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Assessment of a Procedure for Fractionating Organic Phosphates in Soil and Organic Materials using Gel Filtration and H.p.l.c.

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A method for extracting and fractionating organic phosphates is described. In the fractions, inositol hexaphosphate (IHP) was determined with a carbon detector after high pressure liquid chromatography (h.p.l.c.). Sample preparation consisted of a single mild extraction with 0.1M EDTA in 0.3M-NaOH and a preseparation by gel filtration. Adenosine-5-triphosphate (ATP) could also be separated and determined in this way. With a number of samples, clean-up by gel filtration could not prevent deterioration of the anion exchange column, used in the h.p.l.c. determination. IHP was then determined as total P after isolation by two consecutive gel filtrations. Limits of detection were 5–50 μg P/g when determining IHP by h.p.l.c. and 0.05–0.5 μg P/g when determining IHP as total P after isolation by gel filtration. Limits of detection when determining ATP by h.p.l.c. were 5–50 ng P/g. Results of applying the method to soil and various organic materials are given and discussed.

1. Introduction

Methods of analysis of inositol phosphates[1–6] usually involve an acid and/or alkaline extraction followed by precipitation of the inositol phosphates as barium or iron salts. After filtering and redissolving, the inositol phosphates are chromatographed on an anion exchange column. Detection is 'off-line' as total P after destruction. In the course of the analysis, some decomposition and loss of IHP can occur.[2] Also, degradation to penta and lower esters is possible.[3,7] Inositol phosphates can also be present in high molecular weight complexes with soil organic matter[5,8–10] and plant proteins[11,12] These complexes can be quite stable and resistant to extraction and/or precipitation. Total IHP present may thus be higher than that found with standard methods. An alternative to existing methods was sought in a single complete and mild alkaline extraction of IHP with the aid of a strongly chelating agent, followed directly by rapid chromatographic analysis, with 'on-line' detection.

2. Materials and methods

For gel permeation a column of 40 cm length and 2 cm^2 cross-sectional area was used (Pharmacia, type K16/40). A high pressure liquid chromatograph was constructed from a membrane pump (Orlita DMP 1515), a sample injection valve (Valco C20), a steel column (Handy Harman LiChroma tubing 0.25 × 0.083 in) of 30 cm length. Swagelock low-volume connections were used. The column effluent was monitored with a moving-wire detector (Pye-Unicam LCM-2) or a spectrophotometric flow-through detector (Cecil CE-212). The moving-wire liquid chromatographic detector, which in essence is a carbon detector, has been described in detail.[13] The steel column was fitted with a water jacket which could be set and controlled at a temperature between 10 and 80°C. Pump pulsations were attenuated with a Bourdon tube and capillary restrictor in series.[14]

The extraction and separation procedure used for the organic P compounds from various materials is shown schematically in Figure 1. The choice of the type of gel (Sephadex G-25 and G-100)

