Journal of Chromatography, 347 (1985) 267–273
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 013

DETERMINATION OF ETHYL PHOSPHITE, PHOSPHITE AND PHOSPHATE IN PLANT TISSUES BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY

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(Received July 1st, 1985)

SUMMARY

A sensitive method of analysis of some phosphorus oxyanions (ethyl phosphite, phosphite and phosphate) in plant material was developed. The plant extracts were purified by anion-exchange high-performance liquid chromatography. The oxyanions were then converted into their *tert.*-butyldimethylsilyl derivatives and evaluated by capillary gas chromatography using a flame ionization detector. This method allowed the quantitation of amounts as low as 40 nmol of phosphite and phosphate and 100 nmol of ethyl phosphite per gram of fresh plant tissue.

INTRODUCTION

Aluminium ethyl phosphite (fosetyl-Al), a systemically active compound known as Aliette®, is used increasingly to control diseases caused by Phycomycetes[1,2]. Ethyl phosphite and its degradation product in plant tissues, phosphite (Fig. 1), have a greater effect on fungal growth *in vivo* than *in vitro*. In view of the low fungitoxicity *in vitro*, ethyl phosphite has been reported to stimulate the defence responses of infected grape leaves, tomato leaflets[3] and tobacco stems[4]. Moreover, this induced defensive reaction is highly dependent on the phosphate content of the treated tissues[5].

In order to obtain precise information about the location and concentration of this compound in healthy and infected plant tissues, its rate of uptake by host or fungal cells, its rate of degradation and the exact mode of competition with phosphate

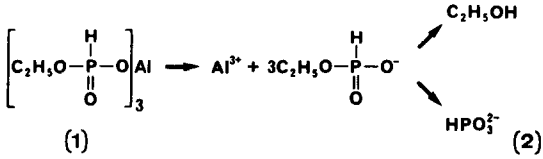

Fig. 1. Structural formulae and metabolic pathway of aluminium ethyl phosphite (1) and phosphite (2) in plant tissue.

0021-9673/85/$03.30

ions, we developed a sensitive and quantitative analysis of the three anions ethyl phosphite, phosphite and phosphate.

The speed, accuracy and sensitivity afforded by gas–liquid chromatography (GLC) offers definite advantages over other analytical methods. Among the derivatization techniques for oxyanions already reported for GC using a flame ionization detector, silylation is the most appropriate. Hashizume and Sasaki[6] first reported the GC detection of the trimethylsilyl derivative of orthophosphate. Similar studies have employed other silylating agents[7–10]. Recently, Mawhinney[11] reported the conversion of inorganic oxyanions into their *tert.*-butyldimethylsilyl derivatives by using N-methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA), a stable silylating agent.

However, application of this method to the analysis of biological materials necessitated purification before derivatization and GC analysis. Anion-exchange chromatography using a standard high-performance liquid chromatographic (HPLC) apparatus with UV detection, which has been described as a suitable method for separation of inorganic anions in water[12], was used for the purification step.

The purpose of this study was to determine the applicability of GC in combination with anion-exchange chromatography to the quantification of fosetyl-Al, its metabolite phosphite and phosphate in plant material.

EXPERIMENTAL

Standards and reagents

Standard samples. Phosphorous and phosphoric acids of analytical grade (Fluka, Buchs, Switzerland) were used to prepare aqueous stock solutions. Ethylphosphorous acid was obtained from fosetyl-Al (technical grade; Rhône-Poulenc Agrochimie, Lyon, France) after removal of the aluminium cations by a batch technique using a Dowex 50W-X8 cation-exchange resin (hydrogen form) (Bio-Rad, Glattbrugg, Switzerland).

HPLC buffers. Potassium biphthalate or sodium salicylate of analytical grade (Prolabo, Paris, France) were used to prepare buffered solutions (10 m*M*, pH 4.0). Water of HPLC grade (Fisons, Loughborough, U.K.) was used throughout. The buffers were prepared daily.

Silylating agents. Dimethylformamide (DMF) for gas chromatography was purchased from Prolabo and MTBSTFA was obtained from Chrompack (Middelburg, The Netherlands).

Extraction of biological material

Tomato and cowpea leaves were first frozen in liquid nitrogen and extracted twice by cold 0.1 *M* trichloroacetic acid (TCA) (1:5, w/v). TCA was eliminated by five washings with diethyl ether.

High-performance liquid chromatography

Aliquots of 2 ml (equivalent to 200 mg fresh tissue) of plant extract previously filtered on a 0.45-μm filter (Millipore, Molsheim, France) were injected in the HPLC apparatus.

