Scale-up potential of ion-pair high-performance liquid chromatography method to produce biologically active inositol phosphates

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Abstract

This study was undertaken to evaluate the possibility that an analytical ion-pair HPLC procedure used to determine phytic acid (IP6) and its degradation products (IP3–IP5) can be transformed to a preparative purification method. A commercial phytic acid (CPA) preparation was separated into its component fractions of IP3, IP4, IP5, and IP6 on two C_{18} columns (1.8 and 4.2 ml) using 51% methanol containing 0.6–1% tetrabutylammonium hydroxide as ion-pair reagent and 0–0.025 M formic acid (pH 4.3) as eluent at 1.7 and 3.0 cm/min linear velocity, respectively, and 40°C. Elution was monitored at 40°C by a refractive index detector. Reproducible separation of CPA into four well-resolved peaks on these columns was possible after optimizing method variables, particularly the concentration of ion-pair reagent in the injected sample (>1.5%). The same separations were obtained after CPA loads were scaled up 25 times on a steel column (15 cm×19 mm I.D.), packed with Ethyl C_2 sorbent (10 µm) and on a 25 cm×21.2 mm I.D. C_{18} column, but at a reduced linear velocity to increase the resolution. Therefore, this optimization of separation not only is useful for analysis of phytic acid and its degradation products but also it provides key parameters for scale up for further fractionation and characterization. Published by Elsevier Science B.V.

Keywords: Preparative chromatography; Inositol phosphates; Phosphates; Phytic acid

1. Introduction

Phytic acid, inositol hexaphosphate, and some of its degradation products with 3–5 phosphate groups (tri-, tetra-, and pentaphosphates) are functional components with health-promoting characteristics [1]. Some inositol phosphate isomers have pharmacological significance and are used as the active ingredients in drugs for the prevention and treatment of many ailments [2]. The aim of this research is to develop methods to prepare sizable quantities of these potentially highly valuable products by specific enzymatic conversion of phytic acid to inositol phosphates followed by large-scale separation of optically pure functional isomers.

Xu et al. [3] recently reviewed the methods used for the analysis of inositol phosphates. Anion-exchange and reversed-phase chromatography are the main methods used for the analysis of inositol phosphates in foods. Reversed-phase chromatography separates components on the basis of hydrophobicity and because of its resolution, it is an excellent and widely used technique for the analysis

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and laboratory purification of biomolecules. Although ion-exchange chromatography is powerful and can be readily used to separate individual isomers of inositol phosphates [4], ion-pair methods are simpler and easier to perform. Lehrfeld [5] conducted extensive studies on the ion-pair reversed-phase high-performance liquid chromatography (HPLC) method originally developed for estimation of phytic acid and inositol phosphates in foods by Sandberg and Ahderinne [6]. Also, Matthaus et al. [7] made some modifications to the method of Sandberg and Ahderinne [6] including omitting formic acid from the mobile phase.

A large-scale HPLC method for separation of inositol tri-, tetra-, penta- and hexaphosphates (IP3, IP4, IP5 and IP6, respectively) may be achieved through optimizing key parameters in the analytical separation methods that already exist. Therefore, the objective of this study was to evaluate the possibility that an analytical ion-pair HPLC procedure for phytic acid and its degradation products could be developed into a useful preparative tool for large-scale purification of inositol phosphates by optimizing separation variables.

2. Experimental

2.1. Materials

Sodium phytate, dodecasodium salt hydrate, phytic acid (40%, w/w, solution in water) and tetrabutylammonium hydroxide (40% solution in water) were purchased from Aldrich (Milwaukee, WI, USA). HPLC-grade reagents and solvents were used throughout this investigation. Unless noted otherwise, chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. The HPLC system

The "Alliance" chromatography system from Waters Associates (Milford, MA, USA) was used for analytical and semi-preparative HPLC separations. Elution was monitored at 40°C with a Model 2410 differential refractometer connected to a Waters Millennium 2010 Workstation (v. 1.05) for the integration of the proportions of various eluted peaks

(Waters). The refractometer can measure extremely small changes in refractive index (RI) to detect the presence of sample. The difference in RI between the mobile phase solution and the injected sample solution is expressed in refractive index units (RIU).

