High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance (HR-MAS-NMR) as quick and direct insight of almonds

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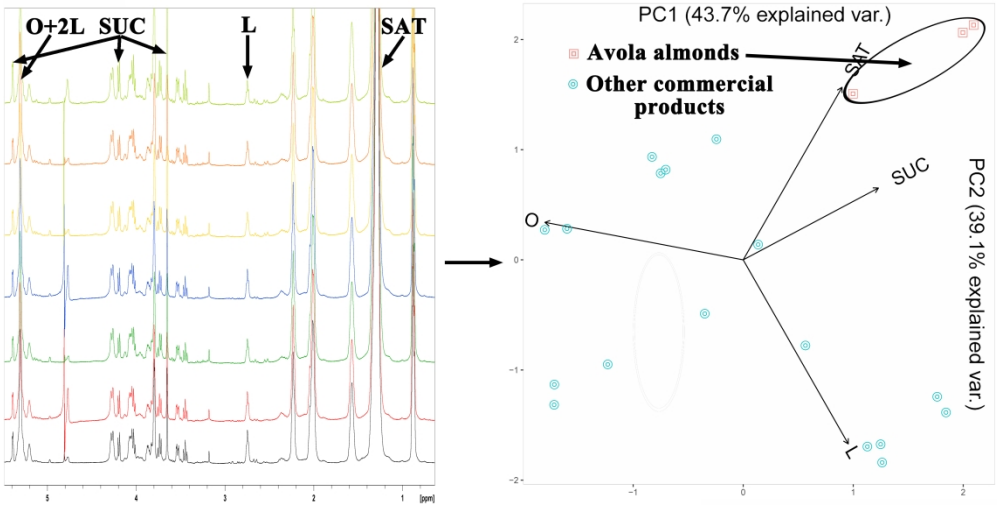

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High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance (HR-MAS-NMR) as quick and direct insight of almonds

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Abstract

Almonds are the tasty seeds of *Prunus dulcis* plants globally appreciated for the pleasant palatability and remarkable nutritional value, therefore it is very spread as snack and as basic ingredient of the confectionery products. The HR-MAS-NMR is a simple spectroscopy able to directly and quickly explore the chemical composition of powdered seed samples dispersed in D_2O. ^1H spectra witness the remarkable presence of triglyceride fatty esters together with sucrose; other minor water soluble metabolites are also detectable. This very rough approach is effectively providing chemical profiles featuring almond samples. In this analysis we were able to statistically distinguish the "Avola" almonds from other marketed products submitted to the same analysis. This is just a first investigation based on the main compounds but it might pave the way toward the quantitative evaluation of many other compounds in the almond therefore implementing the HR-MAS-NMR knowledge of these precious seeds.

Keywords: Food composition, HR-MAS-NMR, almonds, PCA, Quantification.

1. Introduction

Almonds (*Prunus Dulcis*) belong to the *Rosaceae* family and are globally appreciated for their sensory and health features; as it is an important element of the Mediterranean diet and main ingredient of many confectionery industries, almond trees are the most grown on a global basis (Siriwardhana Wijeratne et al. 2006; Chen et al. 2005). Many Mediterranean regions are characterized by these plants bearing a considerable commercial value (Martins et al. 2003; Cicero et al. 2015; Albergamo et al. 2017; Costa et al. 2018). The edible fruit presents three distinct parts: 1) the inner kernel or meat (sold with or without the brown skin), 2) the middle shell portion, and an outer green shell cover or hull. All these parts are deeply studied for the chemical composition being the meat the most important consumable food whereas the outer parts can be recycled as "useful waste" (Esfahlan et al. 2010). Almonds are a rich source of essential fatty acids, carbohydrate and protein and is a highly nutritional source of vitamins, minerals (Gallier et al. 2012) and antioxidant species claimed to slow aging processes (Franklin et al. 2017). This accounts for the careful chemical analyses of almonds aimed to elucidate the beneficial effects of derived products upon the human health (Zeeshan 2010; Geng et al. 2016).

