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Development of a Specific Fluorescence Post-column Derivatization Method Coupled with Ion-Pair Chromatography for Phytate Analysis in Food

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ABSTRACT: Phytate in plants (inositol phosphates, InsPs) affects mineral bioavailability. However, methods for their quantification may lead to variable results, and some are nonspecific (spectrophotometric techniques). In this study, ion-pair high-performance liquid chromatography (HPLC) was coupled with post-column derivatization to allow fluorescence detection (FLD, $\lambda_{excitation}$324/$\lambda_{emission}$364 nm) of InsPs. The fluorescence derivatization reaction, the main input of this study, was based on a ternary complex among phytate, iron(III), and 1,10-phenanthroline. Phytic acid ($InsP_6$) and three other InsPs ($InsP_{3-5}$) were analyzed in peanuts and soybean products after extraction in HCl 0.66 M, followed by purification on strong anion exchange cartridges. The novel method, named IP-HPLC−FLD, selectively separated $InsP_{3-6}$ with linear ranges between 600 and 2000 mg 100 g^{-1} ($R^2 > 0.99$). The limits of detection and quantification were between 120−180 and 340−540 mg 100 g^{-1}, respectively. As the relative standard deviations were under 10%, the IP-HPLC−FLD method is suitable for phytate analysis.

KEYWORDS: *inositol phosphates, specific complexation, fluorescence detection, cereals, legumes*

1. INTRODUCTION

Phytic acid (also called myo-inositol hexakisphosphate, $InsP_6$) is the major form of phosphorus storage in plants. $InsP_6$ has six acidic phosphate groups around a cyclohexane ring. Each of the phosphate groups is esterified to the inositol ring and together they can bind up to 12 protons.[1] In the literature, different terminologies such as phytin, phytate, and phytic acid can be found, leading to confusion. Maga (1982)[2] stated that the term phytin implies a calcium−magnesium salt of phytic acid, whereas phytate refers to the mono- to dodeca-anion of phytic acid.

Phytate contents vary depending on the plant species or the different parts in seeds (whole seed, bran, and cotyledon/albumen). For example, Pires et al. (2023)[3] reported 0.35−9.42 g 100 g^{-1} in almonds, 0.22−6.69 g 100 g^{-1} in walnuts, 2.10−7.30 g 100 g^{-1} in wheat bran, 2.56−8.70 g 100 g^{-1} in rice bran, 0.57−3.35 g 100 g^{-1} in sorghum, and 6.39 g 100 g^{-1} in maize. These raw materials are used for human consumption at household level and can also be processed at industrial scale for manufacturing foods such as "Ready-to-Use Therapeutic Foods" to fight against malnutrition in risk areas.[4]

During food processing, $InsP_6$ can be degraded or dephosphorylated into smaller phosphoric esters of myo-inositol ($InsP_1$ to $InsP_5$). For example, $InsP_6$ was degraded during quinoa, amaranth, and canihua fermentation (with around 64−93% reduction in flours and 12−51% reduction in grains)[5] and germination with the proportion of degradation varying between 2 and 66%, depending on the plants and processing parameters (temperature and duration).[6] Other processes were also reported in the literature to decrease phytate content: water soaking (43−49% of reduction in legumes by leaching),[7] microwave and ultrasound-assisted extraction (around 35% of phytate reduction in legumes by leaching),[8] extrusion of cereal brans with moisture content at 20% and temperature at 115 °C (26−64% reduction),[9] or autoclaving at 121 °C for 20 min (65−70% reduction).[7]

Inositol phosphates (InsPs) are considered antinutrients because they strongly bind metallic cations of calcium, iron, potassium, magnesium, manganese, and zinc, thus reducing their bioavailability.[1,10,11] Their binding capacity is dependent on the number of phosphates on the ring. For instance, myo-inositol pentakisphosphate ($InsP_5$) presents lower binding capacity on iron at physiological pH compared with $InsP_6$. $InsP_3$ and $InsP_4$ alone, depending on the isomer, have even lower iron binding capacity but are able to interact with $InsP_6$ and thereby contribute to the negative effect on mineral absorption.[12] Concerning zinc and calcium, absorption is highly influenced by $InsP_5$ and $InsP_6$, while the other InsPs (lower phosphorylated) have no effect.[13] Phytate can also bind proteins either as a binary protein−phytate complex or as a ternary complex, where protein is bound to a mineral ion which is itself bound to a phytate.[14] To ensure nutrition security and reduce the prevalence of diet-related non-

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communicable diseases, such as anemia, in developing countries, the use of accurate methods for phytate quantification in foods appears to be a crucial factor in evaluating the extent of their impact on nutrient bioavailability. Indeed, the main sources of phytate in the human diet in developed and developing countries are cereals and legumes, including seeds and nuts, which represent about 40 and 60% of the total caloric intake, respectively.[15,16]

Analytical methods for phytate quantification in foods are numerous, from conventional techniques such as colorimetric methods or potentiometric titrations to separation techniques such as capillary electrophoresis, high-performance ionic chromatography (HPIC), and high-performance liquid chromatography (HPLC).[12,17,18] However, phytate analysis still faces several challenges, calling for more advanced modification and development.[19] Indeed, colorimetric methods are simple and cost-effective but suffer from uncontrolled variations due to the matrix composition and nonspecificity due to interferences from other phosphate-containing compounds and free phosphorus. Concerning chromatographic techniques, HPIC, for example, is known to separate enantiomers that are difficult to identify due to the lack of standards. Among reference HPLC methods, phytate analysis can be performed on a reverse-phase column with the use of a refractive index detector (RID).[20−22] However, this method does not isolate the $InsP_6$ peak from the solvent front. Lee and Abendroth (1983)[23] proposed ion-pair liquid chromatography (IP-HPLC) using a reverse phase C_{18} column to improve this method and the overall separation. Lehrfeld (1989)[24] optimized this method using a reversed-phase macroporous polymer column. Many slight modifications of the latter method are reported in the literature regarding the separation of phytate.[25−28] While this method is easy to implement, unfortunately, the use of RID is nonspecific to InsPs because other components may also be detected during analysis. Moreover, this detection method presents a lack of precision and is highly sensitive to the temperature.