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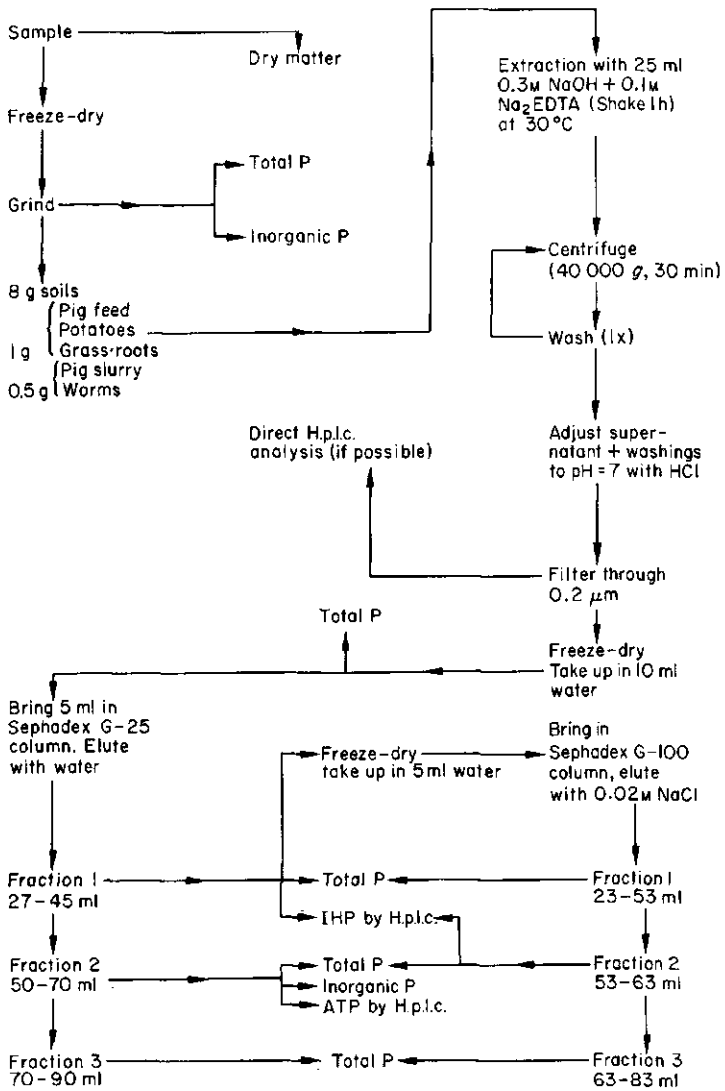

Figure 1. Extraction and fractionation procedure for the determination of IHP. H.p.l.c. = high pressure anion exchange chromatography.

used was based on data on ionic exclusion of various organic P compounds from gel columns.[8] An anion exchange resin (Aminex A-28, BioRad) was used for the high pressure liquid chromatographic separation of IHP. IHP was eluted isocratically at a column temperature of 50°C with a solution containing 0.43M-NaCl, 0.001M-$Na_2B_4O_7$ and 1% ethanol. The injection volume of the samples was 1 ml.

Total organic P was determined from the difference in total P and inorganic P. Total P was determined after destruction with Fleischman acid (HNO_3/H_2SO_4, 1:1 by volume) as reduced phosphomolybdate.[15] Inorganic P was selectively extracted with a solution of HF (40%), HCl (36%) and $TiCl_4$ (10:200:9 by volume) and determined in the extract.[16,17] It was found that the time necessary to obtain a constant extinction of the blue phosphomolybdate complex increased when the Ti concentration in the final solution was more than 0.01M. At 0.05M it took 4 h to obtain a constant extinction reading. Accuracy was not affected, however, as long as sufficient time was allowed to

obtain a constant extinction reading on the spectrophotometer (Pye-Unicam SP 6). Organic P was also determined after extraction with a solution of 0.1M-Na_2EDTA/0.3M-NaOH. A sample quantity of 0.1–10 g was shaken for 1 h at 30°C with 25 ml of the extractant. Organic P was found from the difference between total and inorganic P in the extract. Compared with the results of the HF/HCl/Ti method, between 90 and 100% of total organic P was extracted in this way from the samples.

The IHP used as the reference compound was obtained from BDH Chemicals Ltd. [$C_6H_6(OPO_3Na_2).xH_2O$]. Analysis by ^{31}P-n.m.r. showed it to be almost pure myoinositol hexaphosphate.[4] ATP (adenosine-5-triphosphate), AMP (adenosine-5-monophosphate) and other chemicals mentioned were obtained from Merck.

Sample materials were chosen for comparison with literature values of IHP content (e.g. soils and animal wastes) and their possible contribution to soil phytate (roots, worms, animal wastes).

