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Analytical Methods

Fast determination of bioactive phytic acid and pyrophosphate in walnuts using microwave accelerated extraction

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ABSTRACT

Bioactive compounds phytic acid (IP6) and pyrophosphate (PPi) are minor components of walnuts with the ability of being inhibitors of urolithiasis, among others. Since simultaneous analysis of IP6 and PPi have known drawbacks, a new method to determine their content in walnuts has been developed with emphasis on their extraction from walnuts by microwave-assisted extraction (MAE). Acid content of extracting solvent, extraction time and temperature were optimized. After extraction, compounds were purified by selective adsorption/desorption on an anion exchange solid phase extraction and analyzed by inductive coupled plasma/mass spectrometry. A mixture of H_2SO_4 and HCl as solvent to extract both, IP6 and PPi, provided results slightly higher than those determined by conventional extraction with no statistical difference. The possible hydrolysis of phytic acid by MAE was analyzed. Compared with the conventional acid extraction method, significant improvement is achieved by the MAE method reducing extraction time from 3 h to 10 min.

1. Introduction

Phytic acid (myoinositol hexaphosphate, IP6) and inorganic Pyrophosphate (PPi) are bioactive compounds present in many plant sources, such as cereals and legumes, including nuts. Frequent nut consumption is likely to have beneficial health effects due to the particular lipid profile content, particularly for walnuts. Nuts are also rich in other bioactive macronutrients that have the potential to positively affect metabolic and cardiovascular diseases (Ros, 2010; Sabate & Wien, 2013). Phytic acid is one of the bioactive compounds found in nuts and has been described to be desirable in the human diet due to the different health properties such as antioxidant and anticancer effect (Schlemmer, Frølich, Prieto, & Grases, 2009). These beneficial health effects are more significant for people from developed countries because of the higher incidence of cancer which is associated with higher fat and lower fibre-rich food intakes. Such populations generally do not suffer from mineral deficiencies (Kumar, Sinha, Makkar, & Becker, 2010).

IP6 and PPi have chemical structural similarities and can act as an effective inhibitor of insoluble calcium salts growth in urine and soft tissues because its affinity for calcium compounds, thus, preventing the formation of kidney stones (urolithiasis) and decreasing crystal growth by binding to the crystal surface (Grases et al., 2007; Terkeltaub, 2001).

The analysis of different matrices containing both, phytic acid and pyrophosphate, has paid attention in the last decades but it presents drawbacks like poor separation, reflected in the tedious analytical techniques actually described and used in many laboratories. The quantitative extraction of phytate and pyrophosphate from foodstuff is not selective and always co-extract with cations, protein, or other nutrients leading to erroneous estimation, damage to the analytical instruments, or both (Ray, Shang, Maguire, & Knowlton, 2012).

For the determination of phytic acid, various methods have been developed. Frequently used measurement techniques include colorimetric method (Latta & Eskin, 1980), LC–MS (Tur et al., 2013), HPLC (Perello, Isern, Munoz, Valiente, & Grases, 2004; Skoglund, Carlsson, & Sandberg, 1998), HPIC (Blaabjerg, Hansen-Møller, & Poulsen, 2010), ^{31}P NMR spectroscopy (Kemme et al., 1999), synchronous fluorescence method (Chen, Chen, Luo, Ma, & Chen, 2009), inductively coupled plasma mass spectrometry (ICP-MS) (Muñoz & Valiente, 2003), IPC-ICP-MS (Helfrich & Bettmer, 2004) and enzymatic (March, Villacampa, & Grases, 1995). Indirect deter-

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mination of phytate after hydrolysis has been also used for the analysis of pharmaceutical formulations (March, Grases, & Salvador, 1998). The traditional procedures employed for PPi determination are mainly based in chemical (Chang & Denq, 1985) and enzymatic methods (Baykov & Avaeva, 1982). Chromatographic methods (Yoza et al., 1991) or electrophoretic (Hénin, Barbier, & Brack, 1999) separation of PPi have also been reported. A method for the determination of IP6 and PPi in urine by SPE-ICP-MS techniques was developed in our laboratory achieving a simple and easy measurement to simultaneously analyze IP6 and PPi (Muñoz, López-Mesas, & Valiente, 2010). But non-laborious method for the extraction and purification of the compounds from a fatty sample has not been developed.

