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Determination of phytic acid by gas chromatography–mass spectroscopy: application to biological samples

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Abstract

A GC–MS method is reported for the determination of phytic acid based on purification by anion-exchange chromatography, enzymatic hydrolysis of phytic acid to myo-inositol and derivation to trimethylsilyl derivative, with scyllo-inositol as an internal standard. Analytical features of the method are: limit of detection 9 $\mu g\ l^{-1}$ phytic acid, linear working range 18–500 $\mu g\ l^{-1}$ phytic acid, and coefficient of variation 1.9%. The method has been successfully applied to a variety of biological samples: various rat organs (kidney, liver, brain and bone), human plasma and urine and kidney stones. A comparative study of sample treatments, including deproteization, lipid extraction and the presence of a chelator, is also reported. Phytic acid amounts found in rat organs ranged from 1.07 g kg^{-1} for bone to 32.0 g kg^{-1} for brain. Phytic acid in human plasma was of the order of 0.14 mg l^{-1}. In kidney stones, phytic acid was found in calcium containing stones.

Keywords: Phytic acid

1. Introduction

Myo-inositol hexakis (dihydrogen phosphate), known as phytic acid, is a constituent of the human diet [1]. Antinutritional consequences have been attributed to high levels of phytic acid ingestion [2]. Phytic acid has been related to poor mineral absorption [3]. Recently, a number of beneficial effects on human health have been attributed to this compound, including its action as anticancer agent [4–6], and its inhibition of the crystallization of pathological calcium salts [7]. Phytate may also participate in endochondral ossification impeding the mineralisation of vesicles, a process believed to be regulated by enzymatic phytate hydrolysis [8]. The undesirable crystallisation processes to form a stone can take place in the kidney or in the urinary tract. Experiments undertaken to demonstrate the relation between the crystallization of calcium salts in tissues and phytic acid are in their initial stage with animal experimentation. However, increasing the urinary excretion of phytic acid is recommended to diminish the risk of calcium (oxalate and phosphates) kidney stone recurrence [9]. Most studies of the biological effects of phytic acid are supported by the evaluation of the pathological evolutions of experimental animals undergoing different treatments, and metal analysis in organs. They rarely include data on phytic acid content in organs. A study on the relative biodistribution of dietary ^{3}H radiolabelled phytic acid in rats has been published. Much of the radioac-

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tivity after 24 h was in the liver, kidneys, muscle and skin [10]. Nevertheless, despite the high interest in phytic acid from a biomedical viewpoint, there is still a lack of knowledge on real phytic acid content in animal organs, probably due to the difficulties of its determination.

From the analytical point of view, trace amount of phytic acid have been determined by liquid chromatography. Conductimetric detection with ion suppression [11], and refractive index detection [12] have been used. Other alternatives to determine phytic acid involve the hydrolysis of phytic acid, and the determination of the hydrolysis products, phosphate or inositol. Phosphate used to be determined photometrically [13]. Inositol can be determined by GC after derivation. Several derivatization reagents have been proposed, e.g. trimethylchlorosilane [14,15], heptafluorobutyrylimidazole [16] and trifluoroacetic anhydride [17]. Such methods for inositol determination have mainly been applied to the analysis of food samples and human samples.

From a comparison of the analytical characteristics of published methods, it can be stated that GC determinations show better sensitivity, and because of this, GC has been selected for the present study.

The goal of this work is to provide analytical procedures to determine the phytic acid content in biological samples of interest for animal experiments, and in human samples such as plasma, urine, biopsy samples and other pathological samples, such as kidney stones. This can contribute to the elucidation of the action of phytic acid and the possible relation of low phytic acid content in human samples with pathological states such as renal lithiasis [18].

