Metabolic profiling of complex mixtures using novel NMR-based approaches and chemometrics: Pomegranate juice as a case study

Thesis

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Abstract

Pomegranate juice is a complex mixture of structurally diverse compounds appearing in various concentrations and the composition of final product depends on several factors such as variety, geographical origin and adulteration. It is therefore an excellent system for assessing the potential of an analytical method for rapid, targeted and untargeted analysis. Here we tested the ability of 1D and 2D NMR spectroscopy techniques for the determination of pomegranate juice constituents and for evaluating the impact of various factors on its composition. The NMR spectra assignment was performed using the novel NOAH sequences and spiking with model compounds. Several metabolites including sugars, organic acids and amino acids were identified and quantified. Several internal standards were tested with potassium hydrogen phthalate and dimethylmalonic acid found to be the most appropriate, while $MnCl_2$ was successfully tested as a relaxation agent for the reduction of the experimental time. Among the pulse sequences that were tested for their quantitative potential, the simple pulse-acquire, the z-stored inverse gated decoupling, the Carr-Purcell-Meiboom-Gill (CPMG) and the QEC-HSQC experiments gave the best results. 1D and 2D NMR-based untargeted analysis was able to differentiate between various pomegranate cultivars and geographical origins, as well as detect the adulteration with apple juice. This study provides the proof of concept for 1D and 2D

NMR methods in the targeted/untargeted analysis of pomegranate juice and can be extended to other complex matrixes.

Dedication

This thesis is dedicated to those who have created innovative NMR techniques and provided new alternative analytical solutions with advantages for solving modern problems in food science, and also allowing NMR to be more widely applied in diverse domains.

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Fields of Study

Major Field: Food Science and Technology

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Chapter 1. Introduction

Food Analysis

Food analysis is a diverse and interdisciplinary field of research that has a significant health, societal and economic impact. It aims to characterize food products in terms of chemical composition, safety, quality, sensory perception and nutritional value. Food analysis approaches are used by industry, government/control agencies and academia. The molecular composition of a food product is generally very complex and depends on several factors, including genetic and geographical origin, environmental/climatological conditions, the types of farming, breeding and processing practices and addition of adulterants or presence of contaminants. As a result, the global chemical composition profiling and/or the analysis of individual compounds and their relevance to food quality, authenticity and other properties can be very challenging. In general, there is no perfect method for the analysis of all different food components in all products and current analytical methods for food evaluation are usually complementary to each other. For that reason, the development of more powerful and cost-effective analytical tools for increasing our capabilities to analyze foods rapidly and with high accuracy is a continuous and demanding research effort. The most common analytical methods for food quality assessment are mass spectrometry (MS) usually coupled to liquid (LC) or gas chromatography (GC), capillary electrophoresis (CE), infrared

spectroscopy (IR) and nuclear magnetic resonance (NMR) spectroscopy. In addition to those molecular analysis methods, other methodological approaches of biological origin, such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) are also used extensively for food analysis[1].

Nuclear Magnetic Resonance spectroscopy

NMR is a technology based on the magnetic properties of several atomic nuclei, and it is an emerging methodology for the analysis of complex mixtures, such as foods. In a typical NMR experiment, the spin nuclear magnetization of a sample that contains NMR active nuclei and is located inside a strong field NMR magnet, is excited using radio-frequency pulses. During its relaxation back to equilibrium, a signal is generated, recorded and Fourier transformed to provide the sample's NMR spectrum. NMR is a non-destructive analytical method that can determine and quantify a large number of compounds simultaneously and it is characterized by high reproducibility. It can be applied to samples of all states of matter, although most food-related applications involve liquids and solids. And under carefully chosen experimental conditions[2], it is an accurate and versatile quantitative tool. The main disadvantage of NMR compared to other technologies used in food analysis is its relatively low sensitivity. The sensitivity of the experiment depends on the instrumentation, mainly the type of NMR probe, the strength of the magnetic field, the type of the experiment (nucleus, pulse sequence, acquisition parameters) and the nature of the sample. These factors also affect the spectrum resolution. More information about the principles of NMR spectroscopy as applied on foods can be found elsewhere[3]. The most common nuclei studied in food analysis are

hydrogen, deuterium[4], carbon-13[5] and phosphorus-31[6].The NMR techniques applied to foods include high-resolution (HR) liquid state NMR[7] and HR solid state NMR[8 9], which are mainly used to obtain frequency domain spectra; low resolution, also known as low-field NMR (LF-NMR) or benchtop NMR[10], that has been mostly applied to provide time-domain signal (TD-NMR); and MRI[11] which produces images based on the differences in proton spin density and/or relaxation times between food components, mainly water and lipids. NMR has been applied for the analysis of several different categories of foods, such as fats and oils[12], beverages, fruits, vegetables, dairy[13] and meat products. Representative recent applications include food authentication, quality control, production monitoring/improvement and sensory evaluation. Liquid state NMR is ideal for the analysis of small and medium size molecules, and thus finds applications in the determination of lipids, sugars, antioxidants and other common food ingredients, although more research is required for improving our capabilities for compound identification and quantification. Larger molecules such as polysaccharides and proteins can be also studied using liquid state NMR, although several challenges may exist. In such cases, solid state NMR can be applied, to overcome limitations associated with samples of limited or no solubility.

NMR and chemometrics

The development of multivariate statistical analysis methods and their coupling to NMR spectroscopy was a game changing step in the field of food analysis. Chemometrics rendered NMR a very powerful tool for achieving a holistic and unbiased food

assessment and explore interactions and relationships between chemical profile and food properties, rather than a technique used only for compositional analysis. As an emerging field of "omics" research, metabolomics has been investigated in a large and growing body of literature. The two main approaches for metabolomics are untargeted and targeted analysis. In targeted analysis, metabolites are predefined and quantified, while in untargeted spectral comparisons are performed. Untargeted and targeted approaches are often both used at difference stages in a metabolomics study. Very recently, Markley et al. published a comprehensive review on the NMR-based metabolomics. It has been noted that mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are so far the most prevalent instruments for metabolomic determination[14].

Unsupervised pattern recognition techniques, such as principal component analysis (PCA), are applied as exploratory data analysis methodologies to observe the total variance among samples, reveal patterns and also remove potential outliers. Supervised techniques, such as partial least squares discriminant analysis (PLS-DA) are applied to investigate purposely the variations that arise from specific variables between pre-assigned groups/classes and determine the impact of specific variables on the model. Essentially, PCA reduces the dimension of original data obtained from spectra, which is normally huge. It then allows plotting, visualization and clustering data sets. PCA is a powerful clustering technique, which is able to determine different sample groups, target the principal variables that shows the most difference, and detect the correlation between these variables. Similarly, PLS-DA is applied for groups separation, however, enhanced in the classification ability by labeling different classes of samples. That's why PCA is

called unsupervised classification, and PLS-DA is supervised. Model evaluation for supervised approaches needs to be conducted by applying cross-validation, external validation and other suitable methods. If classification is achieved, identification of statistically significant markers and other critical compounds is normally carried out. PCA and OPLS-DA generate a scores plot and a loadings plot. **Figure 1** shows the scores and loadings plots of passion fruit juice upon thermal treatment[15]. Other statistical approaches that are used or they have the potential to be applied in NMR-based food analysis are linear discriminant analysis (LDA), artificial neural network (ANN) and independent components analysis (ICA). There are various platforms, such as R packages, MATLAB, SAS and various software that can be used for multivariate statistical data analysis. Advances in big data analysis and machine learning, as well as the development of databases are expected to increase the number and the quality of NMR applications in complex food analysis-related problems. Although multivariate statistical analysis is an invaluable tool for marker identification, univariate analysis, such as t-tests and ANOVA are still tools of specific importance. The representative food categories applying NMR are alcoholic beverages and fermented products, coffee and tea, fruits and vegetables, olive oils, milk and dairy products, honey, fish and meat[16]. Faraget al. conducted a comparative study of MS-based and NMR-based hop metabolomics. LC-MS and NMR showed comparable results in terms of cultivar differentiation, with bitter acids composition being the most influential compositional feature. The comparative MS and NMR analysis revealed the advantages and limitations of the two different platforms used in metabolomics, which helps with better utilization

of instrumentations for hop and similar products' research[17]. There are excellent reviews in literature that provide comprehensive descriptions and detailed information about chemometrics and their combination with NMR[18].

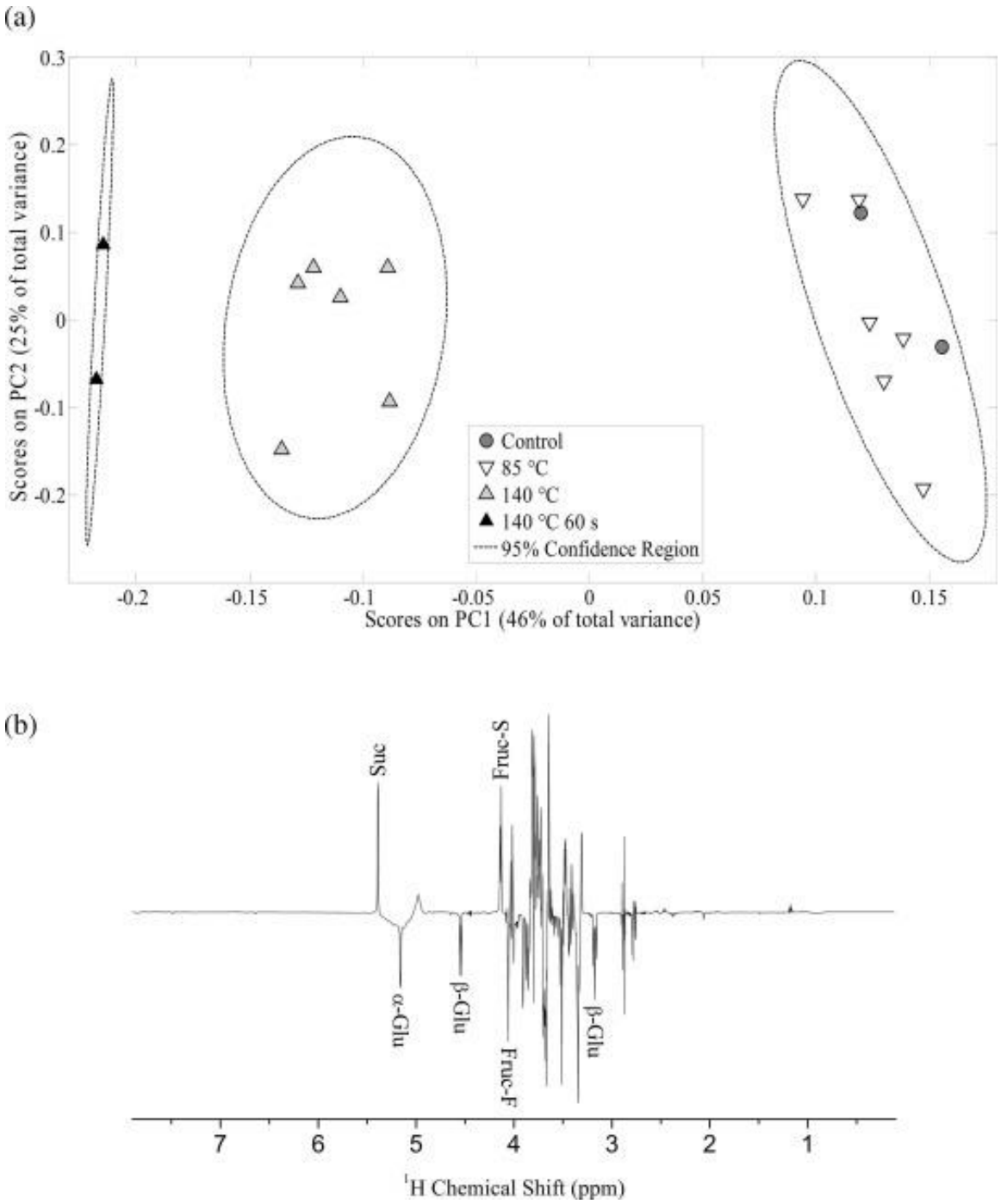

Figure 1. The scores and loadings plots of passion fruit juice upon thermal treatment (Soares et al. 2017).

Food compositional analysis with NMR

HR liquid state NMR spectroscopy represents the majority of applications in food analysis and involves the use of instruments with superconducting magnets that generate strong magnetic fields. The main reasons for the popularity of this approach are the high quality of NMR spectra obtained, which are characterized by excellent resolution and higher sensitivity compared to other NMR-based approaches, as a result of the strong magnetic fields and the sharp signals. However, HR solution state NMR is still less sensitive compared to other analytical techniques, and the limits of detection fall in the μM range, allowing thus the analysis of only a portion of the molecules that exist in a food. Additional advantages of NMR include the inherent quantitative nature of the methodology, especially for 1D applications, facile sample preparation, and the ability to utilize high throughput technologies that are routinely available for these applications. The most common nucleus utilized in HR solution state NMR studies is proton, mainly due to its high sensitivity, the short T_1 relaxation times and the enormous amount of structural information provided by the ^{1}H NMR spectrum of common food components. Interpretation of important NMR observables such as chemical shifts, *J* couplings and signal areas, which are all visible in a typical ^{1}H NMR spectrum, render ^{1}H the nucleus of choice for most food-related applications. However, even in HR analysis, ^{1}H NMR spectra often suffer from low resolution and extensive overlapping between peaks, since many foods are complex mixtures of hundreds of organic compounds. Another challenge with ^{1}H NMR food applications is water/solvent suppression. Water exists in significant amounts in most food samples, and other solvents may also exist in the final NMR

sample as a result of extractions and/or chromatographic separations. It's important to suppress the signals of such solvents in order to obtain NMR spectra of good quality, because protons from water and organic solvents present in high concentration in the NMR sample, produce signals that interfere with those of the analytes of interest. Various suppression methods, such as presaturation, watergate, jump and return, and excitation sculpting have been developed and described in the NMR literature[19 20 21], with presaturation being one of the most commonly used water suppression approaches. For metabolomics type of analyses the first increment of the noesy pulse sequence with presaturation is the most popular water suppression technique used, because it provides spectra with minimal baseline distortion compared to other methods[22]. When a mixture of solvents is present in an NMR sample, the solvent suppression can be very challenging, especially when proton exchange is involved leading to the appearance of broad solvent signals. In such cases, shaped pulses for off-resonance presaturation may be applied. The type of the NMR probe used plays also an important role in water suppression, with inverse probes generally being considered more effective compared to X-nuclei-observe probes. However, when working with inverse cryoprobes and strong magnetic fields, radiation dumping arising from the magnetization of water during suppression may become a serious problem. In such cases, the selection of a room temperature probe can be a good choice, especially when working with samples for which analyte amount or concentration is not an issue. Lyophilization can be used for the physical removal of water from food samples, whereas vacuum and/or gas nitrogen can be used for organic solvent removal. However, this is not always the best option, because it may increase the

sample preparation time, cause degradation or oxidation of sensitive food ingredients, reduce the concentration of volatiles and also induce additional factors of variance in chemometrics-related studies.

