Oenological Influences on the D/H Ratios of Wine Ethanol

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Within the European Union the determination of chaptalization of wine involves the comparison of the D/H ratios of ethanol with the ratios of authentic wine samples that are similar to the suspect wine in terms of geographical origin, grape variety, and vintage. In the frame of a databank project comparison, wines are produced under official control on a small scale. To clarify the influence of the different production conditions between commercial wines and these databank wines, wines that were produced under varying conditions were investigated by the ^{2}H NMR method. None of the parameters under investigation, such as yeast strain, fermentation temperature, or wine fining, showed a significant influence on the $(D/H)_I$ ratio of wine ethanol, which is the most indicative parameter for the determination of the addition of extraneous sugar to wine. For the $(D/H)_{II}$ ratio, different values were found for different yeast strains used for fermentation and a slight decrease was observed with increasing fermentation temperature. At increasing points of fermentation yield an increase of the D/H ratios was found in the present alcohol. The total increase of the $(D/H)_I$ ratio throughout the fermentation was approximately 1 ppm, so that with a fermentation yield of more than 50% no statistical difference could be observed.

Keywords: *Chaptalization; ^{2}H NMR; D/H ratios; wine databank; wine fining; oenological influences*

INTRODUCTION

Within the European Union, the addition of sugar to grape must or young wine (chaptalization), to increase the genuine content of alcohol, is only permitted under certain conditions. The conformity of sugar additions with existing regulations depends on the individual wine-growing region and the potential alcohol content of the must (EC Regulation 822/87, 1987). Since wine that has been enriched gives a better sensorial impression in general, the alcohol content directly influences the commercial value of wine. Therefore, the illicit addition of sugar is a traditional problem within the wine industry with a very strong economic influence. Consequently, the demand existed for a method of analysis that is suitable for the reliable detection of enrichments of musts and wines with extrinsic sugar.

In 1990 the European Union adopted a method for the detection of chaptalization, which is based on the site-specific determination of the deuterium content in wine alcohol with the SNIF-NMR technique (site-specific natural isotopic fractionation−nuclear magnetic resonance) (EC Regulation 2676/90, 1990). This method was developed in the early eighties by Martin et al. and is protected by a patent for commercial applications (Martin and Martin, 1981, 1983, 1987). Deuterium (D, ^{2}H) is a stable isotope of hydrogen (^{1}H). In natural organic compounds 150 deuterium atoms on average appear among 1 million hydrogen atoms. The deuterium content and its distribution in organic material is not random or always the same so it may deliver information about the type and origin of the material. This has been shown for different molecules, such as benzaldehyde, glycerol, and ethanol (Hagedorn, 1992; Hermann, 1999; Martin et al., 1985). The characteristic site-specific distribution of deuterium within a molecule can be visualized by high-resolution ^{2}H nuclear magnetic spectroscopy.

For the ^{2}H NMR analysis of ethanol, tetramethylurea (TMU) is used as an internal standard for the calculation of the D/H ratios. The labeling of the positions and their respective D/H ratios were established by Martin et al. (1985). The $(D/H)_I$ ratio is the D/H ratio of the methyl group; the $(D/H)_{II}$ ratio reflects the D/H ratio of the methylene group. As an intramolecular parameter the *R* value was defined, which sets in relation the deuterium contents of the methyl group and the methylene group. In natural alcohol the methylene group is highly enriched in deuterium compared to the methyl group.

Ethyl alcohol obtained from different raw materials shows particularly big differences in the D/H ratio of the methyl group. Alcohol produced from beet sugar has a $(D/H)_I$ ratio of ∼92 ppm, and alcohol derived from cane sugar ∼109 ppm; instead wine alcohol averages at ∼101 ppm. Therefore the addition of extraneous sugar, beet root or cane, to grape must leads to significant alterations in the $(D/H)_I$ ratio of the resulting alcohol. On the other hand the $(D/H)_{II}$ ratio is mainly affected by the deuterium content of the fermentation water (Martin and Martin, 1988).

