Identification of Amino Acids in Wines by One- and Two-Dimensional Nuclear Magnetic Resonance Spectroscopy

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Amino acids are minor compounds in wines, but they have a profound influence on wine quality, and amino acids composition can be used to differentiate wines according to the vine variety, geographical origin, and year of production. The NMR signals of amino acids in NMR spectra are overlapped by the signals of other compounds present and especially by the signals of dominant compounds such as water, ethanol, and glycerol. In this work we used 1D ^{1}H and ^{13}C, 2D homonuclear COSY, TOCSY, and 2D heteronuclear HSQC and HMQC pulse sequences, also with an incorporated WET pulse sequence element that allows the simultaneous suppression of several frequencies. Complete ^{1}H and ^{13}C NMR assignments for 17 amino acids commonly present in wine and of γ-aminobutyric acid at pH 3 have been achieved in wine sample of Sauvignon from the Coastal wine-growing region of Slovenia, vintage 1994.

Keywords: *NMR; wine analysis; amino acids; WET suppression*

INTRODUCTION

Complex chemical analysis of agricultural and food products is becoming of great importance due to the general endeavor of achieving adequate quality of agricultural and industrial production. Among the already established methods, NMR spectroscopy has an outstanding position in this field because it is nondestructive, selective, and capable of simultaneous detection of a great number of low molecular mass components in complex mixtures. Wine consists of several hundred components present at different concentrations. The dominant ones are water, ethanol, glycerol, sugars, organic acids, and various ions. Amino acids are present at much lower concentrations. The composition of amino acids is of great importance in wine production because they act as a source of nitrogen for yeast during fermentation. They also have an influence on the aromatic composition of wine (*1*). Amino acids in wine are of various origins. Some are indigenous to the grape and can be partially or totally metabolized by live yeast at the end of fermentation or released from dead yeast; others are produced by enzymatic degradation of grape proteins. Amino acid composition can be used to differentiate wines according to vine variety, geographical origin, and year of production (*2*, *3*). Therefore, great interest has been taken by oenologists and food chemists in the determination of amino acids in wines. The most popular and widely used methods are HPLC (*4*) and capillary electrophoresis (*5*). Although these methods are more sensitive than high-resolution NMR spectroscopy, their disadvantage lies in the time-consuming preparation of samples before measurements. Separation, derivatization of amino acids, and preconcentration are common steps in such procedures. On the contrary, sample preparation for NMR spectroscopy is simpler and less time-consuming.

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Use of one-dimensional ^{1}H and ^{13}C NMR spectra of wines in combination with pattern recognition techniques was shown to be very promising (*6*) for the differentiation of wines from various regions. Normally the request is not only to know whether some wines are similar but also which compounds make differences. NMR data of amino acids in wine are scarce and far from being complete. So far, ^{13}C chemical shifts of 12 amino acids in wine have been determined at pH 2 (*2*, *7*). In our preliminary work only partial ^{1}H and ^{13}C chemical shift assignment of six amino acids was given due to the difficulties caused by the strong signals of water, ethanol, and glycerol, which overlap the weak signals of amino acids normally present at the concentration range of only 1−150 mg L^{-1} (*8*).

The purpose of this work is the complete assignment of ^{1}H and ^{13}C NMR resonances of 17 amino acids commonly present in wine (*3*, *9*) and of γ-aminobutyric acid at pH 3, because the expected pH value of natural wine is ∼3−4. The assignment is the result of the successful use of 1D and 2D homo- and heteronuclear NMR experiments using the WET technique (water suppression enhanced through T_1 effects) (*10*) for the suppression of large signals. Unambiguous assignment of ^{1}H and ^{13}C NMR resonances of amino acids is necessary for the selection of appropriate signals in fast and simple one-dimensional NMR that can serve as parameters in the chemometric classification of wines according to the provenance, vine variety, and year of prodution. The wine sample used in this work was Sauvignon from the Coastal wine-growing region of Slovenia, vintage 1994.

MATERIALS AND METHODS

Apparatus. Spectra were recorded on a Varian INOVA-600 NMR spectrometer located at the NIC (Slovenian National NMR Center, Ljubljana, Slovenia). The spectrometer was equipped with a 5.00 mm indirect detection pulsed field gradient probe (ID-PFG), operating at 600.126 MHz for ^{1}H and at 150.914 MHz for ^{13}C.

10.1021/jf0008137 CCC: $20.00 © 2001 American Chemical Society
Published on Web 12/20/2000

Procedure. Two milliliters of wine was freeze-dried and dissolved in 0.7 mL of deuterium oxide, transferred into 5 mm NMR tube, and measured. The time of freeze-drying was 20 h. After the freeze-drying, close attention is required to minimize the contamination of the dried sample with atmospheric humidity.

