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GAS CHROMATOGRAPHIC DETERMINATION OF PHOSPHORUS IN NUCLEIC ACIDS AND NUCLEOTIDES AS TRIS(TRIMETHYLSILYL) PHOSPHATE

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SUMMARY

Conditions for the quantitative conversion of phosphate to tris(trimethylsilyl) phosphate and for its gas chromatographic analysis have been studied. Anomalies in the response of the flame ionization detector were observed, leading to a decrease in the detector linear concentration range. A procedure for the determination of phosphorus in nucleic acids and nucleotides has been proposed and tested. The results of this gas–liquid chromatographic analysis have been compared with those of the spectrophotometric method.

INTRODUCTION

A number of methods have been proposed for the determination of phosphorus[1], the widest use being made of spectrophotometry. Recently, a gas–liquid chromatographic (GLC) determination has been developed[2–6] based on the conversion of the organophosphate into inorganic phosphate, preparation of a volatile tris(trimethylsilyl) derivative and its GLC analysis.

Determination of phosphorus in nucleic acids plays an important role. The chief advantage of the GLC determination of phosphorus is the possibility of combining it with simultaneous determination of the other components of the nucleic acids, so that their composition can be established in a single experiment.

The present work aims at examination of conditions for quantitative phosphorus analysis, in connection with research in the GLC analysis of nucleic acid components carried out in our department[7], and attempts to explain some anomalies which have been encountered in this phosphorus determination.

EXPERIMENTAL

The GLC measurements were carried out with a commercial gas chromatograph (Model C; Carlo Erba, Milan, Italy). Glass columns, 180 cm × 4 mm I.D., were used. The columns were packed with silanized Chromosorb W (60–80 mesh), with 5 wt.% OV-101 stationary phase. The measurements were performed isothermal-

ly at 155°. The flow-rates of nitrogen carrier gas, hydrogen, and air were 20, 27, and 300 ml/min, respectively.

The silyl derivatives were prepared from dried samples in bis(trimethylsilyl)-acetamide (BSA) or bis(trimethylsilyl)trifluoroacetamide (BSTFA) in ampoules closed with silicone rubber septa and heated in an electric block.

For removal of Na^+ or K^+ from the hydrolysates, a column, 6.5 cm × 5 mm I.D., containing Dowex 50 cation exchanger, was employed. The hydrolysate was eluted from the column with distilled water, in an amount five times greater than its volume.

RESULTS AND DISCUSSION

Phosphate anions react with silylating agents —hexamethyldisilazane (HMDS) + trimethylchlorosilane (TMCS), BSA or BSFTA— to form the volatile tris(trimethylsilyl) phosphate [$(TMS)_3PO_4$]. The substance yields a single elution curve, whose retention index on the OV-101 stationary phase is 1282.6 at 155°.

The silylation is complete when the sample is entirely dissolved. Among the inorganic phosphates studied only ammonium phosphates and phosphoric acid were quantitatively silylated. The worst silylation yield was obtained with Na_3PO_4 (roughly 5%); NaH_2PO_4, K_3PO_4, K_2HPO_4, and KH_2PO_4 were silylated to about 30%. Various solvents (benzene, diethyl ether, carbon disulphide, pyridine, dimethylformamide, and acetonitrile) were tested, but they did not influence the yield of the silylation reaction.

However, the course of the silylation reaction is considerably affected by the temperature. Phosphoric acid reacts immediately with BSTFA at laboratory temperature, while silylation of ammonium phosphate takes 12 h at this temperature. The optimum temperature for ammonium phosphates is 150°, at which the reaction is completed within 10–15 min.

As was found from the measurement of the dependence of the relative molar response, $RMR_{(TMS)_3PO_4/naphth.}$, on the amount of silylating agent, a five-fold molar excess of BSTFA is sufficient for complete conversion. However, it is more advantageous to employ a ten- to twentyfold excess, thus preventing solidification of the sample (the m.p. of the reaction product N-trimethylsilyl-trifluoroacetamide (MSTFA) = 46°) and eliminating the negative effect of moisture.

On standing, an unknown reaction product was formed in the samples, making the quantitative evaluation of the tris(trimethylsilyl) phosphate peak more difficult. This substance was isolated and identified by infrared and mass spectroscopy as a polysiloxane.

In the GLC analysis of tris(trimethylsilyl) phosphate, some anomalies in the flame-ionization detector response were encountered. It was observed that the response is linear only within a region from 0.4 to 24 μg tris(trimethylsilyl) phosphate for the instrument employed. With higher amounts, peak splitting and eventually complete inversion occurred (Fig. 1). Similar anomalies were described by Lovelock[8], Pollard *et al.*[9], and Garzó and Fritz[10]. Cooling of the detector flame during burning of Si components is the probable cause. During combustion of Si-containing substances, solid SiO_2 is formed and is deposited on the electrodes, thus decreasing the detection sensitivity. It also causes a high noise value and therefore the detector must be frequently cleaned.

