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Article

Comprehensive Chemical Characterization of the Pistacia vera Fruits through Original NMR Quantification Methods

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Featured Application: Double-phase extraction of seeds, followed by the quantitative NMR analysis through the innovative MARA-NMR, can open up new frontiers in the metabolomics field.

Abstract: (1) Background: *Pistacia vera* is a dry fruit spread all over the world, commonly known as pistachio, and is very appreciated as a snack, as a precious ingredient for confectionery industries and also as a blessed touch for gourmet dishes; (2) Methods: The simultaneous NMR profiles concerning the hydrophilic and hydrophobic phases, enabled by a specific throughput over grinded pistachio seeds, led to the comprehensive chemical characterization of these nuts coming from different territories; (3) Results: The customized extraction method is described in detail as well as the employed nuclear magnetic resonance (NMR) experiments. The chemical quantification takes advantage from the innovative multi-assignment recovered analysis (MARA-NMR) technique able to provide experimentally robust panels for both hydrophobic and hydrophilic chemicals. Beyond the assessed repeatability and the interesting statistical discrimination, this paper adds information about the quantitative and qualitative composition of pistachio as inferred by NMR data. This updated background can pave the way toward the development of wider and wider chemical panels improving the knowledge about the pistachio's composition. This approach can be easily extended to other matrices.

Keywords: qNMR; Pistacia vera; double-phase extraction; MARA-NMR; food authenticity; geographical discrimination

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1. Introduction

Geographical and genetic characterization of food products is a key topic in order to protect aliments with specific history and quality from homologous goods belonging to different cultures and oversold in world markets [1]. Dried fruits deserve great consideration as many recent papers highlight their worthy value as part of healthy diets, therefore it is also crucial to figure out how to define the authenticity of the consumed products [2]. Specifically, pistachio (*Pistacia vera*) belongs to the family of the *Anacariacee* and is a very spread nut often used as snack, candy ingredient or as gourmet dressing with potential beneficial effects such as the protective action against cardiovascular diseases [3,4]. Indeed, beyond the presence of a good recognized fatty acid balance between saturated, mono- and poly-unsaturated fatty acids, other antioxidant compounds and vegetal proteins seem to contribute to these beneficial consequences [5,6]. Pistachio is mainly grown in Iran, Turkey, USA, Greece, Syria and in Italy, where it is cultivated in Sicily close the volcano Etna in the area of Bronte [7]. Chemical studies on pistachio kernels mainly regarded the fatty acids and sterols content [8–10], whereas some targeted analyses concerned also pigments, polyphenols and other aromatic species [11,12], all analyzed by chromatographic techniques. Among such previous studies, Arena et al. [12] performed a nice comparison of pistachio samples coming from Iran, Turkey, California and Sicily in terms of the polyphenolic profile, elucidated by comprehensive two-dimensional liquid chromatography (LC × LC), thus obtaining a bidimensional map usable for a direct comparison. However, longer analysis time, as well as analytical challenges related to system and method development are the main drawbacks.

On the other hand, looking for a comprehensive chemical profiling of pistachio, some of us have explored an innovative, direct, and efficient shotgun mass spectrometry (MS) technique called iknife, able to provide the real-time identification of the geographical origin of pistachio nuts in only a few seconds, after the building of a mass spectral database and the use of chemometrics [13]. Here, we would like to complete this information with a parallel study adding supplemental information about the pistachio chemistry through the innovative nuclear magnetic resonance NMR approach over both hydrophilic and hydrophobic phases. Despite the claimed low analytical sensitivity, NMR allows a quick scan of samples for a first global glance of the metabolic profile. The additional benefit, especially if compared with the abovementioned shotgun-MS techniques, is the possibility to quantify one or more groups of identified analytes with particular attention to marker compounds. In fact, nuclear magnetic detection is strictly proportional to the number of magnetically equivalent protons so that the NMR signals strictly depend on concentration regardless the analyzed sample or chemical groups [14]. This is the reason why, in principle, it is possible to quantify substances without any standard reference (absolute intensity) or quantify many species with just one internal reference (this is often the case in food and metabolomic analyses). In the last two decades, NMR fulfilled the basic requirements for food analysis, such as minimal chemical treatment [15] and quick acquisition of a great amount of data [16], often unveiling complementary information with respect to the other analytical techniques [17–21].

Specifically, the chemical profile of pistachio seeds was disclosed through several NMR analyses independently, focusing on the oil composition made by triglycerides [22] and on the water soluble phase rich in peculiar saccharides, amino-acids and other interesting metabolites [23–25].

Nowadays NMR studies appear usually coupled to the multivariate statistical methods in order to assess geographic origin and/or food authenticity [22,26–29]. This paper aims to study *Pistacia vera* samples by NMR taking advantage on the chance to easily extract both water soluble and hydrophobic fractions; it leads to an easy obtainment of a comprehensive chemical analysis with two NMR studied phases for any starting raw material. The compounds identified in both fractions were all included in a statistical model by using Principal Component Analysis (PCA) in order to obtain clustering of five different origins according to variations in the content of specific markers of authenticity. Beyond the statistical analysis for the provenance discrimination, we think that the main result is the specific chemical profiles featuring several lots sold in any market.

