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Chemical and Biological Technologies in Agriculture

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HRMAS NMR spectroscopy applications in agriculture

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Abstract

The relatively recent and advanced high-resolution magic angle spinning (HRMAS) NMR technique enables the direct application of NMR spectroscopy to semi-solid and gel-like samples. It combines the advantages of both solid- and liquid-state NMR by allowing to concomitantly measure intact and non-manipulated samples. Based on both 1D and 2D homo- and heteronuclear NMR spectra, HRMAS evaluates the composition of fresh semi-solid samples with a similar resolution as that of classical liquid-state NMR techniques. The enhanced spectral quality still obtained for semi-solid samples is mainly due to the MAS system, whose rapid spinning and sample orientation minimize the anisotropic processes that prevent the acquisition of meaningful NMR spectra for non-liquid materials. Moreover, HRMAS allows us to use edited pulse sequences which, especially in the case of biological tissues or agrofood products, may provide a simultaneous information on polar and non-polar components without the need of preliminary sample extraction. Additionally, this technique may differentiate molecular species according to their degree of mobility in hydrated matrices. The evident versatile potential of the HRMAS NMR makes this technique particularly useful for life science molecular studies. Despite the focus of HRMAS has been greatly devoted on clinical biomedicine, materials chemistry, and metabolomics, there are already enough studies that show useful applications on agricultural issues. This report reviews the latest representative studies that employ HRMAS NMR on systems related to agricultural chemistry, requiring the characterization and dynamics of soil components, plant tissues, agrofood products, and in vivo organisms.

Keywords: HRMAS NMR, Soil organic matter, Plant tissues, Agrofood products, Metabolomics, Soil fertilization, Environment

Introduction

Advanced and reliable analytical techniques are demanded to face up the ever more challenging issues related to the wide field of agricultural chemistry. The main topics of large interest are the following: (i) the improvement of knowledge on the complex chemical processes occurring in soil and involving its humic and mineral components; (ii) the unraveling of processes related to the biological stimulation of plant growth and relationship between soil components and plant roots; (iii) the bioavailability and fate of pollutants in the agro-environment; (iv) the assessment of quality, geographical origin, and traceability of agrofood products; (v) the development of practices in bio-agriculture aimed to improve crop quality. Although the complexity of these problems calls for a multidisciplinary analytical approach, the most determinant and critical responses commonly reside in chemical and molecular information. Nuclear magnetic resonance (NMR) and liquid or gas chromatographic systems coupled to advanced mass spectrometers (LC-MS or GC-MS, respectively) are the most used analytical methods to obtain accurate and detailed molecular information. Despite the poor sensitivity in respect to mass spectrometry, NMR spectroscopy enables to reach a complementary structural and conformational information of molecular systems in a non-destructive way.

Conceptually, the NMR phenomenon occurs when nuclei of magnetically active (spin number $\neq 0$) atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Following the pulse

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interaction between the applied electromagnetic radiation and the dipolar moments of the nuclei subjected to the static field, the resonance energy is relaxed producing a free induction decay that contains detailed information about the structure, dynamics, reaction state, and chemical environment of the target molecular material [1, 2]. The combination of phase- and length-modulated pulses specific of the selected NMR pulse sequences enables a controlled manipulation of nuclear spins that ultimately provides structural and dynamical information and reveals through-bonding or through-space correlations among nuclei, in either mono- or multidimensional systems [3].

An interesting aspect of NMR spectroscopy is its versatility since it enables to examine samples in either liquid- or solid-state, by selecting the proper NMR probe where the samples are placed. However, both techniques are not devoid of inherent disadvantages. NMR spectroscopy of solid materials suffers for strong dipolar and quadrupolar interactions, chemical shift anisotropy (CSA), and magnetic susceptibility. Despite the adoption of different technological solutions for reducing the effects of these phenomena, they are still responsible for the low resolution of solid-state spectra and the exclusion of any direct analysis of hydrogen nuclei. Conversely, totally dissolved samples for liquid-state NMR spectroscopy contain molecules that tumble isotropically, thus fully minimizing the anisotropicity of solid matter and enabling acquisition of high-resolution NMR spectra. Nevertheless, the use of liquid-state NMR spectroscopy for the molecular identification of agricultural matrices (plant roots, leaves and fruits, animal tissues, soils, etc.) requires preliminary procedures such as extraction, purification, and concentration, which are not only time-consuming, but also entail a sample manipulation possibly conducive of loss and/or degradation of components [4].

In the late 1990, the new high-resolution magic angle spinning (HRMAS) technique was introduced to enable NMR applications directly on heterogeneous semi-solid and gel-like samples [5]. HRMAS is a combination of solid- and liquid-state NMR techniques since it allows us to obtain spectra with a resolution similar to that of liquid-state spectroscopy but on intact and non-manipulated semi-solid materials. In fact, these samples maintain a sufficient degree of molecular mobility to afford an efficient minimization of factors that affect NMR measurements of solid materials. The principles of HRMAS have been extensively discussed previously [6–10]. Briefly, each semi-solid sample is reduced to small pieces, fit into a HRMAS rotor, and added with a small amount of deuterated solvent to ensure both the molecular spin mobility and the deuterium lock. The rotor is then tightly sealed by a screwed-in insert to prevent dehydration or loss of solvent during spectral acquisition. The rotor is finally capped (Fig. 1), loaded into the HRMAS probe, and spun at a relatively moderate rate (ν_R) around an axis inclined of an angle of 54.7° (the so-called "magic angle") to the static magnetic field (B_0). The MAS system permits to suppress the dipolar interactions, the chemical shift anisotropy (CSA), and the magnetic susceptibility arising at the interface between the solid and the liquid phase. These extensive spectral-broadening interactions are described in a Hamiltonian that contains an angular dependence of $(3\cos^2\theta - 1)/2$, where θ is the angle between the static magnetic field and the internuclear vector responsible for the interactions. When the sample rotates around an axis inclined of 54.7° to the magnetic field, all broadening factors are theoretically nulled [7, 8].

The high resolution of HRMAS spectra is due to the reduction of H–H dipolar couplings to few hundred Hz in many semi-solid samples, in respect to the 20–50 kHz usually found for rigid solid materials. In fact, the still significant segmental molecular motions in semi-solid matter allow the MAS system to efficiently minimize dipolar couplings [8]. Moreover, the signal resolution achievable by HRMAS is sufficient to perform most of 1D and 2D homo and heteronuclear NMR experiments, inasmuch as those conducted by liquid-state NMR

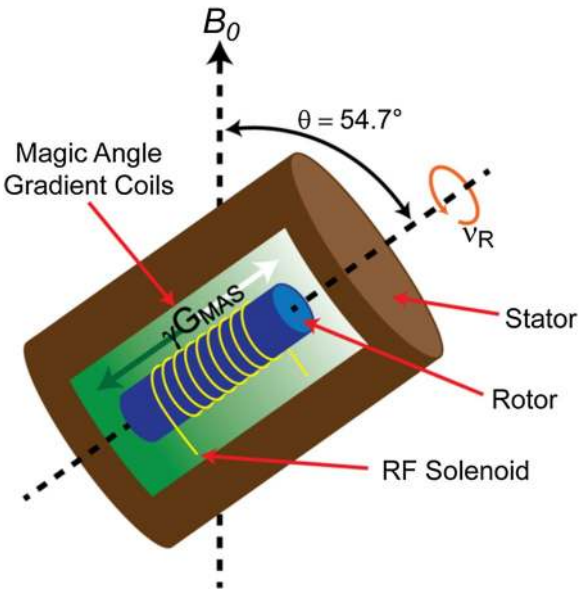


Fig. 1 Scheme of HRMAS stator. The *figure* shows the disposition of both RF solenoid and gradient coils as well as indicates the inclination of *rotation axis* at the magic angle ($\theta = 54.7°$) in respect of static magnetic field (B_0). During HRMAS acquisitions, each sample has to be spun at a moderate rate (ν_R) around an axis inclined at the magic angle. (*Figure* reprinted with permission of authors, © 2012 Alam TM, Jenkins JE. Published in Advanced Aspects of Spectroscopy (Intech Open) under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/48340)

spectroscopy. Therefore, HRMAS represents the best solution to also study complex molecular systems, such as membrane proteins, large molecular-weight polymers, whose dipole–dipole interactions are too strong to be sufficiently averaged by the simple molecular motion as that expected in liquid-state NMR spectroscopy. It is also critical in HRMAS spectroscopy that the spin rotation has to reach a value at least of the same order of magnitude as the line-broadening interactions, while avoiding an excessively fast rotation that may hasten sample degradation and reduce the measurements reliability. A relatively moderate spin rate ranging between 3 and 6 kHz is thus recommended, in order to reach best compromise between resolution and sample stability. An advantage of the HRMAS technique over solid-state MAS resides in the presence in HRMAS probes of gradients coils (Fig. 1), which may not only further improve spectral resolution, but also select coherence pathways and permit diffusion experiments to differentiate molecular species according to their degree of mobility in hydrated matrices. Moreover, edited (namely "filtered") pulse sequences, such as T_2- and diffusion-based sequences, can be used in HRMAS systems [10–12]. This allows HRMAS spectroscopy to provide information on both polar and non-polar components of biological tissues or agrofood products without preliminary separations though extractions.

