From sample preparation to NMR-based metabolic profiling in food commodities: The case of table olives

Stavros Beteinakis [1] | **Anastasia Papachristodoulou**[1] | **Emmanuel Mikros**[2] | **Maria Halabalaki**[1,*]

[1] Division of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Athens, Greece

[2] Division of Pharmaceutical Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Athens, Greece

Correspondence
Maria Halabalaki, Division of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Panepistimiopolis, Zografou, 15771 Athens, Greece.
Email: mariahal@pharm.uoa.gr

Funding Information
European Union (ERDF) and Greek national funds through the Operational Program "Competitiveness, Entrepreneurship and Innovation", under the call "STRENGTHENING RESEARCH AND INNOVATION INFRASTRUCTURES" (PlantUP) 5002803
European Union (EU) Olive-Net project (Horizon2020-MSCA-RISE-2016-734899)

Abstract

Introduction

Nuclear magnetic resonance (NMR)-based metabolic profiling has been widely used in food and plant sciences. Despite its simplicity and inherent reproducibility, the determination of the appropriate pre-processing procedures greatly affects the obtained metabolic profile.

Objectives

The current study represents a detailed guide of use for untargeted NMR-based metabolic profiling of table olives (*Olea europaea* L.).

Methods

Greek Kalamon table olives from different geographical origins were selected as reference materials. Differently treated samples were extracted using different solvents and/or solvent systems. Chemical profiles were evaluated with high-performance thin layer chromatography (HPTLC). Different deuterated solvents and sample concentrations were evaluated for the recording of optimal quality spectra.

Results

The methanol extract of freeze-dried table olives was found to contain the most representative secondary metabolites, in higher concentrations, as well. The optimal deuterated solvent for the NMR analysis was methanol-d_4, while final sample concentration should be within the range of 10 to 15 mg/mL. Multivariate data analysis was also used to estimate and confirm the variation and clustering caused by different characteristics of the samples.

Conclusions

Results of the present study make evident the necessity for thorough planning and method development prior to any extensive metabolomic study based on NMR spectroscopy. Pre-processing and sample preparation stages seemed to greatly affect the metabolic profile and spectral quality in the case of table olives, which by extrapolation could apply to other food commodities. Nevertheless, the nature of the samples must be fully described in general, in order to proceed to solid conclusions.

Keywords

extraction | NMR-based metabolic profiling | *Olea europaea* L | protocol optimisation | sample preparation | table olives

1INTRODUCTION

A new arrow has been introduced to the quiver of analytical methodologies and technologies related to the analysis of microbial, plant, animal and human metabolomes, with the first two being more complex and certainly less specific than the latter.[1] These tools accompanied by the "omics" approaches are being successfully applied in the research of food science and nutrition. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy have extensively been employed in research involving food profiling, biomarker detection, authenticity control and issues related to food quality or safety.[2,3] The aforementioned approaches have recently adopted the label «Foodomics» influenced by the materials they investigate. Changes in the metabolome directly transmit the effect of genetic alterations, disease or acquired influences on the studied biological

systems.[4] Over the last decades NMR tends to become an analytical technique of choice for metabolomics, and specifically foodomics approaches, due to a cluster of advantages that it demonstrates. Despite its lower sensitivity compared to other methods, the high reproducibility and speed of NMR, combined with its inherent quantitative nature and easier sample preparation have made the technique a preferable option over other platforms.[5,6]

In plant and food metabolic profiling everything starts with the procurement of the initial material, storage and sample preparation steps that require certain procedures such as grinding, lyophilisation or loss of humidity under liquid nitrogen.[7] Prior knowledge of the food matrix is a prerequisite during these steps and any mismatches could lead to inconsistent results, even with the optimal analytical method.[8] Furthermore, metabolic quenching, most commonly with lyophilisation or rapid cooling, is essential if the food material is not subjected to analysis directly after sampling.[9] In turn, homogenisation of the raw or dried sample, aside from increasing reproducibility, allows better access to the metabolites.[9]

In the case of liquid state NMR, the metabolome of homogenised foods must be recovered using appropriate procedures. It is worth mentioning that both the extraction solvent and procedure selected determine the size and the quality of the detected metabolome, similarly to plant metabolomics. Different chemical classes of compounds respond in alternate ways to certain extraction parameters and therefore the choice of extraction method is closely associated with the scope of the study, for instance targeted or untargeted profiling. Thus, it can be a source of qualitative and quantitative bias, annulling one of the greatest advantage of NMR, which in principle is not affected by a compound's chemical nature.[9] As far as extraction solvent is concerned, alcohols, such as methanol (MeOH), usually combined with water (H_2O), seem to be able to accomplish satisfactory metabolite coverage.[10,11] In any case, experimental design is adjusted to the food matrix and not vice versa.[12,13] Provided that the extract has been recovered, the following significant step in plant or food metabolomics is the sample preparation prior to NMR analysis. The choice of deuterated solvent and final sample concentration significantly affects the obtained profile, as it was described in the study of *Vitis* wood samples by Halabalaki *et al*.[14] A significant objective in metabolic profiling is to acquire good quality spectra with narrow, symmetrical peaks across the field and minimal or no overlapping that eventually lead to meaningful outcomes.[15,16]

Therefore, the goal of the present study is to develop a workflow which will provide a detailed sequence of optimisation steps towards the development of an untargeted NMR-based metabolic profiling approach. As a reference material, we investigated the case of table olives which, together with olive oil, are typical ingredients of Mediterranean diet comprising the main source of fat. The final protocol could find applications in various matrices, especially food commodities, as well as their by-products.

Table olives are mainly comprised of H_2O and fat, but they are also highly abundant in hydroxytyrosol, tyrosol and their derivatives.[17] Specific levels of these exact compounds have presented olive oil with a health claim regarding the protective effect of the product against oxidation over blood lipids resulting in cardio-protection for the consumer, which by extent raises the value of table olives as a food.[18] The intricacy of table olives as a matrix, with a range of different chemical classes of compounds, makes them a challenging analytical target in need for a

carefully selected experimental design, as many parameters need to be taken into consideration.[17,19] Ultimately, the choices are based on the final goal in each case. As far as olive drupes from Greece are concerned, phenolic,[20,21] organoleptic and microbiological profiles[22,23] have been investigated, while quantitative studies on individual molecules have also been completed.[24,25] NMR spectroscopy in combination with chemometrics has been successfully used in the authentication of a wide range of food commodities, like olive oil, meat, honey and saffron.[26–29] Nevertheless, to our knowledge, our recently published research is the only one reported using the NMR-based metabolic profiling tool for the quality assessment of table olives,[30] with one more applying NMR fingerprinting,[31] while other analytical methods represent a handful of studies.[32–35] Furthermore, in the current study high-performance thin layer chromatography (HPTLC) analysis was used as a complementary analytical technique to facilitate and accelerate the sample preparation procedure. This fast and cost-effective approach could be ideal for a primary chemical profile screening of the numerous studied samples produced during the pilot approaches including extraction. Regarding NMR-based profiling, sample preparation parameters like deuterated solvent and sample concentration were tested with regards to the obtained spectral quality.

2EXPERIMENTAL

2.1Reagents and materials

Table olives belonging to the Kalamon cultivar were handpicked and provided by Greek producers. In total, 30 distinct samples were available, from which three biological replicates (sample coding: TO-1, TO-2, TO-3) were randomly selected and analysed in the present study. All samples were stored in their brines in dark conditions and at room temperature (RT, 25°C) until use.

Solvents used for the extraction of the drupes and HPTLC analysis – MeOH, H_2O, ethyl acetate (EtOAc) and dichloromethane (DCM) – were of high-performance liquid chromatography (HPLC) grade and glacial acetic acid (AA, > 99.7%) was of analytical grade (Fisher Scientific, Loughborough, UK). Deuterated NMR solvents used – chloroform-*d* ($CDCl_3$, purity 99.8% D), methanol-d_4 (CD_3OD, purity 99.8% D) and dimethylsulfoxide-d_6 (DMSO-d_6, purity 99.8% D) – were acquired from Euriso-Top GmbH (Saarbrücken, Germany). Hexamethyldisiloxane (HMDSO, NMR grade, ≥ 99.5%) was purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). NMR tubes (D600-5-7, 5 mm diameter and 7 inches length) with polytetrafluoroethylene (PTFE) caps were obtained by Deutero GmbH (Kastellaun, Germany).

