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Research Article

Qualitative And Quantitative Analysis Of Organic Acids In Georgian Wine Lees By LC-MS/MS Method

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Abstract: In the XXI century, the interest and demand for natural compounds have increased significantly, compared to the previous period, especially for those natural compounds that have antioxidant, antimicrobial, and anti-inflammatory properties. Accordingly, organic acids are noteworthy in this direction since studies have confirmed some organic acids' antioxidant, antimicrobial, and anti-inflammatory effects. It is known that wine from vine products is one of the rich sources of organic acids. Therefore, the waste products of wine production - including wine lees are interesting from this point of view. Winemaking is one of the most successful directions of agriculture in Georgia. Many waste products, such as grape pomace, grape seed, and wine lees, accompany wine production. It was the reason to use the waste product - wine lees- to study organic acids. Hence, the research aims to assess the potential of using a currently wasted winery product - Georgian wine lees as a source of biologically active ingredients. The objective of the current study was to identify the biologically active substances (organic acids) in the waste product of winemaking - wine lees obtained from widely distributed grape varieties in Georgia and to determine their quantitative content. By the LC-MS/MS method, we identified organic acids in 6 samples of wine lees made from different varieties of Georgian grapes (Saperavi, Kisi, Rkatsiteli) and with different technologies (traditional Kakhetian, factory conditions) and determined three main organic acids (tartaric acid, lactic acid, and citric acid) quantitatively. The results of the current research showed that the grape variety and the winemaking technology affect the qualitative and quantitative content of organic acids in the wine; in particular, three organic acids were found in the Saferavi wine made by the factory method, and 2 organic acids in the wine made by the traditional Kakhetian (Georgia) method. Organic acid's quantitative content is also different. Also, the content of organic acids in the wine obtained from different varieties of grapes is different. The research showed that the quantitative content of organic acids in Kisi wine Lees is higher than the other studied varieties. However, the content of organic acids in all three types of wine is significant. The study results support the potential of using a currently wasted product - Georgian Wine Lees - as a source of biologically active ingredients.

Keywords: Organic acids, Waste products utilization, Wine lees, Natural products, LC-MS/MS

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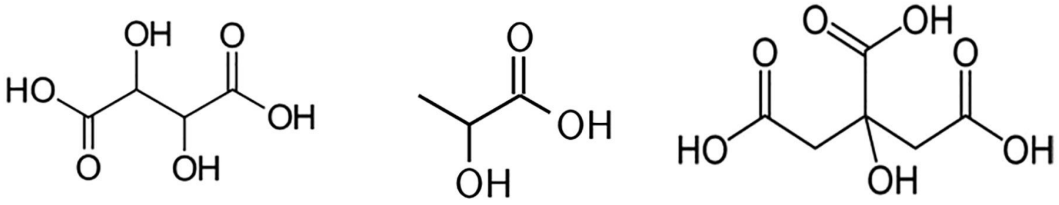
1. INTRODUCTION

