

# CHEMOMETRIC APPROACH TO DETERMINATION OF THE AUTHENTICITY OF DIFFERENT TYPES OF SERBIAN AND MACEDONIAN BRANDY

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*The isotope ratio mass spectrometry (IRMS) was used to determine the value of the stable isotope carbon ( $^{13}\text{C}/^{12}\text{C}$ ), hydrogen ( $^2\text{H}/^1\text{H}$ ) and oxygen ( $^{18}\text{O}/^{16}\text{O}$ ) in the molecule of ethanol, produced by alcoholic fermentation of fresh fruit (apples, pears, plums and grapes) from the Republic of Serbia and Macedonia, with and without the addition of sugar from sugar beet. IRMS analysis of alcohols with known botanical and geographical origins enabled the creation of authentic database for Serbian and Macedonian brandy. Regression analysis has been shown as very effective in prediction of the amount of added sugar beet in fruit brandy based on  $\delta D$  values. Statistical data analysis was performed by comparing stable isotope values for different types of brandies.  $^{18}\text{O}/^{16}\text{O}$  ratio values proved to be very useful in determination of the geographical origin.*

**Key words:** Authenticity, fruit brandy; isotopic analysis; regression analysis; IRMS analysis.

## INTRODUCTION

The use of stable isotopes of carbon, hydrogen and oxygen in examining frauds in food products has increased exponentially in the past three decades. Alcoholic beverages are often a subject of counterfeiting because they are very appreciated in the world market. Falsification of brandy occurs in different ways, such as addition of industrial ethanol, sugars that do not originate from fruits, addition of water or prohibited substances, and blending of undeclared brandies originating from different geographical areas. The use of isotope ratio mass spectrometry (IRMS) provides information on botanical and geographical origins, which are often considered as important characteristics of alcoholic beverages [1-3]. This method is based on measuring stable isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ ,  $^2\text{H}/^1\text{H}$  and  $^{18}\text{O}/^{16}\text{O}$ ) of a product or a specific component [1]. It has been observed that the isotope ratios of a given molecule vary depending on its origin. This variability is linked to the isotope abundance of the starting material and to the isotope fractionation associated with the various physical processes, chemical reactions and/or biochemical pathways involved during the formation of the molecule [4]. As fruit grows, it incorporates atoms of hydrogen, carbon and oxygen from its environment into carbohydrates. Each of these elements exists in more than one form, called stable isotopes. The stable isotopes of carbon are referred to  $^{12}\text{C}$  and  $^{13}\text{C}$ . About 98.9% of all carbon is  $^{12}\text{C}$ , while 1.1% is  $^{13}\text{C}$ . However, these ratios change depending on the geographic region and weather conditions [5].

The amount of heavy isotopes in water and carbon dioxide, and even their distribution in the sugar and ethanol molecule are significantly influenced by geo-climatic conditions of the provenance region, the type of plant (species or subspecies/cultivar) and the mechanism of photosynthesis [6].

Chemometrics is a statistical approach to the interpretation of patterns in multivariate data.

Two general applications of chemometrics technology exist: to predict a property of interest and to classify the sample into one of several categories.

One of the chemometrics techniques is regression analysis and it is widely used for estimating the relationships among variables [7-10]. Regression analysis offers the possibility to find the relationships between dependent and independent variables in the form of mathematical equations. Simple linear and multiple linear regressions are most often used for definition of relationships, because they include one dependent variable and one (simple linear regression) or more than one independent variable(s) (multiple linear regression) [11]. In this paper preliminary results of the characterization of Serbian and Macedonian brandy of different varieties and geographical origins are presented. The isotope ratios ( $^2\text{H}/^1\text{H}$ ,  $^{13}\text{C}/^{12}\text{C}$  and  $^{18}\text{O}/^{16}\text{O}$ ) of various sorts of apples, pears, plums and grapes were determined in order to establish a database of authentic values for possible application in adulteration and authenticity testing.

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## EXPERIMENTAL

**Plant material:** Nature fruits (N = 11) of various sorts of apples, pears, plums and grapes were collected from different geographical regions of Serbia (Northern and Central Serbia) and Central Macedonia. The samples of fruit for each cultivar and region are presented in Table 1.

Table 1. Mean value of  $\delta^{13}\text{C}$ .

