

The intra-laboratory study of EIM-IRMS[®] (Ethanol Isotope Measurement–Isotope Ratio Mass Spectrometry) method performance with CS-qNMR (Cumulative Screening–quantitative NMR)

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Abstract. In our earlier publications we proposed the possibility of using new EIM-IRMS[®] instrumental technique and analytical parameter δD_n wine ethanol, which represents δ^2H value of non-exchangeable hydrogen stable isotope ratio in ethanol, to improve detection of illegal wine production practices such as addition of sugar and/or water to grape must prior to alcoholic fermentation. This article shows a comparative performance study between EIM-IRMS[®] and CS-qNMR methods in order to evaluate potential and demonstrate the precision of two isotopic concepts and their degree of detection of illegal wine production practices. Three batches of laboratory wine samples were prepared from three different types of grapes ($N=24$ samples). Sample results obtained from both methods are compared and a statistical data processing was carried out. Based on the results obtained using the EIM-IRMS[®] method we detected 11 out of 21 of previously adulterated wine samples which represents a success rate of 52.4% detection of illegal practices (beet sugar, water or beet sugar syrup additions). In addition, wines from the Republic of Serbia (90 samples) were examined as well using the EIM-IRMS[®] method and appropriate equipment to confirm their authenticity.

Keywords: Authenticity, EIM- IRMS[®], CS-qNMR, Watering, Chaptalization

1 Introduction

Over the past 30 years, wine has become a product of strategic importance that has greatly influenced the shaping of the modern living standard of people across the globe, and as such has become a part of official politics because of the part in which it participates in gross social income.

From that perspective, the production of wine and the entire world wine industry has changed dramatically over the past 30 years. Although, in the beginning of its existence, the EU has identified ways of establishing the quality and authenticity of wine, it has adopted the necessary measures, procedures, technical and industrial standards and passed them into official regulations; in the beginning of the 21st century, these measures did not come in handy and timely with the changes and all challenges which took place in the real production and market sector.

While in New World countries, wine production began to increase due to the development of different concept of primary viticulture production based on large plantations using modern automated agro-technical measures, Europe also had to adapt to the increasing

volume of imported wine, which was becoming very competitive in quantity and quality. This has caused imported wines from New World countries to become more affordable than domestic wines on European market. Such an expansion boom in wine production from the New World countries in the early 2000's resulted in European wines lagging behind on sales racks as non-competitive ballast for the entire wine sector in Europe. For these reasons, a new measure of distillation of these surpluses and their conversion to bioethanol, which as such was sold as cheaper raw material - (fuel) in the US market, began to be introduced.

Parallel to this, questions have been raised about controlling the spending of EU taxpayers' money on these types of operations. The European Union has realized that only by consolidating and reforming the wine sector in the old continent, can it remain competitive and hold a high place in the global wine sector. The reforms included not only steps that would lead to an even greater increase in European wine production through "grabbing up" schemes and further consolidating of vineyard estates, but also projections related to the marketing and sale of European wines to new world markets such as People's Republic of China and Russian Federation. As

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one thing led to another, so in the course of several years, the Chinese market has become the second biggest world wine market, just behind the US and with a tendency for further increase of sales of European and other wines from the rest of the world.

By raising the standard of living of Chinese citizens, new issues of authenticity of wine, and generally authenticity of food, consumed in the Chinese market have also been imposed. Also, the political situation and the cooling of relations between People's Republic of China and United States and the beginning of the "Customs war" between those two great countries have raised additional questions about the quality of goods imported into China and the added interest of Chinese authorities to gain greater insight into what goods and what quality is offered to Chinese citizens.

For that reason, in recent years Chinese scientific community started to be more involved in scientific research and became a relevant factor and serious partner in international scientific cooperation with other scientific communities throughout the world. One of such cases is scientific project "EU-China Safe" where European and Chinese experts are working together on development of new rapid analytical solutions for detection of food fraud through counterfeit and adulteration of food (www.euchinasafe.eu).

On the other hand, a similar story applies to Russian Federation, where, in addition to EU and US sanctions being imposed, Russia is also a part of a broader Euro-Asian economic union that includes all former Soviet Union Republics, so that goods from Europe, US and the rest of the world freely arrives on the Russian market.

In regards to Russian Federation, this issue of wine quality and authenticity is also highly relevant in two aspects: Russia as a wine importer and Russia as a wine producer. In both cases, state control authorities, pursuant to legally binding methodologies for the detection of commodity frauds, must/are supposed to protect the internal market and local producers (prizing of domestic production), consumers (health aspects), treasury (tax evasion) and, last but not least, to protect the mortality of people and the state.

There are already numerous published scientific articles which reference the information that in 1990's EU has introduced regulations on authenticity control of wine and spent billions of euros to build up the system of the national reference laboratories (NRL) for detection of illegal wine production practices and protection of European wine with designated origin (Commission Regulation (EEC) No. 2676/90).

This official analytical model of analysis on which the whole system was built on is based on SNIF-NMR[®] isotopic concept which has a central place and role in official measurements performed by European Office for Wine, Alcohol and Spirit Drinks (BEVABS).

First, officially adopted stable isotope method for the analysis of wine in EU was determination of the site-specific D/H ratio by NMR for wine ethanol that mainly provides evidence of the addition of beet sugar. NMR and/or Isotope Ratio Mass Spectrometry determinations provide information on the botanical and geographical

origins, which are often considered, by consumers and regulations, as important characteristics of food products (Martin et al., 1983; Kelly et al., 2005; Calderone et al., 2008).

As stated previously by numerous experts and scientists: "SNIF-NMR[®] method is based on the measurement of deuterium/hydrogen (D/H) ratios at the specific sites of the ethanol molecule. To detect the addition of sugar or modified sugar syrups with this method the sample must be fermented under controlled conditions and the resulting alcohol distilled off. The (D/H) ratios are determined at the methyl (D/H)_I and methylene (D/H)_{II} sites of the ethanol molecule. One of the first applications of SNIF-NMR[®] was the detection of the adulteration of wine. Results obtained since then from a survey of all EU wine-growing regions have begun to be checked to ascertain whether they can additionally provide a means for identifying the provenance of European wines" (Ogrinc et al., 2003; Martin et al., 1981, 1988, 1988, 1996; Monetti et al., 1994, 1996; Day et al., 1994, 1995; Rossmann et al., 1996).

European Commission has formed BEVABS, as a specialized central laboratory of the Joint Research Centre (JRC), which coordinates the work of all NRL throughout the EU member states. To monitor wine authenticity, the measured values are compared with those from the respective authentic wines listed in the European Union Wine Databank (Commission Regulation (EC) No. 2729/2000; Zeleny et al., 2007; Gimenez-Miralles et al., 1999; Christoph et al., 2003; Rossmann et al., 1996, 1998).

