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SAMBURESTI WINES CHARACTERIZATION IN TERMS OF THEIR STABLE ISOTOPE CONTENT

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Isotopic fingerprints ($\delta^{13}C$, $\delta^{18}O$ and D/H ratios) were investigated by IRMS and SNIF-NMR techniques in two wine varieties, Cabernet Sauvignon and Merlot from Samburesti vineyard from four consecutive harvest years (2011 to 2014) to asses the capacity for discrimination of their vintage and variety. Mutivariate statistical analysis such as linear discriminant analysis (LDA) was used for variety and vintage authentication. Isotope ratios were identified as providing the maximum discrimination between the wine samples across different harvest years and categorised on the basis of Samburesti vineyard, providing an excellent prediction rate of 96.43%.

Keywords: Wines, Isotopic markers, Multivariate statistical analysis, Variety, Vintage

1. Introduction

Authentication of attributes such as origin represents a critical element in wine compliance certifying with national and international regulations. Starting from the fact that the term "origin" is of considerable importance in direct

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correlation with the quality of the wines, their classification according to geographical origin and variety has become a topic of significant interest to the producers and consumers community.

The chemical composition of wines is affected by multiple factors, including production area, grape variety, soil type, climate (*terroir*) and oenological and vine growing practices. These factors play an important role in differentiating wines according to their year of harvest and geographical origin.

The measurement of the light elements isotopic ratios (H, C, N, O and S), have been shown to be a useful tool in determining the geographical origin of food products [1-3] thereby, achieving their verification of compliance with the legislation and label declaration. In the case of wine, the determination of stable isotopes ($R = {}^{2}H/{}^{1}H$ or ${}^{18}O/{}^{16}O$ or ${}^{13}C/{}^{12}C$, reported as $\delta^{2}H$, $\delta^{18}O$ or $\delta^{13}C$, where $\delta = (R_{sample} - R_{reference})/R_{reference})*1000$) of various constituents such as ethanol and water, often provides information about the origin of the raw materials, which are considered representative indicators not only for the location but also for their botanical origin [4-7].

The natural ratio of stable isotopes in plants is not constant, it depends on the content of isotopes in the original molecules used by plants for photosynthesis (water and carbon dioxide) and on the isotopic fractionation that occurs along the biosynthetic pathways. Both precursor molecules are characterized by specific isotopic ratios of the hydrogen, carbon and oxygen elements. The amount of heavy isotopes in the water and carbon dioxide, and even their distribution in the molecules of sugar and ethanol is significantly influenced by the geo-climatic conditions, the region of origin, type of plant (species or subspecies/grower) photosynthesis mechanism and year of harvest.

In general, the isotope content of wine's water depends on the environment from where it came. The main natural influences on the water isotopic composition are represented by water-rock interaction, transit period and evaporation processes. For grapes that are subjected to the process of irrigation, the water can differ significantly from the local precipitation water isotopic signature/fingerprint. Consequently, the values of the isotopic ratios of hydrogen and oxygen from plants (vines) are directly related to the water accumulated in the soil, the isotopic composition ($\delta^{2}H$ and $\delta^{18}O$) of rainfall providing information about the local climatic and geographical conditions, such as temperature, rainfall, humidity, altitude or latitude [8]. In other words, $\delta^{2}H$ and $\delta^{18}O$ values of vines water, grapes and wine may reflect the area from where the wine originates.

In addition, observations regarding the process of photosynthesis, which influences the ${}^{13}C$ content of constituents, have led to a classification of plants into three categories defined in terms of isotopic fingerprint, which allows to control the "botanical origin".