Fruit	Variety	Geographical origin	Mean * $\delta^{13}\text{C}$
Apple	Idared	North Serbia	-27.95
	Jonathan	Central Serbia	-27.49
	Golden Delicious	Central Serbia	-27.42
Pear	William	Central Serbia	-27.29
	William	North Serbia	-27.74
	Kifer	Central Serbia	-27.15
Plum	Stragari	North Serbia	-27.13
	Stragari	Central Serbia	-26.74
Grapes	Black grapes	North Serbia	-27.57
	Black grapes	Macedonia	-27.27
	White grapes	Macedonia	-26.64

The samples were collected directly from different orchards. All samples were cleaned of mechanical impurities, stems and seeds, and milled in a blender. The average amount of 4 kg of samples were put into a bucket with a lid and alcoholic fermentation was carried out using yeasts that had been added at the beginning of fermentation in the appropriate amount (0.5 g). The following fermentations were done under the same conditions for all samples. In order to create a database that would indicate the addition of sugar in the production of brandy that is produced from sugar beet, and not from fruit, for each probe we added a certain quantity of sugar beet in order to account for a range of commonly added percentage of sugar (5 to 50% of sugar in the weight of fruit) in spirits that were available in the market. Sugar beet was added a week after the beginning of alcoholic fermentation. Sugar derived from sugar cane was added to one of the samples. Distillation of samples was carried out up to 95 vol% ethanol, but all samples were not distilled at the same time. The samples that contained the highest amount of added sugars had the longest time of fermentation, so that all of the added sugar could be converted into ethanol. For the determination of stable isotopes it is important that the sample contains 95–99 vol% of ethanol, because then the most reliable results can be obtained. The samples were prepared for analysis by distillation on the apparatus with Vigreux column. Distillate fractions were collected, distilled at a temperature of 78°C to 78.5°C, while all the previous distillates and residual were rejected. The content of ethanol in the distillate collected was determined by Karl-Fischer method. Zeolite was added to the sample in order to obtain min 95 vol% of ethanol in the sample. Samples were stored in 2 ml vials with standard caps and septa.

**Sample preparation for stable isotope ratio mass spectrometry (IRMS) measurements:** The stable carbon ( $^{13}\text{C}/^{12}\text{C}$  or  $\delta^{13}\text{C}$ ), hydrogen ( $^2\text{H}/^1\text{H}$  or  $\delta\text{D}$ ) and oxygen ( $^{18}\text{O}/^{16}\text{O}$  or  $\delta^{18}\text{O}$ ) isotope ratio in the molecule of ethanol were determined using an Elemental Analyzer (Flash EA 1112 HT), which is connected to a mass spectrometer to measure the isotope ratio (Thermo Finnigan DELTA V Advantage). Using a liquid autosampler AS300S, 0.1  $\mu\text{L}$  of distillate was injected into the Flash EA 1112 HT. The gases obtained by the combustion or pyrolysis of the distillate were introduced into the Thermo Finnigan DELTA V Advantage IRMS spectrometer using the ConFlo III Interface. The calibration of used gases was done by using certified reference materials. **The results of our  $\delta^{13}\text{C}$ ,  $\delta\text{D}$  and  $\delta^{18}\text{O}$  analysis of ethanol were reported using conventional  $\delta$  notation, equation [1] relative to Vienna-Standard mean Ocean Water (V-SMOW) for  $^2\text{H}$  and  $^{18}\text{O}$ , and reference material BCR 656 for  $\delta^{13}\text{C}$ .**

$$\delta = 1000 \cdot \left( \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \quad (1)$$

**FlashEA 1112 HT parameters for determination  $\delta^{13}\text{C}$ :** reactor temperature: 1020°C; GC temperature: 45°C; carrier flow: 90 mL/min;  $\text{O}_2$  flow: 250 mL/min;  $\text{O}_2$  injection time: 1 s; autosampler delay: 23 s. **FlashEA 1112 HT parameters for determination  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ :** reactor temperature: 1400°C; GC temperature: 90°C; carrier flow: 100 mL/min; syringe size: 0,5  $\mu\text{L}$ .

