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Targeted and Nontargeted Wine Analysis by ^{1}H NMR Spectroscopy Combined with Multivariate Statistical Analysis. Differentiation of Important Parameters: Grape Variety, Geographical Origin, Year of Vintage

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Supporting Information

ABSTRACT: The authenticity, the grape variety, the geographical origin, and the year of vintage of wines produced in Germany were investigated by ^{1}H NMR spectroscopy in combination with several steps of multivariate data analysis including principal component analysis (PCA), linear discrimination analysis (LDA), and multivariate analysis of variance (MANOVA) together with cross-validation (CV) embedded in a Monte Carlo resampling approach (MC) and others. A total of about 600 wines were selected and carefully collected from five wine-growing areas in the southern and southwestern parts of Germany. Simultaneous saturation of the resonances of water and ethanol by application of a low-power eight-frequency band irradiation using shaped pulses allowed for high receiver gain settings and hence optimized signal-to-noise ratios. Correct prediction of classification of the grape varieties of Pinot noir, Lemberger, Pinot blanc/Pinot gris, Müller-Thurgau, Riesling, and Gewürztraminer of 95% in the wine panel was achieved. The classification of the vintage of all analyzed wines resulted in correct predictions of 97 and 96%, respectively, for vintage 2008 (n = 318) and 2009 (n = 265). The geographic origin of all wines from the largest German wine-producing regions, Rheinpfalz, Rheinhessen, Mosel, Baden, and Württemberg, could be predicted 89% correctly on average. Each NMR spectrum could be regarded as the individual "fingerprint" of a wine sample, which includes information about variety, origin, vintage, physiological state, technological treatment, and others.

KEYWORDS: *^{1}H NMR spectroscopy, grape variety, geographical origin, year of vintage, German wine, multivariate statistical analysis*

INTRODUCTION

Nuclear magnetic resonance (NMR) has been used for structure analysis of pure compounds over decades. The determination of D/H isotopic ratios by SNIF-NMR in ethanol produced by fermentation of sugars from C3 or C4 plants is used for the detection of adulterations in wines and fruit juices.[1−3] First quantitative analysis of contaminants such as diethylene glycol[4] and natural compounds such as sugars, sugar alcohols, glycerol, and sugar acids[5,6] in wine by ^{13}C NMR was reported in the mid 1980s. The limit of detection was 10 mg/L for diethylene glycol. Sugar compounds with concentrations exceeding 1 g/L could be determined directly in wine, whereas those with concentrations below 1 g/L could be measured in wine concentrates. Ten to fifteen years later, the extraordinary potential of NMR has started to be recognized and exploited in the analysis of mixtures in the context of biofluids, contaminants,[7] foods,[8−10] and beverages.[11−15] This was possible due to the availability of high-throughput automation technology, the increase of sensitivity, and modern water suppression NMR sequences such that simple proton NMR has become applicable, providing highly information rich data. Not only is qualitative information about substances in complex mixtures such as foods and beverages accessible, but also quantitative determination of compounds is possible about a linear range of up to 4−5 magnitudes in only one spectrum.

The most important questions in enology and prevention of adulterations in wine are grape varieties, geographical origin of wine, and year of vintage. Many attempts had been made to analyze the year of vintage, for example, in Montepulciano d'Abruzzo wines.[16] Also, grape varieties were analyzed by several techniques, for example, using aroma compounds by principal component analysis (PCA) and linear discriminant analysis (LDA),[17] by combination of mass spectrometry (MS) based electronic nose (eNose) with visible (VIS) and near-infrared spectroscopy (NIR),[18] and by reduced Fourier transformed infrared FTIR data (FOSS WineScan FT120) in combination with PCA/LDA.[19] The ultraviolet region has been used for the discrimination of types of wines from the Spanish designation of origin La Mancha. PCA and soft independent modeling of class analogy (SIMCA) were used for developing classification models (origin, grape variety, aging process).[20] The composition of the anthocyanins and their relations in red wine is a good tool for the differentiation of red wine varieties, measured by HPLC[21,22] as well as proposed by FTIR.[23] The shikimic acid content of wines, especially of Burgundy grapes,

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Table 1. Experimental Design of All Wine Varieties Measured (Numbers in Parentheses Are EU Database Wines)

	BAD	WT	RHH	MSR	RHP	unknown	total
Riesling	86 (10)	8 (2)	16 (16)	24 (24)	9 (9)	6 (1)	149 (62)
Spätburgunder (Pinot noir)	58 (11)	9 (6)	1 (1)	4 (4)	2 (2)	5 (2)	79 (26)
Weißburgunder (Pinot blanc)	51 (5)	4	2 (2)	1 (1)	2 (2)	1	61 (10)
Müller-Thurgau	39	4	8 (8)	3 (3)	1 (1)	1	56 (12)
Grauburgunder (Pinot gris)	37		4 (4)			6 (1)	47 (5)
Schwarzriesling (Pinot Meunier)	14 (1)	5 (1)				1	20 (2)
Dornfelder	1	1	6 (6)	4 (4)	5 (5)	2 (1)	19 (16)
Gewürztraminer	8 (4)	6 (2)			1 (1)		15 (7)
Lemberger	5	6 (3)				3 (1)	14 (4)
Silvaner	4	1	7 (7)		2 (2)		14 (9)
Sauvignon blanc	11	1					12
Kerner	1	3 (2)	3 (3)	2 (2)	3 (3)		12 (10)
Auxerrois	9 (2)			1 (1)		1	11 (3)
Chardonnay	7	1	1 (1)				9 (1)
Trollinger		9 (6)					9 (6)
Regent	6				1 (1)		7 (1)
Scheurebe	3		4 (4)				7 (4)
Gutedel	6 (4)						6 (4)
Portugieser	1		2 (2)		2 (2)		5 (4)
Cabernet Sauvignon	3				1 (1)		4 (1)
Müller	4 (4)						4 (4)
Elbling				3 (3)			3 (3)
Muskateller	1	1					2
Nobling	2						2
Bacchus			1 (1)	1 (1)			2 (2)
Traminer	1	1					2
Siegerrebe			1 (1)				1 (1)
Huxelrebe			1 (1)				1 (1)
Merlot	1						1
total	359 (41)	60 (22)	57 (57)	43 (43)	29 (29)	19 (5)	574 (198)

allows a prediction of grape variety.[24] Red wine cultivars were differentiated by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) of extracts of phenolic compounds investigated.[25]

