Authenticity testing of Cypriot Wines by using different spectroscopic techniques

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ABSTRACT

The authenticity of 141 wines produced in Cyprus from both indigenous and other vine varieties was investigated by a holistic approach, using advanced technology such as SNIF-NMR and IR-MS for the determination of the stable isotopes and ICP spectroscopy for some heavy metals. The spectroscopic characteristics were evaluated statistically using different chemometric methods. The dependency of the deuterium/hydrogen ratio of the methylene site in the ethanol molecule (D/H)II and also the $\delta^{18}O$ values of the wine water, were the most useful discriminators. Isotopic results allow us to have a complete idea about the regional variability of the isotopes. Among the metals, Ni followed by Pb were the ones with the highest discrimination value. The determined values were within the allowed concentrations in food for human consumption, according to results of studies carried out in different countries and the proposed method of analysis which is expected to be adopted by the OIV committee. The preliminary results of the study of the correlation between the load of heavy metals and isotopes in wines showed a dependence on the grape variety but not the geographical location of the vineyard. This is probably due to the close proximity of wine regions in Cyprus.

Keywords: SNIF-NMR, IR-MS, ICP, Cypriot wines, Stable isotopes, Heavy metals, Chemometrics.

INTRODUCTION

Sophisticated stable isotope analyses are proved to be useful tools in the study of authenticity of food and beverages. Especially for wines, the official method requires the determinations of the site-specific $^{2}H/^{1}H$ ratios of ethanol in wine, the measurements of $^{18}O/^{16}O$ in wine water, and also the $^{13}C/^{12}C$ ratio measurements on wine ethanol [1,2]. Using these methods, scientists from European countries have announced several interesting results about the authenticity and geographical origin of wines [3,4].

Also, there are some publications which connect the load of metals with the origin of the samples [5-7]. In a previous publication we reported the use of the concentration of trace elements to differentiate the Cypriot spirit “zivania” from other alcoholic spirits [8]. We believe that this basis for differentiation a traditional

product is related to the variety of grapes produced in Cyprus and the geological and special climatic conditions existing in the island of Cyprus. However, the determination of heavy metals in wines associated with consumer safety has not been studied systematically. Heavy metals are toxic to biological systems because of their negative impact or physiological functions of some enzymes, and the determination of these elements is of considerable importance due to their potential toxic effects.

According to European Regulations, the State General Laboratory of Cyprus collaborates closely with the Wine Products Council (which is the competent authority for the implementation of Regulation 555/2008) and the Viticulture and Oenology Section of the Ministry of Agriculture, National Resources and Environment, for the establishment of the European Wine Databank which is coordinated by the Joint Research Center at ISPRA. At the same time the Cyprus National Bank of Wines is well in process of completion. The isotopic ratios of Deuterium, Carbon and Oxygen are determined in order to certify the origin of wines and wine products.

The work presented here is a part of a project that is co-funded by the European Regional Development Fund and the Republic of Cyprus through the Research Promotion Foundation (Project ΥΓΕΙΑ/ΤΡΟΦΗ/0609 (ΒΙΕ)/09). For the complete study of authentic Cypriot wine the partners in the project are carrying out different studies for the mapping of the isotopic, metabolic, antioxidant and elemental profiling of local wines from both indigenous and other vine varieties. The study includes both the determination of parameters related to authenticity (stable isotopes of deuterium, oxygen and carbon) [9,10] and factors that affect the health of consumers (heavy metals). Also, the investigation whether the metals Cd, Ni, Pb, Cr could be considered as good indicators of wine authenticity, since they are neither metabolized nor modified during the whole process of vinification. The data were evaluated using PCA, CA and RDA chemometric techniques [11,12].

MATERIALS AND METHODS

SAMPLES ANALYSED:

The total number of samples examined was 141 (94 red and 47 white wines), that includes 105 authentic wines and 36 commercial wines (25 red and 11 white) from the Cypriot market.

The authentic wines belong to the Cypriot Wine Databank according to EU Reg. 2729/2000, from 3 different wine-growing regions (Lemesos, Larnaca and Pafos) and 5 vintages (2006-2010):

- Vintage 2006: 25 samples (16 red and 9 white)
- Vintage 2007: 23 samples (13 red and 10 white)
- Vintage 2008: 23 samples (12 red and 11 white)
- Vintage 2009: 20 samples (16 red and 4 white)
- Vintage 2010: 14 samples (12 red and 2 white).

