Preliminary Investigation on the Characterization of Durum Wheat Flours Coming from Some Areas of South Italy by Means of ^{1}H High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance

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This paper describes the application of ^{1}H HR-MAS NMR (high-resolution magic angle spinning nuclear magnetic resonance) to the analysis of durum wheat flours coming from geographical areas of southern Italy. It is possible to obtain one-dimensional spectra with >80 resonances. With this technique it is possible to avoid the pretreatment of the samples commonly used in other analytical techniques. Using one- and two-dimensional NMR methods, some preliminary spectral assignment were carried out. Finally, it has also been possible to apply a multivariate statistical analysis to spectral data in a trial of discriminating between the samples.

Keywords: *NMR; durum wheat flours; analysis*

INTRODUCTION

In the past few years there has been an ever increasing interest in the application of high-resolution NMR spectroscopy to food science [see, for example, Belton et al. (1995, 1996)]. Among the main goals achieved in testing the analytical performance of NMR application in foods is the analysis of site-specific natural isotope fractionation (SNIF-NMR) (Martin et al., 1991a,b; Martin and Martin, 1995; Day et al., 1995) by ^{2}H NMR, which has become an official CEN method for the recognition of sugar enrichment and watering in wine and fruit juices since 1990. Alongside the information obtained by means of stable nuclear isotope ratios, SNIF-NMR allows the detection of the geographical origin of these kinds of beverages. Recently, multivariate data analysis of one-dimensional proton NMR spectra has permitted one to obtain qualitative or semiquantitative results for fruit samples (Vogels et al., 1996). The geographical origin of extra virgin olive oils coming from some regions of Italy has also been detected by employing high-resolution (600 MHz) ^{1}H NMR spectra (Sacchi et al., 1996). Other examples concerning the evaluation of the adulteration of edible oils (Zamora et al., 1994; Husain et al., 1993; Wollenberg et al., 1990; Gunstone et al., 1993) are based upon the use of ^{1}H or ^{13}C NMR spectra. Finally, the literature also reports a few papers regarding the analysis of other foods (Belton et al., 1993) such as milk (Mine, 1997; Gutierrez et al., 1996; Wahlgren et al., 1995) and meat (Renou, 1995). This work is related to a research program devoted to the study of the possible applications of high-resolution NMR spectroscopy to investigating typical Apulia agricultural products and those of nearby regions of southern Italy. In this work we investigated durum wheat flours, since Apulia is a main producer of this economically important raw material in Italy. Durum wheat flours are used for the production of dry pasta. The quality of the latter is strongly determined by that of the former (protein content, gluten strength, color, micotoxin and chemical levels), which, in turn, depends on the kind of plant, the geographical origin, and the technology of production. Consequently, an important task is to find analytical techniques that allow characterization of these flour features. NMR spectroscopy could be one of the analytical techniques that could reduce the main drawbacks connected with the preparation of the samples for analysis, such as the possible modification of sample composition or the growth rate at the time of the analysis itself. As far as our knowledge is concerned, there are only a few papers in the literature in which flours have been studied by NMR spectroscopy, and all of them are one-dimensional ^{13}C CP-MAS studies (Ha et al., 1997). Even if ^{13}C CP- MAS NMR has the advantage of avoiding sample manipulation, the information that can be inferred from these spectra is strongly limited by low resolution due to the presence of very broad signals, probably generated by high molecular weight components.

In the present work, we have tested the one-dimensional proton high-resolution magic angle spinning (HR-MAS) NMR technique to combine the typical advantages of solid and liquid-state NMR techniques. The aim is to avoid as much as possible the manipulation of samples and to obtain spectra with enough information to allow us a preliminary investigation devoted to verifying the possibility of selecting some spectral features for use as input variables for the multivariate statistical analysis by the principal component algorithm (Wold et al., 1987). To realize that, the CPMG

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Table 1. Agronomic Form of Durum Wheat Flour Trials

trial location	altitude (m Sim)	soil	sowing date	previous crop	fertilization (kg/ha) presowing N	presowing P_2O_5	tilling N	weed control treatment	harvest date
Francavilla F. (Brindisi)	130	loam	Dec 6, 1996	tomato	36	92	52		June 23, 1997
San Severo (Foggia)	80	loam calcareous	Nov 20, 1996	wheat	36	92	52		June 25, 1997
Montelongo (Campobasso)	300	clay	Nov 15, 1996	sunflower	45	115	52	tralcoxidim + bromoxinil + MCPA	July 3, 1997
Matera	400	clay	Jan 17, 1997	wheat			92		July 4, 1997

and NOESY (Jeener et al., 1979) pulse sequences, modified for water suppression, were used. TOCSY (Braunschweiler et al., 1983), $^1H-^{13}C$ HMQC (Bax et al., 1983), and proton *J*-resolved (Aue et al., 1976) NMR spectra of some samples were also collected to get a partial assignment of resonances. These measurements, also, were done using an HR-MAS probe.