For the characterization of the geographic origins, the authentification potential of combined isotopic (SNIF NMR) and trace element analysis (IRMS) was used to predict the origin of 10 regions and 4 years of production of Bordeaux wines.[26] Also, by combination of SNIF NMR and IRMS (^{13}C, ^{18}O) data Slovenian wines could be discriminated between coastal and continental regions[27] as well as Spanish wine from the Valencia region.[28] By chemical characterization of Italian wines from different geographical locations in the Apulia region of southern Italy combined with ICP-MS and ^{1}H NMR, the wines were divided in three groups by multivariate data analysis according to their geographical origin.[29] High-performance ion chromatogaphy exclusion (HPICE), ICP-OES, and ^{1}H NMR measurements were carried out in combination with chemometrics on wine samples from Slovenian and Apulian wine-growing areas.[30] Swiss wines could be differentiated into four main regions, Valais, Tessin, eastern Switzerland, and western Switzerland, by means of multi-isotopic analysis (^{1}H, ^{2}H, ^{18}O) combined with chemometric methods.[31] Polyphenol-rich extracts were used for the classification of Greek wines according to variety, geographical origin, and vintage using NMR-based metabolomics.[32] Metabolite profiles of white wines were determined using GC-TOF-MS and ^{1}H NMR.[33] Metabolomic studies used ^{1}H NMR in combination with multivariate statistics also in wine fermentation processes.[34−39] Metabolomic analysis of German white wines using NMR techniques including phenolic extracts correlated to sensory attributes, varieties, and vintages.[40] Red wines of the three varieties Cabernet Sauvignon, Merlot, and Pinot noir from various geographical origins from Europe and the United States were correctly classified in a range of 96% of samples by HPLC-QTOFMS in combination with several chemometric tools.[41]

In most of these examples for the differentiation of the geographical origin, year of vintage, and grape variety, combinations of chromatographical, spectroscopic, and analytical data have been used for chemometric statistical analysis. Nowadays, it is possible to achieve these objectives only by NMR data and multivariate statistical analysis.[42−47] The information on latent parameters such as grape variety, origin, and vintage is coded in multivariate patterns of multiple parameters in the NMR spectra rather coded in single spectrum markers. Therefore, each NMR spectrum could be regarded as a personal "fingerprint" of each wine sample including all information about variety, origin, vintage, physiological state, technological treatments, and others.

This study combines ^{1}H NMR spectroscopy under an 8-fold suppression of water and ethanol with several steps of multivariate statistical analysis to differentiate between several grape varieties in German wines by targeted as well as nontargeted analysis. The study for the first time highlights answers to the most important questions in enology and prevention of adulterations in wine with a high rate of

predictivity in a large amount of German wines: grape variety, geographical origin of wine, and year of vintage. There are no or only a few attempts to achieve these goals up to now.

MATERIALS AND METHODS

Samples. Authentic samples of pure grape variety wines of the years of production 2008 and 2009 were taken from wine manufactures by official wine inspectors of CVUA Karlsruhe in the Federal State Baden-Württemberg. Samples of wines were given by the official wine research institutes of Baden-Württemberg, Wine Research Institute Freiburg, and Wine Research Institute Weinsberg. Micro-vinified wines according to protocol of EU regulation 2729/2000 for EU Wine Data Base were collected from official wine research institutes in Baden-Württemberg and Rheinland-Pfalz (EU Database Wines). No samples had been blended with any other variety, other vintage, or wine from other regions. The wines derived from the following German geographic wine regions: Baden (BAD), Württemberg (WT), Pfalz (RHP), Rheinhessen (RHH), and Mosel-Saar-Ruwer (MSR). Most of the wines were from the vintage 2008 (n = 318) and 2009 (n = 265). Wines of the following grape varieties were analyzed: Riesling (149), Pinot noir (Spätburgunder 79), Müller-Thurgau (56), Pinot blanc (Weißburgunder, 61), Pinot gris (Grauburgunder 47), Pinot Meunier (Schwarzriesling 20), Dornfelder (19), Gewürztraminer (15), Silvaner (14), Lemberger (14), and some others in minor numbers (Table 1).

Mixture Samples. Different mixtures of pure grape wines (100%) were carried out in steps of 5−10% per volume in laboratory scale.

^{1}H NMR Spectroscopic Analysis of Wine Specimens. For NMR sample preparation, 100 μL of phosphate buffer (1 M KH_2PO_4, 0.1% 3-(trimethylsilyl)propionic acid sodium salt (TSP) as internal standard, D_2O, and NaN_3) were added to 900 μL of wine, and the pH was adjusted to 3.10 exactly (±0.02) (BTpH Combined Titration pH Unit, Bruker BioSpin GmbH, Germany). From the prepared mixture, 600 μL was filled into a 5 mm Wilmad NMR tube (Wilmad Labglass Inc., Vineland, NJ, USA). NMR was performed under full automation for the whole process on an AVANCE III 400 at Bruker BioSpin GmbH, Rheinstetten, Germany, equipped with a 5 mm 1H/D-TXI probehead with z-gradient, automated tuning and matching accessory, and BTO-2000 for temperature control. Samples were measured at 300.0 K. After automated sample transfer to the magnet by a BACS-60 autosampler, a 5 min waiting period was applied for temperature equilibration prior to the start of any NMR experiment. Automated tuning and matching, locking and shimming, and calibration of the 90° hard pulse P(90°) including adjustment of the 25 Hz presaturation pulse was done for each sample using the standard Bruker routines ATMA, LOCK, TOPSHIM, and PULSECAL to optimize NMR conditions. Four ^{1}H NMR experiments were performed for each sample in automation procedure.

Experiment 1 (ZGPR). Experiment 1 was a standard single-pulse experiment with a continuous wave irradiation during the relaxation delay (RD) for presaturation of the water resonance, that is, *RD − P(90°) − acquisition* of the free induction decay (FID). A 25 Hz RF field was used for presaturation. The relaxation delay and acquisition time were set to RD = 4 s and AQ = ∼3.99 s, respectively, resulting in a total recycle time of ∼7.99 s. After application of DS = 4 dummy scans, NS = 8 free induction decays (FIDs) were collected into TD = 65536 (64K) complex data points using a spectral width SW = 20.5187 ppm and a receiver gain RG = 1. FIDs were multiplied with an exponential function corresponding to LB = 1 Hz prior to Fourier transformation.