2.3. Analytical reversed-phase separation method

The analytical reversed-phase (RP) HPLC separation method of the commercial 40% phytic acid, which contains phytic acid and substantial amounts of its degradation products (IP3, IP4, IP5), and sodium phytate standard was based on the ion-pair HPLC of Sandberg and Ahderinne [6] as modified by Lehrfeld [5] and Matthaus et al. [7]. The reversed-phase analytical columns used were a Nova-Pak 5 μm C_{18} 60 Å, 15 cm$\times$3.9 mm I.D. (Waters) and an Aqua 5 μm C_{18} 125 Å, 25 cm$\times$4.6 mm I.D. (Phenomenex, Torrance, CA, USA). The commercial phytic acid solution (CPA) or phytate standard was eluted from the columns using a mobile phase containing 51% methanol in deionized water, 0.6 or 1% tetrabutylammonium hydroxide (TBAOH) and 0, 0.025 or 0.05 M formic acid at a flow-rate of 0.2–1.0 ml/min. The pH of the mobile phase was adjusted to 4.3 using 5 M sulfuric acid. The mobile phase was heated to 42°C before pumping into the column and elution was monitored at 40°C. CPA samples were diluted 1:50 to 1:200 in the same mobile phase of the HPLC run then filtered through a 0.22-μm Millex-HV Filter (Millipore, Bedford, MA, USA) before their injection.

2.4. Standard curve for phytate

Sodium phytate was used for the calibration of the HPLC system (Aqua column). The mobile phase of 51% methanol contained 1% TBAOH and 0.025 M formic acid (pH 4.3). The calibration was carried out by plotting different RI-detector responses after injections of 20 μl of 2.5, 3.6, 7.2, 10.0, 15.0 and 20.0 μmol/ml sodium phytate solution.

2.5. Semi-preparative ion-pair RP-HPLC separation

RP separation of CPA was performed on a steel column (15 cm$\times$19 mm I.D.) packed with Ethyl C_2

sorbent (10 μm) and on a 25 cm×21.2 mm I.D Aqua C_{18} column (Phenomenex) at a flow-rate of 1.0–4.0 ml/min. The mobile phase in reservoirs was maintained at 40°C then heated to 43°C prior to its pumping into the column. CPA loads were scaled up 25 times from 0.05 to 1.28 mg. The separation eluent was 51% methanol containing 0.6% TBAOH (pH 4.3). TBAOH concentration in the filtered, injected samples (25–100 μl) was 3–5%.

2.6. Statistical analysis

Regression analysis of the variables, determined in duplicates, was performed using the software package of Statgraphics (Rockville, MD, USA).

