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Authenticity of the Traditional Cypriot Spirit "Zivania" on the Basis of 1H NMR Spectroscopy Diagnostic Parameters and Statistical Analysis

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A previous publication (Kokkinofta et al. *J. Agric. Food Chem.* **2003,** *51,* 6233–6239) discussed the use of inductively coupled plasma spectroscopy to differentiate between the traditional Cypriot alcoholic beverage zivania and other spirits similar in alcoholic content collected from different countries. In the present paper 1H NMR spectroscopy is applied to confirm the previous conclusions and to obtain additional physical–chemical characteristics that may be used to differentiate zivania from other similar beverages. NMR spectroscopy gave a satisfactory degree of prediction and classification between zivanias and other distillings. The validity of quantification of the method was tested using comparative GC data. It appears that chemical analysis can be very helpful for identifying the unique geological and climatic conditions existing in the island of Cyprus that lead to an authentic product.

KEYWORDS: Zivania; alcoholic beverage; NMR spectroscopy; classification; statistical analysis

INTRODUCTION

Zivania is currently a popular alcoholic drink with increasing importance to the Cypriot economy. Details of its properties and production are given in our previous publication (*1*). Given the proven commercial value of the product, it is necessary to establish zivania as a traditional spirit with a unique composition.

Specifically, inductively coupled plasma spectroscopy (ICP) was used to differentiate between the traditional alcoholic beverage zivania and other spirits similar in alcoholic degree. In this paper it has been shown that canonical discriminant analysis (CDA) and classification binary tree (CBT) analyses revealed the occurrence of grouping between the analyzed samples, according to their metal content. Furthermore, the combined Mg, Zn, and Cu content of soils in Cyprus were deemed to be characteristic. These promising results prompted us to initiate a research activity using complementary methodologies aiming to increase the predictive capability of the ICP methodology. From a search of the literature, many methodologies appeared to apply: SNIF-NMR/IRMS (*2–7*), 1D and 2D NMR spectroscopy (*8–11*), GC, GC-MS (*12–14*), FT-IR (*15,*

16), UV (*15*), capillary electrophoresis (*17, 18*), rheological properties (*19*), molecular techniques (*20*), SPME-GC (*21*), sensory analysis (*22, 23*), ICP, ICP-MS, ICP-OES (*1, 24–26*), flame atomic absorption spectrophotometry (*27*), and HPICE (*25*).

1H and 13C NMR spectroscopy as well as the site-specific natural isotope fractionation (SNIF) NMR technique are among the most applicable methodologies and are considered to be the most powerful tools for the characterization of the geographic origin and authenticity of alcoholic beverages (*9–11, 28, 29*).

1H NMR methodology has been chosen because it combines several advantages: (i) simplicity; (ii) rapidity; (iii) reproducibility; (iv) quantitative character; (v) nondestructive application; and (vi) automation. The only disadvantage of the method is the expensive instrumentation. Sample preparation involves mixing of the alcoholic beverage with a specified weighted amount of pyrazine. Pyrazine was added to quantify the NMR data. Thus, the area of a particular peak was directly compared to the area of the pyrazine peak, which corresponded to a known mass. Pyrazine gives only a single peak at ∼8.85 ppm, which does not coincide with any of the observed peaks. Pyrazine was used successfully in our effort to develop a novel analytical method to detect adulteration of virgin olive oil by other oils as described in our previous publication (*28*). Deuterated benzene stored in a coaxial tube was used as an external

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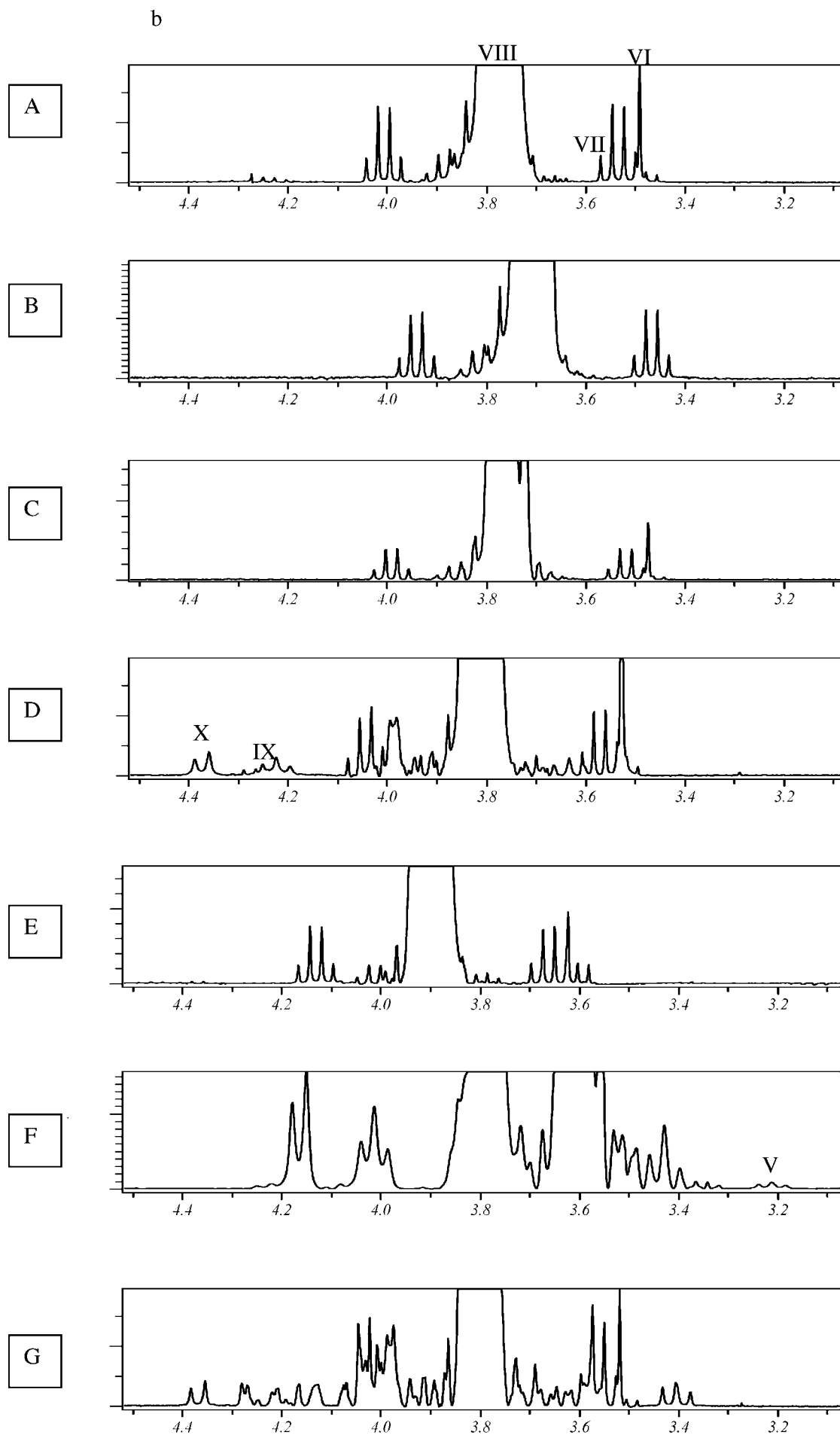§ National and Kapodistrian University of Athens.
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Table 1. Sample Identification and Diagnostic Parameters Derived from NMR Experiments