MATERIALS AND METHODS

Sample Preparation. Durum wheat flour samples were taken from various fields of selected cultivars, which differed from one another by geographical origin, kind of soil, and time of harvest. In Table 1 some of the features of these samples are reported. Figure 1 shows the geographical origins of the samples analyzed.

All of the durum wheat flour samples used for NMR inspection were prepared in the same way. This was done by adding an excessive amount of D_2O (33 mg) to nearly 40 mg of flour to obtain a homogeneous samples with similar degrees of swelling and solvation. NMR spectra were run on freshly prepared samples, but for few of them it took 24 h from their preparation until a proper control could be obtained.

NMR Spectra. All NMR measurements were performed at the proton frequency of a Bruker AVANCE 400 spectrometer operating at 9.395 T using a high-resolution HR-MAS probehead suitable for 4 mm rotors. Spectra were recorded at 300 K, with deuterium field/frequency lock on the deuterated water signal. Samples were brought to the magic angle with respect to the direction of the static magnetic field and spun at 4200 Hz to minimize the chemical shift anisotropy effects.

For every sample, CPMG and one-dimensional version of NOESY pulse sequences were employed, both modified by adding a presaturation sequence for water suppression. In both cases, very effective suppression of the large residual water peak was achieved. Consequently, better spectral dynamic range and better recognition of signals resonanting near the water peak around 1.5 ppm was achieved. Moreover, the use of CPMG pulse sequence permitted the removal of the broad resonances due to high molecular weight components, such as protein and starch, which do not provide information for the consequent multivariate analysis. On the contrary, their overlapping of sharp resonances reduces the precision of the areas and the intensities measured. Furthermore, they produce distortion of the spectral baseline. For these reasons the CPMG experiment was supposed to give additional information with respect to NOESY experiment. On the other hand, NOESY gives the best water suppression; therefore, both pulse sequences were applied for all samples. The experimental conditions were as follows: base spectral frequency = 400.13 MHz; spectral width = 6000 Hz (~15 ppm); 32 K time domain points; 2.733 s of acquisition time; presaturation pulse length = 1.5 s; pulse length corresponding to a flip angle of $\pi/2$ radiants = 10.3 μs; number of scans = 256. Additional acquisition parameters for CPMG were an evolution time of 20 ms, and in NOESY spectra a mixing time of 80 ms was employed. FIDs were processed with multiplication by a line broadening factor of 0.3 Hz, before Fourier transformation (FT). Spectral baseline corrections were done by using a fifth-degree polynomial function.

TOCSY spectra were acquired using TPPI phase cycle (Redfield et al., 1975). Acquisition parameters were as follows: 2048 time domain points (F_2 dimension); 300 increments (F_1 dimension); 8 transients per increment; 80 ms of spin lock; 2 s of presaturation pulse length for water signal suppression. FIDs were weighted, in both dimensions, by sine bell squared functions shifted by $\pi/2$ radiants before complex Fourier transformation to obtain a 1024 × 1024 data point spectrum.

$^1H-^{13}C$ HMQC spectra were acquired with TPPI phase cycle, 1024(F_2) × 200(F_1) data points, 2 s of presaturation pulse length for water signal suppression, 64 scans per transient, an evolution time of 3.45 ms, 2 s of presaturation pulse length for water signal suppression, and GARP pulse sequence for carbon-13 broad band decoupling ($\pi/2$ soft pulse length = 80 μs) (Shaka et al., 1980). FIDs were weighted using a sine bell squared function shifted by $\pi/2$ radiants in both dimensions and zero filled in the F_1 dimension to obtain a spectrum of 1024(F_2) × 512(F_1) data points.

J-resolved spectra were acquired using the following conditions: 8192(F_2) × 64(F_1) time domain data points; 16 scans per transient; 2 s of presaturation pulse length for water signal suppression. FIDs were weighted, in both dimensions, by sine bell and zero filled in the F_1 dimension to obtain a spectrum of 8192(F_2) × 256(F_1) data points. The FT was performed in magnitude mode.

Multivariate Data Analysis. One of the aims of our work was to assess the geographical area of durum wheat flours. To do this, we utilized the pattern recognition method. This technique can be useful to provide rules enabling us to delimit variable intervals for samples of known origin (learning set) and to classify unknown samples (test set) by assigning each sample to a given class or to no class at all (outliers). We will consider every sample as a point in an *n*-dimensional space (*n* = number of variables). Principal component analysis (PCA) has been used to recognize patterns. In PCA analysis points are projected on a plane or in a three-dimensional space after the rotation of the original coordinate system. In the rotated system the samples have new coordinates that are linear combinations of the old ones. The rotation angle is chosen such that the first PC has the maximum variance, the second PC, which is orthogonal with the first one, the second maximum variance, and so on.