2. Materials and Methods

2.1. Reported Tables

Table 1 lists the quantified chemicals with the used abbreviation throughout the paper, Table 2 reports quantifications measured over analyzed samples, whereas all the extended data are reported as Table A1 and in the Supplementary Materials: Tables S1–S3.

Table 1. List of abbreviations for compounds contained in pistachio seeds and found in the PWS (hydrophilic phase) and in PO (hydrophobic phase), the detailed features are reported in Table A1 in the Appendix A.

Substance	**Symbol Code**
Trigonelline	TRG
Formiate	**FA**
Inosine	**INO**
Uridine-di-phosphate	UDP
Uridine	URI
Indole-3-Acetic acid	I3AA
Phenylalanine and Triptophan	PHE + TRP
Tyrosine	TYR
Gallate	**GA**
Fumarate	**FUMA**
Shikimic acid	**SHA**
Quercetin-3-*O*-Glucoside	Q3G
Rafinose	**RAF**
Sucrose	**SUC**
Galactose	GAL
Glucose	**GLC**
Xylose	XIL
Fructose	FRC
Proline	**PRO**
Myo-inositol	**MYI**
Carnitine	CAR
Choline derivates	**CHN**
***N*-Methyl-4-trans-hydroxy proline**	**MHP**
γ-ammino butirrate	**GABA**
Asparagine	ASN
Aspartate	ASP
Citrate	**CA**
Malate	**MAL**
Glutamate	GLU
Arginine	**ARG**
Acetate	ACA
Isoleucine	ILE
Leucine	**LEU**
Valine	VAL
Alanine	**ALA**
Propan-1,2-diol	PDO
Piruvate	PA
Acetyl-lactate	ACLC
Lactate + Threonine	LAC + THR
Tri-acyl-glycerols	TG%
1,2-Di-acyl-glycerols	**1,2 DG%**
1,3-Di-acyl-glycerols	**1,3 DG%**
Saturated fatty esters	SAT%
Oleic fatty esters	O%
Linoleic fatty esters	**L%**
Linolenic fatty esters	**Ln%**
Methylated vegetal sterols	**STR**

Metabolites in bold are used for the final statistical analysis.

Table 2. Average quantification of metabolites whose code is explained in Table 1. The nine sample types with the inter-variability are reported. Extended quantification for each sample is reported in Table S2.