It is notable that even after 48 h of freeze-drying some water remained in the lyophilisate. Becuase prolonged drying is not acceptable for a procedure that should require as little time as possible, we chose to limit the drying to 20 h and apply subsequently signal suppression methods. In such a way we gain time without losing sensitivity.

Samples of all of the individual compounds we studied were prepared in concentrations of 10 mmol L^{-1} in D_2O (Aldrich). The purity of the D_2O used was 99.9 atom % D. The pH of all samples was set to 3 with DCl (Aldrich) (37 wt %, 99.5 atom % D) and measured by glass electrode. 3-(Trimethylsilyl)-1-propanesulfonic acid, sodium salt (TMSP) (CIL), served as an internal chemical shift standard. The temperature during all experiments was 298 K.

For one-dimensional (1D) proton spectra 128 transients were accumulated with 40000 data points over a 6999.7 Hz bandwidth. The digital resolution is ±0.4 Hz.

For simultaneous suppression of large signals we used the WET suppression technique (*10*). Suppression was used on the water signal at 4.80 ppm, on the ethanol signal at 3.64 ppm, on the glycerol at 3.62 ppm, and on the so far unassigned signals at 3.55 and 3.53 ppm. In all WET suppression experiments the same 5.2 ms SEDUCE (*11*) shaped pulse was used.

^{13}C spectra were acquired with 29504 data points over a 29996.3 Hz bandwidth with a 4.0 μs (45°) radio frequency pulse. The digital resolution was ±0.9 Hz. The number of transients was 24000, and the time needed for measurement was 6 h.

Gradient-selected COSY (*12*, *13*) (GCOSY from the Varian standard pulse sequence library) and gradient-selected COSY with incorporated WET pulse sequence element (WETGCOSY) spectra were acquired with 2048 data points covering a spectral width of 6999.1 Hz. Sixty-four scans were acquired for each of 256 increments. In the GCOSY experiment 1 s was used for the relaxation delay and as presaturation time for water suppression. Spectra were processed with a sinebell function in both dimensions. Time needed for GCOSY was 5 h and 20 min and for WETGCOSY, 5 h and 30 min.

TOCSY (*14*, *15*) spectra with water suppression by presaturation (TNTOCSY in Varian pulse sequence library) and WETTNTOCSY spectra with incorporated WET pulse sequence element were acquired with 2048 data points over a 8000 Hz bandwidth. One hundred and twenty-eight scans were acquired for each of the 256 increments. Duration of the spin−lock in both cases was 60 ms. TNTOCSY spectra were processed with sinebell function and sinebell shift constant in both dimensions. For the processing of WETTNTOCSY spectra a Gaussian function was applied in both dimensions. The measurement time for TNTOCSY was 22.5 and that for WETTNTOCSY, 23 h.

Gradient-selected HSQC (*16*) (GHSQC from the Varian standard pulse sequence library) and gradient-selected HSQC with incorporated WET pulse sequence element (WETGHSQC) spectra were acquired with a relaxation delay of 1.0 s. GARP decoupling was applied during acquisition. Sixty-four scans with 1792 data points were acquired for each of the 512 increments covering a spectral width of 6999.1 Hz in the F2 dimension and 22598.9 Hz in the F1 dimension. GHSQC spectra were processed with an exponential line broadening, sinebell function, and sinebell shift constant in the F1 dimension. In the F2 dimension a sinebell function and sinebell shift constant were applied. WETGHSQC spectra were processed with a sinebell function in both dimensions. The measurement time was 21 h for GHSQC, and that for WETGSQC was 22 h.

Two-dimensional gradient-selected HMQC (*17*) (GHMQC in Varian pulse sequence library) spectra consisted of 4096 data points over a 7500 Hz bandwidth. One hundred and four scans