3. Results and discussion

3.1. Effect of flow-rate, load and injection volume on separation

The methods of Sandberg and Ahderinne [6] as modified by Lehrfeld [5] and Matthaus et al. [7] were used to separate the 40% CPA into four peaks as shown in Fig. 1. The mobile phase used in these preliminary separations was 51% methanol containing 0.025 M formic acid and 0.6% TBAOH (pH 4.3) at a flow-rate of 0.2 and 0.5 ml/min for the Nova-Pak and the Aqua C_{18} columns, respectively. These flow-rates are equivalent to velocities of 1.7 and 3.0 cm/min for the columns, respectively. Fig. 1A shows the effect of volume of injected sample on the separation of CPA using the Nova-Pak C_{18} column. In these chromatograms, CPA preparation was separated into IP3, IP4, IP5, and IP6 with tentative retention times of 5.2, 6.0, 7.4 and 10.3 min, respectively. Negative peaks with retention times of 4.2, 5.7, 6.7 and 9.1 min preceded these peaks of inositol phosphates, respectively. CPA was not well separated into its four component fractions at increased injection volumes of the samples, particularly at more than 20 μl injections (at 1:100 and 1:200 dilutions). Also, increasing injection volume, which increases CPA load, increased the height of negative peaks and led to the appearance of hollow peaks particularly with the 20 μl injections or higher. Hollow peaks have no inositol phosphates but
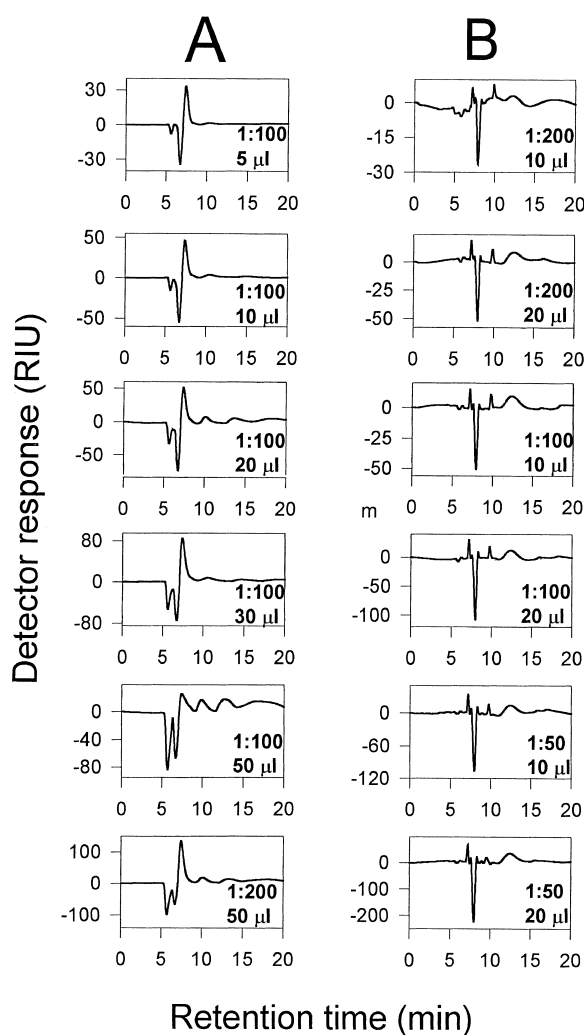

Fig. 1. Separation of a commercial phytic acid preparation to its component fractions of IP3, IP4, IP5 and IP6 on (A) Nova-Pak and (B) Aqua C_{18} columns at various sample concentrations (dilution 1:50 to 1:200) and injection volumes (5–50 μl). Mobile phase: 51% methanol containing 0.025 M formic acid and 0.6% TBAOH (pH 4.3). The flow-rates of the separations were 0.2 and 0.5 ml/min for these columns, respectively. Retention times of IP3, IP4, IP5, and IP6 components were tentatively 5.2, 6.0, 7.4 and 10.3 min, respectively, for A and 5.8, 6.5, 7.3 and 10.1 min for B, respectively.

produce major RI response. Comparing the chromatograms of the 50 μl injections that were diluted 1:100 and 1:200, it appears that decreasing sample size reduces the intensity of hollow peaks.

Fig. 1B gives the separation of the CPA using the

Aqua C_{18} column at two levels of injection volumes and CPA concentrations in the injected samples. Retention times of IP3, IP4, IP5, and IP6 components were conceived to be 5.8, 6.5, 7.3 and 10.1 min, respectively. In all runs, CPA preparation was well separated into its four component but a few negative peaks occurred either before or after the peaks of inositol phosphates, especially the sharp one that followed the appearance of the IP5 peak. The higher the concentration of the sample the more negative the detector response was that followed the elution of this IP5 peak (Fig. 1B). The separation of CPA on the Aqua column was also performed at 1.0 ml/min and gave satisfactory resolution (see Fig. 3). However, separation was not as superior as that obtained at 0.5 ml/min for the Aqua column indicated in Fig. 1B. Therefore, a satisfactory separation can be achieved for the Aqua column at 0.5–1.0 ml/min when the injection volume is at 20–30 µl and the CPA concentration is increased to the level that improves the resolution of CPA peaks without producing too much of a negative response before or after elution of these components (e.g., 1:200).

