Metabonomic fingerprinting of olive fruits for breeding purposes

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Abstract

High-resolution magic angle spinning (MAS) Nuclear Magnetic Resonance spectroscopy has been used to obtain detailed metabolic fingerprint of olive flesh as protons in different chemical environment appear as spectral lines at different chemical shifts. Univariate and multivariate statistical analyses has been carried out in order to establish a procedure to characterize and classify olives in a breeding program.

INTRODUCTION

Spain is the most important producer of olive oil in the world and therefore the achievement of high quality cultivars is a major issue in Spanish breeding programs. Accurate and reliable analitical techniques are key tools for the characterization of quality, mainly as fat content, which is currently determined by means of low-resolution Nuclear Magnetic Resonance (LR-NMR) spectroscopy [1]. Within the last few years, the development of a technique called high-resolution magic angle spinning (MAS) NMR spectroscopy has made feasible the analysis of small pieces of intact tissues with no pretreatment and the simultaneous analysis of a variety of chemical components. Nuclei of hydrogen atoms in different chemical environment appear as a spectral line at different chemical shift (measured in parts per million from a reference). Thus, a HR-NMR spectrum can be considered as an n-dimensional object whose dimensions could be simply the spectral intensity distribution or the concentrations of individual measurable metabolites [3]. When these complex data sets were first interpreted using multivariate analysis the concept of Metabonomics was born [2].

This study aims at establishing a robust procedure for metabolite fingerprinting in olive flesh as a reference within the breeding program of the "Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica" (IFAPA).

MATERIALS AND METHODS

Olea europaea L. fruits were obtained from three different parentals (cv. Picual, cv. Arbequina and cv. Frantoio) and six descendants (Table 1).

Table 1 – Summary of experiments.

Samples (Num. fruits)	NMR equipement (Magnetic field strength in Tesla)	NMR output	Objetive
Picual (4) Arbequina (4) Frantoio (4) Descendants (23)	Bruker Biospec (4.7 T)	LR-NMR spectra	Relative fat content assesment
	Bruker AMX (11.75 T)	1D HR-NMR spectra	Spectroscopic fingerprinting
		2D HR-NMR spectra	Correlation between peaks of the same metabolite

For each sample both low-resolution (LR) (Bruker BIOSPEC 47/40 spectrometer) and high-resolution (HR) (Bruker AMX-500 spectrometer) ^{1}H NMR spectra were obtained at LPF-TAG. Currently at IFAPA water and oil content determination is performed by dehydration in oven at 105ºC and by comparison of NMR signal intensity to standards (Figure 1).

Figure 1 – Olive fruis dehydration in an oven (left), sample preparation in bags containing 10 fruits (middle) and Bruker Minispec equipment at IFAPA for low resolution oil content measurement.

For HR-NMR spectra pieces of flesh (2x2x2 mm) were extracted from olive fruits. These samples were packed into a 4mm HR-MAS zirconium oxide rotor with 50μl cylindrical insert at an angle of 54.7º (magic angle) from the direction of the main magnetic field and spun at 4000 Hz spinning rate (Figure 2). The so-called magic angle allows averaging out the