Heteronuclei, such as ^{13}C, are also used extensively in food analysis, because the ^{13}C nucleus provides much higher resolution and spectral simplicity compared to ^{1}H NMR. The main challenge with ^{13}C analysis is the long experimental times due to the low sensitivity and the long T_1 relaxation times of ^{13}C nuclei. This is not a very big problem when dealing with samples available in high amounts, or when focusing on compounds that are present in high concentration, since in this case ^{13}C NMR spectra with high signal to noise ratio (S/N) can be obtained in minutes by acquiring just a few scans. However, the low sensitivity of ^{13}C can be an important limitation when sample amount and/or analyte concentration is an issue. X-observe NMR probes, where the inner coil is used to excite ^{13}C or other heteronuclei, can provide heteronuclear NMR spectra with improved sensitivity. In most cases ^{13}C experiments are conducted using proton decoupling and thus carbon spectra consisting of singlets are acquired. Although proton decoupling increases sensitivity and spectral simplicity, the elimination of signal multiplicity eliminates important structural information from *J*-coupling with protons that can only be retrieved by heteronuclear 2D NMR experiments. ^{31}P NMR has also provided some very interesting applications in food analysis, due to the high resolution and sensitivity of ^{31}P NMR experiments. The main problem with the ^{31}P nucleus is that only a small number of phosphorus-containing food ingredients can be studied, such as for example phosphates, phospholipids, ATP and ADP. However, the derivatization of compounds possessing

labile protons that can be replaced by phosphorus, using a suitable phosphorylated reagent, opened up a new field for food-related ^{31}P NMR analysis. A similar nucleus-tagging approach has also been developed using 4-fluorobenzoyl chloride and ^{19}F NMR. Derivatization steps are generally not very common in NMR, except for the ^{31}P and ^{19}F tagging protocols mentioned above. The main drawback of such approaches is that they are destructive, in the sense that derivatization reactions alter the molecular composition and the sample matrix, and thus sample recovery for other analytical methodologies is not possible.

In addition to the standard 1D pulse sequences, other approaches have also found application in HR solution state NMR food analysis. A characteristic example is diffusion NMR, where the translational diffusion of molecules is studied. Translational diffusion can be a valuable tool for mixture analysis, molecular weight estimation and for studying biological processes such as molecular binding and aggregation. The results obtained from diffusion NMR studies can be presented either as diffusion coefficient values of molecules or as Diffusion Ordered NMR Spectroscopy (DOSY) spectra, after applying Inverse Laplace Transformation. DOSY is a pseudo-2D plot where compounds are separated along the horizontal axis based on their chemical shifts, like in a typical 1D experiment, and across the vertical axis according to their diffusion coefficients.

Other advanced NMR techniques with a great potential in food analysis include TOCSY combined with chemical shift-selective filters (CSSFs), an innovative quantification method with high selectivity. CSSF deals with the inevitable signal overlapping present in the 1D ^{1}H NMR spectra of complex food sample matrices and

compared to the conventional 2D TOCSY, it is faster and provides higher spectral resolution. Schievano et al. quantified the most commonly present sugars in honey using CSSF-TOCSY without sample extraction[23]. Also, regular 2D NMR experiments can sometimes have difficulties in resolving the signals of complex isomeric mixtures and CSSF-TOCSY combined with INEPT provides a solution for this problem based on the resolution provided by 1D ^{13}C NMR spectra[24]. Gouilleux has published several articles comprehensively discussing the principle and applications of Ultrafast 2D NMR as a real-time reaction monitoring tool[25], in screening analysis[26] and in combination with other techniques[27]. Ultrafast intermolecular single quantum coherence spectroscopy has also been applied for the analysis of viscous liquid foods by Cai et al[28]. In a review by Giraudeau et al., it was argued that quantitative Ultrafast 2D NMR, although not yet supported by a vast number of research applications, has a promising future in food analysis[29]. Other methods that have been used for NMR spectral elucidation of metabolomic mixtures, such as a novel selective TOCSY approach, are also promising tools on food analysis.

HR liquid state NMR is the approach that is most commonly coupled to chemometrics, compared to other NMR methodologies. For this reason, the development of experimental protocols that produce reliable and reproducible NMR spectra are of specific importance. It is difficult or even impossible to provide a general sample preparation protocol applicable for all cases, due the large differences in the structure and properties of various food ingredients and the significant variations observed between different food matrices. However, a usual sample preparation procedure for NMR

analysis only involves dissolution of the food product into the appropriate NMR solvent and any necessary buffer. Characteristic examples of such simple preparation procedures include the analysis of edible oils[30], juices[31] and beverages[32]. In several cases though, some type of analyte isolation and purification steps, such as extraction[33], degassing[34], and centrifugation[35] may need to be included. The solvent selection for final suspension and for extractions depends on the polarity of the compounds of interest. The most common solvents are D_2O or H_2O:D_2O for polar molecules, DMSO-d6 and CD_3OD for semi-polar and $CDCl_3$ for non-polar ingredients. In several cases, the use of solvent mixtures such as CD_3OD:H_2O, CD_3OD:H_2O:$CDCl_3$, acetic acid:H_2O and acetonitrile:H_2O is recommended. These mixtures are usually monophasic, but this depends on choosing the correct solvents ratio and suitably matching the food matrix. In water based solvent mixtures, a buffer may be used to correct for unnecessary pH dependent variations in chemical shifts. This is of specific importance when NMR is coupled to untargeted chemometrics analysis, where accurate spectral comparison is a very critical step. The buffer solution can be used during the extraction but in case there are concerns related to its impact on the extraction procedure[16], it can also be added directly to the extract. Often, a centrifuge step is needed after the final NMR sample preparation step to avoid the presence of macromolecules that have a negative impact on spectral resolution. Alternatively, the use of the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence, which allows the elimination of the NMR signals of large molecules without performing a physical separation can be applied. Although this pulse sequence is

currently used mostly for the analysis of biofluids, it has great potential for some HR food-related applications as well.

In addition to qualitative analysis, NMR has been also applied for targeted-quantitative food analysis. This is very significant in food science, since the absolute or relative concentration of a food components is often needed. Because NMR is a signal ratio method, an internal standard needs to be added when the measure of absolute concentrations is the goal. The most common internal standards for polar solvents such as D_2O include trimethyl-silyl-propanoic acid (TSP),4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), 3,5-dinitrobenzoic acid, benzoic acid, maleic acid, dimethyl sulfone and duroquinone[36]. For non-polar solvents, such as $CDCl_3$, 2,6-di-tert-butyl-4-methylphenol (BHT) is a good choice. In any case, appropriate internal NMR standards that are not overlapping with analyte signals, are non-toxic and have short relaxation times are often difficult to found. Alternative approaches are the electronic reference to assess in vivo concentrations (ERETIC) and the pulse length-based concentration determination (PULCON).

Challenges in NMR-based food analysis still exist in terms of the analysis of minor food components that do not produce strong signals in the NMR spectrum, and for molecules that have complex structures and thus generate spectra with increased complexity. Minor compounds that are still not fully resolved with NMR include various oxidation products of lipids, polyphenols, sterols and vitamins. In addition, although NMR has been successfully used for the quantification of various classes of small molecules, the analysis is usually limited by the low resolution of the ^1H NMR spectra,

where many components overlap, or the low sensitivity of the ^{13}C NMR spectra, where minor compounds cannot be detected under the common experimental conditions/analysis times. Both resolution and sensitivity are improved when analyses are performed at higher magnetic fields and are thus expected to be less important as food scientists obtain more access to high-field NMR instrumentation. On the contrary, LF-NMR instruments that use small size cryogen-free permanent magnets instead of superconducting magnets, which may fit better in an industrial environment and has been proposed as an alternative and more practical choice. For that reason, the development of techniques appropriate for LF NMR are always very important. Also, the potential of water suppression techniques for quantifications has not yet fully investigated. A similar challenge exists for a 2D NMR approaches. which can be an effective alternative when dealing with signal overlapping issues, since the 2D spectra are characterized by high resolution. Although 2D techniques are mainly used to assist the spectral assignments of various analytes and build trustworthy HR liquid state 1D NMR food analysis applications, they have the potential to become attractive tools for low field NMR applications as well, since they offer higher spectral resolution. The combination of the sequences with other NMR tools, such as non-uniform sampling (NUS), which significantly reduce the NMR experimental time, further increases their potential applications in food analysis. Only a small number of applications currently exists in literature for quantitative 2D NMR[37].

NMR in Food authentication

Typical applications of HR liquid state NMR spectroscopy in food authentication deal with geographical characterization, varietal discrimination and the detection of possible adulteration. For this type of research, the integration of NMR spectroscopic data with univariate and multivariate statistics and/or other methods of analysis is often necessary. As an example, **Figure 2** displays the biplot obtained by PCA for the discrimination between virgin olive oil samples produced by two different olive tree cultivars, Koroneiki and Cypriot, using fatty acids as descriptors for the differentiation[38]. Several review articles have been published already discussing applications and technical details of NMR spectroscopy applied for the authentication of foods. For example, Mannina and Sobolev published in 2011 a concise review of olive oil authentication and geographical/varietal origin discrimination using high resolution NMR spectroscopy. The authors discussed targeted analysis, metabolic profiling and metabolic fingerprinting, and also included some reports where metabolomic fingerprinting and targeted profiling were combined studies[39]. The authors discussed NMR and statistical protocols, providing a thorough description of the application of NMR on olive oil characterization. Consonni and Cagliani described, in a review article published in 2010, the application of NMR and chemometrics to assess the quality and authenticity of traditional food products which are produced from specific locations, or made with special recipes. The most typical example is wines labelled with Protected Designation of Origin (PDO) and/or protected geographical indication (PGI) under European Union system, which indicates their unique origin. The authors discussed geographical origin, quality and authenticity of

food, covering a great variety of commonly consumed food products[40]. Recent representative studies discussing the application of NMR in food authentication include reports on the influence of location on the biochemical composition of wild sea buckthorn[41], the varietal classification of sweet melon and correlations between variety and sensory characteristics[42], the determination of adulteration in hempseed oil[43], the authentication of cinnamon spice[44], the geographical/varietal characterization of wines[45] and the determination of the authenticity of roasted coffee[46]. The vast majority of the studies described so far are based on ^{1}H NMR analysis, however, other nuclei such as ^{13}C and ^{31}P have also been used in food authentication studies. For example, Monakhova et al. investigated the adulteration of sunflower lecithin with soy lecithin using ^{31}P and ^{1}H NMR. The authors analyzed both polar and non-polar lecithin extracts, reporting several phospholipid and other compounds, including linolenic acid and stachyose were useful for authenticity verification[47]. In another study, Kamal et al. used ^{13}C NMR-based metabolomics for the biochemical profiling of Asian soy sauces[48], while a study by Erich et al. paired ^{13}C NMR with ^{1}H NMR in a chemometric analysis aiming to discriminate between conventional and organic milk products[49]. Wei et al. applied similar ^{13}C NMR metabolomic approaches to achieve the classification of green coffee beans according to their variety and origin, and overcome spectral misalignment issues in the ^{1}H NMR classification analysis, as the ^{13}C NMR spectra were found to be less or not at all affected by molecular interactions. Thus, a clear separation of variety and origin was evident on the PCA and OPLS-DA analysis plots derived from the ^{13}C NMR spectra, with sucrose, caffeine and chlorogenic reported as the compounds responsible for the classification[50].

The drawback of this approach is the longer experimental times associated with ^{13}C NMR analysis. Lee et.al characterized the profile of green tea cultivated in three different areas with distinct climate conditions using ^{1}H NMR. The geographical origins of the green teas were also discriminated and the compounds responsible for differentiation were identified. By studying chemical composition correlations to the regional climate conditions, the authors concluded that succinate syntheses of green tea is correlated with, and it is promoted by high temperature, long sunlight exposure and high rainfall[51]. Clausen et al. characterized sour cherry juice made from seven different clones/cultivars using ^{1}H NMR and also carried out their sensory evaluation. The correlation of metabolites profiled by NMR and sensory attributes was investigated, noting that glucose and malic acid were directly correlated to sweetness and sourness attributes. They also found some correlation between other juice metabolites and sensory features[52]. To discriminate different cultivars of mango juice, Koda et al. performed a metabolomics analysis of band-selective ^{1}H NMR data. Because the dominating signals of sucrose, fructose and glucose were removed, using selective excitation they were able to increase considerably the sensitivity of the obtained spectra, and thus extract valuable information on minor juice components. The compounds contributing significantly to cultivar classification were identified as the amino acids arginine, histidine and phenylalanine[53]. A study by Vigneau and Thomas explored various model calibration, feature selection and data pre-treatment methods for authenticating orange juice adulterated by clementine juice, based on ^{1}H NMR obtained compositional data. Correspondingly, they found that

the repeated stratified cross-validation, Covsel, logarithmic transformation and pareto scaling led to models of optimum performance in their study[54].