Experiment 2 (NOESYGPPS). Experiment 2 comprised a one-dimensional ^{1}H NMR pulse sequence with suppression of the water and the ethanol signals, that is, *RD − t_{G1}− P(90°) −4 μs − P(90°) − t_m − t_{G2}− P(90°) − acquisition* of the FID. The settings for the parameters RD, P(90°), AQ, and TD were kept similar to the ones from experiment 1; DS = 4 dummy scans and NS 32 scans were used, and the mixing time t_m was set to 10 ms. A shaped pulse was applied during RD with a frequency spectrum of eight highly selective bands to achieve highly selective suppression of the water signal and the seven individual lines of the ethanol triplet and quartet, leaving the rest of the spectrum undistorted. Therefore, the receiver gain could be increased to RG = 16, resulting in a signal-to-noise increase per single scan of ∼6.8 compared to ZGPR. Additional defocusing gradients G1 and G2 were applied during t_{G1} and t_{G2} = 1 ms to improve the signal suppression quality. FIDs were multiplied with an exponential function corresponding to LB = 0.3 Hz prior to Fourier transformation.

Experiment 3 (JRES). Experiment 3 included a two-dimensional J-resolved spectrum with multiple suppression applying the same shape during RD as in NOESYGPPS, that is, *RD − P(90°) − t_0 − P(180°) − t_0 − acquisition* of the FID incrementing t_0 for acquisition in the second dimension. RD, P(90°), and the receiver gain RG were kept similar to the values in NOESYGPPS. After DS = 16 dummy scans, for each of the 40 t_0 increments NS = 4 FIDs were collected into TD = 16384 (16K) complex data points during an AQ of 0.6144500 and hence covering a spectral width of SW = 16.6612 ppm. In F1, the 40 t_0 increments corresponded to an acquisition time of 0.3837921s and a spectral width of 0.1302 ppm (52.112 Hz), respectively.

Experiment 4 (QUANT). For quantification of the ethanol content, a simple one-pulse experiment was applied with a 0.3 μs pulse and a relaxation delay of 5 s. NS = 8 FIDs were collected into TD = 65536 (64K) complex data points using SW = 20.5187 ppm and RG = 1. FIDs were multiplied with an exponential function corresponding to LB = 0.3 Hz prior to Fourier transformation. All spectra were processed in full automation using TOPSPIN 2.1, Bruker BioSpin GmbH, Germany.

Quality Assurance. The following daily procedure was introduced for quality assurance: (a) check of the temperature calibration for the measurement temperature T = 300 K; (b) preparation and measurement of two replicate samples from one specifically selected wine used as standard reference throughout the study. Respective data were used for control of the integrity of the overall automation procedure, quality of preparation (from line positions and intensities), and overall spectral quality (e.g., shimming, water suppression, automated processing).

Multivariate Statistical Analysis, Chemometrics: Nontargeted Wine Analysis. *Numerical Data Analysis Platform.* For metabolite identification, AMIX 3.8 in combination with the reference spectral database BBIOREFCODE.2.0.0, both products of Bruker BioSpin GmbH, Germany, was used. Statistical analysis was done under MatLab 7.6 (R2008a) from MathWorks, Natick, MA, USA. MatLab standard routines and routines from the MatLab Statistical Toolbox were combined with in-house developed algorithms.

Data Reduction and Preprocessing of the ^{1}H NMR Spectra. The generation of input variables for statistical analysis was done via bucketing of the NOESYGPPS spectra. Bucketing was done within 0.5−9.5 ppm, dividing the region into 500 sequential segments ("bins"), obtaining an integral for each of them. The regions of acetic acid and residual water and ethanol have been excluded (those regions are irrelevant for the questions under investigation).

Multivariate Statistical Data Analysis. The potential to predict the grape variety, the sample origin, and sample vintage from NMR data was validated using a combination of established multivariate statistical tools[48,49] including principal components analysis (PCA), linear discriminant analysis (LDA), and multivariate analysis of variance (MANOVA) together with cross-validation (CV) embedded in a Monte Carlo resampling approach (MC). As classification rule, a test set object was assigned to the class with minimum distance between test set object and respective class mean, that is, assignment according to the nearest class mean (NCM).

PCA/LDA Subspace NCM Classification (PCA/LDA/NCM). First, a model set was subjected to PCA for dimension reduction considering only the subspace, which explains 99.9% of the variance in the data. Then, LDA was applied to the projected model set to define a refined subspace with maximum class separation. MANOVA provided the dimensionality of the respective class means, that is, dimensionality of the discriminating PCA/LDA subspace. NCM classification based on comparison of distances between test-set objects and class means of model set classes measured in the PCA/LDA subspace was used for assignment of class membership.

