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FLOW INJECTION ANALYSIS

PART II. ULTRAFAST DETERMINATION OF PHOSPHORUS IN PLANT MATERIAL BY CONTINUOUS FLOW SPECTROPHOTOMETRY

J. RŮŽIČKA and J.W.B. STEWART*

Centro de Energia Nuclear na Agricultura (CENA), C.P. 96, 13400 Piracicaba, S.P. (Brasil)

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Flow injection analysis is a new approach to automation of chemical analyses, based on continuous flow measurement. This very simple method utilizes rapid injection of an aqueous sample into a continuously moving carrier stream of a reagent. The injected sample forms a zone which is then transported towards a detector, which continuously records the absorbance, or changes of electrode potential, etc. As the sampling period is very short, usually less than two seconds, a very high sampling rate can be achieved. This in turn allows the use of a quickly moving carrier stream, which in contrast to the AutoAnalyser concept, does not need to be segmented by air. The advantages of this approach were discussed in the first paper of this series [1], where the first results obtained on model systems were reported. The purpose of the present work was to prove the practical usefulness of this new approach for routine analysis, and to investigate the interrelation of such important parameters as sample volume, tube diameter, tube length, peak height and sampling rate, so that guidelines could be established for the best design of the manifold, and so that the limitations of flow injection analysis could be recognized.

The determination of phosphate after acid digestion of plant organic matter, was chosen, as this analysis is one that is frequently required in agricultural and environmental research, and also because the number of samples which have to be analysed annually in the CENA laboratories approaches a figure of 15,000. Furthermore the same method could be used to determine phosphorus in water samples, soil extracts, blood samples, etc. [2—8], all of which are generally determined by automated methods owing to the number of analyses required. Any improvement in this sampling rate would have obvious significance to many commercial and routine laboratories.

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*UNDP/IAEA Experts in Radiochemistry and Soil Science, respectively, assigned to Project BRA/71/556, on leave from the Chemistry Department A, Technical University of Denmark, Lyngby, Denmark, and from the Department of Soil Science, University of Saskatchewan, Saskatoon, Canada.

The method is based on wet oxidation of the plant organic matter by a mixture of nitric and perchloric acids, and subsequent spectrophotometric measurement of phosphate in an acidified solution of ammonium molybdate containing ascorbic acid [2].

EXPERIMENTAL

Sample injection

Samples were injected manually, by means of disposable 1-ml plastic syringes through a septum located on a plastic tube in which the carrier stream of a reagent was being pumped by a peristaltic pump. The overall arrangement is shown in the flow diagram (Fig. 1). Details of the injection technique and of the construction of the injection port were reported previously [1].

Apparatus

This consisted of a peristaltic pump model 8511, 60 r.p.m. (Polymetron, Hombrechtikon, Switzerland), a spectrophotometer model DB, connected to a recorder model 1005 (both Beckman Inc., Fullerton, California) and a flow-through cuvette (Helma, type 178-QS; light path 10 mm, volume 0.08 ml). The dual-beam mode was used, the carrier stream liquid serving as a blank in a 10-mm stationary cuvette.

The manifold was entirely made of polyethylene and tygon tubing, of the noncollapsible wall-type. A Y-shaped connector was made in a small perspex block, and a mixing coil was made by winding the required length of plastic tube around a larger glass tube with a diameter of *ca.* 5 cm. The connections were made by short pieces of tygon or silicone rubber tubing into which the transmission tubing just fitted tightly. As both the length (Fig. 3) and the inner diameter (Fig. 6) of the tubing have an important effect on the flow pattern, the length of the analytical line between the point of injection and the flow cell should be measured. Moreover, as the inner diameter of the plastic tubes can vary slightly from the manufacturers' specifications it is advisable to check by measuring, at room temperature, the length of the tube which is filled by exactly 1.00 ml of water. The most suitable tubing for routine analysis was

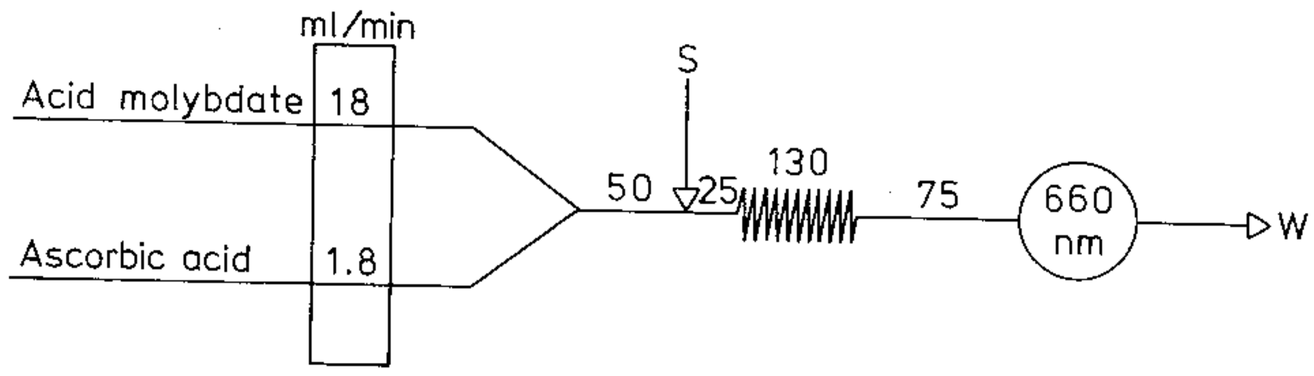


Fig. 1. Flow diagram. Molybdate and ascorbic acid are pumped and jointly form a carrier stream of reagent into which 0.50 ml of the sample (S) is injected. After passage through a mixing coil the absorbance of the molybdenum blue is measured in a flow cuvette at 660 nm and the stream continues to waste (W). The total length of the analytical line is 230 cm, of which the coil constitutes 130 cm. For tube diameter, see text.

found to be polyethylene tubing of 0.95—1.20 mm i.d. (140 cm ml^{-1} of water to 95 cm ml^{-1} of water).

Reagents

All chemicals were A.R. grade, and distilled—deionized water was used for all experiments. The acid molybdate reagent consisted of 0.005 *M* ammonium heptamolybdate in 0.4 *M* nitric acid—5 % ethanol mixture. Unless otherwise stated, the ascorbic acid was an aqueous 5 % solution.

Plant material

Bean samples (*Phaseolus vulgaris* L) were taken from field trials, dried for 48 h at 65 °C, and then ground in a Wiley mill to pass a 0.5-mm sieve.

Decomposition procedure

Either a 1.0 or 0.5 g sample of dried, ground, plant material was placed in a 100-ml Kjeldahl flask, then 10 ml of concentrated nitric acid were added and the flask was heated for 2 h; then 2 ml of perchloric acid (72 %) was added and the mixture was heated for a further 1—2 h. This mixture was cooled and then diluted to 70 ml with water.