				chemical shift		integral (ref to pyrazine = 1)					ratio of intensities						
label[a]	sample	origin	botanical origin	CH$_2$ G	CH$_3$ J	CH$_2$ S	CH$_3$ T	CH$_3$OH cont M1	CH$_3$- of higher alcohols F	CH$_3$- sat right V	M/R	N1/P	R6/P	R7/P	R4/P	R1/P	R2/P
1.1	zivania	Kykkos monastery	grape marc	3.79	1.33	11.10	17.90	0.04	0.06	0.09	5.08	0.11	0.00	0.00	0.00	0.00	0.00
1.2	zivania	Kykkos monastery	grape marc	3.78	1.33	13.91	21.58	0.03	0.10	0.11	5.40	0.07	0.00	0.00	0.00	0.00	0.00
1.3	zivania	Kykkos monastery	grape marc	3.77	1.31	10.05	16.81	0.03	0.06	0.09	5.55	0.11	0.00	0.00	0.00	0.00	0.00
1.4	zivania	Kykkos monastery	grape marc	3.81	1.35	11.32	18.64	0.04	0.05	0.10	5.86	0.06	0.00	0.00	0.00	0.00	0.00
1.5	zivania	Kykkos monastery	grape marc	3.81	1.35	13.04	21.12	0.04	0.06	0.11	5.69	0.07	0.00	0.00	0.00	0.00	0.00
1.6	zivania	Kykkos monastery	grape marc	3.81	1.35	12.20	19.03	0.05	0.05	0.10	5.55	0.09	0.00	0.00	0.00	0.00	0.00
2.1	zivania	Kaminaria	grape marc	3.81	1.35	12.37	19.15	0.03	0.05	0.10	4.73	0.11	0.00	0.00	0.00	0.00	0.00
2.2	zivania	Kellaki	grape marc	3.81	1.35	16.37	24.46	0.11	0.08	0.13	11.39	0.07	0.00	0.00	0.00	0.00	0.00
2.3	zivania	Chrysoroyatissa	grape marc	3.82	1.36	18.34	26.53	0.08	0.08	0.14	8.97	0.91	0.00	0.00	0.00	0.00	0.00
2.4	zivania	Omodos	grape marc	3.78	1.32	11.80	18.34	0.09	0.07	0.10	11.61	0.06	0.00	0.00	0.00	0.00	0.00
2.5	zivania	Ag. Varvara	grape marc	3.79	1.34	16.46	24.07	0.06	0.11	0.12	7.91	0.96	0.00	0.00	0.00	0.00	0.00
2.6	zivania	Fterikoudia	grape marc	3.78	1.33	15.18	22.51	0.04	0.07	0.11	4.71	0.04	0.00	0.00	0.00	0.00	0.00
2.7	zivania	Lagoudera	grape marc	3.81	1.36	12.48	21.06	0.04	0.10	0.11	4.44	0.21	0.00	0.00	0.00	0.00	0.00
2.8	zivania	Pelendri	grape marc	3.82	1.37	15.35	23.76	0.05	0.08	0.12	5.64	0.07	0.00	0.00	0.00	0.00	0.00
2.9	zivania	Sodap	grape marc	3.78	1.33	11.50	18.57	0.04	0.06	0.10	5.70	0.09	0.00	0.00	0.00	0.00	0.00
2.10	zivania	Loel	grape marc	3.78	1.32	10.53	18.41	0.04	0.05	0.09	6.39	0.34	0.00	0.00	0.00	0.00	0.00
2.11	zivania	Vinco	grape marc	3.77	1.32	11.15	16.61	0.03	0.05	0.09	5.84	0.16	0.00	0.00	0.00	0.00	0.00
2.12	zivania	Grusaders	grape marc	3.78	1.33	12.42	20.07	0.04	0.06	0.09	3.62	0.06	0.00	0.00	0.00	0.00	0.00
2.13	zivania	Vounil	grape marc	3.8	1.33	13.73	20.86	0.04	0.06	0.11	5.65	0.12	0.00	0.00	0.00	0.00	0.00
2.14	zivania	Damaris	grape marc	3.79	1.33	12.39	21.74	0.02	0.10	0.12	3.72	0.08	0.00	0.00	0.00	0.00	0.00
2.15	zivania	Keo	grape marc	3.79	1.33	13.76	21.62	0.05	0.06	0.10	5.66	0.12	0.00	0.00	0.00	0.00	0.00
2.16	zivania	with extracts	grape marc	3.76	1.3	15.26	22.77	0.07	0.07	0.12	7.74	0.48	0.03	0.13	0.20	0.17	0.03
3.1	zivania	red	grape marc (addition of cinnamon)	3.8	1.34	9.06	13.99	0.03	0.05	0.07	4.05	0.04	0.16	0.00	0.18	0.15	0.14
3.2	zivania	red	grape marc (addition of cinnamon)	3.7	1.24	10.20	15.17	0.03	0.05	0.08	2.18	0.26	1.12	0.00	0.11	0.10	1.03
3.3	zivania	red	grape marc (addition of cinnamon)	3.69	1.24	9.89	14.47	0.03	0.04	0.07	2.67	0.26	1.02	0.00	0.11	0.09	0.94
4.1	raki zinovia	Cyprus	grape marc	3.78	1.32	10.85	18.49	0.01	0.02	0.10	1.93	0.05	0.00	0.00	0.00	0.00	0.00
4.2	yeni raki	Cyprus	grape marc	3.78	1.32	10.93	16.12	0.01	0.04	0.08	2.30	0.03	0.00	0.04	0.06	0.07	0.00
4.3	rakea	Boulgary	grape marc	3.77	1.31	10.88	16.87	0.01	0.05	0.09	1.78	0.20	0.00	0.00	0.00	0.00	0.00
4.4	rakea	Boulgary	grape marc	3.75	1.29	7.93	12.75	0.01	0.03	0.07	1.40	0.06	0.00	0.00	0.00	0.00	0.00
4.5	tsipouro	Greece, Macedonia	grape marc	3.78	1.3	10.11	15.10	0.04	0.06	0.07	6.08	0.05	0.08	0.00	0.00	0.06	0.11
4.6	tsipouro	Greece, Agio Oros	grape marc	3.69	1.22	3.56	5.85	0.01	0.02	0.03	3.81	0.00	0.00	0.51	0.85	0.64	0.00
4.7	tsipouro	Greece, Tyrnavos	grape marc	3.66	1.19	0.76	1.43	0.01	0.01	0.01	15.81	0.00	0.00	0.00	0.00	0.00	0.00
4.8	tsipouro	Greece, Macedonia	grape marc	3.77	1.31	11.98	17.63	0.04	0.07	0.09	5.88	0.05	0.07	0.00	0.00	0.05	0.10
4.9	tsipouro	Greece	grape marc	3.77	1.31	9.97	14.66	0.06	0.06	0.08	10.48	0.16	0.00	0.00	0.00	0.00	0.00
4.10	tsikoudia	Greece, Crete	grape marc	3.68	1.2	2.29	3.32	0.01	0.01	0.02	4.69	0.81	0.00	0.00	0.00	0.00	0.00
4.11	grappa	Italy	grape marc	3.81	1.35	9.12	15.67	0.16	0.05	0.08	25.53	0.16	0.00	0.12	0.16	0.19	0.00
4.12	grappa bianca	Italy	grape marc	3.78	1.31	9.88	16.06	0.03	0.07	0.08	12.39	0.04	0.00	0.00	0.00	0.00	0.00
4.13	segrel	Spain	grape marc	3.77	1.32	15.68	22.72	0.35	0.10	0.12	39.57	0.08	0.00	0.00	0.00	0.03	0.00
5.1	vodka	Absolute	rectified EtOH	3.7	1.24	3.94	6.96	0.00	0.00	0.04	0.00	0.02	0.00	0.00	0.00	0.00	0.00
5.2	vodka	Russia-Stolichnaya	rectified EtOH	3.77	1.31	8.37	14.31	0.01	0.00	0.07	0.43	0.01	0.00	0.00	0.00	0.00	0.00
5.3	vodka	Sweden	rectified EtOH	3.77	1.31	9.56	13.91	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.4	vodka	Finlandia	rectified EtOH	3.81	1.35	11.01	16.73	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.5	vodka	Holland-Royalty	rectified EtOH	3.76	1.3	8.91	13.18	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.6	vodka	Austria-Zaranoff	rectified EtOH	3.76	1.3	8.42	14.75	0.01	0.00	0.07	0.26	0.02	0.00	0.00	0.00	0.00	0.00
5.7	ouzo	Greece-Mitilini	rectified EtOH + anisole	3.77	1.31	11.50	17.98	0.00		0.09	0.00	0.00	0.00	0.08	0.16	0.11	0.00
5.8	gin	England	rectified EtOH + juniper	3.8	1.34	11.67	17.11	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.9	arak	Jordan	rectified EtOH + anisole	3.79	1.34	9.69	16.66	0.01	0.01	0.09	0.88	0.00	0.00	0.00	0.00	0.00	0.00
5.10	Three Castles	France	rectified EtOH with juniper	3.76	1.3	9.84	14.78	0.01	0.00	0.07	0.21	0.00	0.00	0.00	0.00	0.00	0.00
5.11	Gantous and Abou Raad	Liban	rectified EtOH with anisoel	3.81	1.36	11.67	18.31	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.12	anis	Spain	rectified EtOH with anisole	3.61	1.15	8.99	12.21	0.00	0.00	0.06	0.00	0.00	0.00	2.93	4.76	4.41	0.00
5.13	sambuca	Italy	rectified EtOH with anisole	3.6	1.14	11.16	15.06	0.01	0.00	0.08	0.00	0.00	0.25	3.41	4.93	5.84	0.25
5.14	sambuca	France	rectified EtOH with anisole	3.6	1.14	9.28	12.66	0.01	0.00	0.06	0.00	0.15	0.00	3.06	4.90	4.79	0.00
6.1	tequila	Mexico	sap from agave	3.76	1.3	13.36	20.31	0.09	0.09	0.11	12.99	0.04	0.00	0.00	0.00	0.00	0.00
6.2	tequila	Mexico	sap from agave	3.76	1.3	8.78	14.53	0.03	0.03	0.08	5.61	0.03	0.00	0.00	0.00	0.00	0.00
6.3	rum	Jamaica	molasses[b]	3.76	1.3	8.15	14.27	0.01	0.00	0.08	0.17	0.01	0.00	0.00	0.00	0.00	0.00
6.4	rum	Cuba	molasses[b]	3.76	1.3	7.88	13.25	0.00	0.01	0.07	0.14	0.01	0.00	0.00	0.00	0.00	0.00
6.5	rum	France	molasses[b]	3.76	1.3	8.87	13.50	0.02	0.00	0.07	4.94	0.00	0.00	0.00	0.00	0.00	0.00
6.6	tsouika	Romania	plum brandy	3.71	1.24	6.22	9.04	0.09	0.05	0.05	22.13	0.13	0.00	0.00	0.00	0.00	0.00
6.7	barack	Hungary	apricot brandy	3.76	1.3	9.74	14.67	0.07	0.06	0.08	11.50	0.13	0.00	0.00	0.00	0.00	0.00
6.8	becherovka	Czech	herb liqueur	3.77	1.31	10.08	15.06	0.01	0.03	0.08	0.54	0.04	0.00	0.00	0.00	0.00	0.00
6.9	masumi	Japan	sake (rice)	3.7	1.21	3.95	5.86	0.02	0.01	0.03	3.48	0.03	0.77	0.00	0.00	0.00	0.65
6.10	tsouika	Romania	plum brandy	3.74	1.28	8.84	12.89	0.19	0.05	0.07	30.52	1.06	0.00	0.00	0.00	0.00	0.00
7.1	eau-de-vie	Cyprus	grape marc[c]	3.91	1.46	15.79	24.42	0.02	0.06	0.13	2.34	0.23	0.00	0.00	0.00	0.00	0.00
7.2	eau-de-vie	Cyprus	grape marc[c]	3.9	1.45	14.65	23.75	0.03	0.24	0.12	3.53	0.07	0.00	0.00	0.00	0.00	0.00
7.3	eau-de-vie	Cyprus	grape marc[c]	3.92	1.46	17.18	26.35	0.01	0.06	0.13	2.29	0.26	0.00	0.00	0.00	0.00	0.00
7.4	eau-de-vie	Cyprus	grape marc[c]	3.9	1.45	16.59	25.89	0.03	0.26	0.13	3.71	0.08	0	0.00	0.00	0.00	0.00