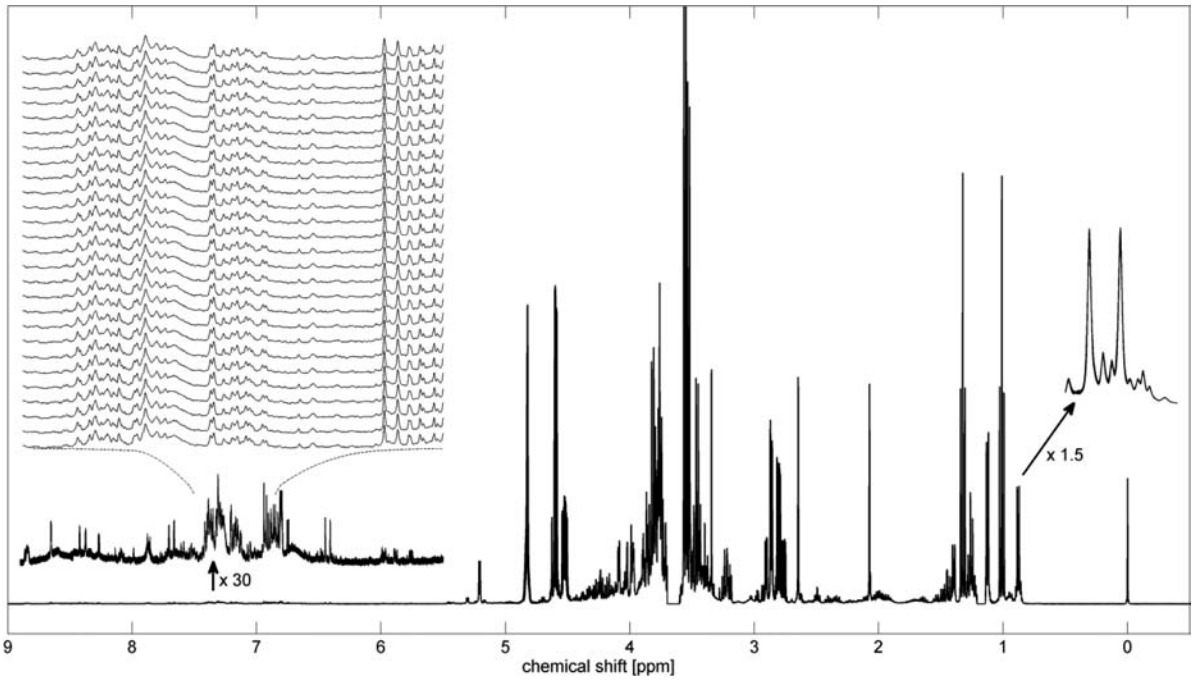

Figure 1. Reproducibility of ^{1}H NMR in white wine, 27 replicates in overlay modus.

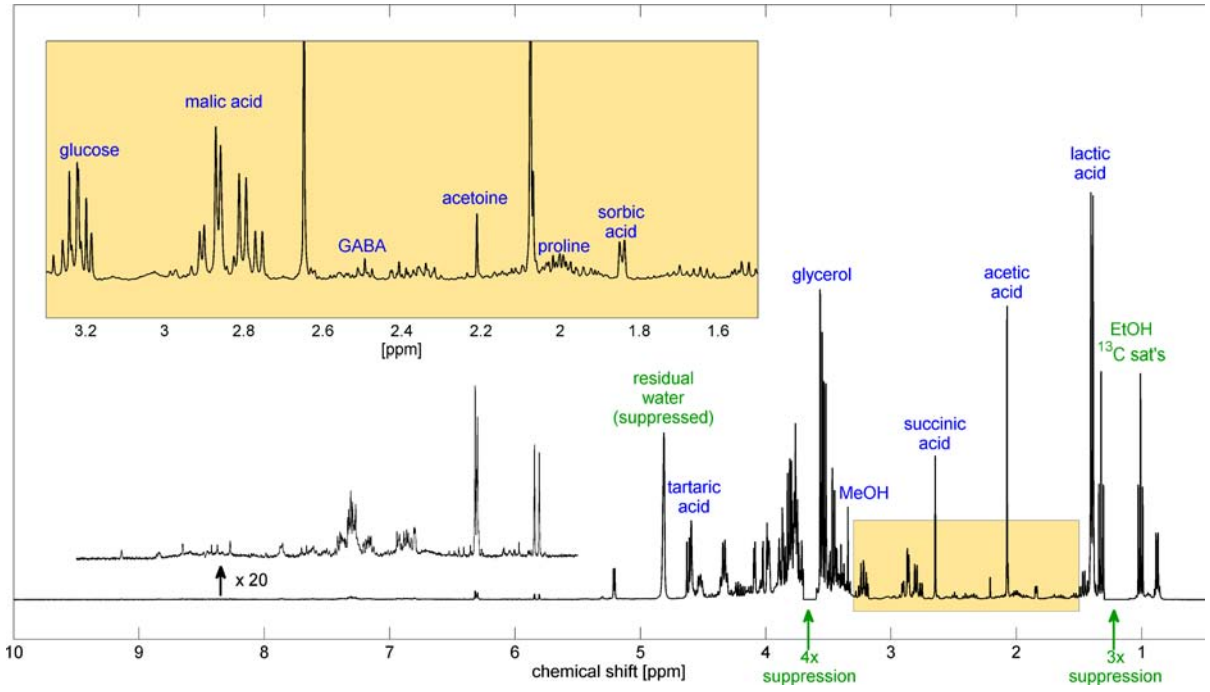

Figure 2. Proton spectrum of wine with 8-band suppression (details in magnification).

MC Embedded CV (MCCV). For validation of the predictivity of the PCA/LDA/NCM, a CV with six randomly selected disjunct subsequent test sets was done. To avoid any segmentation bias, CV was repeated 100 times with new random segmentations for each CV step. Finally, rates of correct and false class predictions were calculated for each class to set up a confusion matrix.

Multiple Univariate Testing for Spectral Differences between Different Wine Parameters. To select spectral regions with significant differences between the different wine parameters a nonparametric version of one-way analysis of variance (ANOVA), the Kruskal–Wallis test was applied. This test evaluates whether the expectation values of the means of different statistical samples are different. Unlike ANOVA, the Kruskal–Wallis test does not assume the normality of the statistical samples. Because the Kruskal–Wallis test operates on a single variable, it has to be applied multiple times, scanning intensities at each individual ppm value for spectral differences.

Targeted Analysis/Quantitation. Sixteen compounds were identified and quantitated in each wine spectrum: methanol, lactic acid, citric acid, malic acid, succinic acid, acetic acid, fumaric acid, tartaric acid, 3-methylbutanediol, acetone, alanine, shikimic acid, caftaric acid, 2,3-butanediol, glycerol, and ethanol. In-house developed Matlab routines were used for signal detection and signal fitting. Absolute quantitation was obtained by using an external reference sample and the PULCON method.[50]

RESULTS AND DISCUSSION

^{1}H NMR Spectroscopy under Multiple Suppression of Water and Ethanol, Stability, and Reproducibility. A wine sample, which was repeatedly prepared the same way on each of 27 successive days, showed good stability of NMR spectroscopy with regard to shift position and intensity (Figure 1). The width of the TSP internal standard was <1 Hz. In the 27 replicate samples, the variability of the position of tartaric acid was limited to ±2 Hz. The stability of the chemical shift is the result of exact preparation of the pH of the sample (3.10 ± 0.02). Integration of ^{13}C satellites of ethanol results in 1.6% relative standard deviation (approximately 60 Hz off to ethanol presaturation); the integrals of the regions of malic acid and α-glucose have relative standard deviations of <1%.

