Regional Origin Assignment of Red Wines from Valencia (Spain) by ^{2}H NMR and ^{13}C IRMS Stable Isotope Analysis of Fermentative Ethanol

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The use of the stable hydrogen and carbon isotope ratios of fermentative ethanol as suitable environmental fingerprints for the regional origin identification of red wines from Valencia (Spain) has been explored. Monovarietal *Vitis vinifera* L. cvs. Bobal, Tempranillo, and Monastrell wines have been investigated by ^{2}H NMR and ^{13}C IRMS for the natural ranges of site-specific $^{2}H/^{1}H$ ratios and global $\delta^{13}C$ values of ethanol over three vintage years. Statistically significant interregional and interannual ^{2}H and ^{13}C abundance differences have been noticed, which are interpreted in terms of environmental and ecophysiological factors of isotope content variation. Multivariate discriminant analysis is shown to provide a convenient means for integration of the classifying information, high discriminating abilities being demonstrated for the ^{2}H and ^{13}C fingerprints of ethanol. Reasonable differentiation results are achieved at a microregional scale in terms of geographic provenance and even grapevine genotypic features.

Keywords: *Wine authentication; origin assignment; fermentative ethanol; stable isotope ratio analysis; ^{2}H NMR; ^{13}C IRMS*

INTRODUCTION

As a consequence of the enforcement of specific rules for the quality assurance of wine (geographic indications, designations of origin) and for the control of the wine trade within the European Union in relation with the origin adulteration of shipments, considerable efforts are being carried out at present for the identification of distinctive criteria to be used for the regional origin typification of European wines. As a relevant wine-exporting region, Valencia (Spain) is particularly concerned by these regulations. Stable isotope ratio analysis (SIRA) methods have been recognized in recent years to provide the most innovative source of chemical information for the authenticity assessment and for the origin assignment of agrofood products (Schmidt, 1986).

^{2}H NMR determination of the site-specific $^{2}H/^{1}H$ ratios of fermentative ethanol in wine (Martin et al., 1983) was initially adopted by the European Union as an official method for the proof of beet and C4 plants (maize and cane) sugar addition (EC Regulation 2676/90, 1990). Results obtained since then for the survey of all EU wine-growing regions have begun to be checked to ascertain whether they can additionally provide a means for the regional origin identification of European wines (Martin et al., 1988; Monetti et al., 1994, 1995; Day et al., 1994, 1995a,b). For this purpose, the ^{13}C IRMS determination of the $\delta^{13}C$ value of ethanol (Monetti et al., 1995; Rossmann et al., 1996) and the ^{18}O IRMS measurement of the $\delta^{18}O$ value of water in wine (Holbach et al., 1994, 1996) have been shown to add relevant information as to the confirmation of wine origin. For the widespread application of isotopic methods for wine authentication it is necessary to know both the reference mean values and the sources (and natural ranges) of variation of the different bioelements' stable isotope ratios within a given wine-producing area. Accordingly, a system of European data banks has been installed to be used for reliable comparison (EC Regulation 2348/91, 1991a). However, the knowledge of the natural stable isotope characteristics of a number of European viticultural areas is still underdeveloped. In particular, the way in which the various ecophysiological factors (plant water status, water-use efficiency) influence the stable isotope composition of *Vitis vinifera* L. products at a local level under the complex and changing environmental conditions of the Mediterranean basin has not been sufficiently investigated.

Results reported for the stable isotope ratio analysis of fermentative ethanol in Italian wines have demonstrated the occurrence of a great deal of within-region spread of $^{2}H/^{1}H$ ratios and $\delta^{13}C$ values, which accounts for a heavy interregional overlapping when attempts are made to distinguish wine geographic origins (Monetti et al., 1995). The scattering of stable isotope ratios can be, at least in part, attributed to the microclimatic irregularity of most Mediterranean viticultural regions (Monetti et al., 1994; Giménez-Miralles, 1998). However, insufficient data are at present available concerning the stable isotope survey of other southern European wine-growing areas at a small regional level (Gigliotti et al., 1993) to support this assumption. As a matter of fact,

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identifying the sources of variation of the stable isotope ratios of wine ethanol at a microregional scale should be considered of primary significance to go forward in our understanding of the relevant sources of variability at a large-sized interregional level. In this regard, research has been undertaken in this work to determine the natural ranges of ^2H and ^{13}C content variation of fermentative ethanol in monovarietal red wines produced in three microclimatically distinct viticultural areas within Valencia, corresponding to three different Denominaciones de Origen (Certified Brand of Origin) (DO Utiel-Requena, DO Valencia, and DO Alicante), over a three-year period (1991−1993), to check the feasibility of using stable isotope ratios as suitable environmental fingerprints for wine regional origin authentication.

MATERIALS AND METHODS

Plant Material. Ninety-six monovarietal red grape (*V. vinifera* L.) samples were collected at technological ripeness during vintage seasons 1991, 1992, and 1993 in three well-defined wine-growing areas of Valencia (Spain) corresponding to three Denominaciones de Origen (DO): region of Utiel-Requena (DO Utiel-Requena, cvs. Bobal and Tempranillo), regions of La Vall d'Albaida and La Costera (DO Valencia, cv. Monastrell), and regions of L'Alt Vinalopó and Les Valls del Vinalopó (DO Alicante, cv. Monastrell). The survey was performed according to the official European databank sampling procedure (EC Regulation 2347/91, 1991b). A number of local vineyards were selected in each viticultural region so as to represent the whole grape production of the considered area. In each vineyard site, one batch (10−12 kg) of fresh and healthy grape bunches was collected from several spread grapevines. Each sample examined represents therefore one local vineyard.