The system used consisted of an U6K injector (Waters Assoc., Milford, MA,

U.S.A.), a single M 6000 A HPLC pump (Waters Assoc.) and a M 450 UV–visible detector (Waters Assoc.). The column (250 × 4.6 mm I.D.) packed with Ionospher^{tm} A (Chrompack) was operated at ambient temperature with a flow-rate of 2 ml/min and a pressure of 1320–1360 p.s.i. The optical density was monitored at 308 nm and recorded on a 730 Data Module (Waters Assoc.).

After HPLC purification, the fractions were passed through a microcolumn system consisting of three C_{18} Sep-Pak cartridges (Waters Assoc.) and a microcolumn containing a 1-ml resin bed of Dowex 50W-X8 (hydrogen form). The microcolumn employed was a pipette tip containing a small plug of glass wool at the bottom. The columns were connected in series, the three C_{18} cartridges being above the cation-exchange microcolumn. Samples were eluted with distilled water and evaporated to dryness at 45°C under vacuum. The final residue was redissolved in methanol and transferred to a reaction vial.

Derivatization

Samples placed into reaction vials were first evaporated to dryness with a stream of nitrogen gas. To these vials were then added successively 100 μl of DMF and 300 μl of MTBSTFA. Aliquots were then subjected to GC analysis.

Gas chromatography

A Girdel 30C gas chromatograph equipped with a flame ionization detector was used. The column was a fused-silica capillary (25 m × 0.22 mm I.D.) wall coated with CP^{tm}-Sil 5 (Chrompack). The injector and detector temperatures were 250 and 270°C respectively and the oven temperature was programmed from 100 to 200°C at a rate of 3°C/min. The carrier gas was helium with a column-head pressure of 0.7 bar. The splitless injection technique was used with a purge activation time of 0.5 min and an injection volume of 1 μl.

A SP-4200 computing integrator (Spectra-Physics, Orsay, France) was used to record and quantify the peaks.

RESULTS AND DISCUSSION

GC separation and quantitation of oxyanions

The separation of the *tert.*-butyldimethylsilyl (tBDMS) derivatives of ethyl phosphite, phosphite and phosphate in their acid forms is shown in Fig. 2. Each oxyanion derivative yielded a sharp chromatographic peak without tailing. No visual evidence of decomposition of the derivatized compounds could be found during GC analysis. Phosphite appeared to be stable toward oxidation, giving a peak distinct from phosphate. A small peak corresponding to phosphite was obtained when a reaction mixture of ethyl phosphite was injected. As was confirmed by anion-exchange chromatography, this is due to traces of phosphite (3%) present in the fosetyl-Al technical material.

The tBDMS derivatives of the three compounds were eluted in order of increasing molecular weight on CP^{tm}-Sil 5.

The sensitivity proved to be excellent; the minimum detectable amounts of phosphite and phosphate were 20 pmol and for ethyl phosphite, 50 pmol per injection volume (1 μl). The response curves for each compound were linear over the range of

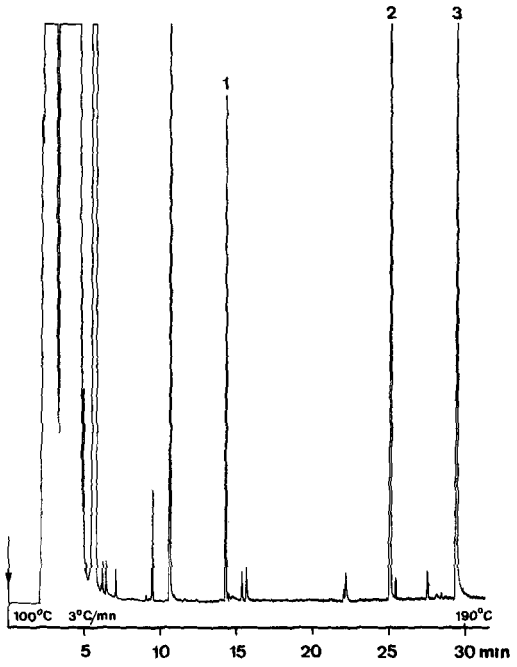

Fig. 2. Chromatographic separation of ethyl phosphite (1), phosphite (2) and phosphate (3) tBDMS derivatives on a fused-silica capillary column wall coated with CP^{tm}-Sil 5 (25 m × 0.22 mm I.D.). Temperatures: column, 100 to 200°C at 3°C/min; injector, 250°C; detector, 270°C. Carrier gas: helium (0.7 bar). Splitless sampling: volume injected, 1 μl. Each peak represents 200 pmol of each oxyanion.