Provenance	**California**		**Turkey**			**Iran**		**Greece**	**Sicily**
Sample Group	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**	**S_8**	**S_9**
FA	0.21 ± 0.01	0.18± 0.05	0.05 ± 0.03	0.11 ± 0.01	0.03± 0.02	0.11± 0.01	0.11± 0.01	0.04± 0.01	0.05± 0.01
GA	nd	nd	0.23 ± 0.01	0.27 ± 0.04	0.34 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
FUMA	0.57 ± 0.01	0.44 ± 0.02	0.38 ± 0.02	0.86 ± 0.01	0.76 ± 0.01	1.35 ± 0.02	1.41 ± 0.06	0.56 ± 0.04	0.79 ± 0.02
SHA	0.48 ± 0.02	0.45 ± 0.06	2.4 ± 0.01	4.9 ± 0.01	3.6 ± 0.02	0.75 ± 0.03	1.2 ± 0.1	2.85 ± 0.05	1.59 ± 0.09
RAF	24.1 ± 0.2	23.4 ± 0.6	20.0 ± 0.3	26 ± 1	24.6 ± 0.7	25.6 ± 0.5	35.4 ± 0.8	19.3 ± 0.3	21.1 ± 0.4
SUC	92.0 ± 0.4	64 ± 3	38 ± 2	64.0 ± 0.8	55 ± 1	63 ± 1	41 ± 1	40.0 ± 0.7	36.0 ± 0.5
GAL	5.4 ± 0.2	6.2 ± 0.1	4.7 ± 0.2	5.2 ± 0.3	4.9 ± 0.2	4.6 ± 0.1	6.4 ± 0.4	4.7 ± 0.1	3.9 ± 0.1
GLC	8.1 ± 0.6	29 ± 2	5.29 ± 0.05	6.5 ± 0.2	6.5 ± 0.3	4.74 ± 0.9	3.7 ± 0.1	6.3 ± 0.2	4.24 ± 0.02
PRO	3.8 (1)	4.4 ± 0.1	6.2 ± 0.4	10.5 ± 0.2	10.0 ± 0.2	3.7 ± 0.2	3.7 ± 0.1	5.0 ± 0.1	3.6 ± 0.1
MYI	6.7 ± 0.3	8.2 ± 0.7	8.0 (8)	13.7 (4)	10.43 (9)	4.7 (2)	6.6 (1)	5.6 (2)	3.4 (2)
MHP	25.53 ± 0.08	24.6 ± 0.1	12.5 ± 0.1	19.7 ± 0.1	15.0 ± 0.1	30.9 ± 0.2	27.9 ± 0.3	13.6 ± 0.3	9.7 ± 0.1
GABA	4.07 ± 0.04	5.72 ± 0.06	2.9 ± 0.2	7.6 ± 0.1	4.7 ± 0.6	7.3 ± 0.1	8.0 ± 0.1	3.6 ± 0.1	4.19 ± 0.08
CA	10.5 ± 0.2	7.8 ± 0.2	13.08 ± 0.08	15.1 ± 0.5	17.6 ± 0.3	15.5 ± 0.4	15.0 ± 0.1	7.0 ± 0.3	9.2 ± 0.2
MAL	3.83 ± 0.04	2.9 ± 0.7	3.0 ± 0.3	5.8 ± 0.3	7.2 ± 0.4	4.4 ± 0.2	4.7 ± 0.4	5.0 ± 0.2	5.6 ± 0.2
ARG	5.76 ± 0.04	6.8 ± 0.1	4.6 ± 0.2	11.5 ± 0.3	8.6 ± 0.2	9.3 ± 0.3	12.3 ± 0.3	4.5 ± 0.2	3.21 ± 0.2
ILE	0.61 ± 0.01	0.75 ± 0.02	0.29 ± 0.01	0.53 ± 0.01	0.31 ± 0.01	0.45 ± 0.01	0.58 ± 0.01	0.45 ± 0.02	0.29 ± 0.01
ALA	5.68 ± 0.06	5.6 ± 0.3	3.5 ± 0.4	8.5 ± 0.7	4.90 ± 0.05	2.41 ± 0.02	4.73 ± 0.06	4.0 ± 0.2	1.93 ± 0.04
1,2DG%	2.4 ± 0.3	4.8 ± 0.3	2.0 ± 0.1	1.9 ± 0.1	2.2 ± 0.1	2.07 ± 0.06	1.85 ± 0.06	2.30 ± 0.03	2.2 ± 0.1
1,3DG%	3.0 ± 0.3	3.3 ± 0.3	1.90 ± 0.07	1.8 ± 0.1	1.94 ± 0.05	1.0 ± 0.5	1.61 ± 0.06	1.1 ± 0.4	1.7 ± 0.5
O%	54.0 ± 0.7	57 ± 1	71.9 ± 0.7	71.2 ± 0.2	70.5 ± 0.5	56 ± 2	59 ± 2	70 ± 3	70 ± 2
L%	28.4 ± 0.8	25 ± 1	13 ± 1	13.5 ± 0.4	14.0 ± 0.6	27 ± 2	27 ± 1	12 ± 1	15 ± 1
Ln%	0.7 ± 0.1	0.65 ± 0.05	0.36 ± 0.02	0.45 ± 0.03	0.45 ± 0.02	0.52 ± 0.07	0.52 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
STR	10.1 ± 0.1	8.38 (8)	5.86 (8)	6.0 ± 0.2	6.3 ± 0.3	8.7 ± 0.8	8.2 ± 0.2	7.5 ± 0.1	7.3 ± 0.1

Cells labelled as nd are not detected or below limits of quantification also taking into account the measured inter-variabilities.

2.2. Reagents and Materials

Water (H_2O), deuterated water (D_2O), deuterated chloroform ($CDCl_3$), tetramethylsilane (TMS), salts for the preparation of the phosphate buffer solution (PBS, pH = 7.2), 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) and methanol (CH_3OH) were all purchased at reagent grade from Merck Life Science (Merck KGaA, Darmstadt, Germany).

2.3. Samples

Pistachio nuts, from five different geographical origins (California, Iran, Turkey, Greece and Italian Bronte), were kindly provided by Pistì S.r.L. (Bronte, CT, Italy). Nine different varieties are chosen as follows, two from California, two from Iran, three from Turkey and just single samples from Greece and Italy as indicated in Table 2 (and detailed in Table S2 in Supplementary Materials). Out from each of the nine varieties, five production lots were collected, so that 45 samples were sorted out in 5 groups according their specific provenance around the world (in Table S2 sample names label the sample variety according to the first number, and the lot according to the second number, whereas the provenance is indicated above).

2.4. Sample Preparation

Ten grams of pistachio nuts were grinded and 300 mg of the resulting oily powders were placed inside a test-tube. To pursue the comprehensive analysis of both hydrophilic and lipophilic compounds, we adopted a Folch-like extraction [30]. After several trials the solvent mixture $CDCl_3$:H_2O with modified volume ratios was adopted. Specifically, 1.5 mL of H_2O, 1 mL of $CDCl_3$ (1:1 water/chloroform weight) were added to the sample powder; test tubes are screw-capped, put in an ultrasound bath for 40 min in the dark (700 Watt, never over T = 30°). Finally, samples were centrifuged for 40 min at 4200 RPM with a working temperature of 10 °C to prevent further heating. Figure S1 (Electronic Supplementary Materials) shows the separation into the centrifuge tube between the upper water soluble fraction (PWS, pistachio water soluble) and the lower green pistachio oil fraction (PO). An aliquot of 630 µL of the upper hydrophilic phase is poured into the NMR tube together with 70 µL of the D_2O phosphate buffer solution (PBS, pH = 7.2) with a small amount of TPS and methanol both used as quantitative and frequency references. The resulting 10% D_2O/H_2O solution is suitable for the NMR analysis. The green bottom-standing $CDCl_3$ solution (PO fraction) is directly analyzed through the NMR analysis as well.