magnetic field inhomegeneities caused by the sample itself and thus, achieving fine resolved peaks.
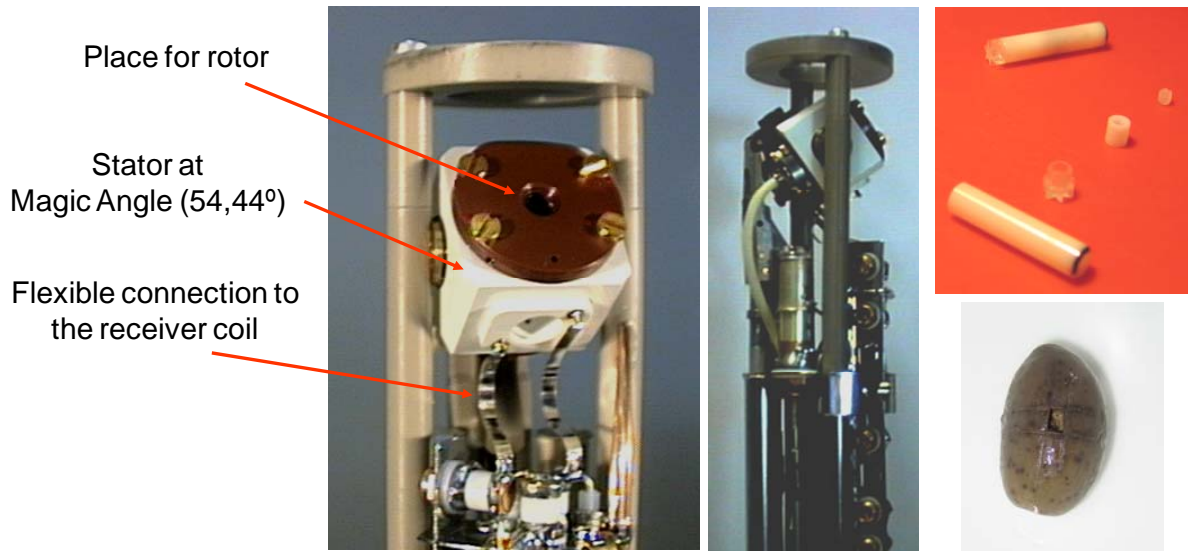

Figure 2 - Instrumentation and sample.

One-dimensional HR-NMR spectrum was acquired using a standard 1D pulse sequence (NOESYPRESAT) with water peak suppression. The position of each spectral band is known as chemical shift. It is measured in frequecy terms, in parts per million, from the position of the added reference substance 3-trimethylsilylpropionic acid (TSP). Spectra were reduced into consecutive integrated spectral regions of 0.04 ppm width for avoidance of pH efect on chemical shift (Lindon et al., 2001) and individually normalized with respect to the total sum of the whole spectrum. Two-dimension correlation spectroscopy analyses, ^{1}H-^{1}H 2D experiments (COSY90), were performed as assistance for future identification of the metabolites responsible for the spectral lines appearing in the one-dimension HR-NMR spectra. Samples were maintained at 4°C during data acquisition in order to limit degradation.

Analysis of variance (ANOVA) was performed to test for significant differences between means of the groups of interest for each relevant spectral variable (chemical shift). A Bonferroni's test (post-hoc multiple comparison test) for each chemical shift indicated which groups were particularly different at 0.05 and at 0.01 significance level.

Spectral variables selected on the basis of their significant differences between groups according to ANOVA results were introduced into a forward stepwise discriminant analysis performed with software package STATISTICA (StatSoft, Inc. 2003). Discriminant function

analysis was performed by reviewing all variables and evaluating which one will contribute most to the discrimination between groups. Classification functions were used directly to classify spectra under a supervised mode being a spectrum allocated into the group for which it has the highest classification score. The percentages of correct classification were calculated as a measure of the model performance. Analysis of covariance between cases labeled according to breeding program provided insight into the causes of misclassification. A cluster analysis with the chemical shifts that entered the discriminant model was applied to study the reliability of the results under an unsupervised mode. In any case validation of the analitical procedure with higher number of samples is a primary objective.