All this indicates that HRMAS NMR spectroscopy is a versatile and effective technique for studying complex molecular systems. Despite that most of HRMAS applications had been focusing on biomedical studies and materials chemistry [7, 13, 14], a body of literature has already pointed out that HRMAS may result determinant in investigations related to agricultural issues. Hence, this review aims to report on the most interesting applications of HRMAS NMR in agricultural chemistry.

HRMAS applications in agriculture

Soil humic matter, environmental studies, and agricultural practices

NMR spectroscopy has been repeatedly applied to elucidate the distribution of carbon molecules in soils of different origins [15]. However, HRMAS may be particularly useful in the quest of clarifying the soil complex chemical processes due to better capacity to characterize the molecular structures of soil humic and humo-mineral components than solid- or liquid-state NMR. In fact, HRMAS was proved to enable the identification of molecular structures at the solid-aqueous interface of a whole soil through the acquisition of 1D, selective-1D, and 2D NMR spectra [16]. The same authors observed that the detection of aromatic signals may be significantly enhanced when the soil is swollen in dimethylsulfoxide (DMSO) rather than in water. This was attributed to the capacity of DMSO and other dipolar aprotic solvents to cleave hydrogen bonds and solvate aromatic components [17]. A 3D HRMAS spectroscopy was applied to examine tomato cutin and then compared to solution-state spectra of soil extracts from the A_h horizon under an oak forest [18]. The authors claimed that the molecular structures identified in the soil humic matter may resemble the biomolecules observed in the tomato cutin.

The HRMAS technique was further employed to identify the components of a peat humic acid mostly involved in the organomineral complexes with both kaolinite and montmorillonite clay minerals and how the interactions were influenced by both clay mineralogy and several solution properties, such as pH, ionic strength, and the presence of Na^+ and Ca^{2+} cations [19]. A study on complexes between calcium-exchanged montmorillonite and either fulvic acids or soil alkaline extracts was conducted by ^{1}H HRMAS NMR and showed that aliphatic and, partially, aromatic components bound preferentially to the clay surface [20]. These results seemed to confirm that a mechanism of organic matter preservation in soil occurs by adsorption on clay minerals of hydrophobic products of biological tissues degradation [21].

The decomposition process of ^{13}C- and ^{15}N-enriched pine and wheatgrass litter was followed by HRMAS NMR spectroscopy [22]. While the 1D ^{13}C spectra of fresh plant tissues well differentiated the composition of fresh pine and grass materials, the differences became negligible after decomposition and suggested a similar accumulation of recalcitrant carbon species, thus again supporting a mechanism of progressive selection of hydrophobic degradation products regardless of the plant origin [21]. Moreover, this study also highlighted the advantage of the use of diffusion-based filter to selectively identify and monitor the signals of fast-diffusing components developed during degradation process [22]. Analogously, ^{13}C and ^{15}N labeled soil microbial biomass and leachate degradation were monitored by HRMAS over time in order to better understand the degradation process [23]. This work also showed that the biomass degradation pathways are similar to those of plant material under similar degrading conditions, thus suggesting that the difference between recalcitrant carbon from different sources is negligible after decomposition. Moreover, ^{1}H–^{15}N HSQC (hetero single quantum coherence) 2D NMR spectra suggested that degradation either selectively preserves or produces specific peptides incorporated in yet unknown structures [23].

A variety of natural organic matters (NOM) (wood, kerogen, bitumen, and whole sediments) were subjected to both Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and NMR techniques [24]. Interestingly, this work showed that HRMAS applied

to the whole NOM samples was superior to the traditional liquid-state NMR measurements of extracts from the same NOM materials [24]. Another study combined HRMAS and DRIFT (Diffuse reflectance infrared Fourier transform spectroscopy) techniques to identify the relationship between the structure of soil humic substances and long-term (40 years) soil management practices, including organic and/or mineral fertilizations [25]. The application of T_2-based (namely CPMG or spin-echo) NMR filters to reduce signals heterogeneity in ^{1}H spectra of humic substances allowed to develop cluster analyses to differentiate humic matter as a function of soil treatments [25]. In the same line, conventional and diffusion-edited HRMAS proton spectra were applied to evaluate the long-term stabilization mechanisms of organic materials in soils undergone solid cattle manure and crop residues amendments and fractionated into free, intra-macroaggregate, intra-microaggregate, mineral-associated, and dissolved organic matter [26]. The authors compared the peaks detected in 1D HRMAS spectra of HF-treated soil with those of a bovine albumin protein and cultured soil microbes and suggested that the material associated with the clay mostly resembles the molecular components derived from microbial biomass [26]. Such finding was accompanied by a further study in which diffusion-edited HRMAS NMR spectra of HF-treated soil aggregates of different dimensions were related to the role of microbes in conservation tillage systems [27]. A tight relationship between soil hydrophobicity and humic composition was revealed by relating HRMAS spectra of soil humic fractions and physical parameters such as soil wettability, pore size distribution, and soil aggregate stability [28]. These results confirmed previous works that the hydrophobicity of humic fractions can affect aggregate wetting and slaking by modifying the pore size distribution with a shift from micro- and mesopores to ultramicropores. The HRMAS technique was also applied to monitor the modifications of both chemical and biological properties of SOM upon the strong pedoturbation caused by colonies of snow vole (*Chionomys nivalis Martins*) in a mountain environment [29]. The authors reported that snow vole activity was capable to significantly affect the humification process and SOM cycling, with effects on the hormone-like bioactivity of humic fractions.

HRMAS NMR spectroscopy was also applied in environmental chemistry studies, such as the interactions between a pesticide and soil mineral components. The adsorbed or intercalated forms of herbicide 4-chloro-2-methylphenoxyacetic acid on a highly hydrated clay, such as hydrotalcite, were clearly distinguished by ^{1}H-HRMAS. In fact, the adsorbed herbicide gave sharp signals indicating high mobility, while intercalated herbicide gave very wide unresolved spectra due to its strong interaction with the solid matrix [30]. The chemical shift drift and line broadening of HRMAS signals also served to reveal the interactions occurring between the herbicide trifluralin and a soil sampled from the A_h surface horizon under an oak forest [16]. A HRMAS investigation on organomineral complexes obtained by coating either montmorillonite or kaolinite clays by peat humic acids showed that these humic-clay complexes, especially in acidic conditions, were responsible for the increasing sorption of the non-ionic and hydrophobic phenanthrene contaminant [19]. The advanced NMR pulse sequence STDD (saturation transfer double difference) was used to prove the pH-dependent (D_2O or sodium phosphate D_2O buffer solution at pH7) interactions between a peat soil and several contaminants, including 1-naphthol and the pesticides trifluralin, acifluorfen, and 4-nitro-3-(trifluoromethyl) phenol [31]. The authors created epitope maps (Fig. 2) on the basis of HRMAS STDD results, which revealed the involvement of pesticides functional groups in the contaminant-soil associations and enabled a prediction of predominant sorption mechanisms. Recently, this study was extended by exploiting results from diffusion, relaxation, cross polarization, and saturation transfer difference NMR experiments, which permitted to follow the sequestration of contaminants from the liquid phase into the whole soil [32].

The HRMAS technique has been successfully applied to follow the effects of inorganic and organic contaminants in biological tissues. In a study of the influence of arsenic-contaminated irrigation water on *Lactuca sativa* L. grown in either sandy or clay loam soils, HRMAS was used to verify how fresh lettuce leaves were affected by the arsenic uptake and soil type [33]. This work showed that the metabolomic approach developed by HRMAS enabled the detection of the significant changes in phenolic compounds contained in As-treated plants. The leaves of lettuce plants exposed to the pesticide Mancozeb were monitored by HRMAS [34]. The metabolomic profile of the plant leaves revealed that the pesticide affected the concentration of a number of lettuce metabolites, thus indicating a large oxidative stress and a Krebs cycle up-regulation at a late stage of plant growth. The effect of the exposure of two organochlorine pesticides lindane and chlordecone on the maize root metabolome was recently explored by HRMAS [35]. The metabolomic profiles revealed some exposure-dependent changes in the amount of several maize metabolites, such as carbohydrates, amino acids, tricarboxylic acid cycle intermediates, and fatty acids, which were included in a diagram of biosynthetic pathways [35].