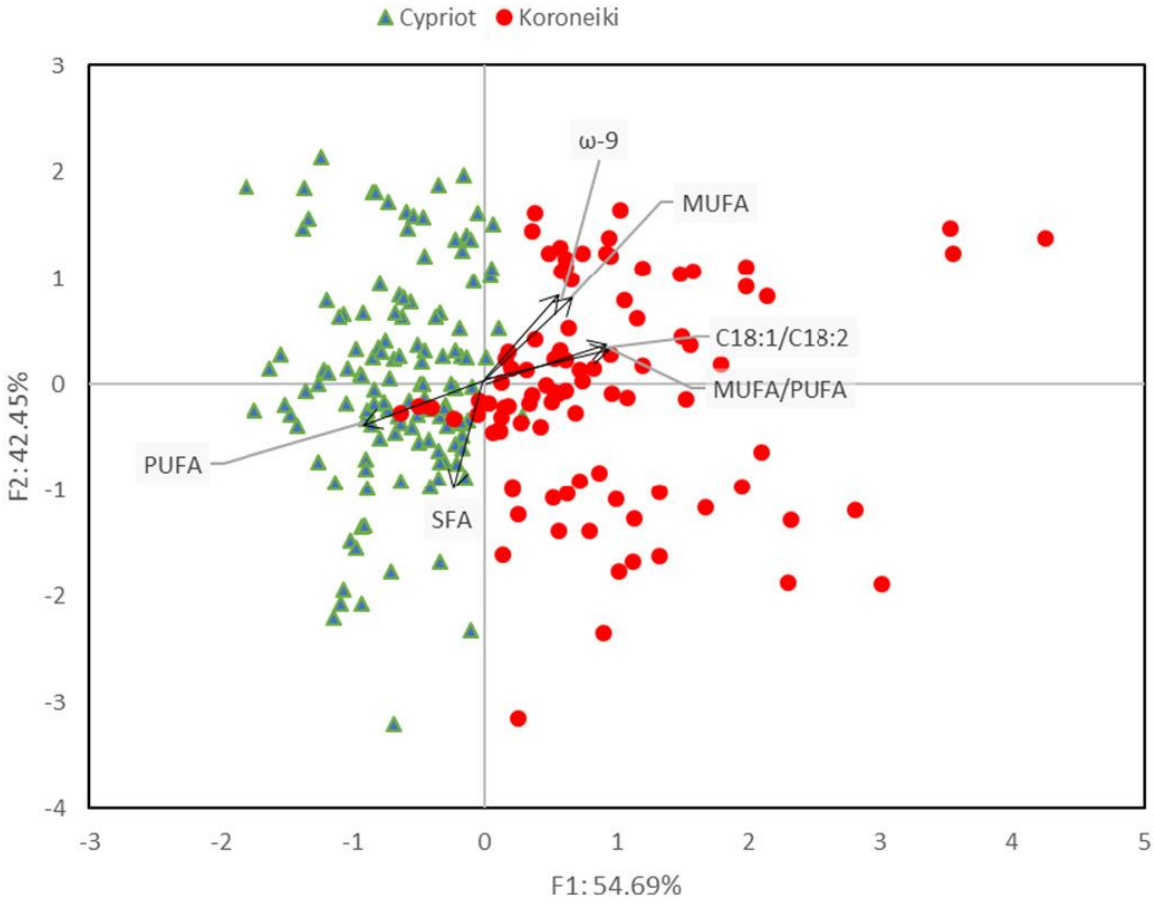

Figure 2. Biplot obtained by PCA for the discrimination between virgin olive oil samples produced by two different olive tree cultivars (Kritioti et al. 2018).

Food authentication has become one of the most serious issue in regard to food quality and safety. NMR plays an important role in adulteration detection. The most extensively reported application of NMR on food adulteration studies are food in liquid form, such as oils or beverages. Among those commonly adulterated food, fruit juice is said to be more often as it can be adulterated easily but it's not easy to inspect by tasting or appearance[55]. Despite many advantages of NMR techniques, such as simple pretreatment, fast, non-destructive, it's not yet widely used in society. NMR's application in food authentication

is limited to date as it is expensive and requires costly maintenance. To be available to more inspection and regulatory institute around the world for food adulteration detection, the price needs to be further decreased to be more affordable[56].

Pomegranate juice

Pomegranate (*Punica granatum L.)* is an ancient fruit originated from Persia (Iran), and has carried a rich cultural symbolism in addition to consumption for its flavor and nutrition. Various pomegranate products, including pomegranate juice (PJ), have been widely and commonly consumed by people around the world for a long time. It has been reported that the total juice and juice drink sales in US fell to $ 19.2 billion in 2018 and is predicted to fall below $ 19 billion in 2019 mainly due to consumers' concern on high sugar content and calories. However, PJ consumption and sales continue growing although traditional juices have struggled. Young generations of consumers look for juice products with novel flavors, functional benefits and clean labels. With PJ meeting these requirements, it is not surprising that its consumption has grown rapidly[57].

Numerous studies on physicochemical[58 59 60]and compositional analysis of PJ have been published due to the growing popularity. In addition to being applied for product evaluation, compositional analysis at the molecular level can also be used for the development of new breeding strategies and new cultivars rich in health-promoting compounds such as anthocyanins, as well as for optimizing or monitoring the effects of processing protocols. The components of pomegranate fruit and juice are shown in **Table 1**. A discussion of published studies on profiling sugars, organic acids, amino acids and other groups of compounds will be presented later.

The composition of pomegranate fruit and thus PJ, is affected by many factors, such as variety, maturing stage, storage conditions, growing environment and juice processing protocol. Several studies have been conducted for evaluating differences caused by these factors using proximate analysis, such as Brix, titratable acidity, pH, turbidity, color change, and antioxidant activity. However, only a few studies have been carried out focusing on a more global compositional profiling.

Due to its higher economic value compared to other fruit juices, PJ is a subject of adulteration, which rises considerable public attention. It is found that commercial PJ often can be adulterated with cheaper juices, such as apple juice, grape juice, or cranberry juice[61]. Corn syrup or citric acid have been also found to be added in commercial PJ labeled as 100% pure juice to adjust the taste of poor-quality PJ[62]. The detection of adulteration can be a challenging task, because of the compositional similarities between PJ and potential adulterants, as well as because of the various factors that affect PJ composition and increase the complexity. Several attempts have been made to develop methods for the detection of the adulteration of PJ. HPLC, GC-MS, IR, and UV-VIS were suggested to be used for the authenticity determination.

Although NMR is rapidly becoming a key technology in food analysis, it hasn’t been used in PJ compositional analysis and authentication. Only one study published by Chater et al. in 2019 used NMR for quantifying fructose, glucose and citric acid in PJ[63].

Table 1. Characteristics of pomegranate fruits and pomegranate juices (El-Nemr et al. 1990).

Characteristics	Values
Average fruit wt.	284 g
Average size	313 cm^3
Specific gravity	0.91 g/cm^3
Peels	38 % of fruit wt.
Internal lamella	10 % of fruit wt.
Edible parts	52 % of fruit wt.
Juice	78 % of edible parts
Seeds	22 % of edible parts
Fresh juice	
Moisture [%]	85.4
Total sugars [g/100 ml]	10.6
Reducing sugars [g/100 ml]	10.5
Non-reducing sugars [g/100 ml]	0.1
Pectin [g/100 ml]	1.4
Total acidity (as citric acid) [g/100 ml]	0.1
Ascorbic acid [mg/100 ml]	0.7
Free amino nitrogen [mg/100 ml]	19.6
Ash [g/100 ml]	0.05
Minerals [ppm]:	
Iron	0.2
Cupper	1.0
Sodium	3.0
Magnesium	2.4
Potassium	49.2
Zinc	—
Manganese	—

Compositional analysis of PJ

The compositional analysis and the evaluation of physicochemical properties have been carried out on different parts of pomegranate fruits of different varieties all over the world. Pomegranate compositional analysis includes proximate/crude composition by AOAC, AOCS and AACC approved methods in earlier studies, as well as compound

profiling at the molecular level and elemental composition. Crude chemical composition analysis of pomegranate includes moisture, total sugars, total acidity, total free amino acid, total protein, total lipids, total phenolics and has a wide industrial application since pomegranate fruits naturally contain a wide variety of compositions and nutrition facts are desired by consumers. The molecular profiling was either conducted on one category of interest or multiple groups, elucidating a more detailed information on each group[64]. Elemental analysis is usually used for determining minerals[65 66]. In this section, we will discuss the PJ compositional profiling, and emphasize the dominant compounds found/revealed in PJ matrix.

Sugars and organic acids consist the majority of soluble solids in PJ, and HPLC coupled with refractive index detector has been used for sugar profiling in all followed literature. Melgarejo et al. 2000 quantified fructose (6.58 g/100g) and glucose (6.14 g/100g) in PJ made from 40 Spanish pomegranate cultivars. They were able to quantify sucrose at very low level, and only qualified maltose at trace amount[67]. Fadavi et al. 2005 found glucose (3.50 to 5.96g/100g) and fructose (3.40 to 6.40g/100g) in PJ made from ten Iranian pomegranate cultivars. Sucrose and maltose weren't not detected by them[65]. Aziz Ekşi, and İzzet Özhamamcı 2009 analyzed PJ reconstituted from 23 PJ concentrates. They detected glucose (46- 66 g/L) and fructose (48- 70 g/L) and found traces of sucrose in 6 out 23 samples[68]. Dafny-yalin et al. 2010 evaluated the sugar profile of PJ made from 29 Israel pomegranate accessions. They found similar content level of glucose and fructose, both varied from 4.8 to 6.6 g/100 g. In addition, maltose was found at trace amount in a few accessions[69]. They correlated total soluble solids (TSS) and sugar

content determined by HPLC in PJ on the basis that TSS is used to measure sugars, acids and salts proximately. In addition to glucose and fructose, low concentrations of arabinose and sucrose were found in Tunisian PJ as well[70]. To conclude, glucose and fructose have been identified as the major sugars in PJ, while the content varied due to the natural variation and measurement errors. Sucrose, maltose and arabinose were found to exist at very low content, even below quantifiable or detectable level, thus resulted in controversy reports in literature.

Melgarejo et al. 2000 identified organic acid profile of 40 Spanish cultivars. They found citric acid (0.28 g/100 g), malic acid (0.14 g/100g). Oxalic acid (0.03 g/100g) presented at very low content. Tartaric and acetic were found be quantifiable only in some cultivars, while lactic, fumaric acid were not quantifiable in most of the cultivars[67]. Aziz Ekşi, and İzzet Özhamamcı 2009 used enzymatic methods for determining the contents of citric acid (6.6-13.6 g/L), L-malic acid (0.5-0.9 g/L), D- isocitric acid (3.9-86 mg/L)[68]. Dafny-yalin et al. 2010 detected five organic acids in PJ. They were able to correlate titratable acidity (TA) with citric acid and malic contents determined by HPLC due to their predominant content. TA is used for crude measurement of exiting acids in juice. Oxalic and succinic acids showed poor correlation with TA due to the low content and severer natural variations[69]. The ratio of citric acid and malic acid was found to be dependent on pomegranate cultivars from different regions. For example, Hasnaoui et al. 2011 studies organic acids constitution in PJ of 30 Tunisian accessions and found that malic acid (~50% of) was the predominant organic acid followed by citric acid (~23%), succinic acid (~17%), oxalic acid (~7%), tartaric acid (~2%), and ascorbic acid

(~0.3%)[70]. However, Türkyılmaz 2013 studied nine varieties of Turkey pomegranate, and found citric acid being the major organic acid in PJ, followed by malic (6–12%), succinic (5–19%), nonidentified (0–14%) and tartaric acids (0.1–3.7%)[71].

Tezcan et al., 2013 detected 11 amino acids including chiral separated amino acids in both freshly squeezed juices and commercial PJ samples. They found the predominant amino acids in fresh PJ were serine (756 mg/L), proline (773 mg/L) and alanine (363 mg/L), and also a significant decrease of amino acids content in commercial PJ reconstituted from concentrate, especially L-proline. The disadvantage of this research was that not all amino acids were examined[61]. Only a few studies have been carried out on amino acid profile of PJ. A more recent research by Li et al. 2016 carried out amino acids profiling of pomegranate from China. In this study, the major amino acids were found to be glutamine, serine, asparagine, alanine, and γ-aminobutyric acid (GABA)[72]. The results were different from previous study by Tezcan et al., which could be caused because glutamine and GABA were not examined in that study, or because that glutamine and proline both were converted from glutamate during amino acid biosynthesis in fruits. In, addition, the pomegranate fruits' cultivar, growth condition and detection methods of amino acids might also result in the differences.

A variety of studies have been reported on polyphenols, phenolic compounds or anthocyanins in PJ due to its antioxidant activity[73 74 75]. A recent review comprehensively discussed these phytochemicals in PJ and their correlation with potential health benefits[76]. A few studies on volatile composition of PJ have been published where sensory profile was correlated as well[77 78]. Free fatty acids profile of pomegranate seeds

has been studied using gas chromatography in a number of papers due to its high content in unsaturated fatty acid[79 80 81]. NMR has also the potential for fatty acid profiling of pomegranate seeds as well, which might be future work in our lab.