The protection of natural resources has received significant attention in recent years, and the development of technological-organizational systems for resource-saving and secondary use of plant raw materials is especially pertinent. The resolution of the European Parliament (# 52011DC0021, A resource-efficient Europe - Flagship initiative under the Europe 2020 Strategy) is relevant to the issues mentioned above; it clearly states the need for resource protection and suggests several steps to ensure this goal is achieved[1]. The demand for natural goods with plant origins is rising worldwide, even though it is well known that they have been used in medicine for a long time[2]. Consequently, it is a significant and innovative issue to disclose the waste product as a novel, natural resource that can be a source of biologically active compounds. Organic acids have long been utilized in the food industry and are essential for maintaining the nutritional value and quality of food[3]. Except for ascorbic acid, which has significant antioxidant activity, there is little evidence of the bioactive potential of organic acids and even less of their positive impacts on human health[4]. It should be emphasized that organic acids are gaining popularity, and more and more research is being done to determine their beneficial impacts on human health. Some organic acids, such as succinic acid, lactic acid, acetic acid, malic acid, citric acid, glutamic acid, and their salts, are known to aid in the absorption of iron[5]. In addition, research shows that taking citric acid orally improves ketosis[6] and that taking a weak organic acid improves insulin resistance in diabetes mellitus by changing the pH of the intercellular fluid[7]. Furthermore, according to the research, citric and malic acids have a strong protective impact on myocardial ischemia and affect ischemic lesions in patients where these substances were added to their diets[8]. Organic acids are naturally found in various fruits, vegetables, and beverages such as tea and wine[9-16]. Winemaking is one of the most successful and ancient directions of agriculture in Georgia; material and historical evidence and facts discovered by archeologists confirm the facts of the first wine production in Georgia 8000 years ago. Approximately 500 different varieties of grapes are currently grown in Georgia[17,18]. Many waste products, such as wine lees, grape seed, and grape pomace, characterize winemaking. These waste products, especially wine lees, have not been studied for curative-preventive purposes in Georgia today. Certain studies are conducting on the analysis and application of waste products from the cultivation of vines in various fields at The Tbilisi State Medical University[19]. Among them is the study of biologically active substances of wine lees, which will be used in the pharmaceutical and food industries. Three distinct organic acids tartaric acid, lactic acid, and citric acid—were discovered during the investigation; their chemical structures are shown in Figure 1. The goal of the study is to evaluate the potential of using Georgian wine lees, a product that is currently a wasted product, as a source of biologically active components. This research aimed to determine the biologically active compounds (organic acids) in wine lees, a waste product of winemaking made from widely grown grape varieties in Georgia, and to quantify the amount of each of them.



Tartaric acid Lactic acid Citric acid

Fig. 1: Chemical structures of the organic acids found in Wine lees

Figure 1 shows the chemical structure of three organic acids (Tartaric acid, Lactic acid, and Citric acid) that we identified during the current research in wine lees obtained from different Georgian grape varieties. According to the researchers conducted in different countries 29[,30,41], the mentioned organic acids have also been identified in the wine lees obtained from other grape varieties.

2. MATERIALS AND METHODS

2.1. Preparation of wine lees samples

The lees of Saferavi, Rkatsiteli, and Kisi wine (made by the traditional Kakhuri method) was collected in the village of Kvemo Alvani, and the lees of Saferavi and Rkatsiteli wine made in factory conditions was collected in the village of Shroma, Georgia in September and October 2022. Traditional Kakhetian Winemaking technology generally means fermenting, vinifying, and aging a certain amount of grape juice with chacha (husks of grapes/grape skin and pips) in a tank. The first and foremost rule of Kakhetian Winemaking is to delay the wine in the tank on its chacha both during and after the alcoholic fermentation. And, during wine production in factory conditions, alcoholic fermentation takes place without the cool parts of grapes (husks of grapes/grape skin and pips). The grape juice is through in a sieve and transferred to the dish to be served. Various chemical additives and devices are used in factory conditions to give wine stability and transparency. We used a sieve to clean the research raw material from large particles. Then, to remove the liquid (wine), we centrifuged the wine lees for 15 minutes at 5000 rpm. The received thick mass extraction was conducted with distilled water with ultrasonic bath assist, with the following proportion of wine lees and distilled water- 1:10. About 1 g of the sample was added to 10 ml of distilled water and placed in an ultrasonic bath for 10 min. Then this sample was centrifuged for 5 minutes, and at 5000 rpm, the supernatant was removed and saved. The extraction was repeated 5 times. We combined the supernatants obtained during the extraction. The combined extracts were filtered through PTFE filters (25 mm × 0.45 μm) before LC-MS/MS analysis. The sample mentioned above preparation procedure was repeated for all types of wine lees sample

preparation. A total of 5 analytical samples were prepared and analyzed [38].

2.2. *Chemicals, standards, and internal standards*

All chemicals for mobile phase solutions (water, formic acid, and acetonitrile from Sigma Aldrich) used in this analysis were HPLC grade. The following organic acid standards were used in the study: tartaric acid (>98.0%) (Sigma Aldrich), citric acid (>98.0%) (Sigma Aldrich), and lactic acid (>98.0%) (Sigma Aldrich).