Interest in microwave-assisted extraction (MAE) has increased significantly over the past decades as a result of its inherent advantages (reduction in extraction time and solvent volume) over more traditional extraction techniques, such as Soxhlet extraction (Li et al., 2012). Conventional extraction methods have been associated to higher solvent requirements, longer extraction times and increased risk of degradation of thermo-labile constituents. In MAE, the solvent and sample are contained in sealed extraction vessels under controlled temperature and pressure conditions. The closed vessels allow the temperature of the solvent to rise well above its boiling point, which shortens extraction time and subsequently increases extraction efficiency (Eskilsson & Björklund, 2000; Ballard, Mallikarjunan, Zhou, & O'Keefe, 2010).

Development of robust, inexpensive, and reproducible techniques for accurate quantification of undigested phytate in complex food sample is essential to advance knowledge of the content of phytic acid and pyrophosphate. The objectives of the current study were to investigate the effects of MAE on the extraction efficiency and optimize the parameters including microwave power, irradiation time and acid solvents. In the present paper, we disclose a rapid and simple procedure for the extraction and determination of phytic acid and pyrophosphate content in walnuts.

2. Materials and methods

2.1. Chemicals and reagents

Tetra-sodium pyrophosphate 10-hydrate (Panreac, Barcelona, Spain), myoinositol hexaphosphoric acid hexasodium salt from corn (Sigma, Steinheim, Germany), hydrochloric acid (J.T. Baker, Devented, Holland) and sodium dihydrogen phosphate (Panreac, Barcelona, Spain) were of analytical-reagent grade. AG 1×8 200–400 mesh, chloride form, and anion exchange resin was from Bio-Rad Laboratories (Hercules, CA, USA). Purified Milli-Q water of 18 mΩ-cm resistivity was used for the preparation of all reagents.

2.2. Preparation of walnut defatted samples

The walnuts were cultivated in Lleida (Spain) and purchased at a local market. According to the food composition database published by the US Department of Agriculture, 100 g of walnuts contain 15.2 g protein, 65.2 g fat, and 6.7 g dietary fiber (Banel & Hu, 2009). The high content in fat can reduce the efficiency of the extraction and determination of the analytes so, it is important to remove it from the walnuts before any procedure and to analyze if the defatting process entails any loss of the analytes.

The walnuts were shelled and blended, dried at 40 °C, until no further weight loss, and stored into a desiccator until use. Total lipid was extracted from walnut samples by using hexane, based on the modified method of (Saad, Mohd Esa, Ithnin, & Shafie, 2011). To do so, 5 ml of hexane was added to 1 g of walnut and soaked for 16 h under a hood. Then after filtration, walnuts were evaporated to dryness using a vacuum pump apparatus.

2.3. Microwave-assisted extraction (MAE)

MAE experiments were carried out at a CEM Mars5 Digestion Microwave System (Matthews, USA) which allowed accurate control of pressure, power and temperature and using 100 mL sealed perfluoroalkoxy Teflon reactor vessels. As it is well known, the solvent composition, extraction time and microwave temperature affect the extraction process so those parameters were studied. Moreover, the possible hydrolysis of phytic acid must be considered (March et al., 1998). In order to minimize the hydrolysis during the MAE process, different acid conditions and microwave parameters have also been investigated.

To perform the experiments, defatted samples were transferred into microwave extraction vessels and hydrochloric acid, sulphuric acid or a mixture of both (at selected concentrations) was added. Extraction was carried out during a certain time at selected temperatures. Three replicates of each sample were extracted at the same time. The obtained creamy extracts were then centrifuged at 5000 rpm for 10 min. The supernatants were collected and used for determination of phytic acid and pyrophosphate.

In order to verify the obtained results, the traditional solid-solvent extraction method from the AOAC (AOAC, 1990) was followed. To do so, 0.66 M HCl was used as extraction solvent. The extraction was carried out at room temperature with constant shaking at medium speed in an orbital mixer for 3 h. The obtained creamy mixture was then processed in the same way as was the creamy extract obtained by microwave extraction.

2.4. Separation and analysis of IP6 and PPi by SPE-ICP technique

The separation and analysis methodologies followed were described by Muñoz et al. (2010). Before separation, the sample was diluted 25 times and then adjusted to pH 6.0 using NaOH solution. To separate IP6 and PPi, 1.0 mL of the obtained cream was transferred quantitatively to a SPE cartridge, packed with 0.2 g of AG 1×8 resin, previously conditioned with 2 mL of HCl 10 mmol L^{-1}. First phosphate and some other matrix components of sample were eluted with 50 ml of HCl 50 mmol L^{-1}. Then PPi was eluted with a second 5 ml HCl 100 mmol L^{-1} eluting. Finally the column was washed with 2 mL of HCl 2 mol L^{-1} to elute phytic acid (IP6). The clean-up process was run by gravity, at a flow rate of 0.33 mL min^{-1}.