The study included wines from both indigenous (Xynisteri, Maratheftiko, Oftalmo, Mavro, Giannoudi) and other varieties (Cabernet Sauvignon, Shiraz).

DETERMINATIONS:
Samples were analyzed to determine the alcoholic grade, the isotopic ratios (D/H)I, (D/H)II, R, 13C/12C, 18O/16O and the heavy metals Cd, Ni, Pb, Cr.

***PREPARATION OF SAMPLES*:**
For stable isotope determinations all the samples, both for analysis by SNIF-NMR and IR-MS, were distilled to extract the ethanol using ADCS (EUROFINS) system. The water content in the distillates was measured by the Karl-Fischer (Metrhom) apparatus. The alcohol obtained was analyzed according to the method described in European Regulation 2676/1990 [2,4], which has been validated and accredited by the laboratory.

For the determination of heavy metals by ICP, an aliquot of 20 ml of each wine sample was diluted in 100 ml of distilled water and then filtered. Additional dilutions took place, in order the level of metals become measurable by ICP. Standard solutions were prepared from monoelemental stock solutions (MERCK) using HNO_3 as diluent [7,8].

***EXPERIMENTAL PROCEDURES*:**
The isotopic measurements were carried out by following the standard method described in the European Regulation 2676/90 for wines:

- Alcoholic grade was measured with a DMA-5000 density meter.
- NMR spectra were recorded on a BRUKER 400MHz NMR spectrometer, using X-WIN NMR 3.1 software for the processing of the spectra and EUROSPEC (EUROFINS) software for the calculations.
- IR-MS determination of 13C/12C was carried out with a EuroEA 3024- Iso Prime (GV) instrument and the determination of 18O/16O was carried out by using a Multiflow Unit with Liquid Handler.
- Heavy metals were measured using a Thermo Inductively Coupled Plasma spectrometer. All determinations were made twice and the mean was retained.

DATA ANALYSIS:

Pattern recognition analysis use of statistical methods for the processing of the results [4]. It was performed by means of the statistical package SCAN, Software for Chemometric Analysis [13].
Each wine sample (object) was considered as an assembly of 9 variables represented by the data. These variables (called “feature”) formed a “data vector” which represented a sample. Data vectors belonging to the same group, as defined by geographical origin were analyzed. Pattern recognition tools were used in this work as follows:

Principal Component Analysis (PCA):

This procedure was used mainly to achieve a reduction of dimensionality by detecting clustering and enabling the detection of “markers”.

Cluster Analysis (CA):

This technique comprises an unsupervised classification procedure which involves measurement of either the distance or the similarity between objects in a cluster. Clusters are defined by an algorithm. Objects are grouped in clusters in terms of their similarity or nearness.

Regularized Discriminant Analysis (RDA): The purpose of the regularization is to reduce the variance related to the sample-based estimates and performs well even the dimensionality of the sample space is small, compared to the dimension of the measurement space. It uses a complex biasing scheme to get better class covariance matrix estimates. The biasing is controlled by two parameters: lambda and gamma and their values can be chosen by cross-validation.

The **Data analysis** was performed in the following steps:

i. Preliminary data analysis by Principal Component Analysis and Hierarchical Cluster Analysis, using the complete data set.
ii. Classification RDA technique was applied to the complete data set, with a category arrangement:
    Category 1, training set of 94 authentic red wines, Category 2 comprising of 47 authentic white wines, Category 3 consisting of 25 commercial red wines (from the local market) and Category 4 comprising 11 commercial white wines.
iii. The reliability of the classification obtained before was checked. The 141 objects were randomly divided between training set and prediction set.
iv. As it is practical important to know the minimum number of features needed to obtain a correct classification, we chose those that contained the most discriminate information.

RESULTS AND DISCUSSION

Stable Isotope Data was evaluated statistically using different chemometric methods.

PCA was performed using Singular Value Decomposition (SVD) algorithm to calculate all components together. Each principal component is orthogonal and is a linear combination of the original variables. In **Figure 1**, the wines are plotted for principal components 2 and 3 in a scatter plot. The dependency of the deuterium/hydrogen ratio of the methylene site in the ethanol molecule (D/H)II and also the δ18O values of the wine water, were the most useful discriminators. Isotopic results allow us to have a complete idea about the regional variability of the isotope. The separation for wines from Larnaca, Lemesos and Pafos was good, except samples from wine-growing villages in the immediate proximity to Lemesos and Pafos. Similar results were obtained for different vintages.