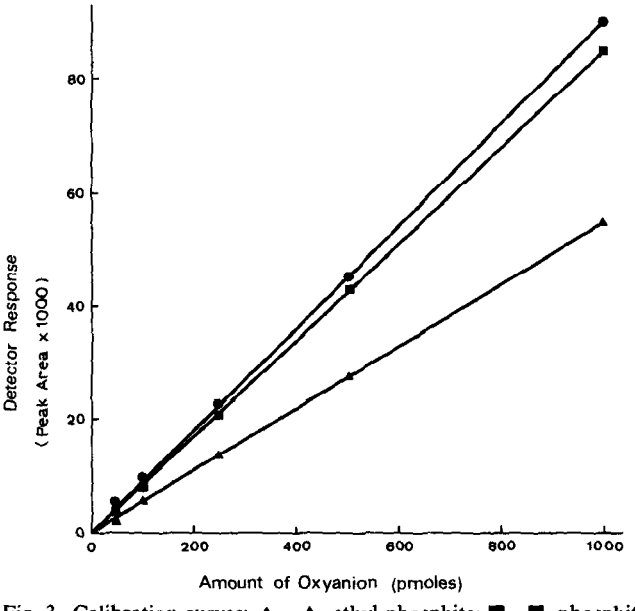

Fig. 3. Calibration curves: ▲—▲, ethyl phosphite; ■—■, phosphite; ●—●, phosphate.

20 pmol to 1 nmol (Fig. 3). In contrast to the derivatives of phosphite and phosphate which possess three tBDMS groups[11], the response to the ethyl phosphite derivative (lower molecular weight) is less strong.

Effect of plant material on derivatization

The formation of the tBDMS derivatives of the free acid forms of the three oxyanions was complete in less than 5 min. However, before application of this GC method to biological material, it was necessary to study its influence on the yield of the derivatization.

A known amount of phosphorous acid was added to a tomato leaf extract and, after evaporation to dryness and silylation by MTBSTFA, different aliquots were injected into the gas chromatograph. The presence of plant material reduced the peak areas of the derivatized phosphite and the response was inversely proportional to the amount of vegetable material (Fig. 4). Many interfering peaks were introduced, and repeated injections led to severe contamination of the injection port. These results showed that a rigorous purification is necessary before the quantification of the silylated oxyanions by flame ionization detection.

Anion-exchange purification

The purification of the biological material was performed by anion-exchange removal of the oxyanions of interest from the biological background. The analysis was accomplished in less than 30 min using a relatively low concentration of an UV-absorbing buffer. Fig. 5 shows the separation profile of a standard mixture of the three oxyanions. The concentration of buffer (10 m*M*) was adjusted in order to reduce the volume of the collected fraction.

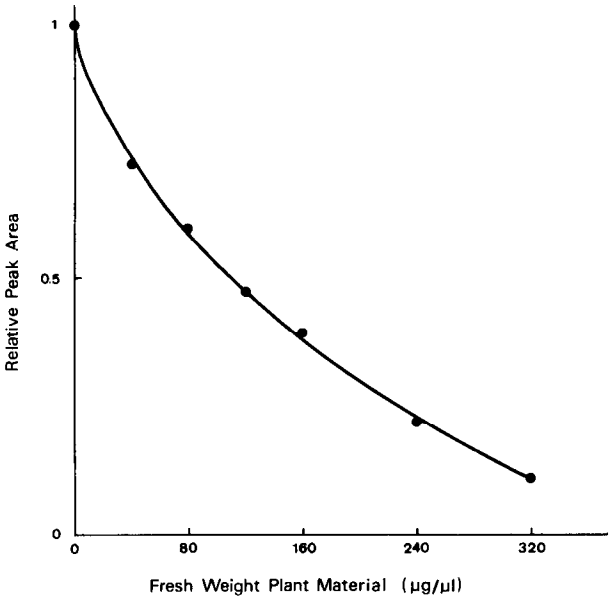

Fig. 4. Dependence of the $(tBDMS)_3PO_3$ peak area on the presence of plant material in the sample. The peak areas were normalized on the $(tBDMS)_3PO_3$ peak area obtained with a standard sample.