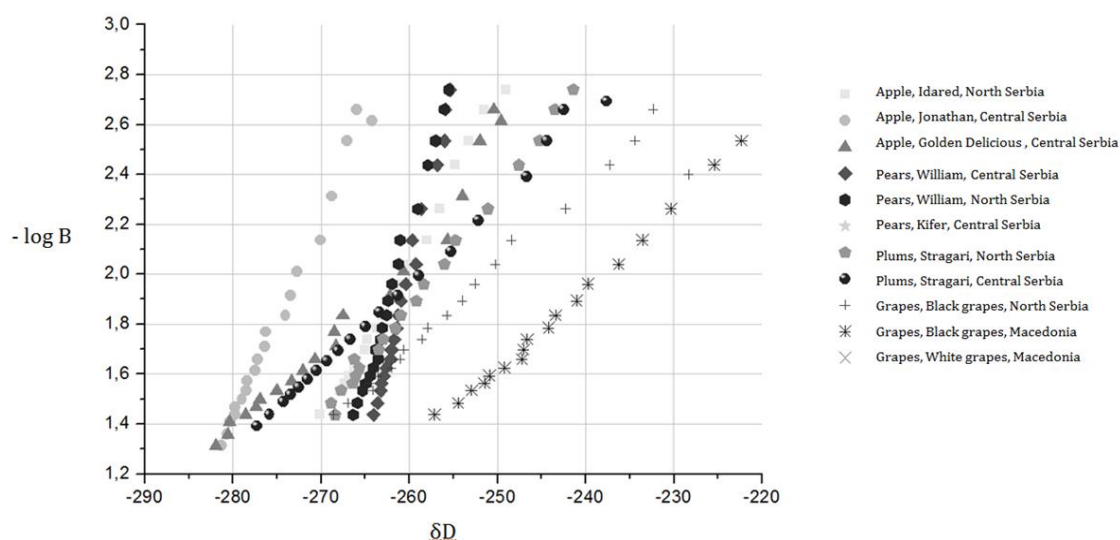
**Statistical analysis:** Statistical calculations were carried out using software package Statistical System Number Cruncher – NCSS 2007 and MATLAB R2009. Linear regression analysis was used to investigate the correlation between the amount of sugar and value of the stable isotope of hydrogen, and for the formulation of a mathematical model

that describes the dependence. The value of percentage of added sugar was transformed in the negative logarithm of the molality ( $-\log B$ ) of added sugar to obtain a linear relationship between the variables.

## RESULTS AND DISCUSSION

**Botanical origin. Carbon-13:** The mean  $\delta^{13}\text{C}$  value of 11 fermented alcohol samples are presented in Table 1. The obtained data confirm that the determination of values  $\delta^{13}\text{C}$  may give insight to from where the ethanol in alcoholic beverages originates. Only fruit was used to obtain the test samples of alcoholic beverages, and therefore all values obtained were in the range of  $\text{C}_3$  plants. Respectively, plants can be classified in three categories according to their photosynthetic pathways. The plants belonging to the first category can fix the atmospheric  $\text{CO}_2$  by carboxylation of ribulose 1,5-diphosphate leading to two molecules of phosphoglycerate (chain of three carbon atoms, hence the name  $\text{C}_3$  plants) [5, 6, 12-14]. The RuBisCo reaction was accompanied by strong  $^{13}\text{C}$  isotope effect causing a large depletion in the carbon-13 content of the plant (carbohydrate  $\delta^{13}\text{C}$  values of these plants range from  $-28\text{‰}$  to  $-23\text{‰}$ ) [15]. Most plants belong to this group (e.g. grape, apple, rice, potato, sugar beet). During the experiment, plum cultivar Kifer was mixed with 5% of the sugar produced from sugar cane. The mean value  $\delta^{13}\text{C}$  for this sample was  $-21.85\text{‰}$ . The sugar cane belongs to the second category of plant, called  $\text{C}_4$  plants. The  $\text{C}_4$  plant fix  $\text{CO}_2$  by phosphoenolpyruvate carboxylase (PEP). The PEP-Carboxylase reaction showed almost no isotope fractionation with respect to the carbon-13 content of atmospheric  $\text{CO}_2$ . Products derived from  $\text{C}_4$  plants showed higher carbon-13 contents than analogous product from  $\text{C}_3$  plants ( $\delta^{13}\text{C}$  values of carbohydrates from  $\text{C}_4$  plants are generally around  $-10\text{‰}$ ) [15]. Cane and maize are the most important representatives of this group from agro-economical point of view. The value that was obtained was between the range of values for  $\text{C}_3$  and  $\text{C}_4$  plants. The reason for this is the use of both types of plants. However, compared to the value for the sample of ethanol, which comes only from plums cultivar Kifer, the value  $\delta^{13}\text{C}$  increased significantly. The chaptalization with cane sugar is easily detectable by IRMS because of the significant increase of the carbon content (resulting from the fermentation of the mixture of  $\text{C}_4$  cane and  $\text{C}_3$  fruit sugar) [4, 6].