2.5. NMR References

As suggested elsewhere [14,31], the addition of any standard compound can be used as a reference for both frequency calibration and quantitative analysis. In the PWS samples, the added MeOH (12.4 mM) is employed for this purpose by setting the sharp singlet resonance to $\delta = 3.34$ ppm. On the other hand we have used the popular TSP standard with a 1 mM concentration in D_2O which, after dilution, relays to 1 mM concentration of the nine-fold methylic signal ($\delta = -0.01 \pm 0.02$ ppm). The use of two references allows to rule out possible macromolecular associations potentially affecting signals at 3.34 and −0.01 ppm. In the PO samples, a little amount of TMS allows the frequency calibration ($\delta = 0.0$ ppm), whereas the 2.35 ppm signal, assigned to the α-CH_2 of fatty esters, is here used as relative quantitative reference.

2.6. NMR Analysis

NMR spectra were run on an Agilent Propulse 500 MHz machine equipped with an OneNMR probe and managed by a ProPulse console. ^{1}H and $^{13}C\{^{1}H\}$ spectra were acquired at the respective 499.74 and 125.73 MHz radiofrequencies and the working temperature was regulated with NMR thermometer at 298 ± 0.1 K (25 °C). All the experiments were launched through the vNMRj software package running on Centos Operating System over an HP personal computer. PWS is analyzed at pH = 7.2 (80 mM of final phosphate concentration). After the standard shimming, tuning and calibration of the 90° pulse (7.7 ± 0.1 µs at 62 dB), the scouting procedure was run to pre-saturate the water signal; after this preliminary scan, the *noesypresat* sequence [32] was applied to optimize solvent suppression and spectra profiles. In order to allow sufficient relaxation time without collecting 'noisy' data-points, we set acquisition time of 1.5 s and relaxation delay of 8.5 s repeated for 128 scans (20 min of experimental time). The total recycling delay of 10 s was enough to consider all the integrated signals fully relaxed after any scan as verified by spectra recorded at 16, 32 and 64 scans keeping the relative integration constant. All the assignments reported in Table S1, Electronic Supplementary Materials are supported by the 1D-DPFGSE-TOCSY [33] and ^{13}C hetero-nuclear techniques HSQC and HMBC [31]. For the PO samples ($CDCl_3$), after the standard shimming, the simple 90° pulse sequence (7.5 ± 0.1 µs at 62 dB) was applied for the ^{1}H experiment with 64 scans, 4 s of acquisition time and 10 s of time delay (total experimental time around 18 min). The total-decoupled $^{13}C\{^{1}H\}$ NMR experiment was run with 32 scans and 25 s of recycle delay to allow the full relaxation. The integration of many regions taken over the 1D experiments conveys complementary information which is processed through the multi-assignment recovered analysis (MARA-NMR), presented in previous studies [34,35]. This procedure led to the comprehensive quantitative analysis.

2.7. Statistical Analysis

In this paper, the statistical analysis is presented just as proof of concept concerning the different chemical panel shown by samples coming from different parts of the world. It would certainly possible to run any other statistical treatment in order to explore detailed features of the metabolic profiles, however, we think that the unsupervised discrimination among the analyzed samples provides a sound image of the samples without artifacts. This statistical evaluation (principal component analysis, PCA) over 25 variables (loadings) throughout 36 samples (scores) is run by R (version 3.5.2) and R Studio (version 1.1.463-2009-2018) software (Vienna, Austria).

3. Results and Discussion

The experimental protocol consists of a priming extraction over pistachio raw material using two immiscible phases: an aqueous phase for the extraction of polar compounds and a chloroform phase for the extraction of apolar compounds. As a consequence, the main goal of this strategy was the simultaneous obtainment of the oil (PO) and the hydrophilic fraction (PWS) which were then analyzed independently, unlike previous studies focusing either on the polar [23–25] or the apolar [22] phase. Figures 1 and 2 display the PO and PWS ^{1}H-NMR profiles, respectively, along with the main identified chemical species. Table 1 reports the full list of metabolites detected either in the PO or in the PWS with the determined quantitative units, while Table 2 reports the average quantitative results over the five lots for nine analyzed pistachio varieties. Particularly, Californian pistachio nuts were sub-classified into two varieties according to a colorimetric analysis; Iranian and Turkey nuts were sub-classified into two and three varieties, respectively, according to their maturation stage. Following the obtained results, the NMR technique showed to be a valuable tool for the detection of quali-quantitative differences among different samples. However, taking into account the limited amount of available samples, the statistical analysis was performed only according to the geography, by including all the available varieties. This allowed us to increase intra-sample variability and maximize the identification reliability of an unknown sample, by properly setting a standard deviation or outlier threshold.

