Progress in Nuclear Magnetic Resonance Spectroscopy 54 (2009) 195–207

Contents lists available at ScienceDirect

Progress in Nuclear Magnetic Resonance Spectroscopy

journal homepage: www.elsevier.com/locate/pnmrs

^{31}P NMR spectroscopy in food analysis

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ARTICLE INFO

Article history:
Received 15 August 2008
Accepted 20 September 2008
Available online 27 September 2008

Keywords:
^{31}P NMR
Phosphorus
Food composition
Food analysis

Contents

1. Introduction

In the past three decades, NMR has found its way into the arsenal of spectroscopic techniques available to food scientists, and has now become a valuable tool in the study of a wide variety of structural and compositional aspects of food chemistry and food analysis. This growing interest has been duly recorded in a series of biannual conferences devoted to "Applications of magnetic resonance in food science" that started in 1992, and continue to date. Proceedings collections from this conference are published in a book series that represents a prime source of reference for the state of the art in the field of NMR applications in food science [1]. Earlier food NMR work was covered in a series of reviews that appeared in Annual Reports on NMR Spectroscopy [2–4]. NMR spectroscopy is currently recognized as an important tool in food

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0079-6565/$ - see front matter
doi:10.1016/j.pnmrs.2008.09.002

science [5,6], food analysis [7,8], and the authentication [9,10], and quality control of foodstuff [11,12]. Low field [13], solid state [14] NMR spectroscopy, and MRI [15] applications in food science have also been reviewed. Reviews of NMR spectroscopy applications in specific topics of food science and food analysis, such as milk and dairy products [16–18], meat [19–21], fruits and vegetables [22], cereals [23], lipids [24], and edible oils [25] have also been published.

Several factors have contributed to the rapidly increasing use of NMR in the field of food science, including advances in high-field magnet and probe design that amplified the analytical capabilities of modern NMR spectrometers, the successful hyphenation of NMR with liquid chromatographic techniques in LC-NMR, and the easier access of food scientists to NMR spectrometers. Although sensitivity reasons rendered the ^{1}H nucleus as the most exploited, other nuclei such as ^{13}C and ^{31}P gained popularity lately, because of their ability to attack specific problems in food science. Specifically, ^{31}P NMR has a long history in food science, with key papers introducing the methodology in meat [26] and milk [27] from a food science perspective dating back to 1985. ^{31}P NMR spectroscopy was of course already established by then as a powerful tool in chemistry, biology and medicine. Gorenstein was the first to summarize the field with an emphasis in biological/biochemical applications [28], while Quin and Verkade focused on chemical characterization and structural analysis [29]. ^{31}P NMR work in food science has been covered as part of general NMR reviews for milk [9], meat [19], and lipids [24], while recently a review of ^{31}P NMR work related to olive oil analysis has been published [30].

In the present work we will focus on publications that use ^{31}P NMR in a food science context, with emphasis on analysis. It should be stressed that most foods originate either from plants or animals, and therefore there may be in certain cases significant subject overlap with biological chemistry, biochemistry and physiology. The authors believe that any attempt to define and discriminate between these borders is not only impractical, but also scientifically inappropriate. This report will attempt to present a comprehensive survey of the literature available on applications of high resolution ^{31}P NMR spectroscopy in the analysis of foods. Also, applications of solid state ^{31}P NMR will be covered for completeness, since it appears that lately this NMR technique is gaining momentum in food analysis research.

2. Phosphorus compounds in foods

Phosphorus is an essential element, and comprises about 1% of the total body weight of adults. Inorganic phosphorus, Pi, and organic phosphorus-containing compounds play a major role in the metabolic reactions comprising life, and are involved in significant functions in the human body. Hydroxyapatite, or calcium phosphate, is the major component of bone and teeth. Phospholipids are important structural components of cell wall membranes. Nucleotides, such as adenosine triphosphate (ATP) are at the heart of the cascade of reactions responsible for energy transfer in all living cells, and several proteins require phosphorylation in order to become active and perform their functional role. Although phosphorus homeostasis in the human body is maintained by several dedicated mechanisms, phosphorus deficiency is rarely a problem in healthy adults [31]. The recommended daily allowance (RDA) of approximately 0.5 g of phosphorus is easily satisfied through food consumption, since foods high in protein, such as meat, milk, eggs and cereals, are also high in phosphorus. This element is very common in both animals and plants, where it serves the same crucial functions as in the human body. Phosphorus in foods is generally easily bioavailable, with the exception of plant seeds, where it is stored in the form of inositolhexaphosphate, IP_6, also termed as phytic acid, or phytate. The digestive systems of most mammals cannot hydrolyze phytic acid, and hence its phosphorus is not directly available. Apart from its natural occurrence in foods, phosphorus compounds are also used extensively as food additives. Lecithins enjoy widespread use as surface active food ingredients, and synthetic lecithins such as ammonium phosphatides are used as emulsifiers to improve the texture of foods such as chocolate and cocoa products. Inorganic polyphosphates are used as acidity regulators in soft drinks, to improve the water-holding capacity of processed meat, and in processed cheeses, dressings, and bakery products. Recently, concerns have been raised on some effects of increased phosphorus dietary consumption in the form of inorganic phosphate additives [32,33], however studies have shown mixed results and this matter is not yet resolved.

3. Experimental considerations

3.1. The ^{31}P nucleus

^{31}P is a spin 1/2 nucleus with 100% natural abundance, and reasonably good natural receptivity, 391 times larger than ^{13}C. The chemical shift range covered by ^{31}P-containing compounds covers more than 700 ppm, from 500 to −200, with 85% H_3PO_4 used as the reference at δ 0.0 [28,29]. However, most of the chemical compounds of interest to food science contain pentavalent phosphorous in the form of phosphate, and thus appear in a relatively narrow region between approx. 20 to −40 ppm. Trivalent phosphorus compounds obtained through chemical derivatization of some food components [34] appear in the range 100–200 ppm. The substituents on the oxygen atoms of phosphate influence the electron density on the phosphorus nucleus, and this has two important consequences for ^{31}P chemical shifts. The first is related to the ^{31}P chemical shift change of the phosphate entity, when metal cations bind to oxygen, and the second consequence is the phosphate chemical shift dependence on pH changes induced by the number of protons bound on the phosphate anion. Although this attribute has been fully exploited, for example to measure intracellular pH in animal [35] and plant tissues, it remains a factor that demands caution when signal assignments in aqueous solutions or tissue are attempted, especially for unknown samples [36]. The long spin-lattice relaxation times T_1 of ^{31}P nuclei, and the variable nuclear Overhauser enhancement (NOE) with adjacent proton nuclei represent two additional factors that influence the ability of ^{31}P NMR to provide quantitative results on the analysis of food materials composition. ^{31}P T_1 relaxation times of the order of 5–15 s require repetition times five times the longest T_1 of more than a minute, thus experiment times can become quite long, especially for low concentration samples. Practical measures used to overcome this problem are: (a) the addition of paramagnetic relaxation agents, such as $Cr(acac)_3$, to lower the T_1 to values lower than 5 s (b) the use of an internal phosphorus standard of known concentration. The latter methodology is less accurate, since it implies that all phosphorus species in a sample have similar T_1 values, which may not be the case. On the other hand, NOE effects can be eliminated by using an appropriate pulse sequence for the acquisition of spectra, such as the inverse-gated decoupling technique [37]. A recent systematic validation of quantitative ^{31}P NMR has indicated that under careful experimental conditions, ^{31}P NMR can compete effectively with chromatographic methods in analytical capability [38].

3.2. Sample preparation

Foods are complex multicomponent systems, necessitating a variety of experimental NMR methodologies to be applied for

the analysis of the phosphorus species they contain. In meat studies, ^{31}P NMR spectra of intact muscle with or without deuterium locking have been used with great success, obtained either by utilizing large bore magnets or horizontal MRI scanners. Phosphorus metabolites are concentrated enough in raw and *post-mortem* meat to allow this type of approach. For carbohydrate-based foods, the low solubility of starch and the low phosphorus content necessitate the use of enzymes to break down the large macromolecular carbohydrate polymers into dextrins, which is an important step for the quantitative analysis of the phosphorus compounds. In a foodstuff such as milk, where phosphorus appears in the form of phospholipids, elaborate experimental procedures have been developed in order to acquire high resolution ^{31}P NMR spectra. These procedures mainly derive from biological applications of ^{31}P NMR spectroscopy, where the acquisition of acceptable NMR spectra is crucial for phosphorus speciation in various tissues and body fluids.