Grapes, beet, potatoes, wheat, etc., belong to the C3 group which fix the atmospheric CO_2 by the carboxylation of ribulose-1,5-diphosphate. This process is accompanied by a strong isotopic effect, causing a significant depletion in the content of ^{13}C (in general, carbohydrate $\delta^{13}C$ values of these plants are ranging from -28‰ to -23‰). A second category of plants (including sugarcane, maize, sorghum, millet) belong to the so-called C4 plant group, containing higher levels of ^{13}C than similar products from C3 plants, and fix the CO_2 by carboxylation of phosphoenolpyruvate (PEP-Carboxylase reaction) leading to four carbon product, oxaloacetic acid. In this case, there is virtually no isotopic fractionation phenomenon in relation with the content of ^{13}C from the atmosphere. Products derived from C4 plants have a higher ^{13}C content than the isotopic content of the C3 plants (carbohydrate $\delta^{13}C$ values range from -18‰ to -12‰). There is also a third group of plants that use the crassulacean acid metabolism CAM for the fixation of carbon dioxide, that consists in the use of the C3 cycle (day) and C4 cycle (night) . The content of $\delta^{13}C$ for the products derived from these plants varies between -24‰ and -15 ‰ [9].

In the specific case of wine, we can say that the determination of the natural composition of stable isotopes is one of the applications which can establish the relationships that corelate the finished product (wine) with its raw materials (water, CO_2) in their natural environment. Geographical and climatic factors significantly influence the stable isotopes ratio (eg. $(D/H)_I$ and $^{13}C/^{12}C$) of water and ethanol of wine, so that they can be used to locate the areas depending on the vineyards climate (cold and dry, cold and wet, warm and humid, hot and dry). From the studies conducted in time, it was observed that there was a decrease in the oxygen isotope content and to a lesser extent the deuterium from wine water as it moves from warm areas to areas with a more temperate climate and from costal to inland.

Because the isotopic ratio value is subjected to environment climate influence, particularly the temperature at which the grapes mature, the values recorded in this report generates credible elucidation of the area of production of a wine (wine area, country, continent). Also, since the isotopic ratios value is dependent on the isotopic composition of water in the soil, and climatic conditions, especially rainfall, investigating these ratios are relevant approaches in order to authenticate the geographical origin of wines.

Starting from these considerations in this study we characterize (using a small number of samples) in terms of isotope content two types of red wines originating from Samburesti vineyard, in order to highlight the influence of climate and harvest year upon their isotopic fingerprint.

2. Study area

The Samburesti vineyard is located in the southern part of the country (in the north part of Olt County), 44°48' north latitude and 24°48' eastern longitude, in the transition area between the ciency hills and extend hillocks which form the southern half of Cotmeana Piedmont in the south part of the Carpathians. Piedmont landscape is dominated by hills with average height and slight inclines, with numerous long plateaus and valleys. Vineyards are located on the south-eastern part of the Samburesti hills, with a grand opening to south with the slopes southwest oriented, bathed in sun and providing a great oenoclimate for the vine.

Samburesti vineyard soils are mostly clay soils, clay loam and loam-clay in depth, at different stages of podzolic. These soils are characterized by a medium to low content of organic substances and minerals, with variable texture (from sandy loam to clay), containing important iron content in the form of colloidal hydroxide which by dehydration imprint to the soil reddish brown or rusty intense color, that represents a important feature for high quality red wines.

The natural characteristic factor is the hilly region with intense insolation throughout the whole year. The vineyards benefit from south southwest or southeast location, most plantations being situated on slopes and plateaus.

Samburesti vineyards are characterized by an average annual temperature of 10.6°C, the thermal resources of the area being given by the global thermal balance between 3338.1 and 3961.9°C and effective thermal balance between 1526 and 2439.8°C, particularly favorable indices for the vine culture. The amount of annual rainfall is over 555.2 mm and the amount of active precipitation is 364 mm.

3. Materials and methods

3.1. Sampling

The present study was conducted using 69 red wine samples from two major varieties: Cabernet Sauvignon and Merlot, originating from Samburesti vineyard. The two varieties are the main varieties of the current manifold for red wine from the vineyard, being a part of the traditional assortment, which brought nationally and internationally recognition to the Samburesti vineyard. The first year of production for the vine plantations was established in 2008 but the current work began in 2011 and lasted until 2014 harvest year. In the first year 6 wine samples from Cabernet Sauvignon were studied, in 2012 10 wine samples, 11 in 2013 and 15 in 2014. For Merlot variety we started the study in 2012 with 4 samples and continued in 2013 with 11 samples and 12 samples for 2014 harvest year.