Almonds, when incorporated in the diet, have been reported to increase the "good cholesterol" (high density lipo-proteins, HDL) in spite of the "bad cholesterol" (low density lipo-proteins, LDL) levels

in humans (Ahmad 2010; Hyson et al. 2002). Extracts of whole almond seed, brown skin, shell, and green shell cover (hull) bear potent free radical-scavenging capacities (Sfahlan et al. 2009; Moure et al. 2007; Siriwardhana Wijeratne et al. 2006); these activities are related to the presence of flavonoids and other phenolic compounds recalling the need of the molecular composition approach (Shahidi et al. 2009). In the field of the chemical studies, nuclear magnetic resonance (NMR) is an irreplaceable tool for the chemical characterization (Rotondo et al. 2012; Rotondo et al. 2014a), elucidation of structures and molecular dynamic behavior (Rotondo et al. 2014b; Rotondo et al. 2015), allowing also characterization of complex mixtures (Salvo et al. 2017). NMR spectroscopy is very efficient for the simultaneous detection and identification of several metabolites (Rotondo et al. 2011, Rotondo et al. 2017) indeed almond oils were deeply analyzed by high resolution NMR (Popescu et al. 2015) as well as the almonds water-soluble extracts (Tanaka et al. 2013).

HR-MAS-NMR spectroscopy opened-up the chance to analyse the solid and semisolid matter (Corsaro et al. 2016) which, in the case of the *Prunus Dulcis* turned out to allow direct chemical changes undergone by almonds upon irradiation processes (Ribó et al. 2004). The aim of this work is to characterize the chemical composition of *Prunus Dulcis* by semi-solid NMR analysis to provide the simultaneous relative quantification of very different chemical species as fats and carbohydrates. To our knowledge this is the first HR-MAS study aimed to draw chemical profiles related to different marketed edible *Prunus Dulcis* seeds, paving the way to deeper investigations on this interesting matrix.

2. Results and discussion

The most important results are obtained by the integration of the ^{1}H pre-saturated experiments and the assignment provided by the used 2D-techniques and by the other literature findings (Figure 1). The different almond samples display a similar qualitative spectral profile (Figure 1S supplemental text). The main relative quantitative values (Table 1) are within the ranges expected by the other literature data (Esfhalan 2010; Ahmad 2012; Barreira et al. 2012).

The principal purpose of the paper is to use the direct HR-MAS-NMR analysis for the first simultaneous relative quantification of the main fatty esters (already observed by Ribó et al. 2004) together with the sucrose. Moreover, with this careful study, it is paved the way to quantify other less represented water-soluble metabolites (such as aspartic acid, proline, phosphocholine derivates and other sugars) respect to the fatty acylglycerols which are certainly the most represented species of the whole almond seeds (Figure 1 and Table 2S). Because of the limited number of samples, for some statistical consideration, we have selected just the saturated (palmitates and stearates, SAT),

mono-unsaturated (oleates, O) and di-unsaturated (linoleates, L) fatty esters together with sucrose (SUC). These four parameters are enough to distinguish "Avola" almonds respect to some commercially available samples as evidenced by a simple PCA statistical sorting (see supplemental text). The "Avola" neat discrimination is mainly possible because of its highest SUC content respect to the fatty fraction. Provided that all the analyzed commercial samples present a chemical fingerprint compatible with a good quality food product, Sicilian "Avola" almonds look featured by higher SUC relative levels and also by a rather high relative presence of O among fatty esters in spite of rather low relative level of L di-unsatured fatty esters. We point out that these are not absolute values, but just relative ratios, therefore, according to the overall data integration, "Avola" type samples are not sweeter, but probably "less fat" (triglycerides are less represented in the total ratio).

In conclusion, without any real dissolution or chemical modification the HR-MAS-NMR provides a first tool for the almond chemical characterization opening up the chance to distinguish different kind of almonds according to the belongings or to the specific industrial treatments. All these elements look very important for the evaluation of this dry fruit endowed with multifunctional nutritional properties.

3. Experimental

Almonds taken from 24 samples of seven different types and grouped in three different belongings (Table 1S) were milled in a stone mortar. Small aliquots of 15-28 mg were homogenized with 100 µL of D_2O, the spectra were acquired at a temperature of 300 K, the mixture was put inside the 4-mm ZrO_2 rotor (detection volume 12 µL), and with a hemispherical Teflon insert for HR-MAS NMR analysis. All NMR spectroscopic studies were performed with a Bruker DMX 500 NMR spectrometer equipped with an HR-MAS accessory and a $^{1}H/^{13}C$ gradient probe and controlled by Topspin 3.2 software package for set-up, acquisition and processing procedures. The sample spinning rate was kept 4 kHz with a magic angle of 56°.