[a] The first digit denotes the group number, and the second is the running number of the samples within the same group. [b] From sugar cane. [c] Low quality.
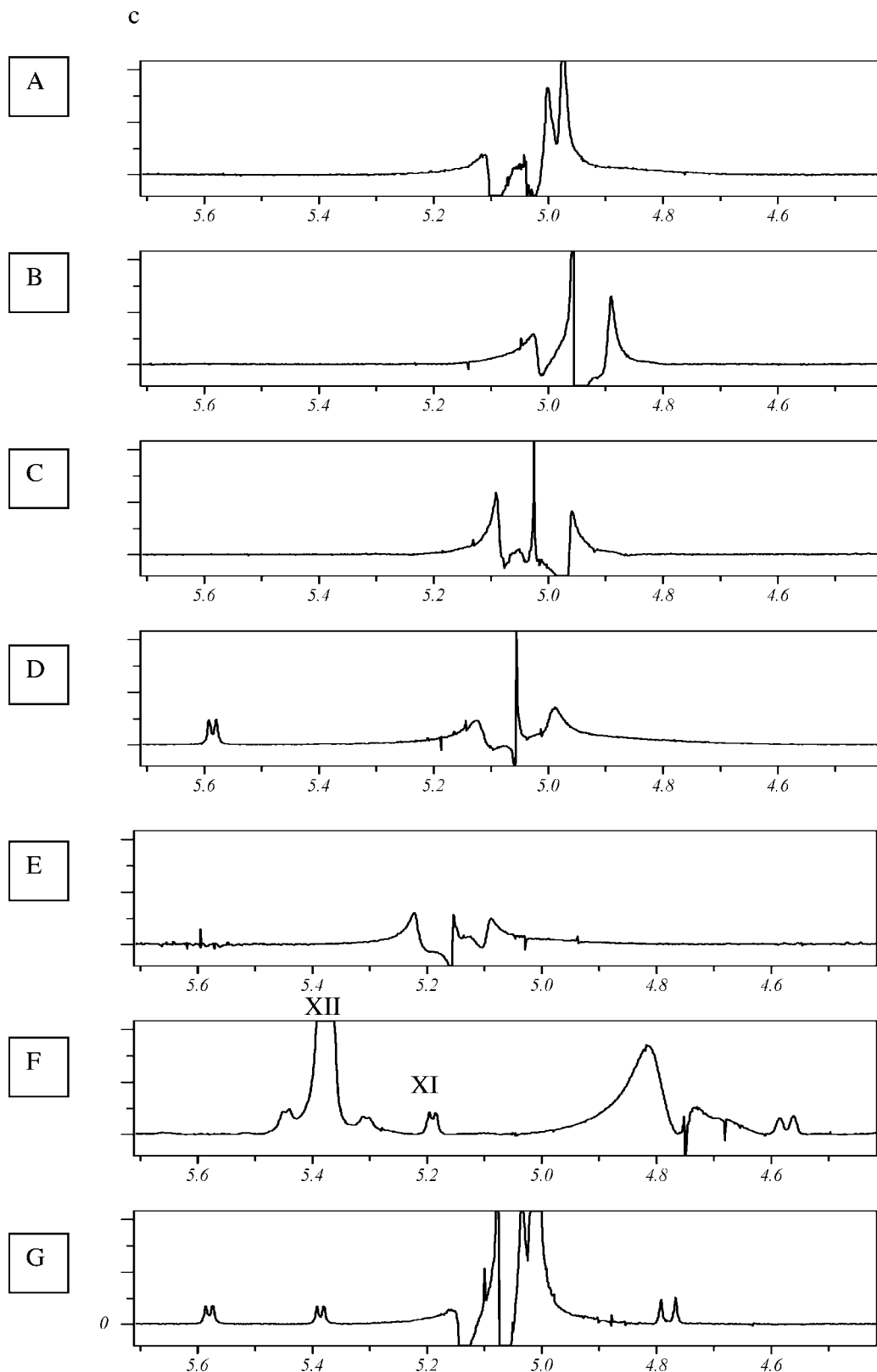
Authenticity of Zivania