Standards

Phosphorus standard solutions were made from potassium dihydrogen-phosphate (50 μg P ml^{-1} requires 0.2195 g of KH_2PO_4 per litre).

Computations

All calibration curves were calculated by means of linear regression analysis with a Hewlett-Packard Programmable Electronic Calculator Model 9810A.

RESULTS AND DISCUSSION

The classical molybdenum blue procedure involves the primary reaction of phosphate with molybdenum(VI) to form 12-molybdophosphoric acid and its subsequent reduction to the heteropolyphosphomolybdenum blue. While the first reaction is very fast, and reaches completion within milliseconds [3], the reduction reaction is slower [2] and even in the AutoAnalyser method, which does not require that reaction equilibrium be reached, double mixing coils and even heated delay coils are used to allow sufficient colour development [4—7]. Thus, although the molybdenum blue method is reliable, as it is not affected by the yellow coloration of some plant digests, it is often replaced by the faster, but less sensitive, vanadomolybdophosphate method. The previous results [1], however, indicated that even the molybdenum blue method could be adapted for flow injection analysis, provided that the concentration of reductant was increased. When the manifold shown in Fig. 1 was used with polyethylene tube of 1.15 mm i.d., it was found (Fig. 2) that the sensitivity of the phosphate determination, *i.e.* the speed of the reduction

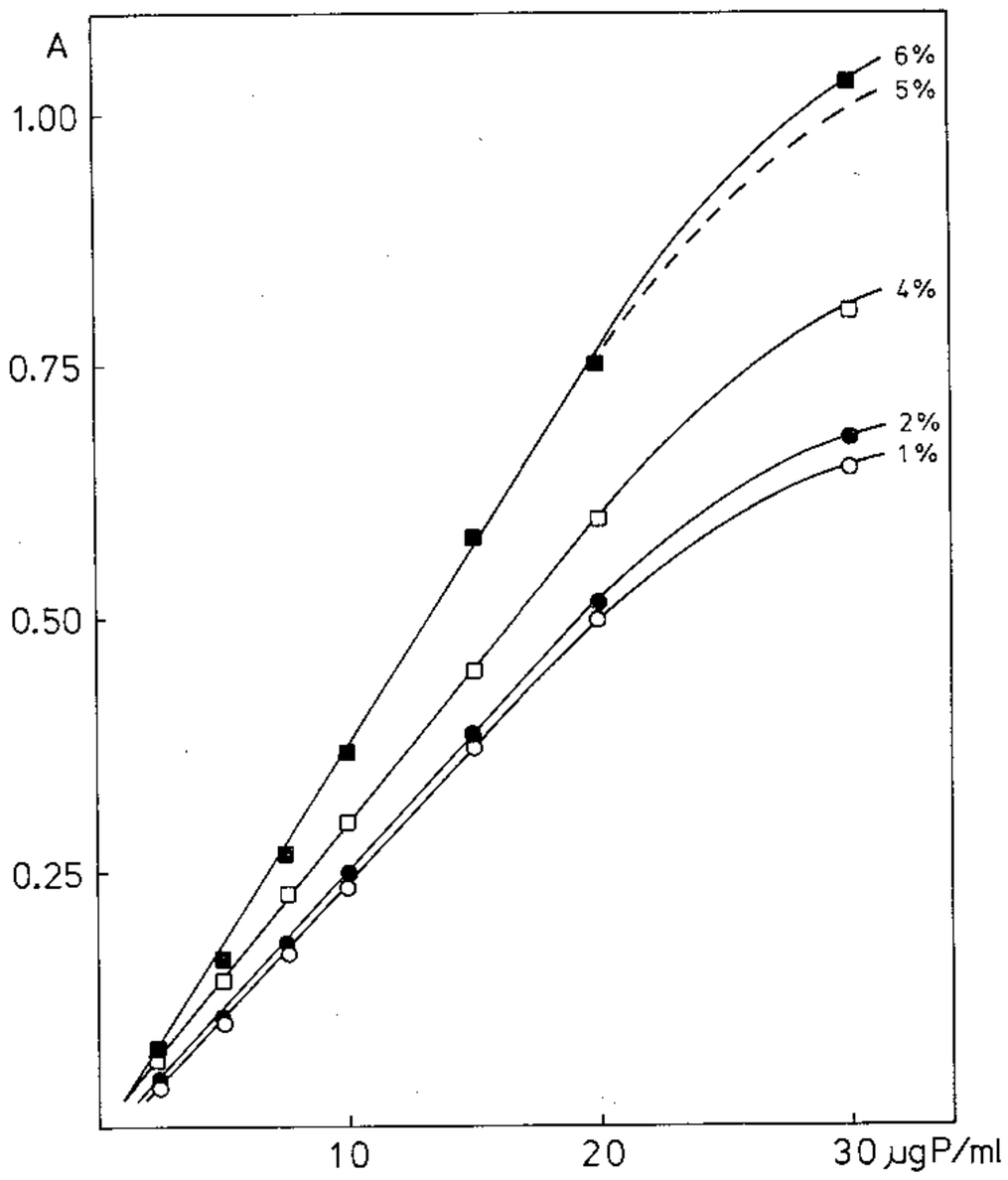
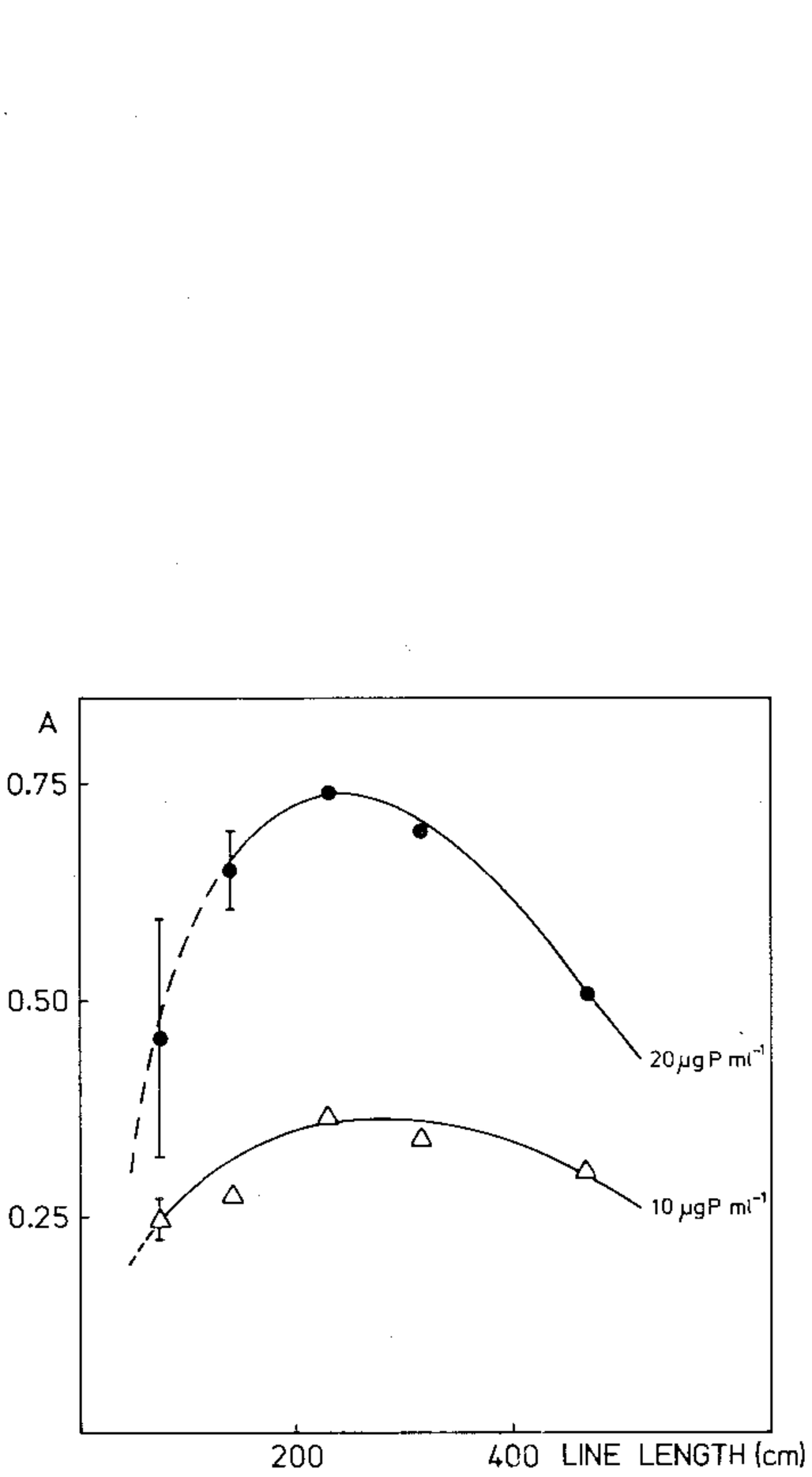