**Hydrogen-2:** Deuterium content ( $\delta\text{D}$ ) concerns the physiology of the plant from which the sugar originates (6). Approximately 85% of the deuterium of sugar is during fermentation transferred into methyl-group and about 75% of the deuterium of water is transferred into the methylene-group of ethanol molecule. The  $^2\text{H}/^1\text{H}$  ratio in methyl-group is therefore significant for the determination of the botanical origin of fermented sugar producing the ethanol. The  $^2\text{H}/^1\text{H}$  ratio in methyl-group is important for the deuterium content of the fermentation water and reflects the climatic conditions related to the geographical origin. The intermolecular ratio of both ratios in the so-called "R-value", also characterizes the origin of the ethanol. After chaptalization of fruit must with sugar beet, the R-value of  $\delta\text{D}$  for the produced ethanol in our experiment decreased significantly depending on the amount of chaptalization (Figure 1).



**Figure 1.** Graphical representation of the  $-\log B$  dependence of values  $\delta\text{D}$ .

Namely, between  $\delta\text{D}$  and  $-\log B$  a linear relationship is present, and this was the reason why the linear regression analysis was applied. For each sample, a mathematical model that best describes the relationship between the amount of sugar and the value of  $\delta\text{D}$  was formulated and used in the production. The independent variable is the  $-\log B$  and the dependent variable is the value of  $\delta\text{D}$ . The mathematical models are presented in Table 2.

**Table 2.** Formulated mathematical models using regression analysis.

Fruit	Variety	Geographical origin	Equation	Mathematical model $-\log B = a \cdot \delta D + b$
Apple	Idared	North Serbia	1	$-\log B = 0.0658 \cdot \delta D + 19.15$
	Jonathan	Central Serbia	2	$-\log B = 0.0803 \cdot \delta D + 23.90$
	Golden Delicious	Central Serbia	3	$-\log B = 0.0388 \cdot \delta D + 12.00$
Pear	William	Central Serbia	4	$-\log B = 0.1473 \cdot \delta D + 40.31$
	William	North Serbia	5	$-\log B = 0.1233 \cdot \delta D + 34.24$
	Kifer	Central Serbia	6	$-\log B = 0.0778 \cdot \delta D + 22.85$
Pear	Stragari	North Serbia	7	$-\log B = 0.0459 \cdot \delta D + 13.81$
	Stragari	Central Serbia	8	$-\log B = 0.0340 \cdot \delta D + 10.81$
Grapes	Black grapes	North Serbia	9	$-\log B = 0.0301 \cdot \delta D + 9.52$
	Black grapes	Macedonia	10	$-\log B = 0.0324 \cdot \delta D + 9.71$
	White grapes	Macedonia	11	$-\log B = 0.0318 \cdot \delta D + 9.54$

All developed mathematical models were validated by the calculation of basic statistical parameters (Pearson's correlation coefficient ( $r$ ), standard error of estimation ( $s$ ) and Fisher's value ( $F$ ) and cross-validation parameters (Table 3). The values of ( $r$ ) and ( $s$ ) indicate that the linear equations obtained are statistically very significant. The value of ( $r$ ) greater than 0.9 indicates a very strong correlation between the parameters. Also, the value of ( $s$ ) quite low, indicating that the linear model described the experimental data obtained very well. Cross-validation is practical for testing significance. The values of the cross-validation coefficient of determination ( $r^2_{cv}$ ) and adjusted coefficient of determination ( $r^2_{adj}$ ) are greater than 0.5, which is a proof of good predictability of the model (16). The predicted residual sum of squares ( $PRESS$ ) is very low (less than one for all of the models). Also,  $PRESS$ -value is less than the total sum of squares ( $TSS$ ), and  $PRESS/TSS$ -value is lesser than 0.4. This indicates the significant predictive power of the models [16, 17]. The practical application of these equations is reflected in the calculation of the approximate amount of added sugar, derived from beet sugar, in brandies during production, based on the values of  $\delta D$ .

