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DETECTION AND QUANTITATION OF OXALIC ACID BY CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

Oxalic acid was extracted from plant tissue and quantified by a rapid and accurate procedure using capillary gas chromatography (GC). Samples derived from leaves, stems, and woody plant tissue were extracted with 3 *M* hydrochloric acid. Oxalic acid in these extracts was esterified with diazomethane in ether and determined as dimethyl oxalate by capillary GC. Recoveries of oxalates added to plant preparations ranged from 92 to 105% as compared to a range of 96–102% for oxalate standards added to 3 *M* hydrochloric acid. Oxalate was detected and quantified in cucumber leaves, rhubarb, woody tissue of American chestnut infected with the fungus *Endothia parasitica* and an agar culture of *Endothia parasitica*. This is the first report of the presence of oxalate in cucumber foliage. The new procedure using diazomethane does not require time consuming purification of the extracts before capillary GC analysis. Unlike alcoholysis, it results in complete esterification. The determination limit for this method of oxalate quantitation is approximately 500 pg using a flame ionization detector.

INTRODUCTION

Measurement of oxalic acid has important applications in areas of medical and plant research. Oxalic acid is thought to have a role in renal stone formation[1,2]. Oxalate is also a plant metabolite, and crystalline deposits of calcium oxalate are found in many plant tissues[3]. Recent studies have demonstrated that aqueous solutions of potassium oxalate, applied to the first two leaves of cucumber, induced systemic resistance to several fungal pathogens[4]. Studies of the metabolism of oxalates and their possible role in induced resistance require sensitive methods for the detection of low levels of oxalates.

Spectrophotometric[5], isotope dilution mass spectrometric[6], electrochemical[7], liquid chromatographic[8], high-performance liquid chromatographic (HPLC)[9], (GC)[10–12], ion chromatographic[13] and enzymatic[14,15] methods have been used to

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separate and quantify oxalic acid. The methods listed above are either less sensitive or inappropriate for crude plant extracts when compared to the method described in this paper.

The present paper describes a simple, rapid, sensitive and reproducible procedure for the detection of oxalates useful for quantitative analyses of these compounds in samples from different origins. The procedures described in this paper include modifications of previously published methods for the extraction of oxalic acid and the derivatization of oxalic acid prior to determination by capillary GC[10–12].

EXPERIMENTAL

Chemicals

All chemicals were of a analytical grade. Diazomethane was generated *in situ* from N-methyl-N-nitroso-*p*-toluenesulfonamide (Sigma) by a standard procedure. Dipropyl oxalate, and diisopropyl oxalate were synthesized by alcoholysis and purified by thin-layer chromatography (TLC) on Silica gel G (0.25 mm) (Analtech). Dimethyl oxalate and diethyl oxalate were obtained from Sigma.

Samples

Cucumber plants (cultivar Wisconsin SMR 58) were grown in a greenhouse. Rhubarb was purchased at a local market. Wood of the American chestnut (*Castanea tentata*) infected with the fungus *Endothia parasitica* was harvested from trees in the Lexington, Kentucky area. *E. parasitica* was grown on an amended potato dextrose agar medium in a standard petri dish[16].

Tissue extraction

A 5 g amount of cucumber leaf tissue was cut into small pieces with a razor blade and homogenized at high speed (Brinkmann, PT 10-35) with 25 ml of 3 *M* hydrochloric acid. The suspension was centrifuged at 25 000 *g* for 5 min and the supernatant filtered through Whatman No. 1 paper. The residue retained by the filter was extracted twice with 15 ml of 3 *M* hydrochloric acid and the combined filtrates were adjusted to 60 ml. Samples (5 g fresh weight) from rhubarb leaves, wood of American chestnut (*Castanea tentata*) infected with the fungus *E. parasitica*, and an agar culture of *Endothia parasitica* were extracted as described above with 60 ml of 3 *M* hydrochloric acid. Aliquots (6 ml) of the 3 *M* hydrochloric acid extracts were used for methyl esterification and subsequent GC analysis.

Alcoholysis of oxalic acid

A 6-ml volume of 3 *M* hydrochloric acid extract was evaporated to dryness under reduced pressure at 40–45°C. Four different oxalic acid esters were made using methanol, ethanol, isopropanol, and *n*-propanol. Dimethyl oxalate was prepared by adding 5 ml of 7% hydrochloric acid in methanol to the evaporated plant extract. The solution was then heated to 60°C for 30 min. Diethyl oxalate and diisopropyl oxalate were prepared by the addition to evaporated plant extract of either 5 ml of 7% hydrochloric acid in ethanol or 5 ml of 7% hydrochloric acid in isopropanol. In the preparation of diethyl oxalate, the solution was heated for 30 min at 70°C, whereas diisopropyl oxalate was prepared by heating the solution for 45 min at 80°C. For the

preparation of dipropyl oxalate, 5 ml of *n*-propanol was added to the evaporated plant extract, and the solution was then heated to 105°C for 3.5 h. After each alcoholysis was complete, 10 ml of water was added. The solution was then extracted with 30 ml (three times at 10 ml each) of chloroform, and the combined chloroform extracts were concentrated by rotory evaporation at 40°C prior to GC and TLC analysis.

