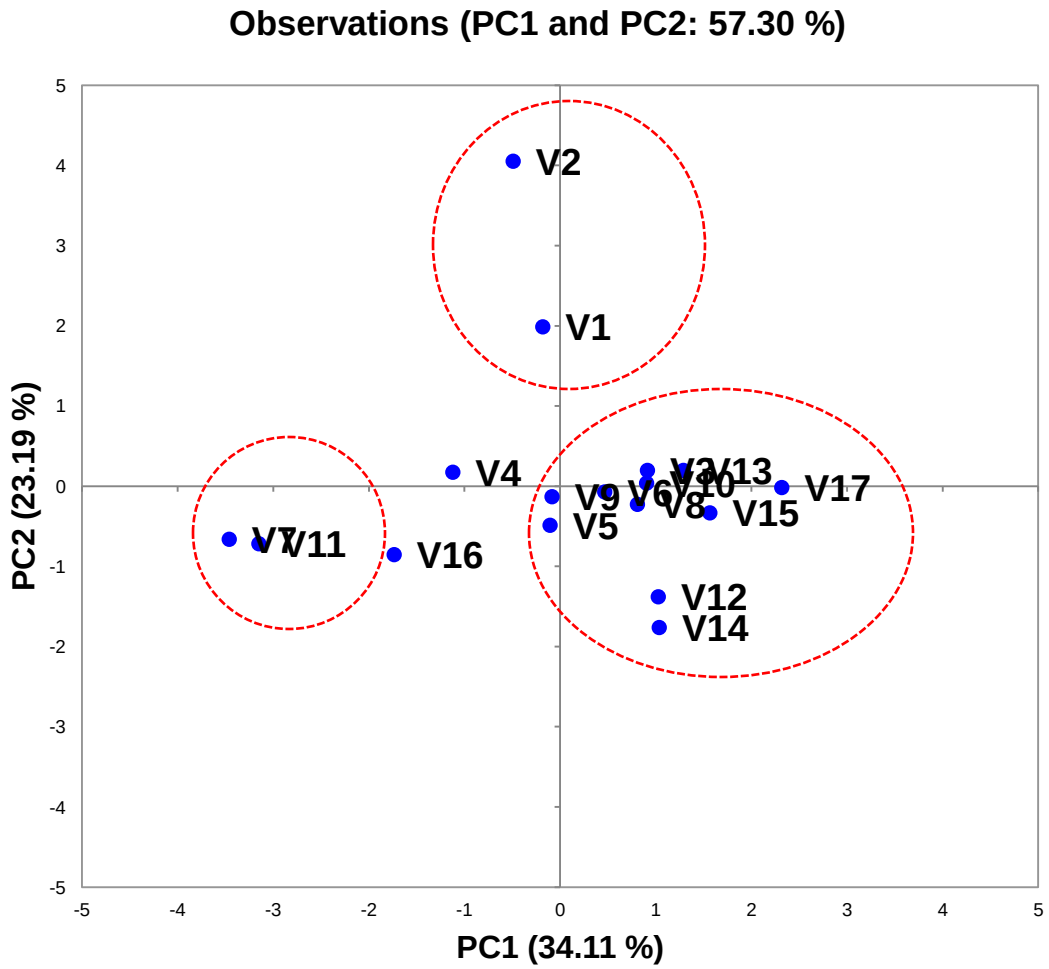
RESULTS AND DISCUSSION

Results for organic acids expressed in mM

Wines	Tartaric (mM)	Malic (mM)	Lactic (mM)	Citric (mM)	Succinic (mM)	Shikimic (mM)	Total acids (mM)
V1	37.4±6.18	2.48±0.43	5.64±1.05	1.88±0.13	4.80±1.16	0.16±0.01	52.37±8.96
V2	28.2±4.94	0.51±0.03	10.9±1.73	4.77±0.23	6.36±2.43	0.12±0.01	50.94±9.36
V3	47.8±10.8	12.9±1.97	4.51±1.20	1.95±0.02	3.87±0.64	0.02±0.00	71.21±16.6
V4	33.1±6.74	9.49±1.71	3.01±0.75	5.44±0.34	3.58±0.29	0.08±0.00	54.74±9.83
V5	36.8±4.94	15.5±0.60	2.56±0.60	2.68±0.02	3.64±0.23	0.04±0.00	61.25±6.40
V6	31.5±3.03	17.5±3.16	1.80±0.60	4.30±0.34	4.51±0.29	0.31±0.04	59.90±7.47
V7	23.5±5.06	5.21±0.60	0.83±0.30	3.42±0.27	1.21±0.23	0.03±0.00	34.19±6.46
V8	44.0±6.52	14.2±0.60	2.63±0.83	1.74±0.07	4.10±0.40	0.08±0.01	66.80±8.42
V9	41.1±4.61	12.3±0.68	3.23±0.90	3.49±0.13	3.58±0.29	0.03±4.45	63.77±11.1
V10	55.7±9.21	7.26±0.43	2.71±0.38	2.82±0.13	4.22±0.40	<LOQ	72.74±10.6
V11	25.7±1.24	7.09±0.94	1.58±0.38	2.95±0.20	0.98±0.03	<LOQ	38.34±2.79
V12	41.8±4.04	22.9±1.20	1.50±0.60	2.42±0.07	3.24±0.40	0.11±0.02	72.06±6.33
V13	54.7±8.43	8.89±1.20	2.56±0.15	2.15±0.07	2.89±0.40	0.29±0.02	71.49±10.3
V14	29.3±2.47	34.4±5.30	2.78±0.68	5.10±0.27	4.22±0.46	0.02±0.00	75.89±9.18
V15	35.3±5.84	20.9±4.70	1.43±0.60	1.95±0.07	5.78±0.35	0.22±0.02	65.59±11.6
V16	32.7±6.40	11.9±1.37	1.65±0.45	5.97±0.40	2.49±0.46	<LOQ	54.78±9.09
V17	44.4±7.30	20.3±5.64	2.93±0.83	3.69±0.27	6.88±1.39	0.07±0.01	78.21±15.4

RESULTS AND DISCUSSION

Principal component analysis (PCA)



CONCLUSIONS

- **Fast** and **simple** CZE-ESI/QTOF-MS for analysis of lactic, succinic, malic, tartaric, shikimic and citric in red wines
- The method was **optimized** and **validated** (determined: linearity, limit of quantification (LOQ), recovery, inter- and intra- day repeatability and reproducibility).
- **Applied on Vranec wines analysis, from various wine regions:**
  - **wide variation of organic acids content,**
  - **relatively high concentration of tartaric acid, typical for this variety.**

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Research Fund of the University “Goce Delčev” – Štip, R. Macedonia for the project titled “*Polyphenolic and aroma profile of Vranec wines fermented with isolated yeasts from Tikveš wine area*”

OHRID LAKE

THANK YOU FOR YOUR ATTENTION