In order to obtain information we have optimized the 90° pulses for the ^{1}H nucleus. We have run: 1) ^{1}H-1D experiments with f1 pre-saturation (zgpr) were acquired with time domain size of 32 k, sweep width of 12 ppm, acquisition time of 2.7 sec, dwell time of 83.2 µsec, relaxation delay of 2.0 sec, dummy scan of 2 and number of scan 16; 2) the ^{13}C-NMR spectra were acquired with time domain size of 64 k, sweep width of 20 ppm, acquisition time of 3.2 sec, dwell time of 50.0 µsec, pre-scan-delay of 6.5 µsec, relaxation delay 2.0 sec, dummy scan of 2 and number of scan 800; 3) in order to confirm and elucidate the chemical nature of the analyzed matter ^{1}H-2D-COSY

experiment were acquired with size FID of 128, time domain size of 3 k, sweep width of 12 ppm, acquisition time of 0.3 sec, dwell time of 83.2 μsec, pre-scan-delay of 6.5 μsec, relaxation delay of 1.3 sec, dummy scan of 2 and number of scan 16.

As reference signal for the frequency calibration we have adopted the terminal methyl group of the fatty ester chains (δ = 0.88 ppm) whereas as quantitative relative reference we have chosen the α-CH_2 group of the fatty esters resonating at δ = 2.25 ppm. On these bases we could readily extract the relative concentration of fatty esters and of the main sugar sucrose. Other metabolites are tentatively quantified according to well known assigned integrations. The overall discussion is based just on the four main metabolites. All the quantification measurements of the used four metabolites were verified for their uncertainty by accomplishment of many different experiments over three homologous samples.

To quickly identify the differences among the almond samples we have run the simple statistical analysis by principal components (PCA); as we had just 24 samples we have reduced the number of quantitative variables to the main four species. By the use of R software, the first two dimensions were used to display the sample mutual relationship (supplemental text, Figure 2S).

4. Conclusions

HR-MAS-NMR analysis is a straightforward tool to investigate almonds and almond seeds without chemical modification or dissolution opening up the way for a direct relative quantification of species of different nature such as the hydrophobic triacylglycerol esters and the hydrophilic carbohydrates and amino-acids. This preliminary study exploits the relative presence of the three main fatty esters and sucrose in order to discriminate several marketed samples from “Avola” almonds grown and collected in Sicily, provided that all the analysed samples showed a chemical profile compatible with good quality products. “Avola” almonds showed definitely higher relative concentration of sucrose respect to the fatty esters. This study paves the way toward the quick elucidation of the almond chemical nature with also a straightforward strategy for geographical characterization and traceability.

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Captions

Table 1. Main chemical composition in relative molecular % respect to the total fatty esters for the 24 almond seeds samples.

Figure 1. HR-MAS ^{1}H-NMR experiment for an “Avola” type almond sample; proton assignment is also graphically labeled with the following labels: sucrose (SUC), linoleate esters (L), oleate esters (O), phospho-choline derivates (PCHO), asparagine (ASN), malate (MAL), proline (PRO).

Table 1. Main chemical composition in relative molecular % respect to the total fatty esters for the 24 almond seeds samples.

Sample	Saturated fatty esters (SAT) %	Oleate fatty esters (O) %	Linoleate fatty esters (L) %	Sucrose (SUC) relative %
S_1_1	11.3±0.6	61.5±0.4	27.2±0.6	79.8±0.8
S_1_2	12.9±0.6	59.3±0.4	27.9±0.8	77.9±0.8
S_1_3	12.4±0.6	60.1±0.4	27.5±0.6	76.2±0.8
S_1_4	11.8±0.6	60.9±0.4	27.3±0.6	78.3±0.8
S_1_5	11.3±0.6	61.5±0.6	27.2±0.6	79.8±0.7
S_2_1	9.2±0.6	68.9±0.4	21.9±0.6	70.7±0.6
S_2_2	9.9±0.6	67.7±0.6	22.4±0.6	73.5±0.8
S_2_3	10.1±0.6	67.5±0.4	22.5±0.6	72.8±0.8
S_3_1	9.3±0.6	69.7±0.6	21.0±0.6	77.1±0.8
S_3_2	9.1±0.6	69.5±0.4	21.4±0.6	68.7±0.8
S_3_3	9.1±0.6	68.9±0.4	22.0±0.6	72.5±0.8
S_4_1	12.5±0.6	69.3±0.4	18.2±0.6	70.1±0.8
S_4_2	11.8±0.6	70.2±0.5	17.9±0.7	71.8±0.8
S_4_3	10.8±0.6	71.6±0.4	17.6±0.7	72.9±0.8
S_5_1	12.6±0.6	69.1±0.5	18.3±0.7	70.6±0.8
S_5_2	10.5±0.6	72.1±0.4	17.4±0.7	73.9±0.8
S_5_3	11.9±0.6	69.9±0.6	18.2±0.7	70.8±0.8
S_6_1	12.0±0.6	69.9±0.4	18.0±0.7	71.4±0.8
S_6_2	14.1±0.6	66.6±0.4	19.3±0.7	70.3±0.8
S_6_3	11.6±0.6	67.5±0.4	20.9±0.7	78.2±0.8
S_7_1	12.7±0.6	69.6±0.4	17.7±0.7	106.8±0.6
S_7_2	10.2±0.6	73.3±0.4	16.4±0.7	113.7±0.5
S_7_3	11.0±0.6	72.2±0.4	16.8±0.7	111.0±0.6