Phospholipids (PL) extracted from foodstuff have the natural tendency to self-aggregate in both polar and apolar solvents. Due to the resulting chemical shift anisotropy effects, ^{31}P NMR spectra become broad and unsuitable for quantitative analysis. In 1979, London and Feigenson demonstrated that an aqueous solution of EDTA combined with detergents is able to provide narrow ($\sim$1 Hz) ^{31}P NMR phospholipid lines, however chemical shifts are detergent and pH dependent [39]. Sotirhos et al. showed that exploiting the sensitivity of high field spectrometers it was possible to obtain reasonably resolved ^{31}P NMR spectra of phospholipids in chloroform/methanol without any pretreatment [40]. Meneses and Glonek [41] described a biphasic chloroform/methanol/water–EDTA solvent system that led to sharp phosphorus signals, with EDTA used to chelate paramagnetic cations that bind to PLs and lead to signal broadening. Unfortunately, spectral reproducibility with this biphasic solvent system is hindered by the strong dependence of PL chemical shifts on the amount of water and methanol present in the chloroform-rich phase, and on PL concentration. A single-phase solution providing reproducible spectra can be obtained using the chloroform/methanol/water–EDTA solvent system [42], provided that exact quantities of the three components are used. However this approach is experimentally difficult to realize because of the low boiling point of chloroform and methanol. In a detailed study of possible monophasic solvent systems suitable for phospholipids NMR analysis, Bosco et al. [43] made a thorough investigation of various solvent systems and proposed using a solution of triethylamine, dimethylformamide, and guanidinium chloride (Et_3N/DMF–GH+). The superior chemical shift dispersion and reproducibility of this solvent system was demonstrated by analyzing crude lipid extracts from different biological sources [44]. Fig. 1 compares representative ^{31}P NMR spectra of a sample of commercial lecithin obtained using the biphasic ($CHCl_3$/CH_3OH/H_2O–EDTA) and the monophasic (Et_3N/DMF–GH+) solvent systems described above. Table 1 presents the ^{31}P chemical shifts of the most important PLs for food analysis, in the same two solvent systems.

Puppato et al. [45] have recently contributed a detailed study of the pH and temperature dependence of PL chemical shifts when using sodium cholate as detergent. This particular solvent system is widely used for the study of PLs extracted from tissues and body fluids [46], although its application in food systems has been limited. The main disadvantage of this methodology is that samples prepared in this way are only suitable for ^{31}P NMR spectroscopy, since the high concentration of the cholate salt results in signal crowding of the ^{1}H and ^{13}C NMR spectra and precludes multinuclear NMR approaches using the same sample. The application of ^{31}P NMR spectroscopy for the study of PL as micellar and membrane components in body fluids, cell and tissue has been recently reviewed [47].

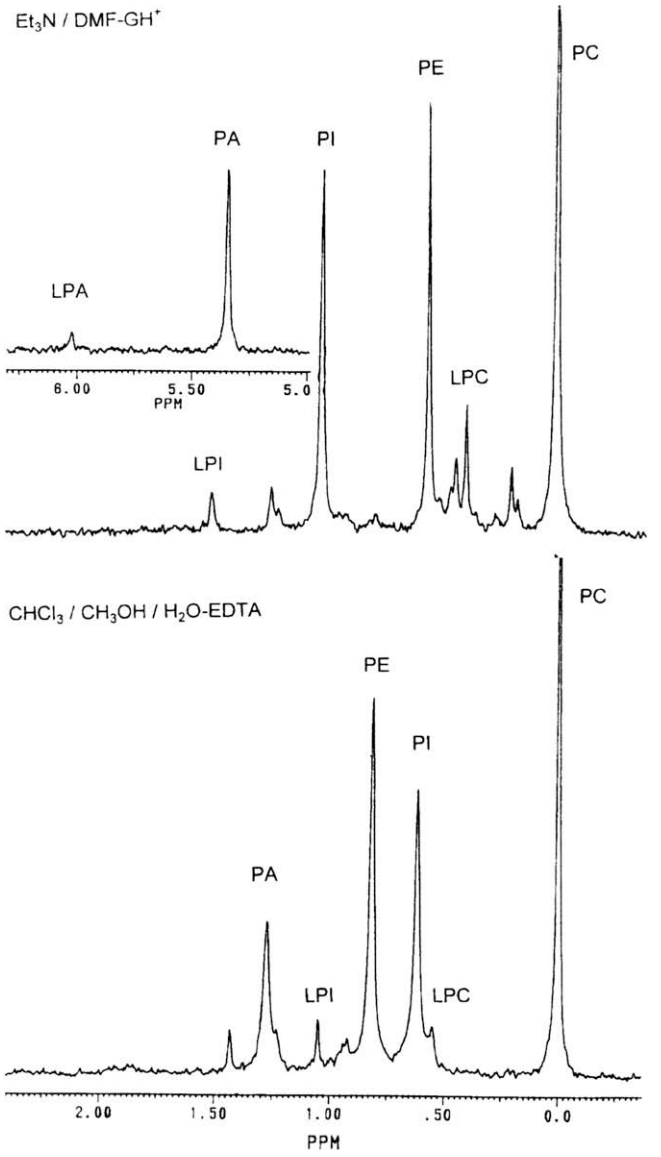

Fig. 1. ^{31}P NMR spectra (121.5 MHz) of commercial lecithin in $CHCl_3$/CH_3OH/H_2O–EDTA (10/4/1) and Et_3N/DMF–GH^+/ (0.3/1) solvent systems; from Ref. [43], used with permission.

Table 1
^{31}P chemical shifts (ppm) of the most common phospholipids in two solvent systems used for the analysis of phospholipids in foods, referenced to external 85% H_3PO_4 [43]

Compound	$CHCl_3$/MeOH/H_2O–EDTA	Et_3N/DMF–GH^+
Phosphatidylcholine, PC	−0.84	0.38
Lyso-phosphatidylcholine, LPC	−0.28	0.83
Phosphatidylserine, PS	−0.21	0.92
Phosphatidylethanolamine, PE	0.00	0.94
Sphingomyelin, SM	−0.09	1.22
Phosphatidylinositol, PI	−0.28	1.40
Phosphatidylglycerol, PG	0.57	1.63
Lyso-phosphatidylinositol, LPI	0.19	1.89
Phosphatidic acid, PA	0.30	5.72
Lyso-phosphatidic acid, LPA	0.91	6.39

4. Applications

4.1. Vegetable oils

Although most vegetable oils do contain small amounts of phospholipids right after extraction, the refining procedure used

to lower their acidity, to improve their organoleptic properties and make them suitable for consumption results in the elimination of phospholipids from the final oil product. Extra-virgin olive oil is a high value edible oil which is extracted from olives using only physical procedures, and thus retains its PL content intact. Recently ^{31}P NMR has been used to measure the phospholipid content of greek extra-virgin olive oil, identifying PA, PI, and the hydrolyzed lyso– analogues, LPA and LPI as the most abudant PLs [48]. Significant quantities of phospholipids were also identified in crude olive-seed oil and in the solid residue precipitate of "veiled" olive oil, while they are absent in refined olive oils. The authors also showed that washing the PL solution with a citric acid-Cs^+ salt resulted in a more efficient removal of paramagnetic cations and better signal to noise ratios in the PL ^{31}P NMR spectra [48].