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b

Authenticity of Zivania

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Figure 1. Expansions of representative spectra from (**A**) zivania from Kykkos monastery, (**B**) Swedish vodka, (**C**) Mexican tequila, (**D**) Italian grappa, (**E**) Cypriot eau-de-vie, (**F**) Italian sambuca, and (**G**) red zivania: (**a**) methyl region; (**b**) ethyl region; (**c**) −OH region.

reference. The sample preparation, the experimental time, and processing take <0.5 h. The validation of the method was checked with GC-obtained results.

Different chemometric methods were employed to analyze the 66 alcoholic beverages from different countries characterized by similar alcoholic grade. More specifically, results obtained from NMR were analyzed statistically using two different types of analytical methods, that is, CDA and CBT. The application of chemometric methods for characterization or classification of products according to origin, quality, variety, type, or other features has already attracted considerable attention from researchers (*30−36*).

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Table 2. Spectral Regions and Associated Parameters Used in the Statistical Analysis

region	peak identification	chemical shift (ppm)	associated parameter
I	−CH₃ group of higher alcohols (other than ethanol)[a,b]	∼1	F (integral)
II	upfield ¹H–¹³C satellite triplet of the CH₃– group of ethanol[b]	∼1.1	V (integral)
			P (intensity of the middle peak)
III	CH₃– group of ethanol[b]	∼1.3	J (chemical shift of the triplet)
			T (integral)
IV	CH₃CO– group of ethyl acetate	∼2.2	N1 (intensity)
V	unidentified peak resonances	∼3.2	R6 (intensity of the middle peak)
VI	CH₃– group of methanol	∼3.5	M (intensity)
			M1 (integral)
VII	upfield ¹H–¹³C satellite quartet of the CH₂– group of ethanol[b]	∼3.6	R (intensity of the leftmost outer peak)
VIII	CH₂– group of ethanol[b]	∼3.8	G (chemical shift of the quartet)
			S (integral)
IX	unidentified peak resonances	∼4.1	R7 (intensity of the middle peak of the triplet)
X	unidentified peak resonances	∼4.3	R4 (intensity of the rightmost peak of the quasi doublet)
XI	unidentified peak resonances	∼5.3	R2 (average intensity of the doublet)
XII	unidentified peak resonances	∼5.5	R1 (average intensity of the doublet)

[a] 3-methylbutanol 1 (d) or 2-methylpropanol 1 (d) or propanol 1 (t) or butanol 1 (t) or butanol 2 (t). [b] J_{vic} between alkyl protons of alcohols is calculated to be ∼7 Hz.