Fig. 2. Influence of the ascorbic acid concentration (1—6 %) on the determination of phosphate as molybdenum blue. Line length 230 cm; i.d. 1.15 mm.

reaction, increased as the concentration of the ascorbic acid solution was increased from 1 % to 6 %. With a 10 % solution of ascorbic acid, precipitation of molybdate occurred in the conduits of the system, hence 5 % ascorbic acid was adopted as a reasonable compromise between economy of reagent and linearity of calibration line.

Effect of line length

The line length is defined as the portion of the tubing, within which the sample and reagent are mixed, the colour develops and the absorbance is measured. This distance between the point of injection and the flow-through cell should be kept as short as possible, yet should still be long enough to allow sufficient time for colour development. By inserting different coil lengths (65 cm, 130 cm, 195 cm, 240 cm and 380 cm) into an otherwise unchanged analytical line and by running 10 μg ml^{-1} and 20 μg ml^{-1} phosphate standards, Fig. 3 was obtained, from which it is evident that an optimal line length, which allows maximal sensitivity, can be found for a particular chemical reaction. It is interesting to note that an extremely short line length of 75 cm resulted in a large measurement error. The reason can be seen from Fig. 4, curve a (recorded at a high paper speed); the valley at the top of the peak shows that a central portion of the sample zone has not had sufficient time to mix, and develop the colour, with the reagent present in the carrier stream. As the length of the analytical line increases the peak develops fully (Fig. 4, curves b, c) and then, because of dilution, decreases again. Obviously,