The main ingredients of wine are water and ethanol (8.5−15% vol). As for alcoholic beverages, suppression of both water and ethanol signals was used for beer[7] and wine[51,52] analysis. In this work we therefore applied a simultaneous saturation of the resonances of water and ethanol (Bruker Pulse sequence NOESYGPPS1D combined with an 8-fold suppression by application of a respective shaped pulse) as previously described.[53] By presaturationg both of these main components can be suppressed (8 frequencies) (Figure 2). The total NMR methodology was carried out in full automation, demonstrating that this experimental design is perfectly suited for fully automated ^{1}H NMR screening. NMR spectroscopy is not only an instrument for qualitative analysis, screening, and fingerprinting (nontargeted analysis) but also a realistic tool for quantitative analysis of suitable substances in one spectrum (targeted analysis). The overall dynamic range of quantitative NMR spectroscopy is in the range of 5−6 magnitudes.

In this study with 574 samples of wine from five wine-growing areas of Germany with several grape varieties, the main objective was not to find unambiguous information from the NMR spectra deriving from single parameters quantitatively (targeted analysis) for the differentiation of several distinguishing features such as grape variety, origin, or vintage. However, from the available data set some distinguishing information could be derived for the varieties of the grapes, the geographical origins, and the vintage of the wines by several steps of multivariate statistical analysis (nontargeted wine analysis).

Predictivity was tested via MCCV on PCA/LDA/NCM classification on the data sets. This multiple statistical approach was used having been successful in previous studies.[48,49] In particular PCA was used for the dimension reduction, because the number of variables (bins) contained in the bucket data was extremely high for subsequent statistical treatments. In turn, LDA was applied to PCA scores to identify the multivariate subspace for maximum group (grape variety, geographic origin, vintage) separation. Finally, to determine the level of class predictivity, CV was carried out, whereby the data were repetitively segmented in two sets, that is, a training set (used to build a model) and a test set (used to test the prediction ability), such that after completion of CV each spectrum was in the test set once. It should be pointed out that the risk of segmentation bias may occur in CV, and to overcome this potential drawback, the CV was carried out multiple times always starting from a new random segmentation, by using a MC resampling approach. The assignment of samples contained in the test sets to the grape variety classes, etc., was carried out by comparing distances between test objects and class means (obtained by MANOVA using the NCM method).[54]

Classification of Grape Varieties by ^{1}H NMR Spectroscopy and Combined Multivariate Statistical Analysis. Due to enological practice according to protocol EU 2729/2000 the wines for EU Wine Database were microvinified. Therefore, the grapes (25 kg) were destemmed and crushed with no longer time of maceration. Thusly prepared red wines resulted in a reddish color and not a dark red color like wines fermented with a longer time of maceration (mash fermentation, mash heating). Other wines from wine manufacturers and estates were produced through regular enological practice, resulting in specific red wine color. Because of different intensities of red color two types of red wines of each species (Pinot noir (Spätburgunder), Pinor Meunier (Schwarzriesling), Dornfelder) could be recognized: the less reddish wine (rosé-type, Spätburgunder 2 (Pinot noir)) showing less information in the aromatic part of the NMR spectra (5.0−10.0 ppm) and the dark reddish wine (red wine type, Spätburgunder 1 (Pinot noir)) (spectra not shown).

The first approach with multivariate statistical analysis (MSA) was undertaken with five compounds quantitated from the NMR spectra (targeted analysis): shikimic acid, caftaric acid, 2,3-butanediol, glycerol, and ethanol. Multivariate statistical analysis shows a clear differentiation of grape varieties. The validation of the predictivity was done by the PCA/LDA/NCM and CV embedded in a Monte Carlo resampling approach (MC). The overall correct prediction rate of the grape varieties Spätburgunder (SPB), Lemberger (LEM), Silvaner (SIL), Pinot blanc/Pinot gris (Weißburgunder/Grauburgunder) (WGB), Gewürztraminer (GT), Müller-Thurgau (MT), and Riesling (RIE) in the first case is 71% (Figure 3). Wine compounds with the most differentiating

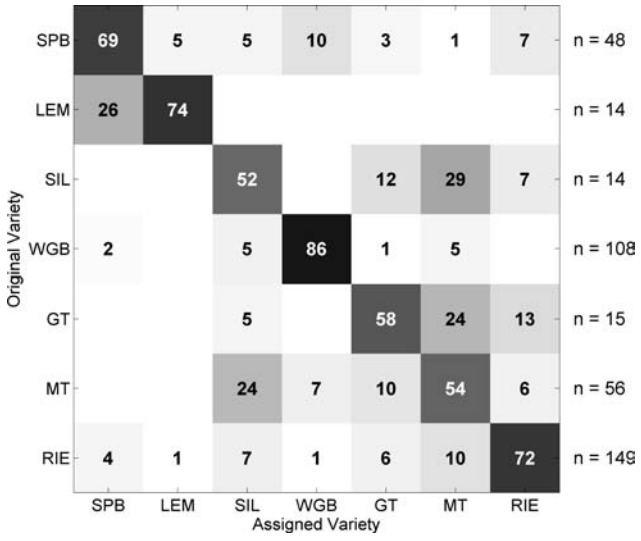

Figure 3. Confusion matrix associated with the predictive results of the Monte Carlo embedded cross-validation (MCCV) analysis for the discrimination of wine grape varieties: classification results for grape variety when using quantification results of five compounds: shikimic acid, caftaric acid, 2,3-butanediol, glycerol, and ethanol. Overall correct prediction rate is 71%. The numbers show the probabilities of predictive outcome for each pair of real and assigned group (diagonal correspond to correct predictions) (RIE, Riesling; WGB, Weiss/Grauburgunder; MT, Müller-Thurgau; SPB, Spätburgunder; LEM, Lemberger; SIL, Silvaner; GT, Gewürztraminer).