Many compounds that are neither chromophores nor fluorophores can be post-column derivatized to form complexes that are detectable by UV or visible absorbance or fluorescence.[29] Chen et al. (2008)[30] described a synchronous spectroscopy fluorescence method in microplates to determine phytic acid in foods based on the formation of a ternary complex between phytate, 1,10-phenanthroline, and iron (Fe^{3+}). Recently, Marolt and Kolar (2020)[18] reviewed that the selectivity of this spectrophotometric method needed to be improved for foods analysis, which can be achieved by the use of HPLC. Therefore, this work aimed to validate a new detection method for quantifying $InsP_{3-6}$ in foods using post-column derivatization adapted to IP-HPLC. The derivatization is based on a complexation specific to phytate, allowing their clear and simple quantification by fluorescence detection (FLD). This novel approach combined the potential of the existing methods (sample extraction and purification, ion-pairing separation, and spectroscopic synchronous fluorescence). The new approach was compared to three conventional methods, which are different in their detection and separation principles (HPIC, ion-pair HPLC with refractive index detection, and a colorimetric method).

2. MATERIALS AND METHODS

2.1. Plant-Based Raw and Processed Samples. Legumes (soybean and peanut) and mixed legume-cereals (soy-maize), either raw or processed (roasted, extruded), were used as samples. They were provided by NUTRISET (Malaunay, France), a company manufacturing nutritional solutions for the treatment and prevention of different forms of malnutrition in southern countries.

2.2. Reagents and Standards. *2.2.1. Reagents.* Methanol, ethanol, hydrochloric acid (HCl), formic acid, sulfuric acid, acetic acid, trichloroacetic acid, ascorbic acid, ammonium molybdate, sodium acetate, sodium hydroxide, tetrabutylammonium hydroxide (TBA−OH, 40%, w/w, solution in water), iron(III) chloride hexahydrate, and 1,10-phenanthroline were purchased from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA). Methanol was HPLC grade, while the other reagents were ACS grade.

2.2.2. Standards. Pure standards of $InsP_{3-6}$ (purity ≥98%.) were bought from CAYMAN (Michigan, USA). The references were the following: D-myo-Inositol-1,2,3,4,5,6-hexaphosphate (CAY-10008415−1), D-myo-Inositol-1,3,4,5,6-pentaphosphate (CAY-10007784−1), D-myo-Inositol-1,3,4,5-tetraphosphate (CAY-60980−1), and D-myo-Inositol-1,4,5-triphosphate (CAY-10008205−1).

A nonpure standard (phytic acid sodium salt hydrate from rice, reference—P8810, batch number BCBZ7573), named InsP mix, with free phosphorus content ≈22.4% dry basis (data from certificate of analysis) was purchased from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA). All standards were dissolved in ultrapure water before use.

2.2.3. Ion-Pair HPLC Mobile Phase Preparation. The mobile phase consisted of 56% HPLC-grade methanol, which was mixed with 44% 0.035 M formic acid. Then, 10 mL of TBA−OH was added, and the pH was adjusted to 4.3 using 72% sulfuric acid. The mobile phase was degassed for 15 min in an ultrasonic bath before HPLC analysis.

2.3. Sample Extraction. Samples were extracted using HCl 0.66 M. 1 g of finely ground sample was mixed with 20 mL of HCl 0.66 M. The extraction was performed overnight at room temperature with continuous agitation using an overhead shaker (Reax 2 Heidolph, Schwabach, Germany) at 30 rpm. Then, the samples were centrifuged at 13,000 rpm (Avanti J-E Centrifuge, Beckman Coulter, Brea, USA) for 10 min at room temperature. Extraction was performed in triplicate for each sample.

2.4. Crude Extract Purification. The supernatant obtained after centrifugation was purified as proposed by Lehrfeld (1989)[24] with slight modifications. Purification was performed on silica-based strong anion exchange (SAX) cartridges [Discovery DSC-SAX solid-phase extraction (SPE) tube, bed wt 1 g, reference 52666-U, Sigma-Aldrich, Missouri, USA]. Briefly, the cartridge was conditioned with 2 mL of methanol, followed by 2 mL of 0.05 M HCl. Then a precise volume of the diluted supernatant was loaded into the cartridge. The SAX phase, after bonding with InsPs, was washed with 2 mL of 0.05 M HCl, and then, InsPs were eluted with 6 mL of 2 M HCl. The eluate was evaporated to dryness using the Genevac EZ-2 Plus—HCl Compatible centrifugal vacuum evaporator (Sp Scientific, Warminster, USA) at 40 °C and 5 mbar. The residue was dissolved in 1 mL of the ion-pair HPLC mobile phase and homogenized in an ultrasonic bath for 5 min. The purified extract was filtered (0.45 μm) and stored at −20 °C until analysis.

2.5. Sample Analysis Using an Existing Spectrophotometric Method. Phytic acid ($InsP_6$) was determined according to the analytical procedure described in the MEGAZYME kit "K-PHYT 05/17".[31] This procedure was based on measuring free and total phosphorus after enzymatic treatment of the crude extract with phytase and alkaline phosphatase and colorimetric reaction with ammonium molybdate.

$InsP_6$ content was determined by calculating the difference between total and free amounts of phosphorus and using a conversion factor of 28.2% (molar mass proportion of phosphorus in phytic acid).

2.6. Sample Analysis Using Existing Chromatographic Techniques. *2.6.1. Ion-Pair HPLC Method with Refractive Index Detection.* Crude and purified extracts were analyzed according to the procedure described by Lehrfeld (1989).[24] An HPLC 1290 infinity II System (Agilent, USA) was used for analysis with a polymeric reversed-phase column (PRP-1, 150 × 4.1 mm, Hamilton). The column temperature was set at 40 °C. The flow rate, using the ion-pair HPLC mobile phase, was set at 0.5 mL min^{-1}, the injection

