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Metabolomic Characterization of Italian Sweet Pepper (*Capsicum annum* L.) by Means of HRMAS-NMR Spectroscopy and Multivariate Analysis

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HRMAS-NMR spectroscopy was used to assess the metabolic profile of sweet pepper (*Capsicum Annum* L.). One-dimensional and two-dimensional NMR spectra, performed directly on sample pieces of few milligrams, hence without any chemical and/or physical manipulation, allowed the assignment of several compounds. Organic acids, fatty acids, amino acids, and minor compounds such as trigonelline, C4-substituted pyridine, choline, and cinnamic derivatives were observed with a single experiment. A significant discrimination between the two sweet pepper varieties was found by using partial least-squares projections to latent structures discrimination analysis (PLS-DA). The metabolites contributing predominantly to such differentiation were sugars and organic and fatty acids. Also a partial separation according to the geographical origin was obtained always by analyzing the NMR data with PLS-DA. Some of the discriminating molecules are peculiar for pepper and contribute to define the overall commercial and organoleptic quality so that HRMAS-NMR proved to be a complementary analysis to standard tools used in food science and, in principle, can be applied to any foodstuff.

KEYWORDS: Sweet pepper; HRMAS-NMR; metabolomics; PLS-DA; traceability; cultivar

INTRODUCTION

Food quality and safety and nutritional value have recently become important topics worldwide, and in the past decade decision making boards, e.g. national governments in close connection with research groups, have made intense efforts to increase the overall foodstuff quality. One of the main task is the identification of peculiar compounds, i.e. genes, proteins, and metabolites, that can be correlated to specific features of the foodstuff, e.g. place of origin, nutritional values, healthiness, type of raw material, cattle breeding, etc. Genomics (*1*−*3*), proteomics (*4*), and metabolomics (*5*) approaches have therefore gained general acceptance in chemical and biochemical foodstuff characterization. They have been successfully applied to a large number of products with the aim of determining the fingerprints for authentication and valorization.

Genomics, proteomics, and metabolomics are considered complementary to each other and have different applicability. Metabolomics takes into account the most abundant low molecular weight compounds, i.e. the metabolome, present in any biological matrix, and is defined as the systematic study of the unique chemical fingerprints that specific cellular processes left behind. This approach was proposed at the beginning of the 1970s for medical and pharmacological applications and made use of the gas chromatographic tool (*6*, *7*). Later, other analytical techniques were considered, and among them nuclear magnetic resonance spectroscopy has found large application. Thanks to its ability of offering a wide range of information on metabolites in a single experiment, NMR has been used for determining the metabolic profile of a large number of fruits and vegetables: tomato (*8*, *9*), lettuce leaves (*10*), potato (*11*), mango (*12*), apple juice (*13*), tea (*14*), and grape berries (*15*).

Recently, the HRMAS-NMR (high resolution magic angle spinning−nuclear magnetic resonance) tool has been proposed as a reliable system based on NMR spectroscopy for assessing the metabolome of foodstuff. It offers the almost unique opportunity of measuring samples without any chemical and/or physical preparation by producing highly resolved NMR spectra. Qualitative and/or quantitative determination of specific molecules can be performed within the same experiment; thermo labile, light sensitive, unstable in general compounds are barely altered, if at all, due to the complete lack of sample manipulation. Furthermore, the full width at half-height of most signals is on the order of about 1 Hz, therefore comparable to the one obtained from liquid sample equivalent so that spectroscopic information are still present. The chemical composition of many foodstuffs has been determined by means of ^{1}H-HRMAS-NMR: cheese (*16*−*18*), meat (*19*, *20*), wheat (*21*), and bread and flour (*22*).

Because the richness of information often results in the high complexity of spectroscopic data sets, the use of chemometric methods to reduce the dimension of the NMR data for visualization

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purpose and to extract meaningful information is required. Multivariate data analysis combined with NMR based metabolomics has been successfully applied to address different issues in food authentication and origin. For example, promising results have been obtained concerning the discrimination of different floral origin honeys (*23*), the classification of Corsican honey (*24*), Brazilian vinegars (*25*), and Korean grapes and wines (*26*), the evaluation of Japanese green tea quality (*27*), the detection of adulteration in orange juice (*28*), and the differentiation of olive oil according to cultivar and geographical origin (*29*, *30*).

Here we present the characterization of sweet pepper (*Capsicum annuum* L.) of different cultivars growing in various zones of Italy by using the HRMAS-NMR spectroscopy combined with chemometric tools. Pepper represents a typical production of Mediterranean regions, and it has been largely studied in the past, in particular for the antioxidants (*31*, *32*), sugars (*33*), organic acids (*34*), fatty acids (*31*, *35*), and capsaicinoids (*36*, *37*) content. However, a comprehensive chemical characterization of sweet pepper has not yet been yielded; in the present work, we report the identification of the major compounds detected by means of ^{1}H- and ^{13}C-HRMAS-NMR spectroscopy. Multivariate analysis by partial least-squares projections to latent structures discrimination analysis (PLS-DA) was established to classify peppers from different cultivars and different geographical origins and to identify the biochemical compounds responsible for the discrimination.

MATERIALS AND METHODS

Samples. During the summer 2009 period, 253 sweet peppers (*Capsicum annum* L.) were collected in Piedmont and Sicily regions. Of the samples collected, 101 samples belonged to cv. "Corno", while 152 peppers belonged to cv. "Cuneo". Among peppers of cv. "Cuneo", 21 samples were harvested in Turin, 99 in "Cuneo", and 32 in the Sicily region; the latter were collected in the only Sicilian area producing this crop. Among samples of cv. "Corno", eight were harvested in the Asti area, 10 in Turin, and 83 in "Cuneo" zone, and none was from Sicily since at the time of this experimentation this variety was not present. Red and yellow peppers were harvested at different stages of maturity: green (fully developed fruit, just before the onset of maturity), breaker (approximately one-half green skin and the other half-red or half-yellow), and red (completely red skin) or yellow (completely yellow skin).

Sample Preparation for NMR Analysis. After harvesting, peppers were transported in refrigerator at +4 °C to the laboratory for NMR analysis. Samples were washed, once with tap water and then twice with distilled, and carefully dried with paper. ^{1}H- and ^{13}C NMR spectra of both varieties were recorded on edible parts of the pericarpus sampled directly with a spatula. To increase the signal-to-noise ratio, by reducing the intense water residual signal at ca. 4.7 ppm, 2D-NMR spectra were acquired on freeze-dried samples. The latter were prepared by leaving smashed peppers for 3 days in a freeze-drying apparatus, then frozen in liquid nitrogen and finally powdered in a ceramic mortar with a pestle. To ensure that the freeze-dried operation does not alter the metabolic profile, we have compared the ^{1}H-HRMAS-NMR spectra, reported as Supporting Information, of freeze-dried samples with those obtained directly taking a piece of pepper, and no difference were found. We have also measured the same pepper three times by sampling in different zones, and the PLS-DA model was not able to discriminate them.