The analysis of the eluted fraction was carried out through ^{31}P analysis by ICP-MS using ^{45}Sc (5 μg L^{-1}) as internal standard. The ICP-MS equipment was PQ-Excell (Thermo Elemental, UK) and conditions were slightly modified to those stablished in Muñoz et al. (2010).

2.5. Statistical analysis

Results are presented as means of triplicate determinations ± RSD. Significant differences between samples ($p < 0.05$) were identified by Analysis of Variance (ANOVA).

3. Results and discussion

3.1. Effect of defatting process and acid solvent selection

Since walnuts are highly rich in fat, it is important to investigate the effect of defatting on the extraction process. In both results, by traditional acid extraction method procedure (AOAC) and MAE method, it can be observed (Table 1) that the non-defatted samples

Table 1
Effect of the different parameters evaluated on the extraction of IP6 and PPi content from walnuts.

Experiment #	Extraction solvent	Defatted	Extraction conditions	Phytic acid content (% dry wt)	Pyrophosphate content (‰ dry wt)
1	0.66 M HCl	Yes	2 h	1.04 ± 0.06	0.13 ± 0.02
2	0.66 M HCl	No	2 h	1.16 ± 0.05	0.06 ± 0.02
3	0.66 M HCl	Yes	3 h	1.04 ± 0.02	0.15 ± 0.03
4	0.52 M H_2SO_4	Yes	1 h	1.05 ± 0.06	0.05 ± 0.02
5	0.52 M H_2SO_4	Yes	0.5 h	1.04 ± 0.03	0.03 ± 0.01
6	0.94 M H_2SO_4	Yes	0.5 h	0.90 ± 0.05	0.08 ± 0.02
7	0.66 M HCl	Yes	100 °C 10 min	0.93 ± 0.08	0.08 ± 0.02
8	0.66 M HCl	Yes	125 °C 10 min	0.95 ± 0.02	0.07 ± 0.01
9	0.66 M HCl	No	125 °C 10 min	1.07 ± 0.03	–
10	0.66 M HCl	Yes	125 °C 20 min	0.99 ± 0.03	0.09 ± 0.02
11	0.66 M HCl	Yes	150 °C 10 min	0.71 ± 0.08	0.46 ± 0.05
12	0.52 M H_2SO_4	Yes	100 °C 10 min	0.99 ± 0.01	0.11 ± 0.02
13	0.94 M H_2SO_4	Yes	100 °C 10 min	0.98 ± 0.01	0.15 ± 0.03
14	0.52 M H_2SO_4 + 0.66 M HCl	Yes	50 °C 10 min	0.97 ± 0.05	0.09 ± 0.01
15	0.52 M H_2SO_4 + 0.66 M HCl	No	50 °C 10 min	1.01 ± 0.05	–
16	0.52 M H_2SO_4 + 0.66 M HCl	Yes	80 °C 10 min	1.06 ± 0.02	0.14 ± 0.02
17	0.52 M H_2SO_4 + 0.66 M HCl	Yes	100 °C 10 min	1.13 ± 0.08	0.15 ± 0.04
18	0.52 M H_2SO_4 + 0.66 M HCl	Yes	110 °C 10 min	1.14 ± 0.02	0.16 ± 0.01

show a higher content on phytic acid, about 0.1%, which means that a small loss of the compound happened during the defatting procedure (comparing experiments 1 with 2 for AOAC, 8 with 9 and 14 with 15 for MAE). According to (Oberleas & Harland, 1986), fat content influences the extractability of phytate from food products and should be lowered below 5% before phytate extraction. Thus, to defat walnuts before the extraction process is mandatory since walnuts fat content is higher than 50% (weight), otherwise will interfere on any posterior procedure (including SPE and ICP-MS), causing inaccurate results.

