HIGH RESOLUTION-MAGIC ANGLE SPINNING NMR STUDY OF OLIVE LEAVES

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1 INTRODUCTION

HR-MAS ^{1}H NMR spectroscopy has proven to be a useful tool for the rapid determination of the metabolic profile of several solid and semisolid foods, such as fruits and vegetables,[1, 2] cheese[3] and meat.[4] During the last few years, olive leaves are increasingly recognized as direct sources of the same natural antioxidants that can be found in olive oil.[5] The use of olive leave extracts as a source of bioactive compounds to be used as food additives is a subject of active research.[6] Furthermore, nowadays olive leaf extracts can be found in pharmacies and supermarket shelves in the form of cap supplements and liquid drops, and as a component of beauty care products. Olive leaves are also marketed as tea bags or in raw form, suitable for the preparation of hot beverages. Thus, olive leaves are emerging as a new and potentially very important economically product for olive tree growing regions.[7, 8] The qualitative and quantitative analysis of olive leaf bioactive compounds (triterpenoids,[9] phenolics[10]) is a very important aspect of both nutritional and economical importance. The range and concentration of phytochemicals in olive leaves depends on several factors including genetic (variety) and pedoclimatic (soil, weather), and varies according to the time of the year during the olive harvesting season. The use of chromatographic techniques in the compositional analysis of phenolics in olive oil, fruit and leaves has been reviewed recently.[11] In this report we present the application of ^{1}H and ^{13}C HR-MAS 1D and 2D NMR spectroscopy for the direct characterization and analysis of bioactive compounds in solid olive leaves, without any pre-treatment. The HR-MAS NMR spectral analysis was performed in three solvents of different polarity (chloroform, methanol, water), but the focus in this report will be the analysis of the $CDCl_3$ HR-MAS NMR spectra and the characterization of the chemical compounds identified.

2 METHODS AND RESULTS

2.1 Experimental procedure

Olive leaves were obtained from Koroneiki variety olive groves in Heraklion Crete during March, 2010. HR-MAS spectra were obtained at room temperature through a Bruker Avance II 400 MHz (9.4 T) spectrometer operating at 400.15 and 100.63 MHz for the ^{1}H and ^{13}C nuclei respectively. About 10 mg of olive leaves previously grinded in a mortar over liquid N_2 were placed into 50 μL zirconia rotors with cylindrical inserts and 10μL of deuterated chloroform were added for locking. ^{1}H HR-MAS spectra were acquired using a

MAS rate of 5 KHz and water suppression to eliminate the large signal due to water in the leaves. A 90° pulse of 7.9 μs, a delay time of 2s and 128 scans were employed.
2D gCOSY HR-MAS experiments were acquired under the following conditions: a spectral width of 4000 Hz in both dimensions, 1 k data points in f2 and 256 increments in f1. An unshifted sinusoidal window function was applied in both dimensions and zero filling in f1 dimension. 2D HSQC HR-MAS experiments were acquired with the following parameters: 80 μs for GARP ^{13}C decoupling, 4000 Hz and 21 kHz spectral widths in the ^{1}H and ^{13}C dimensions, respectively, 1 k data points in f2 and 256 increments in f1. Zero filling in f1 and unshifted squared sinusoidal window function in both dimensions were applied before the Fourier transformation.

2.2 Results

The ^{1}H HR-MAS NMR spectrum of olive leaves of Koroneiki variety using $CDCl_3$ as the lock solvent is reported in Fig. 1. For comparison, the solution state ^{1}H NMR spectrum of a $CDCl_3$ extract of the same sample is also depicted in Fig. 1. The two spectra appear very similar, the only difference indicated being the presence of triglyceride peaks in the chloroform extract.