An example of the application of this type of statistical analysis can be found for olive oil (Dunlop et al., 1995), wine (Armanino et al., 1990), and mineral waters (Caselli et al., 1998).

To perform PCA, we ran CPMG and NOESY 1D spectra on two different samples, prepared in two different rotors, for every type of flour under investigation. The PCA was done using SCANWIN software (Minitab Inc., 1995).

RESULTS AND DISCUSSION

Proton NMR spectra of durum wheat flour measured by HR-MAS technique with CPMG and NOESY pulse sequences, modified for water signal suppression, are shown in Figures 2 and 3, respectively. The CPMG

Figure 1. Geographical location of the analyzed durum wheat flours.

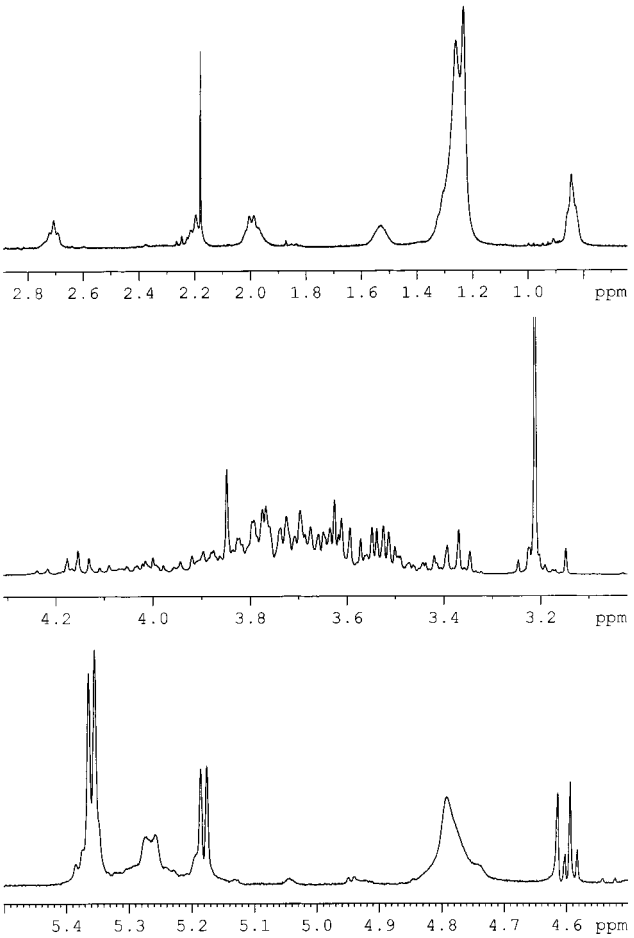

Figure 2. ^{1}H CPMG spectrum of a durum wheat flour sample.

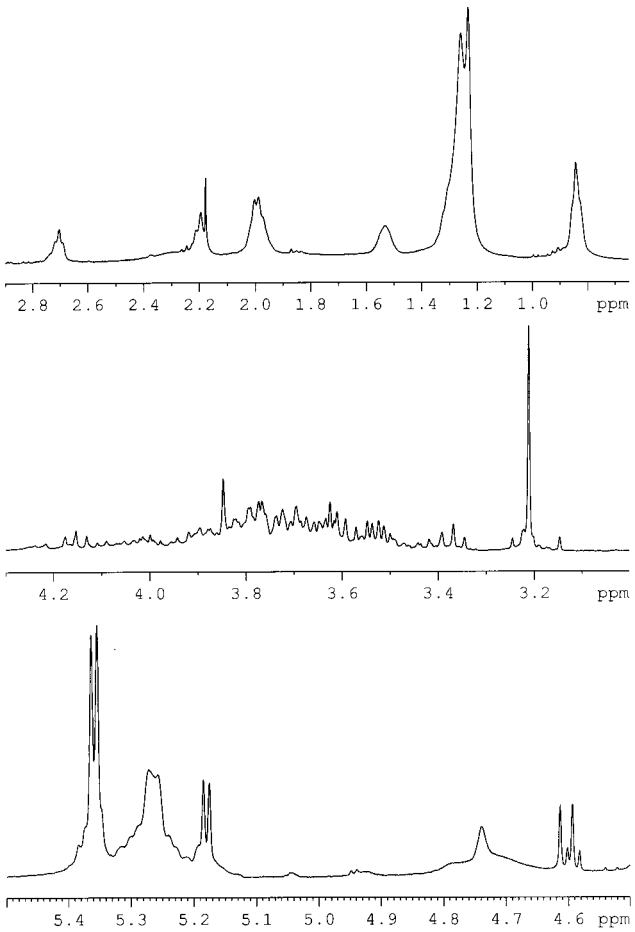

Figure 3. ^{1}H NOESY spectrum.