The major driving force behind BEVABS, which is part of the Food Products and Consumer Goods Unit of the JRC's, stated in Geel, Belgium (previously at Institute for Health & Consumer Protection in Ispra, Italy), is to ensure correct implementation of EU wine quality legislation. This covers aspects such as manufacturing procedures and how much sugar can be added. The regulations are complex and difficult to police. There are national laboratories and wine analysis institutes, but their results must be cross-tested. Being very independent of national influences, the JRC can verify national measurements, certifying that results across Europe are consistent. This ensures better quality of wine and avoids the possibility of damaging trade disputes, which could result from disagreement over analytical results.

At present, the analysts are using very powerful analytical tools like the isotopic techniques to address the problems of adulteration. For this purpose, isotopic analysis (IRMS and SNIF-NMR) is performed on reference wines and the resulting data are entered into a European Wine Databank to be used for quality control, data validation and arbitration of disputes (Alonso-Salces et al., 2007). Specific analytical methods which are based on IRMS and NMR analytical instrumental techniques allow analysts to determine levels of stable isotopes of light elements such as ¹³C, ²H, and ¹⁸O to establish the complete "background" of the analyzed sample. With obtained results and with comparison of those values with reference databases from known authentic products, analysts from BEVABS

and authorized Member State laboratories can determine whether a wine sample conforms to the label on the bottle.

These closed isotopic wine databases have been developed and maintained by BEVABS (JRC), with the help of a network of specialized national reference laboratories which collect and analyze samples from the wine-producing regions on a regular basis. The databases are not publicly available and accessible only to national control laboratories.

According to the most recent EUIPO report from 2020 fraud in wine and spirits sector is damaging the EU economy for a total value of 2.3 billion euros. These losses are presented as Direct Lost Sales, i.e. 5.3% loss of all sales, Total Loss of Sales as 5.2 billion of euros, which has a consequence in Direct Employment Loss of 5.681 jobs and Total Employment Loss of 31.858 jobs and Government Revenue Loss of 2.1 billion euros in 2020(EUIPO, 2020).

In continuation People's Republic of China, Russian Federation, and other big countries in the world did not develop the concept of wine authentication using the European model based on Nuclear Magnetic Resonance (SNIF-NMR[®]) due to the many problems associated with the implementation of such a model in those large countries. A major problem is the inability to access European isotopic databases for wines with designated origin for comparison with real samples of European wine which are sold on their national markets, as well as the long time it would take to build national wine isotopic databases for wines with geographical origin for the protection of domestic wines with designated origin. For time being, authentication is mainly based on the individual efforts of particular scientific institutions in these countries trying to develop new models of detection of illegal wine production practices which is further associated with the time needed to develop new alternative technologies and methodologies that could match the SNIF-NMR[®] concept.

Because of all these reasons Russian scientists developed a new modified NMR method called Cumulative Screening-quantitative Nuclear Magnetic Resonance (CS-qNMR) method, so that they could get the same $(D/H)_I$ and $(D/H)_{II}$ values in wine ethanol in comparison to SNIF-NMR[®]. As the principle of all NMR measurements is basically the same, CS-qNMR and SNIF-NMR[®] methods are different only in choice of internal working standard, calculation over integral intensities of signals instead of calculation over signals heights and without the need for stabilization on ¹⁹F nucleus (Kalabin et al., 2003, 2003, 2018; Ivlev et al., 2019).

Earlier we have published our findings on newly developed isotopic method EIM-IRMS[®] (Ethanol Isotope Measurement-Isotope Ratio Mass Spectrometry) which is based on measuring of non-exchangeable hydrogen stable isotopes in ethanol and showed that EIM-IRMS[®] has a great potential in detecting illegal production practices in wine production as alternative to very expensive and

cumbersome official NMR method. We have published our scientific work based on an inter-laboratory study between four laboratories - Imprint Analytics (Neutal, Austria), IsotoptechZrt (at Atomki Institute in Debrecen, Hungary), China National Research Institute for Food and Fermentation Technologies in Beijing, PR of China (C.N.R.I.F.F.I.), and Cornell Stable Isotope Laboratory (Cornell University, Ithaca, NY, US) (Smajlović et al., 2019).

During 2019, the Republic of Serbia, as a full member of the International Organization for Wine and Vine (OIV) based in Paris (France), submitted to the OIV Sub-Commission "Methods of Analysis" (SCMA) proposal for a new project to consider the EIM-IRMS method for drafting a new OIV resolution. In March 2021, at the 62nd Session of the Sub-Commission "Methods of Analysis" of the OIV, after scientific presentation and performed scientific studies in the previous time, technical experts from 48 countries unanimously accepted the Serbian proposal to pass the EIM-IRMS to step 3 of the step by step procedure for drafting a new OIV resolution on authenticity wine.

In this article, we are presenting results obtained in intra-laboratory comparison study between EIM-IRMS[®] and CS-qNMR.

2 Materials and methods

2.1 Samples

A total of $N=126$ wine samples were considered. For the purposes of this scientific work first $N=24$ of laboratory wine samples were prepared from three different grapes varieties gained from Russian market with labeled origin from Moldova, Peru and Uzbekistan and were used for the intra-laboratory study of EIM-IRMS[®] method performance with CS-qNMR.

Furthermore, industrial wine samples with different geographical origin from Moldova, Thailand, Canada, Italy, France, Armenia, Germany, Vietnam, and Czech Republic ($N=12$) were also analyzed on $\delta^{13}C$ and $\delta(^2H)_n$ (δD_n) in wine ethanol which results are presented in Table 5.

In addition, $N=90$ wine samples from Serbian market were prepared and analyzed using EIM-IRMS[®] (Fig. 1c).

2.2 Samples preparation

Each batch from three different grape varieties was first divided into 8 parts (total of $N=24$). The first part from each batch was fermented as a control sample without any addition, while the other 7 parts were prepared with addition of beet sugar ($N=4$), addition of tap water ($N=2$), or addition of beet sugar syrup ($N=1$) as presented in Table 1. All laboratory wine samples were prepared by alcoholic fermentation under controlled and standard conditions. Other samples were obtained as final bottled wine samples.

Table 1. Sample preparation for fermentation.