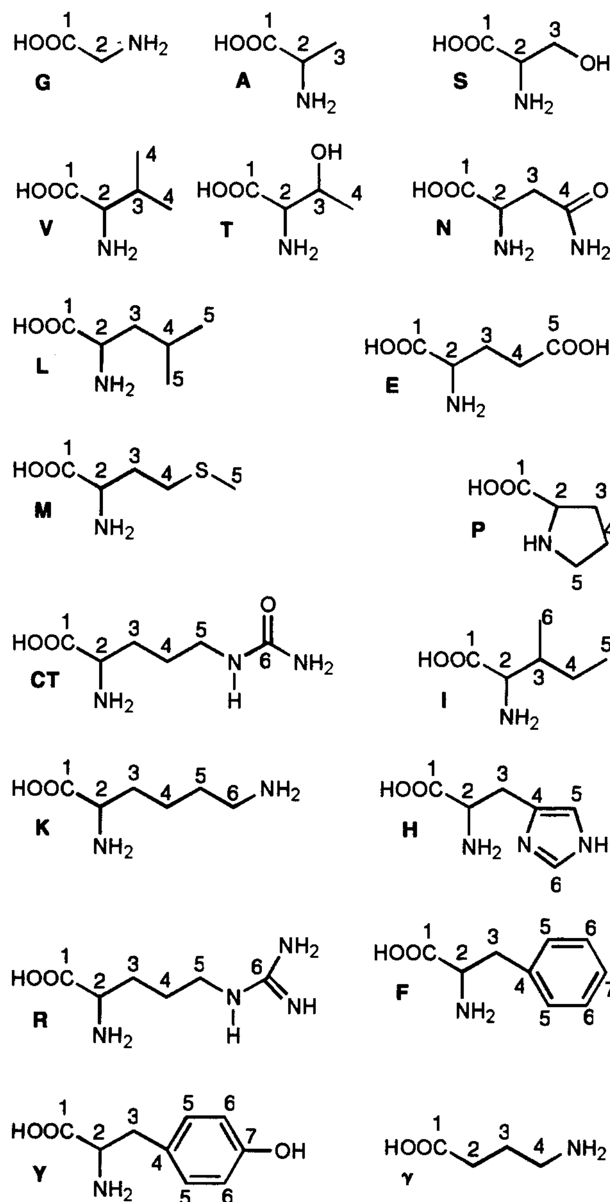

Figure 1. Structures and numbering schemes of the compounds studied.

were acquired for each of the 512 increments. The relaxation delay between scans was 1.5 s, and the evolution time for long-range coupling was set to 62.5 ms. The measurement time was 55 h.

RESULTS AND DISCUSSION

Structures and atom-numbering schemes of the studied compounds are shown in Figure 1. ^{1}H and ^{13}C resonances of glycine (G), alanine (A), serine (S), valine (V), threonine (T), asparagine (N), leucine (L), glutamic acid (E), methionine (M), proline (P), citrulline (CT), isoleucine (I), lysine (K), histidine (H), arginine (R), phenylalanine (F), tyrosine (Y), and γ-aminobutyric acid (γ) from the Sauvignon wine from the Coastal wine-growing region of Slovenia, vintage 1994, as a representative sample, were assigned using homo- and heteronuclear correlation experiments described under Materials and Methods, in combination with the known spectra of individual compounds measured and assigned by us and compared with the published data (*2*, *7*).

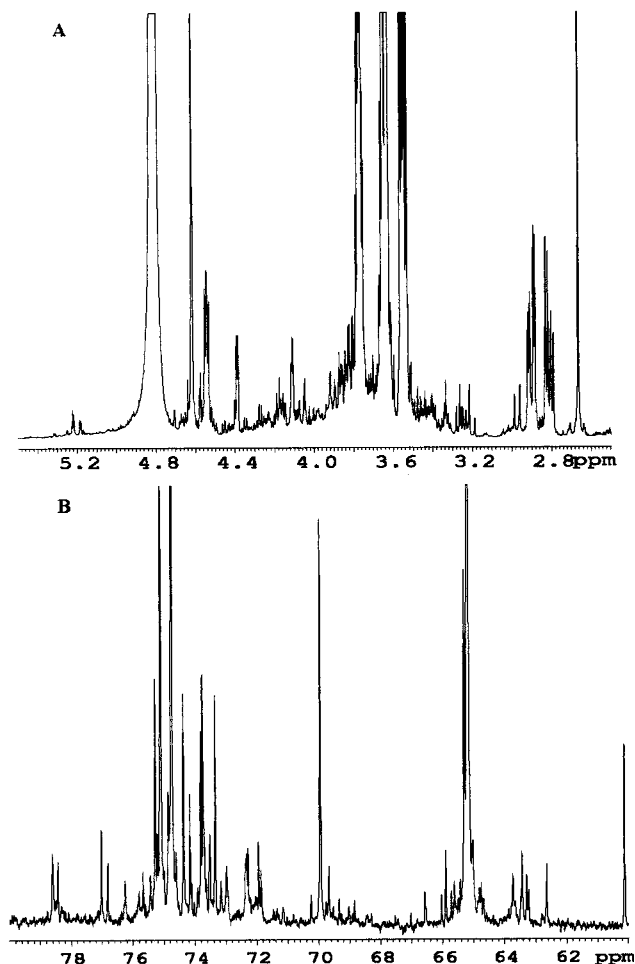

Figure 2. Parts of ^{1}H (A) and ^{13}C spectra (B) of wine (Sauvignon, Coastal wine-growing region, 1994) in the region where the signal overlapping due to the great difference in signal intensities is the most problematic. Both spectra were obtained without suppression of strong signals.