Table 2. Proton and Carbon-13 Chemical Shift Assignment of Lipidic Moiety

moiety	δ^1H[a,b]	δ ^{13}C ^{1}H−^{13}C HMQC	^{1}H connectivities TOCSY
CH$_3$-(CH$_2$)$_n$−CH$_2$−	0.83 bs	14.10	1.22
CH$_3$-(**CH$_2$**)$_n$−CH$_2$−	1.22 bs	23.00	
−(**CH$_2$**)$_n$−CH$_2$−CH$_2$−	1.22 bs	29.65	1.98/5.24
−(**CH$_2$**)$_n$−CH$_2$−CH$_2$−	1.22 bs	32.00	1.50/2.18
−(**CH$_2$**)-CH$_2$−CO−O−	1.50 bs	25.00	2.18
−**CH$_2$**−CH=CH−	1.98 bs	27.20	5.24
−CH$_2$-**CH$_2$**−CO−O−	2.18 bs	34.80	
−CH=CH−**CH$_2$**−CH=CH−	2.69 bs	25.60	5.24
−CH$_2$−**CH**=CH−	5.24 bs	128.00	
−CH$_2$−CH=**CH**−	5.24 bs	130.00	

[a] bs, broad singlet. [b] Gabriel et al. (1987), Jonas et al. (1990), Jengrasiak et al. (1991), Nicholson et al. (1995).

spectrum gave a better baseline versus the NOESY spectrum due to the decay of magnetizations of high molecular weight compounds during the evolution time. In both cases >80 peaks could be distinguished in the spectrum. Some of these have not yet been assigned in the literature, relative to ^{1}H NMR spectra of lipids (Sparling et al., 1989) and flour extract samples (Gruppen et al., 1992). Assignments were confirmed by means of the correlations observed in TOCSY and ^{1}H−^{13}C HMQC spectra, whereas the scalar coupling patterns of several signals were inferred from *J*-resolved spectra (Figure 4−6).

Proton NMR spectra resulted mainly from signals due to lipids and polysaccharides. The signals in the range of 0.8−2.7 ppm and at 5.24 ppm were assigned to the lipids (Table 2). The high-field signals were due to alkyl moieties, whereas the signal at 5.24 ppm was due to olefinic protons. These assignments were confirmed by the carbon-13 chemical shifts extracted from the ^{1}H−^{13}C HMQC spectrum and by the correlations detected in the TOCSY spectrum. This TOCSY showed four correlation patterns: 0.83−1.22; 1.22−1.98−5.24; 1.22−1.50−2.18; and 2.69−5.24. The first two accounted for the CH$_3$−(CH$_2$)$_n$−CH$_2$−CH=CH− moiety, the third for the −CH$_2$−CH$_2$−CH$_2$−CO−O− moiety, and the last for the −CH$_2$=CH−CH$_2$−CH=CH− fragment.

Almost all of the other signals in the spectra were associated with polysaccharides. In particular, it was possible to distinguish four type of signals: 3.0−4.2, 4.6, 5.1, and 5.4 ppm (Table 3). The last three chemical shifts were typically of anomeric protons, the last two being usually due to α(1→6) and α(1→4)-glucopolysaccharides, respectively. This was confirmed by the ^{1}H−^{13}C HMQC spectra's corresponding carbon chemical shifts. These latter were in the range between 96.0 and 99.7 ppm. The signals in the range between 3.0 and 4.2 ppm accounted for H-2−H-6 protons of saccharides as well as the chemical shifts of the corresponding carbons, which ranged between 53.3 and 81.5 ppm. Unfortunately, TOCSY spectra did not help the recognition of further structural information, as there was too much overlap among their correlations. Only one cross-peak between the signals of anomeric protons and signals at 3.5 ppm was easily distinguishable, but it could only be used to confirm what had been previously stated. Proton *J*-resolved spectra also did not provide other information, although they showed the scalar coupling multiplicities of several signals in the range