After 2 h of acid extraction by the AOAC method the result shows no significant differences than 3 h of extraction ($P > 0.05$), although 3 h extraction is the recommended time (experiments 1 and 3 in Table 1). In addition, sulphuric acid was tested as solvent at two different concentrations, 0.52 M and 0.94 M (experiments 5 and 6 in Table 1). It can be observed that with 0.52 M H_2SO_4 the same results as with 0.66 M HCl on IP6 was obtained, while it was lower for PPi. In the same extraction conditions, compared with 0.52 M H_2SO_4, 0.94 M H_2SO_4 reveals a lower yield on IP6 and a higher yield on PPi. Increasing the time from 0.5 h to 1 h with 0.52 M H_2SO_4 did not improve the results obtained (experiment 4). So, H_2SO_4 will be also tested as acid solvent for the MAE procedure.

3.2. Effect of pH

The complexes between phytate and mineral cations are pH sensitive. To minimize the effect of both, metals and proteins, some researchers treated the samples with NaOH –EDTA (Bos, Verbeek, Van Eeden, Slump, & Wolters, 1991; Harland & Oberleas, 1985). For several minerals this complexation shows a maximum around pH 6 (Fruhbeck, Alonso, Marzo, & Santidrián, 1995). In that sense, the influence of adjusting the pH on the column separation was studied. pH was adjusted to 6.0 (above the isoelectric point of the proteins) just before the separation procedure (SPE).

From the results collected in Fig. 1, it is observed that for the no defatted sample, the pH adjustment has a significant effect ($P < 0.05$), obtaining a 0.09% higher content on phytic acid. While for the defatted samples, pH adjustment seems do not have significant difference ($P > 0.05$). Nevertheless, it is worthwhile to emphasize the fact that when the pH adjustments were carried out, the standard deviation was lower. For the defatted samples, the pH adjustment does not show a significant effect because this procedure eliminates the possible phytic acid complex (Fruhbeck et al., 1995). Therefore, in order to gain precision and accuracy in the final outcome, the pH of the diluted aliquot was adjusted to 6.0 with 1 M NaOH just before processing them through the SPE column.

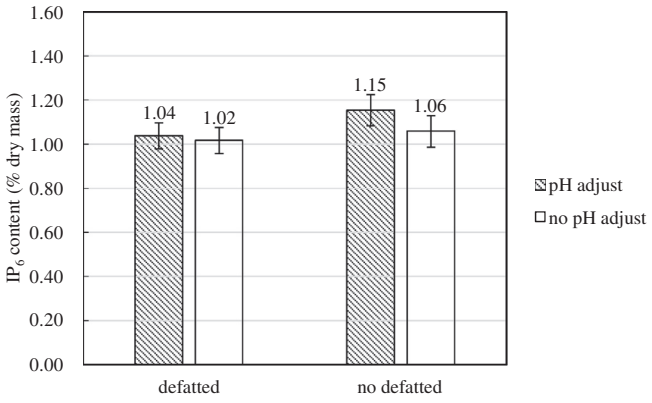

Fig. 1. Effect of pH adjustment on separation of phytic acid from walnut.

3.3. MAE parameters selection and phytic acid hydrolysis

Three main parameters were considered to optimize the conditions for microwave-assisted extraction: acid concentration of extracting solvent, extraction time and temperature. The results obtained by MAE were compared with those obtained by the traditional method. As shown in Table 1, when using the single acid as the extraction solvent, the 0.66 M HCl and 0.52 M H_2SO_4 at the same conditions do not show significant differences for both compounds (experiments 7 and 12). When temperature was raised from 100 °C to 125 °C for HCl, or increased the extraction time from 10 min to 20 min (experiments 7, 8 and 10), no significant differences on the extraction of IP6 and PPi were observed. Increasing the temperature to 150 °C, the extraction of IP6 was decreased and PPi was increased significantly, due to the hydrolysis of IP6 under this condition, confirmed later on by the study of the hydrolysis.

The acids HCl and H_2SO_4 mixture (1:1) was also tested as extracting solvent (experiments 14–18). It is observed that the MAE extraction by this acids mixture show a good recovery of both IP6 and PPi. The best extraction conditions were found to be 0.52 M H_2SO_4 + 0.66 M HCl at 100 °C, 10 min irradiation time, and a solvent volume to walnut ratio of 20 ml/g. Mixtures of HCl/H_2SO_4 provided the best results and slightly higher than those determined by

conventional extraction with the advantage that the extraction time decreased from 3 h to 10 min. This slight increase maybe due to the water contained inside the cells, which absorbs the microwave energy and subsequently begins to evaporate. The vaporization of water generates pressure within the cell wall that eventually leads to cell rupture, thereby facilitating the leaching out of active constituents into the surrounding solvent and improving extraction efficiency and yield, meanwhile, in the conventional method the extraction is produced by the absorption of the solvent into the solid matrix (Ballard et al., 2010).