3. Results and discussion

Results of h.p.l.c. analysis of test solutions of IHP, ATP and AMP using an anion exchange resin are given in Figure 2. As can be seen, the resolution of IHP and ATP is concentration-dependent. This will be a problem in the direct chromatographic determination of IHP whenever the ATP concentration is high. ATP, however, can be analysed without interference from IHP by monitoring the column effluent at 260 nm with a spectrophotometric detector. Other inositol phosphates can also be determined by h.p.l.c., as is shown in Figure 3 for phosphates obtained by hydrolysis of IHP. The affinity of the anion exchanger used (Aminex A-28) for IHP increases strongly at ionic strengths lower than that of a 0.43M-NaCl solution. This allows the injection of large volumes of samples of low ionic strength without appreciably affecting resolution. Theoretically, trace enrichment could be obtained in this way for very large volumes of injection. It can be derived[14,18] that

$$C_{max} = Q . C_0 . V_0 \quad (1)$$

where, C_{max} = the maximum concentration of IHP in the elutant at the end of the column; Q = a constant if the contribution of the injection zone to total dispersion in the column can be neglected and if the distribution isotherm of IHP is linear; V_0 = volume of injection, and C_0 = concentration of IHP in injected sample.

In Figure 4 a plot is given of the volume of injection of a sample containing 10^{-4}M IHP and the corresponding peak height as registered after elution through the anion exchange column. As can be seen, complete linear behaviour as predicted by equation 1 is not observed. This is probably due to the fact that the distribution isotherm of IHP becomes concave above a concentration of 10^{-5}M. A volume of injection of 1 ml was found satisfactory, though perhaps not optimal, in this work.

IHP was extracted from soils and organic materials with a solution of 0.1M-Na_2EDTA in 0.3M-NaOH at 30°C. Recovery (measured as increased total P) of pure IHP added as neutral Na, Fe, Al, Ca or Mg salt and ATP added as neutral Na salt was almost 100%. Also, more than 90% of total organic P in the samples was extracted in this way (see Table 1 under the heading 'EDTA extract'). Solutions of IHP and ATP in EDTA/NaOH at a pH > 5 are very stable even at elevated temperatures. Trace metals which catalyse the chemical and enzymatic hydrolysis of IHP and ATP are effectively masked by EDTA. Pure solutions of ATP are much less stable, while pure solutions of IHP are reasonably stable, especially under strongly acidic or alkaline conditions. At a pH between 4 and 5 IHP is least stable and hydrolysis becomes noticeable within 24 h at temperatures above 70°C. A method of analysis based on extraction with EDTA/NaOH and direct h.p.l.c. was tried with a sandy soil, pig slurry, pig feed, grass roots, potatoes and worms. The extracts were adjusted to pH 7, filtered and then injected directly into the anion exchange column (Figure 1). Only in the case of pig slurry reasonable results were obtained in this way. On injecting the extracts of other samples, an irreversible change in the properties of the Aminex A-28 anion exchange resin occurred. This change was such that under the conditions used IHP was increasingly adsorbed and eventually not eluted at all. The nature of this change was not investigated further and assumed to be caused by