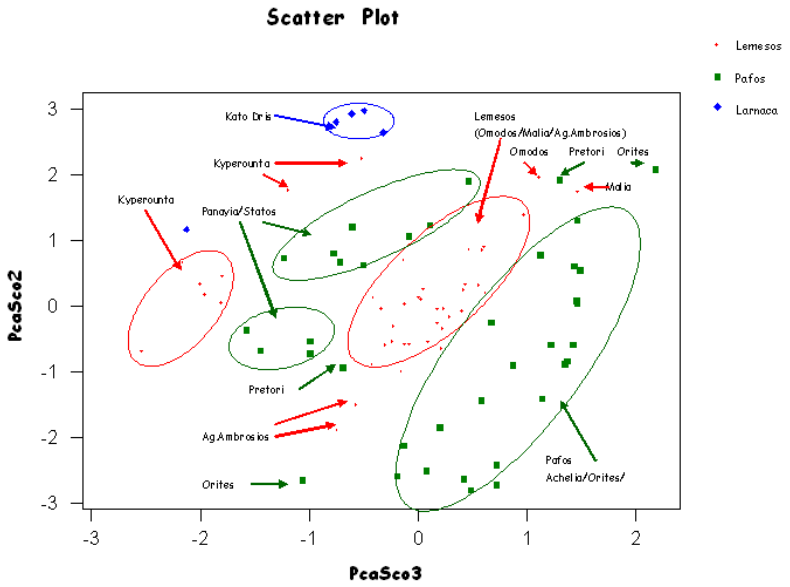

Figure 1: Distribution of Cypriot wines (vintages 2006-2010) in the plane defined by the 2nd and 3rd PCs, using $(D/H)_I$, $(D/H)_{II}$, R, ^{13}C and ^{18}O isotopic ratios.

Similar behaviour was also observed in case of analysis with **RDA** that was applied to the whole data. The results are shown as a dendrogram in **Figure 2**. For model selection, the cross-validate Fk was used with leave-one-out method. The class centroid profile plots show how each group is separated by coordinates. Finally, the reliability of the classification was tested. The percentage of objects placed in the evaluation set was 25%. The samples were randomly divided between the training and the prediction set. The previous division procedure was repeated 10 times, to obtain a good evaluation of recognition and prediction ability. The misclassification matrix calculated without and with-one out-cross validation shows how well the classes are separated. The misclassified samples were originated from wine-growing villages in the immediate proximity to Lemesos and Pafos, as given by the PCA. The analysis gave a total recognition ability of 71.4%. The cross-validated prediction gave similar results.
96%. The cross-validated prediction gave almost similar results.

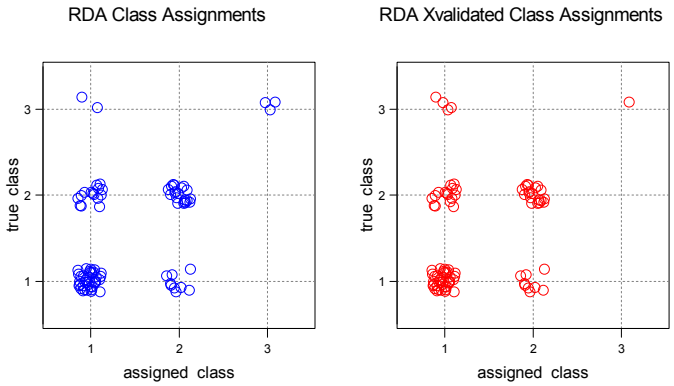

Figure 2: Class assignment plot for Cypriot wines from 3 different regions by RDA (1: Lemesos, 2: Pafos and 3: Larnaca).

Heavy metals concentrations in the examined samples are presented in **Table 1** that shows the mean value, the standard deviation and the range of the measured values for both type red and white wine from indigenous and other varieties. The determined values were within the allowed levels for human consumption, according to results of studies carried out in different countries and the proposed method of analysis which is expected to be adopted by the OIV committee. The concentrations for Pb and Ni, were in higher levels (but still safe) of Cr and Cd, as a result of agricultural treatments in vinegards.

Table 1: Heavy metals load (mean, std, min-max) in wines, according to their type.

