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Determination of phytate and lower inositol phosphates in Spanish legumes by HPLC methodology

C. Burbano,[a] M. Muzquiz,[a] A. Osagie,[b] G. Ayet[a] & C. Cuadrado[a]

[a]*Area de Tecnologia de Alimentos, CIT-INIA, Apdo. 8111, 28080 Madrid, Spain*

[b]*Department of Biochemistry, University of Benin, P.M.B. 1154, Benin City, Nigeria*

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The separation and quantitative determination of phytic acid (inositol hexaphosphate) and lower inositol phosphates (tri-, tetra- and penta-phosphates) was carried out using a HPLC method that included purification by anion-exchange chromatography, and analysis by ion-pair chromatography on a C18 reverse phase column. Samples of the most important legumes in the Mediterranean diet were analysed using this system. The different samples presented a variable content of phytic acid and different relative proportions of lower inositol phosphates.

INTRODUCTION

Phytate, the salt of phytic acid (myoinositol hexakisphosphate), is a naturally occurring plant constituent. The proportion of phytic acid is up to 10–30 g/kg of the dry matter of cereal, legume and oilseeds and constitutes the major portion of total phosphorus in the seed.

Excessive amounts of phytic acid in the diet can have a negative effect on mineral balance because it forms insoluble complexes with essential minerals (Cu^{2+}, Zn^{2+}, Fe^{3+} and Ca^{2+}) and consequently reduces the bioavailability of these minerals; (Forbes *et al.*, 1984). Moreover, phytate has also been shown to interact with the basic residues of proteins, inhibiting a number of digestive enzymes (Reddy *et al.*, 1982).

During storage, fermentation, germination, food processing or digestion in the human gut, phytic acid is enzymatically hydrolyzed by phytases to lower inositol phosphates such as inositol pentaphosphate (IP5), inositol tetraphosphate (IP4), inositol triphosphate (IP3) and possibly the inositol di- and monophosphates (IP2 and IP1). Only IP6 and IP5 have a negative effect on the bioavailability of minerals, the other hydrolytic products formed have a poor capacity to bind minerals, or the complexes formed are more soluble (Sandberg *et al.*, 1989).

Most conventional quantitative methods for determination of phytate are based on the procedure of Heubner and Stadler (1914). These methods involve sample extraction with hydrochloric acid and subsequent precipitation of ferric ion with phytate, and analysis of phosphorus or iron in the precipitate. These methods are inadequate as they lack the specificity to distinguish between the hexaphosphate and its partially dephosphorylated analogues (Xu *et al.*, 1992).

In recent years some high-performance liquid chromatography (HPLC) methods (Camire & Clydesdale, 1982; Knuckles *et al.*, 1982; Graf & Dintzis, 1982; Lee & Abendroth, 1983) have been developed but they have not been very successful in quantifying the different inositol phosphates. The anion exchange column chromatography and ion-pair HPLC methods were shown to be best suited for analysis of inositol phosphates in nutritional studies (Sandberg & Ahderinne, 1986; Lehrfeld, 1989).

The objective of this paper is the determination and quantification of inositol IP3, IP4, IP5 and IP6 in the most important legumes of the Mediterranean diet by an improved HPLC method.

MATERIAL AND METHODS

Materials

Two species of lupins (*Lupinus albus* and *L. luteus*), one variety of lentil (*Lens culinaris* var. Magda 20), two types of beans (*Phaseolus vulgaris* Tolosana and Guerniquesa), two types of chickpeas (*Cicer arietinum* Desi and Kabuli) and two cultivars of faba bean (*Vicia faba* cv Alameda and cv Brocal) from different locations of Spain were used in the present study.

Sample preparation

The legume samples were freeze-dried and ground to pass through a 100 mesh sieve. 0·5 g each of the powder were extracted under mechanical agitation with 20 ml 0·5 M HCl for 2 h at room temperature (Graf & Dintzis, 1982). The extract was centrifuged (700 g, 10 min) and the supernatant decanted, frozen overnight, centrifuged and filtered through MF-Millipore (0·22 μm). The inositol phosphates were separated from the filtrate and concentrated by the ion-exchange procedure of Sandberg and Ahderinne (1986) with some modifications. The filtrate was evaporated to dryness under vacuum (35°C), dissolved in 15 ml, 25 mM HCl, and passed through a Lida strong anion-exchange (SAX) column (quartenary amine bonded silica, 500 mg; Lida Manufacturing Corp, Kenosha, WI, USA) that was connected to a vacuum manifold (Visiprep; Supelco, Bellefonte, PA, USA). The loaded SAX column was washed successively with 10 and 5 ml of 25 mM HCl. The resin-bound inositol polyphosphates (IP3, IP4, IP5 and IP6) were then eluted with ten 1 ml portions of 2 M HCl. The eluent was evaporated to dryness and diluted with 0·5 ml of mobile phase.