**Table 3.** Statistical parameters that indicate the validity of the mathematical model.

Fruit	Variety	Geographical origin	$r$	$s$	$F$	$PRESS$ $S$	$TSS$	$PRESS$ $/TSS$	$r^2_{adj}$	$r^2_{cv}$
Apple	Idared	North Serbia	0.9975	0.0295	3561	0.0214	3.1106	0.0069	0.9969	0.9931
	Jonathan	Central Serbia	0.9944	0.0465	1587	0.0561	3.4716	0.0162	0.9940	0.9838
	Golden Delicious	Central Serbia	0.9827	0.0814	506	0.1580	3.4716	0.0455	0.9655	0.9545
Pear	William	Central Serbia	0.9950	0.0416	1781	0.0401	3.1106	0.0129	0.9929	0.9871
	William	North Serbia	0.9957	0.0387	2057	0.0318	3.1106	0.0102	0.9902	0.9898
	Kifer	Central Serbia	0.9910	0.0588	987	0.0844	3.4716	0.0243	0.9843	0.9757
Plum	Stragari	North Serbia	0.9980	0.0261	4559	0.0152	3.1106	0.0049	0.9959	0.9951
	Stragari	Central Serbia	0.9977	0.0290	3832	0.0224	3.2363	0.0069	0.9969	0.9931
Grapes	Black grapes	North Serbia	0.9807	0.0754	452	0.1744	2.6707	0.0653	0.9653	0.9347
	Black grapes	Macedonia	0.9982	0.0246	5116	0.0147	3.1106	0.0047	0.9960	0.9953
	White grapes	Macedonia	0.9954	0.0396	1964	0.0359	3.1106	0.0115	0.9915	0.9885

For the  $\delta D$  values which are quite low, the use of the described equation will show a high content of added sugar derived from sugar beets, although in some cases that would not be the real situation. Therefore, it is necessary to know where the sample comes from and then consider the results.

**Geographical origin. Oxygen-18.** The isotopic signature of water is usually changed by phase transitions within the atmospheric water cycle. The resulting spatial and temporal differences in  $\delta^{18}O$ - and  $\delta D$ - values in water depend on the amount of precipitation, temperatures, distance from sea (continental effect) and altitude [18]. Measurements of the stable isotope ratios of hydrogen ( $^2H/^1H$ ) and oxygen ( $^{18}O/^{16}O$ ) are applicable for the characterization of geographical origin because they are strongly latitude dependent. The water derived from atmospheric vapor is called "meteoric water". In the meteoric water cycle, a well-known isotopes fraction takes place during the evaporation of the water from the oceans, where depletion in heavy isotopes is observed in the vapor [19]. As the water with a higher content of heavy isotopes is successively removed from the vapor by precipitation, the residual vapor shows a lower content of heavy isotopes. Consequently, precipitation from the residual vapor shows depletion of heavy isotopes. Actually, the precipitation in a tropical area at low latitude shows enrichment of heavy isotopes. On the other hand, snow and ice samples from the Arctic and Antarctic show large depletion of heavy isotopes. In the case of carbon, the isotope ratio in foodstuffs is directly related to their botanical origin.  $C_3$  plants use Calvin photosynthetic pathway to assimilate  $CO_2$ . During this process, the plants discriminate against  $^{13}C$  and therefore possess relatively lower  $^{13}C/^{12}C$  ratios than  $C_4$  plants, which utilize the more energy-efficient Hatch-Slack pathway. Since  $C_3$  plants predominate at higher latitudes and  $C_4$  plants are more common in warmer climates at lower latitudes (such as tropics), there is a gradient of decreasing  $^{13}C/^{12}C$  in plant material from the equator to the poles, which can also be used as a proxy for geographical origin determination. The combination plots of the carbon, hydrogen and oxygen ratio enabled us to designate the geographical origins of alcohol derived from the same kind of cultivars. Mutual dependence of measured isotope ratio for all types of spirits that were analyzed and are shown in two-dimensional rectangular and three-dimensional (spatial) coordinate systems (Figure 2).