2.2Sample handling and extraction

Two different approaches were used in the present study. Three drupes per biological replicate (sample) were picked and subjected in parallel to one of the two following treatments: (i) direct pulverisation or (ii) lyophilisation followed by pulverisation. In both cases, table olives were destoned and only the pericarp was crushed in a porcelain crucible.

Additionally, three different solvents or solvent systems (30 mL) were tested in an attempt to identify the optimal one: (i) MeOH/H_2O: 8:2, (ii) MeOH and (iii) EtOAc. Extracts were obtained by sonication for 15 min at RT and vortex-shaking for 1.5 min using as an initial material 0.3 g of each sample. Following centrifugation at 4000 rpm, the supernatant was recovered and evaporated till dryness and stored at −20°C pending further analysis. Extraction yield was evaluated as a quantitative parameter.

2.3HPTLC analysis

The primary screening of all extracts for the selection of the optimal one was carried out with the HPTLC analytical technique, which in the present study stands as a complementary approach. More specifically, samples were dissolved in 500 uL of MeOH and applied onto 20 cm × 10 cm HPTLC plates (silica gel 60 F254, 0.20 mm layer thickness; Merck, Darmstadt, Germany) using the Automatic TLC sampler 4 (ATS 4, CAMAG®, Muttenz, Switzerland) controlled with VisionCats 2.5 (CAMAG®) software. Parameters used for application are as follows: tracks with 8.0 mm bands, 8 mm distance from the lower edge, 25 mm from the left and right edges, and 13.0 mm between the different tracks. A 25 uL Hamilton glass syringe (NV, USA) and a nitrogen aspirator (Peak Scientific, Inchinnan, Great Britain) were used for the application of 10 uL from each extract. Plate development was accomplished with an automatic development chamber (ADC 2, CAMAG®) using the respective settings: 20 min chamber saturation with pad, 10 min for plate conditioning at 33% relative humidity, using magnesium chloride ($MgCl_2$) as a desiccant, and 5 min for plate drying. Mobile phase used for development comprised of 90% DCM, 10% MeOH and 2% AA. Plate images were recorded at 254 nm and 366 nm prior to derivatisation and visible after derivatisation on a TLC Visualiser 2 System (CAMAG®). Sulphuric vanillin derivatisation reagent [i.e. 5% *w/v* vanillin in MeOH/5% *v/v* sulphuric acid (H_2SO_4) in MeOH 1:1 *v/v*] was sprayed with an automatic derivatiser (CAMAG® Derivatiser) onto the plates for visualisation of the spots.

2.4NMR analysis

2.4.1Sample preparation prior analysis and data acquisition

Following HPTLC analysis, extracts were dried and redissolved in deuterated solvents for NMR analysis, which was applied at the selected extracts. For the optimum concentration assay, the selected extracts based on the qualitative evaluation with HPTLC were prepared at four decreasing concentrations: 25, 15, 10 and 5 mg/mL. Accordingly, various deuterated solvent systems were evaluated for the best deuterated solvent assay: $CDCl_3$, CD_3OD, $CDCl_3$/CD_3OD: 1:1 and DMSO-d_6. HMDSO was added (0.02% *v/v*) as an internal standard and a line-shape indicator. Each sample was dissolved within the eppendorf in 650 μL of the corresponding deuterated stock solution by means of sonication and vortexing. A volume of 600 μL was transferred via a 1-mL Hamilton glass syringe and placed in an NMR tube.

Experimental parameters for proton (^{1}H)-NMR experiments were as follows: spectra were recorded at 305 K on a Bruker AVANCE III 600 NMR spectrometer (Bruker GmbH, Karlsruhe, Germany) equipped with a z-gradient inverse detection 5-mm probe and a BCU for temperature control. The following conditions were used for the acquisition: number of scans (NS), 64; π/2 pulse, ~8 us; time domain (TD), 64 k data points; acquisition time, 2.73 s; relaxation delay, 3.17 s; spectral width, 12019.2 Hz and mixing time, 0.060 s. Pulse programs used were either one-dimensional (1D) nuclear Overhauser effect spectroscopy (NOESY) with water presaturation or simple zg. In both cases, transmitter frequency offset was set at 2880.5 Hz. Spectra were obtained by Fourier transformation (FT) of the free induction decay (FID) by applying exponential multiplication with a line-broadening (lb) factor of 0.3 Hz and zero-filling (size = 128 K) procedure. Resulting spectra were manually phased and baseline-corrected using a polynomial function available in the Bruker TopSpin® 4.0.6 software. Chemical shifts were reported with respect to the signal of the internal standard (IS) set at 0.0 ppm.

2.4.2Processing and statistical analysis of NMR data

NMR raw data were inserted in the MATLAB® suite (version R2018b) for further processing. Spectra (spectral width from −0.5 to 12 ppm) were segmented into 1251 bins with a bin size of 0.01 ppm for multivariate analysis (MVA). Data were normalised using an in-house routine and the area of the peak of the IS was used as a reference value. Normalisation with total intensity was also evaluated. Data were extracted and inserted into the SIMCA® 14.1 software package (Umetrics, Umeå, Sweden), where they were subjected to principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) statistical methods.[36] Prior to PCA, data were scaled using both Unit Variance (UV) and Pareto (Par) scaling with the latter being additionally subjected to logarithmic transformation. Another helpful tool that was used for the visualisation of the alterations between the different treatments was the Biplot chart, where the different plots (scores scatter and loadings plots) are combined in one, in order to enable the analyst to simultaneously view the formed clusters in combination with the significant variables responsible for their formation.

3RESULTS AND DISCUSSION

3.1Selection of the optimal extraction protocol

Approximately 50–60% of processed table olives' composition is water, 20% is fat and the remaining part is comprised of secondary metabolites, sugars cellulose and nitrogenous compounds.[17] Small molecules found in olive drupes belong to different chemical classes, from flavonoids (i.e. luteolin) and phenylethanoid derivatives (i.e. tyrosol, hydroxytyrosol) to tocopherols and triterpenoids (i.e. maslinic acid), thus forming an intricate natural matrix.[19] Due to that complexity, pretreatment, extract recovery and, surely, analysis are challenging tasks that require thorough planning based on the final target of each research endeavour.

Therefore, the initial aim of the current study was the evaluation and determination of the pretreatment's and solvent system's impact on the recovery of secondary metabolites. A schematic representation of the followed methods and experimental parameters is shown in Figure 1. To this

day and to the extent that we may be certain, fresh, lyophilised or oven-dried have been analysed, yet no study investigates the impact of these pretreatments in the profile of the final extract.[24,37,38] On that note, different ratios of MeOH and H_2O have been tested in the extraction of table olives with regards to the recovery of metabolites targeted for quantitative purposes.[39] Other solvents like EtOAc, DMSO or ethanol have been applied in previous studies of table olives,[40–42] but no comparison of extraction solvents has been conducted concerning their effect over the composition of the recovered extract.