2. Materials and methods

2.1. Reagents

All chemicals were of analytical-reagent grade. Granular activated carbon (100 mesh) and Na_2EDTA were purchased from Panreac (Spain), the anion-exchange resin was AG 1-X8 (200–400 mesh) from Bio-Rad (CA,USA), phytic acid (from corn), D-myoinositol-1,4,5-trisphosphate, D-myoinositol-1,3,4,6-tetrakisphosphate, D-myoinositol-1,3,4,5,6-pentakisphosphate, scyllo-inositol (*cis*-1,3,5-trans-2,4,6-hexahydroxycyclohexane), myo-inositol (*cis*-1,2,3,5-trans-4,6-hexahydroxycyclohexane), pyridine (anhydrous), hexane, methanol, chloroform and trichloroacetic acid were from Sigma (MO, USA). Triethylamine (from Panreac) was dried with sodium before use. Derivatization chemicals, 1,1,1,3,3,3-hexamethyldisilazane and chlorotrimethylsilane, were purchased from Aldrich (Germany). Crude phytase from Aspergillus ficcum, 3.5 units mg^{-1} specific activity was from Sigma. A suspension containing 1.0 g crude phytase l^{-1} was prepared in a 3 m*M* HCl solution with magnetic stirring.

2.2. Instrumentation

Gas chromatography was carried out on a Shimadzu QP-5000 gas chromatograph using a 30 m×0.25 mm I.D. fused-silica capillary column SPB-20 (Supelco), and He as carrier. The column temperature was programmed from 120 to 250°C at 8°C min^{-1}. The column pressure was increased from 47.8 to 95.4 kPa at 2.9 kPa min^{-1} in order to keep the gas flow at 0.8 ml min^{-1}. The injector port and the detector interface were kept at 250°C. A perfluorotributylamine standard was run every working session. Injections were carried out automatically in the splitless mode (purge time 1 min). Multiple ions of the fragments with m/z 73, 147, 191, 204, 217, 305 and 318 amu were monitored, as recommended in the literature [19]. Data processing was performed by the CLASS-5000 software (Shimadzu). Peak heights were used for calculation.

2.3. Derivatization procedure

A chromatographic column (16×5 mm) containing 0.2 g of anion-exchange resin was equilibrated with 3 m*M* HCl. Sample solutions at pH 3–4, containing from 0.004 to 0.1 μg phytic acid were passed through the column, where phytic acid was retained. The column was washed with 7 ml of 3 m*M* HCl. Then, 0.5 ml of 3 m*M* HCl and 0.1 ml of the phytase enzyme suspension were added, and the column was closed. Its contents were mixed by rotation at 1 rpm for 1 h at 37°C. The liquid phase was transferred to a vial and the column washed with 2 ml of 50 m*M* HCl. 0.01 μg of scyllo-inositol (in aqueous solution) was added to the vial (internal

standard). Then it was frozen at –20°C and lyophilised. The residue was reconstituted with 1 ml of pyridine (or triethylamine), and 0.2 ml of hexamethyldisilazane and 0.7 ml of chlorotrimethylsilane were added. The solution was maintained at 100°C for 1 h. After reaction, the excess of reagents and organic solvent were blown off under a stream of nitrogen. The solid residue was extracted with 2 ml of hexane. The solution obtained was evaporated and the residue reconstituted in 200 μl of hexane. 1 μl of this solution was injected for chromatography. The calibration graph was obtained from peak height corresponding to the silylated compounds of scyllo- and myo-inositol, at 13.8 and 14.5 min respectively.

2.4. Sample treatment for kidney, liver and brain

Tissue was stored frozen at −20°C to reduce any metabolic activity. For analysis it was lyophilised and pulverised to a uniform blend. The lipids were removed according to Folch's method [20]. 20 mg of sample was twice treated with 3 ml of chloroform–methanol (2:1). The liquid phase was discarded. The solid-phase was homogenised in 5 ml of water using an Ultra-Tunax homogeniser (20 s at 1370 g three times). Then, 0.1 ml of 0.1 *M* Na_2EDTA was added, and the mixture stirred for 1 h. After this time, 0.2 ml of 1 *M* trichloroacetic acid were added to denature the proteins. Solid phase was separated by centrifugation at 1370 g for 5 min. The supernatant was quantitatively transferred to a vial, and the pH was adjusted to 3–4 with NaOH. Using this solution the derivatization procedure described above was followed.

2.5. Treatment for human plasma

Whole blood in 0.3% Na_2EDTA was centrifuged at 1370 *g* for 15 min. 0.5 ml of supernatant was treated with 50 μl of 0.1 *M* Na_2EDTA and 0.1 ml of 1 *M* trichloroacetic acid. The method then continues as in the derivatization procedure.