Fermentation and Ethanol Isolation. Grapes were crushed using a cylinder press, and the resulting juice was submitted to laboratory-scale fermentation using activated *Saccharomyces cerevisiae* K1 yeast (Lalvin, Lallemand Inc., Montreal, Canada) at controlled temperature (~20 °C). Completion of the bioconversion was monitored by determination (Fehling titration) of residual sugars (<2 g/L) using an automated analyzer (Mettler). Fermentative ethanol was quantitatively extracted through distillation using a semi-automated apparatus (Ditta C. Bullio, Milan, Italy), according to the official EC method (EC Regulation 2676/90, 1990). Extraction yield was >98%. The exact ethanol content of distillates (at least 92% w/w) was calculated by subtracting both the water content (Karl Fischer titration) and the amount of volatile compounds (GC determination on a Carlo Erba GC 6000 gas chromatograph using a WCOT fused SiO_2 capillary column, Chrompack, 50 m × 0.25 mm i.d., film thickness = 0.20 μm; FID detection; detector and injector temperature = 240 °C; furnace temperature program, 40 °C for 10 min, 10 °C/min slope for 16 min, 200 °C for 4 min; internal standard, 4-methyl-2-pentanol, Merck).

Stable Isotope Ratio Determinations. *^2H NMR Determination*. The site-specific stable hydrogen isotope ratios ($^2H/^1H$) of the nonequivalent unexchangeable molecular sites of ethanol (methyl and methylene) were determined by quantitative deuterium nuclear magnetic resonance (^2H [^1H] NMR) at the natural abundance level using the internal referencing method (Martin et al., 1985; EC Regulation 2676/90, 1990) on a Bruker AC 300 spectrometer (field = 7.05 T) equipped with a 10 mm specific ^2H probe head (deuterium resonance frequency = 46.07 MHz), ^{19}F field-frequency lock, ^1H decoupling channel, temperature regulation unit, and automatic sample changer. *N,N,N′,N′*-Tetramethylurea (TMU) of known and controlled deuterium content ($^2H/^1H$ = 132.83 ppm) provided by the Community Bureau of Reference (BCR, Brussels) was used as internal working standard. Hexafluorobenzene (Fluka) was added (~150 μL) for the ^{19}F lock signal. The quantitative determination was based on multiple measurements. Ten consecutive ^2H [^1H] NMR spectra were recorded using the following acquisition parameters: 15.0 μs (90° angle) ^2H pulse width, 6.8 s acquisition time, 1200 Hz sweep width, 16 kbit data points, 304 scans, quadrature detection, wide-band ^1H decoupling, temperature = 300 K. Free induction decay (FID) signals were Fourier transformed with sensitivity enhancement (2 Hz line broadening). Signal-to-noise ratio obtained was S/N > 150 for the methyl peak. Both automated peak phasing and integration were used. Data were processed by means of the specific software Wineauto 3.0 (Eurofins, Nantes, France). The $^2H/^1H$ ratios of each molecular site (i) of ethanol, methyl [$(^2H/^1H)_{CH_3}$], and methylene [$(^2H/^1H)_{CH_2}$] were derived from the intensity comparison of the corresponding ^2H [^1H] NMR signals and that of TMU and are expressed in parts per million

$$(^2H/^1H)_i = \frac{N^{TMU}}{N_i}\frac{M}{M^{TMU}}\frac{m^{TMU}}{m^D}\frac{t^{TMU}}{t^D}T_i(^2H/^1H)^{TMU} \quad (1)$$

where N is the stoichiometric number of equivalent hydrogen atoms of each molecular site (i) of ethanol and of TMU, M is the molecular weight, m is the amount of distillate and TMU in the sample, t^{TMU} is the purity (% w/w) of TMU (100% if no correction due to impurities was to be introduced) (Christoph et al., 1991), t^D is the ethanol content (% w/w) of distillate, and T_i is the ratio of the integrated heights of the ^2H [^1H] NMR signals of each molecular site (i) of ethanol and of TMU. Additionally, the relative intramolecular ^2H distribution ratio R (Martin et al., 1985) was calculated as

$$R = 2[(^2H/^1H)_{CH_2}/(^2H/^1H)_{CH_3}] \quad (2)$$

Precision and accuracy of the ^2H [^1H] NMR determination were routinely checked by using reference ethanol standards (BCR, Brussels) for calibration. The repeatability standard deviation (S_r) for the isotopic determination was <0.2 ppm for parameter $(^2H/^1H)_{CH_3}$, <0.3 ppm for parameter $(^2H/^1H)_{CH_2}$, and <0.008 for parameter R.