Due to biological variability the D/H ratio of the methyl group of authentic wine alcohol varies between 98 and 105 ppm. The observed variation depends on the geographical origin, the vintage, and the grape variety. In comparison to that it has been established that the addition of 17 g/L extraneous sugar causes an alteration of only ∼0.9 ppm with respect to the original genuine value (Martin and Martin, 1988; Martin et al., 1986). Consequently, the observed natural range of variation is large compared to the modulation of the $(D/H)_I$ ratio

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caused by enrichment. It is of advantage for analytical purposes that the reported D/H ratios of alcohol obtained from different sugars (beet sugar or cane sugar) are almost stable.

Other influences on the D/H ratios, apart from enrichment, are so manifold and unpredictable that the only way to interpret the D/H ratios of unknown samples is by comparison with authentic wines that are as similar as possible to the suspect wine in terms of geographical origin, vintage, and grape variety. Consequently it is necessary to analyze a lot of authentic wines on a yearly basis in order to obtain a comprehensive net of comparison data. Thus, in the case of a suspect wine it should be possible to refer to isotopic ratios of authentic wines, which are as similar as possible to it, concerning the parameters mentioned above. Depending on the information about the suspect wine a certain range, the so-called authenticity range of the genuine $(D/H)_I$ ratio can be determined and the wine can be assessed in terms of possible enrichment.

A database of authentic wines from the wine-growing countries in the European Union was established and is constantly growing [EC Regulations 2347/91, (1991a) and 2348/91 (1991b)]. The isotopic parameters of the ^{2}H-SNIF-NMR analysis are entered as well as many others such as the geographical origin, grape variety, time of picking, etc. Since 1991 about 1200 wine samples per year have been provided by the Member States and analyzed [Austria, 50 (since 1997); France, 400; Germany, 200; Greece, 50; Italy, 400; Luxembourg, 2; Portugal, 50; Spain, 100; United Kingdom, 2). The distribution of the samples within the Member States must take into account the geographical circumstances and the cultivated grape varieties of each wine-growing area, aiming at a reasonable and representative database.

As well as the SNIF-NMR analysis of the databank wines, their careful production is also a very important part of the databank project, since only their isotopic ratios alone are decisive in the assessment of enrichment. Therefore the thorough microvinification is definitely of as much importance as the NMR analysis. The sample vinification must take place in official institutes, or at least under official control, in a way that should be similar to the regionally established production processes.

In this regard, the fact that these wines are produced by the microvinification of at least 10 kg of grapes, which does not allow the application of all oenological practices that are regularly used in wine cellars, is a limiting factor.

Usually the must is obtained from approximately 20 kg of authentic grapes, picked by official wine controllers at the time of harvest and transferred to the official microvinification facilities. Often the must is pressed from the grapes by manually operated presses. After rapid fermentation (~1 week), initiated spontaneously or by adding dry yeast, in 10 or 15 L fermentation vessels, the young wine is cooled for a couple of days. During this time the yeast and deposits sink to the bottom. Afterward the wine is decanted and sulfur dioxide is added. After further storage at low temperatures the wine is filtrated into bottles. The main aim of this kind of microvinification is not to produce a sensorially valuable wine but to ferment continuously, without any losses, the total amount of fermentable sugars into ethanol.

Consequently, for a suspect wine, the assessment depends on the comparison of commercial samples with the experimental wines analyzed for the data bank, which may have experienced different production procedures or treatments.

Therefore, it was the aim of this study to investigate oenological influences on the D/H ratios of wine alcohol to ensure their correct interpretation. In particular, the treatments of must or wine that may not usually be applied during the production of databank wines were of particular interest. Furthermore, several fermentation parameters were tested as potential influencing factors on the D/H ratios, which are not regulated or fixed either for the production of authentic comparison wines or for the production of commercial wines.

Method. The wines under investigation were produced under varying conditions that were adapted to suit the respective aim of each study. Details are given in each particular section. These wine samples were analyzed according to the official SNIF-NMR method. The procedure of analysis is specified in detail in EC Regulation 2676/90 (1990).

RESULTS AND DISCUSSION

Effect of Cellar Treatment. The clarification or stabilization of the microvinified databank wines with appropriate agents is normally not foreseen. In wine production different types of cellar operations for various purposes are often applied. Therefore the knowledge of possible effects was of interest for their interpretation.