NMR Measurements. Samples were prepared by inserting ca. 25 mg of sweet pepper (fresh for 1D spectra and freeze-dried for multidimensional) in a 4 mm HRMAS rotor with a 50 μL spherical insert. Approximately 25 μL of D_2O phosphate buffer, 0.01 M concentration and pH value equal to 7.2, with 0.5% TSP, i.e. 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt, were then added. HRMAS-NMR spectra were recorded at 298 K with a Bruker AVANCE spectrometer operating at a ^{1}H frequency of 400.13 MHz, equipped with a 4 mm HRMAS dual channel probe head and spinning the samples at 7 kHz. ^{1}H NMR spectra were referenced to the methyl groups signal at δ 0.00 ppm of TSP, while ^{13}C NMR spectra were referenced to the TSP δ 0.00 ppm.

^{1}H-HRMAS-NMR spectra were acquired by using a water suppression pulse sequence, noesypr1D (Bruker library), using 32K data points over a 4807 Hz spectral width and adding 256 transients. A recycle delay of 3 s and a delay for allowing efficient NOE effect equal to 150 ms were used, the 90° pulse length was 5.3 μs, and saturation of water residual signal was achieved by irradiating during recycle delay at δ equal to 4.70 ppm. Each spectrum was FT transformed with 64K data points and manually phased and base-lined, and a line broadening factor equal to 0.3 Hz was applied to the FID prior FT.

^{13}C-HRMAS-NMR spectra were acquired with the power-gated decoupling sequence, zgpg30 (Bruker library), using a 30° flip angle pulse of 5.0 μs. Experiments were carried out using 64K data points over a 22123 Hz (~220 ppm) spectral width by adding 64K transients with a recycle delay of 3 s. Each spectrum was FT transformed with 128K data points and manually phased and base-lined, and a line broadening factor of 0.5 Hz was applied to the FID.

The ^{1}H−^{1}H TOCSY experiment was acquired in the TPPI phase-sensitive mode, with a 4807 Hz spectral width in both dimensions, 100 ms of spin-lock time of 4500 Hz, 2K data points in f_2, and 1K increments in f_1, each with 32 scans. The ^{1}H−^{13}C HSQC spectra were acquired in TPPI phase-sensitive mode, with a 4807 Hz spectral width in f_2 dimension and a 15083 Hz spectral width in f_1. 2K data points in f_2 and 1K increments in f_1, each with 32 scans, were used.

NMR Data Reduction and Preprocessing. All ^{1}H NMR spectra were manually phased, baseline corrected, and aligned by XWINNMR 3.5 software (Bruker Biospin, Karlsruhe, Germany). Each spectrum was divided into intervals equal to 0.06 ppm (buckets) in the range from 0.06 to 9.00 ppm, with the exclusion of the water region from 4.74 to 4.86 ppm, using AMIX 3.5 software (Bruker Biospin, Karlsruhe, Germany). All integrated buckets were scaled to the signal intensity of the peak at 3.81 ppm so that NMR spectra were bucketed in 149 variables.

Multivariate Data Analysis. Experimental data were organized into a 253×149 matrix that was imported into Matlab software (The Mathworks, Natick, MA; version 7.4.0.287) to be processed with different chemometric methods..

First, we investigated if the metabolic information contained in the bucketed NMR spectra were sufficient to provide a reliable discrimination between the cultivars considered. This task was accomplished by building a classification model relating the NMR data to the varietal information; in particular, the PLS-DA method was used for the classification step. However, before any classification model could be computed, it was necessary to divide the available data in two separate sets. The first one, the training set, for the buildup of the classification model, and the second one, the test set, for its validation. Indeed, because the model building step involves changing some adjustable model parameters that have to be defined in order to obtain the best results on the training data, using the same data as a benchmark to evaluate the performance of the model itself would result in biased overoptimistic estimate and, ultimately, in overfitting. On the other hand, leaving aside an independent test set to be used for the evaluation of the model performances provides a more reliable estimate of the classification error on unknown and external samples.

In this work, the division of the available data between training and test set was carried out using the duplex algorithm (*38*), which allows maintainance of a comparable diversity in both sets; the latter are therefore similar in terms of representativeness. The duplex algorithm starts by selecting from the whole data matrix X the two objects that are farthest away from each other according to their Euclidean distance. These objects are put into the training set. Then, among the remaining candidates, the two objects farthest from each other are put into the test set. At the next step, consecutive objects are selected and put alternatively in the training and test sets, the object added being the one farthest away from all the objects of **X** already in the current set. To determine which object is the farthest one, a so-called maximin criterion is used. This criterion is the same as in the Kennard and Stone algorithm (*39*), i.e. the Euclidean distance between each candidate object and its closest neighbor already in the considered set is computed and the object for which this distance is maximal is added.. Eventually, 179 samples (76 from "Corno" and 103 from "Cuneo") were included in the training set, while the remaining 74 samples (25 from "Corno" and 49 from "Cuneo") constituted the test set. The data set splitting can be seen in **Figure 1**, where the projection of the samples onto the first two principal components is shown, together with

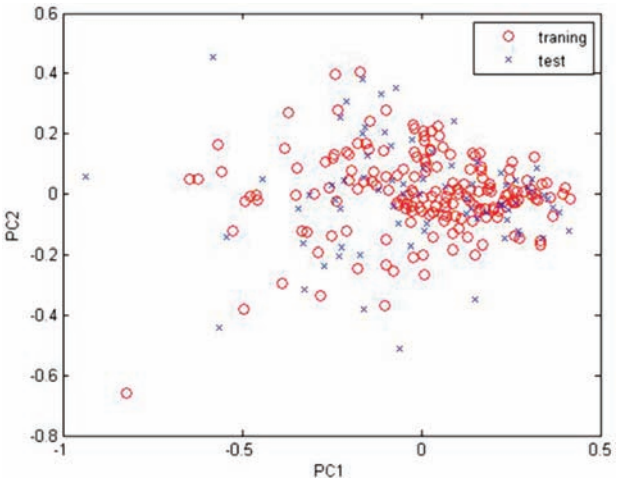

Figure 1. Projection of the test and training samples onto the two principal components.

the training/test labeling. It can be seen how a comparable diversity is preserved in both sets as a consequence of the use of the duplex algorithm.