^{13}C IRMS Determination. The stable carbon isotope ratio of ethanol was determined by dual-inlet isotope ratio mass spectrometry (^{13}C IRMS) using a triple-collector, 90° magnetic sector spectrometer Sira 10 (VG Isotech, Middlewich, U.K.). Ethanol samples were combusted to CO_2 in the absence of air and an excess of oxygen using a manually controlled Isoprep 13 combustion system (VG Isotech) following a method similar to that previously described (Winkler and Schmidt, 1980). Combustion was performed at 800 °C in an oxidation tube containing silver vanadate (Coleman reagent), silver oxide, and silver wolframate on 30−60 mesh Cromosorb P and silver wolframate on MgO. The outlet gases were subsequently passed through a reduction tube containing 40−80 mesh Cu and a silver gauze catalyst at 250 °C to reduce any nitrogen oxides eventually formed. Carbon dioxide was condensed in a steel sample container. Cryogenically trapped CO_2 was transferred to the spectrometer inlet system, further purified by pumping away the remaining noncondensable gases under high vacuum, and allowed to expand at room temperature into the mass spectrometer. Ion currents at m/z 44, 45, and 46 were registered on the three-channel collector and simultaneously integrated for the $^{13}CO_2/^{12}CO_2$ analysis. The relative ^{13}C content of the samples was determined by comparison against a laboratory standard gas through dual-inlet measurements. Results are expressed in $\delta^{13}C$ units as per mil (‰) deviation relative to the PDB international standard (eq 3).

$$\delta^{13}C = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{PDB}} - 1\right] \times 10^3 \quad (3)$$

The laboratory standard gas ($\delta^{13}C$ −10.4‰) was calibrated in the PBD scale using NBS 19 ($\delta^{13}C$ +1.95‰) (Coplen, 1996). Craig's correction was applied for the ^{17}O contribution at m/z 45 (Craig, 1957).

Table 1. MANOVA for the ^{2}H and ^{13}C Fingerprints of Fermentative Ethanol in Valencian Red Wines According to Factors Regional Origin × Vintage Year [Mean Values ± Confidence Intervals for the Means ($P \leq 0.05$)]

source of variation	isotopic variable			
	$(^2H/^1H)_{CH_3}$ (ppm)	$(^2H/^1H)_{CH_2}$ (ppm)	R^a	$\delta^{13}C$ [‰]$_{PDB}$[b]
overall mean (96)[c]	103.7 ± 0.2 (1.2)[d]	131.8 ± 0.4 (1.7)	2.54 ± 0.01 (0.04)	−25.6 ± 0.3 (1.5)
range of variation	100.3−106.6	127.9−135.3	2.40−2.67	−29.4 − −22.6
regional origin effect				
$F_{[2,87]}$[e]	0.98 ns[f]	65.11 **	18.26 **	60.25 **
levels				
DO[g] Utiel-Requena (43)	103.6 ± 0.3 a[h]	130.4 ± 0.3 a	2.52 ± 0.01 a	−25.2 ± 0.3 a
DO Valencia (25)	103.5 ± 0.4 a	132.5 ± 0.4 b	2.56 ± 0.01 b	−27.2 ± 0.4 b
DO Alicante (28)	103.9 ± 0.4 a	133.4 ± 0.4 c	2.57 ± 0.01 b	−24.8 ± 0.3 a
vintage year effect				
$F_{[2,87]}$	16.94 **	2.54 ns	4.17 *	7.38 **
levels				
1991 (33)	103.0 ± 0.4 a	132.0 ± 0.4 a	2.56 ± 0.01 a	−26.2 ± 0.3 a
1992 (33)	104.5 ± 0.4 b	132.5 ± 0.4 a	2.53 ± 0.01 b	−25.5 ± 0.3 b
1993 (30)	103.5 ± 0.4 a	131.9 ± 0.4 a	2.55 ± 0.01 ab	−25.5 ± 0.3 b
interaction (origin × year) effect				
$F_{[4,87]}$	2.85 *	2.23 ns	4.03 **	8.28 **

[a] Relative intramolecular deuterium distribution (Martin et al., 1983). [b] Per mil deviation relative to PDB. [c] Number of samples. [d] SD. [e] Fischer−Snedecor F ratio values based on the residual mean square error. [f] Significance level: ns, *, and ** represent not significant and significant differences at $P \leq 0.05$ and $P \leq 0.01$, respectively. [g] Denominación de Origen (Certified Brand of Origin). [h] Least significant difference (LSD) comparison test ($P \leq 0.01$): same letters denote homogeneous groups; different letters denote a statistically significant contrast between the means.

Determinations were conducted in duplicate. Precision and accuracy of the $\delta^{13}C$ determination were controlled by using additional laboratory standard material. The internal standard deviation obtained for one determination was <0.02‰. The overall repeatability standard deviation (S_r) of the whole experimental procedure, including combustion, was <0.3‰.

Statistical Analysis of Data. Multi-isotopic data were statistically evaluated using the Statgraphics 7.0 software package (Statistical Graphics Corp. STSC, New York). Multivariate analysis of variance (MANOVA) was performed at the 99 and 95% confidence levels, and the significance of the effects associated with the investigated factors was examined using the Fischer−Snedecor F test. Multiple comparisons of mean values were based on Fischer's least significant difference (LSD) test (Haswell, 1992). Linear discriminant analysis (LDA) was performed for the multivariate classification of samples and for the maximization of regional origin discrimination (Krzanowsky, 1988).