2.3. *Qualitative and Quantitative analysis of organic acids by LC-MS/MS*

Determining organic acids in the extracts obtained from the wine lees was conducted by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS). The method of lupin and chickpea with LC-MS/MS, Flores et al. (2012), and Ehling and Cole (2011) were used to determine the organic acids in wine lees[36-37].

2.3.1. *HPLC operating conditions*

The instrumentation used for identifying and quantifying organic acids in wine samples was an Agilent Technologies 6460 triple quad LC/MS Agilent Technologies 1290 infinity. The column was maintained at 30°C during the analysis. A binary gradient was used, with a mobile phase of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B). The gradient used was as follows: 0–1.5 min, 5%; B; 1.5–8.0 min, 25% B; 8–10 min, 35%; 10–13 min, 80%; 13–16 min, 50%, and 16–20 min, 5%; B. The flow rate was 0.7 mL/min.

2.3.2. *Mass spectrometry operating conditions*

The conditions were the following: gas temperature 300°C, gas flow 7 L/min, nebulizer pressure 635 kPa(kilopascal), sheath gas temperature 300°C, sheath gas flow 6.5 L/min, capillary voltage 4000 V, nozzle voltage 500 V and the scanning type was multiple reaction monitoring (MRM). Two MRM transitions for each acid were monitored in negative mode. One was used as a quantifier, and the other as a qualifier (Table.1).

Table1. LC-MS/MS MRM conditions

Compound Name	Precursor Ion (m/z)	Product Ion(m/z)	Dwell (msec)	Collision energy (V)	Retention Time (min)	Polarity
Citric acid	191	87	200	18	2.77	Negative
Citric acid†	191	111	200	10	2.77	Negative
Tartaric acid	149	73	200	8	2.094	Negative
Tartaric acid†	149	87	200	4	2.094	Negative
Lactic acid†	89	43	200	8	2.27	Negative

†Transitions used as quantifiers; m/z - mass-to-charge ratio, msec – millisecond, min-minute, V –voltage

Table 1 shows the chromatography conditions used to identify and quantify the target organic acids. The table shows the precursor ions used in the analysis. It also indicates which transactions were used as quantifiers[36,37]Also; the table contains information about Retention time, an important parameter for identification. According to the most recent SANTE/SANCO validation guidelines, retention times of analyte in a sample and standard solution must not differ more than by 0.1 min.[44]

3. DATA ANALYSIS

Agilent Mass Hunter Workstation software Acquisition B.03 was used for MS data acquisition and analysis. The monitored ions and their retention times, established with the standards of each analyte, are listed in Table 1.

4. STATISTICAL ANALYSIS

All the values were expressed as the mean of three determinations. Additionally, linearity, specificity, accuracy, minimum detectable, and minimum quantitative amount for tartaric acid, citric acid, and lactic acid were determined to check the quality of this analysis.

5. RESULTS AND DISCUSSION

5.1. *Linearity, Limits of Detection (LOD), Limits of Quantification (LOQ), Specificity, Accuracy*

The linearity range was established by injecting five different concentrations obtained by diluting a standard mixture of organic acids. Analytical curves for each organic acid were obtained considering the correlation between the peak area and the respective concentration of the standard using a linear least square model. Information about linear range, correlation coefficients, limit of detection (LOD), and limit of quantification (LOQ) is shown in Table 2. As can be seen from the table, the linearity is satisfactory in all cases with correlation coefficients (R^2 > 0.998). The specificity experiments were conducted to demonstrate the interference of excipients with analyte retention time and peak area. Replicate injections of standards, samples, and blank solutions checked responses' retention time and peak area. The specificity results confirmed no interference of any excipients or/and impurities in the retention time of the analyte.