Successively, a further attempt was made to verify whether, on the basis of the NMR spectra, a discrimination according to the geographical origin of the sample was possible. However, the uneven distribution of the geographical origins among our samples suggested us to proceed considering one cultivar at a time. Furthermore, the selection of an independent test set was also not possible due to the class distribution, therefore only cross-validated results were taken into account.

In all cases, prior to computation of the PLS-DA data, the matrix was pretreated to put the spectra in the most suitable form for the successive data analysis. In particular, each variable was Pareto-scaled, according to eq 1:

$$x'_{ij} = \frac{x_{ij} - \bar{x}_j}{\sqrt{s_j}} \quad (1)$$

where x_{ij} is the value of the jth variable measured on the ith sample and $\bar{x}_j$ and s_j are the mean and the standard deviation of the jth variable, computed over all samples. Pareto scaling was chosen over other pretreatment method (as mean centering or autoscaling) as it allows to upweigh the contribution of lower intensity peaks without overinflating excessively the noise.

RESULTS AND DISCUSSION

Assignment of ^{1}H-HRMAS-NMR Spectrum. The ^{1}H HRMAS-NMR spectrum of fresh sweet red pepper is reported in **Figure 2**, showing the intense peaks belonging to major sugars, i.e. glucose and fructose. Several other minor signals are visible, **Figure 2**, and in order to assign them to the corresponding compounds, multidimensional NMR experiments were performed. Connectivity information obtained from 2D spectra and the use, as guidelines, of chemical shift data reported in literature (*40*), allowed the assignment of a large number of resonances, which are summarized for chemical classes in **Table 1**−**4**, amino acids, carbohydrates, fatty acids, and organic acids respectively.

Figure 3A shows the high field region, from 0.50 to 3.10 ppm, of the ^{1}H-HRMAS-NMR spectrum, which contains signals belonging to the aliphatic groups of amino acids (**Table 1**), organic acids (**Table 2**), and fatty acids (**Table 3**). In particular, the signals arising from valine, isoleucine, leucine, threonine, alanine, glutamate, glutamine, γ-aminobutyrate, arginine, asparagine, acetate, and malate were identified, **Table 1**. Signals in the range from 0.90 to 1.10 ppm arise from methyl or methylene groups of valine, leucine, and isoleucine, and the correct assignment was made based on TOCSY correlations. The latter was of help also in identifying the compounds in the region from 1.40 to 3.10 ppm,

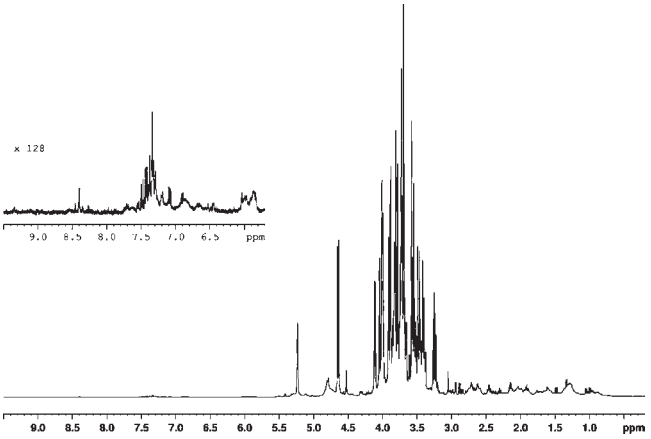

Figure 2. ^{1}H HRMAS NMR spectrum of sweet pepper in phosphate/D_2O buffer with 0.5% of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP).

where several barely resolved multiplets, belonging to amino acids, can be found. In the high field region, apart from sharp resonance signals due to amino acids and organic acids, a series of broad peaks appear. In particular, the signal at 0.88 ppm has in TOCSY spectrum cross peaks with the protons at 1.28, 1.57, and 2.24 ppm. This spin system corresponds to the saturated chains of fatty acids, and we assigned them to the protons of palmitic and stearic acids, which are the most abundant saturated fatty acids in sweet pepper. The cross peaks between the signal at δ = 1.31 ppm and resonances at δ = 2.03, 2.76, and 5.30 ppm are indicative of the presence of unsaturated lipid chains. Linoleic, linolenic, and oleic acids were present in relatively high amount and their signals overlap in the ^{1}H-NMR spectrum, with the exception of the methyl groups so that signal at δ = 1.31 ppm has a broad cross peak with protons at δ = 0.89, 0.92, and 0.96 ppm, which are the terminal CH_3 of oleic, linoleic, and linolenic acids, respectively. In the high field region of the spectrum, one also recognizes the signals pattern of glutamine and glutamate; the multiplets at δ equal to 2.06 and 2.12 ppm, β-CH and β'-CH, respectively, correlate in TOCSY experiment with the signal at δ = 2.35 (γ-CH_2) and 3.77 ppm (α-CH), which forms the typical spin system of glutamate. Its presence is also confirmed by the HSQC spectrum, which yields correlations between protons at δ = 2.12 and 3.77 ppm with ^{13}C resonances at δ = 27.5 and 55.1 ppm, respectively. The signals pattern of glutamine is clearly visible in TOCSY spectrum; in fact, correlations between a series of multiplets at δ = 2.15, 2.46, and 3.78 ppm appear, β-CH_2, γ-CH_2, and α-CH, respectively. This observation is supported by the heteronuclear correlations between protons and corresponding ^{13}C signals in the HSQC spectrum (**Table 1**). The doublet of doublets at δ = 2.69 ppm is not resolved, owing to overlap with the broad signals of fatty acids at δ = 2.74 and 2.76 ppm, and we assigned it to malate. The assignment was assessed based on TOCSY spectrum, where the signal at δ = 2.69 ppm, β-CH, correlates with peak at δ = 2.39 and 4.31 ppm, β'-CH and α-CH, respectively. Pepper contains some organic acids, the most abundant are citric, ascorbic, and malic, and their resonances are expected in this region. The doublets of doublets at δ = 2.86 and 2.96 ppm are assigned to asparagine, β-CH and β'-CH, respectively, on the basis of cross-peaks in TOCSY spectrum with the signal at δ = 4.01 ppm, i.e. α-CH, and with the correlation with ^{13}C signal at δ = 35.6 ppm visible in the HSQC spectrum. Also the correlation between the peak at δ = 4.01 ppm with the ^{13}C resonance at δ = 52.4 ppm in HSQC experiment confirms the presence of asparagine.