In Annex VI of EC Regulation 822/87 (1987) the allowed oenological procedures are described. As part of the list, fining agents and their maximum application concentrations for wine clarification and stabilization are specified.

Today wine fining is done mainly for three reasons: (1) clarification, (2) stabilization and protection against repeated turbidity of already clear or clarified wines, or (3) for sensorial improvement and combined appreciation of wines. Usually the fining material, its type and amount selected in pretrials, is thoroughly mixed with the wine and remains for a certain contact time until its precipitation (Troost, 1980). Afterward the so-called fining precipitate is separated from the wine by decanting or filtration. The principle of fining is based on the coprecipitation, or adsorption, of certain substances on the fining material. Effects on the D/H ratios of ethanol, which has already been formed at the time of application, were not expected due to the physical and chemical properties of deuterated and undeuterated molecules. Since any doubts could be of benefit to a potential wine adulterator, a justifiable demand still existed for this study.

The investigation included 20 different fining materials from two different producers, which were applied to two different wines. Application of the fining techniques took place in 25 L vessels containing the wine. Both wines were German Riesling wines from the cultivation region Mosel-Saar-Ruwer. The first wine was made from healthy grapes, the second from mainly moldy grapes. Intermediate concentrations, according to the recommendations of the producer, were used for the different agents. One SNIF-NMR analysis for each treated wine was performed. Table 1 shows the corresponding isotopic ratios of the wines.

The means, the standard deviations, and the variances are shown in Table 1. The ratios of the untreated

Table 1. Summary of SNIF-NMR Results

Wine 1			
fining agent	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R-Wert
	Producer A		
Na−Ca-bentonite (a)	102.41	124.04	2.420
Ca-bentonite	102.30	124.38	2.432
activated carbon	101.95	124.12	2.432
gelatin/infusorial earth	101.90	124.11	2.436
infusorial earth/gelatin	102.49	124.38	2.425
isinglass + gelatin	102.03	124.27	2.435
K-caseinate	102.10	123.81	2.422
tannin adsorption agent	102.11	124.03	2.431
PVPP	102.07	123.99	2.429
Na−Ca-bentonite (b)	102.09	123.80	2.425
	Producer B		
Ca-bentonite	101.87	123.98	2.437
Ca-bentonite (higher concentration)	102.15	124.29	2.430
bentonite	102.74	124.36	2.421
activated carbon	101.94	124.29	2.439
gelatin/infusorial earth	101.80	124.22	2.440
infusorial earth/gelatin	102.58	124.20	2.424
isinglass	101.90	123.96	2.434
caseinate	102.02	124.31	2.434
tannin adsorption agent	102.07	124.29	2.433
PVPP	102.13	123.96	2.427
mean	102.13	124.14	2.430
standard deviation	0.251	0.185	0.006
variance	0.0629	0.0344	3.6×10^{-5}
wine 1 (native) ($n = 2$)	101.89	124.02	2.433

Wine 2			
	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R-Wert
mean	102.41	124.33	2.428
standard deviation	0.265	0.215	0.0056
variance	0.0702	0.0460	3.1×10^{-5}
wine 2 (native) ($n = 2$)	102.54	124.43	2.429

Table 2. Statistical Values of the Repeated Analysis of One Identical Wine ($n = 13$)

value	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R value
mean	99.48	125.97	2.533
standard deviation	0.224	0.248	0.0047
variance	0.0502	0.0615	2.2×10^{-5}

wines have not been included in the calculation of statistical values. Obviously large differences between single results are not present. The small differences between the measurements can be based on the imprecision of the SNIF-NMR method. For the statistical conformation the variance comparison test (F-test) was used (Kaiser and Gottschalk, 1972). In this case the variance of the repeated analysis of one identical wine (see Table 2) was compared with the respective variance of each experimental series.

Here, the variance of the repeated analysis is a measurement of the variation caused by the method (reproducibility). The application of the F-test showed that no statistical differences between the considered standard deviations are present. So the application of different wine fining techniques to two different wines did not lead to a statistically differentiable variance of the SNIF-NMR results compared to the repeated analysis of an identical wine. This is the case for the $(D/H)_I$ and $(D/H)_{II}$ ratios and the R value. Finally, one can state that each series of wines with different wine treatments can be considered as the repeated analysis of an identical wine.