Sample No.	Sample name	Dry beet sugar addition (g/L)	Tap water addition (% v/v)	Beet sugar syrup addition (60% DM) (% m/v)	Beet sugar addition to 300 mL of grape must (g)	Tap water addition to 300 mL of grape must (mL)	Beet sugar syrup addition to 300 mL of wine (mL)
1	Natural I	-	-	-	-	-	-
2	I-20	20	-	-	6	-	-
3	I-30	30	-	-	9	-	-
4	I-40	40	-	-	12	-	-
5	I-50	50	-	-	15	-	-
6	Natural II	-	-	-	-	-	-
7	II-20	20	-	-	6	-	-
8	II-30	30	-	-	9	-	-
9	II-40	40	-	-	12	-	-
10	II-50	50	-	-	15	-	-
11	Natural III	-	-	-	-	-	-
12	III-20	20	-	-	6	-	-
13	III-30	30	-	-	9	-	-
14	III-40	40	-	-	12	-	-
15	III-50	50	-	-	15	-	-
16	I-water15	-	15	-	-	45	-
17	I-water20	-	20	-	-	60	-
18	II-water15	-	15	-	-	45	-
19	II-water20	-	20	-	-	60	-
20	III-water15	-	15	-	-	45	-
21	III-water20	-	20	-	-	60	-
22	I-sugar syrup	-	-	33.33 mL up to 1 Liter	-	-	10
23	II-sugar syrup	-	-	33.33 mL up to 1 Liter	-	-	5
24	III-sugar syrup	-	-	33.33 mL up to 1 Liter	-	-	10

Table 2. Sample preparation–Distillation.

Sample No.	Sample name	°Bx	Total sugar concentration in grape must (g/L)	Residual Sugar (g/L)	Total potential alcohol (% vol)	Actual Alcoholic Strength (% vol)	Ethanol content in 290 mL wine for distillation (mL)	Distillate Alcoholic strength (%vol)	Distillate volume @ 20°C (mL)	Ethanol content in distillate (mL)	Recovery (%)	Added beet sugar converted to ethanol (g/L)	Beet sugar ethanol in overall wine ethanol (%)
1	Natural I	13.22	132.2	1.78	7.80	7.70	22.32	90.93	20.50	18.64	83.53	0.00	0.00
2	I-20	15.28	152.8	2.72	9.02	8.86	23.91	90.11	26.00	23.43	97.99	19.66	13.10
3	I-30	15.9	159	1.80	9.38	9.28	25.04	91.86	27.00	24.80	99.04	26.78	17.04
4	I-40	16.99	169.9	1.34	10.02	9.95	27.85	90.72	30.00	27.22	97.74	38.14	22.62
5	I-50	17.9	179	2.64	10.56	10.41	30.17	90.03	33.00	29.71	98.46	45.93	26.05
6	Natural II	18.46	184.6	11.21	10.89	10.23	29.67	90.15	32.00	28.85	97.24	0.00	0.00
7	II-20	19.25	192.5	-0.81	11.36	11.41	33.07	92.22	34.50	31.81	96.19	19.92	10.30
8	II-30	20.63	206.3	3.84	12.17	11.95	34.64	91.13	36.50	33.26	96.02	29.07	14.36
9	II-40	21.41	214.1	4.27	12.63	12.38	35.90	93.09	37.00	34.44	95.94	36.44	17.37
10	II-50	22.18	221.8	5.53	13.09	12.76	37.00	91.44	40.00	36.57	98.84	42.88	19.83
11	Natural III	14.83	148.3	6.10	8.75	8.39	24.33	94.15	26.00	24.48	100.61	0.00	0.00
12	III-20	16.67	166.7	3.14	9.84	9.65	27.02	92.59	29.00	26.85	99.37	21.36	13.06
13	III-30	17.51	175.1	6.46	10.33	9.95	28.86	92.53	30.00	27.76	96.20	26.44	15.68
14	III-40	18.52	185.2	5.54	10.93	10.60	28.62	93.06	30.00	27.92	97.55	37.46	20.85
15	III-50	18.98	189.8	6.41	11.20	10.82	31.38	92.89	33.00	30.65	97.69	41.19	22.46
16	I-water15	13.06	130.6	18.74	7.71	6.60	19.14	90.45	20.50	18.54	96.88	-	-
17	I-water20	12.24	122.4	17.15	7.22	6.21	16.77	90.23	18.00	16.24	96.87	-	-
18	II-water15	15.99	159.9	15.49	9.43	8.52	24.71	90.07	27.00	24.32	98.43	-	-
19	II-water20	15.01	150.1	9.25	8.86	8.31	22.44	90.15	25.00	22.54	100.45	-	-
20	III-water15	12.64	126.4	1.32	7.46	7.38	21.40	92.18	23.50	21.66	101.22	-	-
21	III-water20	11.88	118.8	1.68	7.01	6.91	20.04	92.72	22.00	20.40	101.79	-	-
22	I-sugar syrup	16.56	165.6	16.96	9.77	8.77	23.68	90.31	26.00	23.48	99.16	18.22	12.26
23	II-sugar syrup	19.43	194.3	12.77	11.46	10.71	31.06	91.61	34.00	31.15	100.29	8.14	4.48
24	III-sugar syrup	16.67	166.7	3.99	9.84	9.60	27.84	94.21	29.00	27.32	98.14	20.51	12.60

Further, ethanol from all wine samples ($N=126$) was extracted by using EIM-PADS[®] (Ethanol Isotope Measurement–Preparation Automated Distillation System) distillation apparatus which was provided by SG Isotech and with possibility to quantitatively extract ethanol from fermented samples with recovery rate of more than 95% and alcoholic strength of more than 90% vol. and for the needs of isotopic testing. Sample preparation data for the first $N=24$ of laboratory wine samples used in intra-laboratory study between EIM-IRMS[®] and CS-qNMR methods are shown in the Table 2.

2.3 Isotopic measurements

2.3.1 $\delta^{13}C$ determination in wine ethanol

$\delta^{13}C$ values were measured in ethanol obtained by distillation of wine samples. $\delta^{13}C$ analysis, by mass spectrometry, is measured in CO_2 produced from excess oxygen combustion of ethanol obtained by distillation. For measuring isotopic ratios of carbon ($^{13}C/^{12}C$), the technique of mass spectrometry Delta V Advantage with a double collector was used.

Isotopic ratios were measured by the simultaneous measurement of three ion beams ($^{12}\text{C}^{16}\text{O}^{16}\text{O}^+$, $^{13}\text{C}^{16}\text{O}^{16}\text{O}^+$, $^{13}\text{C}^{16}\text{O}^{18}\text{O}^+$) and by comparing the sample to a standard. For expressing the relative difference between isotopic ratios of sample and reference standard, $\delta^{13}\text{C}$ is used, which is defined as:

$$\delta^{13}\text{C} (\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 10^3 \quad (1)$$

where R_{standard} is the absolute isotopic ratio ($^{13}\text{C}/^{12}\text{C}$) of an international standard for carbon and it represents $R = 0.0112372$. By international convention, $\delta^{13}\text{C}$ is always expressed in relation to the value for the standard of calcium carbonate, known as PDB. This standard is a carbonate obtained from the *Belemnitella Americana* fossil. The base of PDB scale is the value $\delta^{13}\text{C} = 0\text{‰}$ for this standard.