RESULTS AND DISCUSSION

Natural Ranges of ^{2}H and ^{13}C Contents of Fermentative Ethanol. The stable hydrogen and carbon isotope ratio determinations were performed on fermentative ethanol as a convenient molecular probe for the multi-isotopic origin characterization of wine (Martin et al., 1986). Results are summarized in Table 1 for the overall mean values (±95% confidence intervals) and for the characteristic variability ranges of the ^{2}H and ^{13}C contents of fermentative ethanol obtained over the whole data set under research. The natural variation ranges of the site-specific ^{2}H/^{1}H ratios are found to reach ∼6 ppm (methyl site) and ∼7 ppm (methylene site), similar to those previously reported for other Mediterranean viticultural regions (Monetti et al., 1994, 1995). Accordingly, the relative intramolecular ^{2}H distribution ratio R is shown to naturally vary between 2.40 and 2.67. With regard to the natural $\delta^{13}C$ value variability, a wide range of ∼7‰ (up to extremely high values of −22.6‰) is observed, which appears to be even larger than those found for far more extensive geographic areas (Rossmann et al., 1996).

Environmental and Ecophysiological Factors of ^{2}H and ^{13}C Content Variation. In principle, the naturally occurring variability of the ^{2}H and ^{13}C contents of fermentative ethanol is to be attributed to interregional and interannual environmental differences associated with the various cultivation areas and vintage years surveyed. The isotopic data set was therefore submitted to multivariate analysis of the variance (MANOVA) for the estimation of the effects of the relevant environmental sources of variation involved (regional origin × vintage year). Results obtained for the F test performed on the individual isotopic parameters (Table 1) demonstrate high levels of statistical significance for the effects associated with both factors investigated, as well as for the interaction between them. On the one hand, the occurrence of natural factors of geographic variation of the ^{2}H and ^{13}C fingerprints of fermentative ethanol in Valencian red wines (even within the limited climatic range of the cultivation areas surveyed) must be admitted, the regional effect being highly significant for the ^{2}H content of the methylene group ($F_{[2,87]} = 65.11$) and for the $\delta^{13}C$ value ($F_{[2,87]} = 60.25$), as well as for the intramolecular ^{2}H distribution ratio R ($F_{[2,87]} = 18.26$), whereas no significant effect is to be found for the ^{2}H content of the methyl site ($F_{[2,87]} = 0.98$). On the other hand, the interannual climatic irregularity typical for Valencia as a Mediterranean region (Table 2) gives rise to a statistically significant interannual variation effect, particularly remarkable for the ^{2}H content of the methyl group ($F_{[2,87]} = 16.94$) and for the $\delta^{13}C$ value ($F_{[2,87]} = 7.38$). Accordingly, significant interregional differences for variables $(^2H/^1H)_{CH_2}$ and $\delta^{13}C$ and significant interannual differences for variables $(^2H/^1H)_{CH_3}$ and $\delta^{13}C$ are revealed by the LSD comparison test (Figure 1). With regard to variable $(^2H/^1H)_{CH_2}$, the regional mean values are found to steadily increase from the northern DO Utiel-Requena (130.4 ppm), through the central DO Valencia (132.5 ppm), down to the southern DO Alicante (133.4 ppm) regions, interannual variations being of secondary importance (Figure 1b). Because the ^{2}H/^{1}H isotope ratio of the methylene site of fermentative ethanol is known to be well correlated to the ^{2}H content of plant water involved in fermentation (grape berry water) (Martin et al., 1986), the interregional variation trend observed for parameter $(^2H/^1H)_{CH_2}$ seems to reflect correctly the latitudinal and continental gradients of ^{2}H content

Table 2. Average Annual Rainfall Amounts for Agricultural Years 1991−1993[a]

viticultural area	station[b]	precipitation (mm) 1991	1992	1993
Utiel-Requena	Utiel-La Noria, 735 m	417.0	260.5	217.0
Valencia (Vall d'Albaida/Costera)	Ontinyent, 350 m	723.7	761.5	981.1
Alicante (Vinalopó)	Monòver-L'Esvarador, 560 m	283.0	271.0	454.0

[a] Data made available by Instituto Nacional de Meteorología (INM). [b] Representative meteorological station for each area and altitude above sea level.