^{1}H one-dimensional NMR spectra of wine are very crowded, and many signals are overlapped (Figure 2A). Because of the different concentration levels of the particular compounds, the signal intensities may vary by the factor of 25. The tails of the dominant frequencies of water at 4.80 ppm, of ethanol at 3.64 and 1.17 ppm, and of glycerol at 3.79 and 3.62 ppm obscure weak signals in the near surroundings of the large ones (Figure 2A). This causes problems with the assignment of ^{1}H signals in the region between 3.6 and 4.8 ppm, in which the signals of H-2 protons of amino acids and the signals of sugars are expected. During the processing of such spectra problems with the appropriate phasing and setting of the vertical scale magnification may arise. Increasing the number of scans does not necessarily result in an increase of weak signal intensities and in clarity of the spectrum; the varying number of scans from 4 to 128 shows that all problems originating from large differences in signal intensities still exist. In any case, the duration of the experiment increases.

Due to the much larger region over which the ^{13}C chemical shifts are spread, the signal overlapping is not so crucial in ^{13}C NMR spectroscopy, although it is still present in the region between 60.0 and 80.0 ppm in which the signals of sugars are expected. In this region the strong signals of ethanol at 60.0 ppm and of glycerol at 65.2 and 74.9 ppm also are present (Figure 2B).

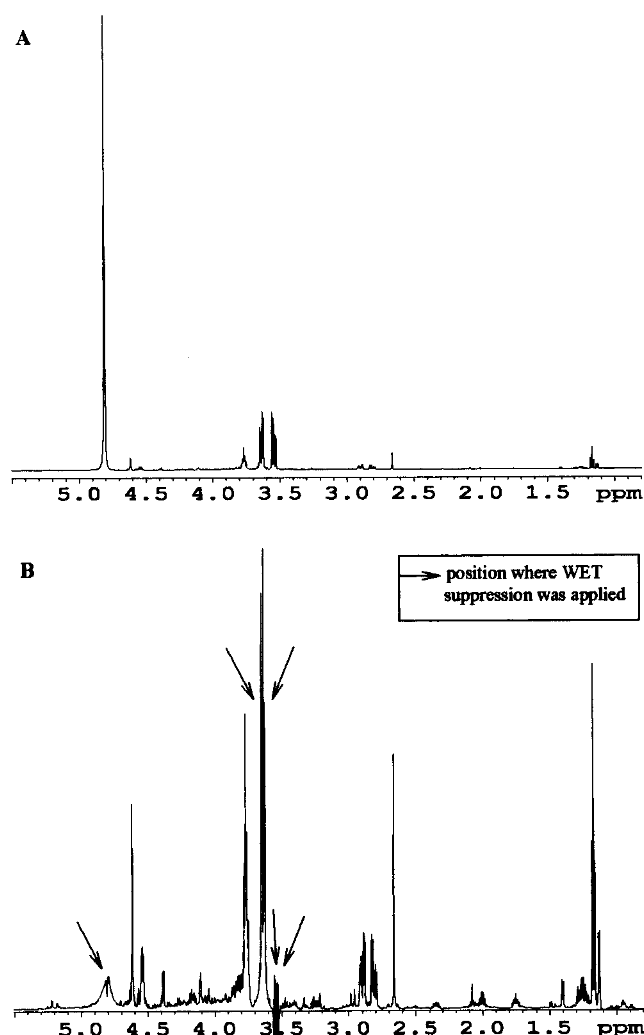

Figure 3. Parts of ^{1}H 1D spectra of wine (Sauvignon, Coastal wine-growing region, 1994) without suppression (A) and with WET suppression of water at 4.80 ppm, of ethanol at 3.64 ppm, of glycerol at 3.62 ppm, and of unassigned signals at 3.55 and 3.53 ppm (B). Both spectra were acquired and processed under the same conditions and with the same vertical scale amplification.

Care should be taken when one is dealing with complex mixtures such as wine with regard to physicochemical properties such as pH, which may influence considerably the chemical shifts, particularly ^{13}C. Regard should also be taken of the concentration levels of paramagnetic species in such samples. Usually, in the preparation procedure some kind of concentration technique is used. Mostly, these techniques are either freeze-drying or vacuum distillation using rotary evaporation unit. A concentrated sample is obtained that contains not only the compounds of interest but also the compounds mentioned above; they may cause the signal shifting and/or broadening.