Table 1. ^{1}H and ^{13}C Chemical Shifts of Assigned Amino Acids[a]

compd	assignment	^{1}H δ (ppm)	multiplicity [J (Hz)]	^{13}C δ (ppm)
		Amino Acids		
alanine (Ala)	α-CH	3.78		
	β-CH_3	1.48	d [7.3]	
arginine (Arg)	α-CH	3.78		
	β-CH_2	1.91		
	δ-CH_2	3.25		
asparagine (Asn)	α-CH	4.01	dd	52.4
	β-CH	2.86	dd [16.9; 7.6]	35.6
	β'-CH	2.96	dd [16.9; 4.4]	35.6
γ-aminobutyrate acid (GABA)	α-CH_2	2.31	t [7.5]	
	β-CH_2	1.92	q [7.4]	
	γ-CH_2	3.02	t [7.5]	
glutamate (Glu)	α-CH	3.77		55.1
	β-CH	2.06	m	27.5
	β'-CH	2.12		27.5
glutamine (Gln)	α-CH	3.78		55.2
	β-CH_2	2.15	m	27.3
	γ-CH_2	2.46	m	31.4
isoleucine (Ile)	α-CH	3.66		
	β-CH	1.98		
	γ-CH_3	1.01	d [7.1]	
	γ-CH	1.26		
	γ'-CH	1.48		
	δ-CH_3	0.94	t [7.6]	
leucine (Leu)	α-CH	3.74		
	β-CH_2	1.75		
	γ-CH	1.75		
	δ-CH_3	0.97		
phenylalanine (Phe)	*o*-CH	7.34		
	m-CH	7.45		
	p-CH	7.39		
threonine (Thr)	α-CH	3.60		
	β-CH	4.25	m	
	γ-CH_3	1.33	d [6.6]	
tryptophan (Trp)	CH-4, ring	7.72	d	
	CH-5, ring	7.20		
	CH-6, ring	7.28		
	CH-7, ring	7.54	d	
tyrosine (Tyr)	*o*-CH	7.19		
	p-CH	6.88	d	
valine (Val)	α-CH	3.65		
	β-CH	2.28	m	
	γ-CH_3	0.99	d [7.0]	
	γ'-CH_3	1.04	d [7.0]	

[a] ^{1}H chemical shifts refer to TSP signal (δ = 0.00 ppm), while ^{13}C ones to TSP, i.e. δ 0.0 ppm.

Table 2. ^{1}H and ^{13}C Chemical Shifts of Assigned Organic Acids and Other Metabolites[a]

compd	assignment	^{1}H δ (ppm)	multiplicity [J (Hz)]	^{13}C δ (ppm)
		Organic Acids		
acetate	CH_3	1.92	S	
ascorbate	CH_2-6	3.73		
	CH-5	4.02		
	CH-4	4.52	d [1.8]	77.4
formate	HCOOH	8.46	s	
fumarate	α,β-CH=CH	6.52	s	
malate (Mal)	α-CH	4.31	dd [9.9; 3.1]	
	β-CH	2.69	dd [15.4; 3.1]	
	β'-CH	2.39	dd [15.4; 9.9]	
		Unsaturated Fatty Acids		
	XH2	1.63		
	XH2−XH=XH−XH2	2.04		
	XH=XH	5.15		
		Other Metabolites		
choline	N−XH3	3.12	s	
creatine and/or creatinine	N−XH3	3.05	s	
cinnamic compounds		6.91		
		7.65		
		6.04		
		7.88		
		7.08		
		7.38		
C4-substituted pyridine	*o*-H	8.40		
	μ-H	7.30		
	o-H	8.55		
	μ-H	7.27		
trigonelline		9.35		
		9.10		
		9.01		
		Tentative Assignment		
cis-olefin		4.44	d [3.8]	
		4.46	d [3.8]	
		4.95	d [3.8]	
		5.02	d [3.8]	
		5.27	d [3.8]	
		5.44	d [3.8]	

[a] ^{1}H chemical shifts refer to TSP signal (δ = 0.00 ppm), while ^{13}C ones to TSP, i.e. δ 0.0 ppm.

Figure 3B shows the middle field region, from 3.20 to 5.60 ppm, of the ^{1}H-HRMAS-NMR spectrum, where the main signals arise from carbohydrates moieties (**Table 4**) strongly overlapping the amino acids α-C*H* peaks. TOCSY is very helpful because it allows the unequivocal assignment of these compounds. Among carbohydrates, the most intense signals arise from the different isomeric forms of D-glucose and D-fructose. Thanks to the ^{1}H−^{13}C HSQC spectrum (data not shown), we assigned all the resonances of α-D- and β-D-glucose and α-D- and β-D-fructose, as well as we distinguished their pyranose and furanose isomers. α-D-Fructopyranose was not observed, most likely due to the low concentration. Sucrose signals are also expected in this region and give rise to the doublet at 5.42 ppm and its correlations. Fresh pepper has a high C vitamin content, and one can clearly observe the ascorbate signals: the doublet at δ = 4.52 ppm and the signals at δ = 4.02 and 3.73 ppm, C*H*-4, C*H*-5, and C*H*-6, respectively. They all correlate in TOCSY spectrum and are known to be the typical spin system for this compound (*40*). Also, the correlation of the doublet at δ = 4.52 ppm with the ^{13}C signal at 77.4 ppm in the HSQC spectrum supports the ascorbate assignment. Finally, in this region are present a series of doublets at δ = 4.44, 4.46, 4.95, 5.02, 5.27, 5.44, and 5.50 ppm, characterized by weak intensities and having the same coupling constant: 3.8 Hz. They show no correlation to each other in TOCSY spectrum, or, if present at all, the cross peaks are hidden by the intense carbohydrates resonances. Only the doublet at δ = 5.50 ppm shows some correlations with protons at δ = 4.12, 2.18, 2.03, and 1.77 ppm; we believe that these signals are due to olefins in *cis* configuration, but no further evidence was found.

Figure 3C shows the low field region, from 5.70 to 9.50 ppm, of the ^{1}H-HRMAS-NMR spectrum of fresh pepper. The signals in this range are the weakest and arise from aromatic groups of amino acids and phenolic compounds. The two singlets at 6.52 and 8.46 ppm belong to fumarate and formate, respectively.