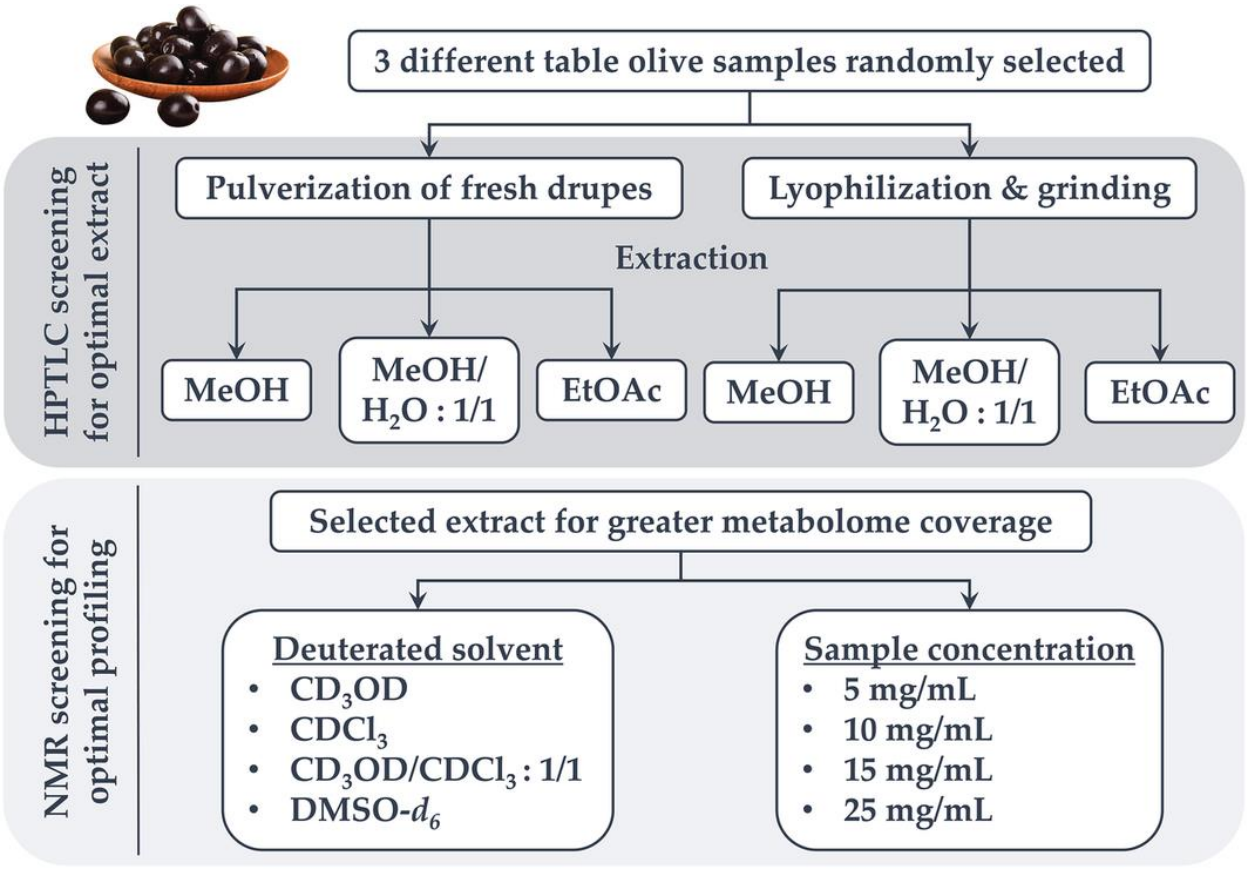

FIGURE 1 Detailed schematic of the tested experimental parameters

Extraction yield and HPTLC analysis, with the latter offering the ability for both qualitative and semi-quantitative (relative quantification) evaluation, were invoked to assess the effect of the different tested parameters. In order to ensure the integrity of any extracted conclusions, three randomly selected samples (biological replicates) belonging to the Kalamon cultivar were examined in this study. Pulverised pericarp from table olives, that either underwent lyophilisation or were fresh, were extracted based on the International Olive Council (IOC) protocol for olive oils[43] with modification to match the different nature of the samples. Aside from the MeOH/H_2O: 8:2 mixture suggested in the aforementioned protocol, pure MeOH and EtOAc were also tested as potential extraction solvents.

Initially, extraction yields were examined. As it can be seen in Table 1, lyophilised samples produced a yield from two-fold in the case of EtOAc to four-fold greater in the cases of MeOH and MeOH/H_2O. Therefore, in terms of absolute yield, EtOAc surpassed MeOH and the

MeOH/H_2O mixture. Given that roughly half of an olive's content is H_2O, a two-fold increase in yield for lyophilised samples was expected, but it seems that the impact is even larger for the two more polar solvent systems. This could possibly be due to the improved adsorption of the solvent in the granulated olive mass after lyophilisation and the better recovery of metabolites, among them verbascoside, hydroxytyrosol, tyrosol, which stand out as characteristic compounds for the studied material.[30,44,45] Therefore, lyophilisation was preferred over direct extraction of fresh drupes. It is also worth noting that weighing accurately the fresh pulverised drupe was significantly harder compared to the refined powder of the dry sample, due to the high-water content and inconsistent sample material. These observations led us to further investigate whether these differences would also reflect in the composition of the derived extracts, qualitatively or quantitatively.

TABLE **1**Yields of olives' extracts after the use of three different extraction solvents and two different pre-treatments (lyophilised vs. fresh-powdered drupe)

Starting material: 0.3 g						
	Fresh		Lyophilised			
Sample code	Extraction solvent	Yield (mg)	Yield (%)	Extraction solvent	Yield (mg)	Yield (%)
TO-1A	MeOH	9.60	3.20	MeOH	41.80	13.93
TO-2A		9.40	3.13		43.70	14.57
TO-3A		17.80	5.93		48.60	16.20
TO-1B	MeOH/H_2O 1/1	6.10	2.03	MeOH/H_2O 1/1	14.60	4.87
TO-2B		3.90	1.30		7.60	2.53
TO-3B		4.90	1.63		13.20	4.40
TO-1C	EtOAc	42.40	14.13	EtOAc	93.40	31.13
TO-2C		44.70	14.90		115.00	38.33

TO-3C		42.60	14.20		100.40	33.47

Sample code: TO, table olive; A, methanol (MeOH) extracts; B, MeOH/water (H_2O) (1:1) extracts; C, ethyl acetate (EtOAc) extracts.

Thus, HPTLC, a fast and accessible method, was selected to provide a deeper insight into the chemical composition of the produced extracts. Due to its ability to screen numerous samples in a cost-effective manner and satisfactorily depict the obtained profiles, it seems that this technique is suitable at least for the qualitative assessment of any pilot study concerning sample pretreatment and extraction.[46] Figure 2 offers an indicative representation of the alternate impact that extraction solvents and lyophilisation demonstrate over the composition.