Standards

The standards used were inositol triphosphate (IP3) and sodium phytate (IP6) (Sigma Chem. Co, St Louis, Mo, USA). A mixture of inositol phosphates was prepared by hydrolyzing an aqueous solution of sodium phytate (6 mg/ml) by autoclaving at 120°C for 1 h. The sodium phytate used consisted of 0·632 mg phytic acid/mg solid. Phosphorus content of sodium phytate was 17·76% determined by ICP atomic emission spectrophotometry. The linearity of phytic acid concentration versus peak area was investigated by 20 μl injections of solutions covering a range from 0·36 to 7·3 mg/ml phytic acid.

HPLC procedure

The inositol phosphates IP3, IP4, IP5 and IP6 were determined by ion-pair C18 reverse phase HPLC. The mobile phase was prepared according to Lehrfeld's method (1989), and a number of adjustments were made following Lehrfeld's indications (pers. comm.). The mobile phase consisted of 515 ml of methanol added to 485 ml of water. Eight milliliters of tetrabutylammonium hydroxide (Fluka, 40% in water), 1 ml 5 M sulfuric acid, 0·5 ml 91% formic acid (Fluka) and 100 μl of a phytic acid hydrolysate (6 mg/ml) were sequentially added. The pH was adjusted to 4·3 by addition of 9M sulfuric acid. The mobile phase was filtered through a Millipore filter (0·45 μm) and degassed under vacuum.

A reverse phase C18 column (Spherisorb ODS 5 μm, 250 × 4·6 mm) heated to 45°C was equilibrated with the mobile phase for 1 h. Analysis was performed with a Beckman System Gold HPLC instrument. Inositol phosphates were detected by refractive index (Beckman, Model 156). The flow rate was 1·2 ml/min and injection volume was 20 μl. Chromatographic analysis was carried out four times on each sample.

RESULTS AND DISCUSSION

The determination of inositol phosphates by HPLC methodology needs a series of interdependent phases: effective extraction, extract purification, separation of individual compounds, and detection and quantification.

The purification of the extract by anion exchange column chromatography allows the separation of IP3, IP4, IP5 and IP6 from lower inositol phosphates (IP1 and IP2). To compare the purification and concentration of phytic acid on a AG 1X8 column (Dowex) and a silica-based quaternary amine column (Sax), a legume sample (lentil) which contained an appreciable amount of partially hydrolyzed phytic acid was analyzed by the HPLC method. Although slightly higher results were obtained with the Dowex column (Table 1), the Sax column offered a lower coefficient of variation (19·4 and 17%, respectively) both being similar to the variation coefficient reported by Lehrfeld (1989). Furthermore, the two columns had similar recoveries when used with other samples, such as Nigerian samples (unpublished data). On the other hand, the Sax commercial column offers other advantages, such as less time-consuming preparation and more reproducibility and so this column was selected to analyze the legume samples.

The technique used to detect and quantitate the inositol phosphates depends on the HPLC chromatographic system used for the separation. The separation can be carried out by anion exchange, reverse phase, ion-pair chromatography, etc and the detection and quantification can be made by refractive index, conductivity or post-column reactions (Xu *et al.*, 1992). We initially applied a reversed phase elution procedure with sodium acetate as mobile phase following the Graf & Dintzis method (1982) and the results indicated that the mixture of inositol phosphates were poorly retained on the stationary phase.

The retention of the inositol phosphates on reverse

Table 1. Inositol phosphates content (% DM) in lentils purified with two types of columns

Column[a]	IP3	IP4	IP5	IP6	Total
Dowex	0·017 (0·011)	0·016(0·007)	0·059 (0·016)	0·342 (0·055)	0·437 (0·085)
Sax	0·016 (0·008)	0·019(0·006)	0·048 (0·005)	0·244 (0·047)	0·328 (0·056)

[a]Four replicate extracts (Dowex and Sax, respectively) of each sample (lentil) and analyzed in duplicate with HPLC (mean values with their standard deviations expressed on a dry weight basis).

phase packings may be increased through the use of the ion-pair reagents allowing the separation of IP3, IP4, IP5 and IP6 (Sandberg & Ahderinne, 1986; Lehrfeld, 1989).

A pH range of 4·15–4·8, and different proportions of methanol (40–60%) in the mobile phase (formic acid: methanol) were used. The results indicated that as pH increased, peak retention time increased. As methanol concentration increased the elution time decreased. Relative to the column characteristics, two reversed phase columns have been used: Spherisorb ODS (250 × 4·6 mm, 5 μm, Teknokroma) and Ultrasphere ODS (250 × 4·6 mm, 5 μm, Beckman) applying different column temperatures (22, 40 and 45°C).