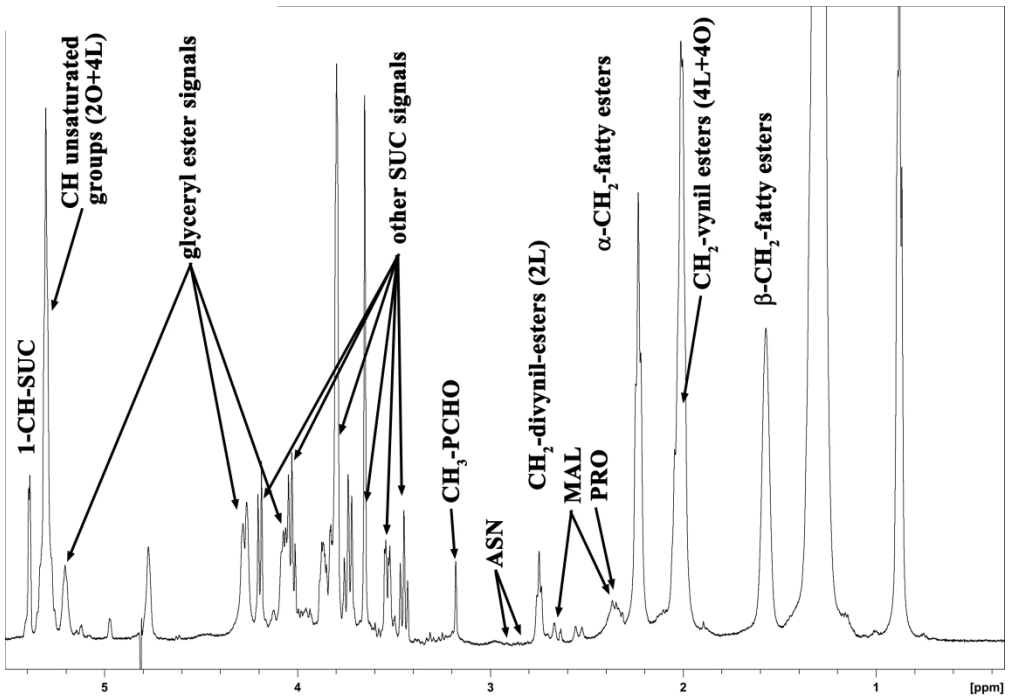

Figure 1. HR-MAS 1H-NMR experiment for an "Avola" type almond sample; proton assignment is also graphically labeled with the following labels: sucrose (SUC), linoleate esters (L), oleate esters (O), phospho-choline derivates (PCHO), asparagine (ASN), malate (MAL), proline (PRO).