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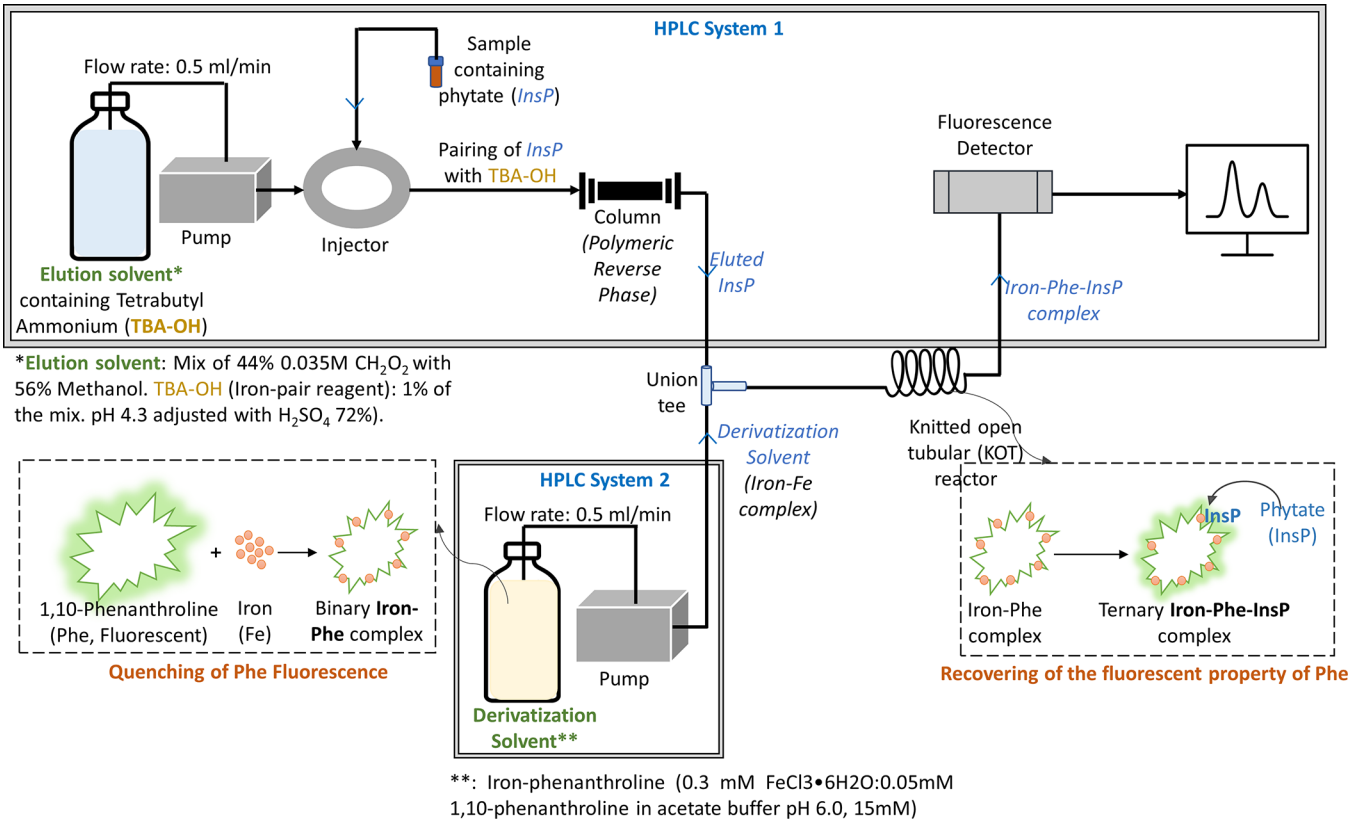

Figure 1. Chromatography principle for synchronous fluorescence post-column derivatization.

volume was 20 μL, and the detector temperature was maintained at 45 °C. The run duration was 30 min.

2.6.2. High-Performance Ionic Chromatography. Crude or purified extracts were also analyzed with HPIC (Dionex, Sunnyvale, CA, USA) using the method proposed by Talamond et al. (1998)[32] with some modifications. An Ion Pac AS11-AC anion exchange column (4 × 250 mm) was used with an IonPac AG11-HC guard column (4 × 50 mm) for separation. The detection was performed with a conductivity detector ICS-6000-DS5 (Dionex, Sunnyvale, CA, USA). An ion suppressor (AERS 500, 4 mm) was also added to the HPIC system. The run duration was 20 min. A gradient elution was used with NaOH and ultrapure water. It was generated automatically by the chromatographic system to reach the following concentrations: 35 to 100 mM from 0 to 11 min, 100 to 35 mM from 11 to 12 min, and 35 mM from 12 to 20 min. The flow rate was set at 0.8 mL min^{-1}, and the injection volume was set at 10 μL.

2.7. Ion-Pair Chromatography Coupled with Post-column Derivatization for Fluorescence Detection. *2.7.1. Post-column Derivatization Solution Preparation.* The reagents were iron(III) chloride hexahydrate ($FeCl_3$ $6H_2O$) dissolved in 15 mM acetate buffer, pH 6 (30 mM stock solution), and 1,10-phenanthroline (Phe) dissolved in 5% ethanol (10 mM stock solution).

Preliminary assays were performed to determine the adequate molar ratios of both reagents. For 1,10-phenanthroline, the concentrations tested were 0.2, 0.25, and 0.3 mM, while for the $FeCl_3$ $6H_2O$ solution, the concentrations were 0.05, 0.075, and 0.1 mM. The standard InsP mix at 0.5 and 1 g L^{-1} was used to determine the adequate molar ratio. The assays were performed in duplicate.

2.7.2. IP-HPLC–FLD Analytical System. An HPLC 1290 infinity II System (Agilent, USA) (System 1) coupled with an HPLC 1100 (Agilent, USA) (System 2) was used for analysis (Figure 1). On system 1, InsPs (injection volume 20 μL) were separated using the ion-pair HPLC mobile phase and the column PRP-1 (150 × 4.1 mm, Hamilton). The derivatization solution was pumped into system 2. The flow rate was set at 0.5 mL min^{-1} on both systems that were connected using a stainless-steel union tee with two inlet ports, one for the mobile phase and the other for the derivatization reagent. The union tee outlet port was linked to a knitted open tubular reactor (dimensions 4 m × 0.25 mm ID) (Biotech, Sweden) to mix the eluted InsPs and the derivatization solution. The FLD parameters were set at 324 and 364 nm for excitation and emission, respectively. The photomultiplier gain was set at 10. The run duration was 30 min.

2.8. Statistical Analyses. First, to determine if all $InsP_6$ standards accurately quantified $InsP_6$ in the samples, a Bland-Altman plot[33] was constructed using data obtained on IP-HPLC–FLD with the pure and the InsP mix standards. $InsP_6$ content determined with the pure standard was considered reference data. The percentages of difference between the two sets of data were plotted, and the upper and lower limits of agreement were determined with a 95% confidence interval (limit of agreement = mean ±1.96 × standard deviation). Second, $InsP_6$ content obtained by the IP-HPLC–FLD method was compared to the data obtained for each conventional method (IP-HPLC–RID, HPIC, and spectrophotometric method) based on Passing–Bablok regression with the significance level set at 5%. All analyses were performed using an XLstat 2022 (Addinsoft, Paris, France).