Table 3. NMR Variables Entered in CDA, *F* Value, Wilks' *λ* Statistic, and Associated *F* Approximation Together with the Probability Level of Significance

	F value	Wilks' *λ*	*F* approximation	$P < 10^{-4}$
F	14.314	0.403	14.314	*
R6/P	13.577	0.166	13.821	*
T	4.706	0.110	10.359	*
J	3.745	0.078	8.647	*
R4/P	12.804	0.032	9.871	*
R7/P	2.695	0.025	8.626	*
G	2.083	0.020	7.665	*

Similar products from other countries have an appellation based on the name given to grape marc (i.e., in Cyprus it is called "zivania"; in Greece, "tsipouro" or "tsikoudia"; and in Italy, "grappa"). Besides, in Greece and Slavic countries such products are also called "rakea" after the Arabic word "arak", which means spirit (alcohol), despite the fact that this distilling has been produced in Greece, Crete, and Cyprus islands since ancient times (*37*).

MATERIALS AND METHODS

Samples of Distillings. The study concerns 66 alcoholic beverages from Cyprus (zivania) and other countries, such as tsipouro, tsikoudia, ouzo, grappa, tsouika, rakea, vodka, gin, and eau-de-vie. The various samples are presented in **Table 1**. The choice of these samples was dictated by the fact that they are seen as competitors to zivania, whereas the European Union classifies them in the relevant legislation in the category of spirits best suited to zivania. Furthermore, eau-de-vie was chosen because it is often used as a cheap substitute for zivania (*38, 39*). For the purpose of comparison, several other samples of extra-European origin were also studied. The botanical origin of the various samples is also indicated in **Table 1**. All samples are fruit-based, with the exception of that of Hong Kong origin, which is rice-based.

Preparation of Samples. A quantity of 0.5 mL of distilling was placed in a 5 mm NMR tube. A known mass (∼14 mg) of 1,4-diazine (pyrazine) was added to quantify the NMR data. Deuterated benzene (7.15 ppm) stored in a coaxial tube was used as an external reference.

Analytical Determinations. Spectra were recorded on a Bruker AC 300 spectrometer operating at 300.13 MHz at a temperature of 300 K. Water resonance was saturated using 0.16 W. Experimental parameters used for ¹H NMR experiments were as follows: pulse duration, 6 *μ*s (18° tip angle); spectrum width, 4500 Hz; digital resolution, 0.2 Hz/point; relaxation delay, 10 s; and acquisition time, 3.64 s. Inverse

recovery experiments have shown that the 13.64 s pulse repetition rate ensures the complete relaxation of the different components and of pyrazine resonance. Reproducibility of the method was checked using several samples run at least six times at interval times of 12 days. The integrated peaks differed by <3%. Spectra were acquired with 128 scans to ensure a signal-to-noise ratio of >600 (calculated as pyrazine peak intensity over rms noise value in the spectral area of 7.2–7.3 ppm). A good resolution was assured when the central minimum of quartet corresponding to the CH₂– ethanol group did not exceed 5% of the maximum. The data were processed using 1D Bruker WINNMR s/w. The area of the peaks was normalized relative to the known weighted quantity of pyrazine.

Biostatistical Methods. CDA is used to achieve the most discriminative variables for the arrangement of samples in a space of reduced dimensionality in a way that maximizes the distances between the a priori formed groups and the independence of the axes of the configuration (*1, 40*).

The CBTs are used to produce a set of simple rules, which will identify any new sample (*41−44*). The construction of a CBT comprises the split of the original set of samples into two parts on the basis of a criterion involving a few variables, usually an algebraic expression of one or two. All variables involved in the construction of a CBT comprise the best diagnostic set for the groups already defined prior to the analysis.

The data are classified into seven groups ($g = 1−7$) by the rationale of distinguishing zivanias among other competitor distillings but also themselves. The group identifier was used as a dependent variable, whereas independent variables are the NMR-derived ones (**Table 1**).

The reduction of error in the classification is monitored by means of a loss function. Several loss functions have been proposed (*45*), two of which were used, that is, towing and gini, on the basis of the better reduction in error they achieved. The goodness of fit (GOF) of the model to the data was estimated at each node with the formula GOF $= 1 − W/T$, where W is the within-group sum of squares and T is the total sum of squares of the dependent variable (*40*). At the end, the CBT forms free nodes, each one corresponding, perfectly or loosely, to an original group.