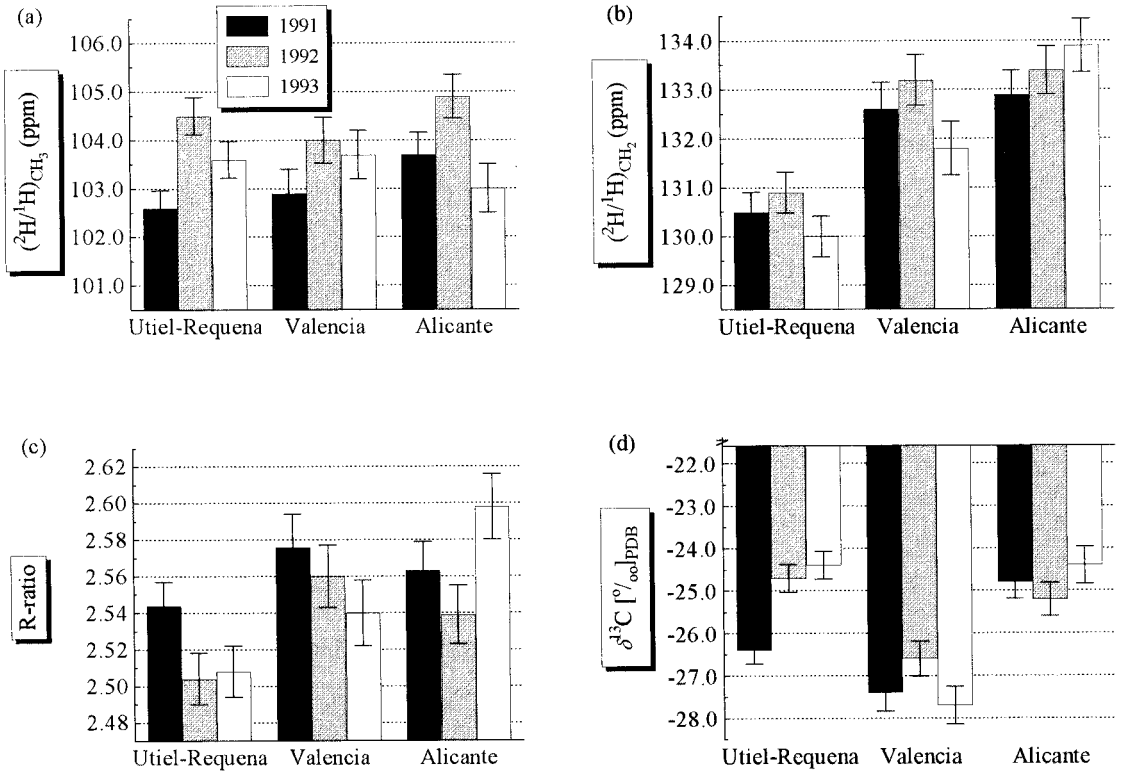

Figure 1. Mean values ± LSD intervals ($P \leq 0.05$) for the ^2H and ^{13}C contents of fermentative ethanol in Valencian wines according to regional origin and vintage year. Non-overlapping intervals denote a statistically significant difference between the means.

variation of the relevant meteoric water supplies available for plant uptake and, hence, of plant water accumulated in the fruits, as well as the corresponding gradient of evapotranspiration rates (Ziegler et al., 1976; White, 1988). However, such a geographic trend of ^2H content variation is not simultaneously reflected to a similar extent on parameter $(^2H/^1H)_{CH_3}$, which is assumed to be mainly related to the ^2H content of grape glucose via fermentation (Martin et al., 1986) (Figure 1a). This observation appears to give an indication of the fact that the ^2H content of glucose accumulated in the grapes is not only determined by the $^2H/^1H$ ratio of the available input water (as defined by the relevant environmental features) but may be further modulated during the process of fixation of hydrogen into the primary assimilates through variations of the extent of isotope fractionation according to the specific ecophysiological responses of the grapevine to such environmental conditions.

In this respect, it is useful to evaluate the $^2H/^1H$ data of fermentative ethanol simultaneously in combination with the analysis of the related $\delta^{13}C$ values, which may provide additional information about the ecophysiological performance of the plant (Farquhar et al., 1988; O'Leary, 1995). In Figure 1d more negative mean $\delta^{13}C$ values are clearly noticed over the whole 1991−1993 period under investigation for DO Valencia (−27.2‰) as compared to both DO Utiel-Requena (−25.2‰) and DO Alicante (−24.8‰) cultivation areas. The observed interregional $\delta^{13}C$ value differences are shown to be related to the water status of grapevines growing under different environmental conditions (which controls the regulation of leaf stomatal conductance and, hence, the extent of photosynthetic ^{13}C discrimination), therefore reflecting remarkable interregional variations of groundwater availability (O'Leary, 1995). Thus, good water supply conditions found over the three-year period for DO Valencia (Table 2) seem to account for a larger extent of photosynthetic ^{13}C discrimination and consequently for less ^{13}C being incorporated into the primary assimilates (and hence into ethanol after fermentation). Conversely, under poor watering or even water-stress conditions (as those noticed for DO Utiel-Requena during seasons 1992 and 1993), fermentative ethanol is found to be ^{13}C-enriched as a result of the expected increase of leaf stomatal closure aimed at saving water resources, with the consequent decrease of ^{13}C discrimination during the photosynthesis of the primary assimilates (O'Leary, 1995). On the contrary, more negative $\delta^{13}C$ values are observed for this viticultural area under better watering conditions (vintage 1991). However, such extreme interannual $\delta^{13}C$ value differences are not observed for DO Valencia and DO Alicante wines (Figure 1d). Most interestingly, the ^{13}C enrichment of