The measurement of the $^{13}\text{C}/^{12}\text{C}$ of ethanol was performed using a continuous flow technique using a Trace GC Ultra peripheral equipped with TriPlus Auto Sampler and Finnigan Combustion III Interface directly coupled to Delta V Advantage IRMS.

The measurement of the $^{13}\text{C}/^{12}\text{C}$ of ethanol at ANA LAB laboratory was performed using Costech Elemental Analyzer directly coupled to the Delta Plus XP IRMS through ConFlo III Interface.

2.3.2 $\delta(^2\text{H})_n$ (δD_n) ethanol determination

SG Isotech Company has provided two fully functional and certified EIMPyro[®] peripherals for continuous flow isotopic analysis with the all following consumables for the purposes of this study and the functionality of this equipment was confirmed by service engineers from the Scientific Research & Education Center of the Peoples' Friendship University of Russia (RUDN University) in Moscow (Russian Federation) and also from ANA LAB D.O.O. (Republic of Serbia) where the equipment was installed.

EIMPyro[®] peripherals was connected to Delta V Advantage IRMS instrument over ConFlo III interface in RUDN University (Russian Federation) and to Delta Plus XP IRMS instrument over ConFlo III interface (ANA LAB, Republic of Serbia). Both configurations were used for determination of δD_n ($\delta(^2\text{H})_n$) values in wine ethanol samples.

The principle of EIMPyro[®] - IRMS is based on the rapid and fully quantitative intramolecular dehydration of ethanol sample over custom made EIM-catalyst, specifically designed by SG Isotech Company, prior to pyrolysis and high precision isotope ratio measurement during a single analysis. There are over 2,000 different known dehydration catalysts which have different catalytic characteristics. That's why SG Isotech has designed and developed special dehydration catalyst for the purpose of EIM-IRMS[®] application which can be used for quantitative dehydration of the sample without isotopic fractionation and with strongly expressed KIE (Kinetic Isotopic Effect). During ethanol dehydration,

ethylene and water are produced and separated in continuous flow. Due to the Kinetic Isotopic Effect (KIE) during sample intramolecular dehydration over EIM-catalyst, on methyl group of ethanol, two hydrogen atoms which vibrate on a longer distance from carbon atom, have bigger probability to go into reaction with EIM-catalyst, then deuterium which is more strongly bonded and closer to Carbon atom of ethanol's methyl group. This phenomenon is a consequence of the difference in masses between hydrogen and deuterium atoms. In that way all deuterium atoms will stay bonded inside of newly formed ethylene. By measuring relative ratio of hydrogen and deuterium in ethylene produced in that specific way, we are measuring non-exchangeable hydrogen stable isotopes in ethanol and thus introduce new analytical parameter, the ethanol δD_n ($\delta(^2\text{H})_n$) value (Smajlović, 2009; Smajlović et al., 2013).

The ethanol δD_n ($\delta(^2\text{H})_n$) value, is related to the Ethanol Standard Material AAWES (Afusali Authentic Wine Ethanol Standard) also provided by the SG Isotech Company and expressed as deviation of the relative ratio of non-exchangeable deuterium and hydrogen atoms in internal ethanol standard (D/H)_n in regard to relative ratio of non-exchangeable deuterium and hydrogen atoms in the AAWES, expressed in parts per 1000 (‰) on SGI Scale, where AAWES has δD_n value of -211.89‰.

2.3.3 NMR measurements

For deuterium measurements NMR JEOL JNM-ECA 600 (JEOL, Japan) spectrometer with an operating frequency for ^1H nuclei 600 MHz and ^2H nuclei 92.102 MHz with 5mm and 10mm sensors, equipped with an auto-sampler and calibrated tubes with a diameter of (4.97 ± 0.013) mm and a length of 178 mm was used.

For the NMR analysis 575 mkl of EtOH was taken and added 25 mkl of dimethyl sulfoxide (DMSO) as internal standard (IS). The D/H ratio in dimethyl sulfoxide was increased by addition of DMSO- d_6 to achieve the same integral intensities of IS and analyte.

The principle of all NMR measurements is the same, but there are some differences in methodology between CS-qNMR and official SNIF-NMR[®] Method (OIV-MA-AS311-05):

1. In CS-qNMR different internal standard is used. In official SNIF-NMR[®] method tetramethylurea (TMU) internal standard is used which requires about 1/3 of total volume of sample. For CS-qNMR dimethylsulfoxide (DMSO) which is enriched in D/H content is used which allows us to decrease the volume of internal standard and increase S/N ratio of the signals;
2. With CS-qNMR integral intensities of signals are used in calculations of the results, while in official SNIF-NMR[®] method heights of signals are used in calculations of the results;
3. With CS-qNMR there is no need for stabilization on ^{19}F nucleus and analysis can be realized at any NMR spectrometer.

3 Results and Discussion

By using EIM-IRMS[®] we have successfully detected previously prepared samples spiked with beet sugar, tap water and beet sugar syrup (Table 3a, Fig. 1a):

1. II-40 – Addition of beet sugar (40g/L) to grape must prior to alcoholic fermentation;
2. II-50 – Addition of beet sugar (50g/L) to grape must prior to alcoholic fermentation;
3. II-water15 – Dilution of grape must with water (15%v/v) prior to alcoholic fermentation;
4. II-water20 – Dilution of grape must with water (20%v/v) prior to alcoholic fermentation;
5. III-20 - Addition of beet sugar (20g/L) to grape must prior to alcoholic fermentation;
6. III-30 - Addition of beet sugar (30g/L) to grape must prior to alcoholic fermentation;
7. III-40 – Addition of beet sugar (40g/L) to grape must prior to alcoholic fermentation;

8. III-50 – Addition of beet sugar (50g/L) to grape must prior to alcoholic fermentation;
9. III-water15 – Dilution of grape must with water (15%v/v) prior to alcoholic fermentation;
10. III-water20 – Dilution of grape must with water (20%v/v) prior to alcoholic fermentation;
11. III-sugar syrup – Addition of 10 mL of 60% (m/v) beet sugar syrup which corresponds to addition of 20 g/L of beet sugar to grape must prior to alcoholic fermentation. In continuation by using CS-qNMR we have successfully detected previously prepared samples spiked with beet sugar (Table 3b, Fig. 1b):
12. III-30 - Addition of beet sugar (30g/L) to grape must prior to alcoholic fermentation;
13. III-40 – Addition of beet sugar (40g/L) to grape must prior to alcoholic fermentation;
14. III-50 – Addition of beet sugar (50g/L) to grape must prior to alcoholic fermentation.