Effect of Must Treatments. Several must treatments were investigated; for instance, must clarification by sedimentation and centrifugation. Neither type of treatment showed statistically significant differences in the D/H ratios.

Also, reverse osmosis belongs to the category of must treatments and has been increasingly used for the enrichment of must during recent years. Hereby the must is pressed against semipermeable membranes, leading to the loss of water. The remaining must is enriched during this procedure. At present an interest exists in the determination of enrichments caused by reverse osmosis. Several samples enriched to different levels were investigated with the SNIF-NMR method, but the D/H ratios of the enriched samples did not vary significantly from those of the untreated wines.

Effect of Fermentation Yield. The fermentation yield of wine is based on the relation between the alcohol present in the wine and the potential alcohol. Wine with a fermentation yield of 100% is completely fermented and no fermentable sugars are present. This study was of particular importance for the judgment of naturally sweet wines.

The adjustment of the residual sweetness is often done by the illicit addition of sucrose. When there is not a complete inversion of the sucrose this can be detected by a simple sugar analysis. If the inversion is complete, on the other hand, which is the case if the sugar is added prior to or during the fermentation, or if invert sugar syrup is added, its detection is much more complicated. One can obtain relevant clues from the ratio of glucose to fructose, which changes characteristically during fermentation, but the method of choice is SNIF-NMR analysis. For this purpose the alcohol present is sepa-

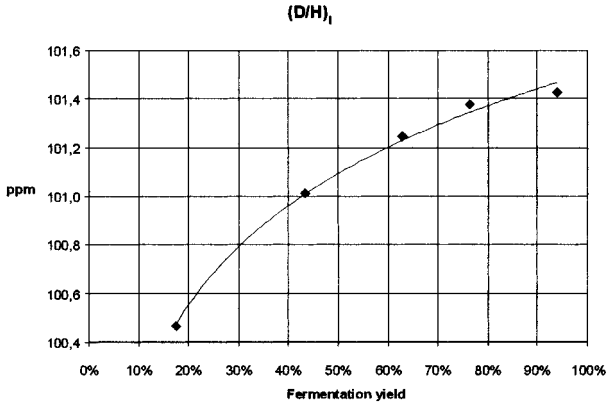

Figure 1. $(D/H)_I$ ratio depending on fermentation yield.

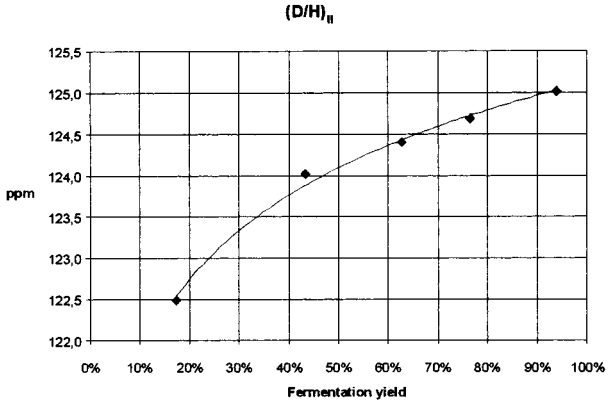

Figure 2. $(D/H)_{II}$ ratio depending on fermentation yield.

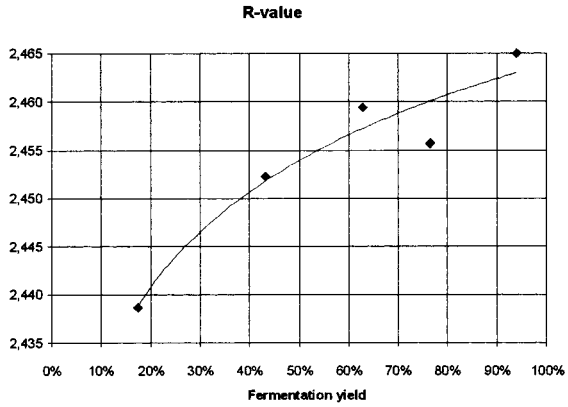

Figure 3. *R*-value depending on fermentation yield.

rated by distillation and analyzed. The sweet distillation residue is fermented completely by the use of dry yeast and distilled a second time. The D/H ratios of the second SNIF-NMR analysis correspond to the residual sweetness. The addition of beet sugar to enhance the residual sweetness can be easily detected by the very low $(D/H)_I$ ratio of the second distillate. The assessment of genuinely sweet wines requires some knowledge about the behavior of the D/H ratios during fermentation.