Although edible oils do not contain any other phosphorus compounds in appreciable amounts, ^{31}P NMR has made significant contributions in oil analysis through an indirect approach inspired from coal (see Chapter 28 in [29]) and lignin [49] research. A phosphitylating agent, I, is used to tag all compounds containing hydroxyl and carboxyl groups and thus afford quantitatively the respective phosphitylated compounds, II, as depicted in Fig. 2. The chemical shift of the phosphitylated compounds II depends on the chemical structure around the –OH group of the original compounds, and thus their concentration can be measured rapidly and accurately by using suitable experimental quantitative ^{31}P NMR protocols [34]. Mono- and diglycerides (DG), free fatty acids, glycerol, total free sterols and polyphenols are minor components of edible oils bearing hydroxyl or carboxyl groups, and thus can be easily studied by ^{31}P NMR spectroscopy. These compounds are also related to the sensory and nutritional properties of oils and are very important for the characterization, authentication and quality control of edible oils, and especially extra-virgin olive oil. The DG and FFA content of a great number of extra-virgin, commercial and refined olive oils from different cultivation regions in Greece has been reported, and oil quality was correlated with the diglyceride (DG) ratio of 1,2-DG to the total-DG isomers (termed D) of the olive oils. [50] A detailed experimental and theoretical study of the kinetics of DG isomerization in olive oil [51] showed that D is an index that is directly correlated with the freshness of olive oil. Further work showed that ^{31}P NMR-derived compositional data in conjuction with chemometrics can be used to detect extra-virgin olive oil adulteration with either seed oils [52], or lampante and refined olive oils [53]. Another application of ^{31}P NMR in olive oil analysis is the quantitative determination of a plethora of phenolic compounds, presented in Fig. 3, that are responsible for the increased sensory, nutritional and antioxidant properties of extra-virgin olive oil [54]. The identification of the different phenolic compounds and sugar derivatives was attained by a combination of ^{1}H and ^{31}P homonuclear and heteronuclear 2D NMR experiments, and spiking the sample with model compounds when necessary [54,55]. Recently, ^{1}H and ^{31}P NMR fingerprinting of minor components of a monovarietal virgin olive oil was used for its geographical characterization applying chemometrics [56]. Cross-validation of the ^{31}P NMR analytical methodology in the determination of olive oil minor compounds has been performed by comparison with standard analytical methodologies [57].

The ^{31}P-tagging NMR methodology has also been applied to study the effect of thermal stressing on saturated [58,59] and unsaturated [60] seed oils. Application of this ^{31}P-tagging NMR methodology is in principle possible in a wide range of food systems provided the components of interest bear hydroxyl or carboxyl groups. In foods containing high amounts of water that might interfere with the phosphitylation reaction, analysis can be performed on the dried food residue, as for example, in the determination of glycerol levels in wine [61]. In foods containing trace amounts of water, it is even possible to use this methodology to measure the water content, as was recently demonstrated in the case of olive oil [62].

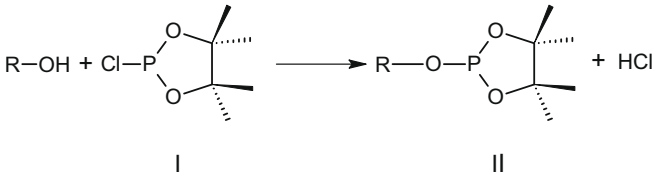

Fig. 2. Reaction of hydroxyl and carboxyl groups of food components with the phosphorus reagent 2-chloro-4,4,5,5- tetramethyldioxaphospholane (I) to afford the phosphitylated products (II).

4.2. Meat

Metabolic reactions in muscle *post-mortem* are a critical factor for meat quality because of their effect on the meat's water holding capacity (WHC) [63] and the loss of extensibility during the onset of *rigor mortis* [64]. Since several important metabolites contain phosphorus, ^{31}P NMR is a powerful tool for the study of both the intrinsic factors effecting muscle metabolism, such as species, age, genetic type, muscular type, and the technological processes followed during meat production, such as feeding, slaughter conditions, storage temperature, and brine injection [19,64]. Most of the work in the field involves high resolution ^{31}P NMR spectroscopy applied to intact muscles, however, recently solid state NMR studies have appeared involving fast [65] and slow [66] MAS spinning rates, and have shown great potential.

Early work demonstrated that ^{31}P NMR spectra of raw intact muscle do indeed contain information on the levels of phosphorylated metabolites and their changes *post-mortem* [35,67]. Vogel et al. published the first application of ^{31}P NMR in a meat science context by studying the *post-mortem* variation of soluble muscle metabolites ATP, phosphocreatine, PCr, phosphomonoesters, PME, and inorganic phosphate, Pi, in beef slaughter carcasses [26]. Fig. 4 depicts a typical stacked plot of ^{31}P NMR spectra recorded continuously from 20 min *post-mortem* until 12 h *post-mortem* on a muscle sample.

An added advantage is that the ^{31}P NMR chemical shifts of ATP, PME and Pi, can be used to measure the intracellular pH of the muscle [35,67]. Most studies use the chemical shift of inorganic phosphorus Pi peak for pH determination because of its favorable pK value. The width of the same signal serves as an indicator of pH heterogeneity in the muscle [64]. The ^{31}P NMR methodology was subsequently used to study the rate of post-mortem metabolism in muscles from several different species of interest to meat science, such as beef [26], rabbit [68], lamb [69], and pork [70]. It was reported that muscle metabolic rates follow the order porcine > ovine > bovine, [69,71], although it should be stressed that interspecies comparisons are complicated by the fact that the metabolic rates depend on many factors, including processing procedures such as pre-slaughter stress, stunning method, cooling, etc.

The effect of fiber composition in muscle metabolism has also been studied in rabbit [72], rat [73], goat [74], pigeon and chicken [75] by ^{31}P NMR spectroscopy. In all cases the rate of ATP and PCr degradation was found to be highest in oxidative and lowest in pure glycolytic muscles, but conflicting results were reported for the rates of pH reduction in different species, indicating that factors other than glycolysis might also play a role [64].

Pork meat is usually classified in three different qualities: normal, pale soft exudative (PSE), possessing low WHC, and dark firm dry (DFD) displaying a high ultimate pH and high WHC [76]. Fig. 5 displays representative ^{31}P NMR spectra from the three types of pork meat obtained at 30 min *post-mortem* [70]. High PME and