The use of 2D homo- and heteronuclear experiments and the suppression of strong signals is a prerequisite for a successful ^{1}H and ^{13}C signal assignment of compounds present in wines. For the suppression of strong signals, the method of choice is WET suppression. We used the refined version based on the original work off Ogg et al. (*10*). It is incorporated in the Varian software. Selective shifted laminar pulses are used (*18*), which target simultaneously the various frequencies that need to be suppressed. The WET pulse sequence

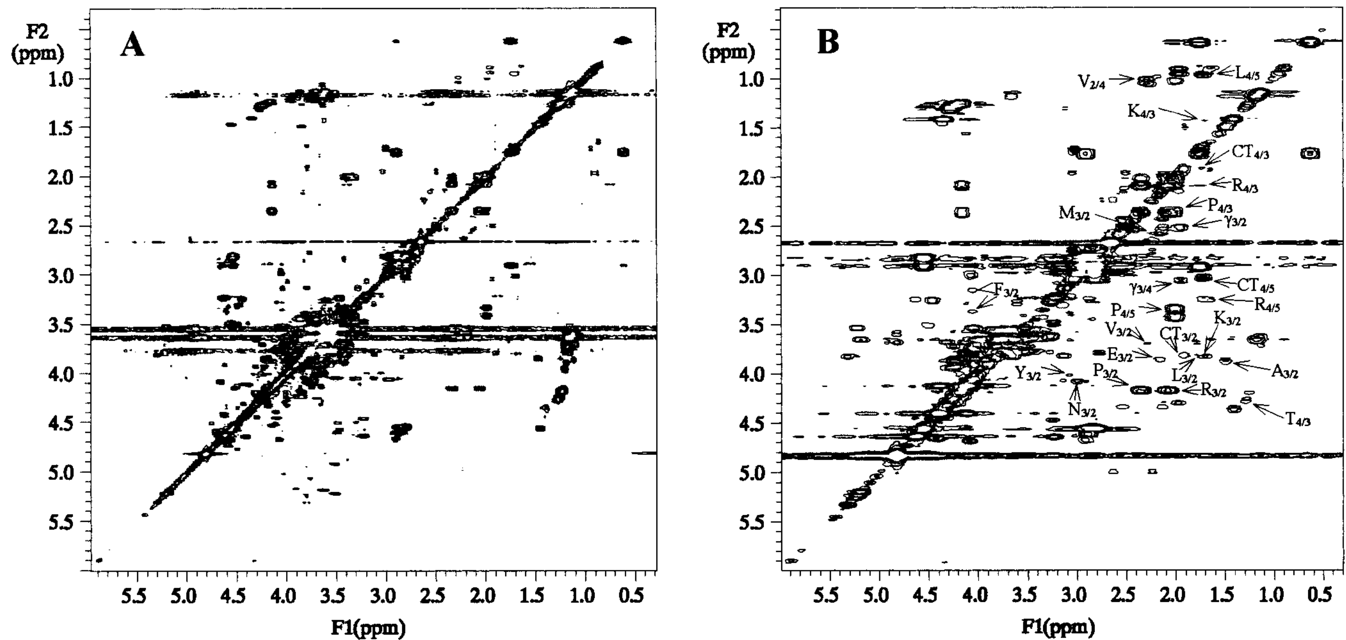

Figure 4. Part of GCOSY (A) and WETGCOSY (B) spectra of wine (Sauvignon, Coastal wine-growing region, 1994) in the region between 0.30 and 6.00 ppm. Suppression of water frequency by presaturation was applied in the GCOSY experiment. In the WETGCOSY experiment multiple suppression of water at 4.80 ppm, of ethanol at 3.64 ppm, of glycerol at 3.62 ppm, and of unassigned signals at 3.55 and 3.53 ppm was used.