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Extended Introduction

Almonds (*Prunus Dulcis*) belong to the *Rosaceae* family and are globally appreciated for their sensory and health features; as it is an important element of the Mediterranean diet and main ingredient of many confectionery industries, almond trees are the most grown on a global basis (Wijeratne et al. 2006; Chen et al. 2005). Many Mediterranean regions are characterized by these plants bearing a considerable commercial value (Cordeiro and Monteiro 2001; Martins et al. 2003; Cicero et al. 2015; Albergamo et al. 2017; Costa et al. 2018). The edible fruit presents three distinct parts: 1) the inner kernel or meat (sold with or without the brown skin), 2) the middle shell portion, and an outer green shell cover or hull. All these parts are deeply studied for the chemical composition being the meat the most important consumable food whereas the outer parts can be recycled as "useful waste" (Esfahlan et al. 2010). Almonds are a rich source of essential fatty acids, carbohydrate and protein and is a highly nutritional source of vitamins, minerals (Gallier et al. 2012; Shi Z et al. 1999) and antioxidant species claimed to slow aging processes (Franklin et al. 2017). This accounts for the careful chemical analyses of almonds aimed to elucidate the beneficial effects of derived products upon the human health (Zeeshan 2010; Geng et al. 2016).

Almonds, when incorporated in the diet, have been reported to reduce colon cancer risk in rats (Davis and Iwahashi 2001) and increase the "good cholesterol" (high density lipo-proteins, HDL) in spite of the "bad cholesterol" (low density lipo-proteins, LDL) levels in humans (Hyson et al. 2002). Extracts of whole almond seed, brown skin, shell, and green shell cover (hull) possess potent free radical-scavenging capacities (Amarowicz et al. 2005; Sfahlan et al. 2009; Moure et al. 2007; Pinelo et al. 2004; Siriwardhana Wijeratne et al. 2006; Siriwardhana Wijeratne and Shahidi 2002; Wijeratne et al. 2006). These activities may be related to the presence of flavonoids and other phenolic compounds. Almond hulls have been shown to serve as a rich source of three triterpenoids (about 1% of the hulls), betulinic, urosolic and oleanolic acids (Takeoka et al. 2000), as well as flavonol glycosides and phenolic acids (Sang et al. 2002; Shahidi et al. 2009). In addition, Sang, Chen et al. (2002), Sang, Lapsley et al. (2002), and Sang, Lapsley, Rosen et al. (2002) isolated catechin, protocatechuic acid, vanillic acid, p-hydroxybenzoic acid, and naringenin glucoside, as well as galactoside, glucoside, rhamnoglucoside of 3β-O-methylquercetin and rhamnoglucoside of kaempferol. In the field of the chemical studies, nuclear magnetic resonance (NMR) is an irreplaceable tool for the chemical characterization (Rotondo et al. 2012; Rotondo et al. 2014a), elucidation of structures and molecular dynamic behaviour (Rotondo et al 2014b; Rotondo et al 2015), allowing also characterization of complex mixtures (Salvo et al. 2017). NMR spectroscopy is very efficient for the simultaneous detection and identification of several metabolites (Rotondo et al. 2011, Rotondo et al. 2018) indeed almond oils were deeply analyzed by high resolution NMR (Popescu et al. 2015; Vigli et al. 2003) as well as the almonds water-soluble extracts (Tanaka et al. 2013).

HR-MAS NMR spectroscopy opened-up the chance to analyse the solid and semisolid matter (Corsaro et al. 2016) which, in the case of the Prunus Dulcis turned out to allow direct chemical changes undergone by almonds upon irradiation processes (Ribó et al. 2004), the aim of this work was to characterize the chemical composition of *Prunus Dulcis* by HR-MAS NMR providing the symultaneous relative quantification of very different chemical species as fats and carbohydrates. According to us this is the first NMR HR-MAS specifically aimed to draw chemical profiles related to different kind of marketed edible Prunus Dulcis seeds, paving the way to deeper investigations on this interesting matrix.