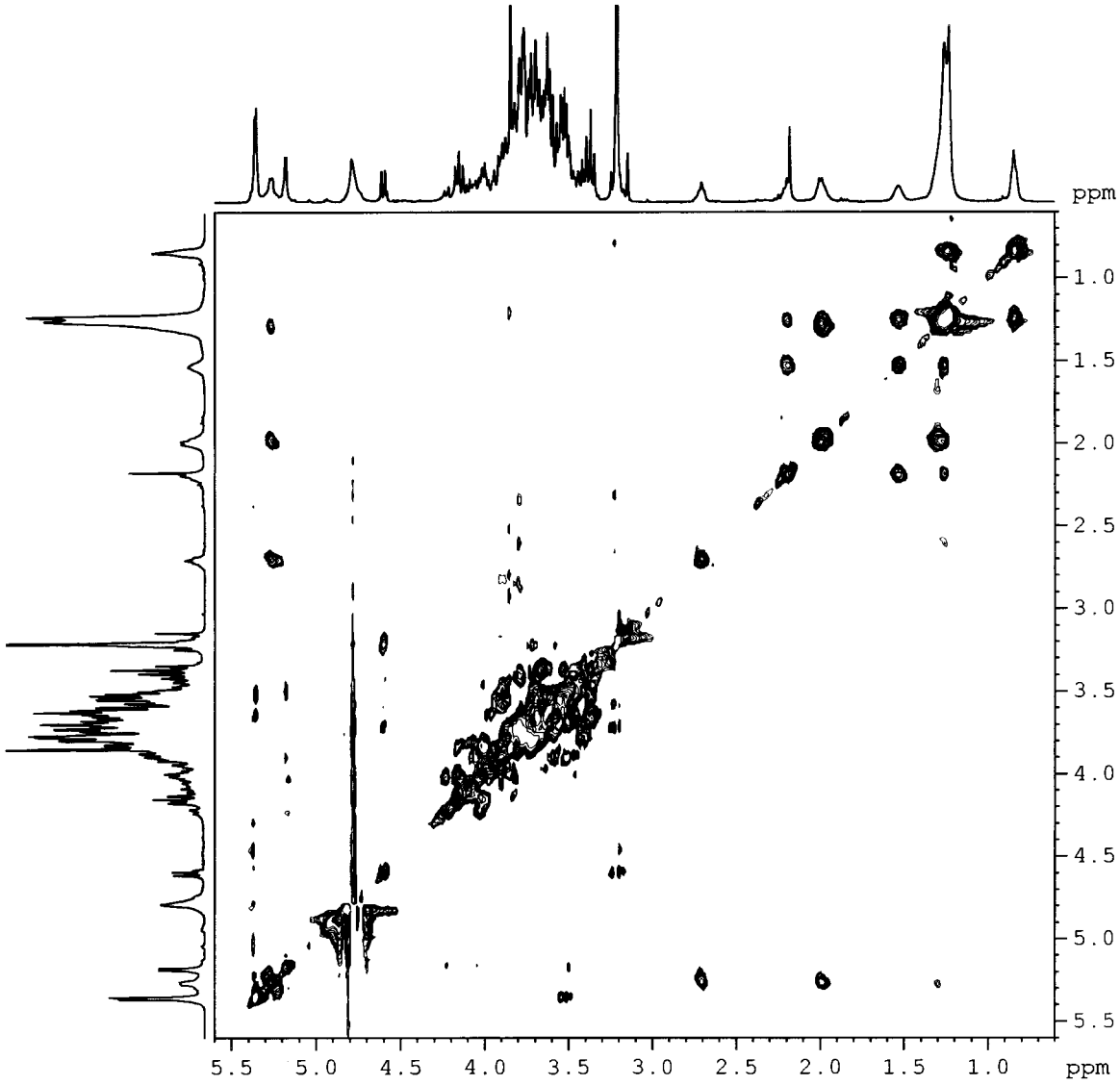

Figure 4. ^{1}H TOCSY spectrum.

between 3.0 and 4.2 ppm. In a few cases, we ran spectra of freshly prepared samples and on the samples stored for a day inside the rotors: no modifications of the intensities of peaks occurred on storing. Therefore, it is possible to state that the time between the preparation of the sample and the acquisition of spectra is not a relevant factor.

Finally, as far as the NMR analysis is concerned, the assignment of resonances could be better obtained if NMR spectra of separated starch, gluten protein, and lipids would be taken into account. However, this was not the aim of the current study. We were primarily concerned with the preliminary investigation of the one-dimensional spectra and their use in statistical analysis.