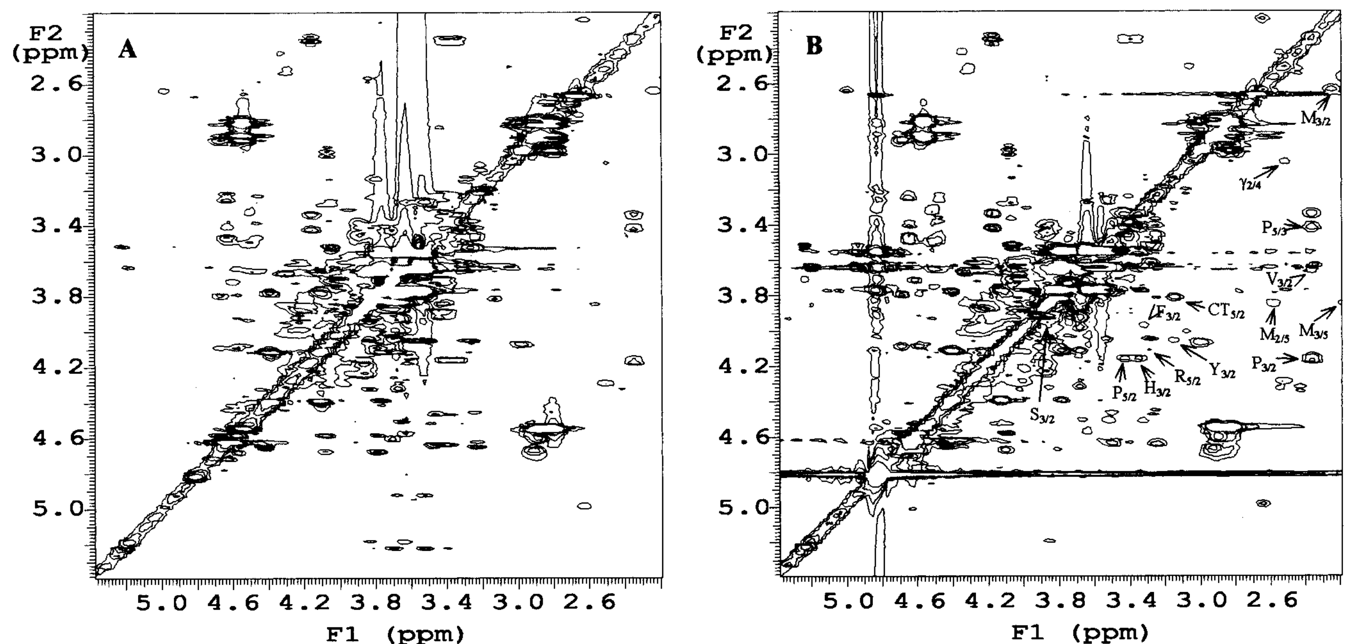

Figure 5. Part of TNTOCSY (A) and WETTNTOCSY (B) spectra of wine (Sauvignon, Coastal wine-growing region, 1994) in the region between 2.20 and 5.40 ppm. Suppression of water frequency by presaturation was applied in the TNTOCSY experiment. In the WETTNTOCSY experiment multiple suppression of water at 4.80 ppm, of ethanol at 3.64 ppm, of glycerol at 3.62 ppm, and of unassigned signals at 3.55 and 3.53 ppm was used.

element is easily incorporated into a variety of 1D and 2D sequences and conventional pulsed-field-gradient experiments.

In Figure 3 1D spectra of 1994 Sauvignon wine without suppression (Figure 3A) and with WET suppression of the strong signals of water, ethanol, glycerol, and two so far unassigned signals (Figure 3B) are shown. Successful suppression of intense signals improves the clarity of the spectrum and the signal-to-noise ratio (Figure 3B). With the increasing vertical scale amplification, the baseline near the strong signals is strongly affected by their tails in the spectrum without suppression (Figure 2A), whereas in the case of suppression of strong signals their influence on the surroundings is significantly reduced (Figure 3B). Nevertheless, when using the signal suppression techniques we have to be aware that some information from the spectra close to the suppressed signals may be lost. The penalty is not significant considering the extra amount of information obtained, and the problem may be overcome by using the combination of different techniques.

The assignment of ^{1}H resonance signals was accomplished by GCOSY (Figure 4A) and TNTOCSY (Figure 5A) experiments using suppression of water

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts of Amino Acids Detected in the Sample of Sauvignon from the Coastal Wine-Growing Region, Slovenia, Vintage 1994

compound		chemical shifts δ												
		C1	H2	C2	H3	C3	H4	C4	H5	C5	H6	C6	H7	C7
glycine	G	174.66	3.611	43.45	–[a]	–	–	–	–	–	–	–	–	–
alanine	A	178.57	3.896	52.23	1.491	18.43	–	–	–	–	–	–	–	–
serine	S	174.44	3.978	58.76	3.873	62.78	–	–	–	–	–	–	–	–
valine	V	176.62	3.694	62.39	2.333	31.80	1.045	20.14	–	–	–	–	–	–
							0.988	20.83						
threonine	T	175.34	3.714	60.90	4.267	68.88	1.367	21.40	–	–	–	–	–	–
asparagine	N	175.57	4.047	53.12	2.986	37.55	–	177.05	–	–	–	–	–	–
leucine	L	177.94	3.823	56.11	1.756	42.28	1.707	26.80	0.942	23.58	–	–	–	–
									0.953	24.09				
glutamic acid	E	175.83	3.918	55.67	2.185	27.73	2.588	31.90	–	179.24	–	–	–	–
methionine	M	176.65	2.637	56.37	2.219	32.26	2.117	31.47	3.863	16.60	–	–	–	–
proline	P	177.27	4.177	63.77	2.358	31.60	2.081	26.39	3.421	48.78	–	–	–	–
citrulline	CT	176.99	3.752	57.31	1.880	30.54	1.554	27.41	3.131	41.64	–	164.46	–	–
isoleucine	I	176.58	3.769	61.73	2.010	37.91	1.460	27.13	0.948	13.58	1.013	16.78	–	–
lysine	K	177.02	3.823	57.03	1.717	29.04	1.451	24.09	1.896	32.24	3.028	41.64	–	–
histidine	H	174.56	4.145	55.69	3.404	28.30	–	130.28	7.429	120.6	8.674	136.82	–	–
arginine	R	174.26	4.111	55.38	2.021	30.18	1.728	26.55	3.265	43.17	–	159.32	–	–
phenylalanine	F	176.35	4.024	58.42	3.312	38.83	–	137.34	7.324	132.06	7.417	131.82	7.366	130.44
					3.141									
tyrosine	Y	177.27	4.074	57.14	3.188	37.55	–	128.69	7.170	133.54	6.890	118.82	–	157.75
γ-aminobutyric acid	γ	181.48	2.504	34.57	1.976	25.10	3.044	41.86	–	–	–	–	–	–