3. RESULTS AND DISCUSSION

3.1. Preliminary Observations on Conventional Methods. *3.1.1. Spectrophotometric Method (Megazyme K-ZPHYT Kit).* The spectrophotometric method, which is used for rapid $InsP_6$ quantification in foods, was efficient in obtaining low variability, with coefficients of variation ranging between 0.27 and 10%. However, this method is nonspecific since phytate quantification is based on the use of phosphorus content and a conversion factor of 28.2% (phosphorus proportion in phytic acid). Therefore, for samples with different ratios of all of InsPs or for those without $InsP_6$, this technique might not be suitable. Moreover, for products enriched in minerals, such as the Ready-to-Use Therapeutic

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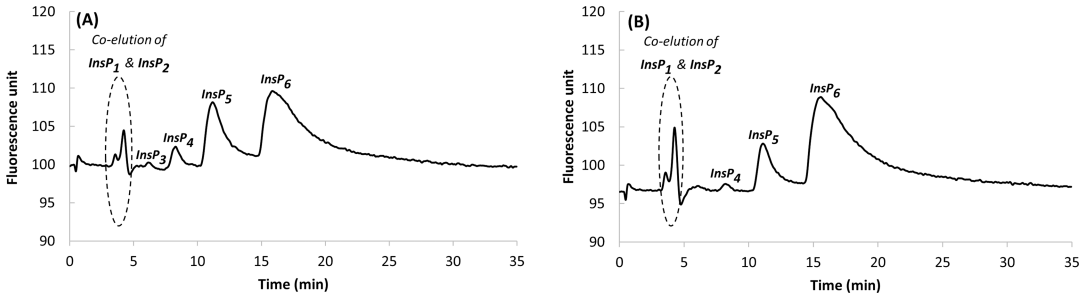

Figure 2. Chromatograms of (A) standard InsP mix (SIGMA) purified through SAX and (B) oat flour by IP-HPLC−FLD.

Foods, a high level of free phosphorus can influence phytic acid quantification.

3.1.2. Conventional Chromatographic Techniques: HPIC vs IP-HPLC RID. 3.1.2.1. Comparison Based on Analysis of Standard Compounds. The chromatograms of the InsP mix standard obtained by IP-HPLC−RID showed only $InsP_5$ and $InsP_6$ with the presence of a negative peak disturbing the identification of $InsP_4$ (Figure S1). Indeed, even if the separation technique is adapted for phytate analysis in plant foods, Lehrfeld (1989)[24] reported that the negative peak is due to air dissolved in the injected sample. In this case, the peak occurred between $InsP_4$ and $InsP_5$, with a mobile phase containing 56% methanol, and before $InsP_6$ when 65% methanol was used in the mobile phase. In our study, we found that this negative peak was also related to the detection with an RID.

The limit of detection (LOD) measured in this study using the RID, after an IP-HPLC separation, was 0.15 and 0.31 mg mL^{-1} for $InsP_6$ and InP_5, respectively. However, it was difficult to determine the LOD of $InsP_3$ and $InsP_4$ because of the occurrence of the negative peak described above. This confirms that the RID detection method is not accurate for phytate quantifications other than $InsP_6$. Moreover, the RID detection is especially disturbed by the presence of saccharides.

Concerning HPIC, it was impossible to clearly identify $InsP_{1-5}$ due to the ability of this chromatographic technique to randomly separate and differentiate isomeric forms of each of these inositol phosphates (Figure S2). Indeed, there are more than 60 isomeric inositol phosphates. Moreover, the standards of many of those isomers are unavailable, and the coupling HPIC−MS is not yet mastered; therefore, many chromatographic peaks remain unidentified. Thus, the HPIC method appears to be challenging for routine $InsP_{1-5}$ analyses in foods but accurate for $InsP_6$, with a detection that we measured 30 times more sensible than RID. This drawback could also explain why numerous studies using HPIC have only published data on phytic acid ($InsP_6$) and none on the content of other InsPs.[34−37]

3.1.2.2. Comparison Based on Samples Analysis. Crude extracts were analyzed to ease the sample preparation procedure. While the injection of standards on IP-HPLC−RID identified retention times and each phytate peak, injection of crude extracts (soybean, peanut, and mixed soy-maize) did not allow phytate elution, leading to a stable baseline without any peaks. This was probably due to interfering compounds in the extract affecting the ion-pair interactions. Regarding HPIC chromatograms, the use of the crude extract generated high random variability of $InsP_6$. On several replicates, up to 10, only 30−40% of the values were repeatable. When the extraction method proposed by Lestienne et al. (2005) and Talamond et al. (2000)[37,38] was applied, which consisted of acidic extraction in a boiling water bath followed by neutralization, similar results were observed on both chromatographic techniques (no peak for IP-HPLC−RID and high variability).

In the literature, only a few studies recommend the purification of the crude extract for phytate analysis using chromatography techniques. SAX cartridges have been used for solid-phase extraction (SPE) to concentrate InsPs and remove inorganic phosphates, as well as other interfering compounds. InsPs were eluted from the SPE using strong acid (2 M HCl).[25,28,39] Carlsson et al. (2001)[40] replaced the use of SPE with a centrifugal ultrafiltration of the extract (to speed up and simplify the sample preparation step) before injection into the HPIC system. However, they pointed out that for conventional HPLC using the RID, certain compounds will disturb the detection of the inositol phosphates, in particular for the detection of $InsP_{1-5}$. Therefore, it was decided to purify all the samples with SAX cartridges, even for the InsP mix standard. Thereafter, the purified extracts/standards were used for all the chromatographic analyses.

3.2. Determination of the Derivatization Reagent Molar Ratio. The static approach performed by Chen et al. (2008)[30] with a spectrofluorometer (synchronous fluorescence reaction based on the formation of a ternary complex between phytate, 1,10-phenanthroline, and Fe^{3+}) was converted into a dynamic reaction (under continuous flow) for post-column derivatization in the HPLC system. Indeed, the latter authors demonstrated that the synchronous fluorescence intensity of the solution was enhanced proportionally with an increase in the phytate concentration.