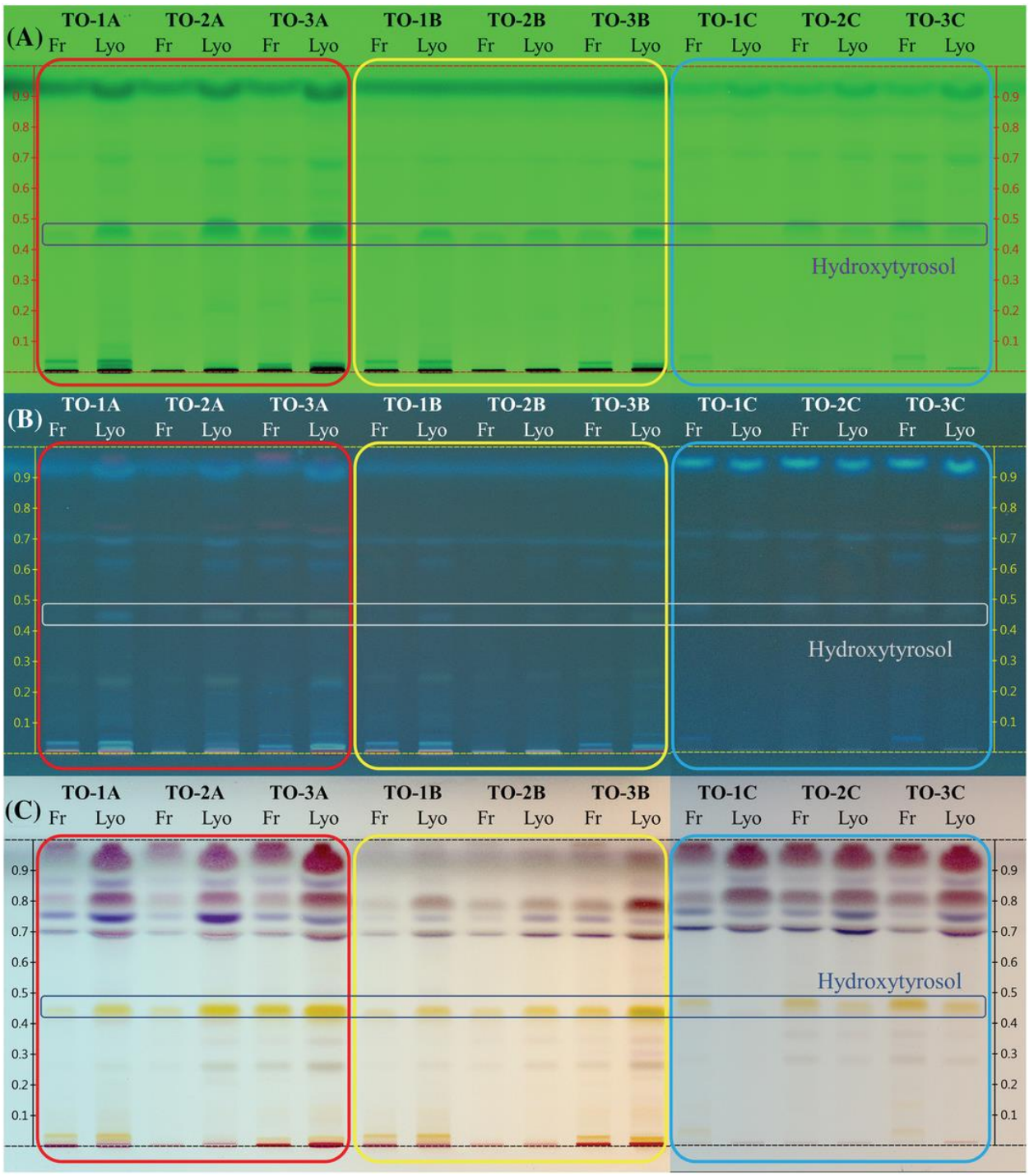

FIGURE 2HPTLC chromatograms (elution system: 90% DCM, 10% MeOH and 2% AA) of all extracts recovered with different solvents MeOH (red), MeOH/H_2O: 8:2 (yellow) and EtOAc (light blue) at (A) 254 nm, (B) 366 nm and (C) visible after derivatisation with sulphuric vanillin. Hydroxytyrosol is indicated. Fr, fresh drupes; Lyo, lyophilised

To elaborate, EtOAc was more successful in the recovery of less polar compounds observed at the solvent front in all wavelengths, while the far more polar mixture of MeOH and H_2O had the

opposite result. The MeOH extract (marked in red) poses as the best solution in the case of an untargeted metabolomics study, as it accomplishes sufficient recovery across all chemical classes. For instance, the strong presence of hydroxytyrosol in this extract is evident in the middle of the plate with several more and less polar compounds detected below and above it, respectively. The stage of HPTLC acts as the cut-off point for this study, where the initial selections were made and we finally proceeded to the next step only with the selected extract – the MeOH one – from lyophilised olive drupes.

3.2Optimisation of sample preparation for NMR profiling

As it is described in Figure 1, the first part of the study is dedicated to the selection of the appropriate and most representative extract through the HPTLC developed method. Although, the second and main part of the current study focuses on obtaining high quality spectra of the selected extract with peaks spread across the field to the maximum extent possible for enhanced resolution. For this reason, two different approaches were used here: (a) investigation of different deuterated solvents and (b) impact of an extensive range of sample concentrations in the recorded spectra. The applied acquisition parameters were evaluated and finally determined based on the variables of aforementioned approaches (see earlier).

3.2.1Determination of the optimal deuterated solvent

The complexity encountered in the chemical composition of plant or food extracts often leads to solubility issues. Diversity and variations regarding chemical classes and concentration levels of contained secondary metabolites are important pieces of the puzzle. The entire scope of untargeted metabolomics approaches is to incorporate the maximum possible number of contained compounds. Hence, the ideal solvent should be able to dissolve the recovered extract in its entirety. Additionally, minimal overlapping is mandatory for optimal resolution across the field. To that end, different deuterated solvents or solvent systems were tested (Figure 3, S1).

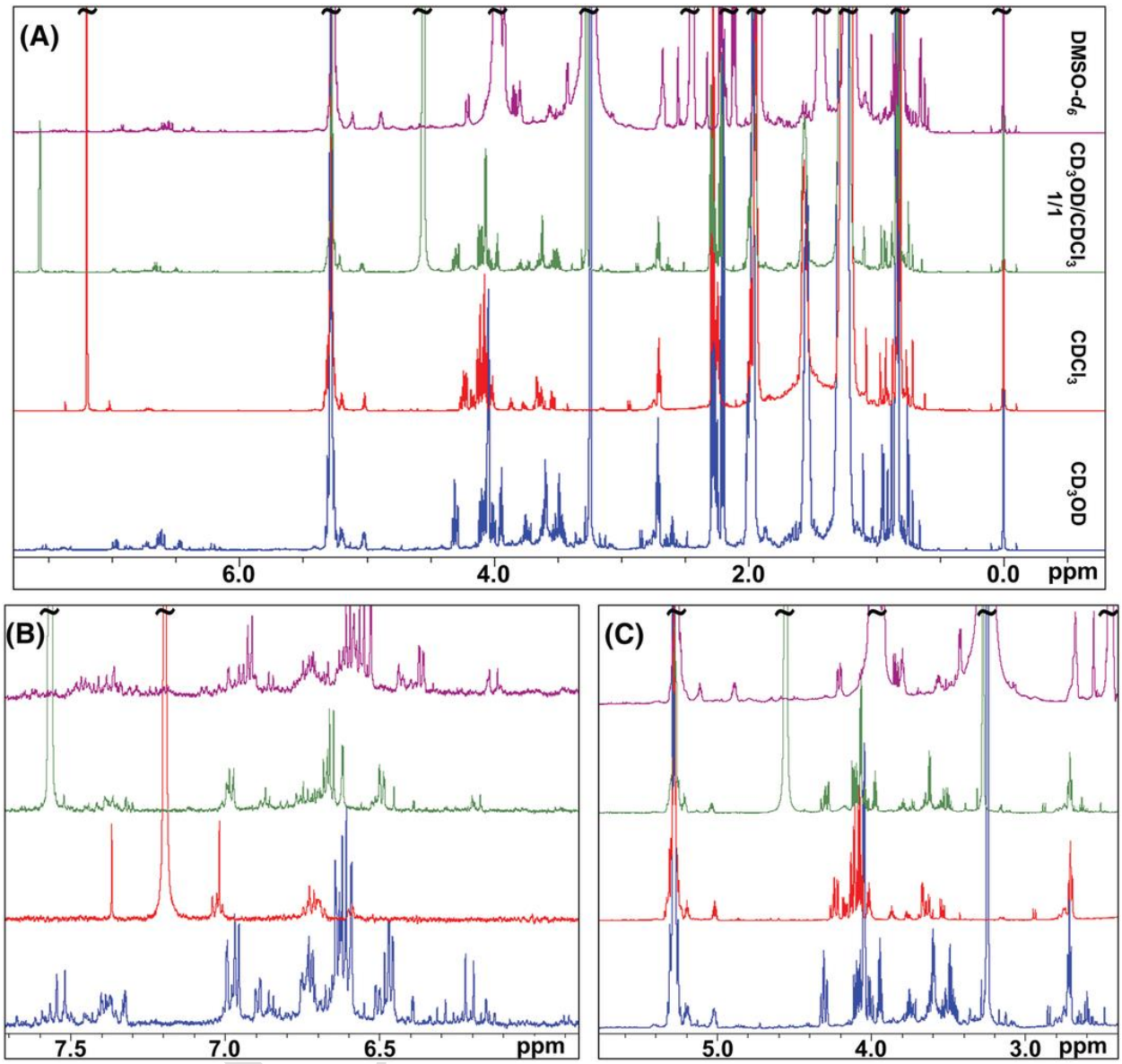

FIGURE 3(A) Overlaid ^{1}H-NMR spectra of sample TO-1A (from lyophilised drupes) dissolved in different deuterated solvents; CD_3OD, $CDCl_3$, $CD_3OD/CDCl_3$: 1:1 and DMSO-d_6 (bottom to top). Sample concentration used was: 15 mg/mL. (B) Zoom-in of the region between 8.0 and 5.8 ppm. (C) Zoom-in of the region between 5.5 and 2.6 ppm

Initially, 15 and 10 mg of the selected extracts (lyophilised-MeOH) were dissolved in deuterated solvents commonly used in NMR spectroscopy, i.e. CD_3OD, DMSO-d_6 and $CDCl_3$. The first two solvents are probably the first choices in the case of a MeOH extract. However, considering the fatty nature of olive drupes, $CDCl_3$ was tested both on its own (Supporting Information Figure S4), as well as in a ratio of 1:1 with CD_3OD (Figure S5). Recorded spectra are presented in Figure 3. Interestingly, compounds from different chemical classes were present in the different solvents based on their respective polarity and solubility.