The possible hydrolysis of phytic acid by the microwave extraction procedure was studied under several work conditions, shown in Table 2.

When comparing the different acid concentrations, it can be observed that the hydrolysis of phytic acid increased as decreasing HCl concentration (comparing experiments 1 with 7 and 4 with 6), in agreement with the known fact that the absorption of microwave radiation of aqueous acid solutions is more effective at lower acid concentrations. The kinetics of heteropolyacid formation was affected by acidity conditions. When comparing at the same molar concentration in acid, it can be observed that at higher temperature higher hydrolysis is observed (see experiments 1–3).

Typically, methods for determining IP6 levels are based in thermal (de Koning, 1994) or enzymatic hydrolysis (March et al., 1995). For the first one, 120 °C in 2 M HCl during 24 h is necessary to reach quantitative hydrolysis. In the present study, at the MAE condition of 150 °C in 0.1 M HCl, 99% of phytic acid was hydrolyzed in 20 min, results comparable to those obtained by (March et al., 1998). When comparing the different inorganic acid at the same microwave treatment conditions, it can be observed that the lower hydrolysis rate was obtained using 0.52 M H_2SO_4, followed by 0.66 M HCl and 0.94 M H_2SO_4 always at 100 °C during 10 min. So, in order to minimize the hydrolysis of phytic acid in the MAE process, temperature of 100 °C during 10 min were recommended.

Finally, a comparison between the phytate content in different foods is shown in Fig. 2 (Harland, Smikle-Williams, & Oberleas, 2004; Macfarlane et al., 1988; Ravindran, Ravindran, & Sivalogan, 1994). As it is seen, the phytic acid content of walnuts (around 11 mg/g, Catalonia walnuts in present experiment) is in the middle range of the selected food and nuts (2–36 mg/g). Walnuts contain less phytate than other nuts, such as peanut and almond, but are rich in polyunsaturated fatty acids, which make it as a valuable source of micronutrients.

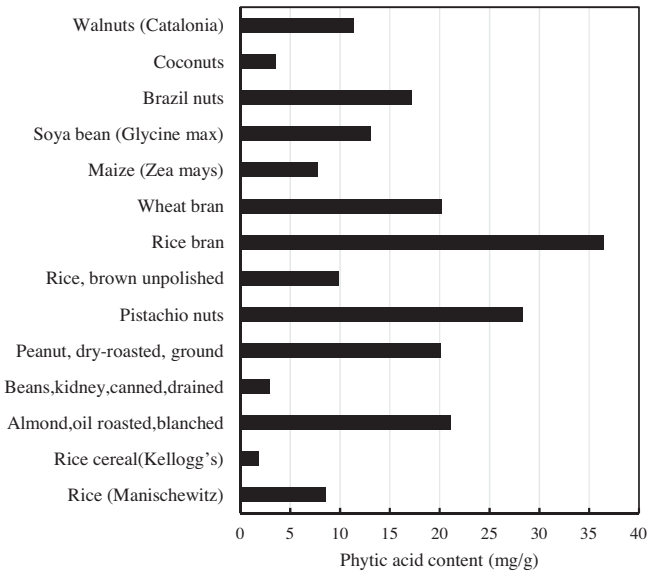

Fig. 2. IP6 content of selected foods.

4. Conclusions

A MAE method has been successfully developed for the extraction of phytic acid and pyrophosphate from a high lipid content food as walnuts. The microwave-assisted methodology proved to be simple and fast since the extraction time required for optimal recovery of total phytic acid and pyrophosphate was significantly reduced from 3 h to 10 min. It is an attractive alternative to conventional extraction methods, such as solid-liquid extraction.

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Table 2
Yield of IP6 hydrolysis reaction.[1]

Experiment	Inorganic acid	Concentration(M)	MAE condition	Hydrolysis (%)
1	HCl	0.1	100 °C 10 min	26
2	HCl	0.1	125 °C 10 min	33
3	HCl	0.1	150 °C 10 min	88
4	HCl	0.1	150 °C 20 min	99
5	HCl	0.78	150 °C 20 min	85
6	HCl	2.0	150 °C 20 min	85
7	HCl	0.66	100 °C 10 min	13
8	H_2SO_4	0.52	100 °C 10 min	7
9	H_2SO_4	0.94	100 °C 10 min	25

[a] Initial concentration of phytic acid, 50 mg/L.

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