3.3. Vinification

To obtain comparable results, every year it was intended to harvest on the same day all the samples, the microvinification process being realised identical for all the studied samples by the Enology Laboratory, Faculty of Agriculture and Horticulture, Craiova University.

3.4. Analysis methods

To characterize the isotopic fingerprint of the two wine varieties hydrogen, carbon and oxygen stable isotopes were determined, their values reflecting specific climatic conditions of the Samburesti area.

Analysis of the stable isotopes of hydrogen, carbon and oxygen was carried out in the laboratories of the National Research and Development Institute for Cryogenics and Isotopic Technologies - ICSI Rm. Valcea, using two advanced techniques:

- Isotope Ratio Mass Spectrometry to determine the $^{18}O/^{16}O$ isotopic ratios of water extracted from wine and $^{13}C/^{12}C$ of ethanol extracted from wine, and

- Nuclear Magnetic Resonance Spectroscopy to determine the isotopic ratios $(D/H)_I$ and $(D/H)_{II}$.

Before the isotopic analysis the wine samples were distilled on an Automated Distillation Control System (ADCS), with Cadiot column spinning bands in order to extract the ethanol and water [7]. The Cadiot columns had temperatures probes and a solenoid valve which regulated the distillate flow. The alcoholic distillate had an alcoholic strength of at least 85% wt., and the loss of alcohol during each distillation was lower than 0.6% vol.

3.4.1. Determination of the $^{18}O/^{16}O$ isotopic ratio of wine water extracted by fractional distillation

For this analysis CF-IRMS mass spectrometer coupled to Delta V Plus Gas Bench II preparation system was used; the basic principle of this type of measurement refers to the water sample equilibration (for minimum 20 hours at $25^{\circ}C$, in which about a mill mole amount of CO_2 is brought into isotopic equilibrium with 500 microliters of water sample usin a specific gas mixture (He + 0.36% CO_2) [10]. Sample preparation is realized automatically, with the aim of transforming the sample into its corresponding gas (ex. CO_2 for oxygen 18), while it preserves the original isotopic composition of the sample. The preparation procedure requires two stages, the first being represented by the sample pre-processing and the second involving the pre-processed samples conversion into their corresponding gases that are carried by the eluent (He) and their introduction into the mass spectromether (MS) with the reference gas (eg. CO_2 99.998% purity).

The $^{18}O/^{16}O$ isotopic ratio is determined by isotope ratio mass spectrometry (IRMS) of ion currents corresponding to m/z = 46 ($^{12}C^{16}O^{18}O$) and m/z = 44 ($^{12}C^{16}O_2$) masses, produced by carbon dioxide obtained after the isotopic exchange with the wine water according to the reaction:

$$C^{16}O_2 + H_2{}^{18}O \leftrightarrow C^{16}O^{18}O + H_2{}^{16}O$$

Due to small variations in the isotopic abundances the ratio's absolute value is not measured, instead the difference from a particular standard is used, the result being express in δ units. The primary standard used for the $^{18}O/^{16}O$ stable isotope ratio is Vienna Standard Mean Ocean Water [11].

3.4.2. Determination of the global $^{13}C/^{12}C$ isotopic ratio from the ethanol extracted from the wine samples by fractional distillation

For the $^{13}C/^{12}C$ isotopic ratio determination CF-IRMS Delta V Plus mass spectrometer coupled to an elemental analyzer EA 1112 HT was used, configuration which has a combustion furnace (combustion process takes place in the presence of oxygen, the oven temperature reach up to 1000°C). The specific purpose of this analysis is to measure the isotopic ratio of the ethanol extracted from the wine. The $^{13}C/^{12}C$ isotopic ratio is expressed as a deviation from the internal standard. The isotopic deviation of carbon 13 ($\delta^{13}C$) is then calculated on a delta scale per thousand (δ/1000) by comparing the results obtained for the measured sample with the ones for the working standard calibrated against the primary international standard VPDB (Vienna Pee Dee Belemnite).