RESULTS AND DISCUSSION

¹H NMR Spectroscopy. Expanded regions from representative ¹H NMR spectra are shown in **Figure 1**. **Figure 1a** depicts the methyl area (1.1−2.6 ppm), **Figure 1b** the expansion of the ethyl part of the spectra (3.1−4.5 ppm), and **Figure 1c** the −OH area. We focused our study on the regions numbered I−XII, which are depicted in **Figure 1** and described in **Table 2**. The variables extracted from the spectra were integrals and
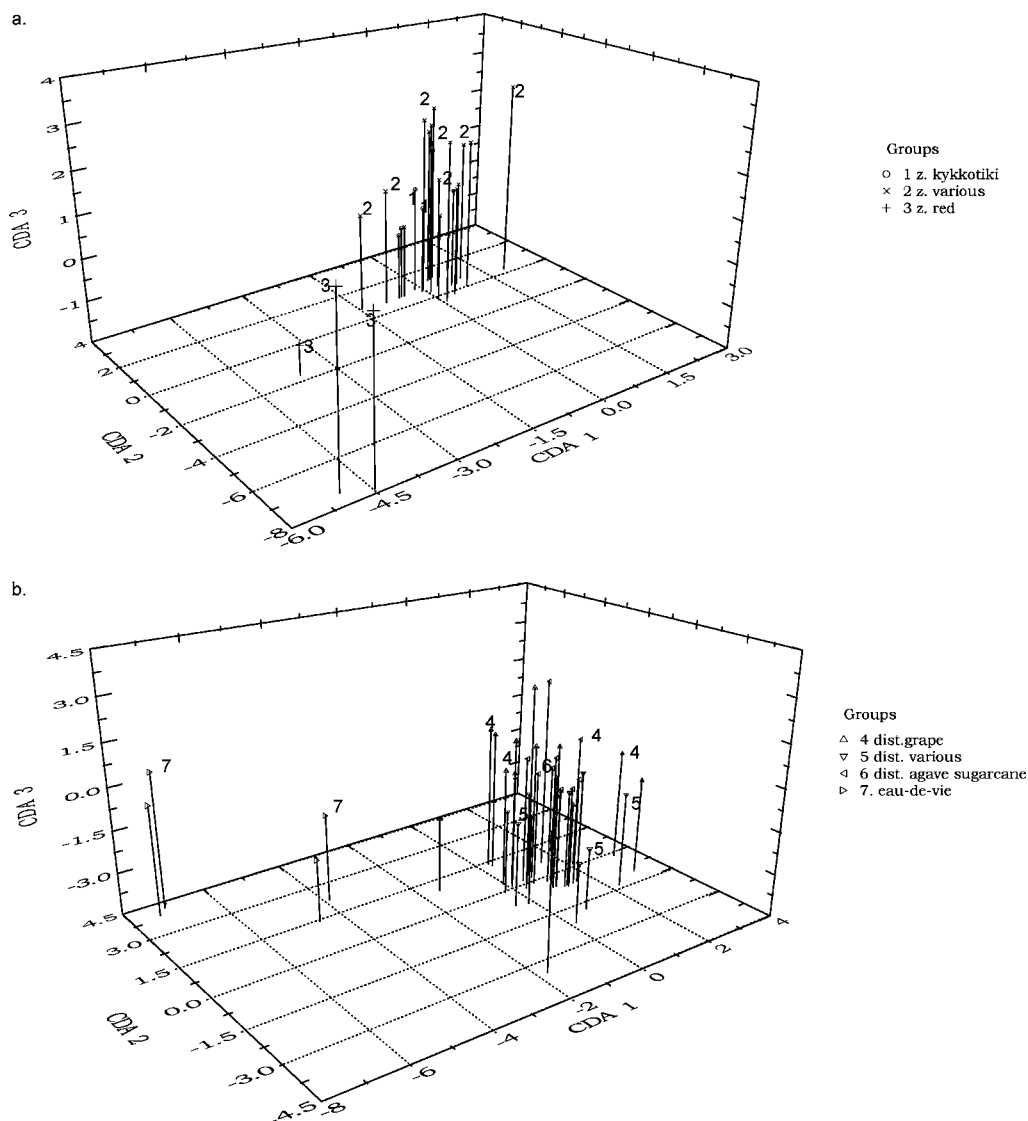
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Figure 2. Three-dimensional configurations of samples. The space was achieved with the important variables of CDA. The variation explained by each axis is 47.9% for CDA 1, 32.32% for CDA 2, and 12.57% for CDA 3 (92.84% in all three CDA axes). Two diagrams are constructed (**a** and **b**) for the first three and the last four groups, respectively, to facilitate inspection.

intensities. In case of overlapping (i.e., methanol peak, region VI) the following procedure was used. The integral of methanol was assumed to be the subtraction of an upfield $^1H-^{13}C$ satellite quartet of the CH_2 included in the overlapped methanol with the downfield $^1H-^{13}C$ satellite quartet of the CH_2. The small doublet observed at the shoulder of the upfield $^1H-^{13}C$ satellite quartet of the CH_2 was ignored because its integration was insignificant. When this was not possible due to the presence of extra resonances in the area (**Figure 1b**, cases D and G), deconvolution of the overlapped peaks was performed. The deconvoluted integral corresponding to methanol resonance was expressed relative to the integral of reference pyrazine peak. Regions V and IX−XIII comprise unidentified peaks that are present probably when taste additives such as anisole, cinnamon, or nutmeg are added (i.e., red zivania, sambuca, or grappa). Furthermore, the lack or the presence of traces of methanol, ethyl acetate, and higher alcohols must be noted in non-fruit-based spirits, for example, those from rectified ethyl alcohol (EtOH).

Quantification of Methanol NMR Data (Expressed in mg/ 100 mL) and Comparison with GC Data. More specifically

the integral of the CH_3- group of methanol was expressed in milligrams per 100 mL of distilling using the formula

$$CH_3OH \ (mg/100 \ mL) =$$

$$\frac{4}{3} \times \frac{W_{pyr}}{MW_{pyr}} \times S_{CH_3OH} \times \frac{100}{0.5} \times MW_{CH_3OH}$$

where S_{CH_3OH} and MW_{CH_3OH} are the integral and the molecular weight of CH_3OH, W_{pyr} and MW_{pyr} are the added weight and molecular weight of pyrazine, and the factor 4/3 expresses the normalization due to the four protons corresponding to the integrated area of pyrazine and the three protons of the CH_3- group of methanol.

The comparison gave a maximum deviation between the two methods concerning the CH_3OH content of ±10%.

Chemometry. The original grouping and the values of the NMR-derived data used as variables in the statistical analysis are given in **Table 1**. The samples were assembled in seven groups. In this grouping three categories are zivanias (Kykkos monastery, $g = 1$; other origin, $g = 2$; and red ones, $g = 3$).
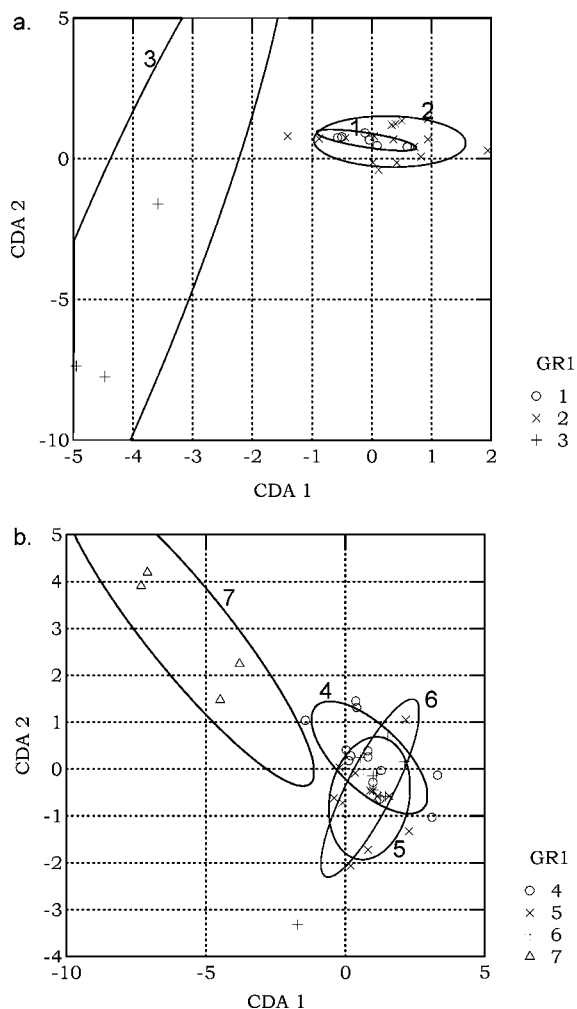
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Figure 3. Relationships of groups in bidimensional CDA spaces showing details in the scattergrams of **Figure 2**. Around the sample points of each group the 95% sample ellipses are drawn. Ellipses are labeled by their group number. The extensive overlap of various groups can be seen. (**a**) The 95% sample ellipse around the group of red zivanias ($g = 3$) is very large, whereas the two groups of zivanias ($g = 1$, zivanias from Kykkos monastery; $g = 2$, group of various zivanias) cannot be separated. (**b**) Groups 4–6 are not separated, but they are distantly located from group 7, eau-de-vie.