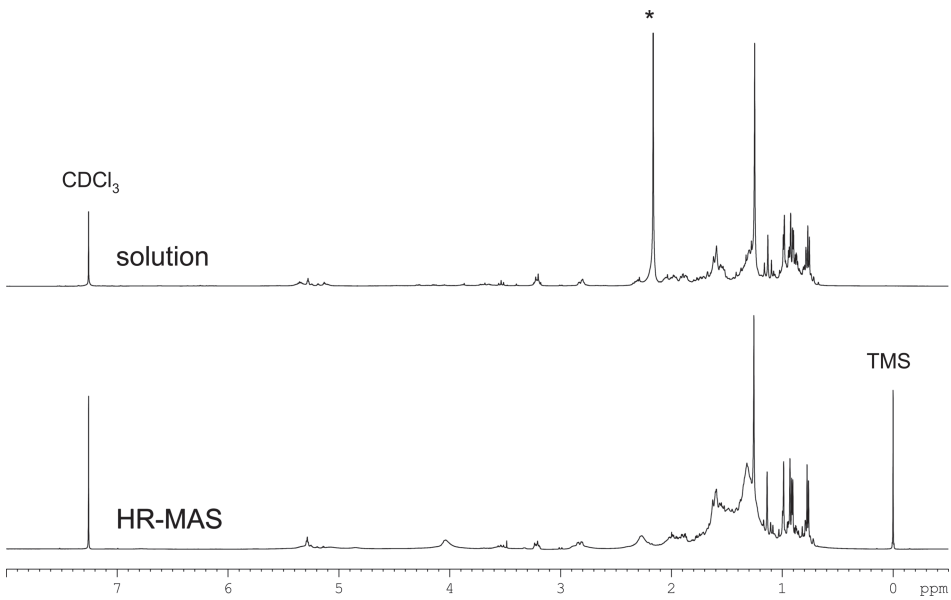
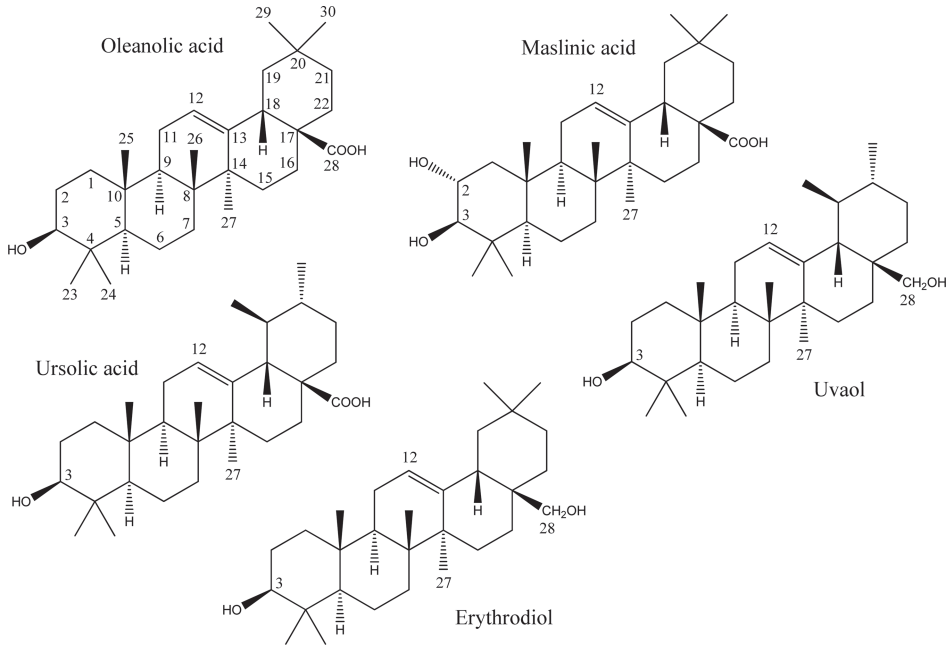


Figure 1 *^{1}H HR-MAS NMR spectrum of olive leaves of Koroneiki variety (bottom) and solution state ^{1}H NMR spectrum of a $CDCl_3$ extract of the same leaves (top). A solvent contaminant is marked with an asterisk (*).*