Our preliminary assays to identify the adequate molarities of 1,10-phenanthroline (Phe) and $FeCl_3{\cdot}6H_2O$ showed that the reaction was mostly dependent on the molarity of Phe. For instance, with Phe at 0.2 mM, $InsP_6$ peak areas at 1 mg·mL^{-1} and 0.5 mg·mL^{-1} were between 123 and 145 (arbitrary unit) and 253−295 (arbitrary unit), respectively. These areas increased from 14 to 26% when Phe concentration was 0.25 mM for both concentrations but with higher variabilities (Figure S3). With Phe at 0.3 mM, the areas were close to the values obtained with Phe at 0.25 mM, and the standard deviations were lower. Finally, the molarities chosen for the derivatization reagents were 0.3 mM and 0.05 mM of 1,10-phenanthroline and $FeCl_3{\cdot}6H_2O$, respectively, in 15 mM acetate buffer pH 6. This ratio between reagents allowed accurate phytate detection by fluorescence (less variability in the peak area) without affecting the chromatographic system stability, mainly the pressure limit. Fluorescence fluorophore detection was preferred over UV−visible chromophore detection (such as Wade reagent) based on its higher

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Table 1. Parameters for IP-HPLC−FLD Validation[a]

compound	linear range (mg mL^{-1})	equation*	R^2	LOD (mg mL^{-1})	LOQ (mg mL^{-1})	$RSD_{intraday}$ (%)	$RSD_{interday}$ (%)
$InsP_6$	$0.3-1_{600-2000}$	$Y = 0.0012x + 0.1634$	0.992	0.09_{180}	0.27_{540}	1.8	6.7
$InsP_5$	$0.3-1_{600-2000}$	$Y = 0.0014x + 0.0557$	0.995	0.07_{140}	0.21_{420}	5.0	8.1
$InsP_4$	$0.3-1_{600-2000}$	$Y = 0.0027x - 0.0125$	0.997	0.06_{120}	0.17_{340}	9.7	5.5
$InsP_3$	$0.3-1_{600-2000}$	$Y = 0.0031x + 0.0177$	0.996	0.07_{140}	0.20_{400}	5.6	<LOD

[a]LOD: limit of detection. LOD = 3.3* standard error of the estimate. LOQ: limit of quantification. LOQ = 10* standard error of the estimate. Values in subscript for the linear ranges, LOD, and LOQ are the correspondences in mg 100 g^{-1}. *: y-axis = InsP concentration in mg/mL and x-axis = area. $RSD_{intraday}$: relative standard deviation in %. Values were calculated after injection of each standard in triplicate at 1 mg mL^{-1}. $RSD_{intraday}$: values were calculated after analysis of the InsP mix standard (SIGMA) at 1 mg mL^{-1} on four different days.

specificity. When the post-column derivatization method was evaluated in this study with Wade reagent for UV−visible chromophore detection, only unreliable results were obtained due to the interference of other compounds and overlapping peaks (data not shown).

3.3. Performances of the Fluorescence Post-column Derivatization. *3.3.1. Selectivity.* Analysis of the InsP mix standard (SIGMA) using FLD after ion-pair HPLC showed peaks of $InsP_{3-6}$ with no interfering peak (Figure 2). However, the resolution of $InsP_6$ was not totally satisfactory. Lehrfeld (1994, 1989)[24,25] also observed a peak tailing for the peak of $InsP_6$. This author proposed modifying the organic solvent by replacing methanol with acetonitrile, which has a higher eluotropic index than methanol ($E^{\circ}_{methanol} = 0$ and $E^{\circ}_{acetonitrile} = 0.072$).[25] In our study, methanol was kept as the solvent because of its lower toxicity. Moreover, we observed that methanol led to a stable baseline compared to that of acetonitrile, probably due to the higher acetonitrile volatility. Finally, methanol is less expensive than acetonitrile, which means that it could easily be used for routine or large-scale analyses of phytates.

Peak tailing was evaluated using the asymmetry factor (As). The As values were 1.62 for $InsP_3$, 0.76 for $InsP_4$, 0.81 for $InsP_5$, and 5.68 for $InsP_6$. While the As values for $InsP_4$ and $InsP_5$ were within the acceptable range (typically 0.8−1.2), the higher value for $InsP_6$ (5.68) indicated significant peak tailing. This was addressed during method development to ensure that such tailing did not compromise the accuracy of the quantification. Despite the higher peak tailing for $InsP_6$, the chromatographic conditions allowed reliable and reproducible measurements. The tailing could also be associated with our internal analytical system (Figure 1) used for post-column derivatization. Employing commercial equipment, such as the Pinnacle derivatization instrument (Restek, USA), specially designed for this reaction, might improve the performance and reduce tailing.

3.3.2. Sensitivity and Linearity. Assays were performed using the InsP mix standard (SIGMA) with a concentration ranging from 0.25 to 10 mg mL^{-1}. When the standard concentration was above 1.5 mg mL^{-1}, the $InsP_6$ peak width became larger, with an unappreciable peak shape. Therefore, the standard concentration range used for the calibration curve was 0.3−1 mg mL^{-1}. This concentration range was also applied to each of the pure standards ($InsP_{3-6}$). The coefficients of determination R^2 found for the calibration curve were around 0.99 for $InsP_{3-6}$. The limit of detection (LOD) and quantification (LOQ) for these InsPs were under 0.3 mg mL^{-1}, which was the lowest concentration of the linear range (Table 1).

In a review, Marolt and Kolar (2020)[18] reported the LOD obtained in relevant studies regarding phytate analysis. Concerning ion-pair HPLC with RID, on C_{18} reverse phase column, the LOD was estimated at 100 mg L^{-1} for $InsP_3$ to $InsP_6$. The LODs obtained in our study with the FLD detector after post-column derivatization were much lower (60−90 mg L^{-1}) compared to the one reported, highlighting the potential of FLD over refractive index detection.

3.3.3. Accuracy and Recovery after Standard Addition. Relative standard deviations (RSDs) of peak areas were calculated to perform the intra- and interday validation. The value obtained for each phytate was under 10% (Table 1).

Analysis of the InsP mix standard (SIGMA) using IP-HPLC−FLD revealed that the $InsP_6$ relative proportion was 40.7% of total phytate. Unfortunately to date, a certified reference material is not available for the validation of inositol phosphate analysis methods. In our study, an in-house control material (ICM) was formulated at laboratory scale by mixing phytate-free ingredients (milk, saccharose, maltodextrin, and a food preservative, ratio 1:1:1:1) with rapeseed oil (at 50%), and the InsP mix standard to formulate two samples with $InsP_6$ content at 250 mg 100 g^{-1} FW (fresh weight) (ICM with low $InsP_6$) and 2000 mg 100 g^{-1} FW (ICM with high $InsP_6$). The phytate-free ingredients were chosen to have a complex sample with various interfering compounds (phosphorus from milk, saccharose, maltodextrin, fat, etc.). After extraction, purification, and IP-HPLC−FLD analysis, the percentage of recovery was 107 ± 1.3% ($InsP_6$ content at 2144.2 ± 25.8 mg 100 g^{-1}) for ICM with high $InsP_6$ and 79 ± 6.8% ($InsP_6$ content at 198.7 ± 17.1) for ICM with low $InsP_6$.