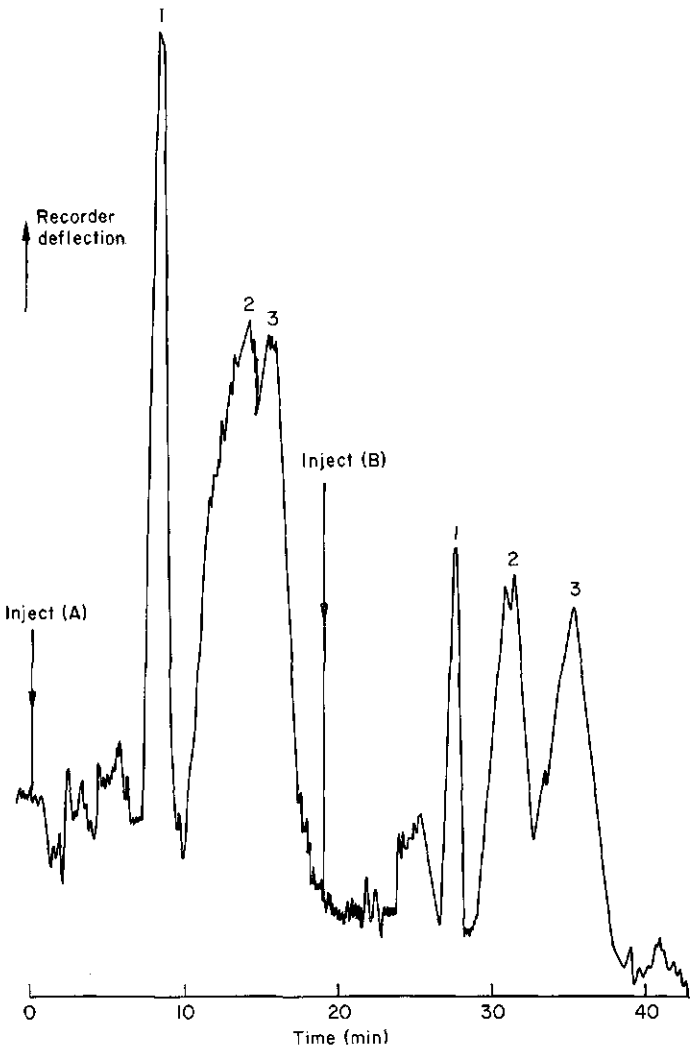

Figure 2. High pressure liquid chromatography of an equimolar mixture of AMP (peak 1), IHP (peak 2) and ATP (peak 3). The concentration of the phosphates is 200 μmol litre^{-1} (A) or 50 μmol litre^{-1} (B). Conditions: anion exchange resin Aminex A-28, elutant 0.43M-NaCl, 0.001M-$Na_2B_4O_7$, 1% ethanol, temperature 50°C, column length 30 cm, internal column diameter 0.2 cm, flow rate 0.25 ml min^{-1}, detector Pye-Unicam LCM-2, wire speed $\times 2$, attenuator $\times 2$, pyrolysis T 800°C.

adsorption to the resin of cation-containing organic complexes. Also, the permeability of the column decreased slowly.

In the case of fresh pig slurry the presence of significant amounts of ATP was suspected as two partly resolved peaks were observed in the chromatogram. To separate IHP from ATP, the EDTA/NaOH extract was filtered through a Sephadex G-25 gel column. The retention behaviour of IHP, ATP, AMP and EDTA in a Sephadex G-25 gel column is shown in Figure 5. IHP is easily resolved from the other organic phosphates and EDTA. ATP is eluted in the centre fraction (50–70 ml) together with EDTA and *ortho*-phosphate (not shown). In Figure 6 results of h.p.l.c. of IHP and ATP in Sephadex G-25 fractions of an EDTA/NaOH extract of dried fresh pig slurry are shown. As can be seen, both IHP and ATP contents are quite significant (pig slurry A in Table 1). Clean-up