Scheme 1

The ^{1}H HR-MAS NMR spectrum in Fig. 1 is dominated by signals due to triterpenoids, (see Scheme 1 for chemical structures) which are the major component of the epicuticular wax of olive leaves and fruit.[12] Other components that were identified include esterified fatty acids, triglycerides, and alkyl esters, in agreement with previous work.[13, 14] Assignment of the signals was made by HR-MAS 2D NMR spectroscopy (gCOSY, g-HSQC) and with the help of standard compounds (ursolic acid, uvaol and erythrodiol). presents The aliphatic part of the 2D gHSQC ^{1}H-^{13}C NMR spectrum of the solid leaves is presented in Fig. 2, indicating the assignment of the resonances of oleanolic acid, the major triterpenoid in olive leaves. The assignment of oleanolic acid signals was in excellent agreement with published data in the solution state.[15]

Apart from oleanolic acid, it was possible to assign several characteristic signals of other triterpenoids in the ^{1}H HR-MAS NMR spectrum of olive leaves. Several expansions of this proton spectrum are depicted in Fig. 3, indicating the signals assigned to specific triterpenoid protons and following the numbering of Scheme 1. The assignment was verified by comparison with solution state ^{1}H NMR spectra of standard compounds uvaol, erythrodiol and ursolic acid.

Five triterpenoids, namely oleanolic acid, ursolic acid, maslinic acid, erythrodiol and uvaol that have been previously reported[12, 16, 17] as constituents of olive leaves have been identified in its proton HR-MAS spectrum. The proton and carbon chemical shifts of the triterpenoids assigned in the present study are summarized in Table 1.

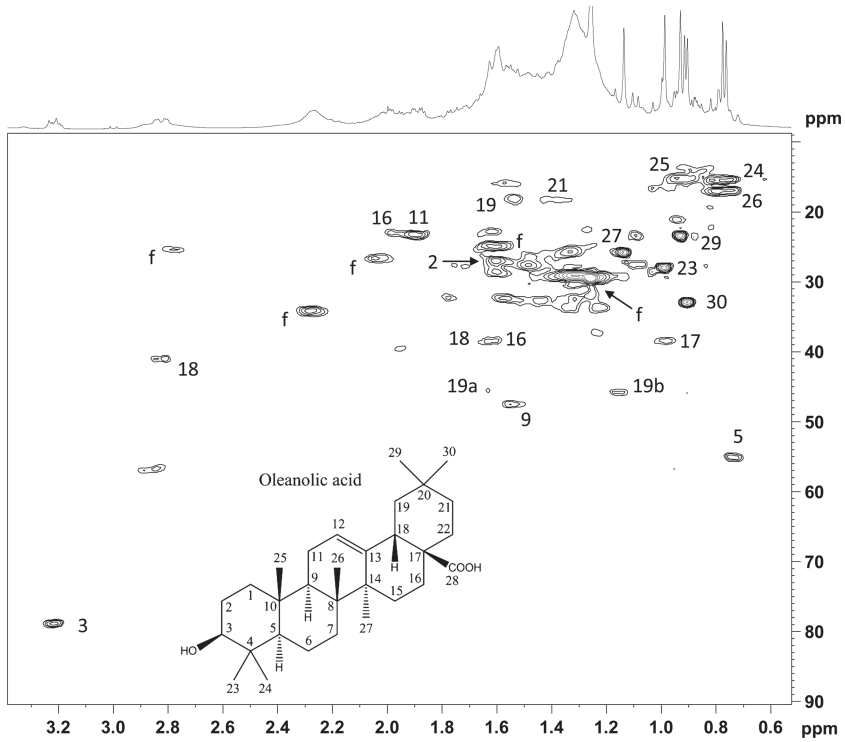


Figure 2 *HR-MAS ^{1}H-^{13}C gHSQC 2D NMR spectrum of olive leaves of Koroneiki variety. Numbers correspond to oleanolic acid protons, while f denotes fatty acid signals.*