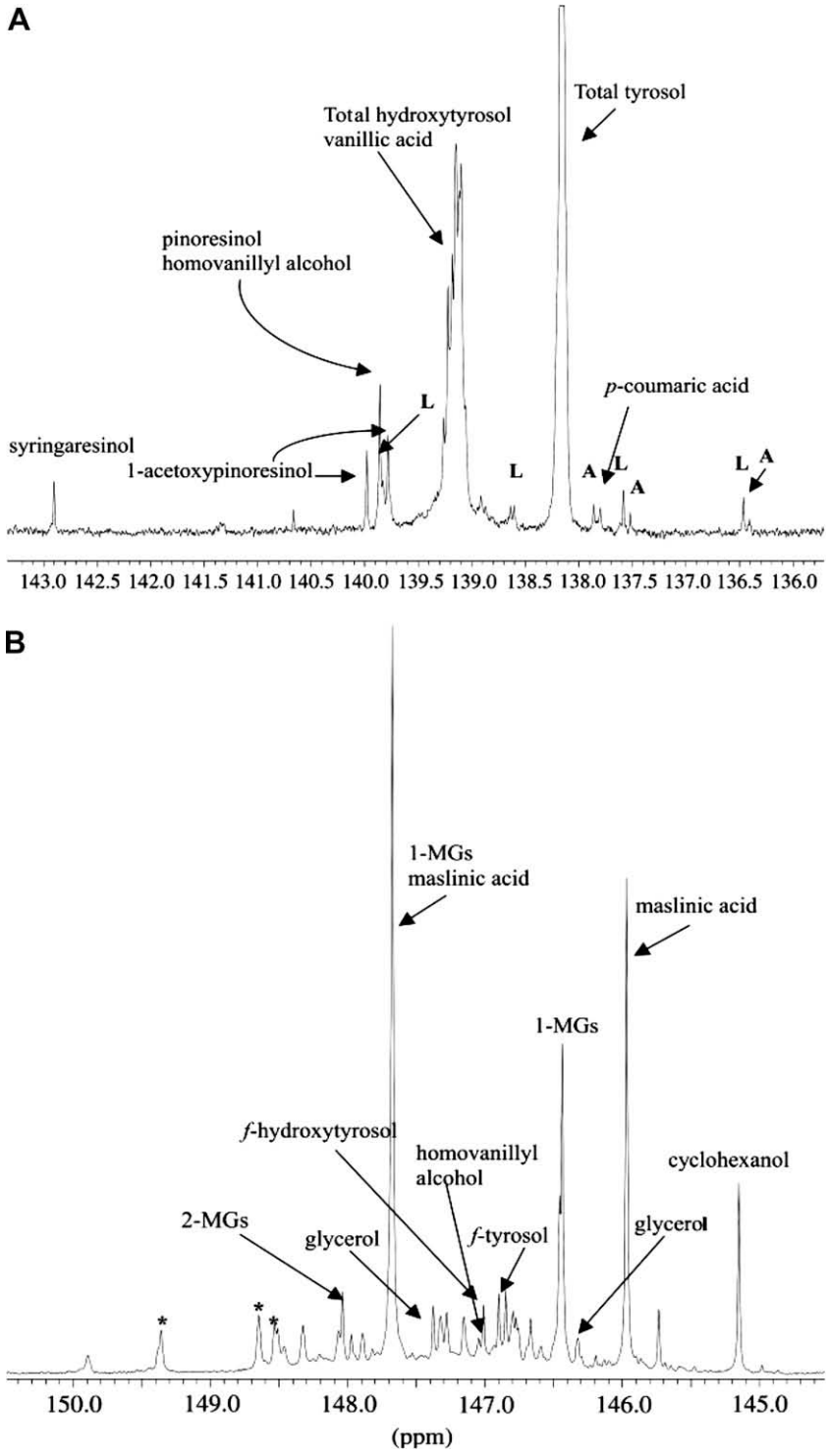

Fig. 3. ^{31}P NMR spectrum (202.2 MHz) of the phosphitylated polar fraction of a virgin olive oil sample in chloroform/pyridine solution: (A) aromatic region; (B) aliphatic region. The labels denote: A, apigenin; L, luteolin; 1-MGs, 1-monoglycerides; 2-MGs, 2-monoglycerides; *f*-hydroxytyrosol, free hydroxytyrosol; *f*-tyrosol, free tyrosol; α, α-D-glucopyranose; β, β-D-glucopyranose. The unidentified signals of the hydrolysis products of oleuropein glucoside (and ligstroside) are denoted by asterisks (*). Reproduced with permission from Ref. [54].

low PCr concentrations denote a DFD-prone meat, while high Pi combined with low PME signals are associated with PSE-prone pork meat, and thus ^{31}P NMR spectra similar to those depicted in Fig. 5 can be used for the early *post-mortem* characterization of pork meat quality.

^{31}P NMR spectroscopy has been used extensively in the study of malignant hypothermia, MH, a genetic syndrome in pigs which is also known as halothane susceptibility [77]. MH effects muscle metabolism leading to PSE pork meat with low organoleptic properties, and has been associated with increased stress susceptibility and high incidences of death during animal transport. Following halothane exposure, animals carrying the halothane gene exhibit decreased levels of PCr and pH compared to non-carriers, as shown by ^{31}P NMR studies both *in vivo* and *post-mortem*, [78–80] and the

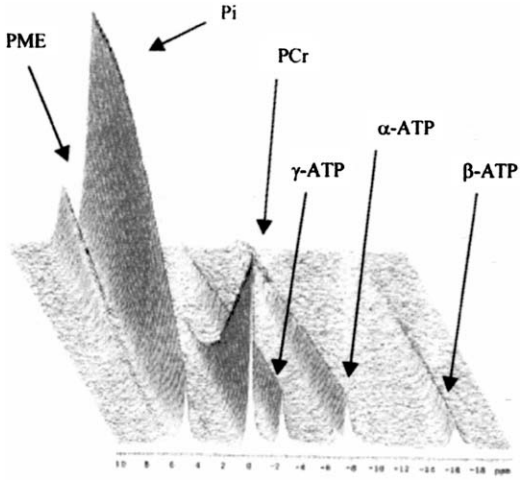

Fig. 4. ^{31}P NMR spectra (121.5 MHz) of pork muscle recorded continuously from 20 m until 12 h *post-mortem*. Due to the metabolic processes involved in the transformation of muscle to meat, signals due to ATP and PCr diminish, while Pi and phosphomonoester (PME) signals increase. Reproduced with permission from Ref. [86].

post-mortem metabolic activity differences are apparent even in the absence of pre-mortem halothane exposure [81]. The PCr/Pi ratio of biopsy muscle samples allowed the discrimination between halothane-positive and halothane-negative pigs, while variations in pH, PCr and ATP are positively correlated with the rate of *post-mortem* metabolism and meat quality traits of the animals after slaughter [81]. In subsequent studies, ^{31}P NMR was used to show that heterozygote MH pigs suffer from impaired muscle metabolism, leading to lower meat quality characteristics [82,83], a situation that can be partially improved by magnesium oxide supplementation [84].

An important animal processing parameter with respect to meat quality that was studied with the aid of ^{31}P NMR spectroscopy is the stunning method (CO_2, electrical, captive bolt pistol or anesthesia) and its effect on the metabolic processes in pig muscle. Apart from apparent ethical considerations, an optimized stunning strategy is important for minimizing the effect of short-term stress immediately before slaughter, a situation known to lead to PSE meat of low quality by increasing the rate of glycolysis. By measuring the levels of various phosphorus metabolites and the pH decrease in pig muscle *post-mortem* Bertram et al. [85] showed that CO_2 stunning is more stressful than generally expected. Another meat processing procedure that was studied by ^{31}P NMR is the chilling of meat immediately after slaughter. Bertram et al. demonstrated that a significant reduction in the *post-mortem* metabolic processes can be achieved under conditions corresponding to tunnel chilling compared with batch chilling conditions [86].

Miri et al. [87] used ^{31}P NMR spectroscopy to study muscle metabolism in isolated perfused rabbit muscles in an attempt to separate the effects of stress hormones and nervous stimulation *in vivo*, *ante-mortem* and *post-mortem*. Electrical stimulation accelerates the rate of pH decrease and the PCr and ATP content *post-mortem*, in agreement with an earlier ^{31}P NMR study on beef muscles [26]. Adrenaline perfusion was reported to have little influence on pH and ATP decrease rates *post-mortem* [86], contrary to results from earlier work [88] suggesting that adrenaline slows down the fall of pH and ATP. Recently, Henckel et al. [89] reported a slower pH decrease rate and a higher ultimate pH *post-mortem* in four different pig muscle types when animals were given adrenaline 16 h prior to slaughter. This ambiguity probably arises as a result of different pH determination methodologies applied to different animals. The muscle pH depends on many different factors, and even on the same animal and muscle, parameters such as fiber type composition were shown to have a significant effect on metabolic rates [90]. ^{31}P NMR in conjuction with perfusion studies has also been applied to show that increased blood potassium levels (hyperkalemia) do not alter the metabolic rates in rabbit muscle [91]. Finally, ^{31}P NMR was used to study the effect of dietary supplements such as magnesium [92] and vitamin E [93] on *post-mortem* metabolism of pig muscle.

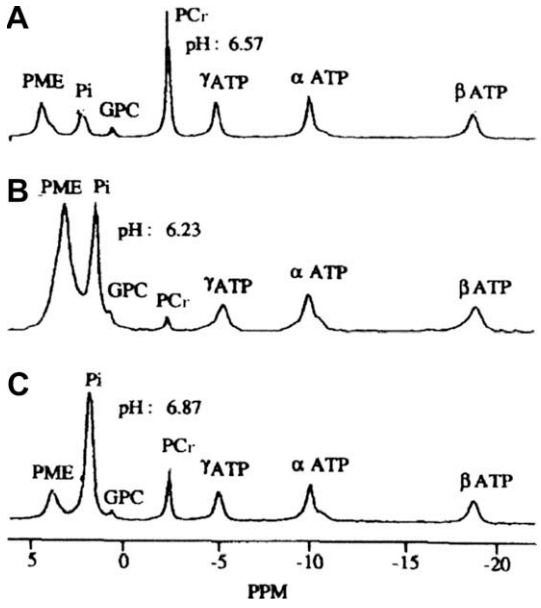

Fig. 5. ^{31}P NMR spectra (161.9 MHz) of raw pig muscle obtained at 30 rain *post-mortem*: (A) normal muscle; (B) PSE-prone muscle; (C) DFD muscle. The labels denote: PME, phosphomonoesters; Pi, inorganic phosphate; GPC, glucerophosphorylcholine; PCr, phosphocreatine; α-, β-, γ-ATP, adenosinetriphosphate. Reproduced with permission from Ref. [70].