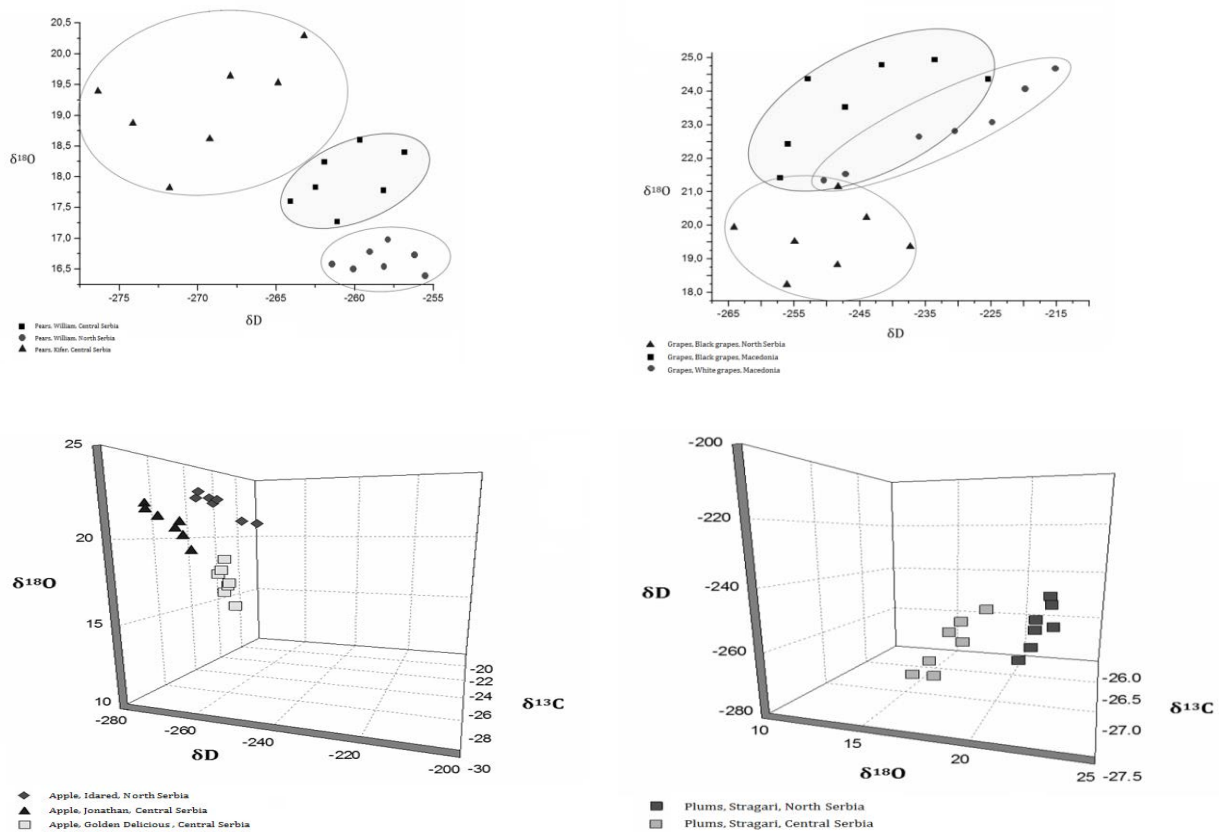


Figure 2. Two-dimensional and three-dimensional view of the stable isotope dependence.