Moreover, in order to test the matrix effect, two samples containing InsPs (peanut and soy flour) were spiked directly with the InsP mix standard (SIGMA) to reach an $InsP_6$ content of 2000 mg 100 g^{-1} FW. The percentage of recovery obtained for these samples was between 103 ± 13.7 and 117 ± 8.4%.

3.4. Influence of the Standard Used for $InsP_6$ Quantification by IP-HPLC−FLD. $InsP_6$ content in some samples was quantified using the pure standard and the InsP mix calibration curves. For the InsP mix standard, the exact $InsP_6$ proportion (40.7%) was used as a correction factor.

The comparison of $InsP_6$ content calculated with the pure standard of $InsP_6$ and the InsP mix standard is shown in Figure 3. Only one outlier was observed beyond the limits of agreement (dashed line in red). The negative difference (between −2% and −5%) revealed a systematic bias related to the standard InsP mix for slightly underestimating the $InsP_6$ content. The absolute mean difference found was 4%. This average percentage of difference indicated an acceptable level of agreement between both standards. Therefore, the application of a correction factor to the InsP mix standard by taking into account the $InsP_6$ proportion in this standard could accurately quantify $InsP_6$ content in foods. Moreover,

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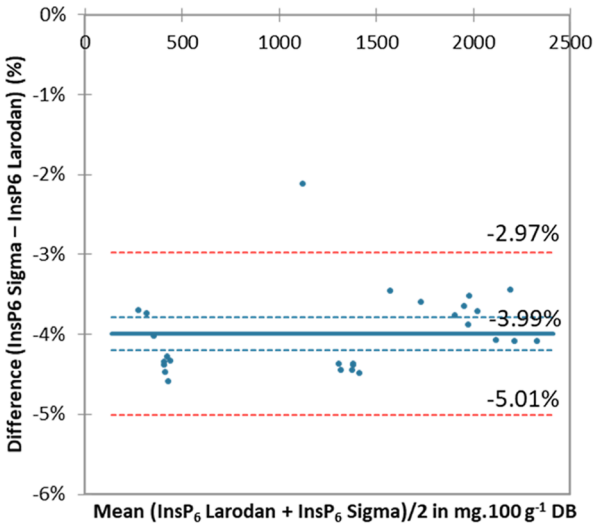

Figure 3. Bland–Altman plot between InsP6 content quantified with InsP mix standard (from Sigma) and InsP6 (from Larodan). The dots are the observations (n = 27) analyzed by IP-HPLC–FLD.

the purification step before analysis was also important because it removed the inorganic phosphate anions, which are known to strengthen the fluorescence intensity. This standard, which is less expensive than the pure standard, can thus be used for routine quantification of $InsP_6$ content.

It is important to point out that InsPs analysis by HPLC requires consideration to all steps: extraction, purification, chromatographic separation, and detection. Therefore, all analytical conditions used in our study as well as the reason for their choice are presented in Table 2.

3.5. Phytate Content in Plants Using IP-HPLC–FLD and Comparison with Existing Methods. *3.5.1. IP-HPLC–FLD Data.* Application of the IP-HPLC–FLD method for phytate quantification in several samples showed that $InsP_6$ was the main phytate in these samples followed by $InsP_5$. $InsP_6$ content in these samples ranged between 358.9 and 2175.4 mg 100 g^{-1} (Table 3). Unfortunately, the amounts of $InsP_3$ and $InsP_4$ in all the samples were under the LOD. Low levels of $InsP_3$ and $InsP_4$ in unprocessed or less processed plant products are usual. Indeed, this result was also observed by Lehrfeld (1989),[24] who found in up to 60 samples, the relative percent of 48–100%, 5–40%, and 1–12% for $InsP_6$, $InsP_5$, and $InsP_4$, respectively. In studies evaluating the degradation kinetics of $InsP_6$ into $InsP_5$ to $InsP_3$ through enzymatic treatment, for example, our method can be employed given its ability to analyze and detect the standards of these InsPs.

3.5.2. IP-HPLC–FLD vs Spectrophotometric Method: $InsP_6$ Content. Passing–Bablok comparison demonstrated significant variation (p = 0.05) for $InsP_6$ content obtained with IP-HPLC–FLD and the spectrophotometric method (Figure 4). Indeed, the spectrophotometric assay is based on multiple assumptions, the main one being that bound phosphorus is only related to $InsP_6$. Therefore, the proposed conversion factor for $InsP_6$ calculation (28.2%, proportion of phosphorus in phytic acid) might generate discrepancies when samples contain other phytate forms ($InsP_{1-5}$). By analyzing the ICMs using the spectrophotometric method, the average values obtained for phytic acid ($InsP_6$) were 3019.1 and 430.5 mg 100 g^{-1} FW for high $InsP_6$ ICM and low $InsP_6$ ICM, respectively. These values were on average 33.7 and 41.1%

Table 2. Synthesis of Experimental Conditions and Reason for Their Choice

analytical stage	innovation	parameter	condition/precision	choice explanation
sample preparation	no	extraction conditions	0.66 M 1 g in 20 mL overnight at room temperature under shaking	to extract samples using the same extractant and molarity like the conventional spectrophotometric method (reference K-PHYT)
		SPE	SAX cartridges	to prevent interferences and remove lipids from high-fat samples. These cartridges have efficient affinity with main InsPs ($InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$)
ion-pair HPLC	no	column	PRP-1, 150 × 4.1 mm, Hamilton	to perform isocratic phytate elution. This column is compatible with nonpolar compounds
		mobile phase	44% 0.035 M CH_3O_2 with 56% methanol. TBA–OH (iron-pair reagent): 1% of the mix. pH 4.3 adjusted with H_2SO_4 72%	to ensure adequate ion-pair formation and peak separation while maintaining system stability
		flow rate	0.5 mL/min	to balance efficient separation and reasonable analysis time without overloading the column
		column temperature	40 °C	to allow consistent retention times and optimal resolution under stable thermal conditions
		injection volume	20 μL	to ensure reproducibility while avoiding overloading the chromatographic column
post-column derivatization	yes	derivatization reagent concentration	0.05 mM $FeCl_3{\cdot}6H_2O$:0.3 mM 1,10-phenanthroline in 15 mM acetate buffer at pH 6	to realize post-column derivatization with suitable pressure. The use of acetate buffer helps to maintain the solution pH value throughout the experimental procedure
		derivatization reagent flow rate	0.5 mL/min	to ensure effective mixing with the eluate while maintaining proper derivatization kinetics
FLD	yes	wavelength	$\lambda_{excitation}$ 324 nm and $\lambda_{emission}$ 364 nm	to allow optimal signal detection