Immages

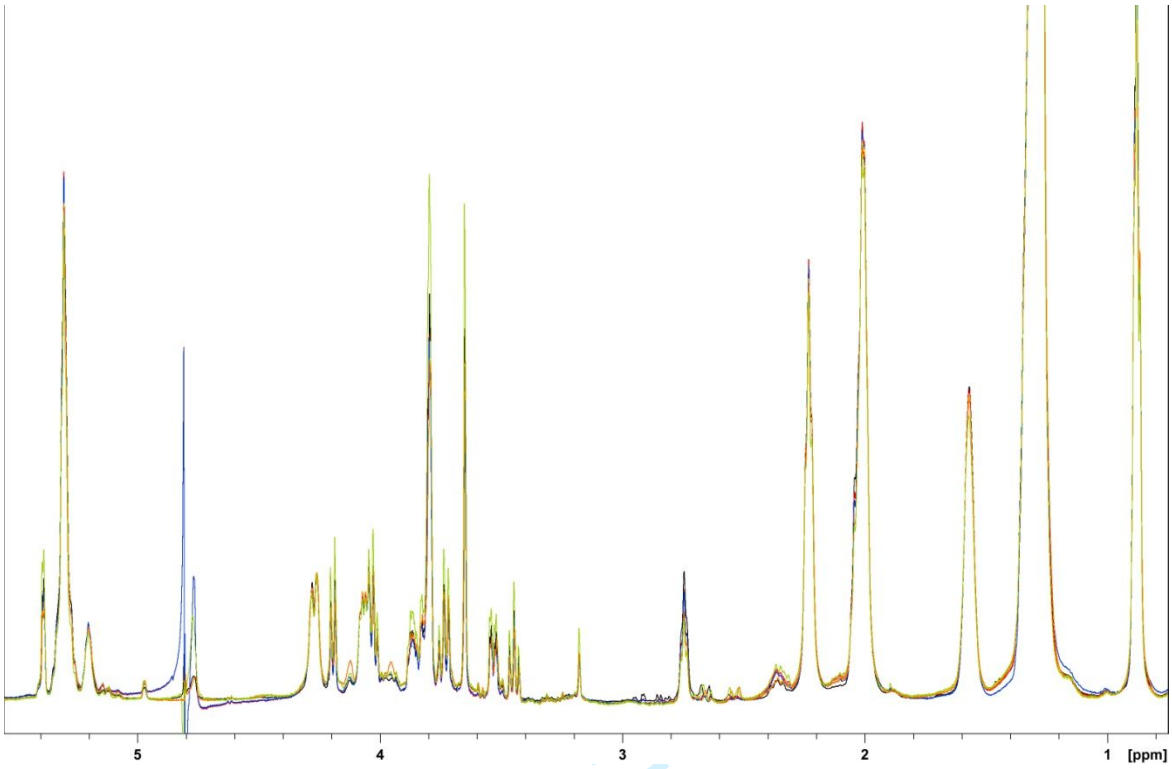

Figure 1S. HR-MAS 1H-NMR traces of the 24 analysed samples showing a pretty centred alignment and allowing the serial integration for the quantification

Table 1S. Specification of the provenience and packaging for the 24 analysed samples.

Samples	Declared Geographical Origin	Labels	Lots
S_1_1	California	package 1 marketed in Europe	1
S_1_2			2
S_1_3			3
S_1_4			4
S_1_5			5
S_2_1		package 2 marketed in Europe	1
S_2_2			2
S_2_3			3

S_3_1		package 3 Marketed in Europe	1
S_3_2			2
S_3_3			3
S_4_1	Italy	package 4 marketed in Italy	1
S_4_2			2
S_4_3			3
S_5_1		package 5 marketed in Italy	1
S_5_2			2
S_5_3			3
S_6_1		package 6 marketed in Italy	1
S_6_2			2
S_6_3			3
S_7_1	Avola Almonds from sicilian cultivars	Almods directly collected from the farm (Siracusa, Sicily)	land 1
S_7_2			land 2
S_7_3			land 3

Table 2S. Extended table of the relative quantification (molecolar % respect to the total tiacylglycerols) for several almond metabolites.

Sample	**Saturated fatty esters %**	**Oleate fatty esters %**	**Linoleate fatty esters %**	**Sucrose relative %**	**Asparagine %**	**Proline %**	**Malate %**	**phospho-choline derivates %**
code	**SAT**	**O**	**L**	**SUC**	**ASN**	**PRO**	**MAL**	**PCHO**
S_1_1	11.3	61.5	27.2	79.8	36.0	4.8	24.2	1.6
S_1_2	12.9	59.3	27.9	77.9	37.5	4.9	25.7	1.5
S_1_3	12.4	60.1	27.5	76.2	35.4	5.0	23.4	1.6