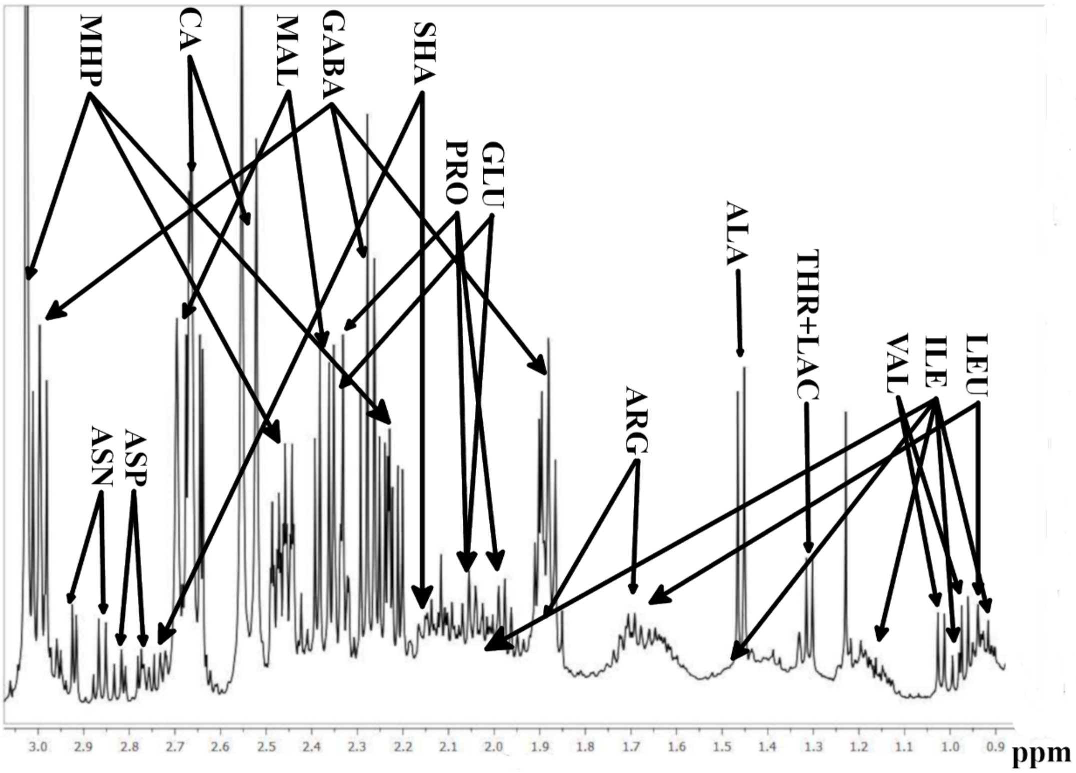

Figure 1. Expansion in the aliphatic region for the 1H-NMR profile at 500 MHz of the water-soluble fraction (PWS) extracted from pistachio powder (sample from Bronte). The main chemical assignment according to the symbol code in Table 1 is driven by arrows; detailed complete assignment is reported in Table S2 (Supplementary Materials).