Occurrence of negative peaks in these types of chromatograms is not desirable because they adversely affect the separation and quantitation of the inositol phosphates. Lehrfeld [5] suggested that negative and hollow peaks can occur in the chromatograms and the negative peak preceding the appearance of the IP3 is caused by contaminants and the depletion of TBAOH in the solvent plug proceeding the sample. Lehrfeld [5] also suggested that another negative peak caused by air can concur with elution of the IP4 peak, which can lead to errors in peak integration. Lehrfeld [5] stated that concentrating the CPA sample and reducing injection volume can minimize this problem. However, the data in Fig. 1 clearly demonstrate that the negative response is proportional to sample load. Furthermore, reducing injection loads generally improve resolution. Sample load is a key parameter influencing throughput since it has a dramatic effect on separation efficiency (α), column efficiency, retention time and even peak shape [8]. Sample load is directly proportional to α and hence the easiness or difficulty of separation. Also, as sample load is increased, the effective plate count decreases. Flow rate is the operational variable which most influences retention time. High flow-rates are possible with separation efficiencies, α, of more than 2.0, while working within the constraints of acceptable back pressure and practical solvent handling capabilities. As α becomes smaller, the flow-rates may have to be decreased to maintain the desired degree of resolution and solute recovery [8].

3.2. Effect of mobile phase and concentration of ion-pair reagent in injected sample on separation

Chromatograms generated using different mobile phases at different concentrations of the ion-pair reagent in the injected samples are presented in Figs. 2–4. Except the chromatograms of the runs using a mobile phase that contained 0.05 M formic acid with 0.6% or 1.0 TBAOH, the chromatograms in these figures clearly illustrate that the CPA sample can be cleanly separated into the four major inositol phosphate peaks. In the ion-pair chromatography of CPA using a mobile phase that was composed of 51% methanol and 0.6–1.0% TBAOH (pH 4.3), the retention times for IP3–IP6 were 2.8, 3.4, 3.6 and 4.0 min when using the mobile phase containing 0.6% TBAOH and 2.8, 3.3, 3.5 and 4.2 min when the mobile phase contained 1.0% TBAOH (Fig. 2). Also, the chromatograms of separations using a mobile phase containing 51% methanol and 0.025 M formic acid, along with either 0.6% or 1.0% TBAOH were identical (Fig. 3). Although the separation of CPA preparation when 0.05 M formic acid and 0.6% or 1.0% TBAOH were included in the mobile phase solution followed the same pattern of separation when the mobile phase contained 0.025 M formic acid and 0.6 or 1.0% TBAOH, the chromatograms were not sufficient. The resolution in these chromatograms was poor since it lacked the clean separation between the inositol phosphates, particularly between the IP5 and IP6 peaks that was observed for the other mobile phases.