Precipitate formation was observed to some extent in every solvent after stabilisation within the NMR tube. As expected, salts and polar compounds were its main constituents in the case of

$CDCl_3$, as opposed to pigments and triacylglycerols (TAGs) in CD_3OD and DMSO-d_6.[47,48] The mixture of CD_3OD with $CDCl_3$, though it presented good solubility, resulted in broad overlapping peaks with poor peak shape. Generally, solvent mixtures are not easily manageable, as they often cause issues with solvent lock and shimming, especially in the case of 1:1 ratio adopted in the present study. Overlaid spectra in different deuterated solvents were very informative with regards to reconstitution of different chemical classes of the extracts, which was evaluated along with the peak resolution and overall spectral quality (Figure 3). In particular, in the $CDCl_3$ spectrum, mainly TAGs are observed, as the absence of aromatic peaks (~6–7 ppm) is evident. Provided that the only solvent mixture tested was discarded due to aforementioned reasons, the final comparison came down to CD_3OD (Figures S2 and S3) and DMSO-d_6 (Figure S6). Nevertheless, based on spectra observation, CD_3OD was opted as the most suitable solvent for profiling of table olives' MeOH extract in untargeted metabolomic approaches. As it is apparent from the particular figure (Figure 3), the peaks were spread well across the field in the respective deuterated solvent, presenting high intensities and by far a better image compared to the other solvents, especially concerning the regions of high importance, such as the aromatic, where hydroxytyrosol, verbascoside and other phenolic compounds can be discerned.

3.2.2Determination of the optimal sample concentration

Provided that the solubility aspect was investigated and led to a solid conclusion, extract concentration was the next parameter to be optimised. As already mentioned, in untargeted metabolomics studies, the aim is to detect as many metabolites as possible that make a group of observations different from another without affecting spectral quality. Consequently, solutions of extracts were prepared at four different – gradually decreasing – concentrations, 25, 15, 10 and 5 mg/mL. All deuterated solvent systems were tested once more in an attempt to verify the superiority of CD_3OD over the others. A visualisation of NMR spectra obtained with CD_3OD is shown at Figure 4.

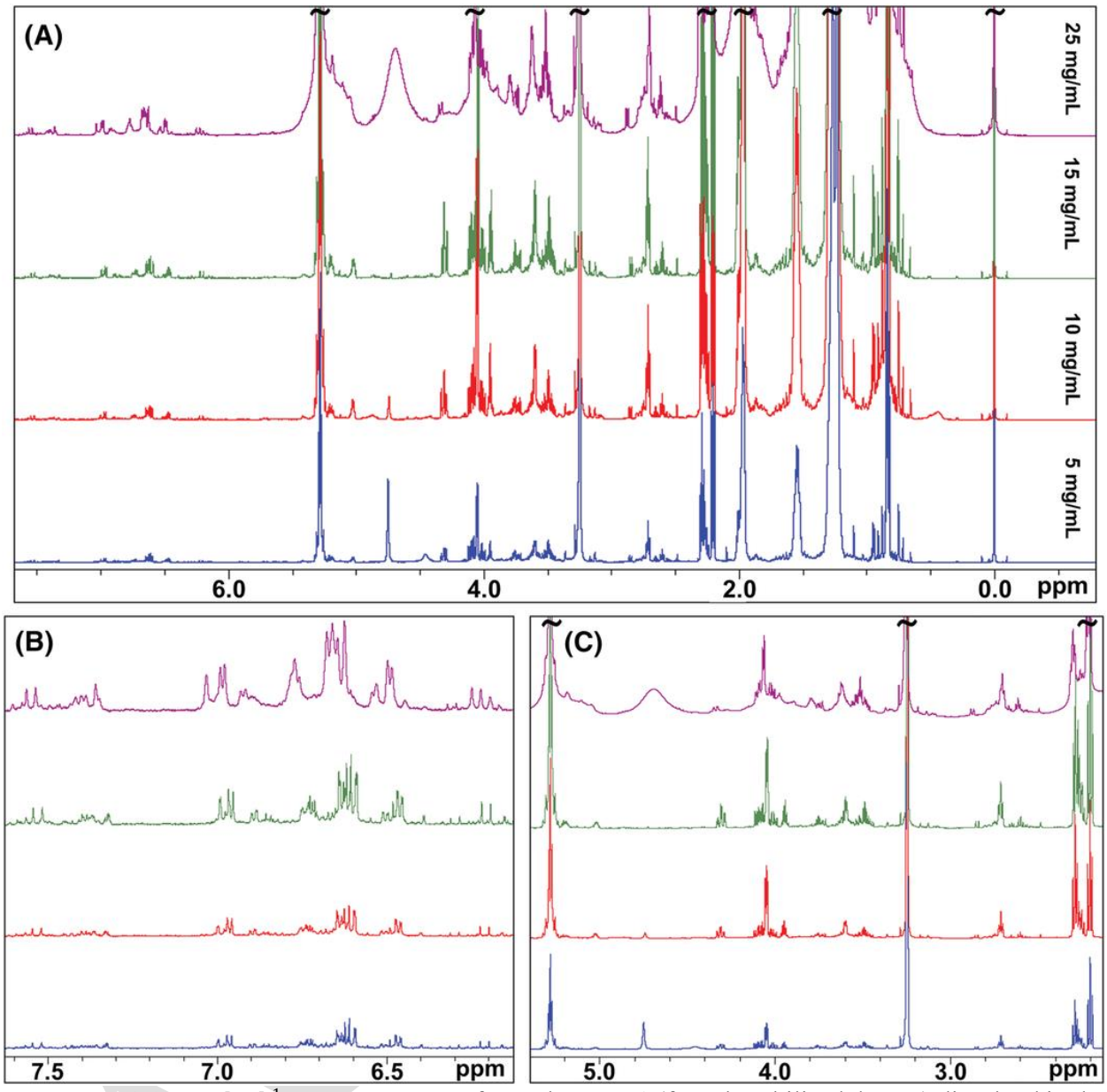

FIGURE 4(A) Overlaid ^{1}H-NMR spectra of sample TO-A1 (from lyophilised drupes) dissolved in the selected deuterated solvent (CD_3OD) in four different concentration levels: 5, 10, 15 and 25 mg/mL. (B) Zoom-in of the region between 7.6 and 6.1 ppm. (C) Zoom-in of the region between 5.4 and 2.2 ppm

In the case of the lowest concentration level spectral quality is poor and the obtained metabolites are at baseline level in many cases, and especially in the aromatic region. Specifically, metabolome coverage is not satisfactory and monitoring of minor compounds cannot be accomplished. Hence, any further statistical analysis is made difficult, whereupon it is rejected. However, the higher extreme value (25 mg/mL) led to spectra with broadened peaks and significant overlapping. The ideal concentration seems to lie within the two medium levels, 10 mg/mL and 15 /mL. Between the two options, it can be observed that the higher one presents slightly better results concerning the peak intensities of the metabolites, although the differences are not really significant to affect

the final selection. Extraction yield is a parameter influenced by the nature of each sample to some extent. Therefore, in our opinion, the starting material should be identical for every sample and any variation of the yield parameter should be attributed to the final analysis. This implies that the weight of the starting material should be determined beforehand in a way that the obtained extract should range between 10 and 15 mg, or at least close to it. Compounds, such as hydroxytyrosol and verbascoside are mentioned in Figure 4, where the richest areas and their fluctuations are also highlighted.