4.3. Fish

The study of muscle physiology and metabolism in fish using ^{31}P NMR is well established. The first application of this methodology appeared as early as 1986, in a study that examined phosphate metabolism in red and white cod muscle *post-mortem* [94]. Most of the published work so far though focuses on biochemical and/or physiology perspectives [95], such as the fish metabolic reaction to environmental stresses (hypoxia [96] or anoxia [97]), and involves *in vivo* ^{31}P NMR spectroscopy, and MR imaging applications [98].

Surprisingly, very few ^{31}P NMR applications from the perspective of food analysis and the metabolic effects on the meat quality of fish have been published. ^{31}P NMR was used to evaluate the degree of freshness of loach muscle, showing that fish bled and washed at lower temperatures were better preserved [99]. Yokoyama et al. studied the changes of high energy phosphate compounds, inorganic phosphate (Pi), and intracellular pH in oyster tissue [100] and carp muscle [101], showing that ^{31}P NMR using a surface coil is able to evaluate tissue freshness non-destructively over a 24 h period *post-mortem*. Finally, ^{31}P NMR was used to characterize phospholipids in rainbow trout liver [102], and phosphorylated metabolites in crayfish extracts [103]. In the latter study, 2D ^{1}H-^{31}P heteroTOCSY experiments were used to aid in the assignment of the spectra of the extracts and the *in vivo* ^{31}P NMR spectrum of live crayfish.

4.4. Milk

Milk is the most important food source of dietary phosphorus for humans. Belton et al. [27] reported the first application of ^{31}P NMR spectroscopy in the study of milk by identifying the signals of inorganic phosphates Pi, phosphoserine (SerP) residues in casein, and glycerophosphorylcholine in spectra of milk and skim milk in bulk. The pH dependence of the chemical shift and the narrowing effect of EDTA on the linewidth of the inorganic phosphorus and phosphoserine signals was noted. Wahlgren et al. [104] extended the number of phosphorus compounds identified in milk by studying concentrated cow's milk, whey and ultrafiltrate. Belton et al. [105] studied the distribution of phosphorus compounds in milk from various species, concluding that the technique may be of value in milk authentication, and also showed that ^{31}P NMR can be used to follow changes during lactation. Belloque et al. showed that ^{31}P NMR can be used for the quantitative determination of inorganic phosphate Pi, casein-bonded phosphoserine SerP, and polyphosphates in milk [106], and reported a good correlation with the classic colorimetric method within the concentration ranges of interest. An analytical methodology using methylenediphosphonic acid as an internal standard was proposed for the quantification of casein in raw, pasteurized and UHT (ultra high temperature) milks [107]. The proposed ^{31}P NMR methodology was validated by comparison with the classical Kjeldahl analysis of total proteins based on nitrogen content, and was found superior to the latter for measuring casein of heat-treated milks, where whey protein interference is observed.

The phospholipids (PL) occurring in both ewe and cow milk were identified and quantitatively determined using ^{31}P NMR spectroscopy with inverse-gated decoupling in a monophasic solvent system [108]. A strict relation between amount and distribution of PL and type of feeding was reported. The microbial quality of raw milk has been shown to affect the hydrolysis of the phosphorylated compounds contained in UHT milk. High levels of the hydrolysis product, α-glucerophosphate, were associated with UHT milks of poor original microbial quality, or expired pasteurized milks, and were ascribed to increased phosphodiesterase activity of bacteria able to survive UHT processing [109]. A subsequent study examined several microorganisms and reported that *P. fluorescens* CECT381 showed significant capabilities in degrading the natural phosphorylated compounds in milk [110].

In a study concerning the content of mineral elements in Ca-fortified UHT milks, de la Fuente et al. [111] applied ^{31}P NMR to quantify the different forms of phosphorus present, and studied the modifications in milk salt balance induced by the Ca enrichment procedure. Hu et al [112,113] examined the potential of NMR spectroscopy as a tool for the analysis of milk without any pretreatment. Using a variety of 1D and 2D NMR experiments, including heteronuclear 2D ^{1}H-^{31}P HMBC spectra, the authors were able to identify lecithin directly in milk samples of various origins [112]. Andreotti et al. compared buffalo milk to cow's milk and reported that these milks are rather similar as far as the phosphorous distribution in small molecules is concerned [114].

Byrdwell et al. [115] used ^{31}P NMR spectroscopy to analyze the sphingolipid composition of bovine milk and reported that bovine milk sphingolipids contain higher levels of dihydrosphingomyelin than bovine brain [116], thus this compound should be considered as a dietary sphingolipid. They also pointed out that ^{31}P NMR might be superior to mass spectrometric methodologies for the quantification of sphingolipid classes in foods. Finally, Lilbæk et al. [117] followed the enzymatic hydrolysis of phospholipids in milk and whey by ^{31}P NMR and studied the resulting modifications in the surface properties of these foodstuffs.

^{31}P NMR spectroscopy in both the liquid and the solid state has been used to characterize casein micelles, and study their static and dynamic aspects in milk. Early work showed that phosphoserine (SerP) residues in caseins give rise to separate signals in ^{31}P NMR spectra, depending on their particular location and chemical environment on the protein. These signals have been assigned and pK information for specific SerP residues has been obtained through the variation of SerP chemical shift with pH [118]. The speciation of phosphorus in enzymatically and chemically modified caseins and its role in casein interactions were studied by applying ^{31}P nuclear magnetic resonance spectroscopy to studies of whole casein [119], while two modified caseins were also identified in commercial caseinates [120]. Hubbard et al. [121] studied the effect of high pressure treatment on skim milk powder dispersions, reporting an increase in the free Pi concentration, which was proportional to the magnitude of the pressure applied. This effect was fully reversible at decompression to ambient pressure, indicating that pressure causes breakdown of casein micelle integrity with concurrent or subsequent release of colloidal calcium phosphate (CCP) species into the serum phase. The effect of heat treatment on skim milk powders has also been studied [122]. Ishii and coworkers [123,124] utilized ^{31}P NMR to study milk serum and various casein micelle fractions isolated from milk by microfiltration. The effect of pH and temperature on the shape and the chemical shifts of the SerP and Pi signals were discussed in relation to micellar calcium phosphate (MCP) and the hydrophobic interactions involved in the dissociation of the casein micelles.

Fig. 6 depicts the solid state MAS ^{31}P NMR spectra of native casein micelles under various experimental conditions [125]. The two narrow peaks at δ 1.2–1.4 have been assigned to SerP in κ-casein, the broader one at δ 3.1 to colloidal calcium phosphate, while the broad resonance centered between δ 2–3 results from immobile phosphorus compounds [125]. Comparison of the ^{31}P magic angle spinning NMR spectra of ovine, caprine, and bovine casein micelles indicated that the micelles from these species are very similar but not identical [126]. In a subsequent study, slow speed MAS ^{31}P NMR spectroscopy was used to further study the composition of bovine casein micelles [127]. ^{31}P NMR has also been included in multinuclear experimental approaches that studied mineral binding to caseins [128] and the effect of heating on mineral equilibria in milk [129].

4.5. Plants

4.5.1. Starch

Starch is a carbohydrate polymer functioning as the major energy resource in plants, where it is found mainly in seeds, roots and tubers, and represents the most important polysaccharide for human nutrition. Phosphorus in native starch is found in three major forms: starch phosphate monoester, phospholipids, and inorganic phosphate [130,131]. Early ^{31}P NMR work showed that in potato [132] and taro [133] starch phosphorus is found in the form of covalently bound monophosphate, located either at the O-6 or O-3 position of the glucopyranosyl residues of starch. This was later verified in a study of both native and phosphorylated potato starch, while wheat starch was found to contain mainly 6-monophosphate esters [134]. Muhrbeck et al. reported that there is an O-6 position preference for native potato starch phosphorylation, the extent of which was dependent on potato variety, while the degree of phosphorylation in the O-3 position was approximately constant [135]. Lim et al. characterized phosphorus in starches from various botanical sources (cereals, roots, tubers, and legumes), and reported that root and tuber starches (i.e., potato, sweet potato, tapioca, lotus, arrow root, and water chestnut) contain mainly starch phosphate monoesters, some Pi, and no phospholipids, while normal cereal starches (i.e., maize, wheat, rice, oat, and millet) contain mainly PL [136]. Also, legume starches (i.e., green pea, lima bean, mung bean, and lentils) and waxy