Concentrations of TBAOH in the injected samples were varied (0.6–3.0%) to study their effects on the reversed-phase ion-pair HPLC separation. When samples were diluted before injection using the mobile phase solution (i.e., no increase in TBAOH), a few negative peaks were eluted before or after the inositol phosphate peaks (the top chromatograms in Figs. 2–4). These negative peaks evidently led to the
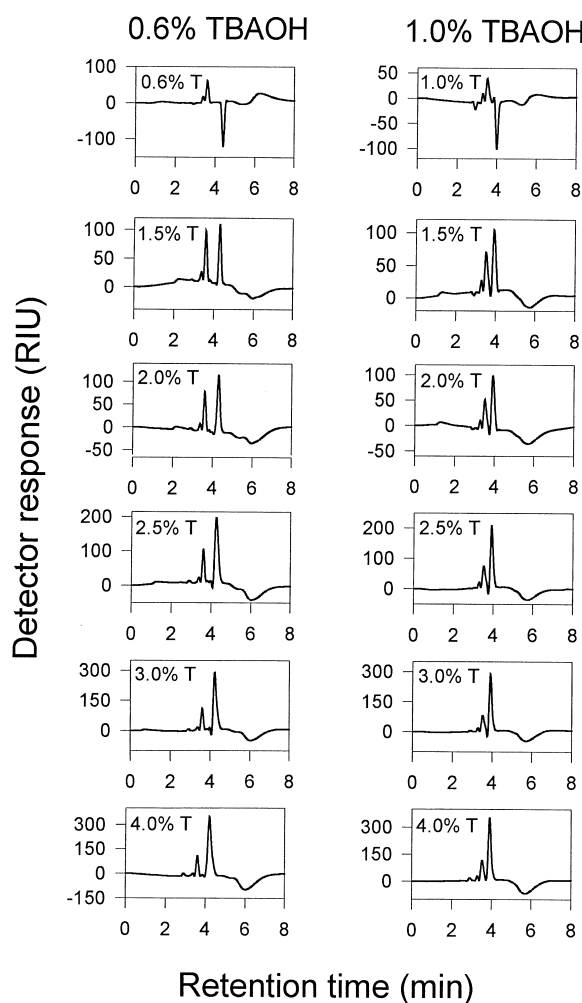
Fig. 2. Reversed-phase separations of the commercial phytic acid preparation on the analytical Aqua column at 1.0 ml/min using various concentrations of ion-pair reagent in samples (% T=% TBAOH). Mobile phase: 51% methanol containing 0.6 or 1.0% TBAOH (pH 4.3). Sample dilution was 1:200 and injection volume was 20 μl. The retention times for IP3–IP6 were conceived to be 2.8, 3.4, 3.6 and 4.0 min and 2.8, 3.3, 3.5 and 4.2 min for the two mobile phases, respectively.
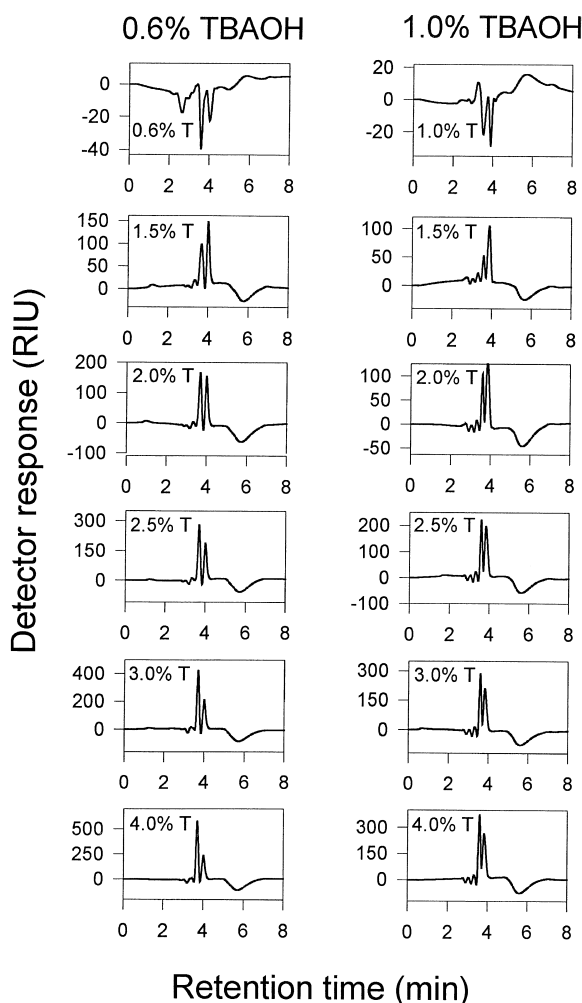
Fig. 3. Reversed-phase separations of the commercial phytic acid preparation on the analytical Aqua column at a flow-rate of 1.0 ml/min using various concentrations of ion-pair reagent in samples (% T=% TBAOH). Mobile phase: 51% methanol containing 0.025 *M* formic acid, 0.6 or 1.0% TBAOH (pH 4.3). Sample dilution was 1:200 and injection volume was 20 μl.

development of hollow peaks that have no inositol phosphates in them but they produce a substantial RI response. Increasing the percent of TBAOH in injected samples 2–5-fold reduced the negative response immensely and improved peak resolution of the CPA components, particularly for IP5 and IP6 peaks (Figs. 2 and 3).