Factors affecting PJ composition

Cultivar and geographical origins

Various methods of genotyping have been used to identify different cultivars. The amplified fragment length polymorphism (AFLP) analysis of DNA was used for successful discrimination of 34 Tunisian pomegranate cultivars[82]. Chloroplast DNA and barcode Genes was applied to investigate the genetic variability of pomegranate[83]. Restriction Fragment Length Polymorphisms (RFLP) coupled with Polymerase Chain Reaction (PCR) techniques was used to differentiate five Spanish pomegranate cultivars[84]. Microsatellite markers were used to characterize 32 Tunisian pomegranate cultivars[85]. Genotyping is a reliable tool for identifying different cultivars, and also can be time-consuming and expensive. What's more, there's barrier between the genotypes and phenotypes, limiting further applications where knowledge on physicochemical properties or metabolites composition at molecular level are required.

Previous research on pomegranate cultivars often focuses on physical-chemical properties and antioxidant activities[86]. Significant differences in various aspects have been found. Total phenolic content is reported to have great variability, which is also determined to have a correlation with antioxidant activity[87]. In addition, it has been shown that the yield of juice production is considerably dependent on the cultivar, which is a crucial aspect for commercial pomegranate juice[88]. In recent years, the development

of metabolomics and multivariate statistical analysis allows a more advanced approach for evaluating and differentiating different pomegranate cultivars, and correlate to specific metabolites within PJ matrix. Targeted metabolite profiling and PCA were used to identify different pomegranate genotypes[89]. Organic acids in PJ was profiled using HPLC/UV, and then used in multivariate statistical analysis for discriminating geographical origins of Iranian pomegranate cultivars[90].

Other factors

In addition to cultivar or geographical origins, a number of other factors on compositional variation of PJ have been investigated, such as maturing stages, climate condition, soil quality and use of fertilizer. Previous studies have reported the physical-chemical characteristics changes of pomegranate during ripening[91 92]. It has been reported that the anthocyanin profile changes during ripening. Although no significant differences in total soluble solids, pH, titratable acidity and skin color were observed within one month in cold storage[93]. It's reported significant changes in total phenolics, total tannins and condensed tannins during the growth and ripening of pomegranate fruit[94]. Climate condition is often correlated with geographical origins. Borochov-Neori and Li, Halilova and Yildiz (2009) conducted a simple study focusing only on the effects of the climate change on proline accumulation in pomegranate. By comparing the proline content in regard to the rainfall volume between year 2007 and 2008, it was argued that there is an increased proline accumulation in pomegranate fruits during hot and dry years[95]. The definition of soil quality is specified by the crop, and there are many controversial indicators to determine the quality of soil. Fertilizers also greatly change the quality of

soil. A number of researches have reported significant influence of soil on the physical-chemical properties of pomegranate fruit. It has been suggested that the yield and quality of pomegranate is significantly increased by using mulches, especially polyethylene[96]. Similarly, it appears that the use of Mn spray significantly affects the anthocyanin content of pomegranate[97]. However, it is well known that anthocyanin composition varies greatly and is affected by many factors.

Post-harvest processing has significant impact on composition of commercial PJ as well where pomegranate fruits undergo various physical-chemical changes to be final juice products. The main processing steps include juice extraction methods, clarification and pasteurization. And there's a gap on extraction methods between in-house made samples by researches and commercially produced samples. In-house made PJ samples are usually pressed only from arils. However, commercially manufactured PJ products are usually pressed from whole fruits or sacs[98 99 73]. The gap brings difficulties on translating results found by researches into commercial applications. Although clarification is usually used to remove macromolecules such as pectn, starch, polyphenols or gums for preventing the formation of cloud juice, it was also found to decrease anthocyanins content significantly[100 101 102 103 104]. Current studies showed that proper pasteurization causes little change on physicochemical property of PJ[100 101 102 105].

Pomegranate juice authentication

PJ has been proved to have various beneficial effects by numerous studies, thus it has been receiving more popularity and increase in consumption. Due to its health-promoting properties, unique taste, attractive appearance and limited production,

adulterants are often added to lower the cost or adjust for the low-quality of certain PJs. Hence under the globalized supply chain system, it's of great importance to establish reliable methods for the detection of adulteration to prevent consumers from being cheated, especially those with health issues who need more its beneficial health effects. However, the significant variations in chemical composition based on cultivars, growing region, ripening stages, climate, storage and processing practices have made the determination of adulteration a very challenging work. So far, researchers have explored many techniques for the detection of adulteration of pomegranate fruit products, mainly juice, and juice concentrates. and molasses.

Genotyping have been used for identifying different pomegranate cultivars but was rarely used for PJ authentication. A study by Marieschi et al. investigated the development of Sequence-Characterized Amplified Regions (SCARs) markers for detecting pomegranate products adulteration by 10 other fruits[106]. Chromatography, spectroscopy and mass spectrometry have been more commonly used for PJ authentication. Borges et al. analyzed wonderful PJ and three other commercial PJs using HPLC-PDA-MS to find specific polyphenol compounds as markers for adulteration with from red grapes[107]. Similarly, Brighenti et al. used the same platform to analyze PJ and peel extract[108]. A comprehensive analysis focused on polyphenol compounds was done in that study, which can be used to determine the adulteration by peel extract[108]. Mass spectrometry-based targeted and untargeted metabolomics was used for the detection of PJ adulteration with apple juice and grape juice in a recent study. By

applying PCA and PLS-DA, the authors were able to detect adulterated PJ to 1%. In addition, a list of markers driving the discrimination were identified[109].

Vardin et al. collected seventeen commercial pomegranate juice concentrates (PJC) samples from Southeast Turkey and they also prepared PJC in the laboratory from fruits of different cultivars. The adulterant in this case was grape juice concentrate (GJC) made from one type of grapes by researchers. Then they conducted tests on titratable acidity, PH, total soluble solids, and performed Fourier transform infrared (FTIR) analysis. Principal component analysis (PCA) was used to find the marker of adulteration. Then partial least square (PLS) analysis was used to predict the concentration of the adulterant in PJC. The absorbance occurred due to the C=O stretching in the infrared spectra was found to be able to differentiate between the pure and adulterated PJC[110]. UV-Visible spectroscopy coupled with multivariate analysis was utilized to detect PJ adulteration with filler juices and water. The authors claimed that they developed a model using in-house made PJ, commercial juices and water/filler juice adulterated PJ samples, which could be a fast, cheap screening method of the detection of filler juices and water in PJ[111]. The new chiral micellar electrokinetic chromatography-laser induced fluorescence method was developed by Tezcan et al.. In this study, three pomegranate fruits and three commercial pomegranate juices were used to establish the method with apple juice as the adulterant. They discovered the amino acid, L-Asn, as the marker[61].

Ja´uregui et al. and Darra et al. have done a relatively more comprehensive research in this direction. They applied HPLC for organic acids and sugars, atomic

absorption-emission spectrometer for mineral analysis, UV-VIS spectrophotometer for proline, and GC/MS for volatile compounds. The main markers for the detection of adulterated PJ with grape juice and peach juice were found[112]. Darra et al. designed a protocol for the detection of PJ adulteration with molasses; first using UV/VIS spectroscopy, then HPLC-DAD, and finally ATR-FRIT[113]. Zhang et al. instead of using a small number of PJ samples, they used 45 samples from the United States and databases of PJ from many other countries to develop the protocol. They considered many possible materials as adulterants, such as sugars, deeply colored fruit juices and peel extracts. An algorithm with 7 steps, including complete chemical profiles, was developed for the determination of authenticity. The ranges of each markers were provided together with the exceptional situations to avoid determining adulteration falsely. The IMAS algorithm protocol was offered with clear instructions and was the most thorough study. It is also the most time-intensive and laborious method[62].

The methods mentioned above had many limitations. They suffered from one or more of the following issues: not sufficient sample size for building a model that can achieve accurate predictions; studied only one type of adulterant, and using only one criterion; profiling only one group of compounds, such as amino acids or anthocyanins; when more examinations on various category of components were performed, this leaded to more time-consuming and costly analysis due to the need of diverse instrumentations.

Chapter 2. Statement of the Problem

Analytical methods, such as chromatography with mass spectrometry, have been used to characterize the composition of PJ and detect biomarkers that can be potentially used for PJ authentication and the profiling of pomegranate fruits for improving nutritional value. Despite the relative success of these techniques they are generally elaborate and time consuming because they require several separation and purification steps that dramatically increase the length of the analysis, while focusing on one nutrient compound at a time. In addition, they often require the use of calibration curves, which also increases the length of the analysis and the use of model compounds that are used as reference standards, which are often expensive or commercially not available. For these reasons, the development of novel and efficient analytical methods that can be used as quick and accurate screening tools for analysis and assessment of PJ is needed. In a 2013 study, the use of NMR spectroscopy for the rapid and reliable analysis of some components in PJ was reported[114]. The NMR spectrum was proved to be very informative and promising tool for the analysis of PJ, thus additional and more rigorous studies are required to assess the application of NMR spectroscopy as a rapid and efficient tool for the analysis PJ. Our studies will focus on investigating the potential of various NMR experiments for the determination of various PJ ingredients and test the ability of NMR, coupled with chemometrics for PJ evaluation and authentication.

Chapter 3. Objectives

Compositional analysis of PJ and q-NMR

1) Perform signal on ^{1}H , ^{13}C and 2D NMR spectra of PJ and determine diagnostic peaks of specific compounds for quantification.
2) Evaluate the potential NOAH super-sequence compared to conventional 2D experiments for complex mixture analysis.
3) Evaluate the performance of multiple NMR pulse sequences as quantitative tools and demonstrate the proof of concept for the application of $MnCl_2$ for reducing experimental time for quantitative experiments.

Untargeted NMR-based metabolomics for PJ authentication

1) Investigate the potential application of NMR coupled with chemometrics for the discrimination of PJ according to the cultivar and the geographical origin and detect PJ adulteration by apple juice.
2) Investigate the potential of 2D NMR-based metabolomics for PJ authentication.

Chapter 4. Metabolic profiling of complex mixtures using novel NMR-based approaches and chemometrics: Pomegranate juice as a case study

1. Introduction

Food consumers are placing emphasis on health and wellness and they seek for food products rich in nutrients and bioactive compounds associated with various health benefits. In the last years, there is an increased interest in the consumption of pomegranate juice (PJ) as a product of high nutritional and commercial value because of its composition in health promoting compounds and its organoleptic properties[115]. The consumption of PJ is linked with several perceived health benefits[116 117 118 119 120] and its growing popularity is reflected on its increased production and consumption worldwide. PJ is considered as a gourmet food with superior properties compared to other fruit juices and thus it is more expensive[115 121]. For that reason and given the complexity of the global food supply chain, PJ may be a subject of adulteration, which is misleading and a disservice to consumers[110]. Also, it may reduce some of its health benefits, while carries a potential safety risk considering that PJ juice becomes popular to people with medical conditions. Therefore, the food industry and food control laboratories need rapid and reliable tools that can be used for the PJ screening and analysis and guarantee its authenticity and thus the reliability of the producers and sellers. Because the composition of pomegranate fruits varies according to the variety and geographical origin[110], such tools can be used for the metabolomic profiling of different pomegranate fruits for the development of new breeding strategies and/or new cultivars for the production of fruits

rich in health promoting compounds with advanced nutritional value, health benefits and traits.

Despite the advances in the development of analytical tools for assessing composition and metabolic profiles of complex mixtures, such as PJ, traditional methods of analysis suffer from many limitations. For example, liquid chromatography requires extraction and purification steps, it is laborious/time-consuming, focuses on one nutrient compound at a time and produces organic solvent waste. Additionally, for compound quantification, which is of specific importance in many scientific fields that involve chemical analysis, traditional methods require high purity internal standards with similar structure characteristics with the analyte, which are often either not available or expensive.

In contrast, NMR spectroscopy is non-destructive, rapid, highly reproducible, requires minimal or no sample preparation and can be efficiently used for the screening of a large number of samples. NMR analysis makes feasible the determination of several different molecules at one snapshot, without using separation, derivatization and purification steps, which provides a significant advantage compared to traditional analysis. Limitations of NMR spectroscopy include low spectral resolution of ^{1}H 1D spectra, low sensitivity, especially for the ^{13}C NMR analysis and the high cost of high field instruments, which are often required to overcome these limitations. Although NMR has mainly been applied for structure elucidation, its applications for quantification have increased dramatically in the past few years due to its inherent advantages[5]. However, there are still many challenges related to the development of NMR as a quantitative tool,

including the choice of internal/external standard and the long relaxation times which affect experimental time. Another challenge is related to the pulse sequences that can be used for quantitation. The simple pulse-acquire and the inverse gated experiments are well established methods for ^{1}H and heteronuclei-based quantifications respectively. However, in food analysis and metabolomics, because of the sample complexity, other sequences must be used in most cases. Characteristic examples are the use of water suppression to deal with overlapping and dynamic range issues, the use of pulse sequences to eliminate signals of macromolecules, and the application of 2D sequences for increasing total spectral resolution. An essential scientific question is therefore if these sequences can generate quantitative results and which experimental factors affect the analysis. Similarly, in most untargeted metabolomics studies, the 1D noesy with presaturation pulse sequence is used. Although this is a reliable approach, it often does not allow the separation of signals in overcrowded spectra. This limitation restricts the wider application of NMR especially with low-field instruments, which due to their low cost and convenient operation, have the potential to become a game-changing platform in chemical analysis.

In this study we applied multinuclear and multidimensional NMR spectroscopy and chemometrics to determine the biochemical profile of PJ using a single direct analysis. The arils of pomegranate fruit are rich in compounds such as organic acids, sugars, and amino acids, while there are controversial data in literature about some of its ingredients. Therefore, PJ is an ideal system to investigate the potential of NMR analysis. The signals of specific biologically important metabolites that exist in PJ were assigned

using the novel NOAH (Nested NMR by Ordered Acquisition using 1H-detection) pulse sequences[122 123] , first time used in mixture analysis. In addition, we performed the first systematic study for the evaluation of the quantitative potential of various NMR experiments for the determination of individual compounds with minimal or no sample preparation and we tested the potential of $MnCl_2$ as a relaxation agent for reducing the length of analysis. Finally, we examined the potential NMR approaches for untargeted analysis related to cultivar and geographical area discrimination and the detection of adulteration. We hypothesized that various PJ cultivars have distinct metabolic profiles and can be detectable *via* NMR spectroscopy and that NMR can detect the adulteration of PJ with apple juice (AJ).