Table 3. EIM-IRMS[®] and CS-qNMR results with intra-laboratory repeatability and reproducibility calculation.

Table 3a. Ethanol δD_n values (‰ vs. AAWES)(Reference material: AFUSALI - Afusali Authentic Wine Ethanol Standard (AAWES) with value -211.89‰).

Sample No.	Sample name	Ethanol δD_n (‰ vs. AAWES)	Intra-laboratory Reproducibility standard deviation sR(‰)*	Intra-laboratory Repeatability standard deviation sr (‰)**	Amount of beet sugar ethanol in overall wine ethanol (%)
1	Natural I	-199.25	0.21	0.21	0.00
2	I-20	-203.31	0.64	1.64	13.10
3	I-30	-204.64	2.11	0.84	17.04
4	I-40	-206.53	2.68	0.89	22.62
5	I-50	-207.79	1.39	1.11	26.05
6	I-water15	-210.58	0.92	0.25	15 ¹⁾
7	I-water20	-214.05	1.44	2.02	20 ¹⁾
8	I-sugar syrup	-215.46	1.28	1.45	12.26 ²⁾
9	Natural II	-207.28	1.69	0.59	0.00
10	II-20	-213.72	0.58	2.03	10.30
11	II-30	-216.32	0.82	1.02	14.36
12	II-40	-218.14	1.18	1.02	17.37
13	II-50	-219.46	1.61	1.20	19.83
14	II-water15	-218.44	0.97	1.30	15 ¹⁾
15	II-water20	-220.61	1.30	0.88	20 ¹⁾
16	II-sugar syrup	-214.91	1.17	1.00	4.48 ²⁾
17	Natural III	-215.11	0.02	0.02	0.00
18	III-20	-222.50	0.48	0.69	13.06
19	III-30	-224.62	2.78	1.80	15.68
20	III-40	-227.80	2.19	0.95	20.85
21	III-50	-228.74	0.95	1.37	22.46
22	III-water15	-223.60	1.33	1.27	15 ¹⁾
23	III-water20	-228.22	1.70	1.51	20 ¹⁾
24	III-sugar syrup	-225.22	0.66	1.32	12.60 ²⁾
Average:			1.25	1.10	

* STDEV of all measurements of the same sample.

** Average STDEV for the same sample.

¹⁾ Dilution with tap water % (v/v).

²⁾ Expressed as percentage of beet sugar ethanol from sugar syrup in overall wine ethanol.

Precision of the measurements represents average value of srand sR: 1.175 ‰.

Table 3b. Ethanol CS-qNMR Results(ppm vs. DMSO)

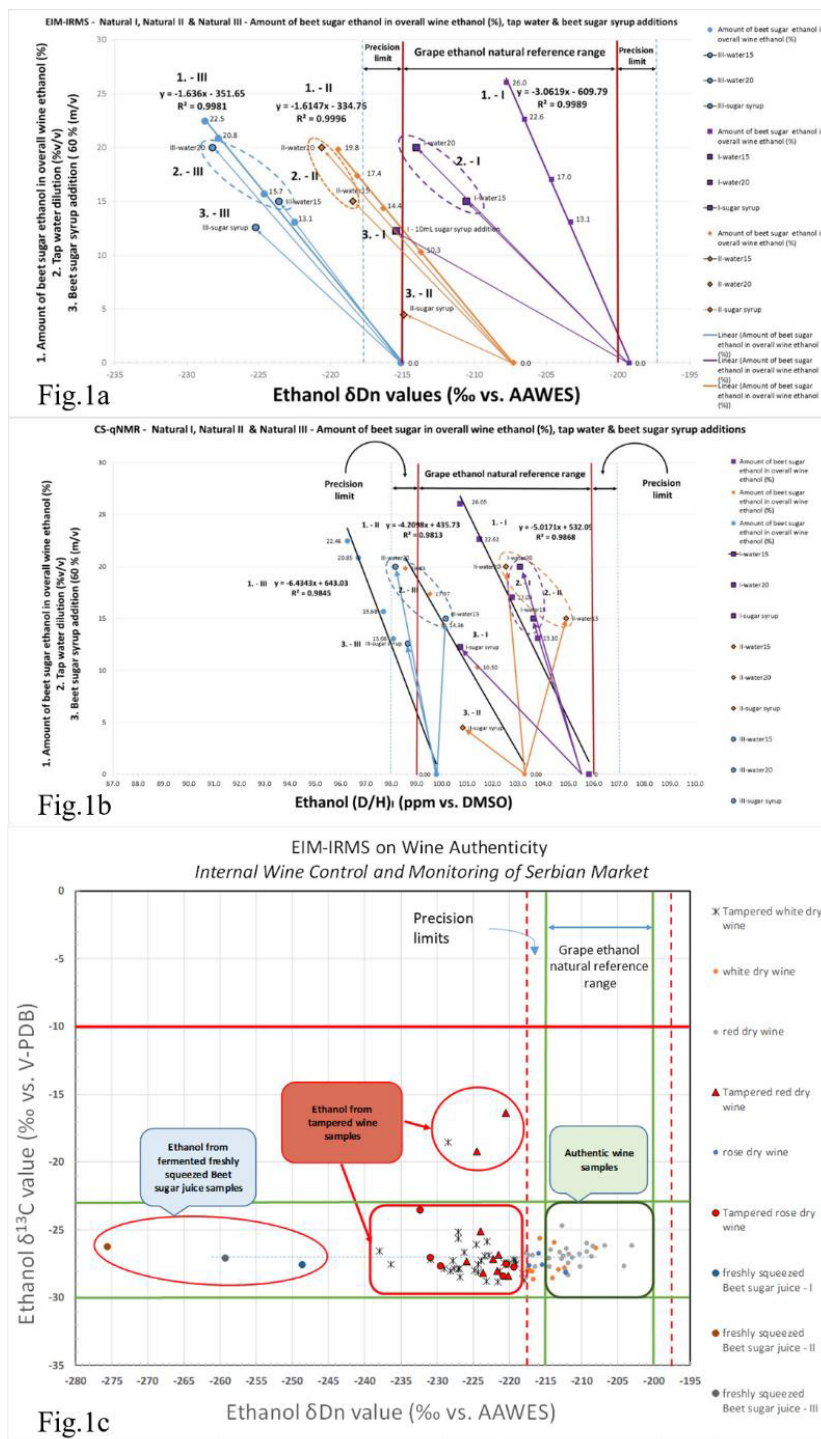
Sample No.	Sample name	(D/H) _i ⁺	(D/H) _{ii} ⁺	R-value	Amount of beet sugar ethanol in overall wine ethanol (%)
1	Natural I	105.81	135.35	2.57	0.00
2	I-20	103.79	136.63	2.61	13.10
3	I-30	102.78	131.65	2.56	17.04
4	I-40	101.47	134.96	2.66	22.62
5	I-50	100.72	134.06	2.69	26.05
6	I-water15	103.61	130.22	2.54	15 ¹⁾
7	I-water20	103.08	127.51	2.52	20 ¹⁾
8	I-sugar syrup	100.72	129.45	2.56	12.2 ²⁾
9	Natural II	103.27	133.87	2.52	0.00
10	II-20	101.41	130.45	2.53	10.30
11	II-30	100.07	134.47	2.67	14.36
12	II-40	99.52	130.26	2.55	17.37
13	II-50	98.56	129.97	2.60	19.83
14	II-water15	104.91	132.72	2.53	15 ¹⁾