In the ion-pair chromatography with a RP-8 phase,

Matthaus et al. [7] used a mobile phase that was composed of 50% methanol and 0.6% TBAOH (pH 4.3) but did not use formic acid in the mobile phase. In our separations using similar conditions (Fig. 2), resolutions of inositol phosphates were satisfactory but the chromatograms contained a few hollow peaks. Lehrfeld [5] described remedies to minimize the occurrence of hollow peaks. Changing methanol concentration from 51% to 50% and longer running
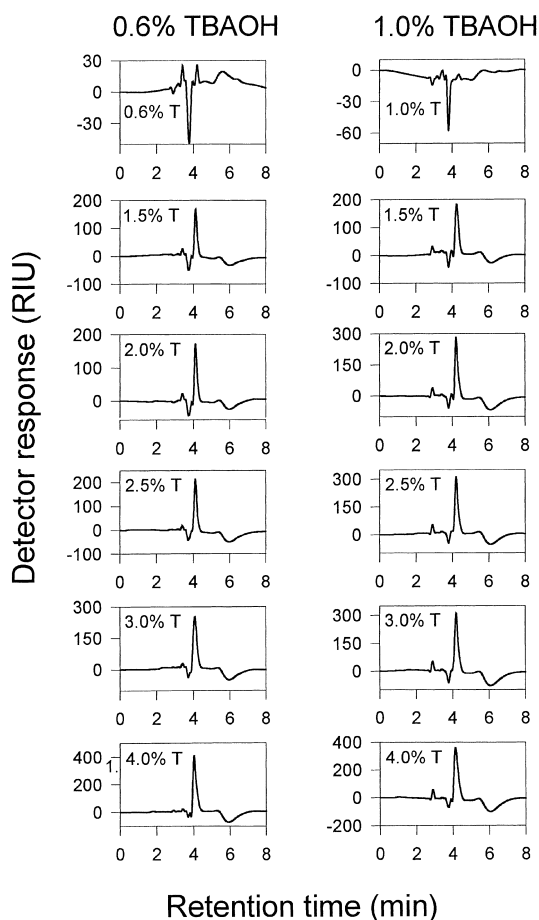
Fig. 4. Reversed-phase separations of the commercial phytic acid preparation on the analytical Aqua column at 1.0 ml/min using various concentrations of ion-pair reagent in samples (% T=% TBAOH). Mobile phase: 51% methanol containing 0.05 *M* formic acid, 0.6 or 1.0% (pH 4.3). Sample dilution was 1:200 and injection volume was 20 µl.

time can shift the position of IP4 by forcing the negative peaks to occur between IP3 and IP4 peaks. However, improvement in the analytical method could be achieved by simpler means such as increasing the concentration of the ion-pair reagent in the injected sample, which eliminated negative peaks and allowed the clean separation of CPA preparation into its component fractions, as illustrated above.

3.3. Reproducibility of chromatograms

Increasing the concentration of the ion-pair reagent in the sample to improve separation efficiency

of components did not change the reproducibility of the runs. Regardless of the amount of TBAOH in the injected sample or in the mobile phases, the replicates of % area integrated from RI detector response for each peak are almost identical. This reproducibility is also supported by the results of regression analysis of the standard curve for the phytate using the analytical Aqua column, which compared well with those obtained by previously reported methods [6]. Plotting the concentration of phytic acid (IP6) with the RI area response obtained from the IP6 peak is another way to confirm that the separation is quite reproducible. A straight line was obtained at a wide range of phytate concentrations. RUI increased linearly with increasing concentration of the standard solutions. The equation of the fitted model is as follows:

$$\text{Area (RIU)} = -725\,935 + 913\,475 \cdot [\text{phytate}]$$

with standard deviation of $2.12 \cdot 10^6$ ($P<0.01$). The correlation coefficient was 0.9563, which indicates a strong relationship between the variables.