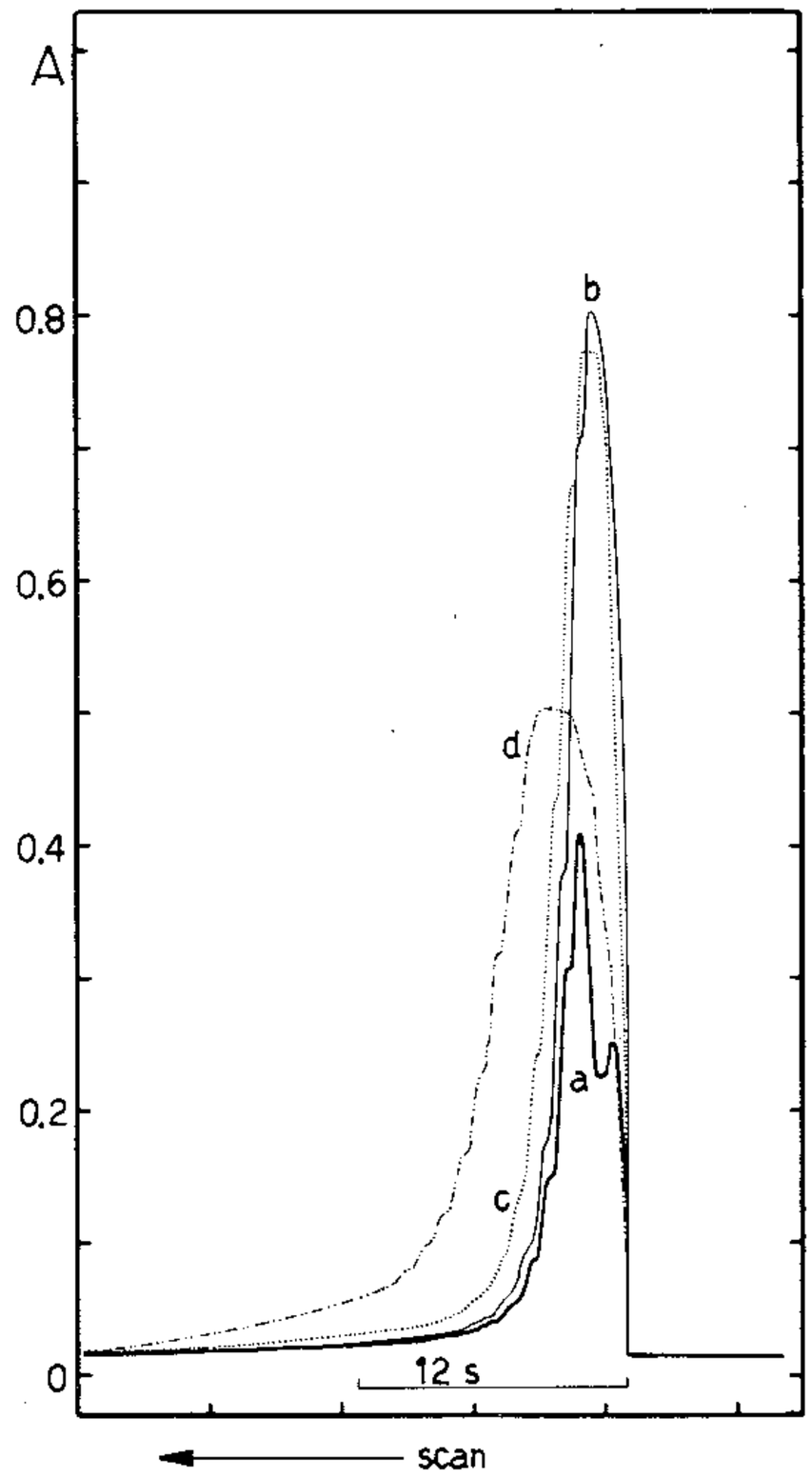


Fig. 3. Influence of the length of the analytical line on the peak height of 10 µg ml^{-1} standards. Tube i.d. 1.15 mm. Optimal line length 250—350 cm. Note the large error of measurement, as indicated by bars, with line lengths below 200 cm (compare Fig. 4).

Fig. 4. Influence of the length of the analytical line on the form of the peak and half-wash time, obtained with a 25 µg P ml^{-1} solution and standard tubing of 1.15 mm i.d. (a) 75 cm ($W_{1/2} \simeq 0.8$ s). (b) 140 cm ($W_{1/2} = 1.1$ s). (c) 205 cm ($W_{1/2} = 1.3$ s). (d) 465 cm ($W_{1/2} = 2.0$ s). Note the valley on top of peak (a) which is caused by insufficient mixing and equilibration of the sample zone.

slow reactions would not be suitable for the flow injection method unless they could be speeded up by heating or catalysis. However, by replacing one coil by another one, the sensitivity of the measurement can be adjusted according to the level of analyte in the samples, so that it remains within the instrument range.

Effect of sample volume

The influence of the sample volume was investigated with line lengths of 230 cm and 415 cm (1.15-mm i.d. tubing). As one would expect, the sensitivity increases with the sample volume until a point is reached where the reagent concentration and degree of mixing are not sufficient to obtain linear calibration curves (Fig. 5, *cf.* Figs. 3 and 4). Decreasing the sample volume decreases the sensitivity and increases the error of the analysis. Thus, by comparing the influence of the line length and of the injected volume, it

became apparent that the optimal sample volume is between 0.4 and 0.6 ml; this offers the best reproducibility and is convenient to inject. Therefore the sensitivity can be more easily and reliably adjusted by changing the line length and tube diameter than by altering the sample volume.

Effect of tube diameter

Tube diameter also has a significant effect on the flow parameters, influencing primarily the half-wash time and thus the maximum achievable sampling rate. To investigate this, the whole line length was made of tubing ranging from 0.80-mm to 1.5-mm i.d.; the total tube length in each experiment was 269 cm. Except for the thinnest tube, where the 25 μg P ml^{-1} standard consistently showed a negative deviation, all calibration curves were strictly linear. When peak heights (absorbances) were plotted *versus* tube volume per ml (Fig. 6), a set of straight lines was obtained with all tubes of internal diameter less than 1.5 mm. Obviously a decrease in the internal diameter of the tubing improves sensitivity and flow parameters, but tubing with internal diameters of 0.80 mm or less were difficult to use, because of the pressure imposed on the system at the moment of injection which could cause leakage at the tube connectors. It should be pointed out, however, that tubing of less than 0.80-mm i.d. could be used with micro samples of less than 0.20 ml provided