Samples	Category	Cd (ppb)	Cr (ppb)	Ni (ppb)	Pb (ppb)
Type	**White Wine**	**1.8** (0.7) 0.8-2.5	**13.9** (3.1) 10.0-18.0	**15.5** (5.5) 8.0-30.2	**19.1** (12.0) 6.0-44.0
	Red Wine	**1.8** (0.8) 0.5-3.0	**14.6** (4.3) 8.8-25.0	**29.0** (12.9) 7.5-68.5	**41.8** (32.3) 9.3-131.8
Variety	**Indigenous**	**1.8** (0.8) 0.8-2.8	**14.2** (3.1) 9.5-21.1	**24.2** (14.5) 8.0-68.5	**35.1** (29.3) 6.0-131.8
	Others	**2.1** (0.8) 0.8-3.0	**14.4** (4.8) 8.8-25.0	**25.8** (10.2) 7.5-48.9	**34.8** (30.5) 9.3-125.0

All the data obtained for 50 samples, were analysed statistically for studying the correlation between the load in heavy metals and the stable isotopes, for authenticity purposes. Different chemometric techniques were applied, after autoscaling, because their units of measurement were different and not comparable between them. The PCA loading plot in **Figure 3**, shows (D/H)II and δ18O values as the most useful discriminator. Among the metals, Ni followed by Pb was that with the highest discrimination value.

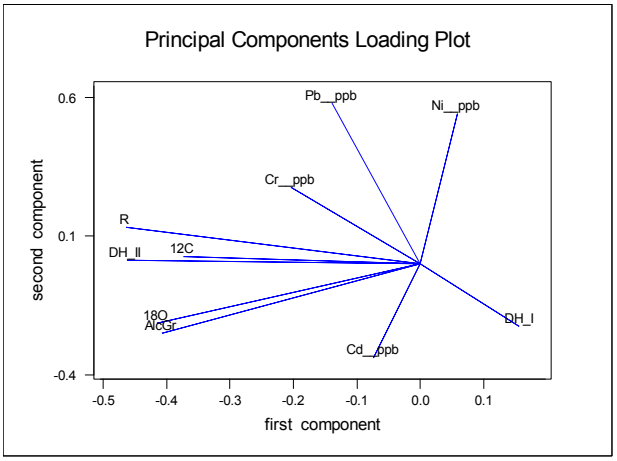

Figure 3: Loading plot for stable isotopes and heavy metals determined in141 samples.

CA is used for searching for natural grouping among the samples. It describes the nearness between samples, using the Euclidean distance between each wine from the rest. The results are shown as a dendrogram in **Figure 4**. Examination of the dendrogram at a similarity level of 75% shows very interesting results: five clusters are formed mainly due to the type of vinification (red or white) and the variety of the grapes and less on geographical origin.

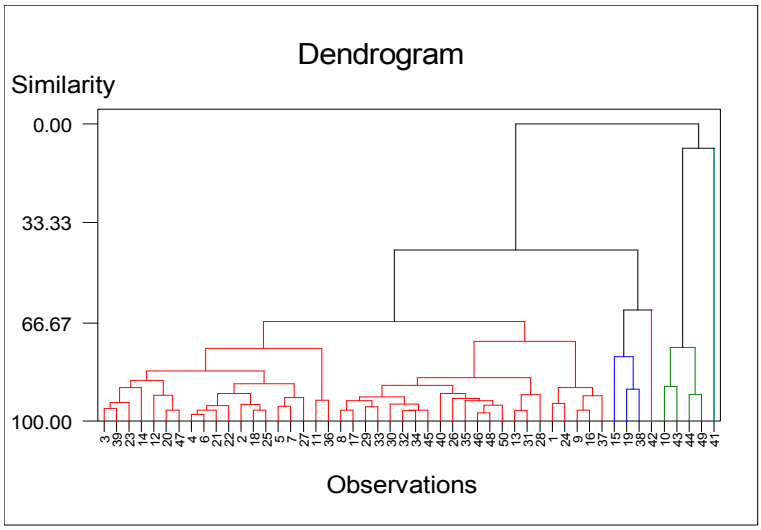

Figure 4: Dendrogram of cluster analysis of 50 wines.

RDA was applied to an initial matrix containing the 50 objects and the 9 variables. The misclassification matrix calculated without and with cross-validation shows how well the classes are separated (see **Table 2** and **Figure 5** for the graphical presentation). The recognition ability for red wines was highly satisfactory; only one sample out of 37 was misclassified. This wine was not produced from indigenous varieties. For white wines, the percentage of correct classification was less successful (only 61.5%). Wines from local variety "xynisteri" was classified correctly. The total recognition ability was 88.0%. The cross-validated error is a measure of the correct prediction, which gave the same results.