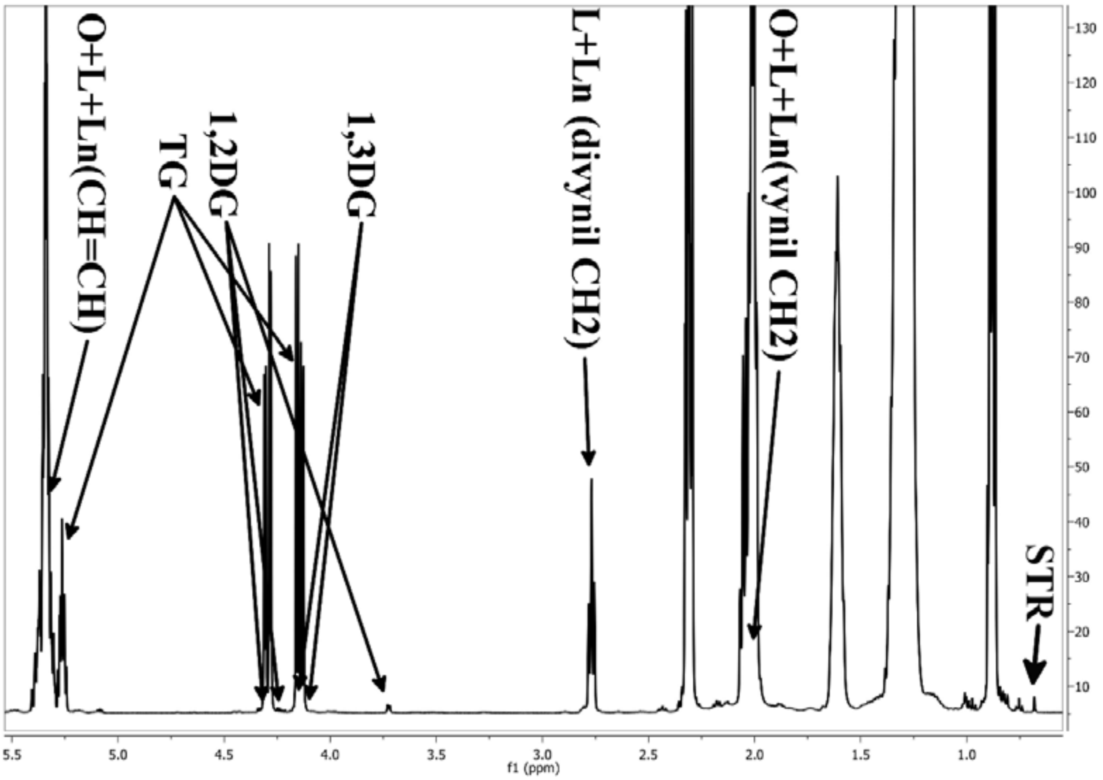

Figure 2. Main spectral region for the ^{1}H-NMR profile at 500 MHz of the $CDCl_3$ soluble fraction (PO) extracted from pistachio powder (sample from Bronte). Arrows display the main assignment connections, according to the symbol codes in Table 1, subsequently processed by MARA-NMR algorithm (see Table S3 for technical details).

3.1. Quali-Quantitative Composition

Looking at Figures 1 and 2, the ^{1}H NMR profile of the PWS sample appears much more complex. Consequently, the complete assignment of the obtained spectrum was not simple and involved 2D and selective experiments (1D-DPFGSE-TOCSY) to support the overall data consistency including the detected spin systems. Such a complexity is essentially related to the intrinsic sample heterogeneity, so that different chemical families were detected, such as aminoacids, sugars, nucleosides, organic acids and alcohols. Provided that these data are generally matching previous findings [22–24] and are consistent with general libraries [36,37], the tedious updated assignment (Table S1, Electronic Supplementary Materials) is updated according to slight mismatches like the fine septuplet structure of the α-CH of MHP (methyl hydroxy proline) and the detected presence of the 1,2-propanediol (PDO). This basic knowledge was necessary in order to enable the robust MARA-NMR algorithm which processed 63 ^{1}H-NMR integrations leading to the quantification of 39 PWS substances (Table 1).

Specifically, sugars were the most abundant compounds of PWS, indeed sucrose is the main component and rafinose is often very well represented. *N*-methyl-4-trans-hydroxyproline was the most concentrated amino acid derivative, followed by glutamate or arginine depending on the specific sample. Some organic acids (citrate and malate) and other less represented amino acids (proline, alanine and γ-aminobutyrate) were also interesting variables for the pistachio characterization. Afterward, the same MARA-NMR approach was adopted for the PO fractions according to the architecture previously built and already investigated for olive oil samples [21,34] able to combine both ^{1}H and ^{13}C profiles. The main hydrophobic components were fatty acid esters, consisting of triacylglycerols (>90% of the total glyceridic fraction), and diacylglycerols (between 2.1% and 5.7% of the total glyceridic fraction). Oleic esters (O%) represent more than 50% of fatty esters, followed by linoleic acid (L%) with a relative content between 13.5% and 28.4%; this is pretty fitting literature data [22]. A small amount of "methylated sterols" was also observed and used for the statistical analysis, since it was considered a potential discriminant feature for the geographical differentiation [28]. Hence, the method has led to the evaluation of six independent chemical parameters out of eight total quantification parameters due to mathematical restrains: the sum of fatty acid esters is set to 100% as well as the sum of di-acyl glycerols and tri-acyl glycerols. The comprehensive analysis of PWS and PO has eventually provided 47 quantitative chemical parameters extensively reported in Table S2. All the identified compounds play a very important role in many metabolic pathways, thus confirming the suitability of the NMR technique for metabolomic studies.

3.2. Statistical Analysis and Geographical Differentiation

In order to keep synthetic and reasonable outputs, unaffected by mathematical artifacts, we have decided to present the unsupervised multivariate analysis (PCA) over just 23 parameters (6 and 17 combined from the PO and PWS variables, respectively). These components were set as "loadings" for the definite principal component analysis (PCA). We highlight that the limited number of independent samples prompts the use of selected parameters with major significance and discriminating power. On another hand, in the case of bigger data-sets (more samples), the whole or wider consideration out of the 47 variables could turn out more effective. The effective geographical chemical distinction is reported in Figure 3 by combining the score and the loading plots: Californian samples are clearly separated from Iranian samples which are far apart from the samples coming from the Mediterranean area (namely Sicilian, Greek and Turkish); these could be also distinguished to each other by supervised (linear discrimination analysis type) clustering. We again point out that, beyond the geographical discrimination, possibly performed by sophisticated and supervised statistical approaches, this double phase extraction, followed by NMR quantification (PO and PWS MARA-NMR), is a promising tool for the comprehensive chemical analysis of pistachio samples and other nuts. According to quantitative results, the American samples display a remarkably higher presence of free glucose, as well as di-acyl glycerol and linolenic fatty esters at the expense of monounsaturated fatty acid esters (oleic acid esters). On the other hand, Iranian samples are featured by the higher presence of trans-methyl-hydroxy-proline. The Sicilian and Greek pistachios contain somewhat less γ-amino-butyric acid, raffinose and arginine, while Turkish nuts contain the higher amount of proline, malate, shikimic acid, gallic acid and oleic acid esters (Figure 3). Such discussion encloses a precious chemical information, complementary to that highlighted by Rigano et al. [13], which majorly focused on lipid compounds. The general aim of such a spectroscopic approach is to understand the intrinsic chemical nature of compounds even though affected by dynamic behavior [38]. Once these phenomena are fully understood, the definite NMR quantification (even though entangled into complicated datasets) is enabled [34].