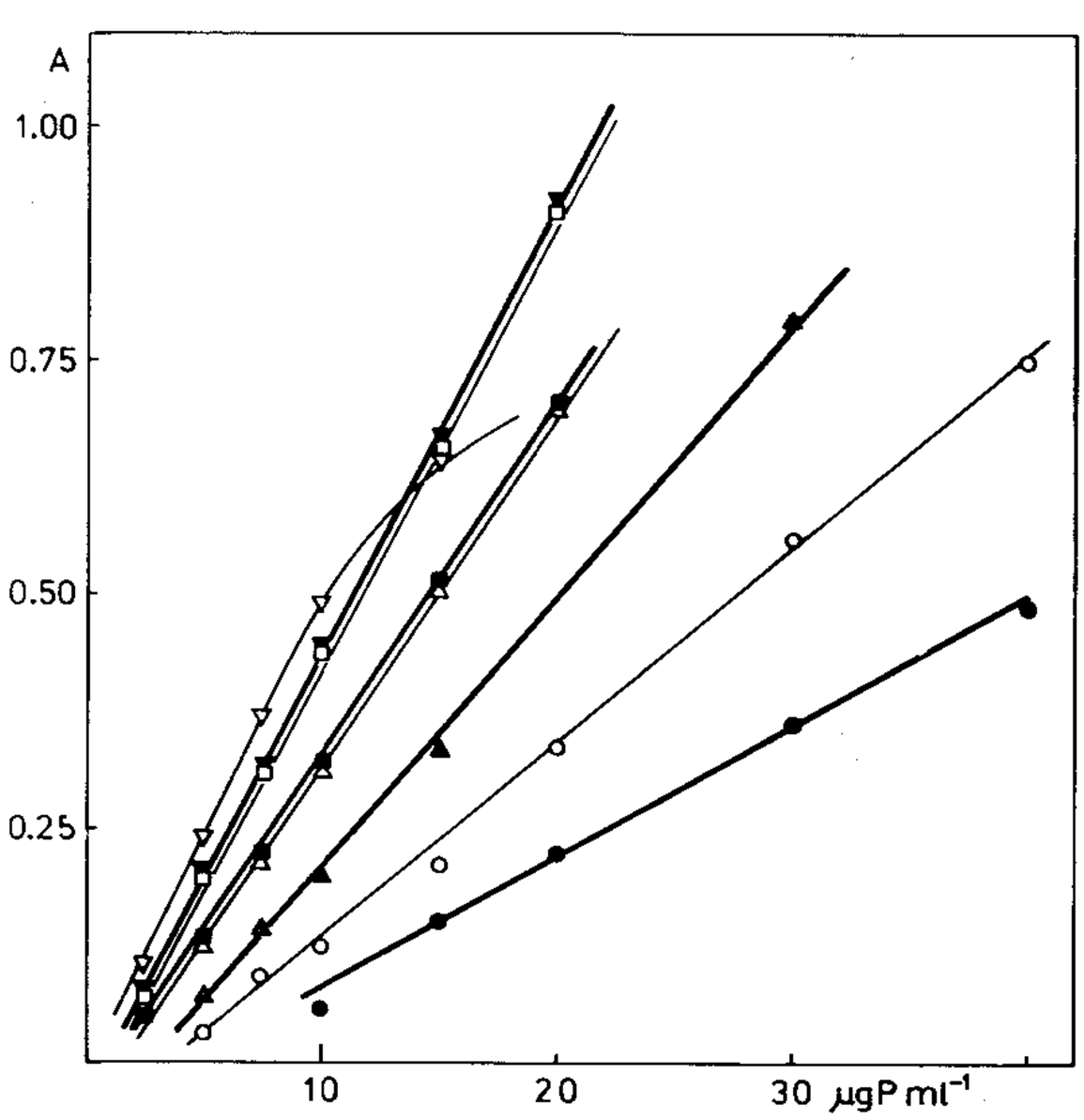
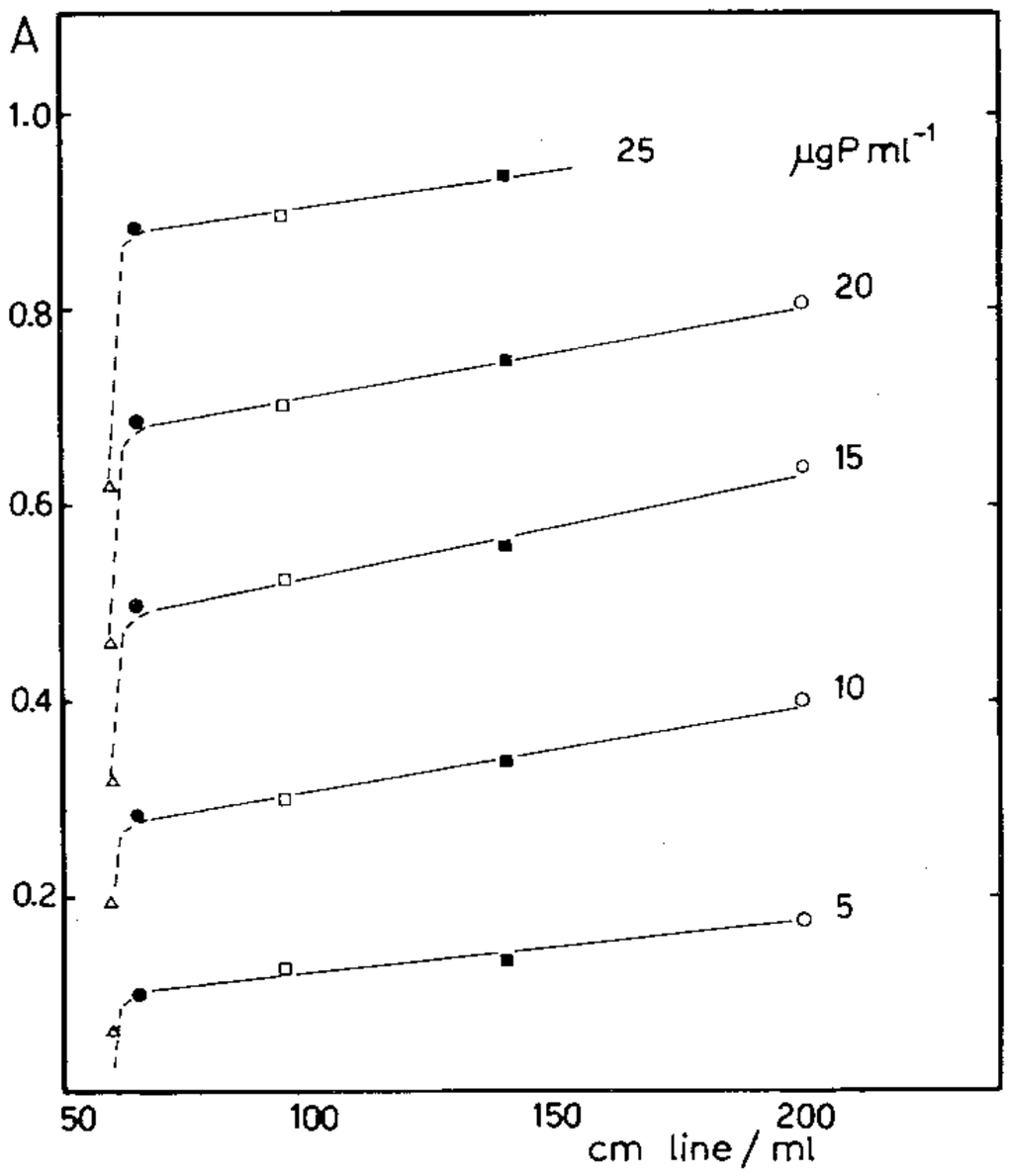


Fig. 5. Relationship between the sample volume and line length, the peak height and linearity of the calibration curve. 1.15-mm i.d. tubing; 5 % ascorbic acid. Short line (230 cm): injected volume (▽) 0.80 ml; (▫) 0.60 ml; (△) 0.40 ml; (○) 0.20 ml. Long line (415 cm): injected volume (▼) 0.80 ml; (■) 0.60 ml; (▲) 0.40 ml; (●) 0.20 ml. Note that a large volume injected in a short line results in a loss of linearity (curve (▽)) while injection of a smaller volume in a longer line extends the linear range (compare curves (▲) and (○) with Fig. 2).