For this study samples of young wine were taken from a large vessel at several stages during their fermentation and then immediately distilled. The fermentation yield was calculated from the original and actual sugar contents, which was controlled by daily sugar analysis (according to Luff-Schoorl, EC Regulation 2676/90, 1990). The results are shown in Figures 1−3. All three measured values increase with the fermentation yield of the must. Curve-fitting was achieved by applying logarithmitical regression. With the consideration that the total fermentation the observed difference in the $(D/H)_I$ ratio was only about 1 ppm, it can be pointed out that from a fermentation yield of 50% or higher no statistically significant difference between fermentation yields can be ascertained. On the other hand the $(D/H)_{II}$ ratio increased during fermentation by up to 3.5 ppm. This effect can be explained by the increase in the deuterium content of the fermentation medium based on the reduction of deuterium-enriched sugars, as described earlier (Martin et al. 1988). The $(D/H)_{II}$ ratio is particularly affected by the isotopic content of the fermentation medium, with a lesser effect, on the $(D/H)_I$ ratio.

Another comprehensive study of wines with genuine sweetness demonstrated that the $(D/H)_I$ ratio of the fermented residual sugar is 0.5−1.5 ppm higher than the $(D/H)_I$ ratio of the original wine alcohol (Christoph, 1994). This observation is also based on the increase in the $(D/H)_I$ ratio with fermentation yield.

Effect of Yeast. Up to now the effect of different yeasts on the D/H ratios has not been investigated in detail. In one study only two strains (*Saccharomyces cerevisiae*) were compared, whereby no differences between the resulting D/H ratios of wine ethanol were observed (Martin et al., 1986). Isotopic fractionation in chemical reactions is also a kinetic phenomenon, based on the different bonding energies between deuterium and hydrogen to carbon (Kalinowski, 1988). Therefore isotopic effects may also occur in the field of enzyme catalysis.

Yeasts that are used for technical fermentations for beer, wine, spirit, and baking belong almost exclusively to the genus *Saccharomyces* and its species *cerevisiae*. For certain applications, for instance, the refermentation of wine, *Saccharomyces bayanus* yeast is utilized. Ever since yeast cultivation has existed, many types have been isolated and brought into cultivation, frequently named according to their geographical origin or to the vineyard from which they were taken (Dittrich, 1977). Yeast is predominantly applied as dried yeast, moderately dried yeast with a final water content of about 8%. Several types of dried yeast, isolated from musts or wines, are available on the market. Besides spontaneous fermentation these yeasts are often used for the production of wine because their application provides some advantages with respect to classical spontaneous fermentation. For example, higher alcohol contents can be reached combined with a reduction of undesired byproducts or off-flavors. After dehydration of the dried yeast material it is added to the must at a concentration of up to 0.3 g/L. The high starting concentrations and the dominating properties suppress the growth of wild yeasts so that the cultivated yeast controls the fermentation exclusively.

Microvinification of databank wines does not require the usage of a certain yeast strain. The model experiments carried out were performed in order to identify the possible effect on the D/H ratios of different yeast strains. Dried yeast agents that are in common use were investigated in depth.