Table 3. MANOVA for the ^2H and ^{13}C Fingerprints of Fermentative Ethanol in DO Utiel-Requena Red Wines ($n = 43$) According to Factors *V. vinifera* Cultivar × Vintage Year [Mean Values ± Confidence Intervals for the Means ($P \leq 0.05$)]

	isotopic variable			
source of variation	$(^2H/^1H)_{CH_3}$ (ppm)	$(^2H/^1H)_{CH_2}$ (ppm)	R[a]	$\delta^{13}C$ [‰]$_{PDB}$[b]
grapevine cultivar effect				
$F_{[1,37]}$[c]	5.48 *[d]	7.58 **	10.89 **	9.62 **
levels				
cv. Bobal (28)[e]	103.9 ± 0.4 a[f]	130.1 ± 0.5 a	2.51 ± 0.01 a	−25.0 ± 0.2 a
cv. Tempranillo (15)	103.1 ± 0.6 b	131.2 ± 0.6 b	2.54 ± 0.02 b	−25.6 ± 0.3 b
vintage year effect				
$F_{[2,37]}$	12.78 **	4.56 *	5.33 **	74.19 **
interaction effect				
$F_{[2,37]}$	0.68 ns	2.52 ns	1.01 ns	38.44 **

[a] Relative intramolecular deuterium distribution (Martin et al., 1983). [b] Per mil deviation relative to PDB. [c] Fischer−Snedecor *F* ratio values based on the residual mean square error. [d] Significance level: ns, *, and ** represent not significant and significant differences at $P \leq 0.05$ and $P \leq 0.01$, respectively. [e] Number of samples. [f] Least significant difference (LSD) comparison test ($P \leq 0.05$): same letters denote homogeneous groups; different letters denote a statistically significant contrast between the means.

fermentative ethanol in DO Utiel-Requena wines found for vintage years 1992 and 1993 as compared to 1991 is shown to be related to a corresponding ^2H enrichment of the methyl site (Figure 1a). Accordingly, it could be analogously assumed that the leaf stomatal regulation of the plant has also a parallel influence on the definition of the ^2H content of the primary assimilates, being responsible for an enhancement of the incorporation of ^2H into sugars synthesized under water-stress conditions and hence for the observed ^2H enrichment of the methyl site of fermentative ethanol. In this respect, the different patterns of interannual variation of groundwater availability observed in all three viticultural regions surveyed could also explain, on the other hand, that high levels of statistical significance are noticed for the interaction (regional origin × vintage year) effect on parameters $\delta^{13}C$ ($F_{[4,87]} = 8.28$) and $(^2H/^1H)_{CH_3}$ ($F_{[4,87]} = 2.85$) but not on $(^2H/^1H)_{CH_2}$ (Table 1).

In summary, when the combined ^2H and ^{13}C fingerprints of fermentative ethanol in Valencian wines are simultaneously evaluated at a small-sized local scale, not only are direct environmental effects detected but, moreover, the specific ecophysiological responses of the grapevine to such environmental conditions (expressed in terms of water status) are made evident, which appear to be the relevant factors controlling the interregional and interannual variation patterns of the stable isotope parameters. Furthermore, in this regard it could be expected that significantly different ecophysiological responses to the environment would arise for different grapevine genotypes growing in a given habitat, which would be reflected in significant stable isotope content variations of fermentative ethanol (Martin et al., 1988). This appears to be the point in the case of *V. vinifera* L. cvs. Bobal and Tempranillo grown in the DO Utiel-Requena region. MANOVA results demonstrate significantly higher average ^{13}C and ^2H (methyl site) and lower ^2H (methylene site) contents over the 1991−1993 period for fermentative ethanol derived from autochthonous cv. Bobal grapes as compared to the allochthonous cv. Tempranillo (Table 3). This observation could be initially interpreted as a result of differences in the degree of ecological adaptation of both *vinifera* cultivars to the irregular water availability conditions of the Utiel-Requena region, which would be expressed through differences in water-use efficiency (Farquhar et al., 1988), accounting for significant plant water status variations between the autochthonous and allochthonous grapevine cultivars. The higher ^{13}C and ^2H (methyl site) contents of fermentative ethanol found for cv. Bobal seem to be in agreement with this interpretation, denoting a decrease of the evapotranspiration rate (higher water-use efficiency) as compared to cv. Tempranillo, whereas the lower ^2H (methylene site) content can be possibly related to the significantly longer vegetative cycle of the former cultivar, leading to an eventual ^2H dilution of grape berry water through late precipitation episodes. Although further research is demanded to support these assumptions, it can be assumed that grapevine genotypic features do represent relevant factors of natural ^2H and ^{13}C content variation of fermentative ethanol in genuine red wines from Valencia.

Discriminant Analysis of Combined ^2H and ^{13}C Data of Fermentative Ethanol. According to the above discussion, the combined evaluation of multi-isotopic data determined on fermentative ethanol appears to offer a convenient means for the regional and even varietal origin identification of Valencian red wines on the basis of the primary dependence of the ^2H and ^{13}C fingerprints upon environmental and ecophysiological factors. For the simultaneous integration of the classifying information individually provided by each isotopic parameter, a multivariate statistical analysis approach based on linear discriminant analysis (LDA) has been used. Optimal combinations of the four isotopic variables investigated [$(^2H/^1H)_{CH_3}$, $(^2H/^1H)_{CH_2}$, *R*, and $\delta^{13}C$] have been obtained (linear discriminant functions), the coefficients of which are computed to maximize the separation between the predefined groups and to minimize the within-group spread (Krzanowsky, 1988). Due to the occurrence of significant interannual effects (Table 1), discriminant functions were individually computed for each vintage year. To test the functions for statistical significance, the eigenvalue associated with each one and the Wilk's Λ statistic (computed as each function is derived) were evaluated. The former statistic provides an indication of the relative importance of the function. The latter one gives an inverse measure of the remaining discriminating information not yet accounted for by the earlier derived functions.