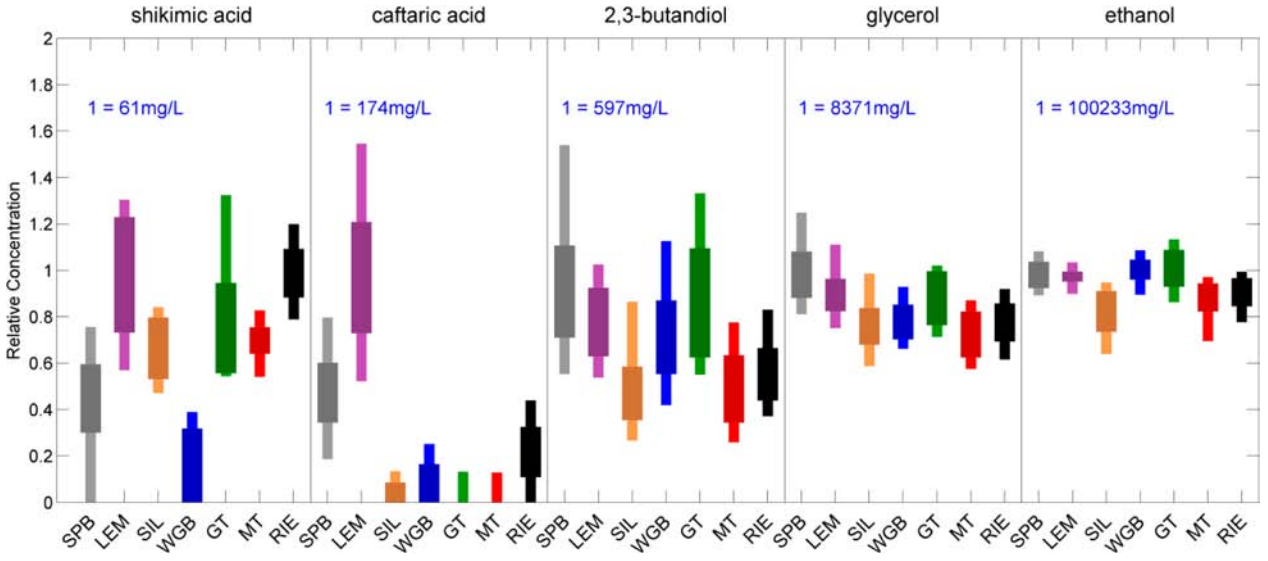

Figure 4. Visualization of concentrations regarding grape variety. Each compound is normalized to the maximum mean of each group.

features are shikimic acid, caftaric acid (ester from caffeic acid and tartaric acid), and 2,3-butanediol. The levels of these parameters are demonstrated in Figure 4. Each compound is normalized to the maximum mean of each group. The low shikimic acid content of wines, especially of the Burgundy grapes Pinot noir (Spätburgunder) and Pinor blanc/Pinot gris (Weißburgunder/Grauburgunder), allows a prediction of grape variety.[24] Caftaric acid level is a primary indication to estimate the oxidation levels that a wine has undergone. For example, pressed wines undergo a high degree of oxidation. Red wines show (Pinot noir (Spätburgunder), Lemberger) high levels of caftaric acid resulting from fermentation processes (mash fermentation, mash heating), whereas white wines present very low levels of caftaric acid. Consequently, caftaric acid content is primarily the result of wine technology and not correlated with grape variety. Also, fermentation product ethanol and fermentation byproduct glycerol are in similar ranges in wines from different grape varieties and cannot be regarded as discriminating factors for grape varieties.

In a second approach with multivariate statistical analysis with 16 compounds quantitated from the NMR spectra (targeted analysis), the prediction rate goes up to 80% (figure not shown). Responsible for this higher degree of the prediction rate are, in addition to the earlier mentioned substances, the following compounds: methanol, lactic acid, citric acid, malic acid, succinic acid, acetic acid, fumaric acid, tartaric acid, 3-methylbutanediol, acetone, and alanine, especially acids and amino acids. In must, arginine is an important source of nitrogen for yeasts through fermentation. Proline is the most abundant amino acid in wine and therefore appears to be a marker for ripeness.[36,55] In white wines from Slovenia the use of the signals of seven amino acids resulted in a good separation of wines according to the wine variety.[11] Metabolic differences in wine from grape varieties from South Korea resulted also partially from amino acids (alanine, proline) and other compounds such as organic acids.[45]

The information on latent parameters such as grape variety, origin, vintage, and technology is coded in multivariate patterns of multiple parameters in the NMR spectra rather than in single spectral markers. The complete spectrum involves more information than all individual compounds together (synergetic effect). In a final approach MSA from the complete NMR spectra (0.5−9.5 ppm) achieved an overall correct prediction rate of the grape varieties of 95%, distinctly higher than the result coded from single compounds. Correct prediction of classification of grape varieties in the wine panel was passed with more than 14 samples per variety (Figure 5). Red wines

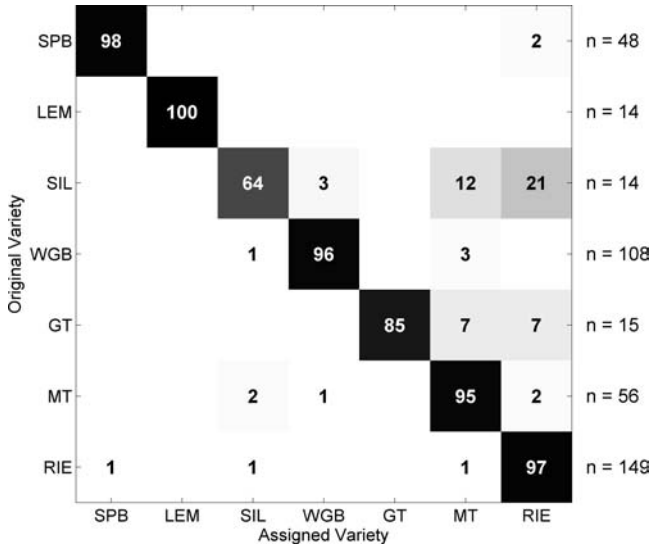

Figure 5. Confusion matrix of the prediction results of grape varieties in wine panel in a Monte Carlo cross-validation (RIE, Riesling; WGB, Weiss/Grauburgunder; MT, Müller-Thurgau, SPB, Spätburgunder; LEM, Lemberger; SIL, Silvaner; GT, Gewürztraminer). Experiment used: 0.5−9.5 ppm, 500 buckets, ethanol/water/acetic acid excluded, 100 Monte Carlo, 6CV, 95.0% mean prediction (±1.6%, 3 times the standard deviation).