Table 2: Quantification and calibration information for organic acids.				
Compound	Linear Range (mg/ml)	R^2	LOD (mg/ml)	LOQ (mg/ml)
Citric acid	0.0014–0.07	0. 9985	0.0004	0.0013
Tartaric acid	0.045–0.35	0.9986	0.0115	0.0350
Lactic acid	0.05–0.45	0.9981	0.0004	0.0014

LOD, limit of detection; LOQ, limit of quantification; r, correlation coefficient

Table 2 illustrates the results from method validation. LOD is the lowest concentration in a sample that can be detected but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy. Generally, a correlation coefficient $R^2 > 0.998$ value is considered evidence of an acceptable fit for the data to the regression line.[15,42,43] To determine the accuracy of the analytical method, three standard concentration solutions were prepared of organic acids (Tartaric acid, citric acid, lactic acid). Their actual concentrations were determined. According to the obtained results, the method's accuracy was established, which, as a result of statistical processing, showed that the coefficient of variability is low, representing the positive side of the method (Table 3).

Table 3. Analytical parameters obtained for accuracy experimentally determined concentration %									
	Tartaric acid			Citric acid			Lactic acid		
	0.045 mg/ml	0.09 mg/ml	0.18 mg/ml	0.0015 mg/ml	0.003 mg/ml	0.006 mg/ml	0.05 mg/ml	0.1 mg/ml	0.2 mg/ml
1	100.1	99.2	99.8	99.9	99.8	99.7	99.8	99.9	99.7
2	99.5	99.7	99.5	99.7	99.6	99.5	99.3	99.4	99.6
3	99.3	99.6	99.4	99.3	99.3	99.3	99.2	99.3	99.3
Mean	99.6	99.5	99.6	99.6	99.5	99.5	99.4	99.5	99.5
%CV	0.4	0.3	0.2	0.3	0.2	0.2	0.3	0.3	0.2

mg/ml - milligrams per milliliter

Table 3 shows the results of the accuracy study during the validation of LC-MS/MS. The accuracy of an analytical method expresses the nearness between the expected value and the value found. In our study, successive analyses (n = 3) for three different concentrations of standard solution were performed to evaluate the accuracy of the method. The study data were statistically analyzed using the formula [% Recovery = (Recovered conc. /Injected conc.) × 100] to study the recovery and validity of the method. The mean recovery should be within 90–110% to be accepted.[15,42,43].

5.2. Qualitative and Quantitative analysis of organic acids by LC-MS/MS

Wine products are known for their high content of organic acids 20[,21,22]. Therefore, we decided to study the organic acid content of the waste product of the winery industry[23,24]. We focused on the quantitative content of three organic acids widely used in the food industry and cosmetics: tartaric acid, citric acid, and lactic acid [24-27]. We chose wine lees made from 3 different Georgian varieties of grapes (Saferavi, Kisi, Rkatsiteli) to identify and measure the organic acids present therein and to correlate the content of organic acids with the grape variety. We also used wine lees produced using various technologies, like the traditional Kakhetian method and factory conditions, in the case of samples from Saperavi and Rkatsiteli. According to the findings from the samples analyzed, Rkatsiteli (traditional) and Saperavi (factory) wine lees contain all three organic acids - tartaric acid, citric acid, and lactic acid – while Rkatsiteli wine lees (factory) contain only one organic acid and Sapefavi (Traditional) only two organic acids (Table 4). These results align with earlier published studies on the various organic acid contents of wine products from various origins [28-30,39,40]. Similar to the results of Georgian wine lees we studied, in different types of wine studied by Castineira A. and et al.[31], organic acids have different qualitative and quantitative compositions. Moreover, according to Scutarașu E.C et al., the technology of wine production affects the content of organic acids in it; this is indicated by the research of Baiano A and et al.[32] on the influence of the production technology[35], where the influence of the wine production technique on the composition and physico-chemical indicators of wine is confirmed, which is correlated with the result of our research on wine on the influence of production technology.

Table 4. Compounds identified of Georgian Wine Lees by LC-MS/MS					
Samples	Name of the Compound	MF	MW [g mol-1]	RT [min]	Peak area
Kisin Kisi	Tartaric acid	C4H6O6	150.086	2.094	81981
	Citric acid	C7H5ONS	192.123	2.203	1137
Rkatsiteli (T)	Tartaric acid	C4H6O6	150.086	2.094	1261
	Lactic acid	C8H8O3	90.078	2.277	119
	Citric acid	C7H5ONS	192.123	2.813	1483
Saferavi(T)	Tartaric acid	C4H6O6	150.086	2.094	1165
	Citric acid	C7H5ONS	192.123	2.732	180
Rkatsiteli (F)	Tartaric acid	C4H6O6	150.086	2.135	4457