RESULTS

One-dimensional LR-NMR spectra showed two regions with visible peaks (Figure 3). The dominant peak corresponds to protons in water molecules (about 5 ppm). Those peaks in the region below 2.5 ppm are associated to main fatty acids (oleic, palmitic, linoleic, stearic and palmitoleic acids) according to literature and simulation software. The ratio between areas of those two regions provides the fraction of oil content with respect to water. However, in future experiments oil content will be assessed by means of signal comparison to standars at IFAPA (Figure 1).
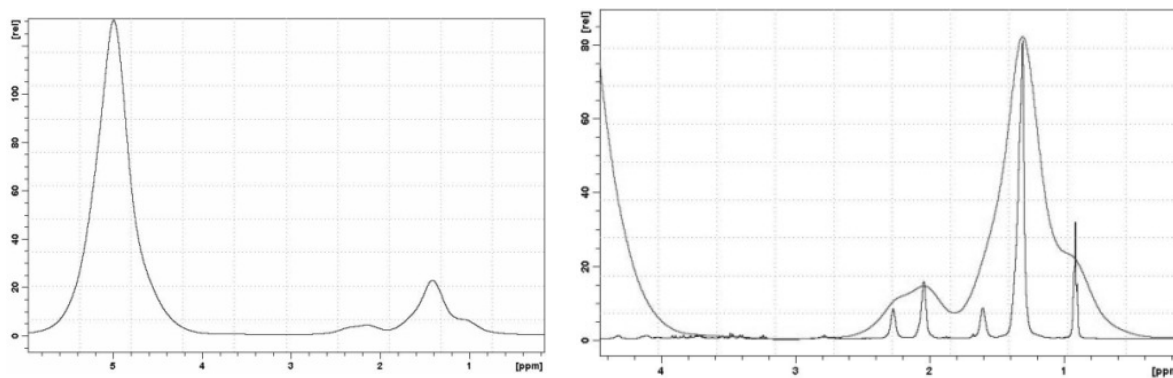

Figure 3 – Example of LR-NMR on the left; and superposition of LR- and HR-NMR spectra from the same olive on the right.

One-dimensional HR-NMR spectrum allows resolving individual peaks which can not be isolated with LR technique (Figure 4). This kind of spectra is a source of information that provides huge amount of data in a single experiment where all the components in the sample are detected independently of their different nature; that is, it provides an n-dimensional metabolic fingerprint of the olive flesh (Figure 4). Signal intensities lying within the region