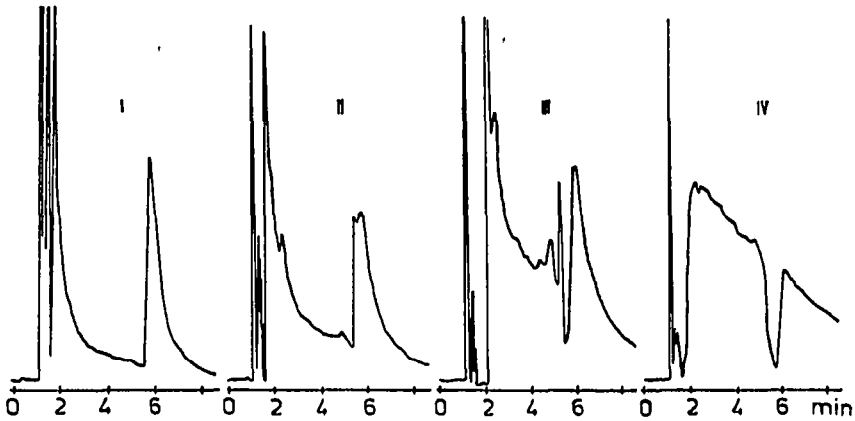

Fig. 1. Deformation of the tris(trimethylsilyl) phosphate peak with increasing sample size. I = 6.2 μg, II = 31.2 μg, III = 124.8 μg and IV = 187.2 μg tris(trimethylsilyl) phosphate.

Under the optimum silylation conditions, *i.e.* heating phosphoric acid or its ammonium salts with a ten- to twentyfold molar excess of BSA or BSTFA for 10 min at 150°, $(TMS)_3PO_4$ was prepared and its *RMR* with respect to naphthalene was measured. The mean value of ten independent measurements was 0.70 $\pm$ 0.02. It was found that the *RMR* values depend considerably upon the gas flow-rate through the detector and on the detector design. By using another commercial instrument with a flame ionization detector (Model F-11; Perkin-Elmer, Norwalk, Conn., U.S.A.), an entirely different value, 0.25 $\pm$ 0.015, was obtained under otherwise identical experimental conditions.

Determination of phosphorus in nucleic acids and nucleotides

For application of the method to organic phosphates, three substances were chosen: ribonucleic and deoxyribonucleic acids and adenosine 5'-monophosphate. It is evident from the above that phosphorus can be determined by GLC after its conversion into phosphoric acid or its ammonium salts. Therefore it is first necessary to release the bound phosphate by hydrolysis, then to remove sodium or potassium ions, neutralize the sample with ammonia and dry it. The treated sample can then be silylated in the manner described above.

The method of King[11], employing concentrated $HClO_4$ and H_2O_2 for RNA and concentrated $HClO_4$ or concentrated HCOOH (advantageous because of its volatility) for DNA, is satisfactory. Purine nucleotides are completely ruptured by hydrolysis with 6 *N* HCl (ref. 12).

Nucleic acids and nucleotides are obtained during isolation in the form of sodium or potassium salts. A column of Dowex 50 was used for removing the sodium or potassium ions from the hydrolysate. The minimum amount required of the eluent was determined using [^{32}P]phosphoric acid.

After the ion-exchange procedure and neutralization, the sample is diluted with water and cannot be directly silylated in this form. It must first be dried, which is performed either by lyophilization or in an oven at 110°. The dried sample can then be silylated and chromatographed by the procedure above.

TABLE I

DETERMINATION OF PHOSPHORUS IN VARIOUS PHOSPHATES

Compound	*Phosphorus content (wt.%)*		
	GLC	*Spectrophotometry*	*Theory*
$Na_2HPO_4 \cdot 12H_2O$	8.2	8.6	8.66
AMP, disodium salt	7.2	8.8	8.64
RNA, commercial	8.8	11.0	10.6
RNA, isolated from rabbit liver, in our laboratory	9.4	12.3	—
DNA, isolated from calf thymus	8.1	10.3	9.05

The results of the determination of phosphorus in various inorganic and organic phosphates by GLC are given in Table I. For comparison, the results of spectrophotometric determinations[11] and theoretical values quoted in the literature[13] are also given. The deviations between the individual methods can be due to loss of tris(trimethylsilyl) phosphate in the chromatographic system, especially due to its decomposition in the injection block. For this reason we are at present testing an all-glass gas chromatograph with capillary columns for the analysis of silyl derivatives.

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