3.4.3. Determination of the $^{2}H/^{1}H$ ratio of ethanol extracted from the wine by fractional distillation

The ^{2}H-SNIF®-NMR (*Site-specific Natural Isotope Fractionation studied by deuterium Nuclear Magnetic Resonance*) principle is represented by the $^{2}H/^{1}H$ isotope ratio transfer from sugar and water by SNIF (Site-Specific Natural Isotope Fractionation) in the methyl and methylene chemical groups of the ethanol molecule. About 80% of the deuterium from sugar is transferred, during the fermentation process, to the methyl group representing the $(D/H)_I$ ratio and about 75% of the deuterium from water is transferred to the methylene group, representing the $(D/H)_{II}$ ratio [12]. Thus, the $(D/H)_I$ ratio is important for determining the botanical origin of the sugar that by fermentation produces ethanol. The $(D/H)_{II}$ ratio gives more information about the deuterium content of the water obtained through grapes fermentation and reflects the climatic conditions related to geographical origin and year of harvest. R represents the relative distribution of deuterium in the two groups, measured directly from the intensities h (peak heights) of the methyl and methylene and characterizes also the origin of ethanol.

The NMR preparation was performed by weighing 3.2-ml alcoholic distillate of the wine sample and 1.3 ml N,N-tetramethylurea reference standard (supplied by the Community Bureau of Reference Geel, Belgium) in a 20-ml glass bottle, adding 150 µl hexafluorobenzene with 10 %(v/v) trifluoroacetic acid as a field-frequency stabilization substance and transferring the mixture into a 10 mm diameter NMR tube. An Ascend 400 Bruker spectrometer was used for the measurements, with a selective deuterium probe-head tuned to a frequency of 61.42 MHz, a fluorine lock channel, and an automatic sample changer. Each sample was measured using 200 scans (the acquisition time for one scan was 6.68 s using a spectral width of 20 ppm). The total acquisition time for one sample was about 4 h. The results, mean values, and standard deviations were calculated with Eurospec (Eurofins-Nantes) software, from ten repetitive experiments with an exponential multiplying factor (LB) equal to 2.

3.5. Statistical analysis

Data analysis was performed to test if significant differences can be highlighted between the harvest years of wine samples originating from the same vineyard. The stable isotopes results were processed using multivariate chemometric techniques such as linear discriminant analysis (LDA). Statistical analysis of data was performed using Microsoft Excel 2010 and XLSTAT Addinsoft version 15.5.03.3707.

4. Results and discussion

In this study were characterized, in terms of stable isotopes, a series of samples of Cabernet Sauvignon and Merlot wines from 4 consecutive harvest years, from 2011 to 2014. The results are summarized in Table 1.

Table 1

Variation of the stable isotopes values for all the studied wine samples

Wine samples		**$\delta^{13}C_{VPDB}$ (‰)**	**$(D/H)_I$ (ppm)**	**$(D/H)_{II}$ (ppm)**	**R**	**$\delta^{18}O_{VSMOW}$ (‰)**
All the wines	Min	-29,74	97,50	126,10	2,52	-2,39
	Max	-23,93	103,00	133,20	2,62	+7,81
	Average	-26,31	100,63	129,24	2,57	+1,21
	SD	1,91	1,38	1,98	0,03	3,15
Cabernet Saugvinon	Min	-29,74	97,50	126,10	2,53	-2,39
	Max	-23,93	103,00	133,00	2,62	+6,50
	Average	-26,43	100,31	129,45	2,58	+0,78
	SD	2,18	1,63	2,16	0,02	3,50
Merlot	Min	-28,28	99,60	126,60	2,52	-0,50
	Max	-24,08	102,30	133,20	2,61	+7,81

Wine samples		**$\delta^{13}C_{VPDB}$ (‰)**	**$(D/H)_I$ (ppm)**	**$(D/H)_{II}$ (ppm)**	**R**	**$\delta^{18}O_{VSMOW}$ (‰)**
	Average	-26,33	100,84	128,72	2,55	+1,45
	SD	1,57	0,70	1,68	0,03	2,63

The effects induced by the weather conditions such as temperature, precipitation (in excess or lack of), relative humidity, are important parameters that must be considered when evaluating the isotopic composition of the wine.