between 3 and 5 ppm (Table 2) and between 9 and 10 ppm include the chemical shifts for phenolic compounds such as verbascoside and demethyloleuropein (Servili et al., 1999) and triglycerides and diglycerides as well as different aldehydes (Sacchi et al., 1996). The intensities (integrals) are proportional to the number of protons present in each functional group. Two-dimensional HR-NMR spectra (Figure 4) show increasing signal dispersion and elucidate the conectivities between signals providing some clues on chemical bonds within specific metabolites.
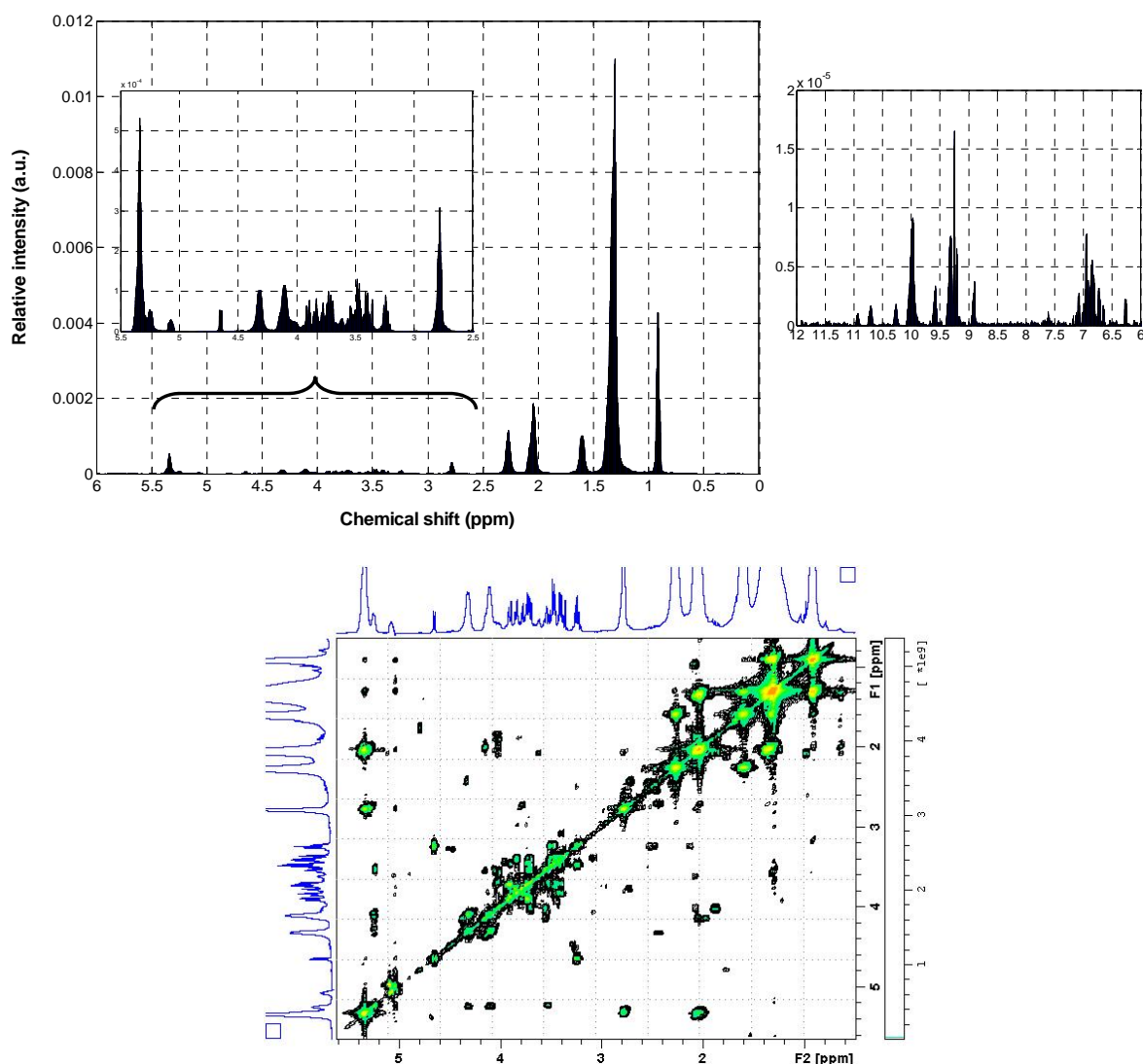

Figure 4 – Upper: Example of HR-NMR spectrum and zoom on relevant spectral regions. Bottom: Example of 2D HR-NMR spectrum, contour lines delimit the correlation between proton populations.

After reduction to buckets of 0.04 ppm witdh 49 spectral variables were selected as having signal intensity above noise level (Table 2). ANOVA revealed that Arbequina and Picual cultivars present the highest number of different signal intensities. In contrast, Picual and Frantoio only differ in one spectral variable. No differences between descendants from Arbequina and Picual were found and therefore, such samples were grouped into the same class for the extraction of classification functions. The spectral variables showing significant differences between groups entered the discriminant analysis.

Table 2 - Selected spectral variables. Bold: variables with significant differences between groups at 5% significance level (underlined: 1% significance level).

Spectral variables (chemical shifts in ppm)												
0,92	1,29	**<u>1,33</u>**	1,57	**1,61**	2,02	**<u>2,06</u>**	2,27	2,79	**3,24**	**3,40**	***3,49***	***3,53***
3,61	**3,69**	**3,73**	**3,77**	**3,81**	**3,85**	**3,89**	**4,01**	4,10	4,30	4,34	***4,63***	***4,67***
5,07	5,23	**5,35**	**6,65**	6,73	6,81	**6,86**	6,94	**7,10**	7,43	7,63	8,89	***<u>8,93</u>***
9,26	**<u>9,34</u>**	9,58	**<u>9,99</u>**	10,27	10,68	10,72	10,92	11,41	11,90			

The next step in the analysis is the definition of a new space with a number of discriminant functions equal to the number of groups, which include those variables with the highest discriminant power. In Figure 4 only three dimensions are represented. The corresponding classification functions achieved 100% successful classification for Arbequina, Picual, Frantoio samples and descendants "FxP 4-62". For "FxP 7-60" descendant 75% were correctly classified whereas only 65.78% of Arbequina and Picual descendants were allocated in the corresponding class. Misclassified samples were assigned to the group of one of their parentals.