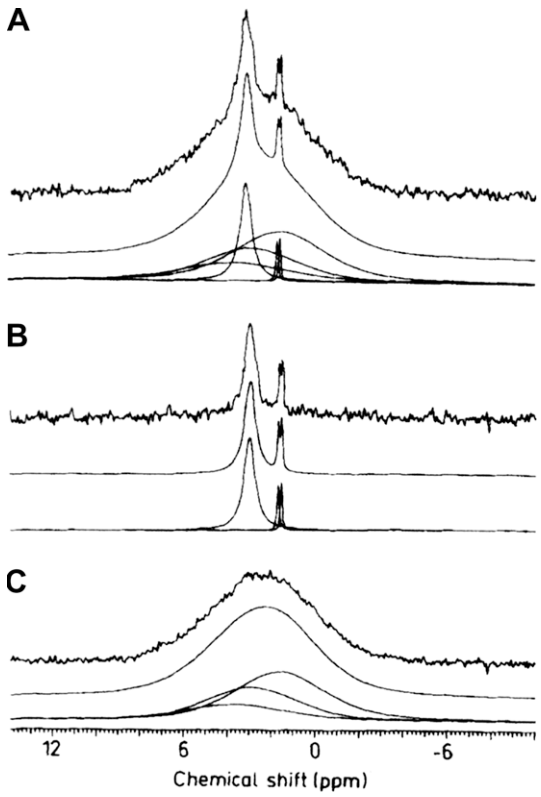

Fig. 6. Magic Angle Spinning ^{31}P-NMR spectra (121.5 MHz) of native casein micelles. Experimental (top) and computer deconvoluted (bottom) spectra are shown for (A) single pulse, (B) spin-echo and (C) cross-polarization experiments. Reproduced with permission from Ref. [125].

starches were found to contain mainly phosphate monoesters [136]. The quantitative ^{31}P NMR analysis of phosphorus compounds in starch was achieved by a methodology that involves the prior hydrolysis of starch enzymically and chemically, followed by the acquisition of the spectra of the resulting dextrin solutions in D_2O containing an internal standard [137]. The total phosphorus content in starches as determined by ^{31}P NMR is in very good agreement with results obtained by the standard colorimetric chemical method.

Modified food starches are important food macroingredients and additives. Starch phosphate mono- and diesters (cross-linked starch) have increased functional properties that can be tailored by controlling the degree of starch esterification and cross-linking [138]. ^{31}P NMR has been used successfully to characterize starch phosphates and correlate their properties as food ingredients to the extent of phosphate substitution [134,139,140]. Phosphorus speciation in oat and barley beta-glucans [141] and resistant wheat starches [142] has also been investigated. The distribution of starch O-6 and O-4 phosphate esters in starch granules [143,144] and amylopectin [145] was also studied by quantitative ^{31}P NMR in order to elucidate correlations with starch molecular structure, architecture and functional properties.

It is worth noting that ^{31}P NMR is a powerful tool for monitoring metabolically important phosphorus compounds during plant growth and development via the analysis of plant tissue extracts [146] or *in vivo* [147]. Although such studies are related to the phosphorus content and speciation in plant foods, the focus of these ^{31}P NMR applications has been almost exclusively in plant biology and physiology. For a recent overview of plant NMR applications involving ^{31}P work, the reader is referred to the excellent review by Rattcliffe et al. [148].

4.5.2. Phytate

Fruits, vegetables and grains contain large amounts of inositol hexaphosphate, also known as phytic acid or phytate. Mammalian digestive systems are not able to hydrolyze phytic acid to produce lower inositol phosphates, and thus phosphorus contained in these foods can only be made available through the action of specific enzymes, phytases, which are naturally present in certain foods and are also produced by fungi, yeasts and bacteria [149]. Furthermore, phytate is known to bind very strongly to essential minerals such as Zn, Fe, and Ca [150], and proteins [151], and thus diets rich in plant fiber can lead to mineral deficiencies in humans and animals [152]. Although initially labeled as an anti-nutrient, phytate has also beneficial effects such as antioxidant [153] and anticarcinogenic [154] activity, and plays a central role in the global phosphorus management [155,156].

An analytical ^{31}P NMR methodology was developed specifically for the quantification of phytic acid in raw and cooked foodstuffs [157]. The ^{31}P NMR spectra of phytate and lower inositol phosphates, produced by phytate hydrolysis are depicted in Fig. 7. Due to molecular symmetry, phytic acid produces four signals in a ratio 1:2:2:1 in the ^{31}P NMR spectrum, however it was found that the chemical shifts depend on solution pH in a nonlinear way [158,159]. The C-2 phosphate peak at pH 4–5 was used for quantification, since it was well resolved not only from the other phytate signals, but also from signals of lower inositol phosphates, and Pi produced by hydrolysis during cooking. The methodology was successfully used for the evaluation of phytate in a range of foods including flours, bread, cereals, soya, etc. [157]. Subsequently, Mazzola et al. [160] suggested some modifications to this experimental protocol, the most important being the use of excess quantities of EDTA to avoid interference from naturally occurring paramagnetic ions that were suspected to provide artificially lower estimates for phytate in certain foods. Good agreement was obtained with an ion chromatographic method, while ^{31}P NMR was also evaluated against other methods for the analysis of phytate and related compounds [161]. This modified analytical ^{31}P NMR spectroscopy protocol was successfully employed to study the phytate content of Turkish and Taiwanese diets [162,163]. Although a higher raw phytate content was reported for the Taiwanese diet, daily phytate and mineral intakes assumed similar values for both the Turkish and Taiwanese diets, and were comparable to those of a typical Italian diet [163]. This is not surprising, since food processing and subsequent cooking procedures are able to initiate the hydrolysis of phytate to lower inositol phosphates, and thus reduce the actual amount of phytate consumed, as compared to the raw food contents. Several studies have shown that the adverse effects of lower inositol phosphates in the body are much reduced compared to phytic acid (see [154] and references therein), thus the use of phytases is an obvious means to lower the risk of mineral deficiencies associated with diets rich in fiber, and to increase phosphorous bioavailability. Because of phytases, leavened bread products containing whole grain cereals have higher phosphate bioavailability than unleavened bread or breakfast cereals. ^{31}P NMR was used to elucidate the mechanism of enzymatic hydrolysis of phytate in whole grain flour and dough, and was found to be superior to the ferric acid method because of its ability to differentiate between phytic acid and its hydrolysis products, the lower inositol phosphates [164].

A study of the breakdown of phytate in wheat and oats showed that it proceeds at similar rates [165], but commercial oats showed no evidence of phytate degradation, due to the inactivation of endogenous phytase by the heat treatment used during processing. The study of phytate degradation by ^{31}P NMR can also be used to characterize the activity of different phytases. Lactic acid bacteria and yeasts isolated from Italian sourdoughs were compared during

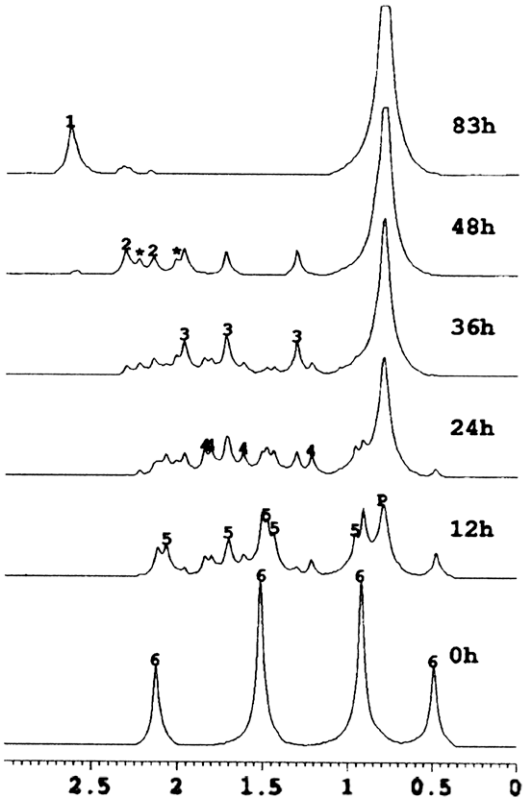

Fig. 7. ^{31}P NMR spectra (161.9 MHz) showing the hydrolysis of pure sodium phytate at the time intervals indicated. The numbered peaks represent resonances due to inositol hexa(6)-, penta(5)-, tetra(4)-, tri(3)-, di(2)-, and mono(1)phosphates and to orthophosphate (P); peaks marked with an asterisk (*) probably represent an additional minor inositol triphosphate. Reproduced with permission from Ref. [169].

wholemeal dough fermentation and sourdough technology using lactic acid bacteria was reported to be superior for breadmaking [166].