In this case, climatic conditions during grape ripening and harvesting led to a significant variation in isotopic values of the investigated wine samples. It was observed the wide range of values recorded for $\delta^{18}O$ wine water the (lowest value -2.39‰ was measured for Saugvinon Cabernet 2014 – a year of excessive rainfall, and the highest recorded value +7.81‰ for Merlot in 2012 – a dry year) (Fig. 1), variation dictated by climatic fluctuations recorded during grape ripening and harvesting.

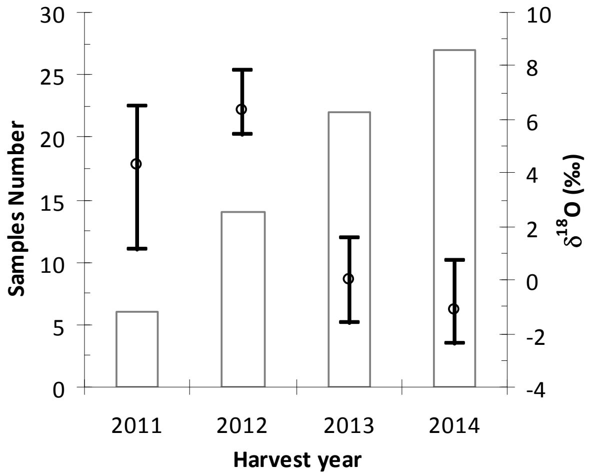

Fig. 1. Diagram of the $\delta^{18}O$ values variation for the wines analyzed, during 2011-2014 harvest years

Regardless of the grapes variety, the $\delta^{18}O$ values of the analyzed wines were recorded between +1.13 ‰ and +6.48 ‰ in 2011, between +5.42 ‰ and +7.81 ‰ in 2012, between -1.64 ‰ and +1.53 ‰ in 2013 and between -2.39 ‰ and +0.74‰ in 2014.

For $\delta^{13}C$ the most depleted value was recorded in 2014 for Cabernet Saugvinon (-29.74 ‰), while the most enriched was -23.93 ‰ in 2012 for the same variety. The $\delta^{13}C$ average value registered in Samburesti region for the 4 studied years was -26.31 ‰ (for Cabernet Sauvignon with a value of -26.43‰ and for Merlot variety of -26.33 ‰).

The same trend, as in the case of δ^{13}C of ethanol extracted from the wine, was found for $(D/H)_I$ ratio, with values between 97.50 ppm for Cabernet Saugvinon in 2014 and 103.00 ppm in 2012, for the same variety.

This differences observed for all the studied stable isotopes are strongly correlated to the harvest periods which were considerably different for each specific year. In 2011 the mean temperature for September (the harvest month) was situated between 20.1 and 22°C and in terms of rainfall, the lowest values (under 10 mm) have been reported in the southwest region that includes Samburesti vineyard. In 2012 the average temperature for the harvest period has decresead with 2°C while the rainfall amount was the same. This decrease was observed in the case of 2013 and 2014 production years (18°C in 2013 and 16°C for 2014) but the rainfall amount has increased considerably, for 2013 ranged between 176mm and 200mm while for 2014 was situated between 51mm and 75mm. Therefore, it can be concluded that the three grape harvesting periods presented different climatic conditions, differences that are stored and traced in the stable isotope values.

In order to achieve a separation of the wine samples according to their variety and harvest years a robust statistical analysis method is required such as a discriminant analysis, due to the fact that this method provides mathematical models for searching differences in physico-chemical variables (Fig. 4).