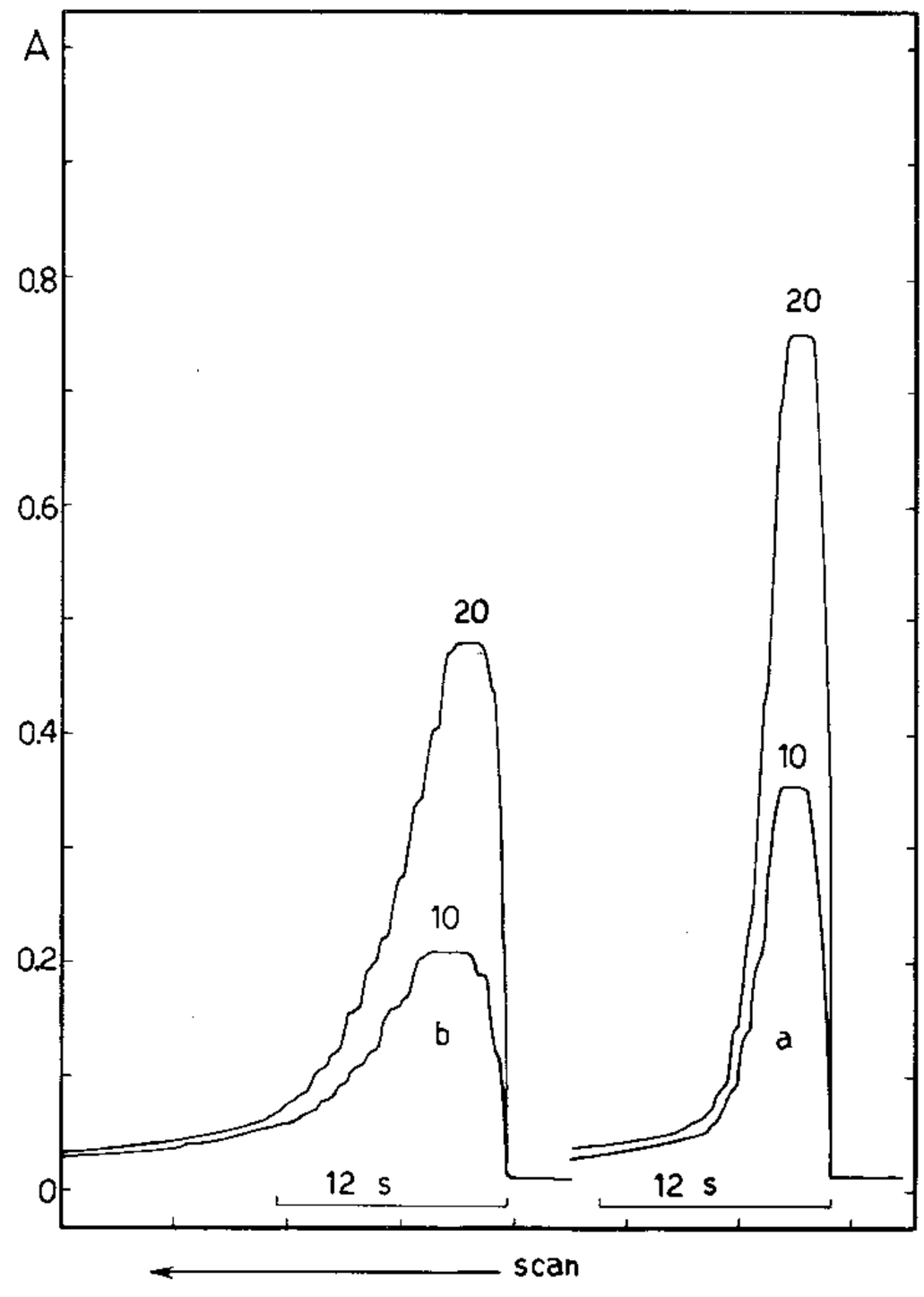


Fig. 6. Relationship between the internal diameter of the tubing and the peak height. Line length, 269 cm (coil 169 cm). Tube diameter is expressed in specific volume (cm ml^{-1}). Tube i.d.: (△) 1.5 mm; (●) 1.4 mm; (□) 1.15 mm; (■) 0.95 mm; (○) 0.80 mm.

Fig. 7. Relationship between the internal diameter of the tubing, peak height and half-wash time. Line length 269 cm; 10 µg P ml^{-1} and 20 µg P ml^{-1} standards. (a) 0.95 mm i.d. (half-wash time, 0.93 s). (b) 1.5 mm i.d. (half-wash time, 3 s). $W_{1/2}$ was obtained by analysis of fall curves.

that special syringes were used to allow precise injection of these small volumes.

Tubing with large internal diameter, however, allows significant sample dilution by the carrier stream, which causes a decrease in sensitivity, an increase in half-wash time and a decrease in the sampling rate. For example, manifolds made of 0.95-mm and 1.5-mm i.d. tubing (Fig. 7 (a) and (b)) had half-wash times of 0.93 s and 3.02 s, respectively, as calculated from the shape of the respective fall curves. For a sample: wash ratio of 1:1.5 and 75 % peaking, maximal sampling rates of 774 analyses per hour (0.95 mm i.d.) and 240 analyses per hour (1.5 mm i.d.) can be theoretically achieved. At these rates, approximately 5 % of the colour of the preceding peak will still be present in the flow cell while the maximum of the next peak is recorded. This computation takes into account the length of the plateau at the top of the peak, but the influence of the residence time on the sampling rate must still be considered.

In practice it is, of course, impossible to reach the highest sampling rate

by manual injection and it is indeed doubtful that rates over 600 samples/h would ever be utilized. Yet, these outstanding flow parameters are very useful; for it can be calculated easily that at a sampling rate of, *e.g.*, 420 analyses per hour in a manifold made of 0.95-mm i.d. tubing with a line length of 269 cm, the carry-over would be less than 1 %. This was checked in an experiment (Fig. 8) where no interaction between 5 μg ml^{-1} and 25 μg ml^{-1} standards was observed at a very high sampling rate of 420 analyses per hour.

Timing of injections

Injection timing is closely related to the residence time of the sample within the analytical line length. Considering the simplest case when only one sample zone is present within the analytical line length at a time, the maximum sampling frequency is determined by the half-wash time and by the line length. Thus, with 0.95-mm i.d. tubing and a line length of 269 cm, the residence time will be *ca.* 6 s, at a pumping rate of 20 ml min^{-1}. If the sample is always injected at the moment when the recorder pen starts to fall, a total sample period of less than 9 s can be achieved (half-wash time $\simeq$ 0.93 s). Thus, at the foot of the next peak only 1.6 % of the colour of the preceding peak will be left in the flow cell. In practice, however, an even higher sampling frequency