Other works have reported the adoption of HRMAS technique to study the effects of different agricultural

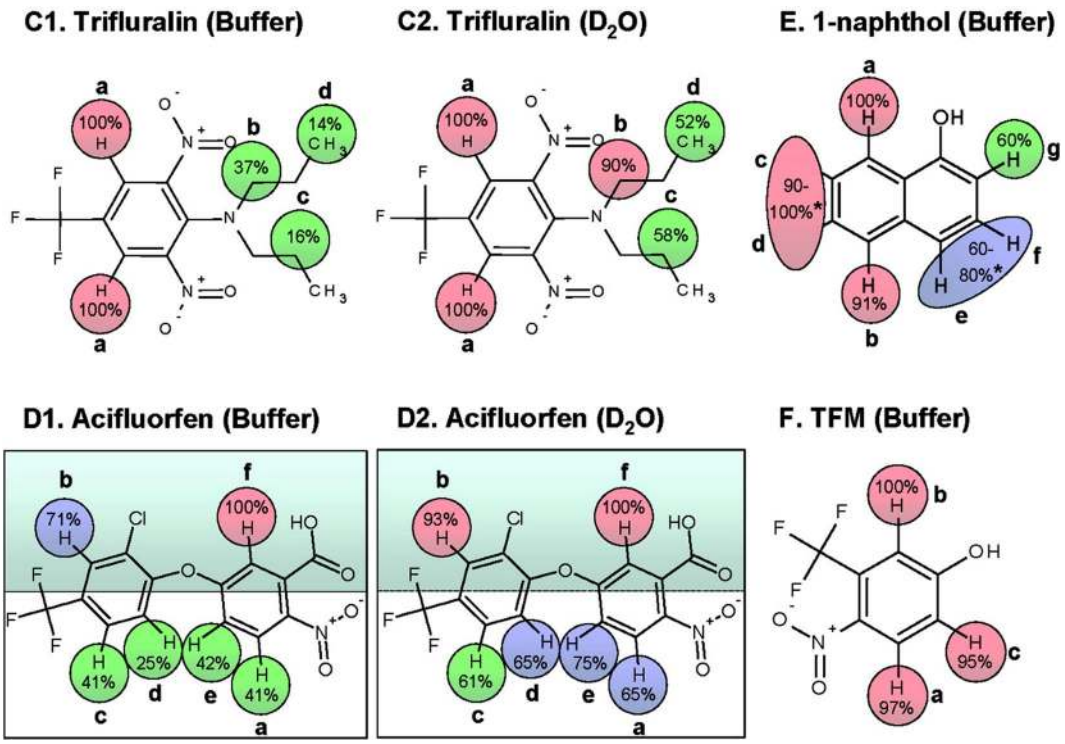


Fig. 2 Epitope maps of the four pesticides dissolved in the pH7 buffer or D_2O. The percentage values for each epitope map are all relative to the strongest binding proton in each pesticide, which is expressed as 100%. Protons which have 80–100% binding are highlighted by *red circles*, protons with 65–79% with *blue*, and 64% and lower with *green*. The *shaded region* in *D1* and *D2* simply highlights the side of the molecule that has the strongest interaction with the soil surface. An *asterisk* (*) indicates that absolute quantification was not possible at these positions due to spectral overlap. [*Figure* reprinted with permission from Shirzadi et al. Copyright 2008 by Environ. Sci. technol. (ACS)]

practices on plants metabolic profiles, thus relating the soil management to agrofood products. For example, HRMAS NMR-based metabolomics was applied with the aim of proving the different compositional qualities of three potato cultivars grown under either conventional or organic system [36]. HRMAS results were also determinant to show that conventional and organic management treatments affect differently the composition of fresh olive fruits [37].

Plants

The possibility given by HRMAS to acquire meaningful NMR spectra directly on whole fresh tissues represents an advantage for studying the molecular composition of plant shoots, flowers, fruits, and roots. Several literature works have used this technique to characterize the complex molecular content and quality of whole plant tissues, and better understand the effects on plants of external stimuli or specific treatments. Multidimensional HRMAS together with CPMAS was applied to characterize cutin, an insoluble component of plant leaf and fruit cuticles. In particular, tomato cutin was shown to contain a structural skeleton composed predominantly by an aliphatic polyester with some olefinic and aromatic moieties [38]. Moreover, these authors inferred that α-branched fatty acids/esters in cutin could be cross-linked to esters of mid-chain hydroxyls [38]. Later, the same authors adopted a similar analytical approach to identify the structure of cutan and a cutin/cutan mixture from the *Agave americana* leaf cuticle [39]. The structure of suburban, a protective biopolymer associated with suberin and located in the periderm of above ground parts of plants or in the endoderm of roots, was also investigated by combining HRMAS NMR to other techniques, such as CPMAS NMR, Scanning Electron Microscopy (SEM), flash-pyrolysis Gas Chromatography coupled to Mass Spectrometry (GC–MS), and FT-IR (Fourier Transform-InfraRed spectroscopy) [40]. The integration of results from these different techniques enabled the authors to propose a model structural unit for the specific suburban from *Betula nigra* bark under study. The algae–fungi symbionts called lichens were successfully differentiated on the basis of chemotaxonomy by using a combination of HRMAS (conventional and CPMG-edited proton spectra) and FT-IR techniques [41]. Despite the difficulty in distinguishing lichens morphologically, the authors

proposed a non-destructive HRMAS-based method enabling unambiguous differentiation results, while concomitantly avoiding a laborious and time-consuming sample pre-treatment [41].

HRMAS allowed to obtain a metabolic profile of both shoots and roots of four genotypes of *Withania somnifera* (L.), which is a plant with several pharmaceutical and nutraceutical applications [42]. The authors were able to detect through HRMAS the presence of withaferin A and withanone molecules, which are secondary metabolites produced by this plant and important natural medicinal compounds [42]. Proton and carbon 1D and 2D HRMAS NMR spectra enabled the identification and assignment of several bioactive triterpenoids and phenolics in Koroneiki olive leaves [43]. HRMAS has been successfully used to quantify the free sugars content in fresh melon fruits, whose results were validated by a quantitative enzymatic assay [44].

The presumed beneficial role exerted by humic acids in plant regeneration was verified by HRMAS on root and shoot calli explanted by pear and quince plants, after treatments with different concentrations of peat humic acids [45]. The authors showed that the selected humic acids induced a positive and dose-dependent response only in pear plants [45]. Recently, HRMAS revealed the molecular composition of *crocus sativus* petals and detected several bioactive compounds such as kinsenoside, goodyeroside A, and 3-hydroxy-γ-butyrolactone [46]. By comparing HRMAS results with those of ethanol extracts of the same petals analyzed by liquid-state NMR, it was observed that extraction favored hydrolysis reactions, thus decreasing the reliability of NMR results in the liquid-state.

An advanced NMR application for the identification of the composition of woody angiosperm plants reports a detailed characterization of ^{13}C-labeled poplar plants, when these were preliminarily grown in $^{13}CO_2$-enriched atmosphere and supplied with [$^{13}C_6$] glucose to roots [47]. The authors used HRMAS and liquid-state NMR techniques to analyze the whole poplar tissues and their ethanol/water extracts, respectively. Remarkably, they exploited the ^{13}C labeling of plants by making use of 3D experiments, such as ^{1}H-^{13}C HCCH-COSY and ^{1}H-^{13}C HCCH-TOCSY, to unambiguously assign several signals. Recently, Mazzei and coworkers applied ^{1}H HRMAS metabolomics to identify the effects on young *Solanum lycopersicum* (tomato) plants of seeds treatment with two bioactive secondary metabolites (6-pentyl-2H-pyran-2-one and harzianic acid) extracted from different *trichoderma* fungal strains [14]. A metabolomic profiling of tomato leaves was achieved with 1D proton and 2D ^{1}H-^{13}C HRMAS NMR spectra (Figs. 3, 4), whereby the most intense proton and carbon signals were duly assigned. The authors observed a significant enhancement of seedlings germination accompanied by a change in leaf metabolome that was treatment-specific, thereby inferring that the method may help in developing new biofertilizers based on fungi extracts [14].