Table 3. ^{1}H and ^{13}C Chemical Shifts of Assigned Fatty Acids[a]

compd	assignment	^{1}H δ (ppm)	multiplicity [J (Hz)]	^{13}C δ (ppm)
	Fatty Acids			
	Saturated Fatty Acids			
C16 palmitic (p)	CH_2-3	1.57		
C18 stearic (s)	CH_2-2	2.24		
	CH_2-4−CH_2-15 (p)	1.28		
	CH_2-4−CH_2-17 (s)			
	CH_3-16 (p)	0.88		
	CH_3-18 (s)			
	Monounsaturated Fatty Acids			
C18:1 (oleic)	CH_2-2	2.36		
	CH_2-3	1.61		
	CH_2-4,7	1.31		
	CH_2-8	2.03		
	CH-9	5.30		
	CH-10	5.30		
	CH_2-11	2.03		
	CH_2-12,17	1.31		
	CH_3-18	0.89		
	Polyunsaturated Fatty Acids			
C18:2 (linoleic)	CH_2-2	2.36		
	CH_2-3	1.61		
	CH_2-4,7	1.31		
	CH_2-8	2.03		
	CH-9	5.30		
	CH-10	5.30		
	CH_2-11	2.74		
	CH-12	5.30		
	CH-13	5.30		
	CH_2-14	2.03		
	CH_2-15,17	1.31		
	CH_3-18	0.92		
C18:3 (linolenic)	CH_2-2	2.36		
	CH_2-3	1.61		
	CH_2-4,7	1.31		
	CH_2-8	2.03		
	CH-9	5.30		
	CH-10	5.30		
	CH_2-11	2.76		
	CH-12	5.30		
	CH-13	5.30		
	CH_2-14	2.76		
	CH-15	5.30		
	CH-16	5.30		
	CH_2-17	2.03		
	CH_3-18	0.96		

[a] ^{1}H chemical shifts refer to TSP signal (δ = 0.00 ppm), while ^{13}C ones to TSP, i.e. δ 0.0 ppm.

We assigned the signals at δ = 7.34, 7.45, and 7.39 ppm to phenylalanine, *o*-C*H*, *m*-C*H*, and *p*-C*H*, respectively, and the two doublets at δ = 6.88 and 7.19 ppm to tyrosine, *o*-C*H*, and *m*-C*H*, respectively. Also the signals of tryptophan are visible in the spectrum: the doublet at δ = 7.54 ppm, i.e. C*H*-7, has a cross peak in the TOCSY spectrum with the doublet at δ = 7.72 ppm (C*H*-4), while the peak at δ = 7.20 ppm (C*H*-5) correlates with the signal at δ = 7.28 ppm (C*H*-6). In the low field region also a series of broad lines are present. The signal at δ = 6.91 ppm has a TOCSY cross peak with the signal at 7.65 ppm; in apple and mango juice, these signals were assigned to phenolic compounds (*12*, *13*), while in tomato juice authors suggested titrated

Table 4. ^{1}H and ^{13}C Chemical Shifts of Assigned Carbohydrates[a]

compd	assignment	^{1}H δ (ppm)	multiplicity [J (Hz)]	^{13}C δ (ppm)
	Carbohydrates			
β-D-glucose (β-Glc)	CH-1	4.65	d [7.9]	97.0
	CH-2	3.25	dd [9.3; 8.0]	75.2
	CH-3	3.49	t [9.1]	76.8
	CH-4	3.40	dd [9.4; 9.0]	70.8
	CH-5	3.43		77.0
	CH_2-6,6′	3.89; 3.74		61.8
α-D-glucose (α-Glc)	CH-1	5.23	d [3.8]	93.2
	CH-2	3.55		72.5
	CH-3	3.73		73.8
	CH-4	3.42		70.7
	CH-5	3.81		72.5
	CH_2-6,6′	3.83; 3.84		61.7
β-D-fructopyranose (β-FP)	CH_2-1,1′	3.57; 3.73		65.0
	CH-2			99.2
	CH-3	3.81		68.6
	CH-4	3.91		70.7
	CH-5	4.00		70.3
	CH_2-6,6′	3.72; 4.01		64.5
β-D-fructofuranose (β-FF)	CH_2-1,1′	3.55; 3.59		63.8
	CH-2			102.6
	CH-3	4.11		76.5
	CH-4	4.11		75.5
	CH-5	3.81		81.8
	CH_2-6,6′	3.80; 3.66		63.5
α-D-fructofuranose (α-FF)	CH_2-1,1′	3.69		64.0
	CH-2			105.5
	CH-3	4.10		83.0
	CH-4	4.00		77.1
	CH-5	4.04		82.4
	CH_2-6,6′	3.69; 3.83		62.1
sucrose (Suc)	CH-1 (Glc)	5.42	[d 3.3]	93.2
	CH-2	3.57		72.1
	CH-3	3.78		73.6
	CH-4	3.48		70.2
	CH-5	3.85		73.4
	CH_2-6	3.83		61.1
	CH-1′ (Fru)	3.69		62.3
	CH-2′			104.7
	CH-3′	4.22		77.6
	CH-4′	4.04		75.0
	CH-5′	3.89		82.2
	CH_2-6′	3.83		63.3

[a] ^{1}H chemical shifts refer to TSP signal (δ = 0.00 ppm), while ^{13}C ones to TSP, i.e. δ 0.0 ppm.

amino acid $-NH_2$ or $-NH_3^+$ groups that exchange protons with the solvent (*9*). To elucidate this point, we measured ^{1}H-HRMAS-NMR spectrum of freeze-dried pepper by using pure D_2O as solvent and preparing the sample under inert atmosphere. This allowed us to obtain a HDO free sample, where the exchange between HDO and NH_2 groups is minimized, if present at all. On the basis of the results of this experiment, we believe that the correct assignment of these signals, i.e. δ = 6.91 and 7.65 ppm, is to phenolic compounds, most likely, as observed for wine extracts (*41*), to cinnamic molecules. This is in agreement with the fact that sweet pepper is rich with such metabolites, predominantly in the form of caffeic, ferulic, and *p*-coumaric acids (*32*). Therefore, we assigned these signals to hydroxycinnamic compounds. Similarly, the pairs of peaks at δ = 6.04 and 7.88 ppm and δ = 7.08 and 7.38 ppm, which correlate with each other in the TOCSY spectrum, can arise from cinnamic compounds. The multiplet at δ = 7.30 ppm has an intense TOCSY cross peak with the signal at 8.40 ppm, suggesting a pyridine structure, but because the