Must (90 L) was obtained from 160 kg of white Italian grapes and prepared for the different experiments. The must was split into 18 portions of about 4.5 L. The fermentation was performed with eight different dried yeast agents in duplicate (yeast 1−7 were *Saccharomyces cerevisiae*, yeast 8 *was Saccharomyces bayanus*). Two

Table 3. D/H Ratios of Fermentation Experiments with Different Yeast Strains[a]

	expt	yeast 1	yeast 2	yeast 3	yeast 4	yeast 5	yeast 6	yeast 7	yeast 8	spontaneous fermentation
$(D/H)_I$ (ppm)	1	100.25	100.57	100.49	100.46	100.40	100.81	100.52	100.02	100.81
	2	100.75	100.59	100.20	100.33	100.61	100.21	100.31	100.31	100.27
$(D/H)_{II}$ (ppm)	1	126.03	126.37	124.40	127.67	126.51	125.41	125.49	126.40	127.30
	2	125.90	126.52	123.68	128.04	127.40	124.86	125.76	126.59	125.66
R ratio	1	2.511	2.513	2.476	2.544	2.520	2.490	2.491	2.527	2.526
	2	2.503	2.513	2.473	2.546	2.530	2.491	2.507	2.527	2.508

[a] All *Saccharomyces cerevisiae* except yeast 8, which was *Saccharomyces bayanus*; also spontaneous fermentation was performed. Each fermentation was done twice (experiments 1 and 2).

Table 4. Results of ANOVA Applied to the Data of Different Fermentations with Different Yeast Strains[a]

	test value	probability	critical value (table value)
$(D/H)_I$	0.496	0.832	3.230
$(D/H)_{II}$	9.463	0.001	3.230
R ratio	21.137	<0.001	3.230

[a] The test value is the result of the ANOVA, the critical value is the table value for the chosen probability of 95%. The probability is the probability for confirmation of the null hypothesis (no differences between the groups).

Table 5. Results of Standardized Fermentation with One Yeast

($n = 6$)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	*R* value
mean	101.03	125.01	2.475
standard deviation	0.131	0.100	0.004

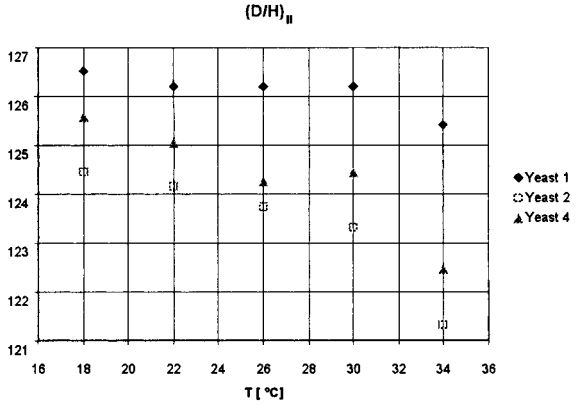

Figure 4. $(D/H)_{II}$ ratio depending on fermentation temperature.

portions were fermented by spontaneous fermentation without addition of any yeast. The production of this experimental series then followed the microvinification process used for the databank wines (pressing, dimension of preparation, decanting, filtration, etc.). The results of the fermentation experiments are given in Table 3.

For the statistical evaluations analysis of variance (ANOVA) was performed and the results are shown in Table 4. The null hypothesis, that there are no statistically significant differences between the different groups (fermentation with different yeast strains), was confirmed only for the $(D/H)_I$ ratio. The $(D/H)_{II}$ ratio and the *R* value depend on the yeast utilized for fermentation.

In individual cases differences of up to 4 ppm were observed for the $(D/H)_{II}$ ratio (Yeast 3, 124.0 ppm; yeast 4, 127.8 ppm). Further fermentation experiments with different musts exhibited that it was not possible to reproduce certain extreme data. No fixed pattern depending on the yeast strain was achieved. Only yeast 3 (*S. cerevisiae*) resulted in low $(D/H)_{II}$ ratios in all cases. As a consequence of the stability of the $(D/H)_I$ ratios and varying $(D/H)_{II}$ ratios, the *R* values—by definition a quotient of the two ratios—behave like the $(D/H)_{II}$ ratio.

To confirm the effect of the yeast on the $(D/H)_{II}$ ratio and *R* value, additional experiments were performed. One must was fermented under standardized conditions with the same dried yeast. The results obtained are shown in Table 5. The standard deviations are smaller than for the repeated analysis of a finished wine. These results must be considered as random because the additional step of fermentation cannot reduce the variation of the method of analysis. It clearly indicates that the influence is due to the yeast and not to other parameters.