Phytases have become very important as food additives in animal nutrition, where they are used as an effective means to increase mineral and phosphorous absorption by farm animals, and to reduce the environmental phosphorus contamination from unabsorbed phytate excreted [167,168]. Kemme et al. proposed the use of ^{31}P NMR spectroscopy as an efficient tool in animal nutrition studies, and performed a detailed analysis of the phytate content of feed ingredients, complete feeds, ileal contents and faeces of pigs and poultry [169]. The use of ^{31}P NMR for the characterization of phosphorus species in environmental and agricultural samples has been recently reviewed [170].

Recently Smernik et al. draw attention to the possible misidentification of the resonances of an unknown compound as phytate in the ^{31}P NMR spectra of NaOH–EDTA soil extracts [171], and suggested spiking with original phytate might be needed to become standard practice, in view also of the well-known pH and ionic strength dependence of most phosphate signals. Since the compound responsible for the misidentification was not characterized, it is not possible at this moment to assess whether this might have affected phytate ^{31}P NMR studies conducted in foods. It is clear, however, that more work is needed towards understanding the complexation of phytate with inorganic cations [150] and proteins [151]. The solid state Magic Angle Spinning ^{31}P NMR spectra of several metal phytates have been reported recently [172,173].

4.6. Food additives

4.6.1. Lecithins

Lecithins are phospholipid extracts of animal or plant origin possessing exceptional surface-active properties, and are used as emulsifiers and dispersing agents in a great variety of foods, such as margarines, bread, chocolate, instant food preparations and baby foods [174]. ^{31}P NMR is a most powerful technique for the characterization and quantification of phospholipid classes in lecithins [24,175], and both the biphasic (methanol/chloroform/water–EDTA) and monophasic (Et_3N/DMF/GH+) protocols have been used. Fig. 8 depicts the ^{31}P NMR spectrum of soya lecithin in a biphasic solvent system, depicting eleven different phospholipids and lysophospholipids that can be easily quantified from such spectra.

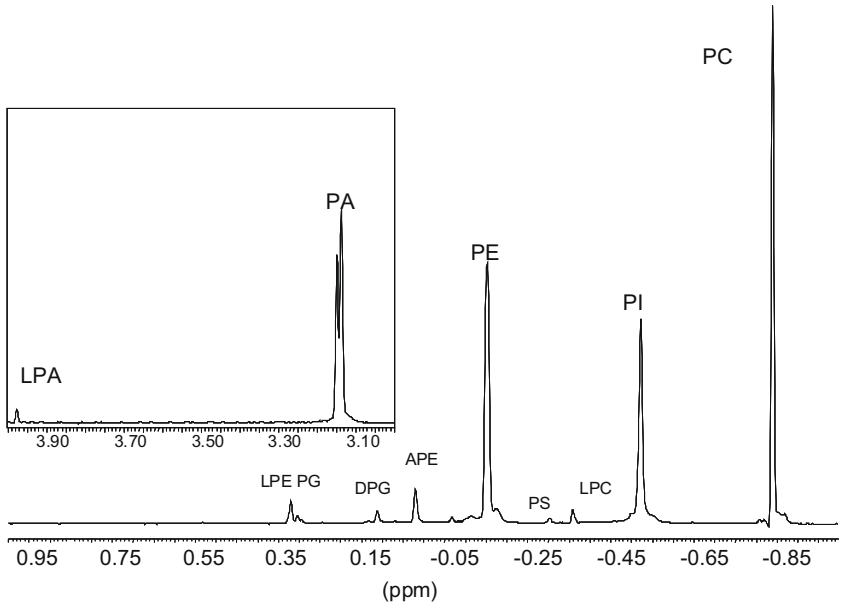

Fig. 8. ^{31}P NMR spectrum (121.5 MHz) of soya lecithin. The labels denote: PC, phosphatidylcholine; PI, phosphatidylinositol; 2-LPC, 2-lyso-phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; APE, *N*-acylphosphatidylethanolamine; DPG, diphosphatidylglycerol; LPE, lyso-phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; LPA lyso-phosphatidic acid. Reproduced with permission from Ref. [24].

Using a two-phase solvent system, Helmerich et al. [176] compared the relative advantages of ^{31}P NMR, TLC and HPLC in the quantitative characterization of phospholipids in various commercial lecithins and flour improvers, and reported that although ^{31}P NMR is less sensitive, it represented the method of choice for quantitative work because all phospholipids and lysophospholipids could be determined in a single experiment. The PL profiles of commercial and laboratory anionic lecithins obtained from different sources (soyabean, canola, egg, butternut) were also reported [177]. Cremonini et al. showed that multivariate statistical analysis of ^{31}P NMR-obtained PL profiles can be used to discriminate between lecithins of different geographical origin and/or extraction conditions [178]. In a related study, the role of phospholipids in the emulsion properties of mayonnaise prepared with normal and phospholipase A2-treated egg yolk was studied using ^{31}P NMR spectroscopy [179].

4.6.2. Proteins

Apart from their nutritional value, proteins play an important role in the physical properties and texture of foods, due to their ability to stabilize gels, foams, doughs and emulsions. Because of their surface-active properties [180], several phosphoproteins naturally occurring in milk, soya, and egg are used as emulsifiers in foods, such as baked products, sweets, desserts, and beer. Plasma and whey proteins obtained as by-products of meat and cheese industries respectively, are gaining interest as functional ingredients in food products. ^{31}P NMR is a well established tool for the study of phosphoproteins (see Chapter 4 in [28]). Ovalbumin is the major constituent of egg white, and an important food ingredient with high functionality including emulsifying properties and foam stability. ^{31}P NMR has contributed towards the evaluation of the emulsifying and structural properties of ovalbumin [181], and the effect of limited proteolysis on emulsion characteristics [182]. The emulsion stability of phosphatidylcholine, PC, and lyso-PC [183], and the interaction of ovalbumin with these PLs [184] and fatty acids [185] have been studied, with a view of clarifying the interfacial adsorptivity of ovalbumin emulsions. These studies take advantage of the fact that separate signals from the phosphate units of PLs and the protein are observed in the ^{31}P NMR spectra of the emulsions. Protein phosphorylation is a well established chemical procedure that provides a means of modifying the functional properties of food proteins and improve their properties for use as food additives [186]. ^{31}P NMR has been used successfully to characterize the chemically phosphorylated sites in