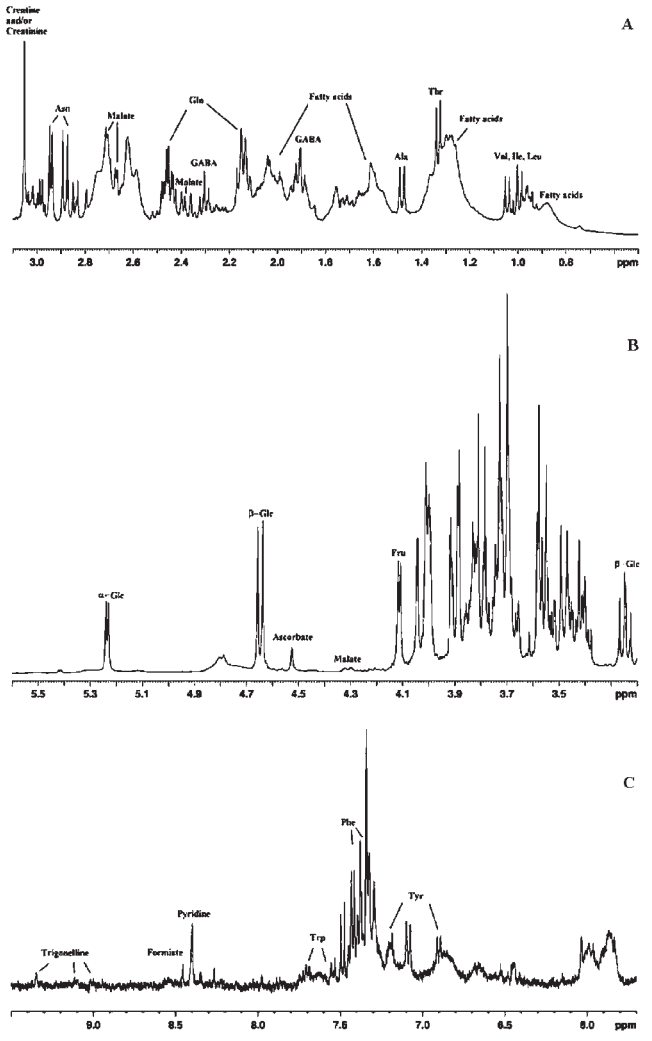

Figure 3. [1]H-HRMAS-NMR spectrum of sweet pepper: (**A**) high field region; (**B**) middle field region; (**C**) low field region.

correlation with the *p*-proton is absent, we assigned these signals to C4-substituted pyridine. Resonances with similar chemical shifts were also found in Parmigiano Reggiano cheese, and the presence of these compounds was proposed as well (*26*). Also the signals at $\delta = 7.27$ and 8.55 ppm, which correlate with each other in the TOCSY spectrum, can be assigned to C4-substituted pyridine. Finally, on the leftmost part of the spectrum, there is another spin system indicative of aromatic heterocyclic compounds; the correlations of the peak at $\delta = 9.35$ ppm with the signals at $\delta = 9.10$ and 9.01 ppm were assigned to trigonelline.

PLS-DA of Spectroscopic Profiles of Sweet Pepper. Multivariate data analysis was applied to the collected ^{1}H NMR spectra in order to discriminate samples according to the variety and to the geographical origin.

Discrimination According to the Cultivar. The training set reported in **Figure 1** was used to build a PLS-DA classification model to discriminate between the "Cuneo" and "Corno" varieties. Pareto scaling was used as data preprocessing to give enough importance to the less intense peaks without overinflating them, as discussed in the Materials and Methods. The optimal complexity of the model, i.e. the number of latent variables (LVs) to be included, was chosen as the one leading to the minimum classification error in 10-fold cross-validation and corresponded to 10 LVs. The model resulted in a very good classification ability (97.2%) and a fairly good nonerror rate both in cross-validation

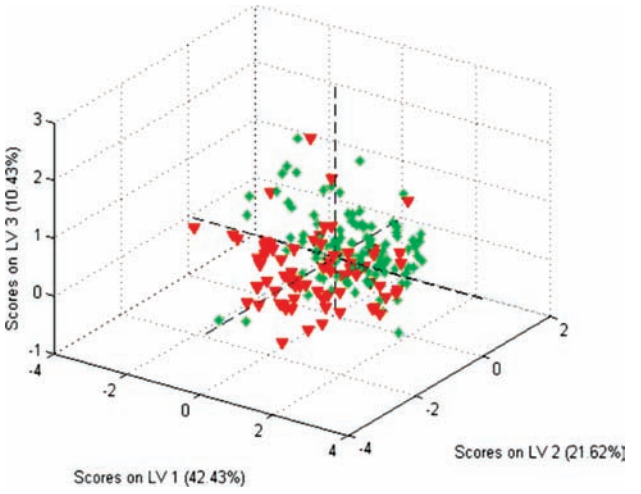

Figure 4. PLS-DA score plot derived from the [1]H-HRMAS-NMR spectra of sweet peppers, triangles represent cv. "Corno", while stars are cv. "Cuneo".

and prediction on the independent test set (83.8% for both). Furthermore, the fact that the cross-validated predictive ability is compared to that on the external validation set is an index that the probability of the model being overfitted is low. The good separation of the two classes can also be seen in **Figure 4**, where the training data are plotted onto the space spanned by the first three latent variables.

To assess which metabolites were mostly responsible for this discrimination, both the VIP scores and the regression coefficients of the PLS-DA model were inspected. In particular, the variable importance in projection (VIP) score of a predictor is a value that expresses the contribution of the individual variable in the definition of the F-latent vector model. Because of the normalization that is used in the definition of the VIP, variables showing values less than 1 are considered not to contribute significantly to the model. The VIP scores for the optimal PLS-DA model are reported in **Figure 5A**, from which it is possible to see that significant intensities are found below 6 ppm. The signal that had the largest VIP coefficient corresponds to the anomeric carbon of sucrose. Also other sugars, such as glucose and fructose, contribute significantly to the discrimination between the two cultivars, as highlighted by the VIP intensities at 3.27, 4.05, 4.11, and 5.21 ppm. Other important metabolites responsible for the classification were the organic acids, in particular malate, ascorbate, and acetate, and the fatty acids, both saturated and unsaturated. Among amino acids, largest VIP coefficients were found for threonine, arginine, and GABA, at 1.89, 3.27, and 1.35 ppm, respectively. To understand how the concentration of the identified metabolites varied with respect to the cultivar, the regression coefficients were also inspected (**Figure 5B**). According to the binary coding adopted, a positive value of the regression coefficient implies that the variable has an higher intensity in the spectra of the cultivar "Corno" than in those of the cultivar "Cuneo", while a negative coefficient accounts for the opposite. **Figure 5B** makes evident that cv. "Corno" peppers have the largest sucrose concentration, with a corresponding regression coefficient of 0.5369. High levels of glucose and polyunsaturated fatty acids were also found for this cultivar. On the other hand, cv. "Cuneo" showed the largest regression coefficients for glucose, arginine, GABA, acetate, and fatty acids.