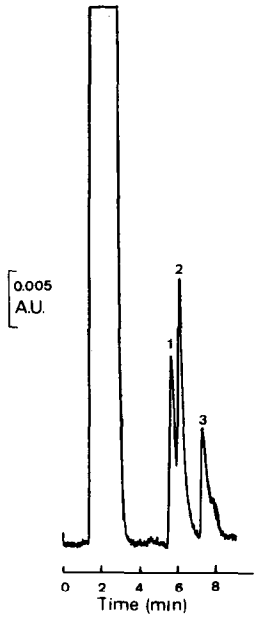

Fig. 5. HPLC separation of a standard mixture of oxyanions: 1 = phosphate; 2 = phosphite; 3 = ethyl phosphite. Column: 10-μm anion-exchange material (Ionospher^{tm} A), 250 × 4.6 mm I.D. Mobile phase: 10 m*M* biphthalate buffer (pH 4.0). Flow-rate: 2 ml/min. UV detection at 308 nm, 0.05 a.u.f.s.

Of the two tested buffers, sodium salicylate and potassium biphthalate, the second was more appropriate, giving better separation and repeatability. Prolonged utilization of sodium salicylate dramatically decreases column performance.

Silylation of a purified HPLC fraction of tomato leaf extract (200 mg) with MTBSTFA gave relatively smaller peaks of each derivative. This is due to the excess of potassium biphthalate and the formation of potassium salts of the oxyanions which are not soluble in the reaction mixture[10].

In order to optimize the yield of silylation, a further clean-up procedure was necessary. Elimination of biphthalate and conversion of the oxyanions into their free acid forms was achieved by a microcolumn system consisting of three C_{18} cartridges and a microcolumn of cation-exchange resin (hydrogen form). The utilization of the C_{18} cartridges allowed the elimination of *ca.* 80% of the biphthalate, and the cation-exchange resin removed the potassium cations.

Known amounts of ethyl phosphite and phosphite were added to plant material previously purified by HPLC and the microcolumn system just before derivatization. GC analysis of the micropreparation showed no decrease in the response of each tBDMS derivative, and the complete absence of interfering peaks.

A series of standard mixtures of ethyl phosphite, phosphite and phosphate were analysed by this total procedure (HPLC purification, C_{18} cartridges + cation-exchange column, silylation and GC analysis). A recovery of the three oxyanions of 85 ± 5.8% (S.D.) was found.

TABLE I

OXYANION CONTENTS OF TOMATO AND COWPEA LEAVES

Leaves were analysed after floating for 48 h on aluminium ethyl phosphite solution (0.56 μmol/ml). Each value represents duplicate analyses of an independent sample. n.d. = Not detected.

Oxyanion	*Amount (μmol/g)*	
	Tomato	*Cowpea*
Ethyl phosphite	n.d.	0.2
Phosphite	1.2	2.0
Phosphate	3.6	12.5

Application to the analysis of plant extracts

Detached tomato and cowpea leaves were placed in petri dishes with the lower surface in contact with an aqueous solution of aluminium ethyl phosphite (0.56 μmol/ml) containing benzimidazole (50 μg/ml). After 48 h under fluorescent light, the leaves were rinsed, washed and extracted as described above. Table I gives the oxyanion contents of the treated leaves.

CONCLUSION

The combined use of HPLC and GC in the analysis of ethyl phosphite, phosphite and phosphate yields an excellent sensitivity and the results are accurate and repeatable. Further application of this method to the quantification of other oxyanions of biological origin or pollutants is possible, therefore providing valuable information about their rôles in the physiology of living organisms.

ACKNOWLEDGEMENT

The authors thank Rhône-Poulenc Agrochimie for supplying technical grade fosetyl-Al.

REFERENCES

1 A. Bertrand, J. Ducret, J. C. Debourge and D. Horrière, *Phytiatr.-Phytopharm.*, 26 (1977) 3.
2 M. Clergeau and A. Beyries, *Phytiatr.-Phytopharm.*, 26 (1977) 73.
3 G. Bompeix, F. Fettouche and P. Saindrenan, *Phytiatr.-Phytopharm.*, 30 (1981) 257.
4 D. I. Guest, *Physiol. Plant Pathol.*, 25 (1984) 125.
5 G. Bompeix, A. Ravisé, G. Raynal, F. Fettouche and M. C. Durand, *Ann. Phytopath.*, 12 (1980) 337.
6 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 15 (1966) 199.
7 C. W. Gehrke and C. D. Ruyle, *J. Chromatogr.*, 38 (1968) 473.
8 W. C. Butts, *Anal. Lett.*, 3 (1970) 29.
9 W. C. Butts and W. T. Rainey, *Anal. Chem.*, 43 (1971) 538.
10 J. Harvey and M. G. Horning, *J. Chromatogr.*, 76 (1973) 51.
11 T. P. Mawhinney, *J. Chromatogr.*, 257 (1983) 37.
12 R. A. Cochrane and D. E. Hillman, *J. Chromatogr.*, 241 (1982) 392.