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Table 3. Phytate Content Determined by IP-HPLC−FLD in Some Plant-Based Samples (mg 100 g^{-1} Dry Basis)[a]

samples	$InsP_6$	$InsP_5$	$InsP_4$	$InsP_3$
mixed soy-maize 1/1 (w/w)	1362.8 ± 17.0	191.7 ± 2.9	<LOD	<LOD
mixed soy-maize 1/1 (w/w) - Extruded	1304.7 ± 35.6	256.2 ± 9.2	<LOD	<LOD
roasting peanut batch 1	366.1 ± 84.4	<LOD	<LOD	<LOD
roasting peanut batch 2	358.9 ± 51.6	<LOD	<LOD	<LOD
roasting peanut batch 3	408.3 ± 11.5	<LOD	<LOD	<LOD
defatted soy flour	2175.4 ± 104.4	379.5 ± 26.0	<LOD	<LOD
extruded soy flour batch 1	1451.4 ± 305.3	395.5 ± 42.6	<LOD	<LOD
extruded soy flour batch 2	2013.4 ± 122.3	385.9 ± 25.2	<LOD	<LOD
extruded soy flour batch 3	1925.7 ± 58.0	396.8 ± 67.8	<LOD	<LOD

[a]Values are mean ± standard deviation (assay performed in triplicate per sample). Batches 1−3 for some raw materials are related to samples from different suppliers or sample processing information (date, processing conditions).

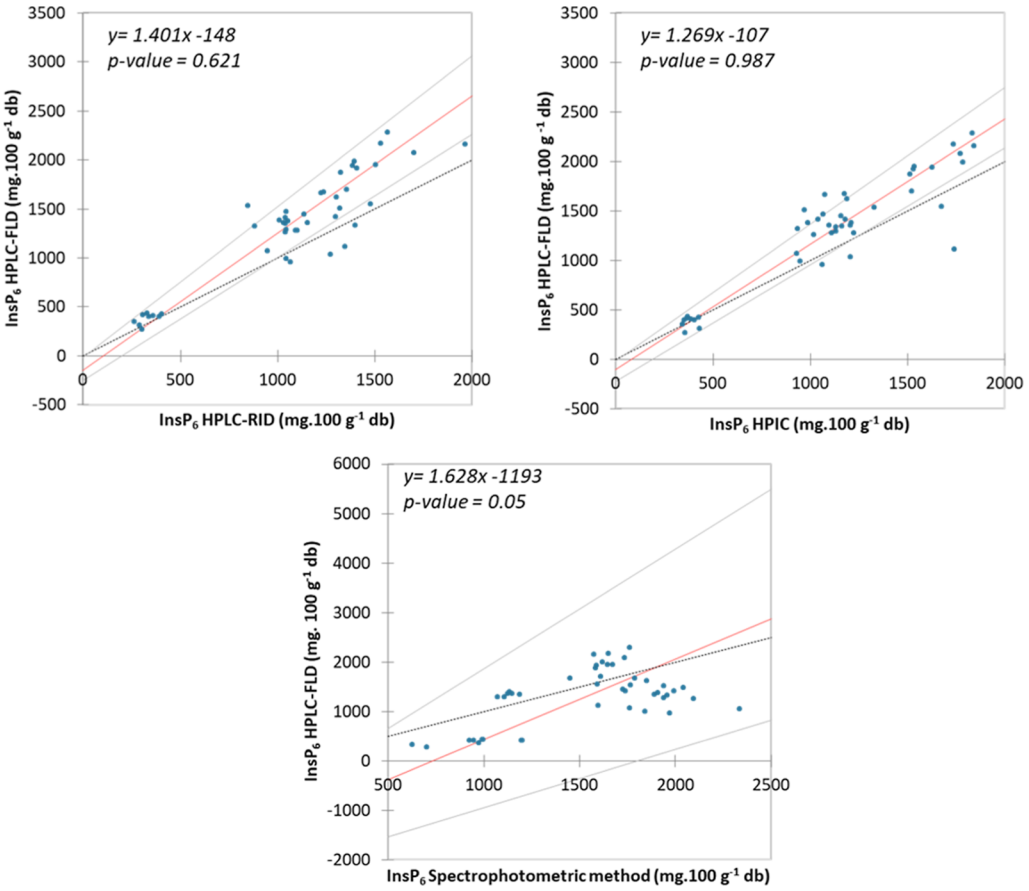

Figure 4. Passing−Bablok regression between phytic acid (InsP6) determined by IP-HPLC−FLD and other existing methods (n = 45, 15 samples analyzed in triplicate).

higher than the targeted $InsP_6$ content for the high InP_6 ICM and low InP_6 ICM, respectively.

In a study on phytate analysis in infant foods, Park et al. (2006)[41] also observed large differences between phytate results obtained with spectrophotometry (realized with Wade reagent) and two different chromatography techniques (liquid and gas chromatography). The spectrophotometry showed a higher phytic acid concentration than that of the chromatographic methods, between 33 and 58%. Talamond et al. (2000)[38] also reported similar differences by comparing anion-exchange chromatographic results to the spectrophotometric method that overestimated concentrations by 26−27%.

3.5.3. IP-HPLC−FLD vs Conventional Chromatographic Methods: $InsP_6$ Content. For the chromatographic methods, despite the deviation from the linearity for samples containing more than 500 mg 100 g^{-1} DB of $InsP_6$, there was no significant variation between data from IP-HPLC−FLD and results obtained by the conventional chromatographic methods (p-value = 0.90 for IP-HPLC−FLD vs HPIC and p-value = 0.62 IP-HPLC−FLD vs IP-HPLC−RID). Thus, the method developed in this study, utilizing ion-pair chromatography with post-column derivatization and FLD, was effective and could be used to determine accurately and reproducibly $InsP_6$ content in plant-based products. Moreover, the satisfactory selectivity of IP-HPLC−FLD for $InsP_{3-5}$ compared with HPIC and IP-HPLC−RID showed the adequacy of this method for other phytate quantification in foods.