Results summarized in Table 4 show the discriminant information associated with the third function derived for each vintage year to be generally negligible (eigenvalue relative percentages 0.4, 2.7, and 2.1%, respectively), contributing insignificantly to the discriminating ability. Consequently, classification results are based on the computation of the two first discriminant functions, which are shown to account for 78 and 21% (vintage 1991), 76 and 21% (vintage 1992), and 61 and 37% (vintage 1993) of the total discriminating power accumulated in the multi-isotopic variables, respec-

Table 4. LDA Results [Discriminating Power (Change in Wilks' Λ and Associated Eigenvalues) and Coefficients of the Two First Discriminant Functions Calculated for Valencian Red Wines According to Individual Vintage Years]

functions derived	Wilks' Λ	X^2	discriminant function	eigenvalue	relative %	unstandardized (and standardized) function coefficients[a] $(^2H/^1H)_{CH_3}$	$(^2H/^1H)_{CH_2}$	R	$\delta^{13}C$	constant
year 1991										
0	0.05	82.9 **[b]	1	6.09	78.4	−4.85 (−4.9)	3.62 (4.2)	−195.92 (−6.8)	1.64 (1.1)	566.08
1	0.37	28.1 **	2	1.64	21.2	−4.30 (−4.3)	4.51 (5.2)	−190.93 (−6.6)	−0.28 (0.2)	329.49
2	0.97	0.8 ns								
year 1992										
0	0.09	67.1 **	1	3.77	76.4	1.05 (1.0)	−1.64 (−1.8)	51.62 (1.8)	1.56 (0.9)	16.13
1	0.43	23.4 **	2	1.04	21.0	1.49 (1.4)	−0.17 (−0.2)	46.69 (1.6)	1.02 (0.6)	−226.52
2	0.88	3.5 ns								
year 1993										
0	0.03	90.2 **	1	5.80	60.9	−1.92 (−1.8)	0.64 (0.6)	−75.81 (−2.4)	1.14 (0.9)	335.41
1	0.18	42.3 **	2	3.52	37.0	−0.0 (−0.1)	1.06 (0.9)	−7.92 (−0.3)	0.79 (0.6)	−92.89
2	0.83	4.6 ns								

[a] E.g.: Discriminant function 1 (year 1991) = 566.08 − 4.85 $(^2H/^1H)_{CH_3}$ + 3.62 $(^2H/^1H)_{CH_2}$ − 195.92R + 1.64 $\delta^{13}C$. [b] Significance level for the X^2 test ($P \leq 0.01$): ns and ** denote, respectively, statistically not significant and significant amounts of discriminating information remaining after derivation of the relevant function.

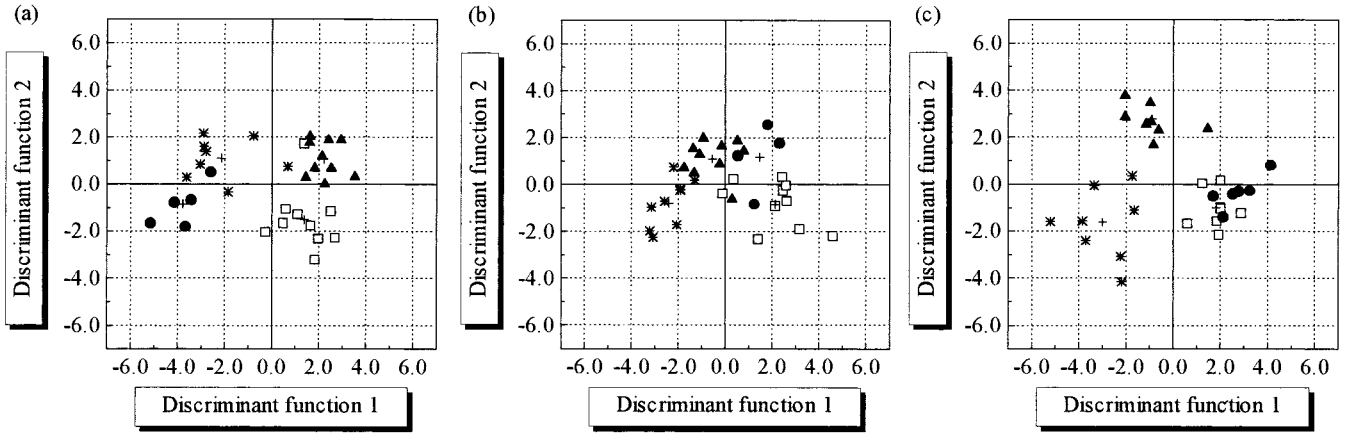

Figure 2. Scatter plot of discriminant scores for vintage years 1991 (a), 1992 (b), and 1993 (c) on the space defined by the two first canonical functions: DO Utiel-Requena cv. Bobal (□); DO Utiel-Requena cv. Tempranillo (●); DO Valencia (*); DO Alicante (▲); group centroids (+).

tively. Once the discriminant functions had been obtained, the relevant discriminant scores were calculated for each sample under research. Visualization of classification and between-group differentiation results was achieved by projecting the set of discriminant scores on the two-dimensional space defined by both discriminant functions (Figure 2). To identify the most significant isotopic variables contributing to the calculation of the various discriminant functions, the relevant standardized function coefficients were derived. Results obtained (Table 4) demonstrate that all four isotopic parameters significantly account for the classification of Valencian red wines according to regional origin or even varietal features.