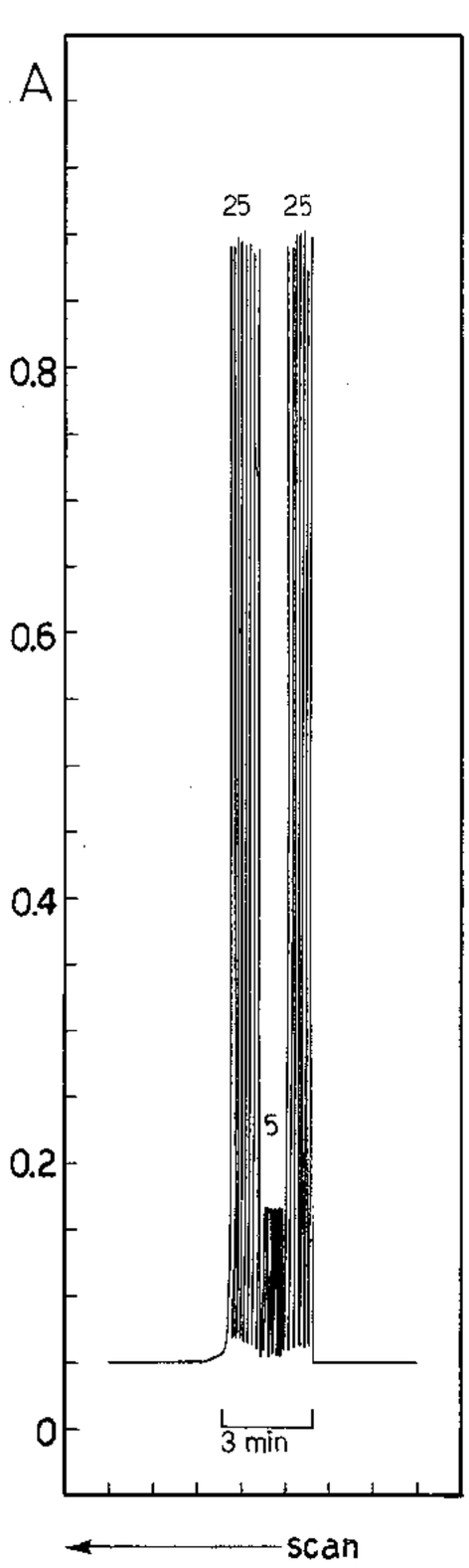


Fig. 8. Flow injection analysis for phosphate (5.0 μg P ml^{-1} and 25 μg P ml^{-1}) at a sampling rate of 420 samples per h. Line length, 269 cm; i.d., 0.95 mm.

can be reached because the injection of 0.5 ml of sample within 2 s increases the flow rate instantaneously to about 35 ml min^{-1}. This slightly decreases the residence time of the sample in the analytical line, further speeds up the washing of the flow cell and thus allows more closely spaced sampling. Consequently, a sampling frequency of 420 samples per hour, corresponding to a time gap of about 8 s, was achieved with this analytical line, with no detectable carry-over between successive samples (Fig. 8).

The above arguments clearly support the choice of shortest analytical line with an i.d. close to 1 mm. Should the line need to be made longer either because of a slow chemical reaction or because several reagents must be added to the carrier stream successively, it would be practical to choose the line length and injection timing so that a sample is never injected at the moment when the maximum of the preceding one is just passing through the flow cell. This can easily be achieved by choosing the line length so that the residence time is a suitable multiple of the half-wash time for that particular system. Thus several evenly spaced sample zones can be present within the sample line at the same time; the spacing of the samples can be maintained by injecting each new sample at the beginning of a fall in the curve.

The speed of injection and the flow rate were not varied in this study. The Polymetron pump accommodated tubes of 1.0, 2.0 and 3.0 mm i.d. which yielded flow rates of 18, 8 and 1.8 ml min^{-1} under the conditions used. Although combinations of these tubes would have given some further choice of flow rates, this type of study was postponed until a peristaltic pump with a continuously variable flow rate becomes available. The influence of the injection speed on the peak height was not studied in detail, because any slow manual injection is subject to a large experimental error. Obviously, the slower the injection, the longer the sample zone, and so the lower the peak and the sensitivity of the measurement. Fortunately, at high injection velocities very good reproducibility can be achieved in manual sampling injection (see below); the particular combination of tube diameter, injection port geometry, needle length and gauge allows the formation of short reproducible sample zones as soon as sample introduction into the carrier stream reaches a certain rate.

Routine analyses

Routine analyses were done with the manifold (Fig. 1) made of 1.15-mm i.d. tubing with a total line length of 320 cm (coil length 200 cm). As the plant digest solutions contained phosphate in the range 8—40 μg P ml^{-1}, 1.15-mm i.d. tubing was chosen in order to keep the absorbance signal within the instrument range. For similar reasons, the line length was long and the wave length was 620 nm. This approach was chosen rather than simple dilution as the same plant digests also had to be analysed for ^{32}P, potassium and calcium. Samples (0.50 ml) were injected manually, so that only one sample was in the line at a time, each being analysed in triplicate. Figure 9 shows a typical recording, with — from left to right — a sequence of P standards, thirty plant digests and the same sequence of P standards. As can be seen from