Furthermore, calculating the % area response of the RI detector of the four peaks, it was found that this CPA solution contained 4.7, 15.1, 40.3 and 42.7 % of IP3, IP4, IP5 and IP6, respectively. To compare these values with other published data, we used the factors they established to convert these RI area values to molar concentrations of the inositol phosphates.

Using the correction factors of Sandberg and Ahderinne [6], these values above were found to be 4.1, 19.8, 38.8 and 37.4 % for proportions above, after multiplying by 2.4, 1.5 and 1.1, respectively. This is in agreement with the results of Lehrfeld [5] who used the protocol of Sandberg and Ahderinne [6] to obtain 4.6, 20.5, 38.2, and 36.7%, of IP3, IP4, IP5 and IP6, respectively, for this CPA solution used in this study. The data are different from the work of Matthaus et al. [7], in which they reported factors of 1.66, 1.28, and 1.1 for IP3–IP5, respectively. Therefore, the optimized method not only can be used for separating and accurately identifying a mixture of IP3–IP5, it has also a great potential for a successful scale up.

3.4. Scale-up of analytical reversed-phase chromatography

The separation of inositol phosphates on the Nova-

Pak column was scaled-up to a semi-preparative scale by using a 15 cm×19 mm I.D. column, packed with Ethyl C_2 sorbent (10 μm). The sample load was increased by increasing CPA concentration five times from 0.5 to 2.5 μmol/ml with five times the injection volume (to 100 μl), to give a total scale-up factor of 25 based on the volumes of the analytical and preparative columns, which are 1.8 and 42.6 ml, respectively. To maintain the linear velocity of the analytical column (1.7 cm/min) constant, flow-rates have to be 4.8 ml/min for the semi-preparative column. However, the initial separations using higher flow-rates of more than 2 ml/min were not satisfactory. In subsequent runs, the flow-rate was reduced from 2.0 ml/min (Fig. 5A) to 1.0 ml/min (Fig. 5B) to improve the resolution of IP3–IP6. Total time involved in these particular separations was approximately 30 and 60 min, respectively. The semi-preparative separation chromatograms were similar to that of the Nova-Pak C_{18} column, where IP3–IP6 were eluted after 46, 57, 73 and 92% of the total separation time, respectively. However, long-term noise cycling of the baseline interfered with peak detection. Cycling occurs when there is a fluctuation in temperature in the eluent entering into the detecting refractometer. Reducing the flow-rate to 1.0 ml/min aggravated this problem since the large size of the column, which resulted in long chromatographic time, made it difficult to maintain the temperature of the mobile phase at 40°C.

Also, the separation of CPA into its four component fractions on the analytical Aqua column (25 cm×4.6 mm I.D.) was scaled-up 25 times to a semi-preparative scale by using a semi-preparative Aqua C_{18} column with larger diameter (21.2 mm) but the same bed length. A velocity of 3.0 cm/min is equivalent to a flow-rate of 10.6 ml/min for this column. However, the flow-rate of the semi-preparative separation was kept at 4.0 ml/min to preserve the life time of the column (back pressure was 1810 p.s.i.; 1 p.s.i.=6894.76 Pa) as well as the detector, until a flow splitter is installed into the column. Fig. 5C–E shows the retention of the inositol phosphates on the preparative Aqua C_{18} column, which was similar to that of the corresponding analytical column as they were eluted after 57, 65, 78 and 96% of the total separation time. This separation with the Aqua C_{18} semi-preparative column is better than the separation with the other C_2 semi-preparative col-
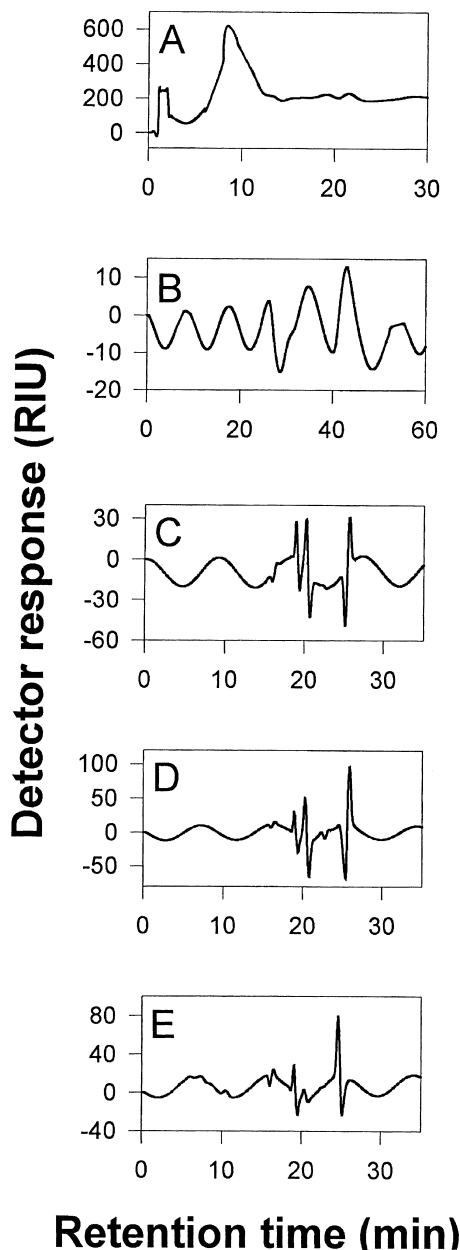