Wirth regard to vintage year 1991 (Figure 2a), it is shown that differentiation on the direction of the first discriminant function is mainly associated with isotopic parameters R and $(^2H/^1H)_{CH_3}$. Variable $\delta^{13}C$ exhibits a larger relative contribution in this direction, whereas its contribution to the second discriminant function is negligible. On this basis, complete resolution between DO Utiel-Requena cv. Bobal [exhibiting lower R values and higher $(^2H/^1H)_{CH_3}$ and $\delta^{13}C$ values] and cv. Tempranillo wines [conversely showing higher R values and lower $(^2H/^1H)_{CH_3}$ and $\delta^{13}C$ values] is found to be able, as well as between cv. Monastrell wines, to differentiate both DO Alicante and DO Valencia cultivation areas. With regard to the second discriminant function, standardized coefficients reported in Table 4 show isotopic parameters R and $(^2H/^1H)_{CH_2}$ to exhibit the most significant contributions. In this respect, resolution in the discriminant space is shown to be possible between DO Utiel-Requena (both grapevine cultivars) and DO Alicante and DO Valencia wines [higher R and $(^2H/^1H)_{CH_2}$ values]. It appears, therefore, that the relevant geographic (environmental) gradient of $(^2H/^1H)_{CH_2}$ ratio variation in Valencian red wines (Figure 1b) is particularly expressed in this case on the second discriminant function direction, whereas genotype-related differences are mainly reflected on the first discriminant function axis in terms of parameters $(^2H/^1H)_{CH_3}$ and $\delta^{13}C$. Apparent classification accuracy results obtained by the resubstitution method (Table 5) are shown to reach 90% for DO Utiel-Requena cv. Bobal and 100% for DO Alicante cv. Monastrell wines.

Table 5. Discriminant Analysis Results for Valencian Red Wines: Percentage Degree of Correct Classification (Resubstitution Analysis) According to Regional Origin

origin (DO[a])	correct result (%) vintage 1991	vintage 1992	vintage 1993
Utiel-Requena			
cv. Bobal	90	80	100
cv. Tempranillo	80	75	83
Valencia	75	90	100
Alicante	100	70	100

[a] Denominación de Origen (Certified Brand of Origin).

With regard to vintage years 1992 and 1993 (Figure 2b,c), a somewhat different pattern of regional origin classification in the discriminant space is found in

comparison to vintage year 1991, as expected from the different patterns of interregional ^2H and ^{13}C content variation discussed above (Figures 1a,d). Thus, the first discriminant function derived is found to be mainly contributed by isotope parameters R, $(^2H/^1H)_{CH_2}$, and $\delta^{13}C$ (vintage 1992) and R, $(^2H/^1H)_{CH_3}$, and $\delta^{13}C$ (vintage 1993), whereas variables $(^2H/^1H)_{CH_3}$ (vintage 1992) and $(^2H/^1H)_{CH_2}$ (vintage 1993) are particularly associated with the second discriminant function (Table 4). In this case, the highest differentiation potential is also shown to be linked to the direction of the first discriminant function, enabling the complete resolution between DO Utiel-Requena and DO Valencia wines (showing higher R and lower $\delta^{13}C$ values). On the direction of the second discriminant function, differentiation is made evident between DO Valencia and DO Alicante wines on the basis of the higher $(^2H/^1H)_{CH_3}$ values noticed for the former (vintage 1992) and of the higher $(^2H/^1H)_{CH_2}$ and $\delta^{13}C$ values found for the latter (vintage 1993). However, no significant discrimination between DO Utiel-Requena cv. Bobal and cv. Tempranillo wines is now found. Complete resolution of all samples according to geographic origin is shown to be achieved for vintage year 1993, even within the limited range of the regions investigated. Group membership prediction rates are found to reach 100% for all three regional origins (Table 5), whereas the overall between-group spread is found to be significantly decreased for vintage year 1992 (Figure 2b).

Conclusion. Variability factors linked to the microclimatic complexity of most Mediterranean viticultural areas may pose a particularly relevant challenge for the stable isotope characterization of wine regional origin. This is demonstrated here for the most important red wine producing regions of Valencia (Spain). $^2H/^1H$ and $\delta^{13}C$ values determined on fermentative ethanol are shown to exhibit large interregional and interannual variations, even at a small-sized local scale. These variations are found to reflect not only differences of environmental conditions, but, moreover, the specific ecophysiological responses of the grapevine to the irregular environmental features and even genotype-related differences. Linear discriminant analysis is shown to be a convenient statistical tool for the integration of the environmental and ecophysiological information provided by the stable isotope fingerprints of fermentative ethanol. Regional origin discrimination results are found to vary noticeably from year to year, no general classification pattern being extrapolable to a number of vintages. Even so, results obtained for the 1991−1993 period allow us to conclude that the approach presented here may be of practical use for the typification of the most important red wine producing areas of Valencia and, hence, for quality control in relation with the regional origin assessment.

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