Pinot noir (Spätburgunder) (red wine type) and Lemberger were predicted 98 and 100% correctly, respectively. Also, white wines Pinot blanc/Pinot gris (Weißburgunder/Grauburgunder), Gewürztraminer, Müller-Thurgau, and Riesling were classified 93% correctly on average. The group Pinot blanc/Pinot gris (Weißburgunder/Grauburgunder) was handled as one variety because of their high similarity. The Silvaner wine was classified only 64% correctly, with 21% to Riesling, 12% to

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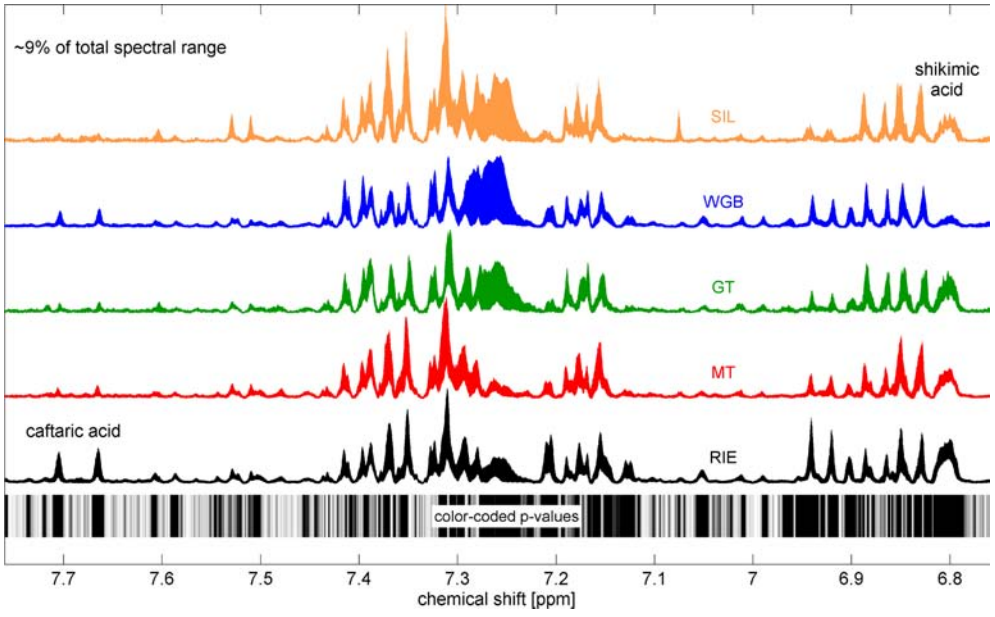

Figure 6. Identification of spectroscopical regions responsible for discrimination of white wine varieties. *p* values according to Kruskal–Wallis test, are symbolized as gray scale (white, high *p* value; black, low *p* value). Low *p* values indicate spectral regions with information for grape variety.

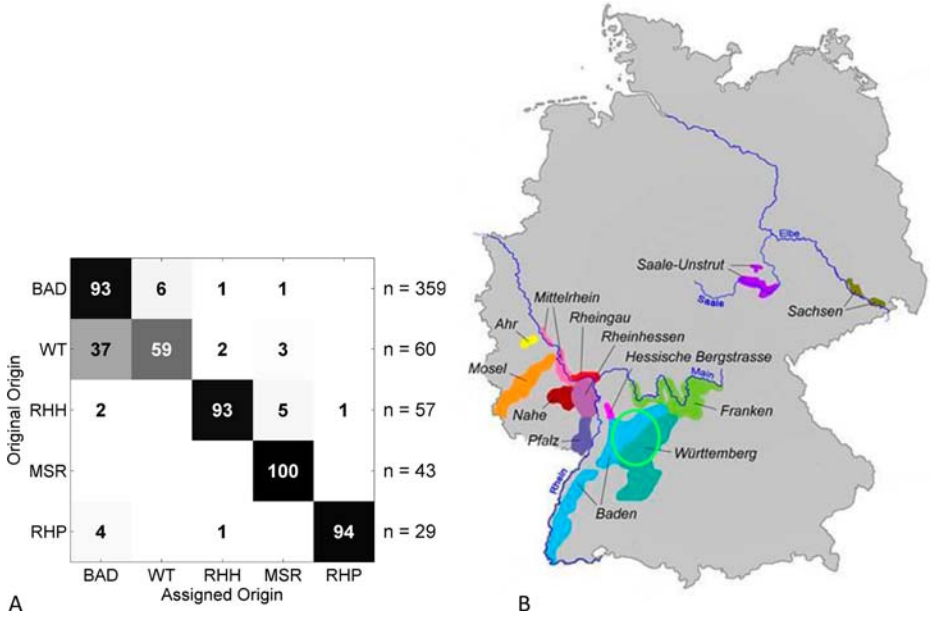

Figure 7. (A) Predictivity of origin of German wines of different wine-producing regions. All information is based on ^{1}H NMR spectrum of 548 samples (BAD, Baden; WT, Württemberg; RHH, Rheinhessen; MSR, Mosel, Saar, Ruwer; RHP, Rheinpfalz). Experimental information: 0.5–9.5 ppm, 500 buckets, ethanol/water/acetic acid excluded, 100 Monte Carlo, 6CV, 89.5% mean prediction (±2.1%, 3 times the standard deviation). (B) German wine-producing regions. Note that Baden and Württemberg are next to one another.

Müller-Thurgau, and 3% to Pinot blanc/Pinot gris (Weißburgunder/Grauburgunder), respectively. No clear separation could be achieved because only 14 samples were analyzed.

The information for discrimination of patterns is located in several parts of the NMR spectra. In general, class differences found in the spectra were typically attributed to subtle intensity differences and not due to the presence or absence of class-specific signals. Spectroscopic regions responsible for the discrimination of white wine varieties can be identified by the Kruskal–Wallis test (Figure 6).

If the grape variety is labeled, mixtures of different grapes in wines are legal up to 15% according to European wine regulation. The labeling of grape varieties is facultative but it is very conventional and important information for customers of German wines and most wines worldwide. Different mixtures of pure grape wines (100%) in laboratory scale were carried out in steps of 5–10% per volume with Riesling versus Pinot blanc/Pinot gris (Weißburgunder/Grauburgunder) (white–white blend). A clear separation of the mixtures could be achieved in the model Riesling versus Pinot blanc/Pinot gris (Weißburgunder/Grauburgunder) with 100 and 99%, respectively. With LDA only the mixtures 40, 50, 60, and 70% in the system Riesling versus Pinot blanc/Pinot gris (Weißburgunder/Grauburgunder) are discarded. The other mixtures are assigned

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to the main groups (figure not shown). These results give a good prediction of grape varieties also in mixtures of different grape wines as shown in the model system.