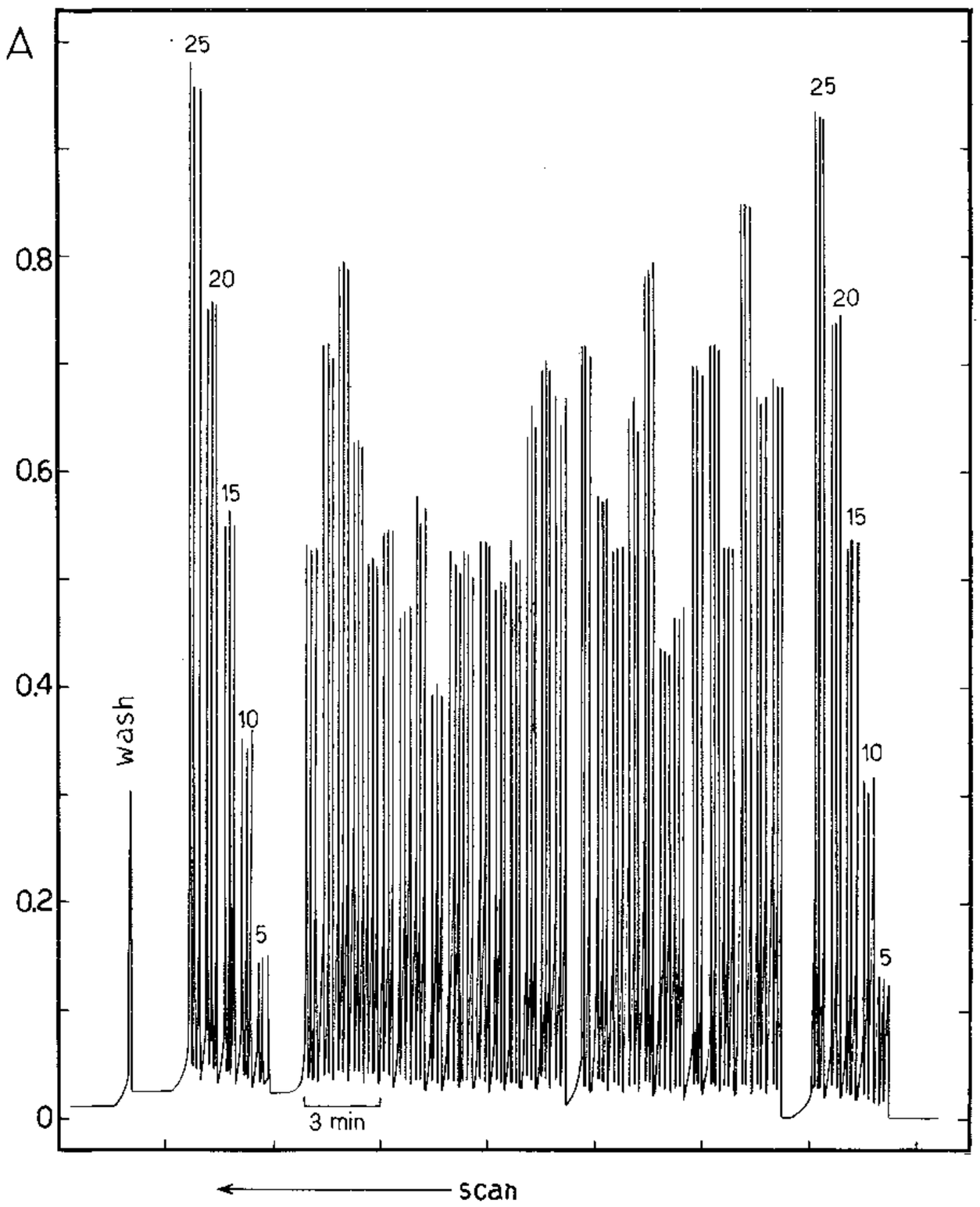


Fig. 9. Routine analyses for phosphorus in plant digests. Line length 320 cm, 1.15 mm i.d. tubing. From right to left: standards of 5.0, 10.0, 15.0, 20.0 and 25.0 μg P ml^{-1}, followed by thirty plant digests and phosphate standards. All samples were analysed in triplicate. Last peak on left is a wash effected by injecting 1 *M* NaOH. Average sampling rate of digested samples was 250 analyses per hour.

the record, the baseline increased slightly during the measurement, because of deposition of molybdenum blue in the flow cell, despite the addition of alcohol to the carrier stream. Therefore, whenever necessary, the system was washed by injecting 0.5 ml of 1 *M* NaOH (the last peak to the left). An average standard deviation of 1.32 % was obtained for this particular series of thirty samples (Table I) which were analysed at an average sampling rate of 250 samples per hour.

In order to keep pace with this rate in routine analyses, two persons were needed for manual injection, one filling and washing the syringes, and the other adjusting the volume and injecting the sample with the same syringe repeatedly three times. As there is no "warm up" or delay period, the baseline can be adjusted immediately and the instrument can be used for short periods at a time as the need arises, with a good economy of reagent consumption and time. Even at the slow sampling rate of 250 analyses per hour, smaller amounts of reagents are used than with the manual method (Table II).

The precision and accuracy, compared to the manual method [8], can be seen from a comparison of the phosphate results obtained on the same 30 samples

TABLE I

Determination of phosphorus in plant digests by flow injection and by a manual method

(Phosphorus expressed as % P in dry plant matter)

Sample No.	Manual method [8] Mean[a]	Flow injection[b]	
		Mean[c]	s_r (%)
1	0.130	0.131	1.07
2	0.130	0.128	0.59
3	0.173	0.160	0.48
4	0.108	0.105	0.26
5	0.157	0.137	0.38
6	0.127	0.134	0.56
7	0.147	0.144	0.64
8	0.094	0.087	1.59
9	0.151	0.149	1.00
10	0.129	0.125	3.19
11	0.102	0.104	0.57
12	0.119	0.112	0.59
13	0.136	0.136	0.69
14	0.127	0.127	2.42
15	0.133	0.134	0.91
16	0.121	0.124	2.73
17	0.089	0.102	2.68
18	0.092	0.097	1.02
19	0.101	0.104	0.58
20	0.100	0.101	2.72
21	0.083	0.100	2.54
22	0.079	0.079	1.81
23	0.106	0.109	2.66
24	0.092	0.092	0.86
25	0.106	0.105	0.56
26	0.101	0.100	1.78
27	0.101	0.119	0.98
28	0.140	0.147	0.51
29	0.131	0.134	2.00
30	0.098	0.102	1.35
Mean value	0.117	0.116	1.32
Difference (μg)	0.2		
(%)	1.0		

[a] Analysed in duplicate.
[b] See also Fig. 9. The regression coefficient for standards in flow injection was 0.99987.
[c] Analysed in triplicate.

TABLE II

Comparison of reagent consumption of manual and flow injection methods

(g of material per analysis)

Method	Reagent			
	Ascorbic acid	Molybdate	Acid[a]	Water
Flow Injection				
225 analyses h^{-1}	0.02	0.03	0.12	5.3
420 analyses h^{-1}	0.01	0.02	0.06	2.8
Manual method [8]	0.02	0.05	0.90	25.0

[a] Manual method, H_2SO_4. Flow injection, HNO_3.

shown in Table I. No statistical difference at the 0.1 % level could be found between the two sets of values. It is interesting to note that the 30 samples in duplicate took two persons 3—4 h manually with pipettes, volumetric flasks, etc., whereas the 30 samples in triplicate were completed in 30 min by the injection procedure.

CONCLUSIONS

Although the flow injection method is still in an early stage of development, it shows great promise, as it allows the routine analyses of phosphate to be made at a sampling rate of 250 samples per hour, with an average standard deviation of 1.32 %. At sampling rates as high as 420 samples per hour, still no carryover was observed, and the flow parameters indicate the possibility of reaching sampling rates in excess of 700 samples per hour for determination of phosphate by the molybdenum blue method. Other advantages compared with manual or even conventional automated continuous flow analysis are equally obvious: the consumption of reagents is minimized, and the cost of equipment is very low, apart from the peristaltic pump. If the analytical requirement is less than a few hundred analyses per day, manual injection is tolerable, but a higher analytical load will, unquestionably, require construction of an automated injector with an appropriate timer. This would allow the inherent capacity of the system to be employed fully.

The great simplicity of construction, low cost and versatility should encourage widespread use of this system. Further, in a similar manner to the AutoAnalyser system, flow injection offers good control of reaction conditions, which allows the analytical use of reactions that do not go to completion or form unstable products. An added advantage is that the flow injection system can be ready instantly for analysis and is therefore useful even when only a few determinations are required.

The optimal flow parameters have now been established on an empirical basis and the usefulness of the system has been demonstrated for one type of analysis; further analytical methods will be adapted for the flow injection method and the possibility of multi-component analysis will be investigated. With regard to the determination of phosphate in lower amounts than in plant material, *e.g.* in water or soil extracts, experiments are under way, based on the addition of antimony to improve the sensitivity.

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SUMMARY

The effect of sample volume, tube length, tube diameter, peak height and sampling rate on the determination of phosphorus in acidic plant digests was investigated, and optimal conditions for the flow injection method are described. Sampling rates of 420 samples per hour were achieved without incurring problems from carryover of samples, and evidence was obtained that rates as high as 700 samples per hour are possible. The flow injection method was proved to be suitable for routine analyses and has obvious advantages over other automated or manual methods in sampling rate, simplicity of design and cost.

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