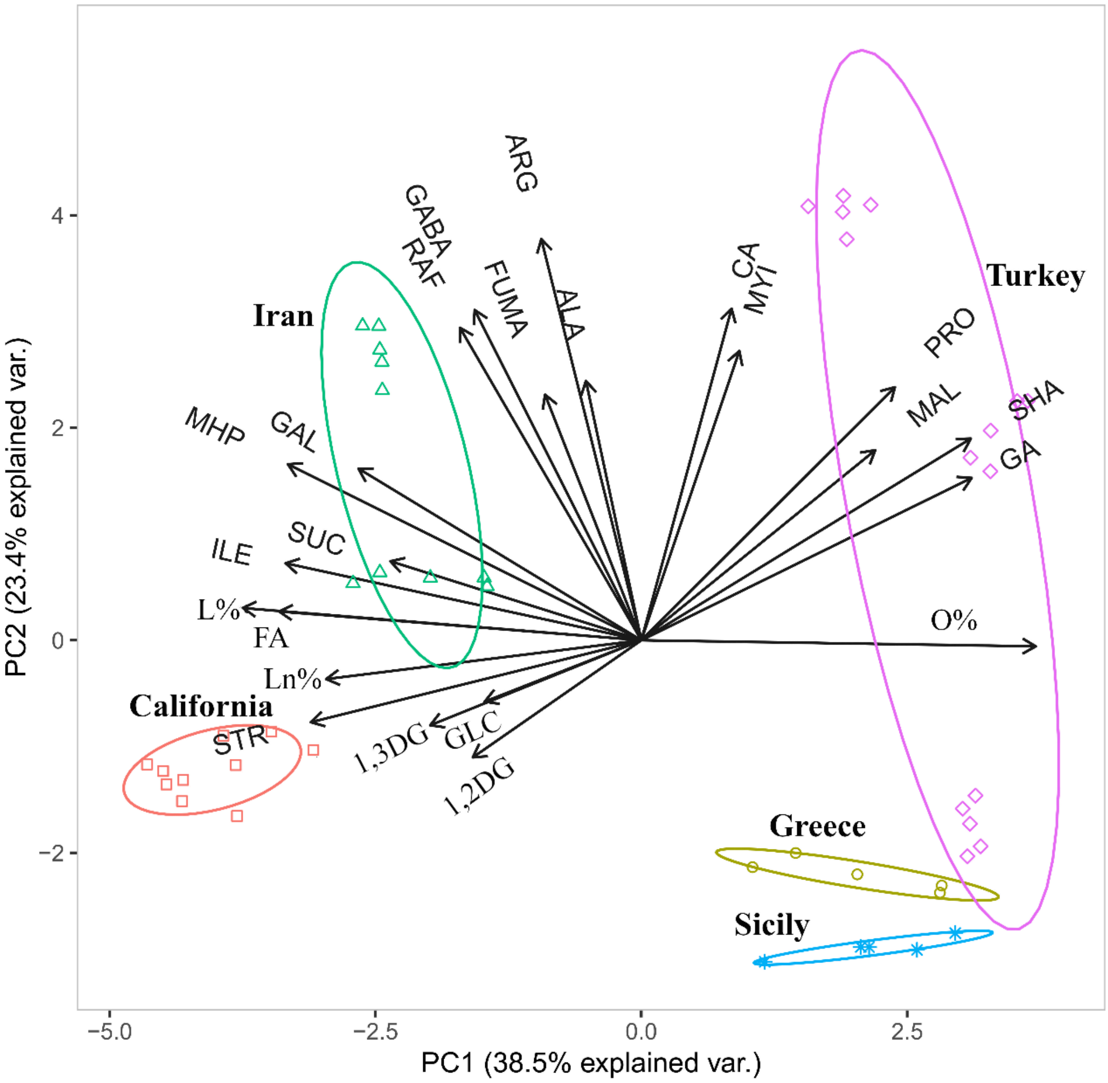

Figure 3. Graphical representation of the principal component analysis (PCA). It is a two-dimensional plot representing samples (marked as shaped spots) according to the most representative variables (loadings: represented by the radial harrows). The plot evidences are the definite clustering according to the different sample belongings.