2. Materials and Methods

PJ and AJ samples: PJ obtained from fresh-pressed pomegranate fruits of three varieties, Wonderful (n = 30), Granada (n = 30) and a Chinese cultivar (n = 17). Wonderful fruits were provided by POM Wonderful, while Granada and the Chinese cultivar were purchased at local grocery store. A total of 40 Commercial PJ samples were obtained with 20 different brands and 2 bottles of each brand. Commercial PJ from USA (n = 12), Middle East countries (n = 20), unknown location (n = 8) as well as Commercial AJ (1 bottle for each brand, n = 6) were purchased from local retail markets or online markets. Because this study does not focus on developing models for adulteration detection but rather providing the proof of concept about NMR's capabilities. The PJ/AJ blended samples were limited by only preparing mixtures of AJ and PJ at a 1:1 ratio.

Sample preparation for NMR analysis: For the untargeted analysis, 0.8 ml of juice were centrifuged at 13,000×g for 5 min. 0.3 ml of the supernatant were placed into a 5mm NMR tube and mixed with 0.3 ml of buffer (100 mM H_3PO_4/KH_2PO_4, 1mM EDTA-2Na[124]) in D_2O which contains 10 mM of Trimethylsilylpropanoic acid d_4 (TSP d_4) and 10 mM NaN_3. The buffer and EDTA-2Na were used to minimize pH-dependent and ionic strength-dependent chemical shifts, respectively. TSP was used for calibrating chemical shifts and NaN_3 was used for preventing microorganism growth. The model/artificial PJ samples were made by mixing glucose, fructose, malic acid, citric acid, glutamic acid and alanine in various concentration, in buffer and PHP, DMA were used as ISs. For quantification measures in PJ samples, the approach followed for untargeted analysis was applied, but the stock buffer solution was containing 10 mM PHP and 10 mM DMA.

Chemicals: D_2O, TSP d_4, Phosphate buffers, EDTA-2Na, NaN_3, $MnCl_2$, Potassium hydrogen phthalate (PHP), Dimethylmalonic acid (DMA), Maleic acid, 3,5-dinitrobenzoic acid, D-glucose, D-fructose, Citric acid, Malic Acid, Alanine, Glutamic acid, Isocitric acid, L-ascorbic acid, sucrose and arabinose were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation: ^1H, ^{13}C and ^1H-^{13}C HSQC experiments for untargeted analysis and quantitative ^{13}C NMR were acquired on 700 MHz Bruker NMR spectrometer equipped with a TXO helium-cooled 5mm probe (Billerica, MA, USA), while the other quantitative experiments and NOAH pulse sequences were run on a 600 MHz Bruker NMR instrument equipped with a TCI helium-cooled 5mm probe (Billerica, MA, USA).

All experiments were performed at 25 ± 0.1°C and the spectra were processed by the Topspin software package provided by Bruker Topspin. Low-field NMR experiments were conducted on an Magritek Spinsolve 80 MHz instrument (Wellington, New Zealand) and spectra were processed with MNOVA software.

NMR experiments: For untargeted analysis a typical one-dimensional ^{1}H NMR spectrum was acquired for each of all samples employing the first increment of NOESY pulse sequence (NOESYPR1D) with presaturation with the following acquisition parameters: 64 scans and 4 dummy scans, 32K data points, 90° pulse angle, relaxation delay 3 s and spectral width 12 ppm. The ^{13}C NMR spectra were recorded using a z-restored spin echo pulse sequence[125] with the following acquisition parameters: 512 scans and 4 dummy scans, 64K data points, 90° pulse angle, relaxation delay 2 s and spectral width 221 ppm. The HSQC experiments for untargeted analysis were run using 1K and 256 data points in F1 and F2 respectively, 8 scans, 2 dummy scans, 90° pulse angle, relaxation delay 1.5 s and spectral width 16 and 165 ppm for ^{1}H and ^{13}C dimension respectively. The spectra were zero-filled to a final size of 1K × 1K prior to Fourier transformation.

To achieve signal assignments, NOAH-BSC experiment was acquired using 2K and 512 data points in F1 and F2 respectively. NOAH experiments were processed using the splitx AU program.

Quantitative experiments were conducted using the simple pulse-acquire (zg), the ^{1}H NMR experiment with presaturation (zgpr), the watergate (zgwg), the excitation sculpting (zges), the Carr-Purcell-Meiboom-Gill (cpmg), the first increment of noesy with

presaturation (noesy1dpr), the inverse gated decoupling version of a z-restored spin echo method and a new quantitative equal carbon response sequence sequence recently developed by Makela et al.[126]. A modification was done on the phase cycling program, which was reported in the original paper to achieve better spectra quality. HSQC experiments acquired with NUS were processed with xht2 command to perform Hilbert transform to re-create imaginary data and make phase corrections when necessary. The T_1 relaxation times for ^{1}H and ^{13}C nuclei were acquired using the inversion recovery experiment.

Multivariate data analysis: For ^{1}H NMR untargeted analysis, the spectral regions δ 0.50–9.50 were integrated into regions (bins) with equal width of 0.005 ppm using the AMIX software package (V3.9, Bruker topspin, Billerica, MA, USA). For ^{13}C NMR based metabolomic analysis, the regions δ 10–190 and a bin width equal to 0.5 ppm were used. The HSQC data for untargeted analysis bins of 0.5 and 0.1 ppm for ^{13}C and ^{1}H were used respectively. The region 5-4.7 ppm where water signal appears was discarded from the data. To adjust for the concentration differences among samples, the bucketed regions were normalized to the total sum of the spectral integrals before applying statistical data analysis. MVSA was carried out with SIMCA-P+ software (version 14.1, Umetrics, Sweden) and AMIX. Data were mean-centered and scaled using Pareto method. Principal component analysis (PCA), Partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structures with discriminant analysis (OPLS-DA) were conducted for sample clustering and sample classification. The OPLS-DA model's confidence level for membership probability was set to 95% and was validated using a 7-

fold cross validation method. The quality of the model was assessed by the values of R^2Y and Q^2.

3. Results and discussion

Compositional analysis

^{1}H and ^{13}C NMR spectral assignment. PJ is a complex mixture consisting of many different compounds, mainly sugars, organic acids, amino acids and polyphenols. These molecules have different chemical structures and physicochemical properties which renders their simultaneous determination challenging and usually a combination of methods is required for their analysis[127]. We hypothesized that NMR offers a valuable tool for their rapid and accurate quantitative determination in a single analysis, without any separation/purification step. This is an important advantage compared to other analytical techniques that are laborious and time consuming, such as HPLC, or they do not provide detailed information at the molecular level, such IR.

The first step required for the compositional analysis of PJ constituents is the accurate assignment of the ^{1}H and ^{13}C NMR spectra. Typical ^{1}H and ^{13}C NMR spectra of a PJ sample with the assignment of specific signals are shown in **Figure 3** and **Figure 4** respectively. The main compounds and functional groups that can be identified by NMR, as found by our study, are carbohydrates, including glucose and fructose; organic acids, such as citric acid, malic acid and succinic acid; amino acids, such as GABA, L-serine, L-alanine and L-glutamine; and polyphenols/anthocyanins. These compounds are considered of specific importance for PJ quality and authenticity[61 62 107 112 108] and their determination with traditional methods of analysis is complicated, since calibration

curves, chromatographic separation and/or derivatization steps are usually required. The chemical complexity of PJ, results in spectral complexity and thus the unambiguous assignment of the 1D NMR spectra can be achieved only by applying 2D NMR and spiking with model compounds.

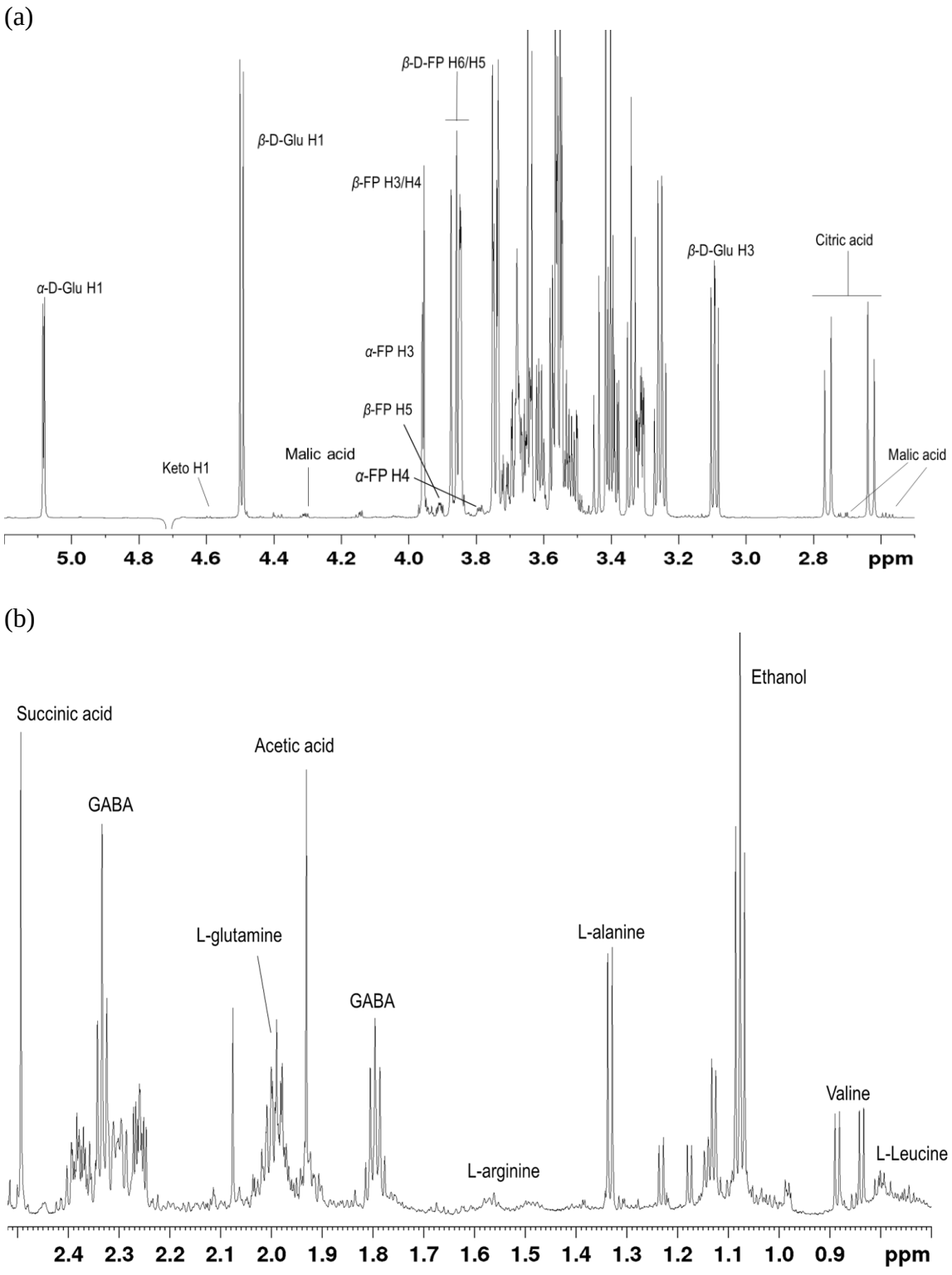

Figure 3. Typical ^{1}H spectrum of a PJ sample with the assignment of specific signals.

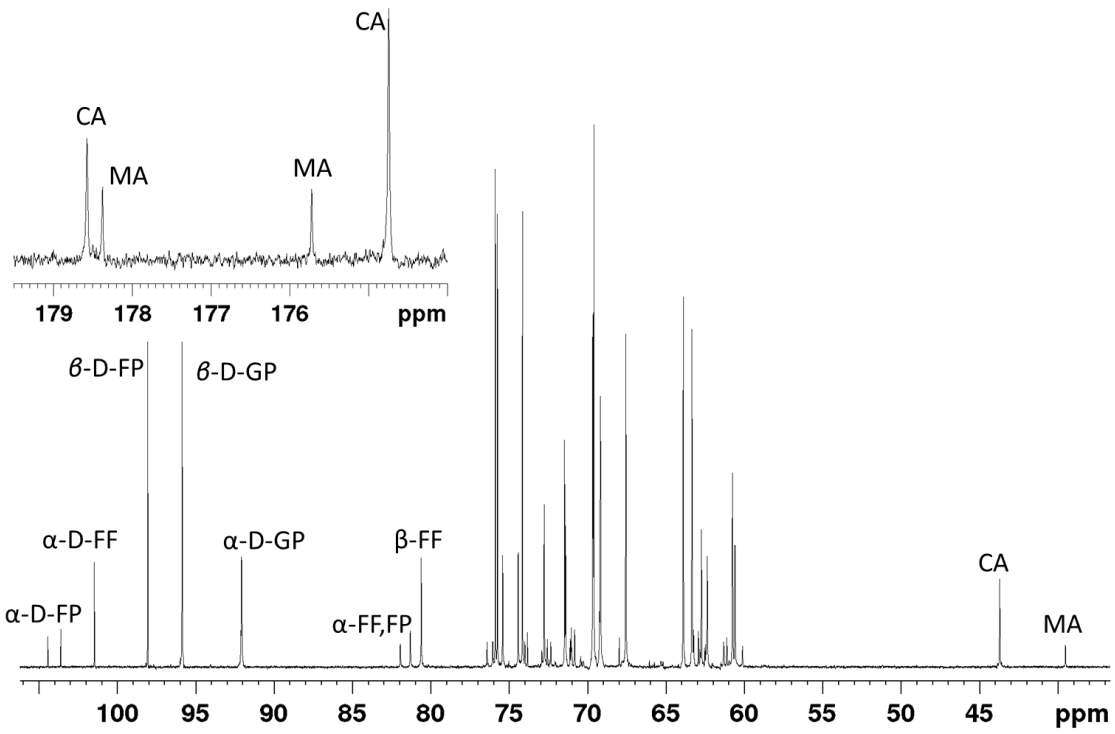

Figure 4. Typical ^{13}C spectrum of a PJ sample with the assignment of specific signals.