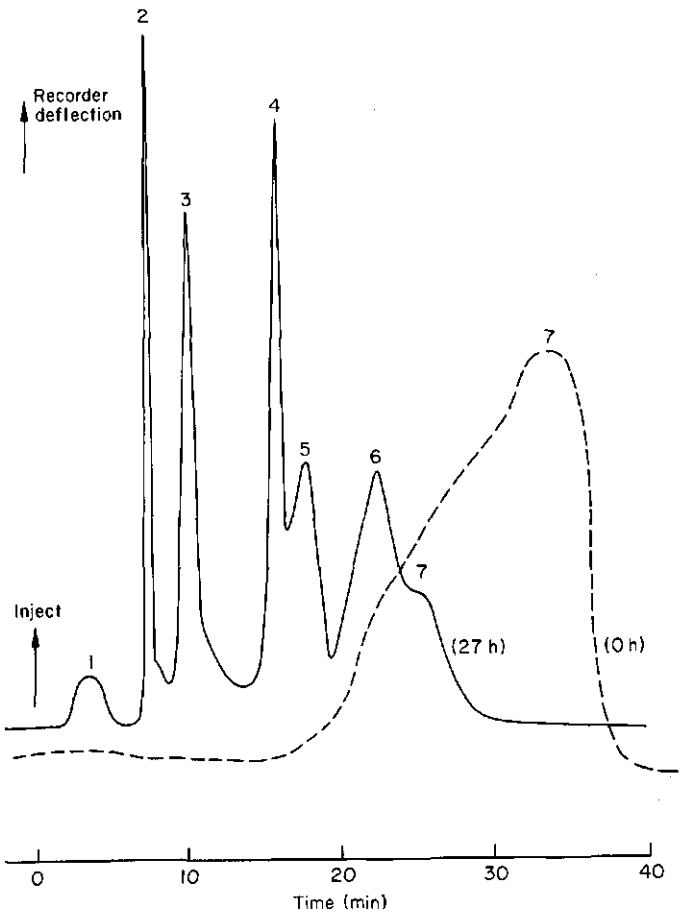

Figure 3. Results of the injection of a solution of 1000 μmol litre^{-1} IHP into an anion exchange column before (dashed line) and after hydrolysis at 100°C for 27 h at pH 8. Conditions as given for Figure 2, except flow rate = 0.15 ml min^{-1} and detector attenuator = $\times 8$. Peak 1 = inositol, peak 7 = IHP, the other peaks are intermediate phosphate esters resulting from hydrolysis of IHP.

by gel filtration also made it possible to analyse extracts of pig feed and potatoes by h.p.l.c. without deterioration of the anion exchange resin. Results are given in Table 1 under the heading LC%. The determination of IHP (and ATP) in the extracts of the other sample materials (soil, grass, roots, worms) was not improved by gel filtration. Even a second filtration of the IHP (or ATP) containing fraction from Sephadex G-25 through a Sephadex G-100 column gave little improvement. In these samples and also, for comparison, in the other samples IHP was determined after filtration of the IHP containing fraction from Sephadex G-25 through a Sephadex G-100 gel column and analysing the second fraction (53–63 ml, Table 1, Figure 7) for total P. The recovery of added IHP in the Sephadex G-100 fraction, determined as increased total P, was 80–90%. The data found for IHP by h.p.l.c. of the Sephadex G-25 fractions of pig slurry, potatoes and soil (shown under the heading LC% in Table 1) show good agreement with the total P values for the Sephadex G-100 fraction containing IHP (G-100-2 in Table 1). It can thus be assumed that only IHP is eluted in this Sephadex G-100 fraction and that possible other organic P compounds are eluted before (high molecular weight) or after (lower molecular weight). A survey of IHP, ATP and total organic P content of the gel fractions of the extracts of the various samples together with some sample characteristics is given in Table 1.