4. Conclusions

An original double-phase extraction of pistachio seeds was proposed for the quick simultaneous NMR analysis of the hydrophilic (PWS) and hydrophobic (PO) solutions; this enabled the reconstruction of the overall chemical composition of these appreciated dry fruits. The patient assignment of many resonances allowed the full use of the innovative MARA-NMR quantification recently published. Samples grown in five different territories around the world were partially discriminated by unsupervised statistical analysis (PCA); however, without pushing mathematical tools for a perfect geographical clustering or definite foodomic conclusions, we here stressed the great value of a pretty quick and trustable procedure able to enlighten the comprehensive quantitative chemical composition of pistachio. As a matter of this fact, several crucial components like Shikimic acid (SHA), N-Methyl-4-trans-hydroxy proline (MHP), and glucose (GLC) present multiple signals sometimes overlapped; however, thanks to MARA-NMR, their quantification is unambiguously performed enabling quantification differences. For instance it is assessed that samples from Iran are rich in MHP, samples from Turkey are featured by more SHA and Californian samples contain more GLC. This study paves the way to comprehensive chemical quantifications supported by simultaneous double NMR analysis of hydrophilic and hydrophobic solutions simultaneously extracted from any studied matter.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/16/5523/s1.

Author Contributions: Conceptualization, A.R.; methodology, A.R.; software, A.R.; validation, A.R. and F.R.; formal analysis, A.R.; investigation, A.R.; resources, A.R.; data curation, A.R.; writing—original draft preparation, A.R.; writing—review and editing, A.R. and F.R.; visualization, A.R. and F.R; supervision, L.M.; project administration, L.M.; funding acquisition, L.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Extended details concerning the text.

Table A1. Detailed list of compounds contained in pistachio seeds which were detected and quantified by NMR either in the PWS or in PO, along with the quantification units and labels for each variable. Variables in bold were used for the principal component analysis.

Substance	Symbol Code	Quantification Units	Analysed Solution
Trigonelline	TRG	concentration (mM)	PWS
Formiate	**FA**	**concentration (mM)**	**PWS**
Inosine	**INO**	**concentration (mM)**	**PWS**
Uridine-di-phosphate	UDP	concentration (mM)	PWS
Uridine	URI	concentration (mM)	PWS
Indole-3-Acetic acid	I3AA	concentration (mM)	PWS
Phenylalanine and Triptophan	PHE + TRP	concentration (mM)	PWS
Tyrosine	TYR	concentration (mM)	PWS
Gallate	**GA**	**concentration (mM)**	**PWS**
Fumarate	**FUMA**	**concentration (mM)**	**PWS**
Shikimic acid	**SHA**	**concentration (mM)**	**PWS**
Quercetin-3-*O*-Glucoside	Q3G	concentration (mM)	PWS
Rafinose	**RAF**	**concentration (mM)**	**PWS**
Sucrose	**SUC**	**concentration (mM)**	**PWS**
Galactose	GAL	concentration (mM)	PWS
Glucose	**GLC**	**concentration (mM)**	**PWS**
Xylose	XIL	concentration (mM)	PWS
Fructose	FRC	concentration (mM)	PWS
Proline	**PRO**	**concentration (mM)**	**PWS**
Myo-inositol	**MYI**	**concentration (mM)**	**PWS**
Carnitine	CAR	concentration (mM)	PWS
Choline derivates	**CHN**	**concentration (mM)**	**PWS**
***N*-Methyl-4-trans-hydroxy proline**	**MHP**	**concentration (mM)**	**PWS**

Table A1. *Cont.*

Substance	Symbol Code	Quantification Units	Analysed Solution
γ-ammino butirrate	**GABA**	**concentration (mM)**	**PWS**
Asparagine	ASN	concentration (mM)	PWS
Aspartate	ASP	concentration (mM)	PWS
Citrate	**CA**	**concentration (mM)**	**PWS**
Malate	**MAL**	**concentration (mM)**	**PWS**
Glutamate	GLU	concentration (mM)	PWS
Arginine	**ARG**	**concentration (mM)**	**PWS**
Acetate	ACA	concentration (mM)	PWS
Isoleucine	ILE	concentration (mM)	PWS
Leucine	**LEU**	**concentration (mM)**	**PWS**
Valine	VAL	concentration (mM)	PWS
Alanine	**ALA**	**concentration (mM)**	**PWS**
Propan-1,2-diol	PDO	concentration (mM)	PWS
Piruvate	PA	concentration (mM)	PWS
Acetyl-lactate	ACLC	concentration (mM)	PWS
Lactate+ Threonine	LAC + THR	concentration (mM)	PWS
Tri-acyl-glycerols	TG%	percent of the total glyceridic fraction	PO
1,2-Di-acyl-glycerols	**1,2 DG%**	**percent of the total glyceridic fraction**	**PO**
1,3-Di-acyl-glycerols	**1,3 DG%**	**percent of the total glyceridic fraction**	**PO**
Saturated fatty esters	SAT%	percent out of the total fatty esters	PO
Oleic fatty esters	**O%**	**percent out of the total fatty esters**	**PO**
Linoleic fatty esters	**L%**	**percent out of the total fatty esters**	**PO**
Linolenic fatty esters	**Ln%**	**percent out of the total fatty esters**	**PO**
Methylated vegetal sterols	**STR**	**molecules out of thousands of total molecules**	**PO**

Metabolites in bold are used for the final statistical analysis.

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