[a] Dashes indicate that the given proton or carbon atom is not present in the molecule.

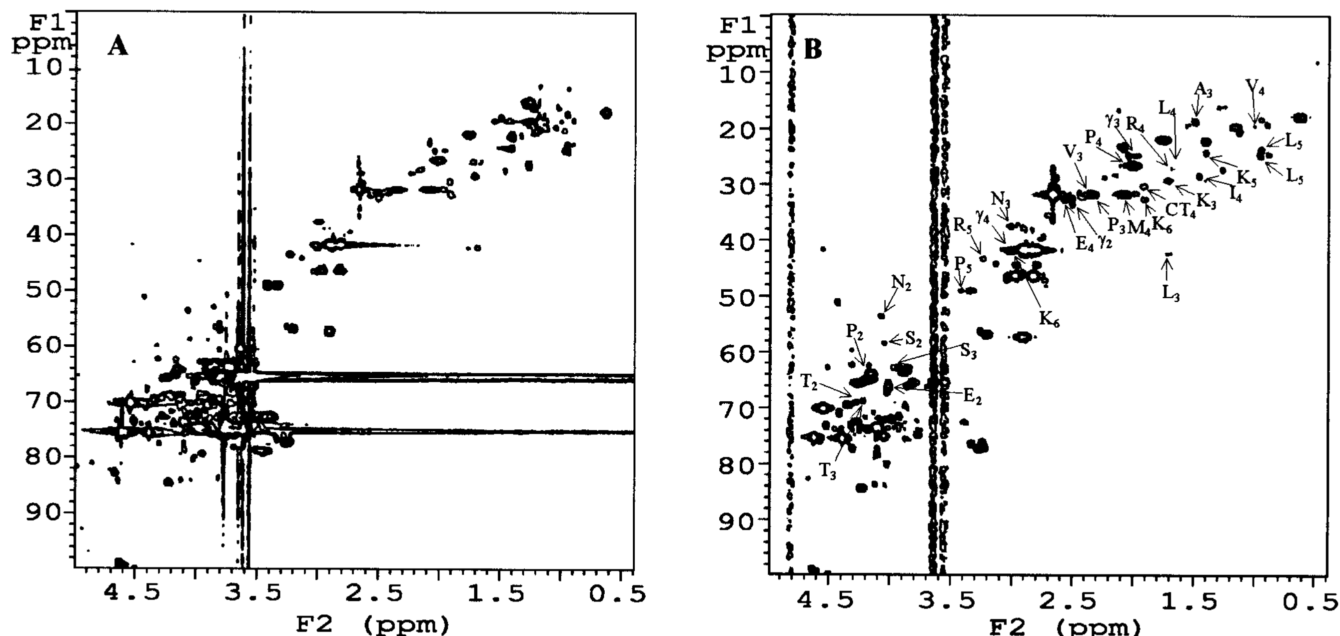

Figure 6. Part of GHSQC (A) and WETGHSQC (B) spectra of wine (Sauvignon, Coastal wine-growing region, 1994) in the region between 0.40 and 5.00 ppm in the ^{1}H dimension and between 0.00 and 100.00 ppm in the ^{13}C dimension. In the WETGHSQC experiment WET suppression was applied on water frequency at 4.80 ppm, on ethanol at 3.64 ppm, on glycerol at 3.62 ppm, and on unassigned signals at 3.55 and 3.53 ppm.

signal by presaturation and by WETGCOSY (Figure 4B) and WETTNTOCSY (Figure 5B) sequences with incorporated WET pulse sequence element. Comparison of Figures 4A,B and of 5A,B shows improvement in clarity in the region between 4.40 and 4.70 ppm, where signals of sugars are expected, and in the region between 3.90 and 4.10 ppm, where H-2 signals of some amino acids are expected. In WETTNTOCSY several signals of amino acids become visible or more reliable. For example, the correlation signals of S between 3.98 and 3.87 ppm, of Y between 4.07 and 3.19 ppm, and of M between 3.86 and 2.64 ppm appear, and correlation signals between 4.14 and 3.40 ppm of H and between 4.02 and 3.31 ppm of F become clearer and more reliable.