15	II-water20	102.53	131.78	2.52	20 ¹⁾
16	II-sugar syrup	100.83	133.19	2.64	4.48 ²⁾
17	Natural III	99.78	131.81	2.60	0.00
18	III-20	98.07	132.99	2.66	13.06
19	III-30	97.68	131.10	2.61	15.68
20	III-40	96.70	129.97	2.66	20.85
21	III-50	96.26	129.82	2.66	22.46
22	III-water15	100.15	129.51	2.56	15 ¹⁾
23	III-water20	98.15	129.72	2.67	20 ¹⁾
24	III-sugar syrup	98.65	133.34	2.66	12.60 ²⁾

*Repeatability standard deviation 1ppm; Reproducibility standard deviation 1,4 ppm.

¹⁾ Dilution with water % (v/v).

²⁾ Expressed as percentage of sugar beet ethanol from sugar syrup in overall wine ethanol.



■ Natural I samples series
 ■ Natural II samples series
 ■ Natural III samples series

Figure 1. Graphical preview of EIM-IRMS[®] and CS-qNMR results on wine authenticity.

Based on the analytical values obtained from both instruments we recalculated the ethanol δD_n values from ‰ to ppm vs. V-SMOW by using the following Eq. (2) (Table 4):

$$D_{(\text{ppm vs. V-SMOW})} = (\delta D_n/6.42) + 155.76 \quad (2)$$

where 1ppm = 6.42‰ and 155.76 ppm represents Deuterium content in the International Standard V-SMOW.

Also we calculated relative average deuterium content of (D/H)_I and (D/H)_{II} in ppm vs. V-SMOW by using the next Eq. (3) (Table 4):

$$D_{(\text{ppm vs. V-SMOW})} = [(D/H)_I + (D/H)_{II}] / 2 \quad (3)$$

Table 4. Bias between EIM-IRMS[®] and CS-qNMR instrumental analytical methods.

Sample No.	Sample name	CS-qNMR			EIM-IRMS [®]				Difference in values (ppm)	Bias (%)
		(D/H) _I *	(D/H) _{II} *	Relative average Deuterium content from methyl and methylene group of ethanol (ppm vs. V-SMOW) [(D/H) _I + (D/H) _{II}]/2	Ethanol δD_n values (‰ vs. AAWES)	Intra-lab. repeatability ySt.dev. (%)	Relative deuterium content in ethylene (ppm vs. V-SMOW) [($\delta D_n/6.42$)+155.76]**	Intra-lab. Repeatability St.dev. (ppm) [$\delta D_n/6.42$]		
1	Natural I	105.81	135.35	120.58	-199.25	0.21	124.71	0.03	4.13	3.43
2	I-20	103.79	136.63	120.21	-203.31	1.64	124.08	0.26	3.87	3.22
3	I-30	102.78	131.65	117.22	-204.64	0.84	123.88	0.13	6.66	5.68
4	I-40	101.47	134.96	118.22	-206.53	0.89	123.58	0.14	5.36	4.54
5	I-50	100.72	134.06	117.39	-207.79	1.11	123.38	0.17	6.00	5.11
6	I-water15	103.61	130.22	116.92	-210.58	0.25	122.95	0.04	6.04	5.16
7	I-water20	103.08	127.51	115.30	-214.05	2.02	122.41	0.31	7.12	6.17
8	I-sugar syrup	100.72	129.45	115.09	-215.46	1.45	122.19	0.23	7.11	6.17
9	Natural II	103.27	133.87	118.57	-207.28	0.59	123.46	0.09	4.89	4.13
10	II-20	101.41	130.45	115.93	-213.72	2.03	122.46	0.32	6.53	5.63
11	II-30	100.07	134.47	117.27	-216.32	1.02	122.06	0.16	4.79	4.08
12	II-40	99.52	130.26	114.89	-218.14	1.02	121.77	0.16	6.88	5.99
13	II-50	98.56	129.97	114.27	-219.46	1.20	121.57	0.19	7.30	6.39
14	II-water15	104.91	132.72	118.82	-218.44	1.30	121.73	0.20	2.91	2.45
15	II-water20	102.53	131.78	117.16	-220.61	0.88	121.39	0.14	4.23	3.61
16	II-sugar syrup	100.83	133.19	117.01	-214.91	1.00	122.27	0.16	5.26	4.50
17	Natural III	99.78	131.81	115.80	-215.11	0.02	122.24	0.003	6.45	5.57
18	III-20	98.07	132.99	115.53	-222.50	0.69	121.09	0.11	5.56	4.82
19	III-30	97.68	131.10	114.39	-224.62	1.80	120.76	0.28	6.37	5.57
20	III-40	96.70	129.97	113.34	-227.80	0.95	120.27	0.15	6.93	6.12
21	III-50	96.26	129.82	113.04	-228.74	1.37	120.12	0.21	7.08	6.27
22	III-water15	100.15	129.51	114.83	-223.60	1.27	120.92	0.20	6.09	5.30
23	III-water20	98.15	129.72	113.94	-228.22	1.51	120.20	0.24	6.26	5.50
24	III-sugar syrup	98.65	133.34	116.00	-225.22	1.32	120.67	0.21	4.67	4.03
				Average:		1.10		0.17		4.98

*Repeatability standard deviation 1 ppm; Reproducibility standard deviation 1,4 ppm.

** Explanations: 1 ppm = 6.42‰ vs. V-SMOW; 155.76 ppm represents Deuterium content in V-SMOW International standard.

Further when we recalculate those values from ppm to ‰ using the correlation that 1ppm equals 6.42‰ at V-SMOW scale, then the repeatability standard deviation (sr) and reproducibility standard deviation (sR) for CS-qNMR equals to sr = 6.42‰ and sR = 8.99‰, which in comparison to EIM-IRMS[®] values are much bigger (EIM-IRMS[®]sr = 1.10‰ and sR = 1.25‰). For such precision limits of NMR method, EIM-IRMS[®] method detects addition of approx. 15% ethanol originating from sugar beet.