The chromatographic system giving the best resolution of inositol phosphates was 0·012 M formic acid and 0·8% tetrabutylammonium hydroxide in 51·5% methanol (pH 4·3), and as a stationary phase the Spherisorb column thermostated at 45°C. In Fig. 1(a) the HPLC profile of a phytic acid hydrolysate is shown, using these conditions. Inositol tri- to hexaphosphates were detected and quantified by refractive index at isocratic conditions, the flow rate was 1·2 ml/min. This system was utilised to make the calibration curves and to analyze all the samples (Fig. 1(b)–(f)).

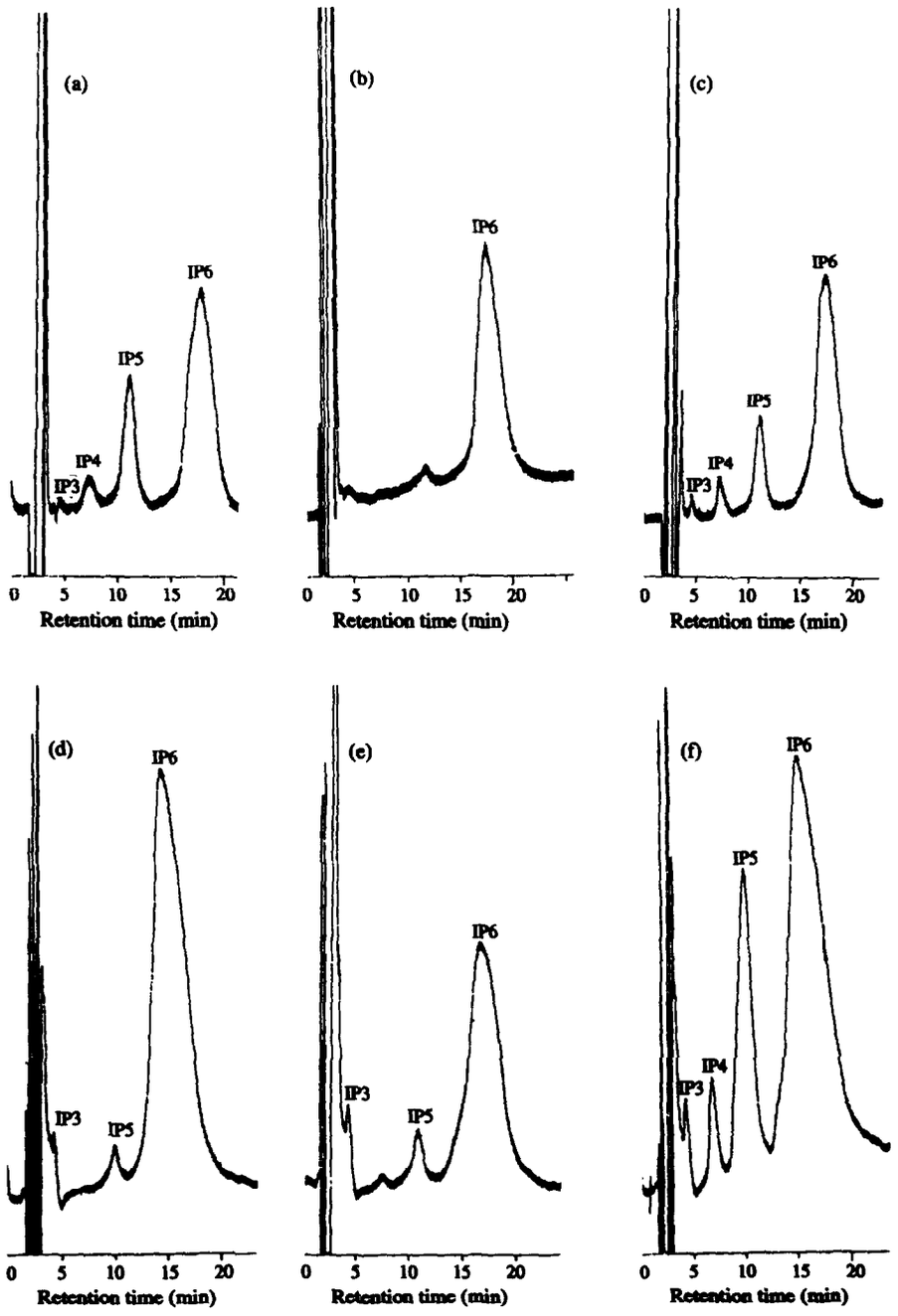

Fig. 1. Reversed-phase HPLC of a phytic acid hydrolysate. (a) *L. albus*; (b) *L. culinaris*; (c) *Ph. vulgaris* var. Guerniquesa; (d) *C. arietinum* type Kabuli; (e) *V. faba* var. Alameda; and (f) ion-pair chromatography. Samples were injected (20 μl) onto a Spherisorb column (250 × 4·6 mm, 5 μm) and heated at 45°C. Mobile phase was 0·012 M formic acid and 0·8% tetrabutylammonium in 51·5% methanol. The pH was adjusted to 4·3 with sulfuric acid. Flow rate: 1·2 ml/min. Detector: Refractive index. IP3, inositol triphosphate; IP4, inositol tetraphosphate; IP5, inositol pentaphosphate; and IP6, inositol hexaphosphate (phytic acid).