The low number of samples used in this study reduces the reproducibility of these results. At this point an unsupervised procedure using a clustering analysis is carried out in order to evaluate the reliability of the classification percentages using the chemical shifts included in the discriminant model. No concordance was found as parentals and descendants were mixed up into the same clusters.
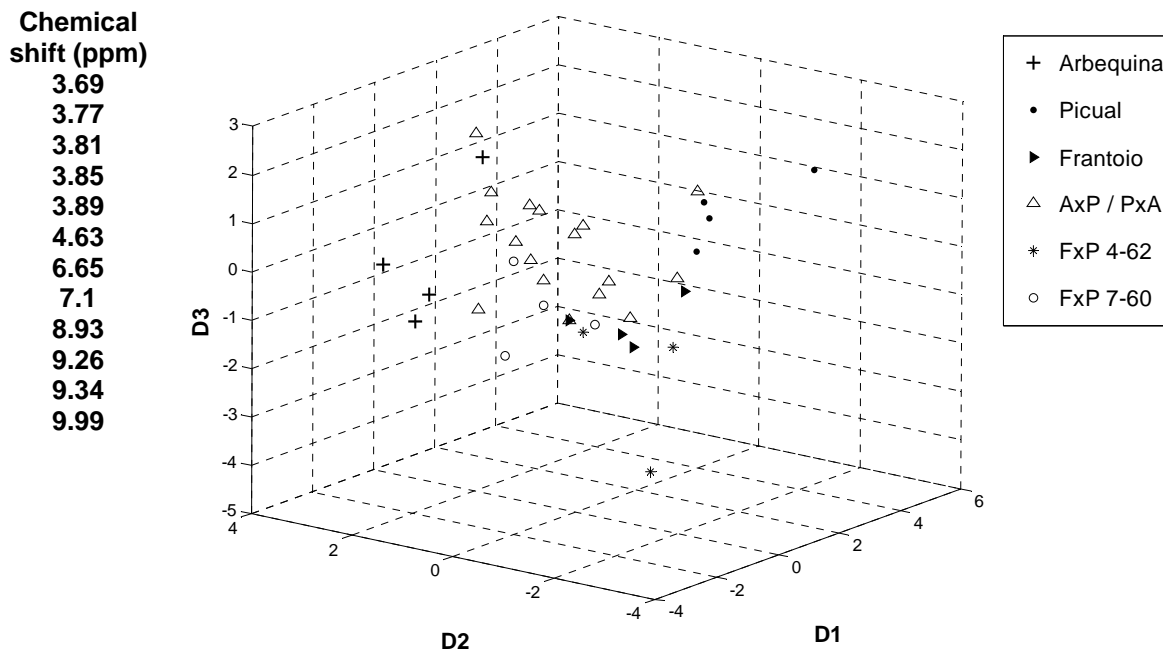
Figure 4 – On the left chemical shifts selected by the discriminant analysis to generate the discriminat functions. On the right representation of the three first canonical scores computed from the three first discriminant functions.

CONCLUSIONS

High resolution NMR spectroscopy provides simultaneously detailed information on the components comprising the metabolic profile of olive flesh. The exploitation of this characteristic through multivariate statistical analysis is of great interest for characterizing and classifying cultivars in a breeding program. The analytical procedure showed in this work need to be validated in the next seasson using larger pools of samples.

Such procedure will be used as reference method for the development of robust calibration new models in a portable NIR equipment prototype for in-field use, which is also showed in the present Conference.

Acknowledgements

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