2.6. Treatment for bone

The femur was pulverised to a uniform blend. 25 mg of the obtained uniform blend were shaken with 0.2 ml of 12 *M* HCl for 3 h. Then, the suspension was diluted to pH 3–4 and filtered through a 0.45 μm membrane filter (cellulose acetate, obtained from Micron Separations). The method then continues as in the derivatization procedure.

2.7. Treatment for renal stones

20 mg of phosphate or oxalate stones were dissolved with 0.5 ml of 12 *M* HCl. 50 mg uric acid or cystine stones were dissolved with 0.5 ml of 5 *M* NaOH. The pH of the obtained solutions was adjusted to 3–4. The method then continues as in the derivatization procedure.

2.8. Treatment for human urine

Urine was acidified with HCl to pH 3–4. 10 ml of sample was purified using a chromatographic column of 20 mm I.D. containing 0.5 g of activated carbon. 0.2 ml of purified sample were analysed as in the procedure.

3. Results and discussion

3.1. Method development

Phytic acid and inositol are natural constituents of most of living organisms [21,22]. The phytic acid determination described here is based on the silylation reaction of myo-inositol with chlorotrimethylsilane and hexamethyldisilazane, as catalyser, in presence of a ternary amine base using scyllo-inositol as internal standard. Under such conditions the formation of hexamethylsilylinositol easily takes place. Consequently, the method of determination can be divided in three parts: (1) separation of phytic acid from free myo-inositol and scyllo-inositol; (2) hydrolysis of phytic acid to myo-inositol; and (3) derivatization of inositols.

3.2. Separation of phytic acid from myo-inositol and scyllo-inositol

The separation of phytic acid from free myo-inositol and scyllo-inositol has been carried out using a strong anionic exchange resin. It is known that phytic acid is strongly retained on such resin at pH

higher than 1.5 [13]. However, inositols are not retained and 5 ml of 3 m*M* HCl was sufficient for complete elution of inositols. This was supported on the results of several recovery experiments carried out with standards, artificial urine (prepared according to literature data [23]), human urine and rat kidney. The phytic acid content in such samples ranged from 8×10^{-3} μg to 2×10^{-1} μg and the added inositols from 4×10^{-2} to 80 μg. The obtained recoveries were about 100%.

3.3. Hydrolysis of phytic acid

The hydrolysis of phytic acid to inositol can be accomplished by heating in acid media for a long time [14] or enzymaticaly [24]. The use of the enzymatic hydrolysis prior to GC determination remains unstudied. Both hydrolysis reactions are compared here. The acid hydrolysis was performed in a closed tube containing 2 *M* HCl and heating at 120°C, the enzymatic one as indicated in the derivatization procedure. Fig. 1 shows the evolution of found myo-inositol in equimolar solutions of phytic acid and myo-inositol as a function of the duration of the hydrolysis. Fig. 1A demonstrates that myo-inositol in acid media undergoes side degradations to non-inositol compound that, obviously, reduce the yield of the derivation reaction (curve b). For phytic acid (curve a) the curve obtained reflects the sum of phytic acid transformation to inositol, and its further hydrolysis to uncharacterised compounds. Consequently, the graph shows a maximum. As can be seen, the maximum was located at 11 h. A loss of sensitivity was obtained after this time (see limit of detection in validation study section). The results obtained from the study of the enzymatic hydrolysis (Fig. 1B) lead to the following conclusions: (1) no interaction of free inositol with the enzyme was detected (curve b); (2) the yield of the hydrolysis reached a maximum for a 0.5 h incubation time (curve a); and (3) phytic acid was quantitatively transformed to myo-inositol. The influence of the amount of enzyme and resin on the yield of the hydrolysis were also studied, for the reported experimental conditions (1 h incubation time), 0.10 mg of phytase crude resulted in a 100% hydrolysis in the analytical range of phytic acid concentrations. Obviously, a 100% hydrolysis could be obtained with less quantity of enzyme but with a longer incubation period. It was also demonstrated that the hydrolysis yield is not dependent on the amount of resin in the range 0.1–0.3 g resin. Bearing in mind the absence of side reactions of inositol, it can be concluded that myo-inositol can be used as standard to prepare the calibration graph when using enzymatic hydrolysis. Nevertheless, if the acid hydrolysis is adopted, phytic acid must be used as standard, and the hydrolysis conditions must be accurately controlled or, alter-