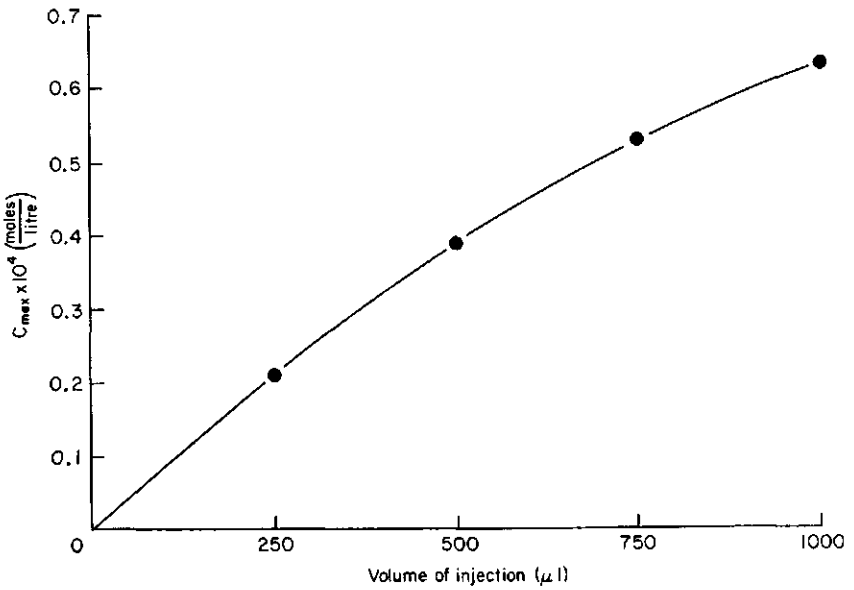

Figure 4. Plot of the maximum peak height (C_{max}) on injection of 100 μmol litre^{-1} IHP (neutral sodium salt) into an anion exchange column against the volume of injection. Conditions as described in Figure 2.

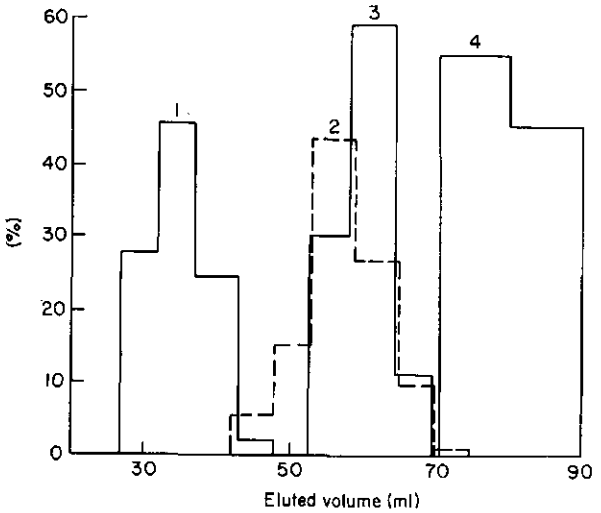

Figure 5. Elution in water of IHP (1), EDTA (2), ATP (3) and AMP (4) from Sephadex G-25. Void volume in column = 30 ml, total column volume = 75 ml.

It was observed that IHP separated from EDTA by gel filtration through Sephadex G-25 decomposed slowly. This is probably due to the presence of a phytase enzyme in the same gel filtration fraction as IHP, reactivated in the absence of EDTA.[19,20]

The detection limit, using the Pye-Unicam LCM-2 carbon detector, was about 5–10 μmol litre^{-1} for both IHP and ATP. Using the Cecil CE 212 spectrophotometric detector, a detection limit of 10^{-2} μmol litre^{-1} was found for ATP. With the analysis scheme used (Figure 1), this results in a maximum attainable limit of detection for IHP of 5 μg P/g (soils) to 50 μg P/g (pig slurry). Determining IHP from total P in a Sephadex G-100 gel fraction gives a limit of detection of 0.05 μg P/g

Table 1. Results of P, IHP and ATP analyses in chromatographic fractions of various samples