The sodium phytate which did not contain inositol phosphates other than the hexakisphosphate was used as an external standard. Because there was no difference in detector response, as shown by the similar RF of IP3 and IP6 (0·11 and 0·12, respectively), the calibration curve was constructed for phytic acid (IP6) concentration versus peak area, and a linear response was evident for the concentration range of 0·36–7·3 mg/ml). The correlation coefficient was 0·998 and the sodium phytate standard was also used for calculation of the IP3, IP4 and IP5.

Table 2 summarizes the results of the inositol phosphate contents (% DM) of the different legumes analyzed in this work and previously published values obtained by colorimetric methods. In this study the highest inositol phosphates total value corresponded to faba beans cv. Alameda (0·88%), and *L. albus* showed the lowest value (0·31%). Great variation could be observed between species (for example *L. luteus* and *L. albus*) as well as within species (i.e. *V. faba*). However, in some legumes the content was similar between different varieties or types (for example beans and chickpeas).

Several authors (Lolas & Markakis, 1975; Duhan *et al.*, 1989) reported that a wide variation in the phytic acid content was related to the varieties analyzed, environmental factors and in some cases the cooking quality of legumes. Bhatty and Slinkard (1989) found that phytic acid was largely responsible for the observed differences in the cooking quality of lentils.

The values of phytate obtained by the iron precipitation method are usually higher than that obtained by HPLC methods. According to Sandberg and Ahderinne (1986), this difference can be explained by the fact that the former causes coprecipitation of other phosphorus compounds, thus increasing the values.

The advantage of the HPLC method described is the ability to quantitate IP6 and lower inositol phosphates separately, which are present in different legumes samples. This method is particularly useful for analyzing the effect of food processing in the phytate content and production of lower inositol phosphates. The results are in good agreement with previous reports based on the application of HPLC methodology (Sandberg & Ahderinne, 1986; Lehrfeld & Morris, 1992), and it was found that the IP6 was the major inositol phosphate in the different samples, ranging from 100% in *L. albus* to 68·9% in *V. faba* cv. Alameda. With the exception of the later variety, the relative proportion of IP3 to IP5 is low, and can vary between different legumes, as it is clearly seen in Fig. 1. It is necessary remember that although phytic acid is involved in reducing the bioavailability of minerals due to the formation of phytate–mineral complexes (Reddy *et al.*, 1988), it also seems to have a beneficial role in the cooking quality of some legumes such as lentils.

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Table 2. Inositol phosphates content (% DM) of different legumes samples

Sample[a]	IP3	IP4	IP5	IP6	Total	Phytic acid: Literature value
Lupin						
L. luteus	—	—	0·06 (0·008)	0·717 (0·120)	0·777 (0·125)	
L. albus	—	—	—	0·314 (0·028)	0·314 (0·029)	0·5[b]
Lentils						
L. culinaris var. Magda 20	0·016 (0·008)	0·019 (0·006)	0·048 (0·005)	0·244 (0·047)	0·327 (0·056)	0·15–1·54[c]
Beans						
Ph. vulgaris var. Tolosana	0·009 (0·000)	—	0·020 (0·005)	0·564 (0·137)	0·593 (0·135)	0·54–1·58[d]
Ph. vulgaris var. Guerniquesa	0·009 (0·001)	—	0·019 (0·004)	0·628 (0·013)	0·656 (0·010)	
Chickpeas						
C. arietinum var. Desi	0·027 (0·008)	0·011 (0·001)	0·068 (0·003)	0·389 (0·015)	0·495 (0·020)	0·74–0·81[e]
C. arietinum var. Kabuli	0·025 (0·009)	—	0·031 (0·002)	0·379 (0·009)	0·435 (0·017)	
Fababeans						
V. faba var. Alameda	0·016 (0·003)	0·047 (0·008)	0·212 (0·015)	0·610 (0·030)	0·885 (0·020)	1·80[f]
V. faba var. Brocal	0·020 (0·012)	0·013 (0·003)	0·075 (0·002)	0·345 (0·056)	0·453 (0·047)	

[a]Two replicate extracts of each sample and analyzed in duplicate with HPLC (mean values with their standard deviations expressed on a dry weight basis).
[b]Camacho *et al.* (1991).
[c]Bhatty and Slinkard (1989).
[d]Lolas and Markakis (1975).
[e]Duhan *et al.* (1989).
[f]Reddy *et al.* (1982).

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