For the statistical analysis we took into account the monodimensional spectra of all the samples examined. Our attention was focused first on the region of signals, due to lipid components (0.7−2.8 ppm), where some well-resolved peaks can be distinguished and, second, the region of saccharides resonances (3.0−3.4 ppm). By taking into consideration all of the resonances of this range as input variables for the PCA, it was possible then to reduce the number of the input variables by eliminating those resonances that were less correlated. This was necessary to avoid bias in the method. On the other hand, knowing the assignment of resonances, it was logical to retain some of the resonances considered. In the analysis of the resonances for inclusion in the statistical data treatment, two major approaches have been considered: (1) the use of relative heights with a standard reference; (2) the use of relative areas. In the first case we faced the problem of different line shape of the spectra due to the different conditions of the magnetic field homogeneity and the different homogeneities of the samples. This problem was observed only in a few cases and mainly for very sharp signals as doublets of anomeric protons, which, as mentioned before, were not taken in account for statistical analysis. For the other signals the differences of line shape, checked by overlapping of the spectra by means of software facilities, were very small. In any case, these effects were partially averaged by the use of two different sets of data for each type of flour (spectra of two different samples for every type of flour), and the residual effects due to this problem are considered in the statistical analysis because they contribute to the dispersion of the data.

On the other hand, the approach making use of the relative areas required the deconvolution of signals because of partial overlapping. This procedure may

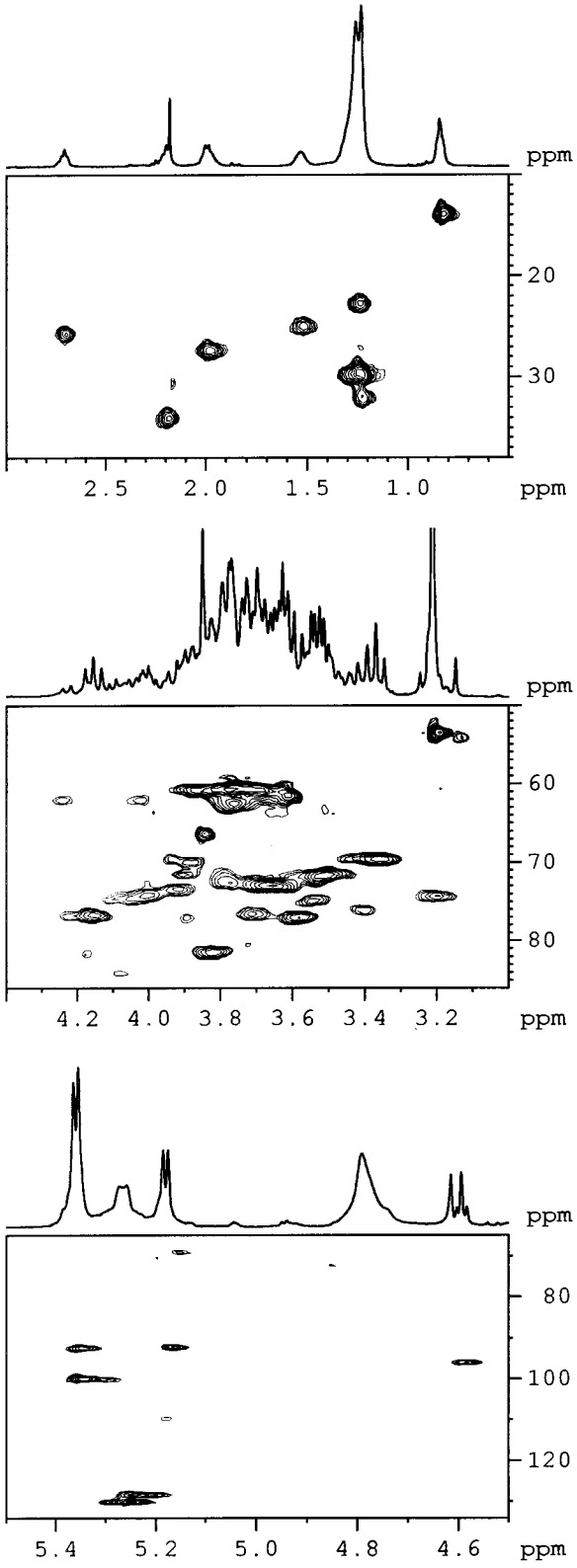

Figure 5. $^{1}H-^{13}C$ HMQC spectrum.