	OM (%)	P in sample (%)			Organic P in gel fractions (%)						LC (%)	
		Inorg.	Org.	EDTA extract	G25.1[a]	G25.2[b]	G25.3	G100.1	G100.2	G100.3	P_{IHP}	P_{ATP}
Reclaimed 'haar' podsol soil (0–20 cm)	5.84	0.032	0.02	0.040	0.009	0.002 (0.031)	0.001	0.005	0.004	0.001	0.005	10^{-4} – 10^{-5}
Reclaimed 'haar' podsol soil (20–40 cm)	8.23	0.003	0.01	0.010	—	0.0005 (0.002)	0.0004	0.003	0.0025	0.001	n.d.	n.d.
Pig slurry A age: 1 week DM 6.35%	73.2	1.63	0.6	2.30	0.24	0.20 (1.65)	0.12	0.032	0.15	0.031	0.13	0.14
Pig slurry B age: 1 month DM 6.8%	—	1.65	0.3	1.90	0.12	0.10 (1.65)	0.09	—	—	—	0.09	0.06
Pig slurry A age: 6 months DM 5.5%	71.3	2.07	0.2	2.13	—	0.05 (2.00)	0.05	0.023	0.010	0.03	0.02	10^{-4}
Pig slurry C age: 2 years DM 3.8%	69.1	2.27	0.2	2.51	0.023	0.1 (2.30)	0.15	0.017	0.005	0.002	n.d.	10^{-5}
Pig feed 1	94.6	0.25	0.41	0.56	0.30	0.00 (0.230)	0.035	—	—	—	0.14	0.007
Pig feed 2	92.8	0.05	0.37	0.37	0.240	0.00 (0.102)	0.013	—	—	—	0.17	0.007
Grass roots (*Poa annua*)	70.1	0.125	0.10	0.210	0.037	0.05 (0.128)	0.017	0.018	0.013	0.004	n.d.	10^{-4}
Potatoes (Irene)	95.0	0.072	0.22	0.230	—	0.04 (0.067)	0.009	0.012	0.085	0.001	0.10	n.d.
Worms (*Lumbricus rubellus*)	94.0	0.37	0.72	0.77	0.110	0.48 (0.160)	0.075	0.071	0.023	0.008	n.d.	0.08

Values in parentheses denote inorganic P in this fraction, etc.
All data refer to dry samples.
LC = by anion exchange chromatography.
n.d. = not detectable.
[a] G25.1 = first fraction of extract after filtration through Sephadex G25 gel.
[b] G25.2 = second fraction.

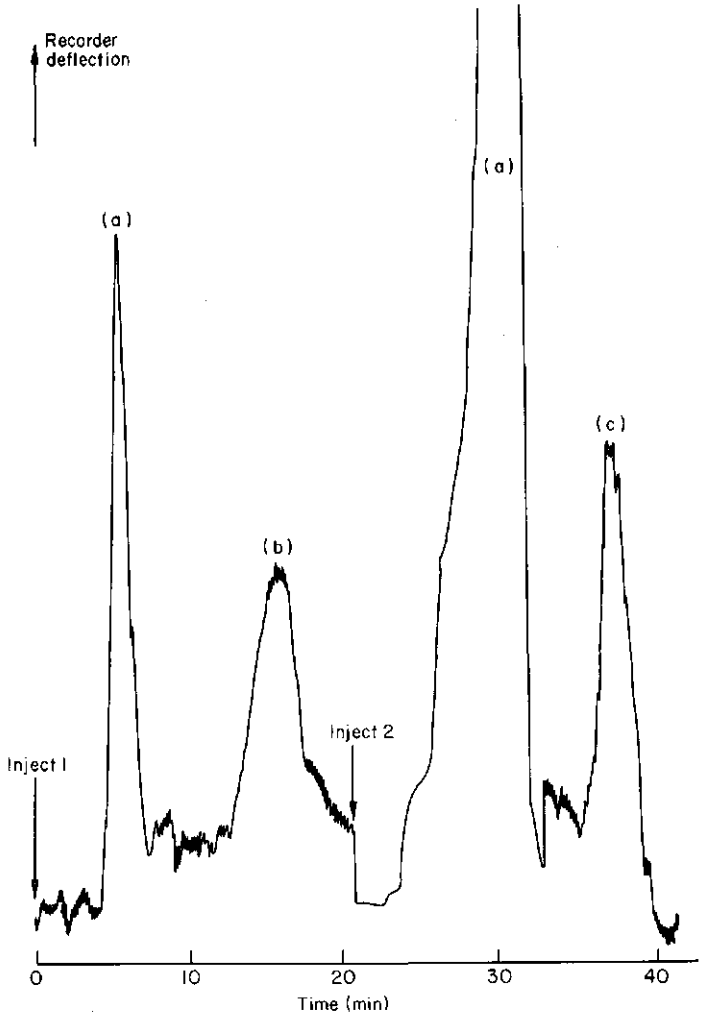

Figure 6. Examples of chromatograms resulting from the injection into an Aminex A-28 anion exchange column of the Sephadex G-25 gel chromatographic fractions of extracted pig slurry A (Table 1). The first gel fraction (27–45 ml, Figure 1) is injected at 1 and the second gel fraction (50–70 ml) is injected at 2. Peak (a) = EDTA, peak (b) = IHP and peak (c) = ATP. Chromatographic conditions are as given for Figure 2, except that peaks (a) were recorded at a wire speed $\times 1$ and attenuation $\times 4$. Flow rate = 0.27 ml min^{-1}.