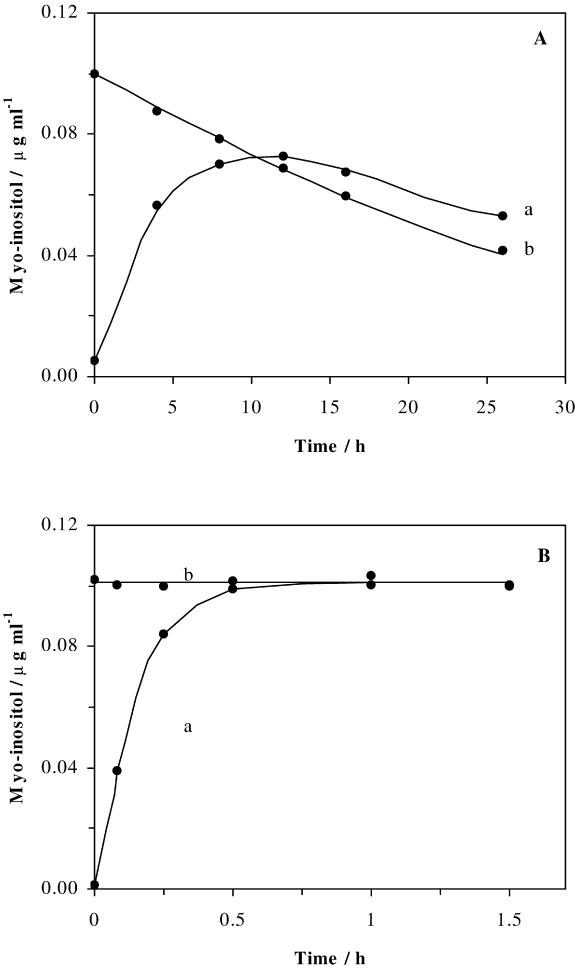

Fig. 1. Yield of the hydrolysis reaction of phytic acid to inositol (curves a) and stability of inositol (curves b) with the time of hydrolysis reaction. (A) acid hydrolysis. (B) enzymatic hydrolysis.

natively, the standard addition method for each sample could be used (Fig. 1A).

3.4. Derivatization

The derivation reaction involves a silylation reaction of the hydroxyl groups of inositol. Pyridine is usually used as solvent. Considering the toxicity of this solvent, its substitution for another organic base was studied. Fig. 2 shows the evolution of the peak height with the time of silylation reaction at several temperatures for pyridine and triethylamine. For pyridine, 1 h reaction time is enough to reach equilibrium at 100°C. An increase of temperature up to 150°C did not increase the yield of the reaction, nevertheless, a lower yield was obtained at 80°C. In triethylamine, the formation of products was less favourable. The yield increased with temperature. Consequently, if the sensitivity is not a critical requirement, pyridine can be substituted by triethylamine. Triethylamine has two advantages: (1) it is a less toxic solvent; and (2) its evaporation is faster. Using the glassware of our laboratory, to blow off the pyridine with a stream of nitrogen, approximately 30 min was necessary, however, to remove triethylamine, 7 min was enough.

Fig. 3 shows a series of chromatograms corresponding to two samples and a standard. Evaluation of peaks at 13.8 and 14.5 min permitted the determination of phytic acid.

3.5. Treatment of biological samples

Rat organs, human plasma, urine and renal stones were analysed. Urine samples had been determined previously [13], but, to our knowledge, the other samples have not been studied from the analytical point of view. In order to select the adequate sample treatment, a study of lipid extraction, protein separation and presence of a cation chelator was carried out (Table 1). Lipid extraction was accomplished by extraction with chloroform–methanol. The chelator used was EDTA. For protein precipitation, trichloroacetic acid, hydrochloric acid and acetonitrile were compared. In general, the three treatments were necessary to obtain satisfactory recoveries. Nevertheless, the lipid extraction was not necessary for kidney. EDTA was added to minimise the interac-