3.3Evaluation of the selected experimental protocol using multivariate analysis (MVA)

For the additional evaluation of the selection of experimental protocols used, the obtained spectroscopic data were submitted after their processing to MVA, in order to explore the alterations observed between the different groups. Solvent peaks (CD_3OD, $CDCl_3$ and DMSO-d_6), as well as baseline noise were excluded from the analysis. As a result, the final datasheet contained only 598 variables (from the initially 1251 variables obtained). Normalisation with the total intensity approach was found more suitable given the great variations observed in the different solvents.

Clear separation between $CDCl_3$ and DMSO-d_6 is observed in the PCA scores scatter plot formed by the first two components (Figure 5). However, samples dissolved in CD_3OD, pure or in a 1:1 ratio with $CDCl_3$, seem to share similar traits. Figure 6, depicting the biplot of the respective PLS-DA model, provides a better insight regarding the metabolite profile obtained with each solvent, verifying the observations of the unsupervised approach. Tighter clustering was expected for the three formed groups. Permutation tests with 500 permutations were also conducted to ensure this models validity (Figure S7). Interestingly, the increased polarity that 50% of CD_3OD contributes to its solvent system with $CDCl_3$ is sufficient for the dilution of many polar constituents of the extracts, hence the overlapping with the samples diluted in pure CD_3OD. This observation is confirmed by the viewed loadings in the biplot, as the majority of variables of the aromatic region (phenolics) are gathered in the bottom right quadrant. On the contrary, loadings show that clustering of the samples dissolved in $CDCl_3$ and DMSO-d_6 is due to TAGs and very few polar compounds only in the case of the latter.

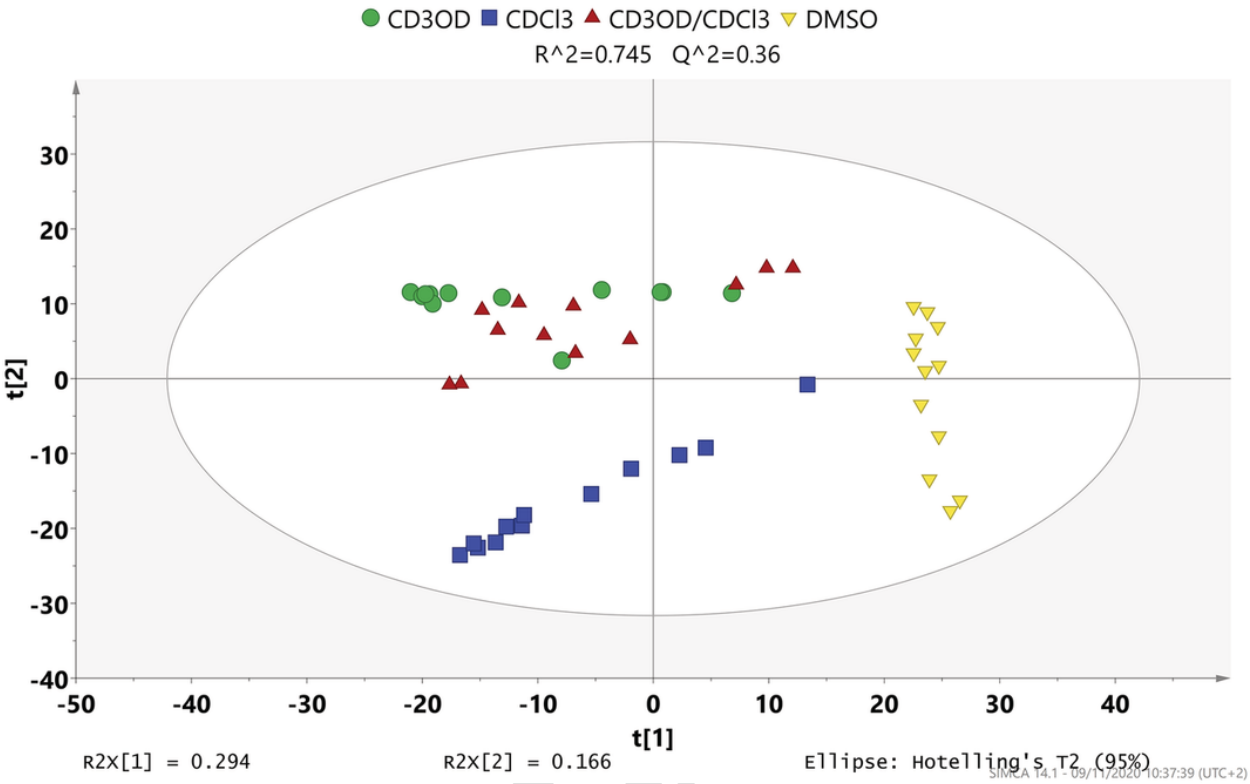

FIGURE 5PCA scores scatter plot of the lyophilised table olives' methanol extracts dissolved in four different deuterated solvents (CD_3OD, $CDCl_3$, $CD_3OD/CDCl_3$: 1:1 and DMSO-d_6) and four examined concentration levels (5, 10, 15 and 25 mg/mL)

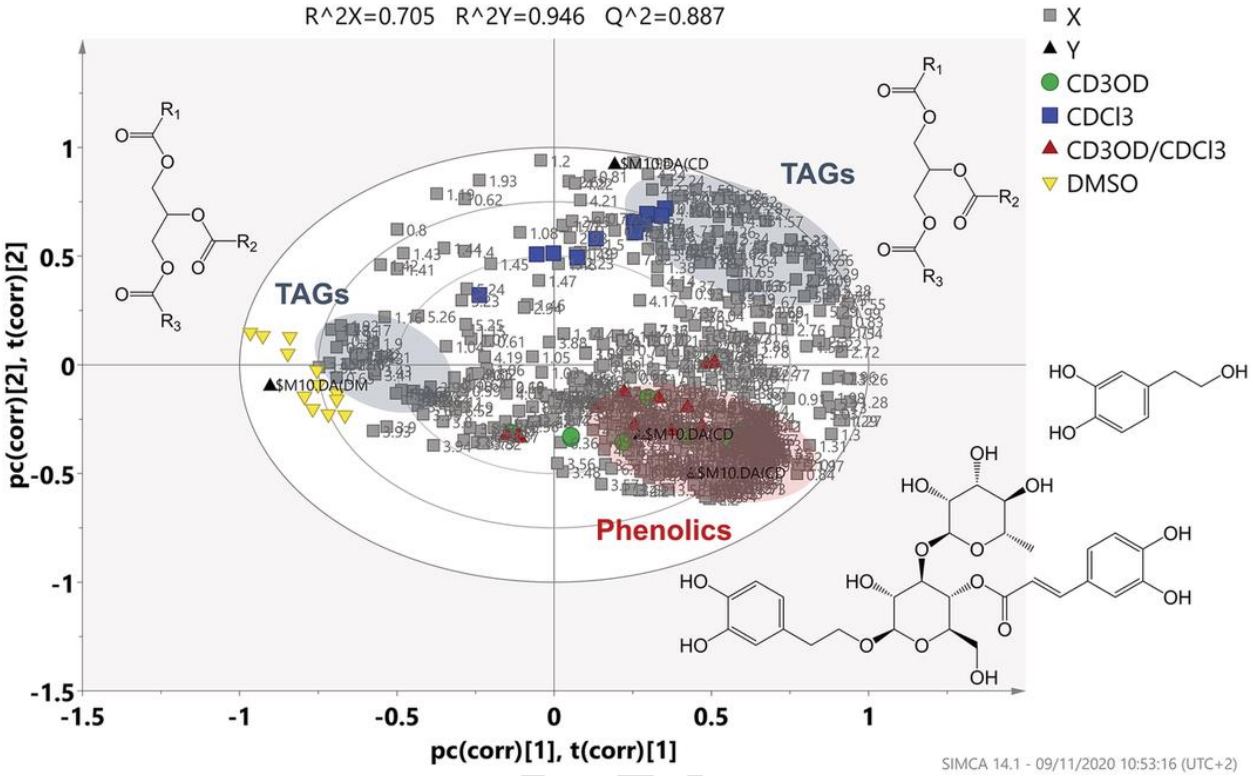

FIGURE 6 Biplot of the respective PLS-DA model with samples dissolved in four different deuterated solvents (CD_3OD, $CDCl_3$, $CD_3OD/CDCl_3$: 1:1 and DMSO-d_6) and the four examined concentrations (5, 10, 15 and 25 mg/mL). Different groups of variables are mentioned in the four different deuterated solvents used. Indicatively, 6.22 ppm: verbascoside, 6.46 ppm: hydroxytyrosol[30]

Several studies have been completed over the last decades concerning metabolic profiling and fingerprinting in food commodities (olive oil, wine, etc.), as well as in plant materials.[9] The inherent complexity of these samples due to the expected biological variability makes this task quite challenging. Moreover, the intricacy is further enhanced due to other parameters affecting their composition, such as processing or handling during analysis. For instance, in the case of table olives these are the debittering process and sample preparation procedures prior to analysis.[49] In such studies, NMR-based metabolomics are widely employed regardless if the objective of the study is targeted or untargeted profiling. However, as in any other analytical platform for that matter, using extracts of plants or food commodities requires thorough planning and optimisation and sample preparation is a crucial parameter that should not be overlooked.