Saferavi(F)	Tartaric acid	C4H6O6	150.086	2.094	1005
	Lactic acid	C8H8O3	90.078	2.277	202
	Citric acid	C7H5ONS	192.123	2.773	2366

RT- Retention Time, MF- Molecular Formula, MW- Molecular Weight

Table 4. shows the organic acids identified in several samples of Georgian wine lees. Acetic acid, lactic acid, and tartaric acid have been identified. The table illustrates that only one organic acid was identified in some samples. Also, it can be seen from the table that the peak area of the organic acid compounds is different in the analyzed samples, which also gives us information about their quantitative content. It should be noted that Kisi wine lees contain 18–80% higher tartaric acid than other samples from a quantitative standpoint. Four samples include citric acid. However, only Rkatsiteli (traditional) wine lees (Figure 2.) and Saferavi (factory) wine lees have lactic acid. According to the quantitative content of total organic acids (Tartaric acid, Lactic acid, Citric acid), Kisi lees has the highest concentration of organic acids (5.2666 mg/ml). In comparison, Saperavi has the lowest concentration (0.0761 mg/ml).

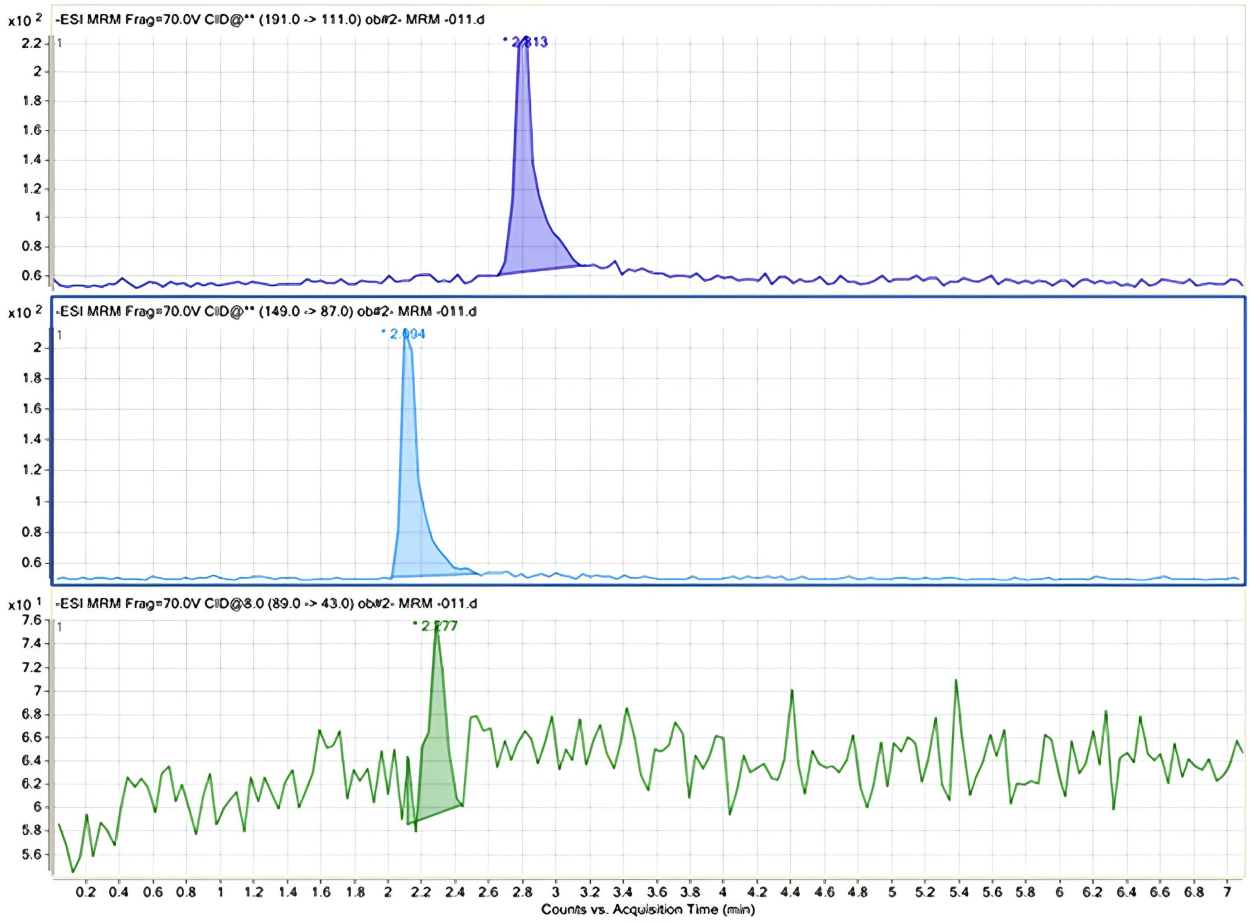


Fig. 2: LC-MS/MS chromatogram of Rkatsiteli wine lees (traditional)

Figure 2. illustrates the LC-MS/MS ion chromatogram of the Georgian Rkatsiteli wine lees sample. Each peak represents the quantitative transition ion (qualitative transition ion not shown): purple-colored peak – citric acid (retention: 2.813 min, Mass transition 191.0 -> 111.0); blue-colored peak – tartaric acid (retention: 2.094 min, Mass transition 149.0 -> 87.0), green colored peak – lactic acid (retention: 2.277 min, Mass transition 89.0 -> 43.0). The only organic acid that can be found in all analytical samples is tartaric acid. Based on the results, we can say that tartaric acid is the dominant organic acid among the studied acids in our samples; its concentration (5.2549 mg/ml) is much higher than other organic acids (lactic acid and citric acid). In addition, we can infer from the results that the grape variety affects the amount of organic acids in wine lees. Table 5 lists the number of organic acids found in the examined wine lees.

Table 5. The concentration of Organic acids in Wine lees samples Organic acid Content (mg/ml)

Sample	Citric acid	Tartaric acid	Lactic acid
Kisi (T)	0.0117	5.2549	nd