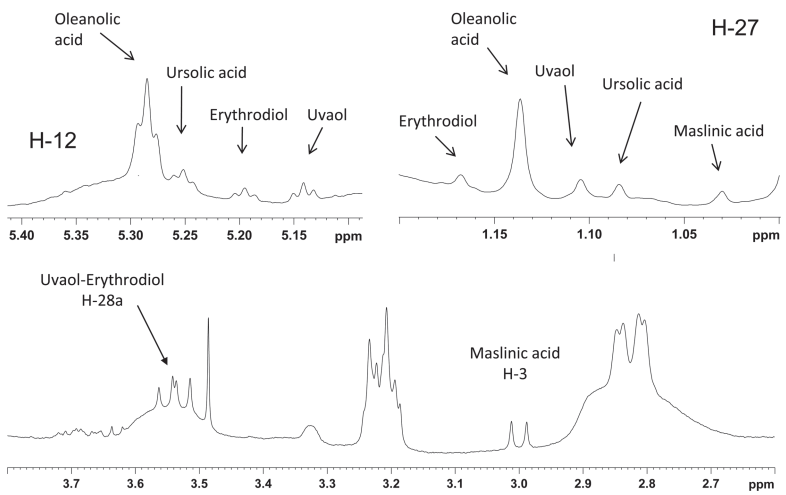


Figure 3 *Expansions of the ^{1}H HR-MAS NMR spectrum of olive leaves: vinyl proton H-12 (top left), methyl group H-27(top right), hydroxyl region (bottom).*

Table 1 *^{1}H and ^{13}C (in parenthesis) chemical shifts of the triterpenoid signals in the HR-MAS NMR spectra of olive leaves*

C/H no[a]	Oleanolic acid	Maslinic acid	Ursolic acid	Erythrodiol	Uvaol
1	0.97/1.63 (38.5)	0.90/1.98			
2	1.61 (27.2)	3.68			
3	3.22 (78.9)	3.00			
4	q[b]				
5	0.74 (55.1)				
6	nd[c]				
7	nd				
8	q				
9	1.53 (47.5)				
10	q				
11	1.86/1.91 (23.2)		1.89/1.94	1.84	1.88/1.94
12	5.28 (122.5)	nd	5.25	5.19	5.14
13	q				
14	q				
15	nd				
16	1.62/1.98 (22.1)				
17	q				
18	2.81/2.84 (41.0)				
19	1.15/1.62 (45.7)				
20	q				
21	1.38/1.52 (18.0)				
22	1.43/1.52 (32.7)				
23	0.99 (28.0)				
24	0.78 (15.5)				
25	0.92 (15.1)				
26	0.76 (16.9)				
27	1.14	1.03	1.08	1.17	1.11

	(25.7)		
28	-	3.55/3.21	3.53/3.20
	(182.2)	(69.8)	
29	0.93		
	(23.4)		
30	0.90		
	(33.1)		

([a] Refers to nubering in Scheme 1. [b] not detected. [c] quartenary carbon)

Having successfully separated characteristic signals of all five terpenic compounds in the HR-MAS spectra of olive leaves, we used signal integration to calculate the % composition of the triterpenoids present in olive leaves of Koroneiki variety. The results obtained were oleanolic acid 67%, maslinic acid 8%, ursolic acid 7%, uvaol 12% and erythrodiol 6%, and are in good agreement with literature values. Kontogianni et al. reported that the concentration of oleanolic acid in leaves of *Olea Europaea* was found approx. 10 times larger than that of ursolic acid by both solution state NMR and HPLC analysis of extracts.[17] Guida et al. used GC to study leaf extracts from three different Spanish olive varieties, reporting that oleanolic acid was the most abundant triterpenoid in the leaves, followed by maslinic acid, and smaller amounts of the other three triterpenoids.[16]

3 CONCLUSIONS

The data presented in this report demonstrate that HR-MAS NMR spectroscopy is a powerful tool for the direct analysis of the composition of olive leaves without the need for any sample pretreatment or solvent extraction steps. The ^{1}H HR-MAS NMR spectrum of olive leaves provides direct information regarding the different terpenic acids and alcohols present in the olive leaf, and can be extended for quantitative analysis by using a suitable internal standard. Further work involving the HR-MAS NMR analysis of other greek and italian olive varieties and commercial samples of olive leaves is in progress.

Acknowledgements

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