The NMR characterization of complex mixtures with NMR is usually conducted by using well established experiments such as COSY, HSQC and HMBC [128]. The most significant limitation when running a series of individual 2D NMR experiments is the long experimental time as a result of their different evolution times and relaxation delays. A revolutionary approach based on NOAH NMR super-sequences that dramatically reduces the experimental time of 2D NMR analysis, was recently introduced by Kupce and Claridge [122 123]. NOAH NMR super-sequences that combine several pulse sequences sharing the same evolution period and relaxation delay, may offer an attractive approach for compound identification in complex food mixtures such as PJ. To our knowledge this is the first application of NOAH experiments in food analysis and we believe that it can be an effective tool for a time-efficient NMR acquisition of food samples and other

complex mixtures. As an example, **Figure 5** shows the HSQC-DEPT spectrum of a commercial PJ obtained using the NOAH-3 (BSC) super-sequence that combines three 2D NMR spectra, namely, HMBC, HSQD-DEPT and COSY. The three spectra were recorded within 35 min in a single experiment and were compared with those obtained by the conventional HSQC-DEPT, HMBC and COSY. They were found to be of the similar spectral quality, except for some minor artifacts in the COSY part of NOHA, which however, did not cause significant problems in the analysis.

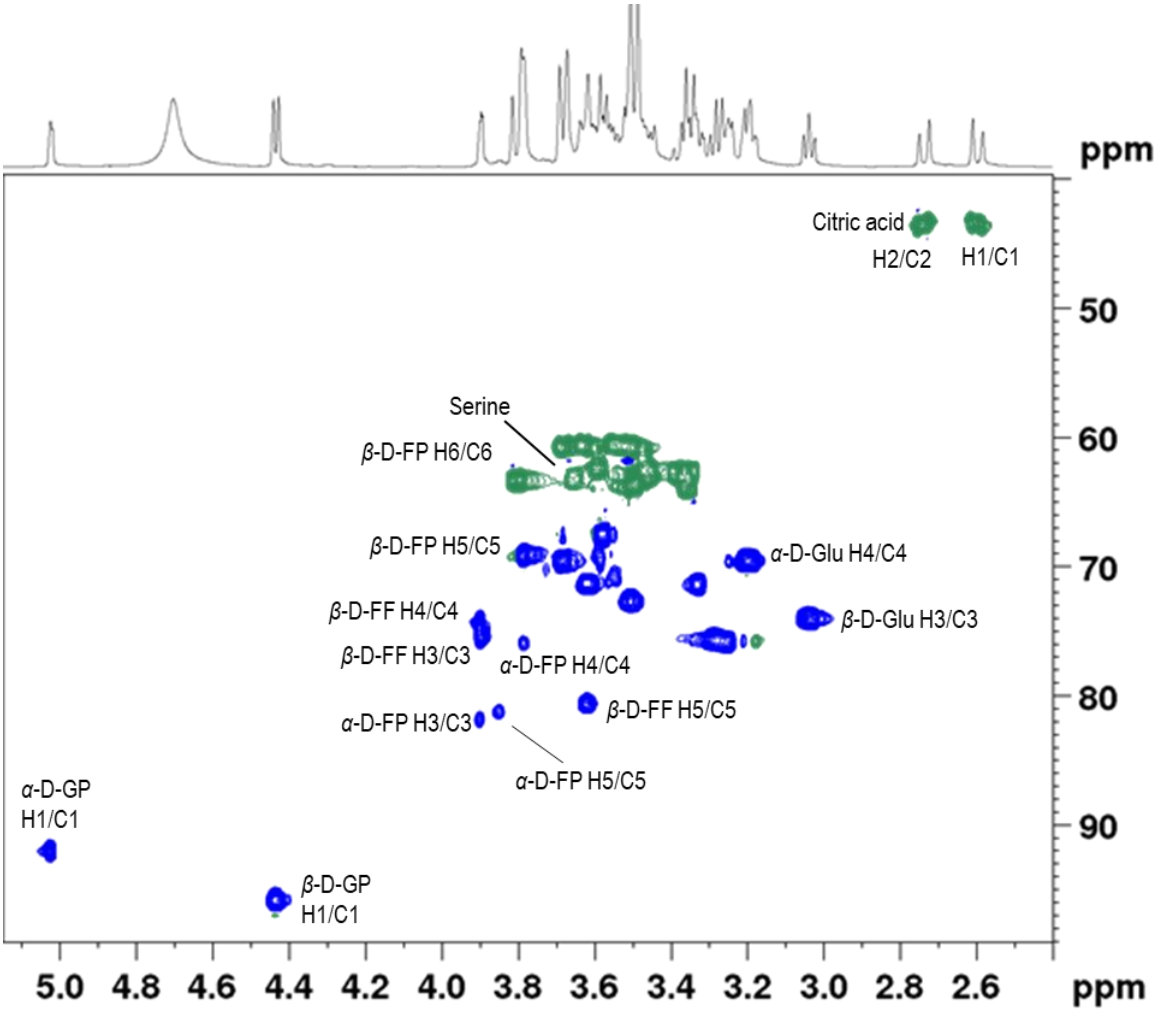

Figure 5. Peak assignment on a HSQC-DEPT spectrum of a commercial PJ obtained using the NOAH-3 (BSC) super-sequence.

To further validate the results obtained by 2D NOHA NMR and assign even more peaks, we performed spiking with model compounds. Isocitric acid[129], and L-ascorbic acid[130], have been mentioned in literature as PJ constituents, although other studies do not mention their existence in PJ[71 131]. Spiking with standards did not confirm their presence in PJ, at least for the detection limits of NMR. Literature is also conflicting in terms of the existence of sucrose and arabinose in PJ[70 64]. Our analysis indicated that small amounts of sucrose and arabinose are present in PJ as indicated by the doublets at δ 5.35 and δ 5.20 that belong to the anomeric hydrogen of sucrose and arabinose respectively. A limitation of the ^{1}H NMR analysis is related to oxalic acid, which has been reported in literature as a minor PJ constituent[130], and it is not detectable with ^{1}H NMR spectroscopy because it does not bear any hydrogens in its structure. The most challenging from the analyzed compounds was fructose. Due to its complex tautomerization and its overlapping with glucose, it was challenging to find a single peak for quantification. In an aqueous solution, such as PJ, fructose forms α/β fructo-furanose (FF) and α/β fructo-pyranose (FP), while traces of acyclic forms may also exist[132]. Fructose can be calculated by integrating the signal area from 4.00 to 3.80 ppm in the ^{1}H NMR spectrum and from the sum of the signals at 104.4, 101.4 and 97.99 ppm in the ^{13}C spectrum. Some errors in fructose quantifications are introduced because α-FF contributes three proton signals, in contrast to the other forms that contribute only two. However, these inaccuracies are expected to be low, because of the small concentration of α-FF. For the determination of minor compounds, the ^{13}C NMR analysis becomes impractical since a tremendous amount of time is required to obtain signals with a reasonable S/N ratio. **Table 2.**

summarizes the individual compounds that can be determined with NMR spectroscopy with the diagnostic signals can be used for quantification. The chemical shifts are pH dependent thus caution is required when comparing spectra obtained by samples with different pH. Samples in this study had a pH of 3.7 using a buffer of H_3PO_4/KH_2PO_4.

Table 2. Compounds determined using NMR spectroscopy and corresponding diagnostic signals for quantification.

Compound	^{1}H Diagnostic signals	^{13}C Diagnostic signal
Sugars		
α-Glucose	δ 5.08, d 3.53 Hz	91.99
β-Glucose	δ 4.49, d 7.94 Hz	95.79
Fructose	δ 4.00 - 3.79	(104.4 +101.4 + 97.99)
Sucrose	δ 5.34, d 3.70 Hz	96.70
Arabinose	δ 5.16, d 3.54 Hz	N/A
Organic acids		
Citric acid	δ 2.75, d 15.54 Hz δ 2.63, d 15.54 Hz	43.42
L-Malic acid	δ 2.71, dd 16.23 Hz δ 2.58, dd 16.23 Hz	38.77
Succinic acid	δ 2.51	N/A
Acetic acid		25.05
Amino acids		
L-alanine	δ 1.33, d 7.16 Hz	15.75
L-Leucine	δ 0.80, d 7.23 Hz	N/A
Valine	δ 0.83, d 7.02 Hz; δ 0.88, d 7.02 Hz	N/A
GABA	δ 1.79, m; δ 2.33, t 7.37 Hz	30.70
Serine	δ 3.79	N/A
Arginine	δ 1.57	N/A
Glutamine	1.99, m	26.07
Other compounds		
Ethanol	1.09, t 6.85 Hz	14.2
Punicalagin	6.53, d 9.89 Hz	N/A

Quantitative analysis

The diagnostic signals identified by 2D NMR and spiking can be used for the fast and accurate quantification of PJ ingredients. The signal integrals can be directly used for relative quantifications, however, for measuring absolute concentrations, the use of an internal standard (IS) is required. Finding an appropriate IS is not always an easy task as various factors including avoidance of signal overlapping and short T_1 values should be taken into account. Here we tested the potential five ISs, namely, 3,5-dinitrobenzoic acid (DBA), potassium hydrogen phthalate (PHP), maleic acid (MA), dimethyl malonic acid (DMA) and trimethylsilylpropanoic acid (TSP). Some of these compounds, such as MA have been previously used for NMR-based quantifications[133], while TSP is mainly used as chemical shift reference. One of the biggest challenges with NMR-based quantifications when using an IS, is the long T_1 relaxation time of these compounds. ISs are generally small symmetrical molecules and thus have long T_1s, which increase the length of the analysis. An effective solution to this issue is the addition of a paramagnetic reagent. For non-polar solvents, such as $CDCl_3$, $Cr(acac)_3$ has been found to drastically reduce T_1[5 134]. However, for polar solvents, such as H_2O:D_2O there are only very limited applications. Tian et al. proposed the use of $MgCl_2$, which was found to be very effective for carboxylic acids and amino acids, when a high concentration of $MgCl_2$ was used[135]. Here we investigated the potential of $MnCl_2$ as a relaxation agent to accelerate the analysis. $MnCl_2$ has been previously used as a contrast agent for MRI applications[136], but to our knowledge this is the first application for NMR-based quantitative studies. After

testing various concentrations of $MnCl_2$, 0.033 mM in the final NMR solution was found to be the ideal. Larger concentrations can cause signal broadening and dramatic variations in chemical shifts. **Table S1** shows the T_1 ^{1}H and ^{13}C relaxation times of the five ISs before and after the addition of $MnCl_2$ as measured by the inversion recovery experiment. Although MA is one of the most common IS for polar solutions, its T_1 relaxation time was found to be 7.12 s in the sample matrixes tested in this study. This would result to extremely long experimental times as a delay of 35.6 s (5 x T_1) between pulses is required for ensuring quantitative results. The addition of $MnCl_2$ causes a significant reduction in T_1 of MA, however, a delay of about 10 s is still required. Based on the T_1 measures and considering the number of signals produced by each compound and their multiplicity, DMA and PHP were selected as the most promising ISs. Spiking experiments of PJ with DMA and PHP showed that there is no overlapping with PJ constituents. The T_1s of various analytes appear in PJ are also reduced significantly (**Table S1)**, indicating the potential of $MnCl_2$ for a wide number of applications. Finally, there was no significant difference in quantifications with and without the addition of $MnCl_2$ (**Table S2**) indicating that there are no matrix effects, at least for the $MnCl_2$ concentrations used here.

The selection of pulse sequence is also a crucial topic for quantitative NMR analysis. Although the simple pulse-acquire 1D NMR experiment and the 1D inverse gated decoupling are considered reliable quantitative tools for proton and heteronuclei NMR-based quantifications respectively[137], little is known about the reliability of other pulse sequences, which have the potential to address issues associated to the analysis of

food samples. The majority of samples analyzed in food science and metabolomics are in water, however, its physical removal is often avoided because it increases the experimental time and may causes degradation or loss of some sensitive or volatile compounds. For that reason, water suppression techniques are used, however their quantitative potential is unknown. Typical pulse sequences for water suppression include the simple 1D ^{1}H NMR experiment with presaturation (zgpr), watergate (zgwg), excitation sculpting (zges) and the first increment of noesy with presaturation (noesy1dpr). Other experiments include the Carr-Purcell-Meiboom-Gill (cpmg), which is often beneficial as it generates spectra clean from the signals of large molecules and the 2D HSQC, which addresses the resolution limitations of the 1D ^{1}H NMR spectra. The latter can be of specific importance when using low field instruments that provide a user-friendly and economic option. All the above 1D sequences contain extra pulses and delays as compared to the simple pulse-acquire experiment and thus their appropriateness for quantifications should not be accepted a priori. Similar concerns arise for 2D HSQC experiments, although promising approaches have been recently proposed[126]. Here we tested the potential of zgpr, zgwg, zges, noesy1d, cpmg, z-stored with inverse gated decoupling and QEC-HSQC for quantitative purposes, as compared to gravimetric method and the simple pulse-acquired experiment. To our knowledge this is the first systematic and comparative attempt of evaluating the quantitative potential of all these experiments and examining the influence of spectroscopic parameters on their performance, namely linearity, accuracy and precision.