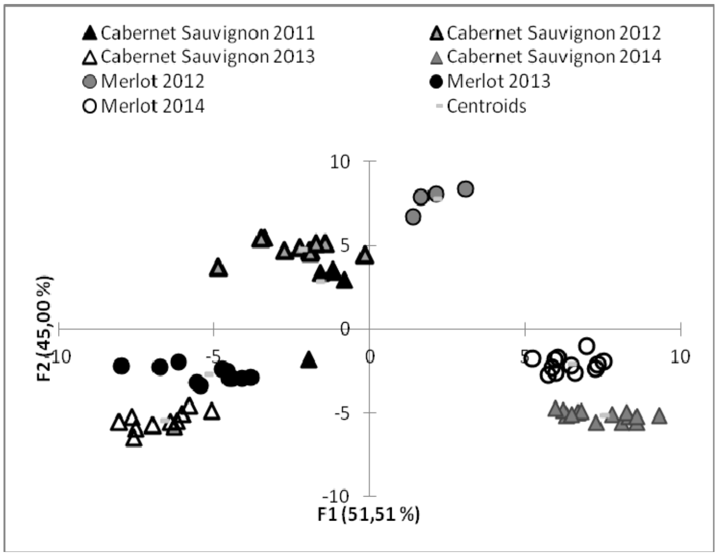

Fig. 2. LDA showing cross-plot of the first two functions for the wine stable isotope data (δ^{13}C, δ^{18}O and D/H ratios) for the variety and harvest year

The discriminant analysis was able to indicate the elements that separate the wines based on their origin. As a classification factor grape variety and harvest year were used and $\delta^{13}C$, $\delta^{18}O$, $(D/H)_I$ and $(D/H)_{II}$ ratios as independent variables. Through the discriminat analysis was obtained a classification of the wines with a correct classification rate of 96.50% and a cross validation rate of 93.94%. Function 1 expresses 51.51% of the variance and provides separation between the main harvest years while function 2 (45.00% of the variance) discriminates variety. All four variables ($\delta^{13}C$, $\delta^{18}O$, $(D/H)_I$ and $(D/H)_{II}$) taken into account provide significant separation. By tracking the isotopic values of the four years in conjunction with the climatic conditions we can observed a significant effect given by the variety as well as the variety – harvest year interaction effects. The most useful variables in discriminating the four harvest years were $\delta^{18}O$ and $\delta^{13}C$. In the case of $\delta^{18}O$ this influence can be explained by the fact that oxygen 18 is correlated to the water existing in the plant tissue and the main source of water is represented by the local meteoric water. In leaves the heavy isotopes of oxygen are enriched due to the evaporation and transpiration process specific for each individual plant, and after the equilibrium phenomen with the fresh water from the xylem (Péclet effect) is distributed to the individual leves in plant reaching in this way to the grape berry. This water represents the principal source of oxygen from the organic matter and this process is accompanied by the thermodynamic effect specific to oxygen. Therefore the organical bound oxygen represents the water source present in the biosynthesis process specific to each type of plant and is related with the local meteoric water isotopic composition and climatic conditions. More specifically, this difference found within the same harvest year between the two studied varieties is explained by the different diffusion constants of the plant stomata. Therefore, $\delta^{18}O$ from wine water can be used as a marker of origin generally used for reconstruction of recent climate conditions. Also, the discrimination given by $\delta^{13}C$ and $(D/H)_I$ between the two types of wine may be due to the photosynthesis phenomenon, with a distinct rate for each variety of grape that lead to well defined assimilation of CO_2 and H^2O for each grape variety.

5. Conclusion

The results of this study report that the stable isotope ratios of wine samples provided a complete picture of the variety variability distribution among four and respectively three different harvest years. The $\delta^{13}C$, $\delta^{18}O$ and D/H ratios were identified as the most useful indicators with the ability to discriminate wines according to variety and harvest year from a single production region from Romania using linear discriminant analysis (LDA) based on their stable isotope content in correlation with their specific climatic features.

One of the most important observations was related to the $\delta^{18}O$ values from wine water which proved to be more efficient and specific in discriminating the four harvesting years. The $\delta^{18}O$ values are strongly related with the annual climatic conditions leading to a better separation between harvest years, while $\delta^{13}C$ alone has less significance for the vintage discrimination.

A better separation was achieved by applying chemometrics analysis to multi isotope data. The excellent prediction rate achieved through linear discriminant analysis in order to classifying wines from a single producing region from Romania according to their vintage year, gives further evidence of the ability of multivariate statistical analysis, based on stable isotopes to show provenance.

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