(soils) to 0.5 μg P/g (pig slurry). For ATP, the attainable limit of detection should be 5 ng P/g (soils) to 50 ng P/g (pig slurry). Due to interference from other compounds resulting in incomplete resolution of ATP, detectability in practice can, however, be less by a factor 10–100. For sensitive and specific determination of ATP, the enzymatic luciferin–luciferase method should be used with which a limit of detection of 10^{-6} μmol $litre^{-1}$ can be attained.[21]

From literature data[22–26] it follows that IHP content of top soils can vary from 1–500 μg P/g. Parameters causing this variation can be: Fe and Al content, age, time under cultivation, moisture level, redox potential, temperature and pH. The variation found in manures and slurries[1,27,28] from 10–7000 μg g^{-1} (dry matter) is mainly due to storage time and IHP and Ca content of feed. With pigs, intestinal hydrolysis of IHP increases and consequently the IHP content of manure decreases with decreasing Ca content of the feed.[28]

In soils, ATP levels are in the range of 2–200 ng P/g.[21] The high ATP levels found in the pig feed

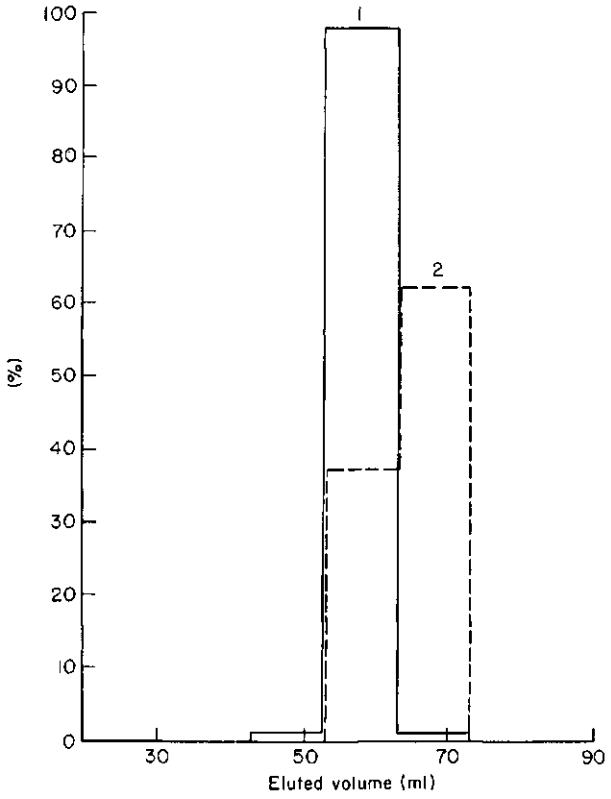

Figure 7. Elution in 0.02M-NaCl of IHP (1) and EDTA (2) from Sephadex G-100. Void volume in column = 23 ml, total column volume = 70 ml.

(Table 1) are probably due to fungal growth which could be observed. Activated sludge can contain about 50 ng P/g as ATP.[21]

For a number of cases the detection limits obtainable with the method used here will be marginal. Nevertheless it may be concluded that the procedure for determining IHP as outlined here (Figure 1) offers a more specific, reliable (less subject to interference and hydrolysis) and often faster alternative to existing methods.[1–6]

An added advantage of the method is that accompanying organic P compounds are also extracted and obtained in separate fractions for possible further analysis and identification. This has partly been done for high molecular weight organic phosphates in pig slurry.[29]

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