Primarily, the current study indicated the variation in the chemical profiles obtained with different solvents during the extraction process and the importance of lyophilisation in the recovered extract, which were initially evaluated using HPTLC analysis. HPTLC is not a technique commonly employed in analytical experimental designs. However, a significant range of advantages arise from its use. Aside from being an easy to use and low-cost option, it requires minimum sample preparation and is substantially faster compared to LC approaches. Furthermore, the availability of a wide range of derivatisation agents could offer a greater coverage of the studied metabolome.[50] These explain the preference towards its selection during the initial screening in this study with the possibility of being used orthogonally to NMR in the future.

In the study of table olives, among the tested solvents, MeOH was selected in this case for untargeted metabolomics, as it was the most appropriate for the recovery and profiling of the largest possible number of metabolites, the ultimate purpose of this research. Determination of the most-suited deuterated solvent, sample concentration level and acquisition parameters finally used for NMR analysis, seemed to be of high-importance in order to achieve the goal of fully depicting the metabolic fingerprints of the samples.

The diverse physicochemical properties of foods' secondary metabolites lead to a really challenging task. However, it all starts with the widely-varied nature of the food commodity itself, which presents researchers with a different obstacle each time. From TAGs in table olives and olive oil, to sugars in honey and ethanol in wine, adaptability is the key in order to overcome certain hurdles that prevent the profiling of minor constituents. As shown in the study of table olives, different extraction solvents should be tested and the recovered profile has to be evaluated when a new matrix is studied for the appropriate optimisations to be made. The final choice is aligned with the final research objective, whether this is targeted or untargeted. The optimisation of extract concentration and the solubility of samples prior to NMR analysis considerably enhanced the quality of the obtained spectra in the current study. Continuous research efforts are made in order to improve the applied experimental protocols from the initial to the last step and further enrich the metabolic profile, to reduce the required experimental time and the complexity of data recovery.

ETHICS STATEMENT

Our research did not include any human subjects and animal experiments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest concerning the publication of this article.

ACKNOWLEDGMENTS

The authors would like to thank the Hellenic Foundation of Research and Innovation, EL.ID.E.K.-Flagship actions 2018-2021: “Creation of national networks in value chains of olive – The foremost flagship initiative in Greece ‘Olive roads’”. Further, the authors would like to acknowledge the European Regional Development Fund (ERDF) and Greek national funds through the Operational Program “Competitiveness, Entrepreneurship and Innovation”, under the call “Strengthening Research and Innovation Infrastructures” (PlantUP, project code: 5002803) and the European Union (EU) Olive-Net project (Horizon2020-MSCA-RISE-2016-734899) for financial support and collaboration opportunities. Finally, the authors would like to thank the Laboratory of Food Microbiology and Biotechnology, Department of Food Science & Human Nutrition, Agricultural University of Athens for providing the samples of table olives.

REFERENCES

1. Dunn WB, Ellis DI. Metabolomics: Current analytical platforms and methodologies. *TrAC - Trends Anal Chem*. 2005;24(4):285-294. doi:10.1016/j.trac.2004.11.021

2. Herrero M, Simõ C, García-Cañas V, Ibáñez E, Cifuentes A. Foodomics: MS-based strategies in modern food science and nutrition. *Mass Spectrom Rev*. 2012;31(1):49-69. doi:10.1002/mas.20335

3. Laghi L, Picone G, Capozzi F. Nuclear magnetic resonance for foodomics beyond food analysis. *TrAC - Trends Anal Chem*. 2014;59:93-102. doi:10.1016/j.trac.2014.04.009

4. Dettmer K, Hammock BD. Metabolomics--a new exciting field within the "omics" sciences. *Environ Health Perspect*. 2004;112(7). doi:10.1289/ehp.112-1241997

5. Wishart DS. Quantitative metabolomics using NMR. *TrAC - Trends Anal Chem*. 2008;27(3):228-237. doi:10.1016/j.trac.2007.12.001

6. Emwas AH, Roy R, McKay RT, et al. Nmr spectroscopy for metabolomics research. *Metabolites*. 2019;9(7). doi:10.3390/metabo9070123

7. Cevallos-Cevallos JM, Reyes-De-Corcuera JI, Etxeberria E, Danyluk MD, Rodrick GE. Metabolomic analysis in food science: a review. *Trends Food Sci Technol*. 2009;20(11-12):557-566. doi:10.1016/j.tifs.2009.07.002

8. Gallo M, Ferranti P. The evolution of analytical chemistry methods in foodomics. *J Chromatogr A*. 2016;1428:3-15. doi:10.1016/j.chroma.2015.09.007

9. Keun HC, ed. *NMR-Based Metabolomics*. Royal Society of Chemistry; 2018. doi:10.1039/9781782627937

10. Kaiser KA, Barding GA, Larive CK. A comparison of metabolite extraction strategies for 1H-NMR-basedmetabolic profiling using mature leaf tissue from the model plant Arabidopsis thaliana. *Magn Reson Chem*. 2009;47(SUPPL. 1). doi:10.1002/mrc.2457

11. Khakimov B, Bak S, Engelsen SB. High-throughput cereal metabolomics: Current analytical technologies, challenges and perspectives. *J Cereal Sci*. 2014;59(3):393-418. doi:10.1016/j.jcs.2013.10.002

12. Rubtsov D V., Jenkins H, Ludwig C, et al. Proposed reporting requirements for the description of NMR-based metabolomics experiments. *Metabolomics*. 2007;3(3):223-229. doi:10.1007/s11306-006-0040-4

13. Schripsema J, Dagnino D. Metabolomics: Experimental Design, Methodology and Data Analysis. *Encycl Anal Chem*. Published online 2014:1-17. doi:10.1002/9780470027318.a9939

14. Halabalaki M, Bertrand S, Stefanou A, et al. Sample preparation issues in NMR-based plant metabolomics: Optimisation for Vitis wood samples. *Phytochem Anal*. 2014;25(4):350-356. doi:10.1002/pca.2497

15. Gowda GAN, Raftery D, eds. *NMR-Based Metabolomics*. First ed. Humana Press; 2019. doi:10.1007/978-1-4939-9690-2

16. Smolinska A, Blanchet L, Buydens LMC, Wijmenga SS. NMR and pattern recognition methods in metabolomics: From data acquisition to biomarker discovery: A review. *Anal Chim Acta*. 2012;750:82-97. doi:10.1016/j.aca.2012.05.049

17. Ryan D, Robards K. Phenolic compounds in olives. *Analyst*. 1998;123(5):31-44. doi:10.1039/a708920a

18. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL-cholesterol concentrations (ID 1639), mainte. *EFSA J*. 2011;9(4):2033. doi:10.2903/j.efsa.2011.2033.

19. Boskou D. Olive Fruit, Table Olives, and Olive Oil Bioactive Constituents. In: Boskou D, ed. *Olive and Olive Oil Bioactive Constituents*. 1st ed. AOCS PRESS; 2015:1-30.

20. Mitsopoulos G, Papageorgiou V, Komaitis M, Hagidimitriou M. Total Phenolic content, phenolic profile and antioxidant activity of leaves and drupes in major Greek olive varieties. *Not Bot Horti Agrobot Cluj-Napoca*. 2016;44(1):155-161.