Influence of Fermentation Temperature. Another fermentation parameter that can potentially affect the D/H ratios of alcohol is the fermentation temperature. Under real conditions the cellar temperature within wine-growing regions lies between 16 and 24 °C. Lower temperatures (11−14 °C) are present in certain regions of the Mosel in Germany as extreme values, combined with fermentation periods of over 6 months. Since glycolysis is an exothermal process, the fermentation good is warmed during fermentation. In large containers temperatures of more than 35 °C are observed, which may cause the heat death of yeast followed by interruption of fermentation under certain conditions. To prevent high fermentation temperatures the fermentation containers can be cooled from outside with water. Without active cooling the fermentation temperature inside the vessel cannot be below room temperature. It is known that completely identical fermentation setups (must, amount, container, yeast) can ferment at different temperatures.

Due to the exothermal glycolysis the real fermentation temperature is not easy to control. It is possible to vary the surrounding temperature of the fermentation vessel in a model experiment. It was decided to carry out this study with small fermentation vessels stored in a water bath at different temperatures. Hereby, the temperature exchange between the fermentation vessel and the water is much higher than when temperature-regulated air is used. This setup for the experiment is clearly of model character but it guaranteed that the fermentation was at the selected temperature.

The fermentation of 600 mL of must was performed in 1000 mL vessels. The must was a pasteurized filtered Rheingau-Riesling. The fermentations were carried out at 18, 22, 26, 30, and 34 °C in a 25 L water bath, adjustable to 0.1 °C. Duplicate fermentations with three commercial dry yeasts were performed. A yeast cell wall agent (0.5 g) was added as fermentation nutrient to all preparations. The fermentation was monitored by the CO_2 loss, and the end of fermentation was checked by residual sugar analysis. Figures 4 and 5 show the results of SNIF-NMR analyses. Each point is the average of a duplicate determination, including fermentation.

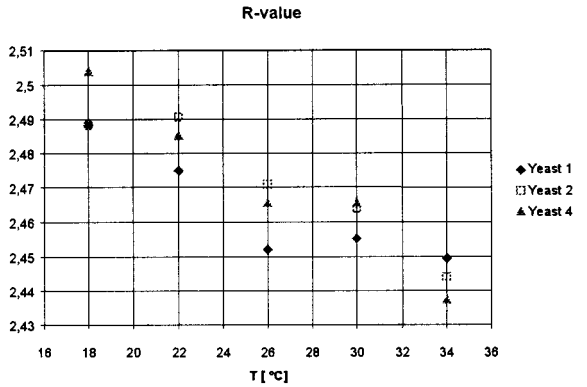

Figure 5. *R* value depending on fermentation temperature.

The $(D/H)_I$ ratio was nearly constant for the different fermentations, therefore it is not displayed. The $(D/H)_{II}$ ratio and *R* value decreased with increasing temperature. A temperature difference of 16 °C caused a decrease of up to 3 ppm for yeasts 2 and 4. The decrease was not that clear for the fermentation with yeast 1. The *R* value declined by 0.06 unit with a temperature increase of 16 °C. Here the decrease was almost equal for all three yeasts.

Confirmation of these results obtained under the above model conditions was performed by the investigation of a series of wines produced close to the established practice at different temperatures during fermentation. The fermentation of fresh-pressed untreated must was carried out in 100 L containers; one was stored in a cool wine cellar, and the other was stored in a warmer room (real fermentation temperature difference ~6−8 °C). Also here the evaluation showed that the $(D/H)_{II}$ ratios and the *R* values decreased with increasing temperature. On the contrary, the $(D/H)_I$ ratio remained constant.

Conclusions. It has been shown that the $(D/H)_I$-ratio is independent of all considered wine and must treatments. This is very important since a supposed adulterator cannot refer to differences between the suspect wine and the experimental wines of the databank during their production as explanation for different $(D/H)_I$ ratios.

It was found that the $(D/H)_{II}$ ratio and the *R* value vary in relation to the production conditions. They are influenced by the yeast strain used for fermentation and the fermentation temperature. Regarding wine authenticity, the amount of additional information that can be obtained from the $(D/H)_{II}$ ratio and *R* value is clearly restricted due to these influences.

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