Rkatsiteli (T)	0.0129	0.0808	0.0849
Saperavi (T)	0.0015	0.0746	nd
Rkatsiteli (F)	nd	0.2856	nd
Saperavi (F)	0.0206	0.0644	0.1442

nd, below detectable limit; T –traditional Kakhetian method, F - Factory conditions. All values are expressed as means (n = 3).

Table 5. illustrates the quantitative content of organic acids in samples from Georgian wine lees from different varieties of grapes produced by different technologies determined by the LC-MS/MS method. Tartaric acid is one of the dominant organic acids among the analyzed acids. Its content is significant, and also, it is present in countable amounts in all five samples. Some samples below the detectable limit present lactic acid and citric acid. Additionally, the same grape variety's wine lees from the Rkatsiteli, which are produced using a traditional method and under factory conditions, and the Saperavi wine lees, which are produced using a traditional method and under factory conditions, were used to demonstrate the composition of organic acids 32-35. However, a deeper investigation is necessary due to the technologies' reliance on the lees' chemical composition.

6. CONCLUSION

The present study examined the potential for utilizing Georgian wine lees as a source of biologically active substances (organic acids). Tartaric acid was found in large concentrations in the Kisi wine lees during the study. Also, the content of organic acids is noteworthy in other analytical samples. In conclusion, the findings suggest the potential of Georgian wine lees, which are currently considered waste, have the potential to be a cheap and natural source of biologically active chemicals. The results also supported the need for further investigation on wine lees and their potential value in producing biologically active compounds.

7. FUNDING ACKNOWLEDGEMENT

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8. AUTHORS CONTRIBUTION STATEMENT

Tamar Kirvalidze, Tamaz Murtazashvili, Lasha Bakuridze, devised the project and the main conceptual ideas. Tamar Kirvalidze, Koba Sivsivadze, and Malkhaz Jokhadze performed the analysis. Tamaz Murtazashvili, Lasha Bakuridze were supervising the findings of the work. All authors discussed the results and contributed to the final manuscript.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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