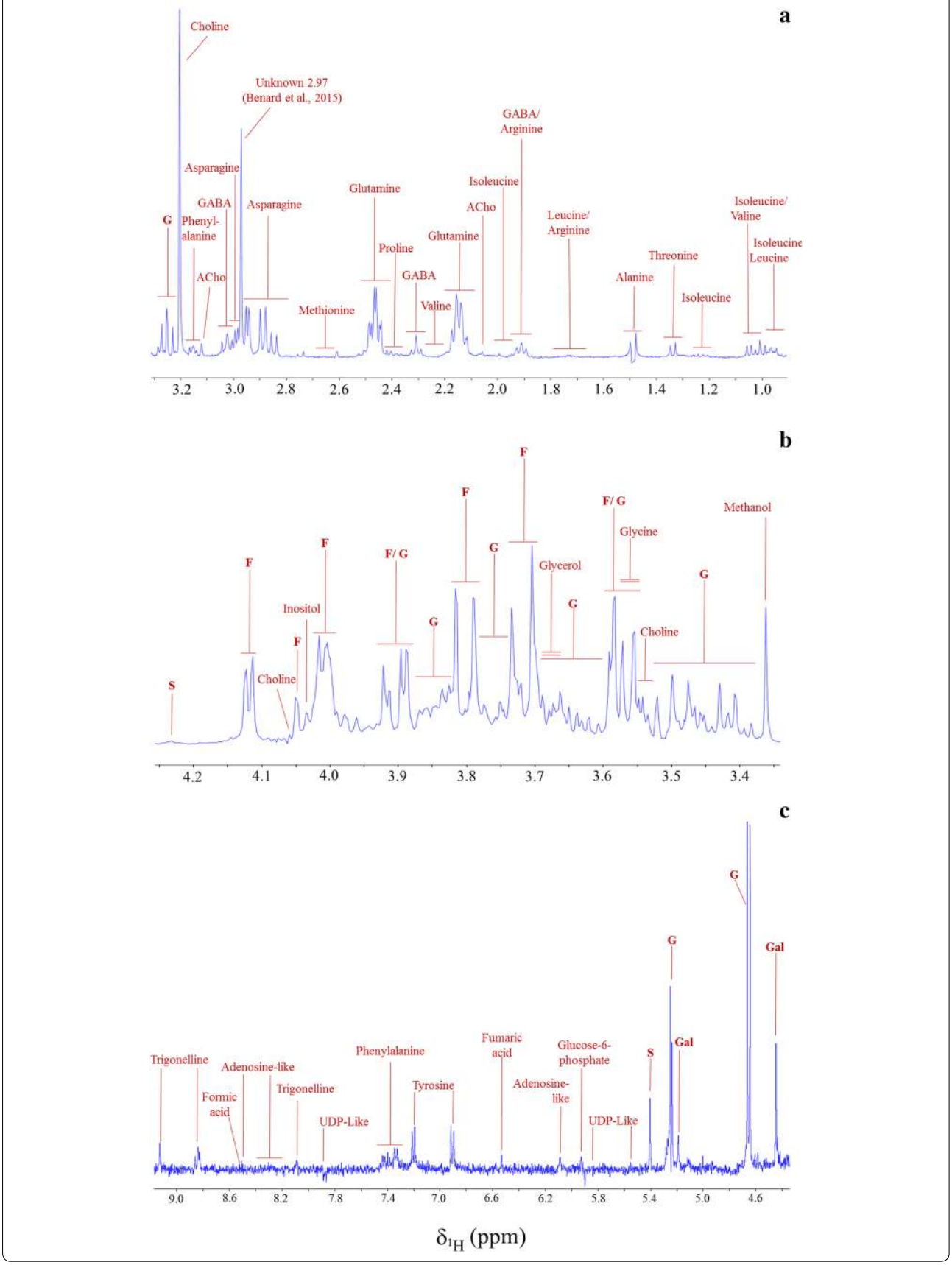
The HRMAS technique has been also applied to evaluate transgenic plants. ^{1}H HRMAS NMR spectra were successfully combined with the Batch Processing multivariate approach in order to model and interpret differences between wild-type and PttMYB76-modified poplar plants, while monitoring the time- or growth-related metabolic fluctuations in poplar plants [48]. Both HRMAS NMR and IR enabled the differentiation of the metabolome of three different cultivars of beans deriving from either wild or transgenic genotype [49]. Moreover, the authors also reported an overexpression of quercetin and myricetin flavonoids in the transgenic beans. Roots and leaves of *swingle citrumelo* were investigated to prove the capability of a selected transgene to induce an overexpression of proline, an amino acid commonly associated with plant responses to certain environmental stress [50]. In particular, the authors used HRMAS to compare the metabolome of wild-type plants with that of transgenic T-35S plants carrying the VaP5CSF129A transgene encoding a mutated enzyme involved in proline biosynthesis. Spectroscopic results showed that the transgenic plants exhibited a genotype-dependent response that entailed different levels of proline, proline betaine, sucrose, and acetic acid [50]. In another study, the composition of wild-type *Arabidopsis thaliana* was compared to that of cellulose-deficient mutant *ectopic lignification1* through HRMAS-based experiments. Interestingly, the mutant form showed a larger abundance of methanol, fatty acids and/or lipids, glutamine, phenylalanine, starch, and nucleic acids [51].

Agrofood products

The quest from producers and consumers for quality of agrofood products, such as edible fruits, vegetables,

(See figure on next page.)

Fig. 3 ^{1}H HRMAS CPMG NMR spectrum of control tomato leaves acquired at a spin rate of 5 kHz. Three spectral regions are shown: **a** 0.8–3.3 ppm, **b** 3.34–4.27 ppm, and **c** 4.4–9.25 ppm. The labels refer to assignment of the most intense signals detected in all treatments (*F* fructose, *G* glucose, *Gal* galactose, *S* sucrose, *ACho* acetylcholine, *GABA* γ-aminobutyric acid, and *UDP* uridine diphosphate). [*Figure* reprinted with permission from Mazzei et al. Copyright 2016 by J. Agric. Food Chem. (ACS)]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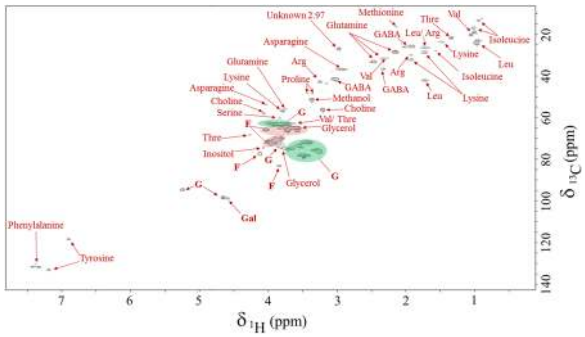

Fig. 4 ^{1}H–^{13}C HSQC NMR spectrum of control tomato leaves optimized for a short-range JCH coupling of 145 Hz. The labels refer to the assignment of the most intense signals detected in all treatments (*F* fructose, *G* glucose, *Gal* galactose, *S* sucrose, *GABA* γ-aminobutyric acid, *Leu* leucine, *Arg* arginine, *Val* valine, and *Thre* threonine). The *ellipsoid regions* circumscribe the dense cluster of F (*red*) or G (*green*) signals. [*Figure* reprinted with permission from Mazzei et al. Copyright 2016 by J. Agric. Food Chem. (ACS)]

cheeses, and cereals, calls for the adoption of reliable analytical techniques and methods that warrant the food nutritional value, traceability, and safety. In this context, the HRMAS NMR capacity to precisely detect and quantify specific compound classes in complex biological materials indicates this technique as an excellent candidate to assess the general quality of agrofood products.

In the case of fruit studies, ^{1}H and ^{13}C HRMAS NMR spectra were helpful in identifying the progressive changes in composition of mango pulp during 19 days of ripening [52]. In fact, ripening produced a decrease of citric acid accompanied by an increase of lactic acid, gamma aminobutyric acid, phenylalanine, and niacin. The effects of relatively low pressures on strawberries during storage were estimated by following the physical tissue damage, and the changes in molecular quality by Magnetic Resonance Imaging (MRI) and HRMAS proton spectra, respectively [53]. HRMAS was used to assess the chemical composition of coarsely ground coffee beans of Arabica and Robusta varieties, as well as the variation in content of Maillard reaction-related compounds and coffee aroma constituents, as a function of coffee roasting temperature from 30 to 215 °C [54].

A metabolomic approach by ^{1}H-HRMAS helped to neatly differentiate several cultivars of apples [55] and sugarcanes [56]. A detailed metabolic profiling of different tissues (flavedo, albedo, seeds, and pulp) of both lemon and citrus fruits was conducted through HRMAS as well as they characterized several compounds associated with the development of mold in the citron flavedo [57]. Recently, HRMAS has also been used to efficiently trace the geographical origin of 60 fermented and dried cocoa beans obtained from major growing areas in Africa, Central/South America, Asia/Oceania [58].