Other grape marc derived distillings comprise the fourth group. Distillings derived from rectified alcohol with the addition or not of anisole comprise the fifth group. Distillings from agaves, sugar cane fruits (other than grape), and rice form the sixth group. Finally, eau-de-vie distillings make up the seventh group.

Twelve of the 14 NMR-derived diagnostic parameters were used in the statistical analysis representing either chemical shifts (J) and (G) or ratios of intensities as (M/R), (N1/P), (R4/P), (R7/P), and (R6/P) or integrated areas with respect to pyrazine peak, (T), (S), (M1), (F), and (V).

In both CDA and CBT analyses the variable bearing the group specification is the dependent one (*40*, *41*). The NMR measurements are used as the independent predictor variables. To retain a very high proportion of the original variation in the stepwise CDA configuration (92.84%), only seven variables are necessary (**Table 3**). The produced configuration is statistically well supported, and the least number of dimensions, that is, the most parsimonious space, that are statistically significant is three ($P < 10^{-4}$). The variable (F) corresponding to the CH_3- group of higher alcohols is the most informative variable, whereas the

Table 4. (**A**) Classification and (**B**) Leave-One-Out Classification Resulting from CDA (Numbers Marked by the Same Letter Are the Same in Both Panels)

	actual grouping	CDA prediction of grouping							% correctly classified
		1	2	3	4	5	6	7	
(**A**) Classification									
zivania (Kykkos monastery)	1	4	1	0	1	0	0	0	67
zivania (other origin)	2	6	10	0	0	0	0	0	63
zivania red	3	1	0	2	0	0	0	0	67
grape-based distillings	4	3	2	0	6	0	2	0	46
rectified alcohol distillings	5	0	0	0	0	10	3	0	77
agave/sugar cane/fruit-based distillings	6	0	1	0	3	2	4	0	40
eau-de-vie	7	0	0	0	0	0	0	4	100
total		14	14	2	10	12	9	4	62
(**B**) Leave-One-Out Classification									
zivania (Kykkos monastery)	1	4	1	0	1	0	0	0	67
zivania (other origin)	2	7	9	0	0	0	0	0	56
zivania red	3	1	0	2	0	0	0	0	67
grape-based distillings	4	4	2	0	3	0	4	0	23
rectified alcohol distillings	5	0	0	0	1	9	3	0	69
agave/sugar cane/fruit-based distillings	6	0	1	1	3	3	2	0	20
eau-de-vie	7	1	0	0	0	0	0	3	75
total		17	13	3	8	12	9	3	49

Table 5. Cut Values, Proportional Reduction in Error (Improvement), and Goodness-of-Fit (GOF) Value of the Variables Entered at Each Node[a]

node	split variable	no. of samples	cut value	improvement	GOF value
1	M/R	66	3.721	0.316	0.316
3	J	28	1.360	1.171	0.367
2	T	38	16.612	0.275	0.563
4	F	22	0.041	0.098	0.625

[a] Nodes are before the split variable shown in **Figure 4**. All values are given at each node of the CBT. **Figure 4** also shows cut values and distribution of samples in the groups.

chemical shift of the CH_2- group of ethanol (G) is the least informative. All other variables are intermediate to them.

The CDA configuration is depicted in **Figure 2**. The group of eau-de-vie ($g = 7$) distillings is the best-formed cluster in this arrangement. The groups of grape, rectified alcohol, and fruit-based distillings ($g = 4$, 5, and 6) are concentrated around the origin of the CDA1−CDA2 principal plane. The red zivanias ($g = 3$) are distinctly differentiated from the other two groups of zivanias ($g = 1$, 2), which are intermingled (**Figures 2** and **3**). The extensive overlapping of sample ellipses of groups 1 and 2 (**Figure 3a**) shows that the NMR variables, derived in this method, are not able to discriminate among zivania types.

In general, zivania types cannot be well separated with this method and they cannot be discriminated from other distillings on the basis of their location in the CDA space (**Figure 3**). Red zivanias are located to the negative end of the CDA1 axis (**Figure 3a**), wheres eau-de-vie (group 7) is differentiated from groups 4−6 and is located on the far negative end of CDA1 and on the positive end of the CDA2 axis. The classification ability of CDA for zivania samples, on the basis of these seven variables, is 64% (60% in a leave-one-out classification), and regarding the whole set of the samples 62% (49% in a leave-one-out classification) are correctly classified (**Table 4**).

The analysis by means of the CBT algorithm on the basis of the 12 variables resulted in the "mobile" of **Figure 4** (*1*, *43−45*). All 12 variables were initially used in the construction of