Fig. 5. Semi-preparative-scale separations of the commercial phytic acid preparation on a 15 cm×19 mm I.D. column packed with 10 μm Ethyl C_2 sorbent at a flow-rate of 2 ml/min (A) and 1 ml/min (B) and on a 25 cm×21.2 mm I.D. Aqua C_{18} column at a flow-rate of 4.0 ml/min. Mobile phase was 51% methanol containing 1.0% TBAOH (pH 4.3). % TBAOH in injected samples was 3% for chromatograms A–D and 5% for chromatogram E. Injection volumes were 100 μl for chromatograms A and B; 50 μl for chromatograms C and E; 25 μl for chromatogram D.

umn presented in Fig. 5A and B. This is not surprising considering the column packing materials and that the volume of the former column (88.3 ml) was more than double the volume of the latter column (42.6 ml). The chromatogram presented in Fig. 5C for the separation of a CPA sample containing 3% of the ion-pair reagent shows that the separation is superior in general but the baseline in the chromatogram still has a minor cycling problem plus the IP3 ($t_R = 16.5$ min) was not well resolved. Two measures were taken to improve this separation: The injection volume was reduced into half while maintaining the sample load at the same level (i.e., diluting CPA to 1:10 instead of 1:20). The retention time of the IP3 peak was unchanged but its resolution was improved (Fig. 5D). Also, increasing the concentration of the ion-pair reagent in the 50 μl injections from 3 to 5% improved the resolution of IP3 ($t_R = 16.5$ min) (Fig. 5E) even better than that of the 25 μl injections. As stated above cycling is hard to avoid considering the size of the column but a higher flow-rate (4 ml/min) did help in minimizing temperature fluctuation.

Scale-up of analytical methods is achieved by increasing the column diameter while maintaining the column bed length and linear flow-rate constant [9]. Loads of chromatographic separations are usually scaled up a number of times equal to the ratio of their cross sectional areas. Ideally, runs are carried out at the same linear velocity (flow-rate in cm^3 divided by the cross section area of the column) to maintain an equivalent time frame for the small and large scale separations [10]. However, many chromatographic separations are scaled up at much lower flow-rates than those based on maintaining linear velocity to increase resolution [8].

4. Conclusions

Using a mobile phase of 51% methanol containing 0–0.025 *M* formic acid and 0.6–1.0% of the ion-pair reagent (pH 4.3) with 1.5% or more of the ion-pair reagent in injected samples gave the desired separation of phytic acid breakdown products into inositol tri-, tetra-, penta- and hexaphosphates. Increased concentrations of the ion-pair reagent eliminated most negative peaks effecting better integration and resolution of inositol phosphates peaks. Optimizing the analytical separation method provided useful parameters for successful scale-up on some semi-preparative columns.

5. Disclaimer

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