A number of interesting works have described the application of HRMAS NMR to edible vegetables. For example, seeds, peel, flesh, and puree of tomatoes were studied by HRMAS to produce a metabolomic profiling of Spanish Almeria tomatoes and identify the molecular markers correlated to ripening process [59]. Later, the same research group used HRMAS to differentiate three varieties of tomatoes and the basis of their composition [60]. In particular, they observed that the content in metabolites relevant for the tomato taste, such as fructose and organic acids, depended on the specific variety [60]. Ritota et al. [61] reported the use of HRMAS to differentiate Italian sweet peppers (*Capsicum annum* L.) as a function of both cultivar and geographical origin [61]. Later, the same research group used a similar ^{1}H- and ^{13}C-HRMAS approach to characterize two Italian cultivars of garlic (*Allium sativum* L.) [62]. They found several organosulfurs, allicin, and some allyl-organosulfurs in garlic samples and confirmed their presence by SPE-GC-MS analysis. Lam et al. [63] suggested that HRMAS-based experiments also enabled to differentiate and describe both structural and metabolic profiles of intact ^{13}C-enriched wheat, broccoli, and maze seeds [63].

Dairy products were also intensively studied by HRMAS NMR. Already in 2004, HRMAS was applied to trace the metabolomic profile of the Italian Parmigiano Reggiano cheese [64] and to correlate its concentration of several free amino acids and low molecular-weight metabolites to the cheese ripening during a 24 months-long monitoring [65]. Furthermore, Shintu and Caldarelli [66] also described, through HRMAS, the metabolic differences of twenty Emmental cheeses from five different countries [66]. A combination of several multivariate statistical analyses of ^{1}H HRMAS results was used to differentiate the Italian "mozzarella di bufala campana" on the basis of the geographical origin of that specific soft-cheese product [67]. Their work relied not only on conventional proton spectra, but also on both T_2- and diffusion-edited ^{1}H spectra, which enabled to isolate and resolve the proton signals of relatively low molecular-weight molecules (long spin–spin relaxation times) and lipidic components (smaller diffusivity), respectively (Fig. 5). Moreover, they found evidence that the relevant abundances of isobutylic alcohol, lactic acid, and acetic acid, were the markers which would help to identify the aged samples of "mozzarella di bufala campana" [67].

The HRMAS has been also employed to examine cereals, cereal-derived, and apicultural products. This technique combined with HPLC analyses successfully discriminated Italian Royal Jellies from Chinese ones [68]. Moreover, an HRMAS approach was recently

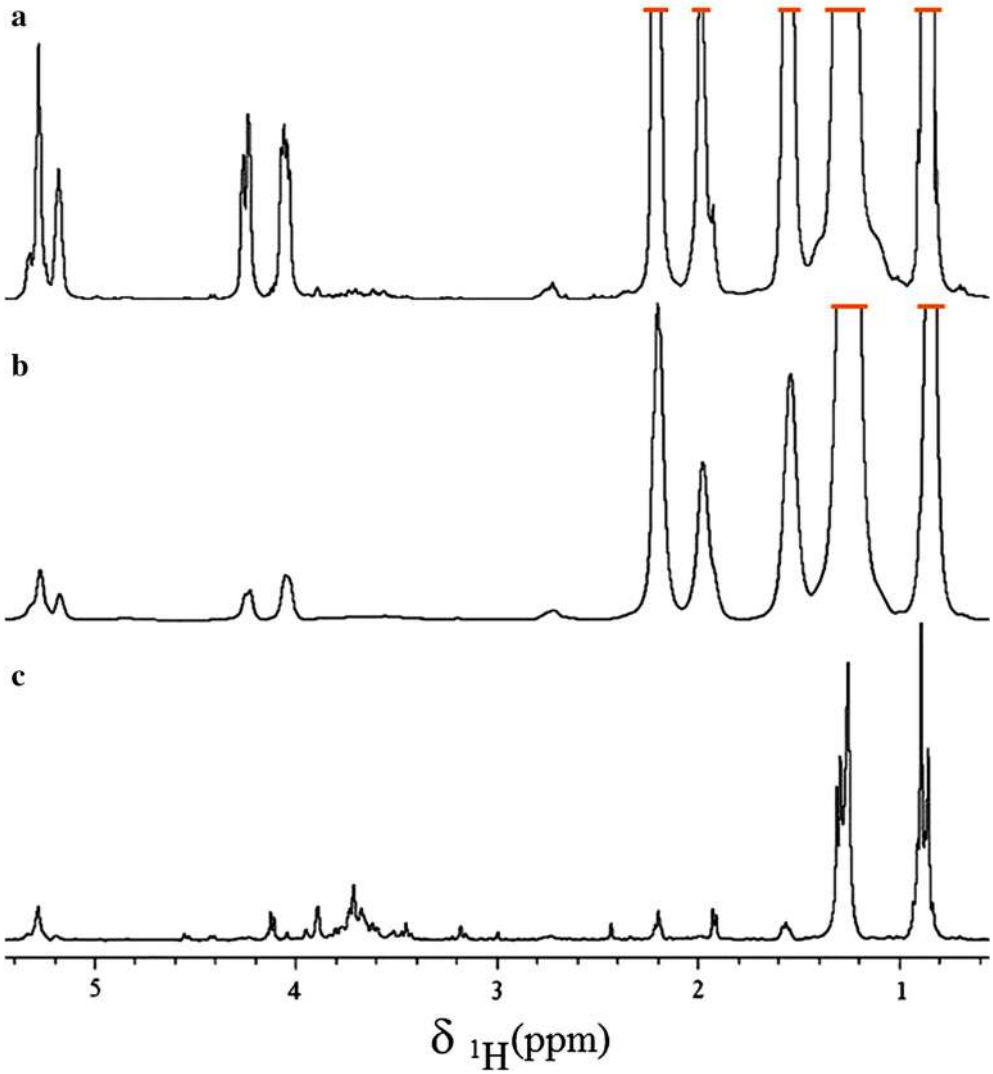

Fig. 5 ^{1}H HRMAS spectra of a sample of buffalo-milk mozzarella: **a** conventional ^{1}H spectrum; **b** diffusion-edited spectrum; **c** CPMG-edited spectrum. [*Figure* reprinted with permission from Mazzei & Piccolo, Copyright 2012 by Food Chem. (Elsevier)]

exploited to distinguish waxy from non-waxy Chinese rice grains, deriving from different *Oryza sativa* L. cultivars [69]. Both ^{1}H and ^{31}P HRMAS NMR spectra were used to elucidate the granule hydration in either wheat- or maize-derived starches at different temperatures [70]. Most interestingly, the authors modulated the MAS spin rate to distinguish between immobilized (water-inaccessible starch domains) and mobile components, thus inferring that their distribution depended on the starch granules assembly [70].

Plant response to biotic and abiotic stress

As for other biological materials HRMAS has been usefully applied to rapidly monitor the plant stress in response to both abiotic and biotic factors, such as drought, salinity, nutrients deficiency, or pathogens influence. For instance, a study by HRMAS of both seedling shoots and roots enabled to identify a neat metabolomic response induced in two cultivars of rice by drought and salinity stress [71]. In particular, the abiotic stress favored the accumulation of free amino acids and saccharides. Concomitantly, the same authors presented preliminary results on the metabolomic changes occurring in rice leaves due to the penetration and colonization of three strains of the *Magnaporthe grisea* phytopathogenic fungus [71].

Both liquid-state and HRMAS NMR techniques showed that drought response in wheat kernels consisted

in a resistance occurring when wheat was subjected to drought early during grain-filling [72]. Moreover, they found an influence on proteins metabolism, and fumaric acid was identified as a potential marker for drought stress in mature kernels. The HRMAS technique was combined with MRI to investigate the poisonous *Jatropha curcas* L. plant infected by a *Jatropha mosaic begomovirus* [73]. This study associated morphological, anatomical, and metabolic profiling of virus-infected tissue of *J. curcas* plant. A twofold accumulation of intermediates of the Tricarboxylic acid (TCA) cycle in virus-infected plants was found, which suggested a larger rate of respiration [73]. In another work, both leaves and fruit peels of *citrus sinensis* plants were proved by HRMAS to be infected by the bacterium *Xanthomonas axonopodis pv. Citri*, which is responsible for the citrus cancer. The authors revealed that the defensive metabolism activated by *Citrus sinensis* induced relevant changes in amino acids, carbohydrates, organic acids, and terpenoids content in the plant [74].