By comparing the values obtained for brandy apple cultivar Idared, which originated from Northern Serbia, with the sort of brandy apple Jonathan from Central Serbia, it can be seen a significant difference in the values for the  $^{18}\text{O}/^{16}\text{O}$  ratio. The average  $\delta^{18}\text{O}$  values of brandy from Northern Serbia is 17‰, while the brandy from Central Serbia is 20‰. The cause of these differences comes from differences in climate, varieties of apples, and the difference in distance from sea and other sources of evaporation. As expected, the obtained data indicate that the strongest effect was of the distance from the equator, because the value of  $\delta^{18}\text{O}$  obtained for a sample of brandy from Northern Serbia is significantly lower than the value of the sample of brandy of Central Serbia. For the apple brandy from Central Serbia, cultivar Delicious,  $\delta^{18}\text{O}$  values are similar to the values obtained from samples of the apple brandy cultivar Jonathan, also from Central Serbia. However, the average  $\delta^{18}\text{O}$  value for the Delicious sort is 22 ‰. If we observe only the location from which the sample comes, we should expect that the samples from Northern Serbia have the same values of  $\delta^{18}\text{O}$ . If there is a difference, it is the result of a different metabolism and physiology of plants, resulting in the adoption of different abilities of water and carbon dioxide. Since, in this case, three different varieties of apples were tested, it was expected to have partly different values for  $\delta^{18}\text{O}$ . On the Figure 2. it can be noticed that data are clustered, belonging to different varieties of fruits and originating from different geographical locations. This confirms that, for each type of fruit spirits, there is a range of  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values, which is due to different environmental conditions and the location where the fruit grew. By comparing  $\delta^{18}\text{O}$  values obtained for samples of grape brandy from Macedonia, it is important to note that they are very different from the values obtained for the Northern and Central regions of Serbia (Figure 2). The average value of  $\delta^{18}\text{O}$  for samples from Macedonia is 24‰, which is significantly more than the value of samples from Central Serbia (19‰). Macedonia's geographical position and closeness to the sea caused a significantly higher value of  $^{18}\text{O}$  in the immediate surrounding plants, thus increasing the availability of plants. When comparing two samples of grape brandy from Macedonia, there is an obvious match of  $\delta^{18}\text{O}$  values, which confirms that they originate from the same location.

## CONCLUSIONS

The number of 11 species of fruit brandies originating from Northern Serbia, Central Serbia and Macedonia were analyzed by IRMS method. A small database of alcohol isotope ratio was constructed in order to determine the botanical and geographical origins of raw material in alcohol. Therefore, a database was formed for the ratio values in stable isotopes of carbon ( $^{13}\text{C}/^{12}\text{C}$ ) indicating the botanical origin. By regression analyses, mathematical models were developed. They enable the prediction of the amount of sugar that was added in a brandy that originates from sugar beets. The result of stable isotope ratio analysis of authentic brandy from the Republic of Serbia indicate that, for the authentication of brandy, it is generally important to use all isotope ratios. Graphical comparison of the results of the dependence between  $^{13}\text{C}/^{12}\text{C}$ ,  $^2\text{H}/^1\text{H}$  and  $^{18}\text{O}/^{16}\text{O}$  allowed the classification of the samples of brandy according to their geographical origin.

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## **ХЕМОМЕТРИЈСКИ ПРИСТУП ОДРЕЂИВАЊУ АУТЕНТИЧНОСТИ РАЗЛИЧИТИХ ВРСТА РАКИЈЕ СРБИЈЕ И МАКЕДОНИЈЕ**

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Одређивањем односа стабилних изотопа у алкохолним пићима могуће је дати веома прецизну оцену квалитета алкохола. Применом хеометријских метода за обраду резултата могуће је потврдити аутентичност различитих врста воћних ракија. Анализом односа стабилних изотопа С, Н и О, применом IRMS методе, у воћним ракијама које су припремљене на традиционални начин, формирана је база података која је садржала неопходне информације за статистичку обраду. У поступку добијања воћних ракија, воћу које је коришћено, додата је растућа количина шећера пореклом од шећерне репе. За све врсте ракија које су анализирани применом линеарне регресионе анализе дефинисани су математички модели који описују зависност између додате количине шећера пореклом од шећерне репе и вредност односа стабилних изотопа  $^2\text{H}/^1\text{H}$ . Применом једначина могуће је израчунати додату количину шећера у непознатом узорку. Дефинисане разлике у вредностима односа  $^{18}\text{O}/^{16}\text{O}$ , испитиваних ракија, указују на географско порекло воћа које је коришћено при производњи ракије. Графичким приказивањем међусобне зависности односа изотопа ( $^{13}\text{C}/^{12}\text{C}$ ,  $^2\text{H}/^1\text{H}$  и  $^{18}\text{O}/^{16}\text{O}$ ) извршена је класификација узорака ракије према врсти воћа и локацији са које потичу.

**Кључне речи:** аутентичност; воћна ракија; анализа изотопа; регресиона анализа; IRMS.

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