S_1_4	11.8	60.9	27.3	78.3	33.7	5.0	23.3	1.5
S_1_5	11.3	61.5	27.2	79.8	36.0	5.0	24.1	1.6
S_2_1	9.2	68.9	21.9	70.7	14.3	5.0	31.9	1.5
S_2_2	9.9	67.7	22.4	73.5	15.8	5.0	34.9	1.5
S_2_3	10.1	67.5	22.5	72.8	15.8	4.9	34.3	1.5
S_3_1	9.3	69.7	21.0	77.1	12.3	5.0	29.7	1.6
S_3_2	9.1	69.5	21.4	68.7	12.3	5.0	29.7	1.6
S_3_3	9.1	68.9	22.0	72.5	16.8	5.0	35.2	1.4
S_4_1	12.5	69.3	18.2	70.1	1.2	4.8	23.3	1.6
S_4_2	11.8	70.2	17.9	71.8	0.9	4.5	23.6	1.6
S_4_3	10.8	71.6	17.6	72.9	0.0	3.5	21.7	1.6
S_5_1	12.6	69.1	18.3	70.6	0.8	3.7	22.6	1.6
S_5_2	10.5	72.1	17.4	73.9	1.8	4.7	24.1	1.6
S_5_3	11.9	69.9	18.2	70.8	0.9	4.4	22.7	1.7
S_6_1	12.0	69.9	18.0	71.4	1.0	4.6	24.0	1.6
S_6_2	14.1	66.6	19.3	70.3	3.5	4.5	26.1	1.5
S_6_3	11.6	67.5	20.9	78.2	47.0	5.0	46.4	2.6
S_7_1	12.7	69.6	17.7	106.8	17.6	5.0	46.5	2.8
S_7_2	10.2	73.3	16.4	113.7	15.5	5.0	46.7	2.9
S_7_3	11.0	72.2	16.8	111.0	16.1	5.0	46.2	2.9

PCA Analysis

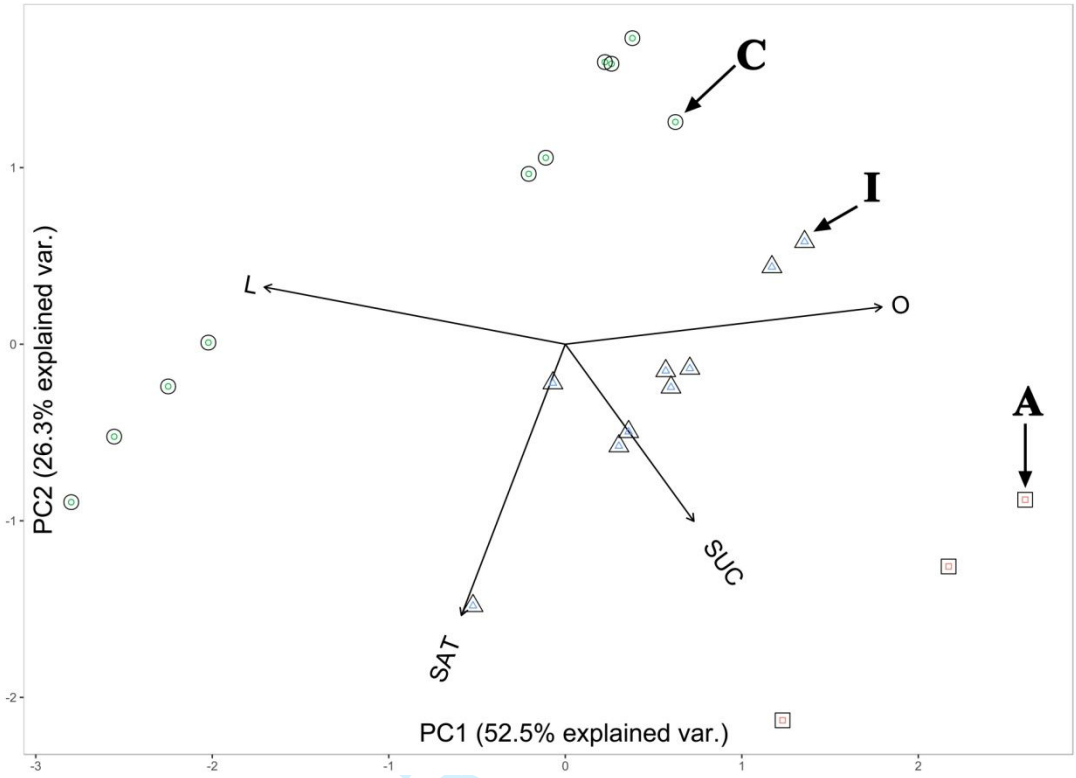

Figure 2S. Principal component analysis for the 24 NMR analysed samples referred to the four most represented quantitative values: the plot separates without any supervision the different declared origin (C=Californian, I=Italian, A = "Avola" species directly collected from the trees).

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