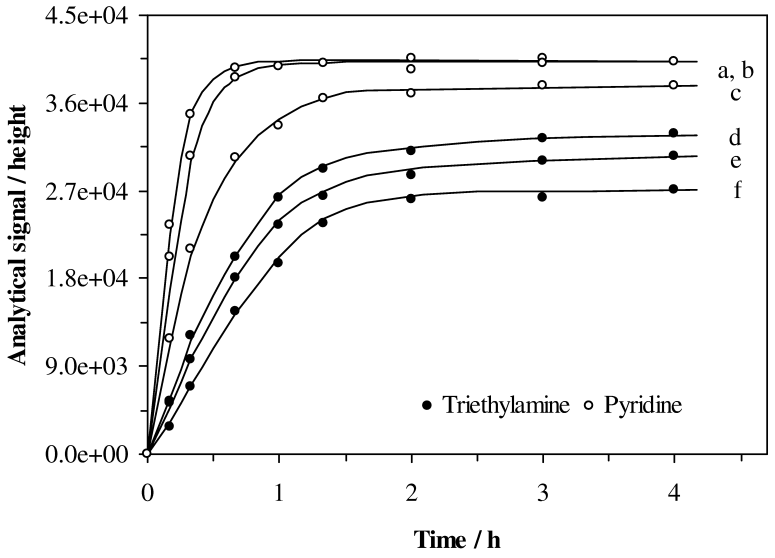

Fig. 2. Evolution of analytical signal (peak height) with the time of silylation reaction for several temperatures for pyridine and triethylamine. The temperatures studied for pyridine were (a) 150°C, (b) 100°C and (c) 80°C. The temperatures studied for triethylamine were (d) 150°C, (e) 120°C and (f) 100°C.

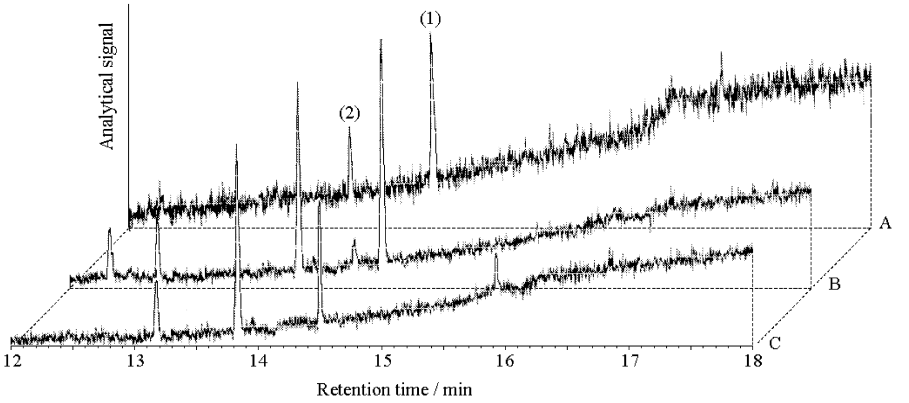

Fig. 3. Typical multiple ion monitoring chromatograms. The analytical signal is the sum of fragments at m/z 73, 147, 191, 204, 217, 305 and 318 amu. (A) standard obtained from 18 μg phytic acid l^{-1} and containing 3 μg scyllo-inositol l^{-1} (referred to the chromatographed hexane solutions). (B) and (C) chromatograms corresponding to kidney and bone respectively (amount of sample indicated in the procedure). (1) silylated myo-inositol and (2) silylated myo-inositol and (2) silylated scyllo-inositol (internal standard).

tions of phytic acid with calcium (and other metallic species). The presence of EDTA was necessary for a 100% recovery, mainly for plasma and kidney. A trichloroacetic treatment to precipitate proteins led to better recoveries than other treatments studied.

Once the optimum sample treatment was selected, several samples (kidney, liver, brain and bone) from different rats were analysed (Table 2). We wish to emphasise the high phytic acid amount found in brain. Moreover, several human plasma, urine (Table 2) and renal stones have been also analysed (Table 3). Seven calculi, characterised by IR spectroscopy [25], were selected as examples of the most frequent renal stones. As can be seen, phytic acid concentrations in the range 0.4 to 1.5 g kg^{-1} were found for calculi containing calcium and/or magnesium. Nevertheless, phytic acid was not detected in organic calculi (uric acid and cystine). Since, phytic acid is an effective inhibitor of calcium crystal formation [18], it has been proposed for treatment of relapsed lithiasis. The mechanism of inhibition is based on the selective adsorption of the inhibitor on the crystal nucleus and/or on the crystal growth active sites. Results in Table 3 show that phytic acid is adsorbed and retained on calcium crystal surfaces during calculus formation in the urinary tract, but that absorption was not detected on uric acid and cystine calculi. This implies a selective interaction of phytic acid with calcium crystals in vivo.