After the successful assignment of ^{1}H chemical shifts of amino acids and γ-aminobutyric acid (Table 1) we performed 2D heteronuclear experiments, which make possible the assignment of the ^{13}C chemical shifts. For this purpose, we used GHSQC, WETGHSQC, and GHMQC pulse sequences. From spectra obtained with the use of the GHSQC sequence we were able to assign most of the ^{13}C nuclei of amino acids (Table 1). Similar to the case of homonuclear experiments, a few very strong signals are present in such spectra (Figure 6A). These are the signals of ethanol at 3.64 ppm in the ^{1}H dimension and at 60.0 ppm in the ^{13}C dimension and of glycerol at 3.79 ppm in the ^{1}H dimension and at 74.9 ppm in the ^{13}C dimension and at 3.62 ppm in ^{1}H dimension and at 65.2 ppm in the ^{13}C dimension. Next

to those signals weak correlation signals of nuclei 2 (Figure 1) of amino acids are expected. As may be seen in Figure 5A, it is almost impossible to make a reliable assignment in that region without the suppression of strong signals mentioned above. As a result of the use of the WETGHSQC pulse sequence, we obtained a very clear spectrum (Figure 6B) in the region between 3.70 and 4.80 ppm in the ^{1}H dimensionand between 50.0 and 90.0 ppm in the ^{13}C dimension. This enabled us to assign signals that became visible and much more evident. Such signals are, for example, that of T at 4.27 ppm in the ^{1}H dimension and at 68.9 ppm in the ^{13}C dimension, of E at 3.92 ppm in the ^{1}H dimension and at 55.7 ppm in the ^{13}C dimension, of Y at 4.07 ppm in the ^{1}H dimension and at 57.1 ppm in the ^{13}C dimension, and many others (Table 1).

For the assignment of the carbonyl resonances of amino acids we used the GHMQC sequence, which gives the correlation between heteronuclei along multiple bonds. The chemical shifts of carbonyl resonances assigned are listed in Table 1.

Comparison of the ^{13}C chemical shifts of amino acids from the literature (*2*) determined at pH 2 and those measured by us at pH 3 shows that most of them change by ~1 ppm. The change of pH value from 2 to 3 results in relatively small differences in chemical shifts of amino acids because isoelectric points of amino acids are at pH $\geq$5.5 except in the case of glutamic acid, which is at pH 3.1 (*19*).

In conclusion, high-field NMR has been shown to be a promising method for the nondestructive analysis of low molecular mass compounds in wine. We have assigned proton and carbon chemical shifts of 17 amino acids and of γ-aminobutyric acid commonly present in wines using 1D ^{1}H and ^{13}C and 2D homo- and heteronuclear experiments in combination with WET pulse sequence for strong signal suppression and with the comparison of the recorded spectra of individual compounds with the literature data. The efficient use of the WET suppression technique for the simultaneous suppression of several frequencies resulted in assignment of weak signals located near the large ones. The technique can be easily used with minimal adaptation for application to similar types of samples. We consider extending this work to the measurement of intensities of the assigned NMR signals and the quantitative determination of amino acids in wines.

ABBREVIATIONS USED

A, alanine; COSY, correlated spectroscopy; CT, citrulline; DCl, deuterium chloride; D_2O, deuterium oxide; TMSP, 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt; E, glutamic acid; F, phenylalanine; G, glycine; GARP, globally optimized alternating-phase rectangular pulses; GCOSY, pulsed field gradient correlated spectroscopy; GHMQC, pulsed field gradient heteronuclear multiple quantum correlation; GHSQC, pulsed field gradient heteronuclear single quantum correlation; H, histidine; HMQC, heteronuclear multiple quantum correlation; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum correlation; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; NMR, nuclear magnetic resonance; P, proline; R, arginine; S, serine; T, threonine; TNTOCSY, total correlation spectroscopy with water suppression; TOCSY, total correlation spectroscopy; V, valine; WET, water suppression enhanced through T_1 effects; WETGCOSY, WET pulsed field gradient correlated spectroscopy; WETGHSQC, WET pulsed field gradient heteronuclear single quantum correlation; WETTNTOCSY, WET total correlation spectroscopy with water suppression; Y, tyrosine; γ, γ-aminobutyric acid.

ACKNOWLEDGMENT

We are grateful to Prof. D. Hadži for critical comments and to S. Golič Grdadolnik for assistance in using the WET pulse sequence.

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Received for review July 5, 2000. Revised manuscript received November 1, 2000. Accepted November 3, 2000. This work was supported by the Ministry of Science and Technology of the Republic of Slovenia.

JF0008137