Table 2: Classification with RDA (all features)

			Assigned classes		
	True class	Total number	1	2	% correct recognition
Red wines	1	37	36	1	97.3
White wines	2	13	5	8	61.5
		% error rate: 12.0			
Cross-validation	True class	Total number	1	2	% correct recognition
Red wines	1	37	36	1	97.3
White wines	2	13	5	8	61.5
		% error rate: 12.0			

CONCLUSIONS

The study reported here shows that the stable isotope ratios of D/H, 18O/16O and 13C/12C of 141 samples provided a complete picture on the regional variability of the isotopes. The dependency of the deuterium/hydrogen ratio of the methylene site in the ethanol molecule (D/H)II and also the δ18O values of the wine water, were the most useful discriminators. The separation for wines from different districts was good, with the exception of samples from wine-growing villages in the immediate proximity to Lemesos and Pafos. Similar results were obtained for different vintages. The method can be used to investigate the authenticity of commercial wines, when they are compared to the authentic samples from the same region.

The determined concentrations of heavy metals Pb, Ni, Cr and Cd that are related to the safety of wines were within the safe levels for human consumption.

Although the correlation of stable isotopes and heavy metals for authenticity purposes is still under investigation, preliminary results show that it was able to differentiate Cypriot wines mainly due to the type of vinification (red or white) and the variety of the grapes and less on geographical origin. This is expected because of the extent of the Cypriot vineyards and their similar climatic conditions.

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REFERENCES

1. G.J. Martin, C. Guillou, M.L. Martin, M.T. Cabanis, T. Yutthay and J. Aerny, Natural Factors of Isotope Fractionation and the Characterization of Wines, J. Agric. Food Chem. 36, **1988**, 316-322.
2. G.J. Martin, B.L. Zhang, N. Naulet and M.L. Martin, Deuterium Transfer in the Bioconversion of Glucose to Ethanol Studied by Specific Isotope Labeling at the Natural Abundance Level, J. Am. Chem. Soc., **1986**, 108, 5116-5122.
3. T. Bigwood, M. Sharman, A. Aldus and M.J, Dennis, “Global Variations in the Deuterium/ Hydrogen Isotope Ratios of Wine”, Journal of Wine Research, **1998**, 9, 155-166.
4. A. Robmann, F. Reniero, I. Moussa, H.L. Schmidt, G. Versini, M.H. Merle, “Stable oxygen isotope content of water of EU data-bank wines from Italy, France and Germany”, Z.Lebensm Unters Forsch A, **1999**, 208:400-407.
5. C.Herrero and B.Medina, Use of Some Mineral Elements in Differentiation of Galicia Wines, Connais. Vigne Vin **1990**, 24, 147-156.
6. S.Frias, J.P.P.Trujillo, E.M.Pena, J.E.Conde. Classification and differentiation of bottled sweet wines of Canary Islands (Spain) by their metallic content, Eur Food Res Technol **2001** 213,145-149.

7. D. Kostic, S. Mitic, G. Miletic, S. Despotovic, A. Zarubica, “The concentrations of Fe, Cu and Zn in selected wines from South-East Serbia”, Journal of Serbian Chemical Society, **2010**, 75, 1701- 1709.
8. R. Kokkinofta, P. Petrakis, T. Mavromoustakos and C.R. Theocharis, “Authenticity of the traditional Cypriot spirit “Zivania” on the basis of metal content using a combination of Coupled Plasma Spectroscopy and Statistical Analysis”, Journal of Agricultural and Food Chemistry, **2003**, 51, 6233- 6239.
9. European Commission: Food Authenticity- Issues and Methologies, Eurofins Scientific **1998**.
10. I.Kosir, M. Kocjancic, N.Ogrinc, J.Kidric, Use of SNIF-NMR and IRMS in combination with chemometric methods for the determination of capitalization and geographical origin of wines, Analytica Ch. **2001**, 429, 195-206.
11. E.K.Kemsley, Discriminant Analysis and Glass Modelling of Spectroscopic Data, John Willey & Sons **1998**.
12. R.I.Kokkinofta – C.R.Theocharis, “Chemometric Characterization of the Cypriot Spirit “Zivania”, Journal of Agricultural and Food Chemistry, **2005**, 53, 5067- 5073.
13. Minitab, Inc. Software for Chemometrics Analysis (Users’ Manual); State College, PA, **1995**.