21. Boskou G. *Antioxidant Capacity and Phenolic Profile of Table Olives from the Greek Market*. Elsevier Inc.; 2010. doi:10.1016/B978-0-12-374420-3.00099-1

22. Mastralexi A, Mantzouridou FT, Tsimidou MZ. Evolution of Safety and Other Quality Parameters of the Greek PDO Table Olives "Prasines Elies Chalkidikis" During Industrial Scale Processing and Stora. *Eur J Lipid Sci Technol*. 2019;121(3):1-13. doi:10.1002/ejlt.201800171

23. Panagou EZ, Tassou CC, Skandamis PN. Physicochemical, microbiological, and organoleptic profiles of Greek table olives from retail outlets. *J Food Prot*. 2006;69(7):1732-1738. doi:10.4315/0362-028X-69.7.1732

24. Alexandraki V, Georgalaki M, Papadimitriou K, et al. Determination of triterpenic acids in natural and alkaline-treated Greek table olives throughout the fermentation process. *LWT - Food Sci Technol*. 2014;58(2):609-613. doi:10.1016/j.lwt.2014.04.005

25. Zoidou E, Melliou E, Gikas E, Tsarbopoulos A, Magiatis P, Skaltsounis AL. Identification of throuba thassos, a traditional Greek table olive variety, as a nutritional rich source of oleuropein. *J Agric Food Chem*. 2010;58(1):46-50. doi:10.1021/jf903405e

26. Rotondo A, Mannina L, Salvo A. Multiple Assignment Recovered Analysis (MARA) NMR for a Direct Food Labeling: the Case Study of Olive Oils. *Food Anal Methods*. 2019;12(5):1238-1245. doi:10.1007/s12161-019-01460-4

27. Böhme K, Calo-Mata P, Barros-Velázquez J, Ortea I. Recent applications of omics-based technologies to main topics in food authentication. *TrAC - Trends Anal Chem*. 2019;110:221-232. doi:10.1016/j.trac.2018.11.005

28. Spiteri M, Jamin E, Thomas F, et al. Fast and global authenticity screening of honey using 1H-NMR profiling. *Food Chem*. 2015;189:60-66. doi:10.1016/J.FOODCHEM.2014.11.099

29. Jakes W, Gerdova A, Defernez M, et al. Authentication of beef versus horse meat using 60 MHz 1H NMR spectroscopy. *Food Chem*. 2015;175:1-9. doi:10.1016/J.FOODCHEM.2014.11.110

30. Beteinakis S, Papachristodoulou A, Gogou G, Katsikis S, Mikros E, Halabalaki M. NMR-based metabolic profiling of edible olives-determination of quality parameters. *Molecules*. 2020;25(15). doi:10.3390/molecules25153339

31. Crawford LM, Janovick JL, Carrasquilla-Garcia N, Hatzakis E, Wang SC. Comparison of DNA analysis, targeted metabolite profiling, and non-targeted NMR fingerprinting for differentiating cultivars of processed olives. *Food Control*. 2020;114(March). doi:10.1016/j.foodcont.2020.107264

32. Bonvehi JS, Coll F V. Original article Detecting vegetable oil adulteration in hazelnut paste (Corylus

avellana L .). Published online 2009:456-466. doi:10.1111/j.1365-2621.2008.01742.x

33. APCC. Apcc Quality Standard Virgin Coconut Oil. 2009;(August):5-6. www.apccsec.org/apccsec/admin/files/11VCO Standard Flyer.pdf%5Cn

34. Kalogiouri NP, Aalizadeh R, Dasenaki ME, Thomaidis NS. Authentication of Greek PDO kalamata table olives: A novel non-target high resolution mass spectrometric approach. *Molecules*. 2020;25(12). doi:10.3390/molecules25122919

35. Moreno-González R, Juan ME, Planas JM. Table olive polyphenols: A simultaneous determination by liquid chromatography–mass spectrometry. *J Chromatogr A*. 2019;1609(460434):1-11. doi:10.1016/j.chroma.2019.460434

36. Eriksson L, Byrne T, Johansson E, Trygg J, Wikström C. *Multi- and Megavariate Data Analysis Basic Principles and Applications*. Third Edit. Umetrics Academy; 2013. www.umetrics.com

37. Michel T, Khlif I, Kanakis P, et al. UHPLC-DAD-FLD and UHPLC-HRMS/MS based metabolic profiling and characterization of different Olea europaea organs of Koroneiki and Chetoui varieties. *Phytochem Lett*. 2015;11:424-439. doi:10.1016/j.phytol.2014.12.020

38. Sahan Y, Cansev A, Gulen H. Effect of processing techniques on antioxidative enzyme activities, antioxidant capacity, phenolic compounds, and fatty acids of table olives. *Food Sci Biotechnol*. 2013;22(3):613-620. doi:10.1007/s10068-013-0122-9

39. Cabrera-Bañegil M, Schaide T, Manzano R, Delgado-Adámez J, Durán-Merás I, Martín-Vertedor D. Optimization and validation of a rapid liquid chromatography method for determination of the main polyphenolic compounds in table olives and in olive paste. *Food Chem*. 2017;233:164-173. doi:10.1016/j.foodchem.2017.04.052

40. García P, Romero C, Brenes M. Bioactive substances in black ripe olives produced in Spain and the USA. *J Food Compos Anal*. 2018;66(September 2016):193-198. doi:10.1016/j.jfca.2017.12.022

41. Bianchi G. Lipids and phenols in table olives. *Eur J Lipid Sci Technol*. 2003;105(5):229-242. doi:10.1002/ejlt.200390046

42. Mastralexi A, Mantzouridou FT, Tsimidou MZ. Evolution of Safety and Other Quality Parameters of the Greek PDO Table Olives "Prasines Elies Chalkidikis" During Industrial Scale Processing and Storage. *Eur J Lipid Sci Technol*. 2019;121(3):1-13. doi:10.1002/ejlt.201800171

43. International Olive Council. Determination of Biophenols in Oive Oils by HPLC. 2017;(29):1-8.

44. Vuthijumnok J. Effect of freeze-drying and extraction solvents on the total phenolic contents, total flavonoids and antioxidant activity of different Rabbiteye blueberry genotypes grown in New Zealand. *IOSR J Pharm Biol Sci*. 2013;8(1):42-48. doi:10.9790/3008-0814248

45. Gião MS, Pereira CI, Fonseca SC, Pintado ME, Malcata FX. Effect of particle size upon the extent of extraction of antioxidant power from the plants Agrimonia eupatoria, Salvia sp. and Satureja montana. *Food Chem*. 2009;117(3):412-416. doi:10.1016/j.foodchem.2009.04.020

46. Mikropoulou E V., Petrakis EA, Argyropoulou A, Mitakou S, Halabalaki M, Skaltsounis LA. Quantification of bioactive lignans in sesame seeds using HPTLC densitometry: Comparative evaluation by HPLC-PDA. *Food Chem*. 2019;288(February):1-7. doi:10.1016/j.foodchem.2019.02.109

47. Gandul-Rojas B, Cepero MRL, Mínguez-Mosquera MI. Chlorophyll and carotenoid patterns in olive fruits, Olea europaea Cv. Arbequina. *J Agric Food Chem*. 1999;47(6):2207-2212. doi:10.1021/jf981158u

48. Montano A, Sanchez AH, Casado FJ, de Castro A, Rejano L. Chemical profile of industrially

fermented green olives of different varieties. *Food Chem*. 2003;82:297-302. doi:10.1016/S0308-8146(02)00593-9

49. Fernández-Poyatos MP, Ruiz-Medina A, Llorent-Martínez EJ. Phytochemical profile, mineral content, and antioxidant activity of Olea europaea L. cv. Cornezuelo table olives. Influence of in vitro simulated gastrointestinal digestion. *Food Chem*. 2019;297(June):124933. doi:10.1016/j.foodchem.2019.05.207

50. CAMAG® Derivatizer | CAMAG. Accessed March 1, 2021. https://www.camag.com/product/camag-derivatizer