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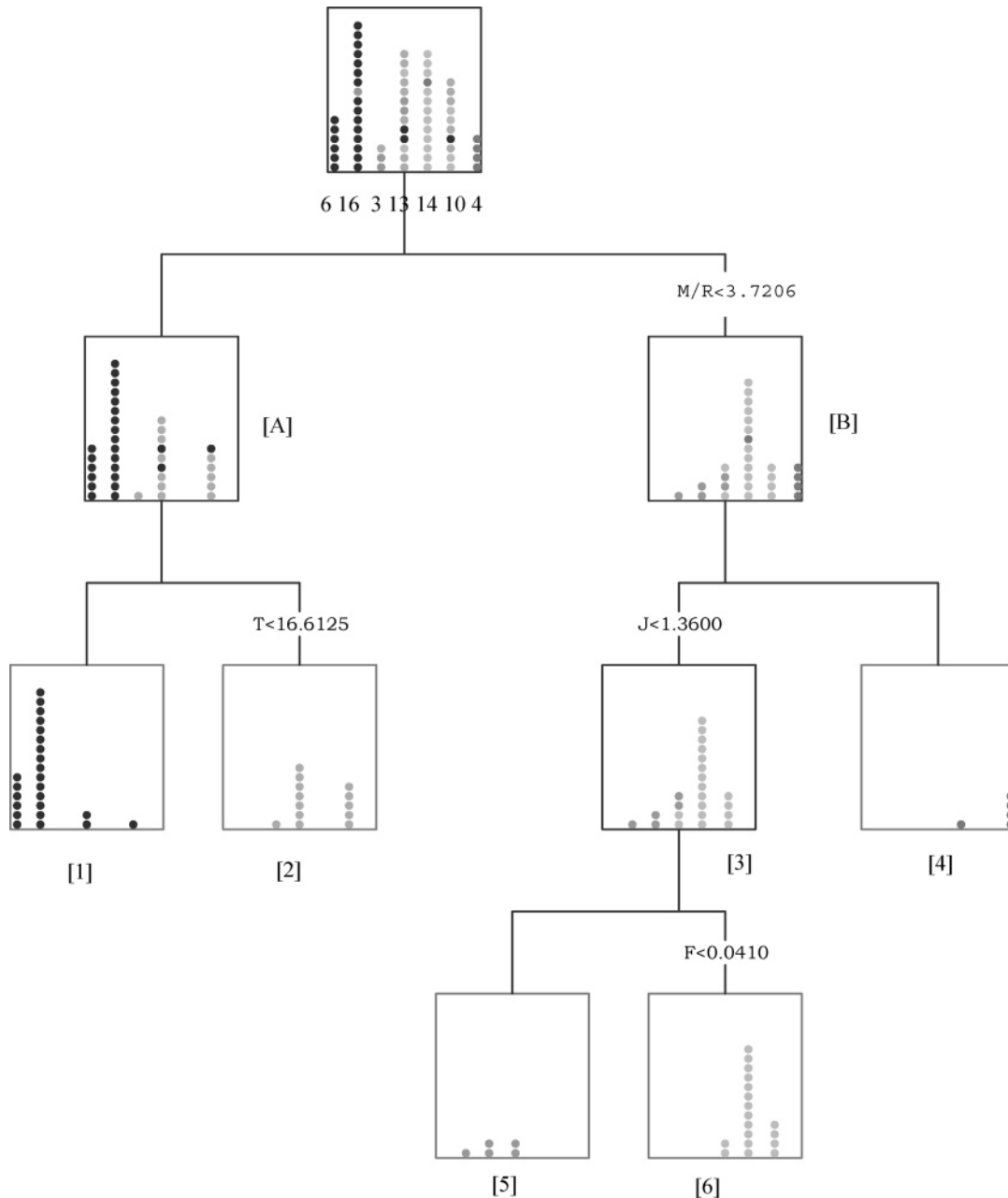

Figure 4. Classification tree of the 66 samples. The numbers in square brackets underneath or next to each node (i.e., box) are used in the discussion. The correspondence between the a priori formed groups and terminal nodes is not perfect. Zivanias are more concentrated on the left part of the diagram in nodes [A] and [1]. Nodes [1], [2], [4], [5], and [6] are terminal nodes. Node [1] is dominated by zivanias, whereas the red zivanias are spread in nodes [2] and [5]. Node [4] corresponds to eau-de-vie. In nodes [2] and [6] are concentrated groups 4–6, which show a leaching to node [5].

CBT, but only 4 of them were informative (**Table 5**). The variables M/R, J, T, and F are entered as informative in CBT. It is worth mentioning that the variable M/R without being selected in CDA (**Table 4**) is the most important variable in CBT, causing the initial split in the tree of **Figure 4**. Similarly, the variable F, unlike in CDA, is the least informative of the four variables included, serving only in the separation of the bulk of group 5 and half of the samples of group 6. The proportional reduction in the classification error of all samples is very good (86.03%). Specifically, the terminal nodes in **Figure 4** collected the samples in the correct groups explaining 86.03% of the variation in the initial data of the 12 variables. This rate was achieved by using least squares as a loss function because the other functions, that is, gini and towing, gave a much lower reduction in classification error (*45*). Groups 1 (zivanias from Kykkos monastery) and 2 (different origin

zivanias) are gathered in node [1], although only one sample from group 2 went to the right branch of the tree and appears in the impure node [5]. The seventh group of eau-de-vie is also well formed in node [4] having only one impurity from group 5 (rectified alcohol group).

The salient feature of the mobile is the early separation of red zivanias from the rest at nodes [A] and [B] and the agglomeration of zivanias from Kykkos monastery with other origin zivanias in node [1] (**Figure 4**). Moreover, the M/R variable improves the classification (**Table 5**). Variables J and F are exclusively used to separate samples in groups 4−6. These groups are not well formed, and they are scattered to several nodes. Group 6 is spread in nodes [1], [2], [3], and [6]. Group 4 is scattered in nodes [1], [2], [5], and [6], although almost half (7 of 13) of its samples are included in node [2]. The most important pattern in the mobile of **Figure 4** is that all zivanias,

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except the red ones, are included in a single node, that is, node [1], and eau-de-vie in another node, that is, node [4].

Variables R1/P and R2/P were introduced in the statistical analysis in an attempt to gather the red zivanias in one terminal node and to cancel their early separation at nodes [A] and [B]. This attempt was proven to be not successful. Nevertheless, red zivanias seem to be well distinct from all of the other samples on the basis of viscosity, as can be found in the literature (*46*).

Conclusion. The validity of quantification of the method was tested using comparative GC data. The former metal-based classification of zivanias, published by the same authors, relies primarily on external factors such as soil and bedrock constitution, whereas the classification reported in this article is mainly based on the chemical composition of the distilled substrate. However, the percentage of correctly classified samples was slightly lower using NMR spectroscopy. This is because metals in general differ with the geographic origin of the substrate to be distilled, and additionally some metals were absent from certain distillings, thus behaving as binary variables.

Nevertheless, although the overall prediction was not excellent, the zivanias were separated from the other distillings. The fact that discrimination between such similar samples as those used here (i.e., zivania and its mainly European counterparts) is possible using a few different constituents provides a reasonable base for proposing the use of our methodology for proving the authenticity and geographic origin of zivania distillates. Additional experiments using different spectroscopic methodologies are under progress. All of these methodologies will be evaluated alone or in combination to provide the best suitable method to differentiate zivania from European counterparts.

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