4. Validation study

4.1. Specificity

Biological samples contain significant amount of myo-inositolmonophosphate as well as a number of myo-inositolpolyphosphates [21]. Thus, the interference from lower esters than phytic acid was studied. To carry out this study D-myoinositol-1,4,5-trisphosphate, D-myoinositol-1,3,4,6-tetrakisphosphate and D-myoinositol-1,3,4,5,6-pentakisphosphate were selected. Working with standards containing 0.5 mg l^{-1} phytic acid it was found that 2.0 mg l^{-1} of triester and tetraester did not cause any significant error. Nevertheless, under the same conditions, myo-inositolpentaphosphate caused a positive interference (found phytic acid was a 15% higher than added phytic acid). The maximum concentration of myo-inositolpentaphosphate that did no interfere when determining 0.5 mg l^{-1} phytic acid was 0.5 mg l^{-1}. Considering that lower esters in biological samples are present at lower concentration than phytic acid [21], it was concluded that the present method can be considered specific for phytic acid.

4.2. Linearity

The linearity of the peak ratio (myo-inositol/scyllo-inositol) with phytic acid concentration was

Table 1
Study of the sample treatment

Sample	Amount of sample[a]	Treatment			Phytic acid			C.V.[c] (%)
		Lipids-extration	Cation-chelator	Protein-precipitation	Added[b]	Found[b]	Recovery (%)	
Rat tissues								
Kidney	20	Cl_3CH:CH_3OH	EDTA	Cl_3CCOOH	–	1.52	–	2.3
					0.5	2.03	102.1	–
		–	EDTA	Cl_3CCOOH	–	1.52	–	2.3
					0.5	2.02	101.0	–
		–	–	Cl_3CCOOH	–	1.36	–	–
					0.5	1.82	92.3	–
		–	EDTA	HCl	–	1.47	–	–
					0.5	1.95	96.1	–
		–	EDTA	CH_3CN	–	1.37	–	–
					0.5	1.83	92.2	–
Brain	20	Cl_3CH:CH_3OH	EDTA	Cl_3CCOOH	–	32.0	–	2.0
					8	40.1	101.2	–
		–	EDTA	Cl_3CCOOH	–	31.8	–	–
					8	39.7	98.7	–
		–	–	Cl_3CCOOH	–	31.6	–	–
					8	39.4	97.5	
		–	EDTA	HCl	–	31.4	–	–
					8	38.7	91.3	–
		–	EDTA	CH_3CN	–	31.1	–	–
					8	38.6	93.7	
Liver	20	Cl_3CH:CH_3OH	EDTA	Cl_3CCOOH	–	3.86	–	2.0
					1	4.86	99.8	–
		–	EDTA	Cl_3CCOOH	–	3.77	–	–
					1	4.75	98.0	–
				Cl_3CCOOH	–	3.78	–	–
		–	–		1	4.74	95.9	
		–	EDTA	HCl	–	3.72	–	–
					1	4.69	97.1	–
		–	EDTA	CH_3CN	–	3.71	–	–
					1	4.68	97.0	–
Bone	25	–	–	–	–	1.07	–	2.4
					0.5	1.59	104	–
Human biological fluids								
Plasma	0.5	–	EDTA	Cl_3CCOOH	–	0.14	–	2.1
					0.05	0.19	100.0	–
		–	–	Cl_3CCOOH	–	0.13	–	–
					0.05	0.17	88.0	–
		–	EDTA	HCl	–	0.14	–	–
					0.05	0.19	97.5	–
		–	EDTA	CH_3CN	–	0.13	–	–
					0.05	0.18	96.0	–
Urine	0.2	–	–	–	–	1.57	–	2.2
					0.4	1.98	102	–

[a] mg for rat tissues and ml for biological fluids.
[b] μg phytic acid g^{-1} of tissue for rat tissues and μg phytic acid ml^{-1} of fluid for biological fluids.
[c] Three determinations.