As it was earlier commented by Kalabin Gannady and his associates: “To date, a significant base of scientific knowledge on the intervals of the content of deuterium in methyl (D/H)_I and methylene (D/H)_{II} groups of ethanol molecule of grape origin has been accumulated according to the results of foreign researches. Taking into account agro climatic (geographical) and technological factors, the minimum and maximum levels for (D/H)_I and in

From obtained ethanol $\delta D_n(\delta(^2H)_n)$ values and (D/H)_I we have calculate intra-laboratory repeatability and reproducibility standard deviations (Table 3) for EIM-IRMS[®] (sr = 1.10‰; sR = 1.25‰) and for CS-qNMR (sr = 1 ppm; sR = 1.4 ppm) respectively.

By comparing obtained values in the same units (ppm) we have calculated average bias of 4.98% between EIM-IRMS[®] and CS-qNMR methods (Table 4).

By converting ethanol $\delta D_n(\delta(^2H)_n)$ values from ‰ to ppm we have observed that EIM-IRMS[®] has better precision in measurements then CS-qNMR which has repeatability standard deviation (sr) of 1 ppm, while EIM repeatability standard deviation sr (expressed in ppm) equals to 0.17 ppm (Table 4).

grape ethanol are 98.0 and 106 ppm respectively, and for the (D/H)_{II} - 121.0 and 136.0 ppm respectively. These ranges also include ethanol extracted from certain other fruits but not sugar beet. Sugar beet belongs like grape to the group of photosynthesis C3 pathway plants and photosynthesis C4 pathway plants (e.g. from corn, sugarcane, sorghum and others). The SNIF-NMR[®] method requires the use of NMR spectrometers, with an operating frequency of at least 400 MHz for 1N nuclei and equipped with a 10 mm tube sensor and a NMR signal stabilization system ¹⁹F. One of the features of the CS-qNMR method is that the stabilization of the resonance conditions with the ¹⁹F signal is not used. Therefore, the measurement of deuterium content in SN2- (D/H)_{II} and SN3- (D/H) and fragments of ethanol molecules will have significant errors in height of the corresponding peaks due to a small drift of the signals. However, this practically does not affect the areas that are measured in the quantitative NMR spectroscopy by the

integral but not by the height intensity. For that reason, with CS-qNMR integral intensities of signals are used. Another feature of the developed method is the use of dehydrated dimethyl sulfoxide (DMSO) as a calibration additive with deuterium content increased by two orders of magnitude. This reduction in 10 times decreases the volume fraction of the calibration of the substance and therefore halves the recording time of the NMR spectrum. The total error of this method provides a precision of the measured values $\pm 3\%$, which is quite sufficient to estimate the botanical origin of ethanol (Kalabin et al., 2018).

Furthermore, EIM-IRMS[®] results from analyzed industrial wine samples (Table 5) showed that for wine samples under 3, 10, and 11 we detected adulterations

since their ethanol δD_n values -237.46% vs. AAWES, st.dev. = 1.16‰; -226.18% vs. AAWES, st.dev. = 0.81‰ and -228.31% vs. AAWES, st.dev. = 1.01‰ respectively, are more negative and out of the proposed natural range for grape ethanol from -200% to -215% vs. AAWES. Even though that for two wine samples under 2 and 9, which originate from Viet Nam and Thailand we obtained ethanol δD_n values of -215.19% vs. AAWES, st.dev. = 0.30‰ and -208.35% vs. AAWES, st.dev. = 0.81‰ respectively and which values are falling into grape ethanol reference range from -200% to -215% vs. AAWES, their ethanol $\delta^{13}C$ values are more positive (-17.75% and -16.80%) which indicates addition of sugar originating from C4 plants (most probably sorghum or sugarcane).

Table 5. EIM-IRMS[®] results of industrial wine samples.

Sample No.	Sample name	Ethanol δD_n value (‰ vs. AAWES)	St. dev. (‰)	Ethanol $\delta^{13}C$ value (‰ vs. V-PDB)	St. dev. (‰)
1	Red wine from Moldova, 2011	-217.40	0.76	-26.86	0.09
2	Merlot, Red wine from Thailand, 2009	-215.19	0.30	-17.75	0.17
3	Red wine from Canada, Niagara Peninsula, 2014	-237.46	1.16	-26.46	0.02
4	Red wine from Toscana, Italy, 2011	-198.76	1.90	-26.93	0.06
5	Red wine from France, 2015	-207.21	1.14	-27.50	0.08
6	Red wine of Armenia, 2015	-200.79	1.70	-28.05	0.09
7	Red wine from Verona, Italy, 2011	-206.83	1.75	-27.00	0.05
8	Red wine from Bordo, France, 2014	-201.20	1.57	-26.92	0.04
9	Red wine from Viet Nam, 2015	-208.35	2.38	-16.80	0.12
10	Red wine from Germany, 2015	-226.18	0.81	-28.60	0.02
11	Red wine from Czech Republic, 2015	-228.31	1.01	-27.30	0.19
12	Red wine from Moldova, 2020	-211.47	1.97	-25.78	0.10

In addition, results for wine samples ($N=90$), analyzed from Republic of Serbia using EIM-IRMS[®], showed that adulterations have been detected in 48 wine samples, since their ethanol δD_n values were more negative than -215% vs. AAWES, taking also into consideration precision limits, i.e. from -217.93% to -237.96% vs. AAWES respectively. This represents 53.34% of the total number of analyzed samples. In continuation in 48 wine samples where we detected additions, 3 samples were found with a presence of ethanol originating from C4 sources (Corn). EIM-IRMS[®] first detected more negative ethanol δD_n values (-220.48% vs. AAWES, st.dev. = 0.56; -224.54% vs. AAWES, st.dev. = 0.96‰ and -228.50% vs. AAWES, st.dev. = 0.77‰) and then by measuring of $\delta^{13}C$ values in wine ethanol we confirmed the EIM-IRMS[®] findings (-16.39% vs. V-PDB, st.dev. = 0.02‰; -19.25% vs. V-PDB, st.dev. = 0.01 ‰ and -18.58% vs. V-PDB, st.dev. = 0.04 ‰) (Fig. 1c).