Due to the concern on iron (Fe) plant deficiency, which is potentially responsible for poor yields and crop quality, any understanding of the metabolomic plant response related to poor Fe uptake is important to design strategies to deal with iron-deficiency. With this aim, Fe-sufficient and Fe-deficient soybean leaf extracts and whole leaves were studied by liquid-state and HRMAS NMR, respectively [75]. The authors observed that Fe-deficient soybeans revealed an enhanced TCA cycle activity, and the activation of mechanisms against the oxidative stress. HRMAS NMR has been recently applied to study the metabolomic changes in the germination of wheat seeds treated with fungicides bavistin and thiram [76]. Both fungicides were found to delay the germination cycle in wheat seeds, while thiram, a non-systemic fungicide, affected the metabolic processes to a larger extent than the systemic fungicide bavistin. Moreover, HRMAS identified the metabolic changes exerted by salinity and silicon (H_4SiO_4-based fertilizers) treatments (either alone or combined) on the quality of four Marmande tomato varieties [77]. In particular, silicon addition appeared to enhance the effect of light intensity on photoassimilates available to fruits, without a clear effect on the organoleptic quality.

In vivo HRMAS NMR spectroscopy

Due to its capacity to produce meaningful spectra of semi-solid and fresh samples, HRMAS represents an eligible technique to perform in vivo analysis of living micro- and macroorganisms. In this respect, Fredlund et al. [78] proposed a pioneering method to in vivo study microorganisms subjected to stress conditions [78]. They focused on the biocontrol of yeast *Pichia anomala J121* and verified how their intracellular metabolites varied as a function of an induced oxygen limitation. Specifically, they tested two conditions: (i) initial aerobic conditions with restricted oxygen access during the yeast's growth period, and, (ii) initial microaerobic conditions followed by anaerobiosis. Biomass production was larger in treatment (i), whereas the specific ethanol production rate upon glucose addition was similar in both treatments, thus implying that oxygen availability affected the respiration, but not the yeast fermentation. Following glucose depletion, ethanol was oxidized to acetate in treatment (i), but continued to be produced in (ii) [78].

HRMAS was later applied to monitor the antituberculosis ethionamide pro-drug activation in a strain of *Mycobacterium smegmatis* [79]. This work showed that both conventional and diffusion-edited ^{1}H HRMAS NMR spectra enabled the detection of a drug metabolite directly inside intact cells of living bacteria. Their experimental work was based on an optimized procedure of filling the 4 mm HRMAS rotor with microorganisms. They were successful to show the feasibility even at moderate magnetic fields of NMR detection in bacterial cells of an unlabeled metabolite at a pharmacologically relevant (i.e., submillimolar) concentration [79]. ^{1}H and ^{31}P HRMAS NMR spectroscopy was also applied to in vivo evaluate the composition of *Aporrectodea caliginosa* earthworms [80]. In particular, a metabolic profiling of studied earthworms was achieved in order to monitor the effects of an acute exposure to the herbicide glyphosate. The authors claimed for a low toxicity of the herbicide as well as they argued its possible trapping into the cutaneous mucus which suggested a role of earthworms in the horizontal dispersion and stabilization of glyphosate in the drilosphere. Moreover, ^{31}P-^{1}H HETCOR (HETeroCORrelation) 2D NMR spectra were helpful in the identification of several phosphorylated metabolites such as phospholombricine and lombricine [80]. *Daphnia magna* crustaceans, which are conventionally used as aquatic model organism in ecotoxicology to evaluate the short- and long-term impact of toxic compounds at physiological and genomic levels, were analyzed by HRMAS NMR [81]. The authors identified the metabolic variations occurring in daphnids as a function of successive life stages and at different physiological statuses. More recently, HRMAS was used to examine in vivo the composition of both a wild type and a mutant (GST2) of the *Drosophila melanogaster* fruit fly [82]. Increased levels of triglycerides observed in the mutant were interpreted as indicative of insulin resistance with implications for mitochondrial disfunction. Finally, also the CMP (Comprehensive Multi Phase), a recent NMR probe enabling the analysis of samples in all phases, including the semi-solid HRMAS-like approach, was used to investigate whole living organisms. For example, it has been recently reported that 1D

and 2D CMP-NMR experiments permitted to evaluate the most abundant metabolites in living *Hyalella Azteca* crustaceans [83]. In addition, the authors studied the survival extent of these amphipods as a function of oxygen availability, temperature, and organism orientation into the NMR rotor. It is noteworthy the strategy to feed with ^{13}C-labeled *Chlamydomonas reinhardtii* algae the *H. Azteca* prior their NMR analysis in order to significantly increase the spectroscopic response for carbon signals.

Concluding remarks

HRMAS is a relatively recent NMR technique that was developed to allow direct examination of semi-solid fresh samples without any pre-extraction. Here, we described the most relevant applications of HRMAS in studies related to agricultural issues. Specifically, we showed how HRMAS provides, by both multidimensional and multinuclear spectroscopy, a clear and informative molecular characterization of complex heterogeneous systems (i.e., soil organic matter, plant-derived materials) and enables to unravel the environmental reactivity of inorganic and organic materials. Moreover, when combined with chemometrics, HRMAS represents a reliable tool to study edible agrofood products and assess, directly and indirectly, their nutritional value, geographical origin, authenticity, genotype, and safety. The HRMAS becomes ever more important for the investigation of metabolic processes in plants as related to specific management practices or to responses to either abiotic stress or phytopathogens infections. Moreover, the possibility to acquire edited spectra (T_2- and diffusion-based ones are the most commonly used) by HRMAS, further enhances the potential of this technique for quantitative analyses of complex biological systems.

The wide variety of materials (including in vivo micro- and macroorganisms) successfully studied by HRMAS suggests that it is the best choice when standard solution- and solid-state NMR techniques are not applicable or unsatisfying. Moreover, the rapidity and relative simplicity of the HRMAS technique, well coupled to the inherent reduction of solvents consumption and waste production, represent further elements in making this methodology very attractive for a wide spectrum of scientific investigations.

Authors' contributions
PM and AP contributed equally in the literature selection and discussion. Both authors read and approved the final manuscript.

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References

1. Levitt M. Spin dynamics: basics of nuclear magnetic resonance. 2nd ed. Chichester: Wiley; 2008.
2. Mazzei P, Piccolo A. Interactions between natural organic matter and organic pollutants as revealed by NMR spectroscopy. Magn Reson Chem. 2015;53:667–78.
3. Jacobsen NE. NMR spectroscopy explained—simplified theory, applications and examples for organic chemistry and structural biology. New Jersey: Wiley; 2007.
4. Valentini M, Ritota M, Cafiero C, Cozzolino S, Leita L, Sequi P. The HRMAS–NMR tool in foodstuff characterization. Magn Reson Chem. 2011;49:S121–5.
5. Maas WE, Laukien FH, Cory DG. Gradient, high resolution, magic angle sample spinning NMR. J Am Chem Soc. 1996;118(51):13085–6.
6. Doty FD, Entzminger G, Yang AY. Magnetism in high-resolution NMR probe design. II: HR MAS. Concept Magn Res. 1998;10:239–60.
7. Alam TA, Jenkins JE. HR-MAS NMR spectroscopy in material science. In Farrukh MA, Editor. Advanced aspects of spectroscopy. Intech Open; 2012. p. 279–306.
8. Beckonert O, Coen M, Keun HC, Wang Y, Ebbels TMD, Holmes E, et al. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. Nat Protoc. 2010;5(6):1019–32.
9. Renault M, Shintu L, Piotto M, Caldarelli S. Slow-spinning low-sideband HR-MAS NMR spectroscopy: delicate analysis of biological samples. Sci Rep. 2013;3(3349):1–10.
10. Farooq H, CourtierMurias D, Soong R, Bermel W, Kingery WM, Simpson AJ. HRMAS NMR spectroscopy: a practical guide for natural samples. Curr Org Chem. 2013;17(24):3013–31.
11. Liu M, Nicholson JK, Lindon JC. High-resolution diffusion and relaxation edited one- and two-dimensional 1H NMR spectroscopy of biological fluids. Anal Chem. 1996;68:3370–6.
12. Courtier-Murias D, Farooq H, Masoom H, Botana A, Soong R, Longstaffe JG, et al. Comprehensive multiphase NMR spectroscopy: basic experimental approaches to differentiate phases in heterogeneous samples. J Magn Reson. 2012;217:61–76.
13. Mazzei P, Piccolo A, Nugnes L, Mascolo M, De Rosa G, Staibano S. Metabolic profile of uterine leiomyomas intact tissue using high resolution magic-angle spinning ^{1}H NMR spectroscopy. NMR Biomed. 2010;23:1–10.
14. Mazzei P, Vinale F, Woo SL, Pascale A, Lorito M, Piccolo A. Metabolomics by proton high-resolution magic-angle-spinning nuclear magnetic resonance of tomato plants treated with two secondary metabolites isolated from trichoderma. J Agric Food Chem. 2016;64:3538–45.
15. Šmejkalová D, Spaccini R, Piccolo A. Multivariate analysis of CPMAS ^{13}C-NMR spectra of soils and humic matter as a tool to evaluate organic carbon quality in natural systems. Eur J Soil Sci. 2008;59:496–504.
16. Simpson A, Kingery W, Shaw D, Spraul M, Humpfer E, Dvortsak P. The Application of 1H HR-MAS NMR spectroscopy for the study of structures and associations of organic components at the solid-aqueous interface of a whole soil. Environ Sci Technol. 2001;35:3321–5.
17. Piccolo A, Campanella L, Petronio BM. ^{13}C–NMR spectra of humic substances extracted with different mechanisms. Soil Sci Soc Am J. 1990;54:750–5.
18. Simpson A, Kingery W, Hatcher P. The identification of plant derived structures in humic materials using three-dimensional NMR spectroscopy. Environ Sci Technol. 2003;37:337–42.
19. Feng X, Simpson AJ, Simpson MJ. Investigating the role of mineral-bound humic acid in phenanthrene sorption. Environ Sci Technol. 2006;40:3260–6.
20. Simpson AJ, Simpson MJ, Kingery WL, Lefebvre BA, Moser A, Williams AJ, et al. The application of 1H high-resolution magic-angle spinning