Sugars and organic acids are key factors in determining the sweet pepper taste features, while C vitamin, phenolic compounds, and carotenoids contribute to the nutritional aspects. On the other hand, it is known that contents of these compounds

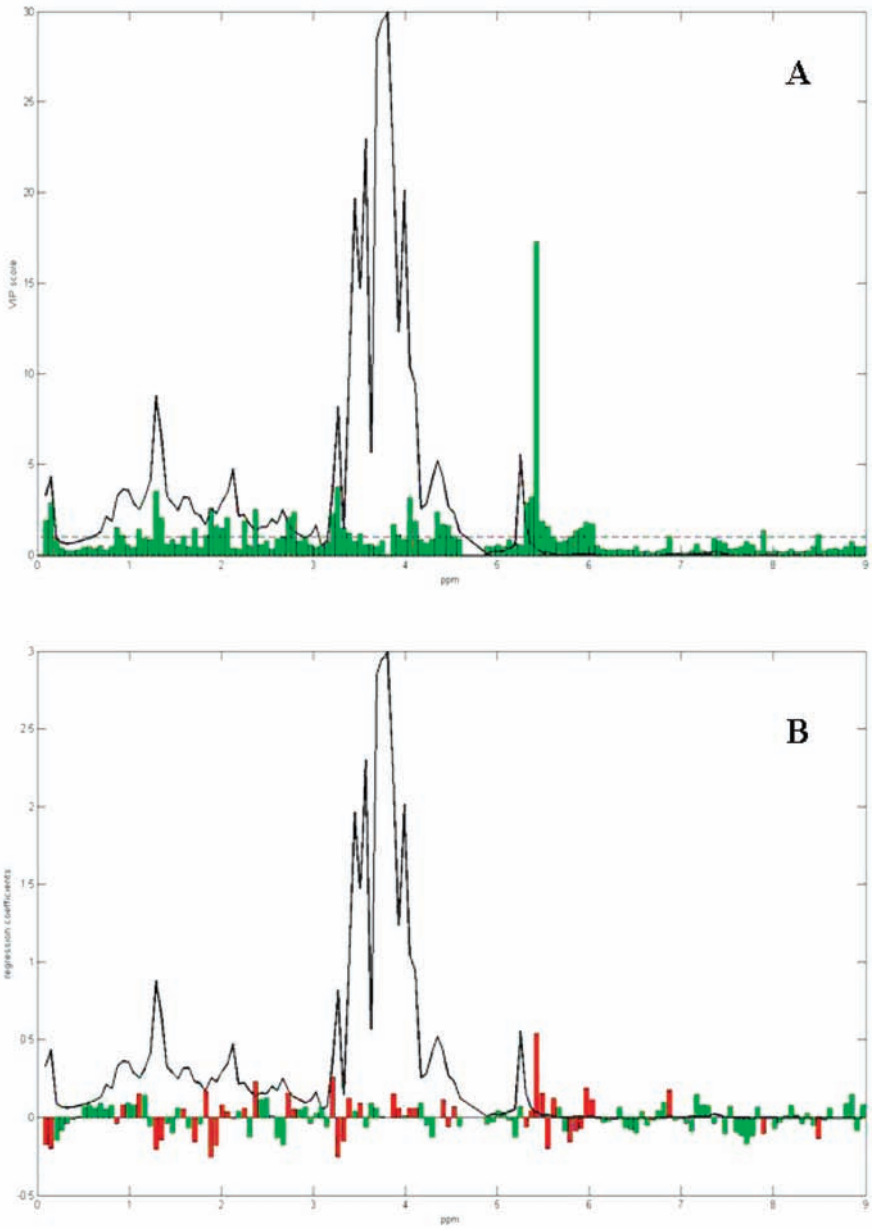

Figure 5. VIP scores plot (**A**) and complementary regression coefficients (**B**) for discrimination according to the cv.

can vary by genotype and ripening stage and are influenced by growing conditions and losses after harvesting (*42*). Results obtained from the PLS-DA analysis of the ^{1}H NMR spectra reveals that the metabolites mainly contributing to the discrimination between the two cultivars considered were sugars and organic and fatty acids.

Discrimination According to Maturity Stage and Color. To elucidate the influence of the ripening degree on the discrimination observed above, we have built a PLS-DA model considering only peppers with homogeneous maturity. **Figure 6** summarized the results for early ripening stage samples, and one can observe that relevant metabolites for discriminating the two varieties are almost the same as those found by analyzing the entire pepper data set, with sucrose, glucose, and fructose being the most significant. As previously observed also organic acids, i.e. malate, ascorbate, and acetate, contribute to the differentiation. The only difference found is the contribution arising from *cis*-olefins. Once again, higher levels of glucose, sucrose, and fatty acids were found in cv. "Corno".

We also considered a PLS-DA model, data not shown, for samples with the same color, and we found that the metabolites discriminating the varieties are again those reported for the entire pepper data set.

Geographical Origin for cv. "Corno". A total of 101 peppers were considered, obtained from three different geographical areas in Piedmont, namely "Cuneo" (83), Turin (10), and Asti (8). The sample distribution among the classes makes the proper external validation of the classification model rather difficult, so that only a 10-fold cross-validation was performed. The corresponding model (13 LVs) was dominated by the most numerous class, i.e. "Cuneo", as shown in **Figure 7A**, where the projection of the samples onto the first 3 LVs is reported. From the point of view of the classification ability, an acceptable discrimination was obtained, with an overall 92.1% nonerror rate in cross-validation (3 samples from Torino and 5 samples from "Cuneo" misclassified). Also in this case, it is possible to assess which metabolites are responsible for such discrimination. VIP scores (data not shown) and the regression coefficients (**Figure 7B**) indicate that samples from Asti were separated according to sugars, mostly glucose and fructose, organic acids, i.e. malate and vitamin C, and some amino acids, including asparagine and glutamine, content. Relatively low