We also detected one example of manipulation with LOT number for the wine produced from the same producer. Two wine bottles from the same wine producer were picked up from two different locations on the market. Labeled LOT numbers were identical (L060117), but the information on the labels indicated that the time of bottling differed for more than 1.5 years between two bottles (12.07.2019 at 19.54h & 17.12.2020. at 11.14h). In wine sample no.1 we obtained ethanol δD_n value of -211.77% vs. AAWES, st.dev. = 0.56‰ and ethanol $\delta^{13}C$ value of -27.15% , st.dev. = 0.03‰. EIM-IRMS[®] detected

illegal adulteration in the sample no. 2 (Ethanol δD_n value of -224.51% vs. AAWES, st.dev. = 0.77‰), which was then confirmed with measurement of $\delta^{13}C$ value in ethanol -19.25% vs. V-PDB, st.dev. = 0.01‰ as confirmed presence of ethanol from C4 origin (Corn or Sugar Cane).

4 Conclusions

In this scientific paper, we did a comparative intra-laboratory performance study between the EIM-IRMS[®] and the CS-qNMR method. Based on the results obtained using the EIM-IRMS[®] method we detected 11 out of 21 of previously adulterated wine samples (Fig.1a) which represents a success rate of 52.38% detection of illegal practices (beet sugar, water or beet sugar syrup additions), while 47.62% of the obtained values remained in the natural ethanol δD_n range from -200% to -215% vs. AAWES. At the same time, using the CS-qNMR method, we detected only 3 of 21 samples of counterfeit wines (Fig.1b), representing 14.28% of the detection success of illegal practices, while almost 85.71% of the values obtained remained in the $(D/H)_I$ natural range of 98 ppm to 106 ppm. Furthermore, we have calculated bias between two methods of 4.98%, taking into consideration that the total error of CS-qNMR provides a precision of the measured values with $\pm 3\%$. Because of bigger sr as well as the fact that the total error of CS-qNMR method provides a precision of the measured values of $\pm 3\%$, NMR results are more scattered (from

112.04 ppm to 120.58 ppm) then those obtained from EIM-IRMS[®] (from 120.12 to 124.71 ppm) (Table 4).

On the other hand, our findings are in complete agreement with the previous statements from Segebarth Nicolas and his associates, including also Claude Guillou, as the team leader of (BEVABS), which reported that by using official OIV SNIF-NMR[®] method: “A sample is considered problematic if its ethanol (D/H)_I value falls out of the natural range. In the case of wine for instance, values under 99 ppm or over 105 ppm are suspicious. If average values for (D/H)_I are considered, then an evaluation of the detection limit for ethanol addition can be made for the different possible ethanol sources. This is only indicative since with this approach the addition of beet ethanol to a wine with high (D/H)_I value (105 ppm) would be detected only if its proportion is superior to 50%! If the geographic origin of the wine is known, then the natural (D/H)_I range can be reduced and the detection limit refined: it is case to case work necessitating supplementary information on the wine (grape growing region for instance). With this method, addition to wine of ethanol from distilled wine or fruit wines (apples, etc.) cannot be detected (all fruit alcohols have similar D/H ranges). If the ethanol added comes from grain, it will be detected only if its proportion is higher than 70% (Segebarth et al., 2008).

This also means that in over 80% of all tested samples (D/H)_I will stay inside the natural range from 98 ppm to 106 ppm. At that point, in all these cases, we are not talking about detection of sugaring or watering of wine, but only if tested wine is the original brand or some other wine. Inside of the natural (D/H)_I range we cannot talk about sugar or water addition, but only about possibility of another wine. Furthermore, for wine without designated origin, where there is no information about its origin used for comparison, SNIF-NMR[®] concept is useless since there is no reference information from isotopic databases for comparison with tested wine samples.

Earlier we have published EIM-IRMS[®] inter-laboratory study between four laboratories and we reported that the precision limits for EIM-IRMS[®] are $r = 2.7\%$ and $R = 2.13\%$ respectively (Smajlović et al., 2019).

Transferring those EIM-IRMS[®] precision limits, which we have reported in our earlier publication, from ‰ to ppm units, where 1 ppm equals 6.42‰, we obtain values of $r = 0.42$ ppm and $R = 0.33$ ppm respectively. By comparing of those values with precision limits from the official OIV-MA-AS311-05 (SNIF-NMR[®]) method published in the OIV Compendium of International Methods of Wine And Must Analysis and which were obtained also through inter-laboratory study between three laboratories, we can observe that precision of EIM-IRMS[®] is better than those reported for SNIF-NMR[®] (Precision limits for (D/H)_I: $r = 0.72$ ppm and $R = 0.99$ ppm and precision limits for (D/H)_{II}: $r = 0.84$ ppm and $R = 1.75$ ppm) (OIV-MA-AS311-05, 2019).

EIM-IRMS[®] determines the content of non-exchangeable stable hydrogen isotopes derived from the methyl and methylene sites of the tested ethanol. In this way, EIM-IRMS[®] quickly identifies illegal wine

production practices regardless of whether it is an illegal addition of sugar, or water, or both. If any of these practices are carried out during wine production, the δD_n value of ethanol would be changed in relation to the natural range for wine ethanol from -200‰ to -215‰ vs. AAVES. As previously suggested by Eric Jamin and his associates, by subsequent verification of the correlation of stable oxygen in wine ethanol and wine water of the tested wine sample using the same IRMS instrument further illegal water addition can be confirmed (Jaminet al., 2003; Perini & Camin, 2013). By comparing the values of δD_n , $\delta^{18}O$ and $\delta^{13}C$ of wine ethanol and $\delta^{18}O$ value in wine water and using the method of rejection, it is very easy to determine which illegal practice was used, illegal addition of sugar, illegal addition of water or both.

Knowing this, another aspect of this whole study is related to the practicality of testing the authenticity of wine at European level. The EIM-IRMS[®] shows better possibility in detecting wine adulteration than the official NMR OIV method in routine laboratory work and that many more problematic samples can be detected by using the EIM-IRMS[®] concept. This is very important given the current market situation, costs and financial losses to which the European Union is exposed each year due to adulteration and counterfeiting of wine and other food products, even though there is already a very costly system of national reference laboratories with the JRC working together to identify illegal wine production practices such as the addition of sugar, water or sugar syrup and protection of wines with a designated origin. From this all one question arises: “Could this all be done in a much more practical way, and with a higher percentage of success, while keeping the overall costs lower?” So far, mostly NRL have dealt with these wine and food authentication testing using stable isotopes, because the official SNIF-NMR[®] equipment used is horribly expensive, not only costly, but also financially demanding, so these types of jobs are entrusted only to national reference laboratories that could withstand that burden. Also due to the high maintenance costs the analysis prices were high and still are, causing the number of samples tested to be lower.

The development of EIM technology, which allows testing of a much larger number of samples, opens up the possibility of raising the authenticity of wine and food in general to a much higher level and entrusting these jobs also to other accredited laboratories in the market which would lower the **prices** of testing, open up the market where authentication testing and illegal practices will become accessible to everyone, and thus increase the security of products from legal manufacturers not only in the European, but also in the world market.

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