NMR for the study of clay-organic associations in natural and synthetic complexes. Langmuir. 2006;22:4498–503.
21. Piccolo A. The supramolecular structure of humic substances. A novel understanding of humus chemistry and implications in soil science. Adv Agron. 2002;75:57–134.
22. Kelleher BP, Simpson MJ, Simpson AJ. Assessing the fate and transformation of plant residues in the terrestrial environment using HR-MAS NMR spectroscopy. Geochim Cosmochim Acta. 2006;70:4080–94.
23. Spence A, Simpson AJ, Mcnally DJ, Moran BW, McCaul MV, et al. The degradation characteristics of microbial biomass in soil. Geochim Cosmochim Acta. 2011;75:2571–81.
24. Zhong J, Sleighter RL, Salmon E, McKee GA, Hatcher PG. Combining advanced NMR techniques with ultrahigh resolution mass spectrometry: a new strategy for molecular scale characterization of macromolecular components of soil and sedimentary organic matter. Org Geochem. 2011;42:903–16.
25. Ferrari E, Francioso O, Nardi S, Saladini M, Dal Ferro N, Morari F. DRIFT and HR MAS NMR characterization of humic substances from a soil treated with different organic and mineral fertilizers. J Mol Struct. 2011;998:216–24.
26. Courtier-Murias D, Simpson AJ, Marzadori C, Baldoni G, Ciavatta C, Fernández JM, et al. Unraveling the long-term stabilization mechanisms of organic materials in soils by physical fractionation and NMR spectroscopy. Agric Ecosyst Environ. 2013;171:9–18.
27. Plaza C, Courtier-Murias D, Fernández JM, Polo A, Simpson AJ. Physical, chemical, and biochemical mechanisms of soil organic matter stabilization under conservation tillage systems: a central role for microbes and microbial by-products in C sequestration. Soil Biol Biochem. 2013;57:124–34.
28. Dal Ferro N, Berti A, Francioso O, Ferrari E, Matthews GP, Morari F. Investigating the effects of wettability and pore size distribution on aggregate stability: the role of soil organic matter and the humic fraction. Eur J Soil Sci. 2012;63:152–64.
29. Pizzeghello D, Cocco S, Francioso O, Ferrari E, Cardinali A, Nardi S, et al. Snow vole (Chionomys nivalis Martins) affects the redistribution of soil organic matter and hormone-like activity in the alpine ecosystem: ecological implications. Ecol Evol. 2015;5(20):4542–54.
30. Combourieu B, Inacio J, Delort AM, Forano C. Differentiation of mobile and immobile pesticides on anionic clays by 1H HR MAS NMR spectroscopy. Chem Commun. 2001;21:2214–5.
31. Shirzadi A, Simpson MJ, Kumar R, Baer AR, Xu Y, Simpson AJ. Molecular interactions of pesticides at the soil-water interface. Environ Sci Technol. 2008;42:5514–20.
32. Masoom H, Courtier-Murias D, Soong R, Maas WE, Fey M, Kumar R, et al. From spill to sequestration: the molecular journey of contamination via comprehensive multiphase NMR. Environ Sci Technol. 2015;49:13983–91.
33. Beni C, Marconi S, Boccia P, Ciampa A, Diana G, Aromolo R, et al. Use of arsenic contaminated irrigation water for lettuce cropping: effects on soil, groundwater, and vegetal. Biol Trace Elem Res. 2011;143:518–29.
34. Pereira SI, Figueiredo PI, Barros AS, Dias MC, Santos C, Duarte IF, Gil AM. Changes in the metabolome of lettuce leaves due to exposure to mancozeb pesticide. Food Chem. 2014;154:291–8.
35. Blondel C, Khelalfa F, Reynaud S, Fauvelle F, Raveton M. Effect of organochlorine pesticides exposure on the maize root metabolome assessed using high-resolution magic-angle spinning 1H NMR spectroscopy. Environ Pollut. 2016;214:539–48.
36. Pacifico D, Casciani L, Ritota M, Mandolino G, Onofri C, Moschella A, et al. NMR-based metabolomics for organic farming traceability of early potatoes. J Agric Food Chem. 2013;61:11201–11.
37. Rosati A, Cafiero C, Paoletti A, Alfei B, Caporali S, Casciani L, et al. Effect of agronomical practices on carpology, fruit and oil composition, and oil sensory properties, in olive (*Olea europaea* L.). Food Chem. 2014;159:236–43.
38. Deshmukh AP, Simpson AJ, Hatcher PG. Evidence for cross-linking in tomato cutin using HR-MAS NMR spectroscopy. Phytochemistry. 2003;64:1163–70.
39. Deshmukh AP, Simpson AJ, Hadad CM, Hatcher PG. Insights into the structure of cutin and cutan from Agave americana leaf cuticle using HRMAS NMR spectroscopy. Org Geochem. 2005;36:1072–85.
40. Turner JW, Hartman BE, Hatcher PG. Structural characterization of suberan isolated from river birch (*Betula nigra*) bark. Org Geochem. 2013;57:41–53.
41. Alcantara GB, Honda NK, Ferreira MMC, Ferreira AG. Chemometric analysis applied in 1H HR-MAS NMR and FT-IR data for chemotaxonomic distinction of intact lichen samples. Anal Chim Acta. 2007;595:3–8.
42. Bharti SK, Bhatia A, Tewari SK, Sidhu OP, Roy R. Application of HR-MAS NMR spectroscopy for studying chemotype variations of *Withania somnifera* (L.) Dunal. Magn Reson Chem. 2011;49:659–67.
43. Manolopoulou E, Spinella A, Caponetti E, Dais P, Spyros A. High resolution-magic angle spinning NMR study of olive leaves. In: van Duynhoven J, Belton PS, Webb GA, van As H, editors. Magnetic resonance in food science: food for thought. London: Royal Society of Chemistry; 2013. p. 164–70.
44. Delgado-Goni T, Campo S, Martın-Sitjar J, Cabanas ME, San Segundo B, Arus C. Assessment of a 1H high-resolution magic angle spinning NMR spectroscopy procedure for free sugars quantification in intact plant tissue. Planta. 2013;238:397–413.
45. Marino G, Righi V, Simoni A, Schenetti L, Mucci A, Tugnoli V, et al. Effect of a peat humic acid on morphogenesis in leaf explants of *Pyrus communis* and *Cydonia oblonga*. Metabolomic analysis at an early stage of regeneration. J Agric Food Chem. 2013;61:4979–87.
46. Righi V, Parenti F, Tugnoli V, Schenetti L, Mucci A. Crocus sativus petals: waste or valuable resource? The answer of high-resolution and High-resolution magic angle spinning nuclear magnetic resonance. J Agric Food Chem. 2015;63:8439–44.
47. Mori T, Tsuboi Y, Ishida N, Nishikubo N, Demura T, Kikuchi J. Multidimensional high-resolution magic angle spinning and solution-state NMR characterization of 13C-labeled plant metabolites and lignocellulose. Sci Rep. 2015;5(11848):1–12.
48. Wiklund S, Karlsson M, Antti H, Johnels D, Sjöström M, Wingsle G, et al. A new metabonomic strategy for analysing the growth process of the poplar tree. Plant Biotechnol J. 2005;3:353–62.
49. Choze R, Alcantara GB, Filho EGA, Silva LMA, Faria JC, Liao LM. Distinction between a transgenic and a conventional common bean genotype by 1H HR-MAS NMR. Food Chem. 2013;141:2841–7.
50. Oliveira CS, Carlos EF, Vieira LGE, Liãoc LM, Alcantara GB. HR-MAS NMR metabolomics of 'Swingle' citrumelo rootstock genetically modified to overproduce proline. Magn Reson Chem. 2014;52:422–9.
51. Wheeler HL, Soong R, Courtier-Murias D, Botana A, Fortier-Mcgill B, Maas WE, et al. Comprehensive multiphase NMR: a promising technology to study plants in their native state. Magn Reson Chem. 2015;53:735–44.
52. Gil AM, Duarte IF, Delgadillo I, Colquhoun IJ, Casuscelli F, Humpfer E, Spraul M. Study of the compositional changes of mango during ripening by use of nuclear magnetic resonance spectroscopy. J Agric Food Chem. 2000;48(5):1524–36.
53. Otero L, Préstamo G. Effects of pressure processing on strawberry studied by nuclear magnetic resonance. Innov Food Sci Emerg Technol. 2009;10:434–40.
54. Ciampa A, Renzi G, Taglienti A, Sequi P, Valentini M. Studies on coffee roasting process by means of nuclear magnetic resonance spectroscopy. J Food Quality. 2010;33:199–211.
55. Vermathen M, Marzorati M, Baumgartner D, Good C, Vermathen P. Investigation of different apple cultivars by high resolution magic angle spinning NMR. A feasibility study. J Agric Food Chem. 2011;59:12784–93.
56. Alves Filho EG, Silva LMA, Choze R, Lião LM, Honda NK, Alcantara GB. Discrimination of sugarcane according to cultivar by 1H NMR and chemometric analyses. J Braz Chem Soc. 2012;23(2):273–9.
57. Mucci A, Parenti F, Righi V, Schenetti L. Citron and lemon under the lens of HR-MAS NMR spectroscopy. Food Chem. 2013;141:3167–76.
58. Marseglia A, Acquotti D, Consonni R, Cagliani LR, Palla G, Caligiani C. HR MAS 1H NMR and chemometrics as useful tool to assess the geographical origin of cocoa beans—comparison with HR 1H NMR. Food Res Int. 2016;85:273–81.
59. Pérez EMS, Iglesias MJ, Ortiz FL, Pérez I, Galera MM. Study of the suitability of HRMAS NMR for metabolic profiling of tomatoes: application to tissue differentiation and fruit ripening. Food Chem. 2010;122:877–87.
60. Pérez EMS, López JG, Iglesias MJ, Ortiz FL, Toresano F, Camacho F. HRMAS-nuclear magnetic resonance spectroscopy characterization of tomato "flavor varieties" from Almería (Spain). Food Res Int. 2011;44:3212–21.
61. Ritota M, Comarini F, Sequi P, Valentini M. Metabolomic characterization of Italian sweet pepper (*Capsicum annum* L.) by means of HRMAS-NMR spectroscopy and multivariate analysis. J Agric Food Chem. 2010;58(17):9675–84.