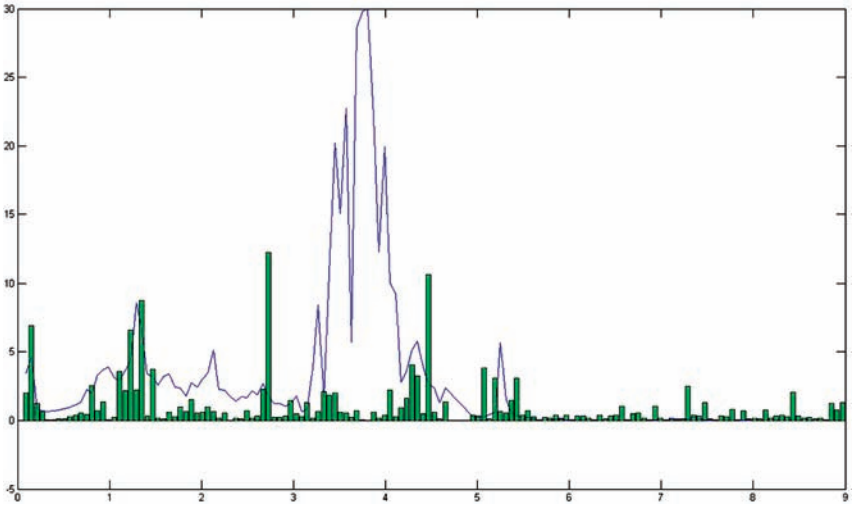

Figure 6. VIP scores plot of the PLS-DA model discriminating between cv. "Corno" and cv. "Cuneo" sweet peppers harvested at an early maturity stage.

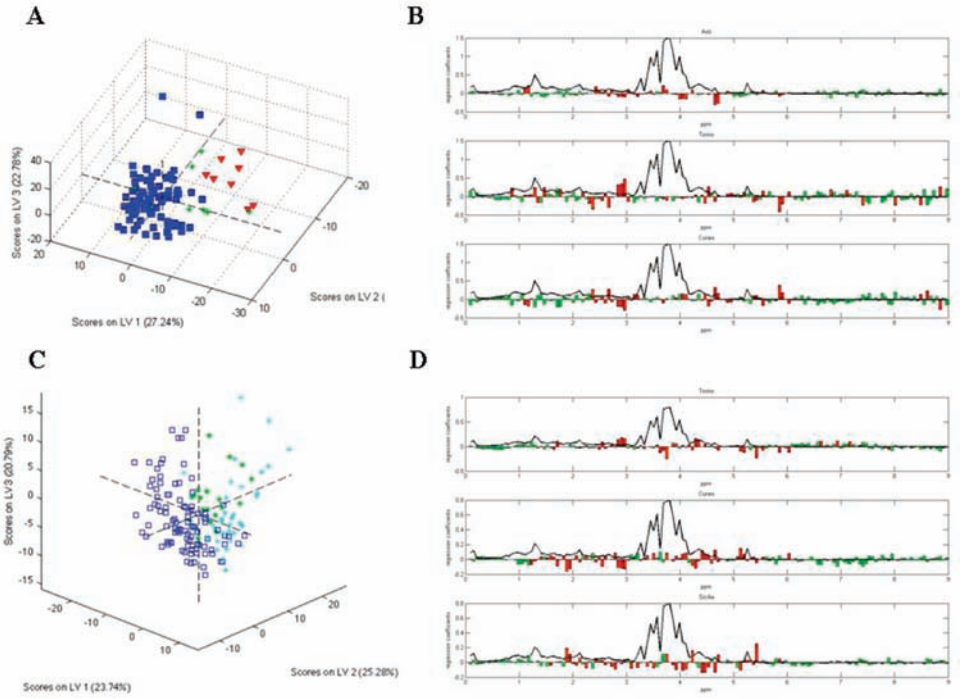

Figure 7. PLS-DA score plot and complementary regression coefficients for discrimination according to the geographical origin for cv. "Corno" and for cv. "Cuneo" samples, (**A**), (**B**), (**C**), and (**D**), respectively. In (**A**), squares are samples from "Cuneo" area, triangles from Asti, and stars from Turin. In (**C**), empty squares correspond to peppers from "Cuneo", stars from Sicily, and finally circles from Turin.

levels of glucose and fructose, as well as of asparagine and vitamin C is found in samples from Asti, while the levels of glutamine and malic acid were higher than those in peppers from "Cuneo" and Turin. cv. "Corno" samples grown in Turin, instead, were discriminated for their high level of asparagine, which was the compound with the highest contribution to the classification, and the relatively high content of ascorbate. Furthermore, they showed a very low concentration of malate, aromatic compounds such as cinnamic acids and phenylalanine, and unknown metabolites, whose signals have a δ 5.90 ppm. Finally, relatively high contents of all sugars, especially glucose, malate, and the same unknown metabolites just mentioned, were found in peppers from "Cuneo". On the other hand, they had a low level of asparagine, malate and vitamin C compared to those of peppers came from Asti and Turin.

Geographical Origin for cv. "Cuneo". Samples were harvested in two different areas: 32 in Sicily and 121 in Piedmont, among which were 99 in "Cuneo" and 21 in Turin. Also in this case, 10-fold cross-validation was performed. The corresponding model included 9 latent variables and resulted in an overall cross-validation non error rate of 94.7% (three samples from Turin and five from "Cuneo" misclassified). The projection of the samples onto the first 3 LVs computed by the model is reported in **Figure 7C**.

In evaluating the VIP scores (data not shown) and the regression coefficients (**Figure 7D**), one can argue that the metabolite mainly contributing to the discrimination model is sucrose, whose VIP value is equal to 11.0418. Sugars levels were relatively low in samples from Turin, while malate and asparagine were present in higher concentration. In "Cuneo" samples, low levels of asparagine, malate, sucrose, and glucose were found, while fructose,

glutamine, and fatty acids were in higher concentration. Peppers from Sicily had sucrose as the major contribution to discrimination; on the contrary, fructose and β-glucose amounts were found to be low. Finally, small concentrations of *cis* olefins and unsaturated fatty acids were found in samples from Sicily, while acetate, glutamine, GABA, and arginine were present in higher amounts.

The high resolution magic angle spinning NMR (HRMAS-NMR) approach was used to yield the metabolic profile of sweet pepper (*Capsicum Annum* L.). The possibility of using a single technique to evaluate, simultaneously, soluble amino acids, small organic acids, insoluble fatty acids, and many other metabolites present, without any extraction, purification, and separation steps, allows this tool to be suitable for the determination of the metabolic profile, in principle, of any foodstuff. ^{1}H-HRMAS-NMR spectra combined with statistical models allowed us to discriminate peppers from different cultivars. PLS-DA analysis was also able to classify peppers according to their geographical origin. The results obtained suggest that HRMAS-NMR could be a very useful tool for pepper characterization, and combined with multivariate analysis, it could be a quick and reliable method for classification studies.

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Supporting Information Available: Comparison between freeze-dried and fresh ^{1}H-HRMAS-NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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