We initially tested the applicability of the sequences for quantitative analysis using an artificial PJ sample containing glucose, fructose, malic acid, glutamic acid and alanine, as well as PHP and DMA as ISs. Suitable peaks for quantification of each compound were chosen based on non-overlapping signals, and then normalized by the number of contributing protons. **Table 3** shows the concentration of the compounds calculated from integrated signal area using an IS, as compared to gravimetric concentrations. This initial screening showed that results obtained with watergate and excitation sculpting had a very significant deviation from the gravimetric measurements for compounds with signals closed to the water signal. This is because the peaks located near to the water peak were influenced by the strong water suppression of these experiments and thus, they were not further investigated for linearity and accuracy. The results obtained by simple pulse-acquire experiment (zg) and the inverse gated decoupling version of the z-stored method were very close to the gravimetric concentrations, as expected. Noesy1dpr has a significant error for glucose but less than watergate and excitation sculpting because of the softer signal suppression approach. Other sources of errors in noesy1dpr may arise from the delays and pulses of the experiment which can cause NOE enhancements and magnetization loss. Zgpr and cpmg, which also have water suppression blocks, generated more accurate results; especially cpmg was very close to the gravimetric concentration. QEC-HSQC gave very good results although a slightly higher concentration of glucose was measured. The QEC-HSQC sequence we used here does not have any water suppression block and thus does not affect the peaks close to water. Water molecules do not have HSQC peaks but

generate noise along F1 dimension. The coding/decoding gradients of QEC-HSQC used for coherence selection to select the desired ^{13}C-^{1}H signals and diphase undesired signals from ^{12}C-^{1}H, can help for the reduction of water peak. In that term the QEC-HSQC is superior to the 1D sequences with solvent suppression. It should be noted that we used a modified phase cycling program compared to the one reported in the original paper, because this generated signals with less phase distortions and better quality. More specifically, the phase of the fifth ^{1}H proton pulse (the ^{1}H pulse before T_1) became *y* instead of *x* and the receiver phase program became *x -x -x x* instead of *x -x x -x*. Also, the peak integrals of QEC-HSQC are inherently normalized by the number of contributing protons, and this should be taken into account during calculations. The repeatability of the methods was tested by running the same sample at the same instrument six times, and the reproducibility was measured by preparing independently six times the same model sample and run it on two different instruments. RSD of each test were calculated and showed acceptable values, as shown in **Table S3**.

Table 3. Comparison of the concentrations measured by q-NMR and gravimetric concentrations of the compounds in model PJ sample.

Compound	Gravimetric (mM)	Zg	Zgig z-stored	Zgpr	Noesy1dpr	Cpmg	Zges	Zgwg	QEC-HSQC
PHP	10	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Glucose	60	61.21	62.04	59.06	48.94	61.24	9.32	22.32	67.86
Malic acid	40	38.10	41.89	37.85	37.91	39.02	6.88	13.82	42.51
Fructose	30	30.43	31.25	31.24	30.56	31.52	26.97	19.88	31.15
Citric acid	40	39.26	41.82	40.04	37.99	38.87	37.49	37.49	43.47
Glutamate	20	19.16	21.40	19.64	18.75	18.95	18.68	18.68	18.44
Alanine	20	20.02	20.57	20.35	19.66	19.98	19.72	20.08	19.98
DMA	10	9.99	20.94	20.10	9.57	9.83	10.15	10.06	9.98

The influence of important spectroscopic parameters of the pulse sequences on the quantitative results were examined. More specifically, the effect of mixing time was examined for noesy1dpr and it was found to cause variations up to 10% in the relative signal areas. Because the signal in the 1D noesy is the sum of the diagonal peak and the associated cross-peaks in the corresponding 2D spectrum, it can be assumed that this can be attributed to relaxation loss during mixing time, as well as the unique dependence of peak integral on mixing time for each spin pair. For cpmg, the effect of loop number and the echo time were investigated, and results showed they affect the results, although no dramatic differences were observed. However, a loop number between 200-50 and an echo time of 1 ms are generally recommended, at least for the samples used here. When using a smaller number of loops and longer echo time the field homogeneity may become a more critical factor, because of the diffusion differences between molecules in the mixture. For the QEC-HSQC experiments, the effect of non-uniform sampling (NUS) was examined. Results obtained by traditional sampling and NUS up to 50% were very close, indicating the potential of rapid and high resolution 2D NMR based quantifications.

The linearity of zgpr, cpmg and QEC-HSQC when working with various concentrations was examined by correlating the known concentrations of model compounds at the range of 1-80 mM, with the integrals of the corresponding signals in the NMR spectra. Five samples containing gradient concentrations of glucose, fructose, citric acid, malic acid, alanine, and glutamate, as well as consistent concentration of the reference compounds PHP and DMA at 10 mM were prepared and analyzed. Linear

regression indicated that methods have good linearity and slopes/correlation coefficients were close to unity in several cases, although there were some exceptions. Over the concentration range between 64.76-2.59 mM of glucose, QEC-HSQC (R^2=0.9994) showed the best linearity, after zg (R^2=0.9999) with cpmg, zgpr and noesy showing acceptable linearity with R^2 of 0.9943, 0.9943 and 0.9918 respectively. All pulse sequences showed adequate linearity for fructose, citric acid, malic acid alanine and glutamic acid and glucose. The zg, zgpr, cpmg, z-stored with inverse gated decoupling and HSQC sequences were also tested in commercially available PJ samples. The QEC-HSQC spectrum of a PJ sample with the correlation peaks that can be used for quantifications is shown in **Figure 6**.

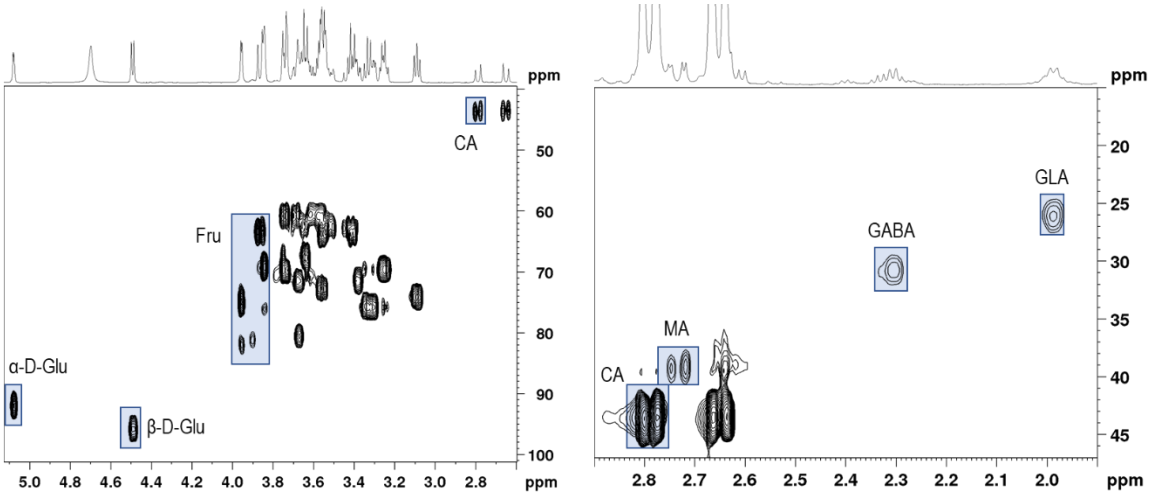

Figure 6. Diagnostic peaks of representative compounds for quantification identified in a typical QEC-HSQC spectrum of a PJ sample.

Untargeted NMR analysis for PJ metabolic fingerprinting

Varietal discrimination. The potential of 1D ^{1}H NMR for pomegranate varietal discrimination was initially explored. Three different fruit types, namely Wonderful (USA), Granade (USA), and a Chinese pomegranate cultivar were analyzed. Although

there are similarities in the biochemical profiles and thus in the NMR spectra of juices produced from different cultivars, PCA analysis showed significant variations in the composition between them. As seen in the PCA scores plot in **Figure 7**, the three cultivars form separate clusters. The first principal component explains 38.2 % and the second explains 22.5 % of the total variance. Although variety is an important factor of variance, the samples analyzed in this study were also differing in geographical area, as some were from USA and some from China. The two American cultivars are separated along PC1, while the Chinese cultivar is separated from Granada along PC2 and from Wonderful along PC1 and PC2, indicating that both factors contribute to the clustering and separation of samples. The more significant loadings from PCA analysis are shown in **Figure 8** and include bins at δ 2.775, 2.675, 2.825, 4.525 which belong to citric acid, malic acid and fructose. OPLS-DA analysis was also performed and resulted to excellent classification for the three groups (**Figure S1A**). The model was validated using CV-ANOVA (**Table S4**) and permutation tests (**Figure S1B**) and was found to have high predictability with no overfitting (permutated model $R^2 = 0.0235$, Q^2=-0.227). The most important variables for the classification of the three varieties as determined by OPLS-DA were similar to the ones identified by PCA.

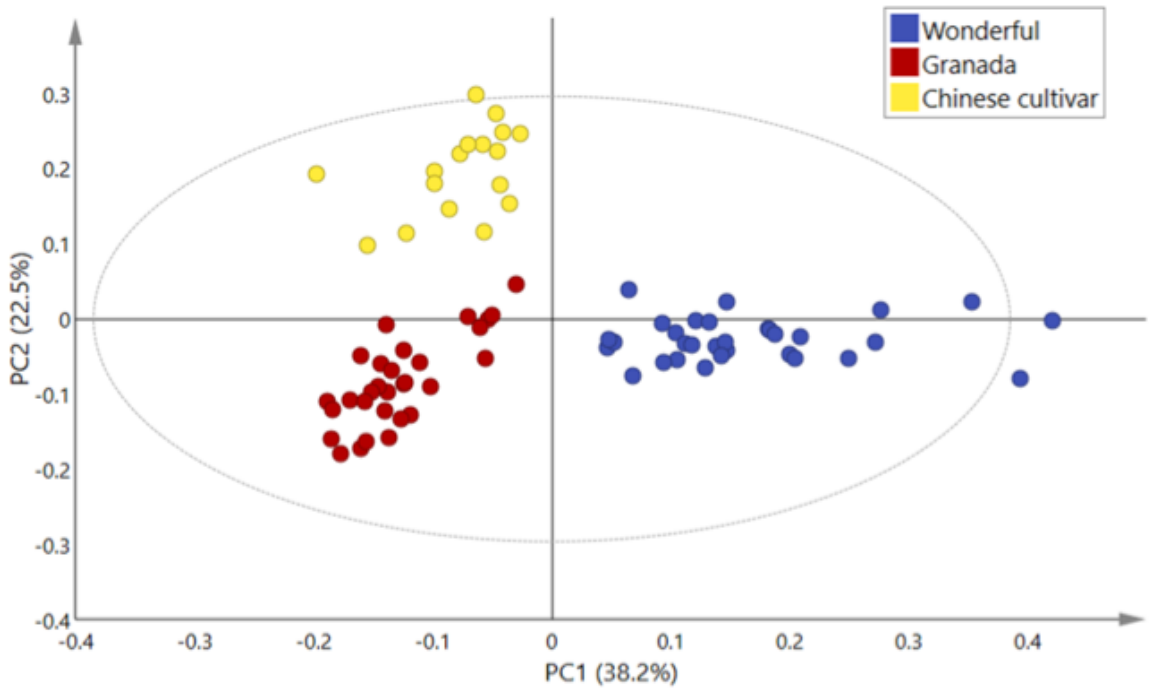

Figure 7. PCA Score-plot of Chinses cultivar, Granada and Wonderful PJ using 1D ^{1}H NMR.

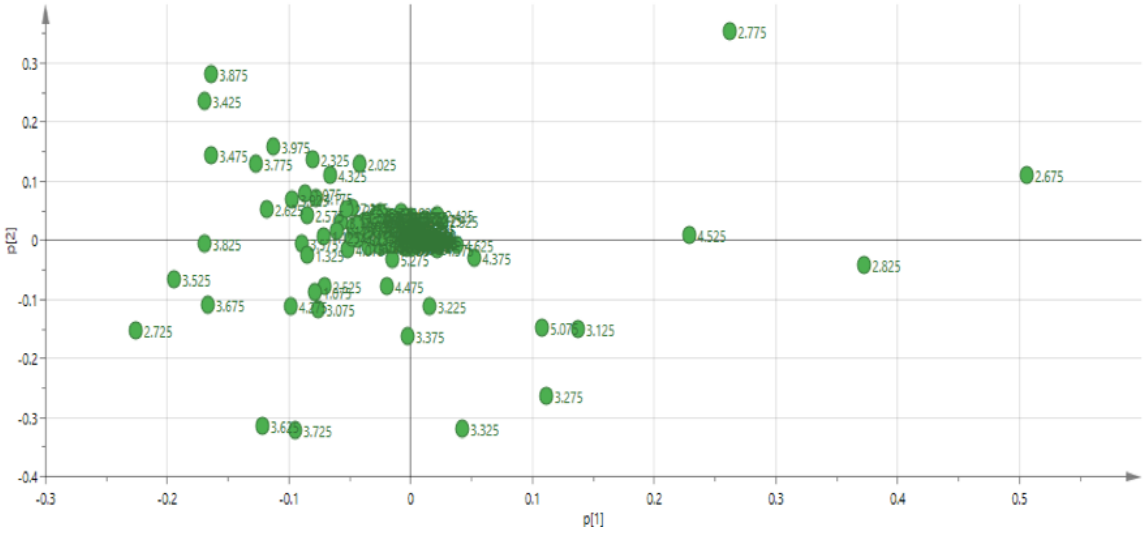

Figure 8. Loading plot of Figure 7 with first and second PCs.