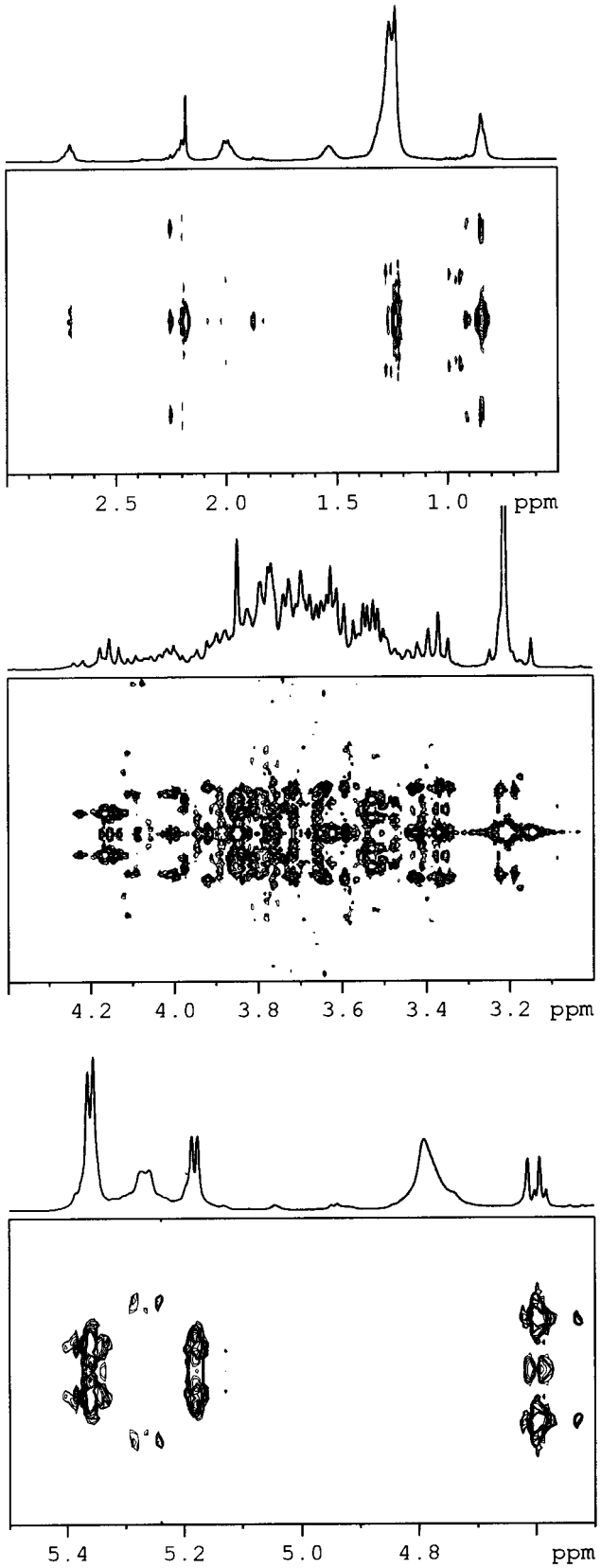

Figure 6. ^{1}H *J*-resolved spectrum.

have introduced some further inaccuracies in the method (the trend of the baseline influences substantially the quality of the fitting), for which is valid the consideration mentioned before about the contribution of these effects to the dispersion of the data obtained by the statistical analysis. Having not found meaningful differences using the two methods, we have only considered the first of the two approaches. Spectroscopic data obtained using NOESY pulse sequence were more useful in discriminating the samples than CPMG pulse sequence data. In the case of the first examined range, the result was [as can be seen in the score plot of the first two principal components (Figure 7)] that this technique has a good deal of repeatability. This result

Table 3. NMR Data of Saccharide Moieties[a]

δ^1H[b]	δ^{13}C[c] ^{1}H−^{13}C HMQC	*J*-res[b]	δ^1H[b]	δ^{13}C[c] ^{1}H−^{13}C HMQC	*J*-res[b]
3.20	74.2	t	3.82	81.5	dd
3.22 s	53.3	s	3.87	70.0	dd
3.36 t	69.6	t	3.91	73.5	dd
3.37	60.5	o	3.93	69.6	dd
3.40	76.0	t	4.00	74.2	t
3.50	71.6	dd	4.01	61.9	t
3.55	75.0	dd	4.15	76.5	t
3.58	77.0	t	4.22	61.9	d
3.60	61.2	t	4.59 d	96.0	
3.65	73.0	o	4.605 d	96.0	
3.71	76.5	o	5.18 d	92.0	
3.75	62.2	o	5.36 d	92.0	
3.77	72.7	o	5.36 d	99.7	
3.82	66.2	dd			

[a] ^{1}H connectivities by TOCSY spectrum: 3.247−4.59; 3.36−5.15; 3.38−3.63−5.36. [b] s, singlet; bs, broad singlet; d, doublet; t, triplet; dd, double of doublet; o, overlapping signal—multiplicity detected by *J*-resolved spectrum. [c] Assignment done on the basis of correlations detected in ^{1}H−^{13}C HMQC spectrum.