Classification of the Geographic Origin by ^{1}H NMR Spectroscopy and Combined Multivariate Statistical Analysis. The geographic origin of wine is one of the most important parameters not only for customer acceptance but also for worldwide trading. Wines are differentiated all over the world in wine with and without geographical origin. According to European wine regulations (Commission regulation (EC) No. 607/2009) the origin of wine is differentiated in protected designations of origin (gU) such as Qualitätswein in Germany, Appellation d'Origine Controlée AOC in France, Denominación de Origen DO in Spain, and Denominazione di Origine Controllata DOC in Italy and protected geographical indication (ggA), a national geographic indication. The wine quality is also intensely influenced by the geographic region where the grapes are grown by environment parameters such as soil geology and composition, climate, water availability, and light exposure. The French expression *terroir* summarizes all natural and cultural parameters that have an influence on the authenticity and identity of the product such as climate (temperature, rainfall, microclimate, insolation, solar energy, ecology), soil (geology, topography, water management, hillside situation), and grape culture (grape variety, grape education, density of plants, yield, enology). The geographic origin of all wines under investigation (n = 548) from the largest German wine-producing regions Rheinpfalz, Rheinhessen, Mosel, Saar, Ruwer, Baden, and Württemberg could be predicted 89.5% correctly on average by means of chemometrics. Most of the regions were classified >90% correctly (Figure 7A). Wines from Württemberg were correlated 59% correctly, but 37% were correlated to the neighboring wine-producing region, Baden. These wine-producing areas are next to one another (Figure 7B). In agreement with the studies on Slovenian wines,[11] also wines from various southern Italy wine-producing regions (Basilicata, Campania) could be differentiated on the basis of the contents in glycerol, butylene glycol, and succinic acid.[42] Three different Aglianico red wines from the Campania region of Italy from different vineyards characterized by various microclimatic and pedological properties were differentiated from each other by six metabolites: α-hydroxyisobutyrate, lactic acid, succinic acid, glycerol, α-fructose, and β-D-glucuronic acid.[57] Most of these compounds are mainly of microbiological origin and may be influenced by microbiolocal activity corresponding to different microclimate (e.g., β-D-glucuronic acid from *Botrytis cinerea* mold). A good separation of international wines (France, California, Australia, South Korea) from Cabernet Sauvignon, Shiraz, and Campbell Early grapes could be differentiated according to grape varieties. The metabolites contributing to the separation were assigned to be 2,3-butanediol, lactate, acetate, proline, succinate, malate, glycerol, tartrate, glucose, and phenolic compounds. For the geographical separation of these international wines mainly the different level of proline was responsible.[44] In metabolomic studies on geographical grapes and their wines from different regions of South Korea, the discriminatory compounds among the wines were sugars and acids but also compounds resulting from fermentation such as glycerol and 2,3-butanediol.[34] ^{1}H NMR has been used for metabolomic analysis of Riesling and Müller-Thurgau white wines from the German Palatinate region.[40] The high-quality wines were characterized by elevated levels of compounds such as proline, 2,3-butanediol, malate, quercetin, and catechin. Riesling wines were characterized by higher levels of catechin, caftarate, valine, proline, malate, and citrate, whereas compounds such as quercetin, resveratrol, gallate, leucine, threonine, succinate, and lactate were found discriminating for Müller-Thurgau. The wines from the 2006 vintage were dominated by leucine, phenylalanine, citrate, malate, and phenolics, whereas valine, proline, alanine, and succinate were predominantly present in the 2007 vintage.

Classification of the Year of the Vintage by ^{1}H NMR Spectroscopy and Combined Multivariate Statistical Analysis. Growth conditions are very important for the overall quality of wines. Wine quality is dependent first of all on the climate where the grapes are growing (terroir, rainfall, humidity, hours of sun, temperature day/night, etc.) and secondly on technological influences. If the year of vintage gives no good conditions for growth and quality of the grapes, the product can only be marginally influenced by technological and enological means. Therefore, the vintage is important information both for trading and customers. Labeling the year of vintage is facultative according to European wine regulations, but is normally declared also in wines from all over the world. In accordance with European wine law mixtures with other wines than the declared year of vintage from the same wine-producing region are possible up to 15%. The classification of the vintage of all analyzed wines (n = 583) from different grape varieties, different enological technologies, and different wine-growing regions by means of chemometrics of the whole NMR spectra data resulted in a correct prediction of 97 and 96% of vintage 2008 (n = 318) and 2009 (n = 265), respectively (data and figure not shown). These investigations resulted from NMR data of the whole spectra where also different species of compounds therefore are responsible. Differences in metabolic fingerprints of grape berries such as sugars, organic acids, and amino acids correlated to differences between vintages in Bordeaux grapevine-growing areas.[56] Whereas amino acids are responsible for differences in wine varieties, also with the signals of glycerol, butylene glycol, and succinic acid could be achieved the separation of wines from the coastal and the continental part of Slovenia.[11] The chemometric classification of wines according to their phenolic profile allows discrimination between Greek wines from different wineries of the same wine-producing zone and between different vintages for wines of the same variety.[32]

With the present study in a targeted and nontargeted approach with ^{1}H NMR spectroscopy coupled with several steps of multivariate statistical analysis, wines from the southern and southwestern wine production regions of Germany could be differentiated by grape varieties with a high degree of predictivity. Also, mixtures of wines from different grapes were separated. In addition, the year of the vintage and the wine-producing region were separated with high degrees of differentiation. The information on latent parameters such as grape variety, geographical origin, and vintage is coded in multivariate patterns of multiple parameters in the NMR spectra rather coded in single spectral markers. Each NMR spectrum could be regarded as the individual "fingerprint" of a wine sample, which includes information about variety, origin, vintage, physiological state, technological treatments, and others.

It is proposed to establish a model of all German wines in a database for wines. Due to the linear range of NMR spectra (5−6 magnitudes) it is also possible to quantitate individual compounds of wine only in one spectrum.

■ ASSOCIATED CONTENT

Supporting Information

Data and ^{1}H NMR spectra of two types of red wines; prediction results of blended wines; predictivity of the year of vintage of German wines, all information based on ^{1}H NMR spectrum of all samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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