Discrimination of commercial PJ samples based on geographical origin. Forty commercially available PJ samples were also analyzed for their biochemical profile. The commercial samples formed different clusters from the home-made samples, further

supporting our previous observation that the type of production has an impact on the metabolome. Interestingly, six samples of unknown geographical origin were grouped with those from Middle East and two unknown samples group with those from USA, as shown in **Figure 9**. The impact of other factors such as organic versus non-organic cultivars, and fresh pressed versus concentrate were examined, however no significant differences were found. Although further studies with monovarietal and single geographical origin samples are required to confirm these results, this study provides a clear proof of concept for the potential of NMR-based metabolomics to capture compositional differences between samples of different origins.

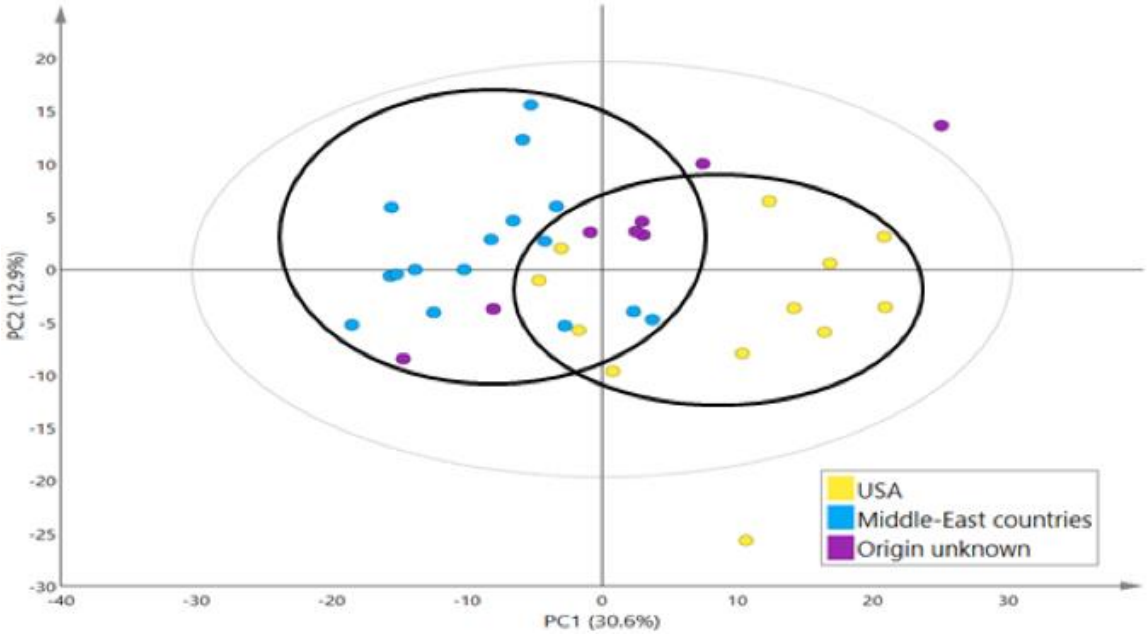

Figure 9. PCA Score-plot based on 1D ^{1}H NMR of all CPJ samples.

Adulteration of PJ with AJ. Adulteration of PJ with other juices is expected because of its higher price and its limited production[138]. Since NMR was able to capture metabolome differences between pomegranate fruits from different varieties and

geographical areas, it is expected to differentiate between PJ and other fruit juices and detect adulteration. Indeed, 1D ^{1}H NMR was proved to be a rapid and robust tool for the detection of adulteration of PJ with AJ. In most NMR-based metabolomics studies the ^{1}H NMR spectra are used, while there are only a few studies using ^{13}C NMR metabolomics. The potential of 2D NMR in metabolomics has not been studied adequately and there are only very few applications. Here we performed a pilot study to compare these three untargeted NMR approaches and we tested the potential of 2D NMR data, obtained for the HSQC experiment and combined with MVSA for juice discrimination and the detection of adulteration. To our knowledge this is the first study in food science involving 2D NMR-based metabolomics. The ^{1}H NMR-based metabolomics analysis was able to discriminate between PJ, AJ and blended/adulterated samples (**Figure 10**). That was not surprising because ^{1}H NMR has been proved to be a powerful tool for the detection of adulteration. Similar results were also obtained by the ^{13}C NMR analysis (**Figure 11**). More importantly, 2D NMR based chemometrics analysis was found to be a powerful tool for separating various groups (**Figure 12**). Group separations and classifications were as efficient as those obtained by 1D NMR data, however 2D analysis has a great potential to be applied for low field instruments, where the spectral resolution in the 1D ^{1}H spectra is not always ideal. **Figure S2**, shows the 1D and 2D spectra acquired for a PJ sample using a low filed, 80MHz instrument. As can be seen the resolution in the 2D HSQC is high, and thus these spectra can be combined with MVSA, however other studies focusing on low field NMR analysis are needed to confirm this. Supervised OPLS-DA analysis indicated that the compounds that are responsible for the

separation between PJ and AJ are malic acid, fructose, and glucose. It worth to not that NMR analysis showed that production type affects the biochemical profile of PJ juice. The type of juice production has been recently found to affect certain compounds in PJ and thus it was of our interest to investigate its influence in the total metabolome, since the aril PJ samples that were produced using a juicer form a distinct sub-cluster and are separated from the homemade samples that were prepared using hydraulic pressing.

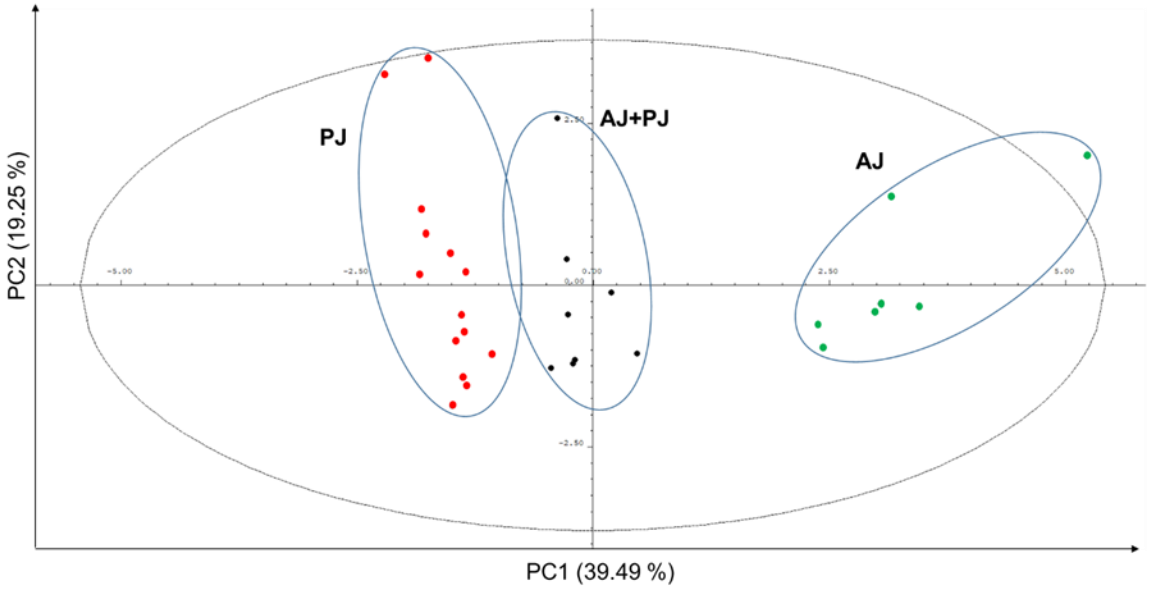

Figure 10. PCA Score-plot based on 1D ^{1}H NMR of all CPJ samples.

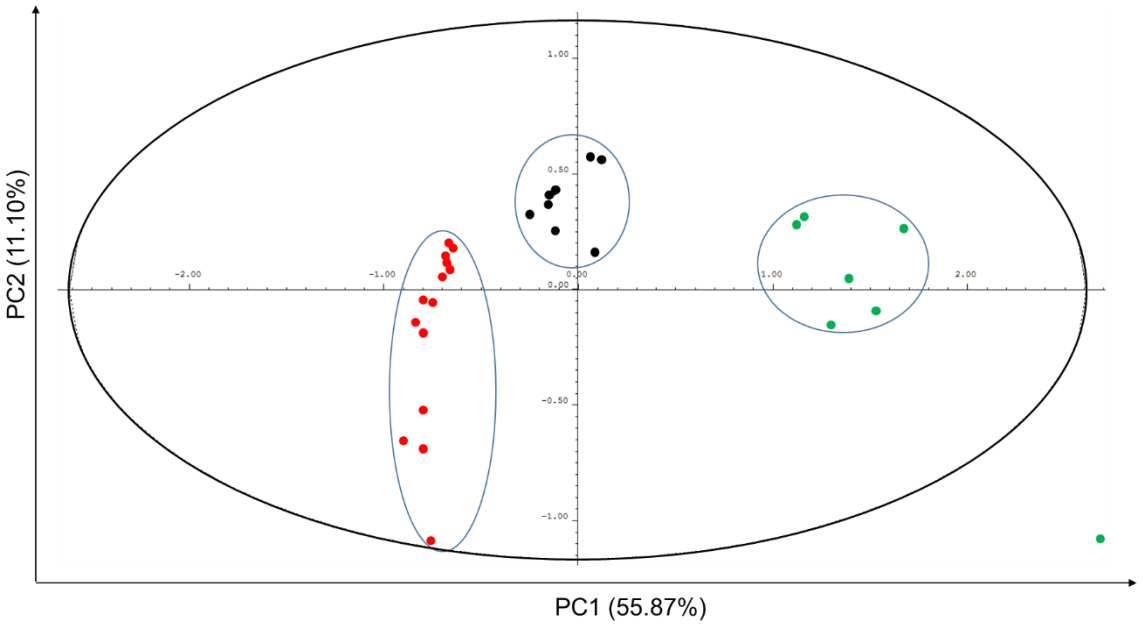

Figure 11. PCA Score-plot based on 1D ^{13}C NMR of all CPJ samples.

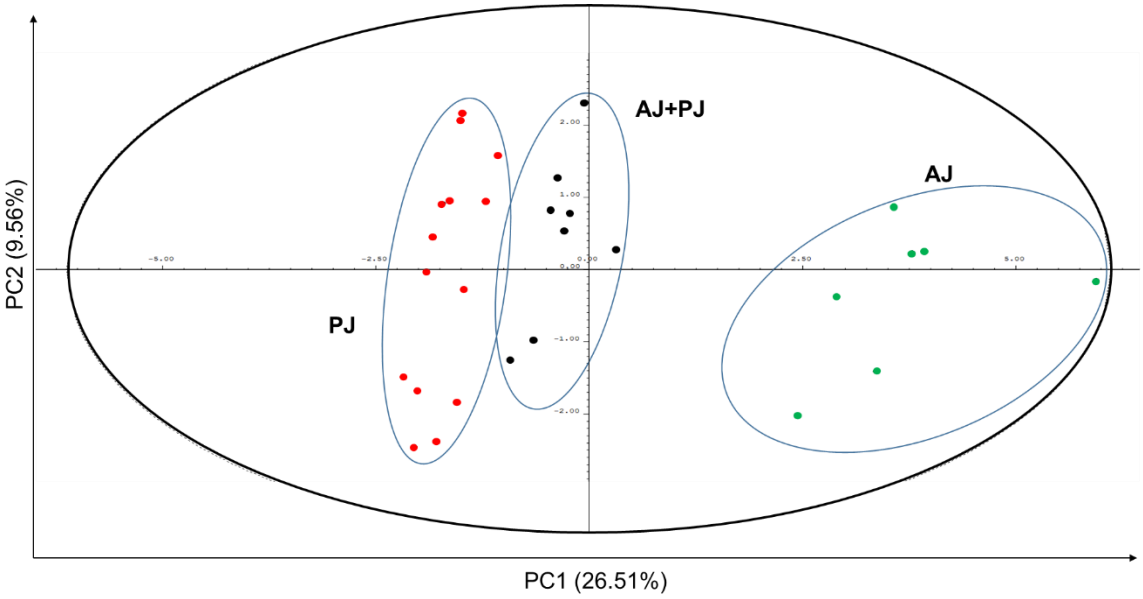

Figure 12. PCA Score-plot based on 2D NMR of all CPJ samples.

It is important to note that the pilot models developed here cannot be used for predictions and classifications of new samples according to variety, geographical area and purity. For that purpose, models built by hundreds or even thousands of samples that

have been certified on terms of their origin and their genetic profile, as well as blends of various ratios are required. In addition, the juice production, processing and storage should be also taken into account for these studies. Nevertheless, this study clearly proves that NMR untargeted profiling captures metabolic variations between pomegranate fruits and the addition of other juices in PJ and can create an opportunity for these applications.

Chapter 5. Conclusions

NMR was proved to be an excellent tool for the targeted and untargeted analysis of PJ. The assignment of the signals from various PJ constituents was performed using NOAH sequences and spiking with model compounds. Several pulse sequences were evaluated for their quantification potential. Sequences with suppression blocks generated significant errors for signals appearing close to the water peak, except for cpmg with presaturation which gave the best results among these 1D sequences. The QEC-HSQC is also a promising tool for quantifications. NUS up to 50% do not have an impact on the quantifications, making this approach even more attractive. NMR-based untargeted analysis enabled the classification of PJ according to the variety and the geographical region, while allowed the detection of adulteration. Importantly, 2D HSQC-based metabolomics generated data of similar separation power as the well-established 1D NMR approaches. This result is crucial for the growth of NMR as an analytical tool, because this idea can be applied on low field NMR instruments which fit even in an industrial environment, and as shown here, they generate HSQC data of high quality. This study sets the basis for future studies focusing on the development of prediction models for the detection of adulteration of PJ with other juices using various blends, as well as the classification of fruits according to the cultivar and geographical area based on samples of known and certified origin. Finally, this successful application of NMR for the fingerprinting of PJ, a complex mixture of many compounds, proves its potential for other similar matrices in food and chemical analysis.

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