62. Ritota M, Casciani L, Han BZ, Cozzolino S, Leita L, Sequi P, et al. Traceability of Italian garlic (*Allium sativum* L.) by means of HRMAS-NMR spectroscopy and multivariate data analysis. Food Chem. 2012;135:684–93.
63. Lam L, Soong R, Sutrisno A, de Visser R, Simpson MJ, Wheeler HL, et al. Comprehensive multiphase NMR Spectroscopy of Intact 13C Labeled Seeds. J Agric Food Chem. 2014;62:107–15.
64. Shintu L, Ziarelli F, Caldarelli S. Is high-resolution magic angle spinning NMR a practical speciation tool for cheese samples? Parmigiano Reggiano as a case study. Magn Reson Chem. 2004;42:396–401.
65. Shintu L, Caldarelli S. High-resolution MAS NMR and chemometrics: characterization of the ripening of Parmigiano Reggiano Cheese. J Agric Food Chem. 2005;53:4026–31.
66. Shintu L, Caldarelli S. Toward the determination of the geographical origin of emmental(er) Cheese via high resolution MAS NMR: a preliminary investigation. J Agric Food Chem. 2006;54:4148–54.
67. Mazzei P, Piccolo A. ^{1}H HRMAS-NMR metabolomic to assess quality and traceability of mozzarella cheese from Campania buffalo milk. Food Chem. 2012;132:1620–7.
68. Messia MC, Sobolev AP, Gómez-Caravaca AM, Lamanna R, D'Amico I, Caboni MF, Marconi E, et al. HR-MAS NMR metabolic profiling, furosine and (E)-10-Hydroxy-2-decenoic acid for qualitative and geographical discrimination of royal jelly. J Apic Res. 2013;52(3):141–8.
69. Song EH, Kim HJ, Jeong J, Chung HJ, Kim HY, Bang E, et al. A ^{1}H HR-MAS NMR-based metabolomic study for metabolic characterization of rice grain from various *Oryza sativa* L. cultivars. J Agric Food Chem. 2016;64:3009–16.
70. Larsen FH, Blennow A, Engelsen SB. Starch granule hydration—a MAS NMR Investigation. Food Biophys. 2008;3:25–32.
71. Fumagalli E, Baldoni E, Abbruscato P, Piffanelli P, Genga A, Lamanna R, et al. NMR techniques coupled with multivariate statistical analysis: tools to analyse *Oryza sativa* metabolic content under stress conditions. J Agron Crop Sci. 2009;195:77–88.
72. Winning H, Viereck N, Wollenweber B, Larsen FH, Jacobsen S, Søndergaard I, et al. Exploring abiotic stress on asynchronous protein metabolism in single kernels of wheat studied by NMR spectroscopy and chemometrics. J Exp Bot. 2009;60(1):291–300.
73. Sidhu OP, Annarao S, Pathre U, Snehi SK, Raj SK, Roy R, et al. Metabolic and histopathological alterations of Jatropha mosaic begomovirus-infected *Jatropha curcas* L. by HR-MAS NMR spectroscopy and magnetic resonance imaging. Planta. 2010;232:85–93.
74. Silva LMA, Alves Filho EG, Choze R, Lião LM, Alcantara GB. 1H HRMAS NMR spectroscopy and chemometrics for evaluation of metabolic changes in *Citrus sinensis* caused by Xanthomonas axonopodis pv. Citri. J Braz Chem Soc. 2012;23(6):1054–61.
75. Lima MRM, Diaz SO, Lamego I, Grusak MA, Vasconcelos MW, Gil AM. Nuclear magnetic resonance metabolomics of iron deficiency in soybean leaves. J Proteome Res. 2014;13:3075–87.
76. Gogna N, Dorai K. HR-MAS NMR-based metabolomic approach to study the effect of fungicidal stress on wheat seed germination. Curr Sci. 2015;108(9):1694–701.
77. Iglesias MJ, García-López J, Collados-Luján JF, López-Ortiz F, Díaz M, Toresano F, et al. Differential response to environmental and nutritional factors of high-quality tomato varieties. Food Chem. 2015;176:278–87.
78. Fredlund E, Broberg A, Boysen ME, Kenne L, Schnürer J. Metabolite profiles of the biocontrol yeast *Pichia anomala* J121 grown under oxygen limitation. Appl Microbiol Biotechnol. 2004;64:403–9.
79. Hanoulle X, Wieruszeski JM, Rousselot-Pailley P, Landrieu I, Baulard AR, Lippens G. Monitoring of the ethionamide pro-drug activation in mycobacteria by 1H high resolution magic angle spinning NMR. Biochem Biophys Res Commun. 2005;331:452–8.
80. Bon D, Gilard V, Massou S, Pérès G, Malet-Martino M, Martino R, et al. In vivo 31P and 1H HR-MAS NMR spectroscopy analysis of the unstarved *Aporrectodea caliginosa* (Lumbricidae). Biol Fertil Soils. 2006;43:191–8.
81. Bunescu A, Garric J, Vollat B, Canet-Soulas E, Graveron-Demilly D, Fauvelle F. In vivo proton HRMAS NMR metabolic profile of the freshwater cladoceran Daphnia magna. Mol Biosyst. 2010;6:121–5.
82. Righi V, Apidianakis Y, Psychogios N, Rahme LG, Tompkins RG, Tzika A. In vivo high-resolution magic angle spinning proton NMR spectroscopy of *Drosophila melanogaster* flies as a model system to investigate mitochondrial dysfunction in *Drosophila* GST2 mutants. Int J Mol Med. 2014;34:327–33.
83. Liaghati Mobarhan Y, FortierMcGill B, Soong R, Maas WE, Fey M, Monette M, et al. Comprehensive multiphase NMR applied to a living organism. Chem Sci. 2016;7:4856–66.