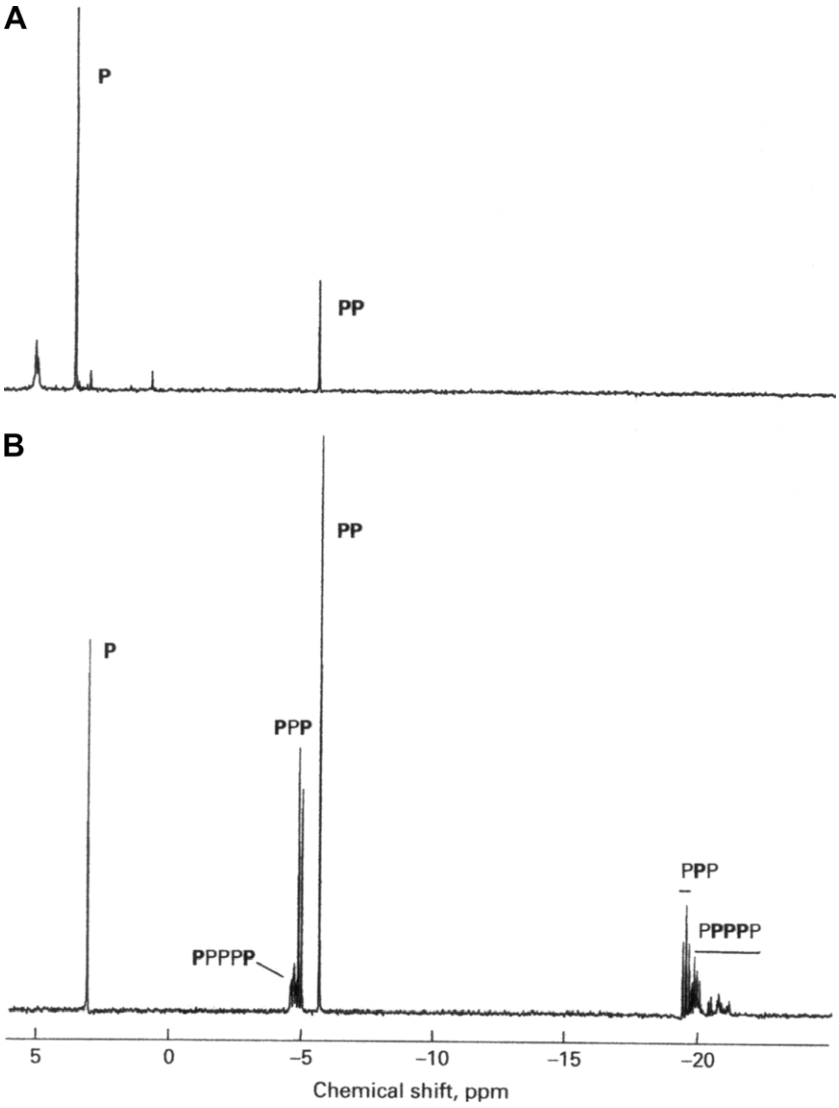

Fig. 9. ^{31}P-NMR spectra (161.9 MHz) of (A) a UHT milk sample and (B) a commercial polyphosphate. The labels denote: P, monophosphate; PP, diphosphate; PPP, triphosphate; PPPPP, polyphosphates. Reproduced with permission from Ref. [106].

casein, whey protein extract and egg-white proteins, showing that besides phosphoesters, phosphodiester and polyphosphate bonds were introduced in egg-white protein [187].

4.6.3. Phosphates

Inorganic oligophosphates, and linear and cyclic polyphosphates are important food additives, used for example as leavening systems in baked products, to improve the water holding capacity of meat, and as stabilizers in UHT and sterilized milk. Gard et al. reported a comprehensive statistical evaluation of ^{31}P NMR for the determination of oligophosphate species, and found that this methodology has accuracy and precision comparable to chromatographic methods and superior to IR and XRD [188]. For higher oligophosphates, ^{31}P-^{31}P homonuclear 2D J-Resolved spectroscopy was suggested in order to facilitate the assignment of the crowded spectral region of the middle phosphate nuclei of the chain [189].

Early work in the field involved the use of ^{31}P NMR to detect whether added polyphosphates were present in frozen foodstuffs, including chicken, fish and ground beef [190], and to follow polyphosphate hydrolysis of chicken during long term frozen storage [191]. In later studies, the hydrolysis of pyrophosphate and tripolyphosphate additives in chicken meat was studied [192,193], showing that hydrolysis depends on the presence of NaCl, the length of the meat ageing procedure and the type of phosphate additive used. The effect of *pre rigor* NaCl salting with or without pyrophosphate addition on rabbit muscle metabolism has also been reported [194]. Recently, diphosphate was successfully identified and quantified using ^{31}P NMR spectroscopy in samples of commercial UHT milk [106,111], by comparing milk spectra with those of several standard polyphosphate compounds. Fig. 9 presents the ^{31}P NMR spectra of a UHT milk and a standard polyphosphate mixture. Some problems related to the NMR quantitation of higher polyphosphates, such as relaxation time differences between phosphate species, and polyphosphate hydrolysis to lower phosphates and Pi were also discussed [106].

4.7. Organophosphorus pesticides

Organophosphorus pesticides (OP) have been widely used in agriculture during the last few decades, replacing organochlorine pesticides due to their smaller persistance in the environment. As a result, traces of organophosphorus pesticides or their degradation products may be introduced in the food chain. Very early, ^{31}P NMR has been proposed as an analytical tool to measure OP levels at the 0.5 ppm level in cole crops such as broccoli and cabbage, where traditional gas chromatographic techniques are prone to errors due to interference with non-phosphorus coextractants [195]. However, most applications of ^{31}P NMR in the field of OP deal with reactivity issues [196], the characterization of OP degradation products [197,198] and metabolites [199], or toxic contaminant analysis of OP formulations [200]. The use of ^{31}P NMR for OP residue analysis in soil, water and other environmental samples has been reviewed [201].

Only recently, ^{31}P NMR has been proposed as an efficient method to determine traces of trichlorfon, an organophosphorus insecticide, in tomato. The ^{31}P NMR methodology has an LOD of 50 mg L^{-1}, a value comparable to that of GC–MS and HPLC analyses, but possesses superior precision [202], while avoiding tedious sample preparation treatment. Despite the low LOD offered by chromatographic techniques, it is clear that ^{31}P NMR spectroscopy is underutilized in the field of OP residue analysis in foods. The increased NMR sensitivity brought about by the use of cryoprobe technology and nanoprobes, make ^{31}P NMR spectroscopy attractive for such studies, since it offers the added advantage of allowing the simultaneous determination of both original pesticides, and their plants metabolites that ultimately end up in food products.

5. Conclusions and future directions

The body of research work summarized in this article is ample evidence that ^{31}P NMR spectroscopy has been an invaluable tool in the analysis of foods and the study of the factors affecting food quality. Matching the heterogeneity of food matrices, a multitude of available experimental NMR approaches for detecting ^{31}P nuclei have been applied to foods and food ingredients, including high resolution, MRI and solid state NMR methodologies. It is expected that ^{31}P NMR will continue to play an important role in food analysis, since its selectivity towards phosphorus-containing compounds, coupled with the increasing sensitivity and analytical capabilities of modern NMR spectrometers, and the successful hyphenation with chromatographic techniques in LC-NMR [203], are advantages that cannot be easily matched by other analytical techniques in food analysis. It is also expected that ^{31}P NMR metabolomic approaches will play an integral role in the characterization of foods and food systems. Phospholipidomics is a fast growing area of research, and although so far mass spectrometry has been the main tool utilized [204], it is envisioned that ^{31}P NMR will, in the future, play an important role in this field, that encompasses many potential applications in food science and analysis. Furthermore, the increasing ability to perform advanced 2D NMR experiments with solid state spectrometers [205], previously only available to the liquid state, is expected to increase the role of MAS ^{31}P NMR applications in the analysis and characterization of solid and semisolid foods.

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Glossary

APE: ***N***-Acylphosphatidylethanolamine
ATP: Adenosinetriphosphate
CCP: Colloidal calcium phosphate
$Cr(acac)_3$: Tris(acetylacetonato)chromium(III)
D: Ratio of 1,2-diglycerides to total diglycerides
DFD: Dark firm dry
DG: Diglycerides
DMF: Dimethylformamide
DPG: Diphosphatidylglycerol
EDTA: Ethylenediaminetetraacetic acid
Et_3N: Triethylamine
GC–MS: Gas chromatography (coupled to) mass spectrometry
GH+: Guanidinium chloride
HPLC: High pressure liquid chromatography
IP_6: Inositolhexaphosphate
IR: Infra-red
LC-NMR: Liquid chromatography (coupled to) nuclear magnetic resonance
LOD: Level of detection
LPA: Lyso-phosphatidic acid
LPC: Lyso-phosphatidylcholine
LPE: Lyso-phosphatidylethanolamine
LPI: Lyso-phosphatidylinositol
MAS: Magic angle spinning
MCP: Micellar calcium phosphate
MH: Malignant hypothermia
MRI: Magnetic resonance imaging
NMR: Nuclear magnetic resonance
NOE: Nuclear Overhauser enhancement
OP: Organophosphorus pesticides
PA: Phosphatidic acid
PC: Phosphatidylcholine
PCr: Phosphocreatine
PE: Phosphatidylethanolamine
PG: Phosphatidylglycerol
PI: Phosphatidylinositol
Pi: Inorganic phosphate
PL: Phospholipids
PME: Phosphomonoester
PS: Phosphatidylserine
PSE: Pale soft exudative
RDA: Recommended daily allowance
SerP: Phosphoserine
TLC: Thin-layer chromatography
UHT: Ultra-high temperature
WHC: Water holding capacity
XRD: X-ray diffraction