Table 2
Analysis of phytic acid in rat tissues and biofluids

Sample	Number of samples	Average of analyzed samples[a]	Range of found concentrations[a]
Rat tissues			
Kidney	5	1.78	1.04–2.80
Liver	4	3.72	3.20–4.10
Brain	3	32.3	30.0–42.0
Bone	3	1.13	1.07–1.21
Human biological fluids			
Plasma	5	0.23	0.14–0.31
Urine	10	1.58	0.43–2.52

[a] μg phytic acid g^{-1} of tissue for rat tissues and μg phytic acid ml^{-1} of fluid for biological fluids.

examined. The regression line obtained from 12 standards (from 0.018 to 0.5 mg l^{-1} phytic acid and 0.04 mg l^{-1} of scyllo-inositol) was:

Peak ratio =

$-(0.001\pm0.032)+(6.932\pm0.115)$

[phytic acid, mg l^{-1}],

$R^2 = 0.9997$

(phytic acid concentrations are referred to the injected hexane solution and slope and intercept are expressed as value±standard deviation).

4.3. Limit of detection

The limit of detection (LOD) was calculated as three times the standard deviation ($n=3$) of the lowest studied concentration of the linear range (Fig. 3A). The LOD found was 9 μg l^{-1} phytic acid (concentration referred to the injected hexane solution).

For comparative purposes, the limit of detection was also calculated under different experimental conditions than the recommended in the procedure. Using triethylamine as solvent (instead of pyridine), the LOD was 19 μg l^{-1} phytic acid. The LOD when

Table 3
Analysis of phytic acid in renal stones by the proposed method

Renal stones	Amount of sample (mg)	Phytic acid			
		Added (mg kg^{-1})	Found (mg kg^{-1})	Recovery (%)	C.V.[a] (%)
Calcium oxalate monohydrate	20	–	0.94	–	2.6
		0.40	1.35	103	–
Calcium oxalate dihydrate	20	–	0.40	–	2.4
		0.40	0.79	98	–
Uric acid	50	–	<0.05	–	–
		0.10	0.095	95	–
Cystine	50	–	<0.05	–	–
		0.10	0.11	110	–
Hydroxyapatite[b] + struvite	20	–	1.07	–	2.9
		0.40	1.47	100	–
Hydroxyapatite[b] + carbonatoapatite	20	–	0.7	–	3.0
		0.40	1.09	98	–
Hydroxyapatite[b] + calcium oxalate dihydrate	20	–	1.53	–	2.8
		0.40	1.95	105	–

[a] Three determinations.
[b] Hydroxyapatite $Ca_5(PO_4)_3(OH)$.

the hydrolysis was carried out at 120°C in 2 *M* HCl for 11 h was 26 μg l^{-1} phytic acid. As can be seen, the enzymatic hydrolysis led to a lower LOD than the acid one and, besides, is faster (from 11 to 0.5 h) and easy to automate.

4.4. Precision

The precision was determined under three different experimental conditions: the proposed procedure, with triethylamine instead of pyridine, and undergoing HCl instead of enzymatic hydrolysis. Measurements of 5 different standards containing 70 μg l^{-1} phytic acid gave coefficients of variation (C.V.s) of 1.9, 3.5 and 3.8% respectively. The C.V. of samples are reported in Tables 1 and 3. As can be seen, for real samples, slightly higher values were obtained (C.V. ranged from 2.1 to 2.8%).

4.5. Accuracy

To test the accuracy of the method spiked samples were analysed. As can bee seen in Table 1, recoveries for rats tissues and human fluids ranged from 104% for bone to 99.8% for liver. For kidney stones (Table 3) recoveries ranged from 105% to 98%.

The higher concentration in urine permitted such samples to be analysed according to a published procedure based on the complete hydrolysis of phytic acid and extracto-photometric determination of phosphate ions [13]. Both methods were comparable with a 95% confidence level (regression graph for 10 samples: $y=1.053x-0.036$, $R^2=0.997$, where y represents obtained concentration by the present method and x concentration by the extractophotometric method). As urine samples were analysed through analysis of phosphate and through analysis of myo-inositol, the molar ratio phosphate/myo-inositol was calculated for each sample. The obtained molar ratio value was 5.93 (SD = 0.31, $n=18$). As can be seen, it was not different to the phytic acid theoretical value (ratio = 6).

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