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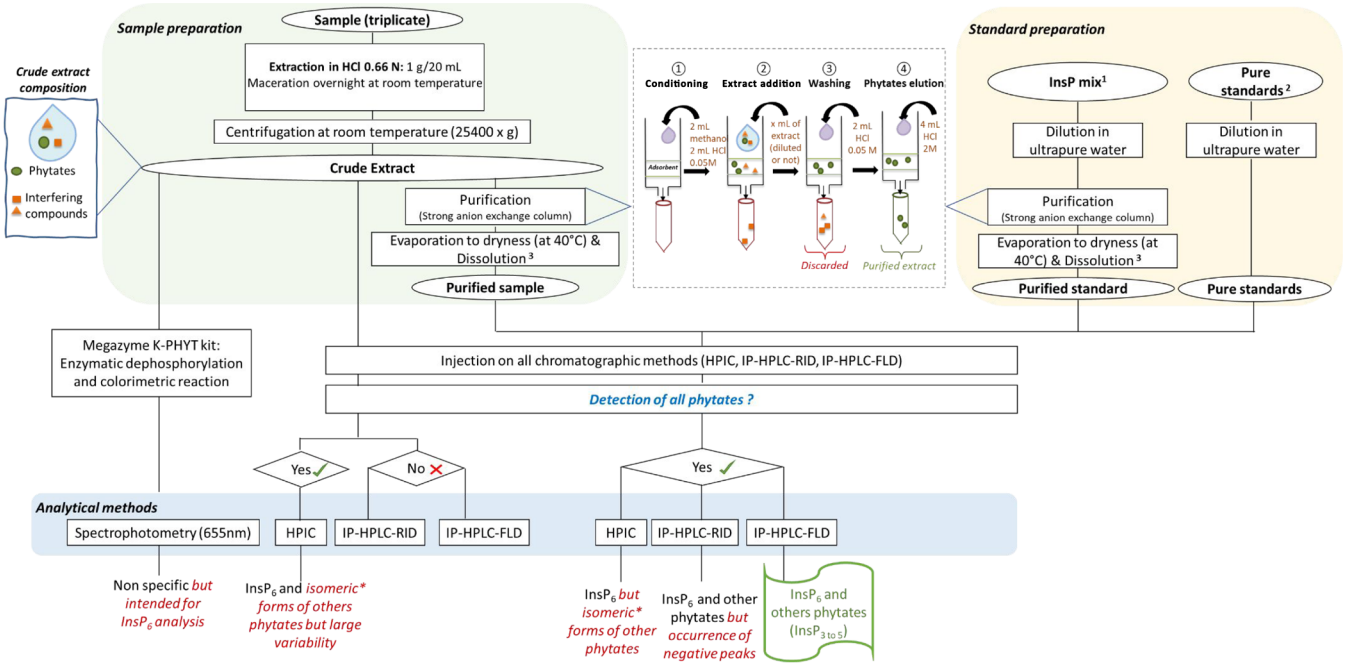

Figure 5. Summary of the research procedure and main conclusions. HPIC; IP-HPLC−RID: ion-pair HPLC method with refractive index detection; and IP-HPLC−FLD: ion-pair chromatography followed by fluorescence post-column derivatization (excitation at 324 nm, emission at 364 nm). (1): phytic acid sodium salt hydrate from rice, reference—P8810, Sigma-Aldrich, Missouri, USA. (2): D-myo-Inositol-1,2,3,4,5,6-hexaphosphate (CAY-10008415−1), D-myo-Inositol-1,3,4,5,6-pentaphosphate (CAY-10007784−1), D-myo-Inositol-1,3,4,5-tetraphosphate (CAY-60980−1), and D-myo-Inositol-1,4,5-triphosphate (CAY-10008205−1). (3): ion-pair HPLC mobile phase: 44% 0.035 M CH_2O_2 with 56% methanol. TBA−OH (iron-pair reagent): 1% of the mix. pH 4.3 adjusted with H_2SO_4 72%. *: difficulty to have all the available isomeric commercial phytate standards.

Figure 5 presents the whole analytical procedure used in this study and the main conclusions observed for the conventional methods and the new method. For the spectrophotometric method, the crude extracts were used directly after extraction and followed the enzymatic and colorimetric reaction proposed in the Megazyme K-PHYT kit. This method is intended for $InsP_6$ quantification. However, our results confirmed its nonspecificity to accurately quantify phytic acid. In general, all of the chromatographic methods used in this study required sample purification to obtain an extract free of interference compounds before injections. For HPIC, purification avoided having high variabilities in the data, and for IP-HPLC, with RID as well as IP-HPLC−FLD, it leads to phytate peak elution and detection.

4. CONCLUSIONS, RECOMMENDATIONS, AND PERSPECTIVES

This study demonstrated the potential of IP-HPLC coupled with post-column derivatization for fluorescence detection (FLD) to selectively and reproducibly analyze InsPs, especially $InsP_3$ to $InsP_6$. Standards of $InsP_3$ to $InsP_6$ were easily analyzed and allowed the method (named IP-HPLC-FLD) performance to be determined (LOD, LOQ, and RSD). The main input of this study was the dynamic synchronous fluorescence post-column derivatization proposed for phytate detection. Samples analyzed in our study using the proposed method showed the predominance of phytic acid ($InsP_6$) compared to the other forms, i.e., $InsP_{1-5}$. Future investigations need to be carried out by analyzing a range of samples (cereals, legumes, roots, and tubers) which might contain different forms of InsPs.

Concerning the analytical conditions, the proportion of methanol in the mobile phase might be adjusted to improve the resolution of the peaks, especially $InsP_6$ resolution.

Since InsPs are known to have affinity with minerals, further studies are needed to ensure the aptitude of the IP-HPLC−FLD method for phytate quantification in complex food matrixes such as the Ready-to-Use Therapeutic Foods which are enriched in minerals.

This method can also be adapted for routine use in commercial laboratories, thanks to its easy implementation, efficiency, and reproducibility. A shorter column should be prioritized to reduce the runtime. Routine evaluation of the system efficiency (column, post-column derivatization, and detector) should be performed using standards. Cleaning the SPE cartridges could also reduce analytical costs for sample preparation while providing a reliable method suited to high-volume commercial assays. The cleaning can be achieved by rinsing the sample with ultrapure water (approximately 20 mL) until a neutral pH is reached, then conditioning with methanol (approximately 4 mL), and drying at room temperature.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c09065.

Figure S1: Chromatogram of the InsP mix standard on IP-HPLC−RID; Figure S2: chromatogram of mix InsP standard as well as pure $InsP_3$, $InsP_4$, and $InsP_5$ on HPIC; and Figure S3: experimental details on the determination of the derivatization reagent molar ratio (PDF)

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Notes

The authors declare no competing financial interest.

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