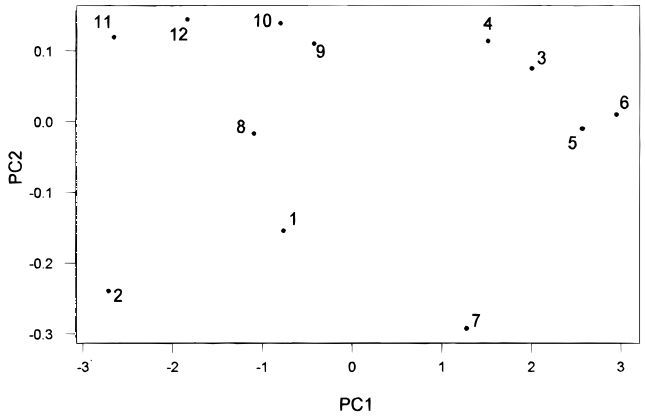

Figure 7. Scatter plot of the scores from the first two principal components PC1 and PC2 for durum wheat flour samples using the spectral data obtained from ^{1}H NOESY sequence in the range 0.7−2.8 ppm: S. Severo Ofanto (1, 2); Matera Fortore (3, 4); Matera Ofanto (5, 6); Montelongo Ofanto (7, 8); Francavilla Fortore (9, 10); Francavilla Ofanto (11, 12).

was confirmed from the analysis of the second spectral range, the corresponding score plot of which is shown in Figure 8. In both cases it was possible to determine that the geographical origin of the samples was a greater source of experimental variance than the quality of the flour sample. In fact, the score plot showed two main groups coming from the southern locations [Matera (3−6) and Francavilla (9−12)]. The other samples from northern locations, being discriminated from these two main groups, showed a more scattered pattern that did not convey other useful information.

This work represents only a preliminary attempt in analyzing a few samples. Greater accuracy should be achievable by taking into consideration a larger number of samples collected over a greater geographical area and a better standardization in the experimental conditions. This kind of investigation is already on its way in our laboratories.

CONCLUSIONS

In this work we have presented a new application of ^{1}H NMR spectroscopy for the determination of the origin of flours by the use of HR-MAS technique. Remarkable results have been the following: first, the measurement

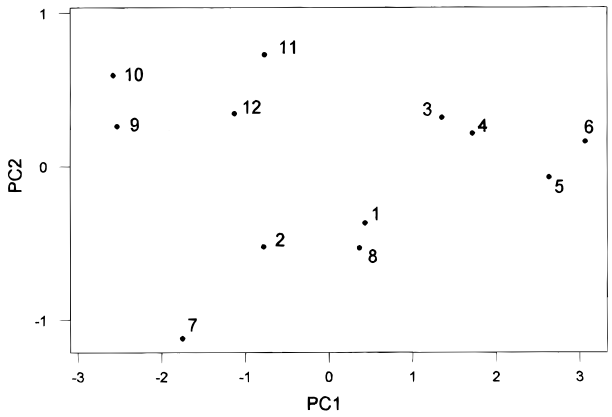

Figure 8. Scatter plot of the scores from the first two principal components PC1 and PC2 for durum wheat flour samples using the spectral data obtained from ^{1}H NOESY sequence in the range 3.0−3.4 ppm: S. Severo Ofanto (1, 2); Matera Fortore (3, 4); Matera Ofanto (5, 6); Montelongo Ofanto (7, 8); Francavilla Fortore (9, 10); Francavilla Ofanto (11, 12).

under investigation is highly feasible because it does not require any pretreatment of the sample apart from the addition of a small amount of deuterated water necessary to produce a homogeneous dough and a field/frequency lock. This means that sample manipulation allows good reproducibility of results. Moreover, due to the high concentration of the sample, measurement time is very short, <15 min of data acquisition being enough to collect spectra with high signal-to-noise ratio. These two features make ^{1}H HR-MAS NMR a very attractive and competitive technique with respect to other analytical techniques such as chromatography (HPLC, GC, etc.). In fact, chromatography usually requires pretreatment of the samples, which makes the total measurement time longer and can introduce modifications in the sample composition itself. Second, the HR-MAS technique permits obtaining one-dimensional spectra with sufficient resolution to distinguish >80 peaks. This means that with one single fast measurement it is possible to obtain all of the information necessary for the multivariate statistical analysis and, furthermore, also information about saccharides and lipids composition. This feature is not matched in other analytical techniques, which usually require different analytical procedures to reach this target.

Also, the HR-MAS technique permits running two-dimensional NMR experiments such as *J*-resolved TOCSY and ^{1}H−^{13}C HMQC to help in the assignment of the one-dimensional spectra. Finally, the method seems to be sensitive in detecting small differences among the samples due mainly to their geographical origin.

In conclusion, we can state that the target of the present work has been totally fulfilled and it will be our purpose in the future to collect a set of samples coming from a more widespread area of production, we hope from the whole country of Italy.

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