Esterification with diazomethane

A 6-ml volume of the 3 *M* hydrochloric acid extract was evaporated to dryness at 40–45°C and 12 mmHg using a vacuum rotary evaporator. Diethyl ether was added to the dry, hydrochloric acid-free residue. The ether solution was then transferred to a graduated 15-ml vial and evaporated under a stream of nitrogen to a volume of 5 ml. To this solution, 10 ml of freshly prepared diazomethane (0.05 mol N-methyl-N-nitroso-*p*-toluenesulfonamide, 3 g potassium hydroxide; 100 ml diethyl ether) was added. The esterification was complete when bubbles were no longer visible and the yellow color of the solution was stable. The vial was closed by a screw cap and stored at 4°C until GC analysis.

TLC

After esterification, solutions derived from plant extracts or pure compounds were chromatographed on silica gel G plates using cyclohexane–ethyl acetate (3:1, v/v) as the mobile phase. Spraying the plates with hydroxylamine hydrochloride–iron (III) chloride resulted in a violet color reaction for mono- and diesters of oxalic acid.

Capillary GC

A Perkin-Elmer Sigma 2000 gas chromatograph equipped with a computer integrator, flame ionization detector and a 25 mm × 0.32 mm fused-silica column coated with methyl silicone (OV-1) at a film thickness of 0.25 μm was used for analyses. A 1-μl aliquot of the 15 ml ether solutions was used for the determination of oxalate content by capillary GC. The split injection port of the instrument was adjusted to a split ratio of 18:82. Nitrogen was used as a carrier gas at a flow-rate of 15 ml/min through the column with an inlet pressure of $1.0 \cdot 10^5$ Pa. The column temperature was held at 70°C initially, then raised at 30°C/min to 220°C with an isothermal hold for 15 min at 220°C. The detector and injector port were set at 270°C. The amount of oxalate in plant extracts was quantitated by comparing the peak areas of the detector response to injections of plant samples and known amounts of a dimethyl oxalate standard. The dimethyl oxalate standard was dissolved in ether prior to injection.

RESULTS

Methods of esterification

Two methods for derivatization of oxalic acid. Alcoholysis resulted in only a partial conversion of oxalic acid to the diester derivative. The relatively high temperatures required for alcoholysis also increased the number of contaminants in plant extracts which often cochromatographed with compounds of interest. Diazomethane was used as an alternative method for esterifying oxalic acid. In contrast to alcoholysis, esterification with diazomethane resulted in the total conversion of oxalic acid to

dimethyl oxalate. Dissolving the evaporated 3 *M* hydrochloric acid extracts in ether before esterification with diazomethane removed many contaminants. This was the only sample cleanup necessary prior to GC analysis.

Capillary GC

Analyses were performed on plant extracts which contained esterified oxalates using different oxalic acid diesters as standards. Fig. 1 shows the chromatogram for dimethyl, diethyl, diisopropyl and dipropyl oxalate at 90°C column temperature. The dimethyl ester of oxalic acid was the most useful ester derivative in quantitating oxalate in plant extracts since peaks of contaminants in plant extracts interferred with the peaks for the diethyl, diisopropyl and dipropyl oxalate, whereas contaminant peaks did not interfere with the peak of dimethyl oxalate. A decrease in the column temperature to 70°C enhanced the separation of the peak of dimethyl oxalate from the solvent front.

Fig. 2 shows chromatograms of dimethyl oxalate obtained from a standard solution and from cucumber leaf extract at 70°C. As shown by this figure, the peak of dimethyl oxalate was well separated from peaks of contaminants in plant extracts.

Recovery of applied oxalates

The results of oxalate recovery studies are shown in Table I. The average recovery for the three different compounds added to cucumber leaf tissue and extracted with 3 *M* hydrochloric acid was 97% and the average recovery for the three compounds added to 3 *M* hydrochloric acid extracts of cucumber leaf tissue was 102%. Treatment of cucumber leaf tissue with 3 *M* hydrochloric acid led to complete extrac-

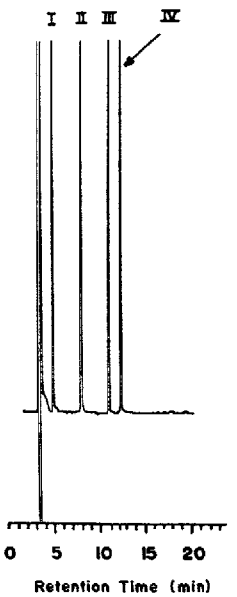

Fig. 1. Chromatogram of different oxalate diesters. Peaks I, II, III, and IV correspond to dimethyl, diethyl, diisopropyl and dipropyl oxalate, respectively.

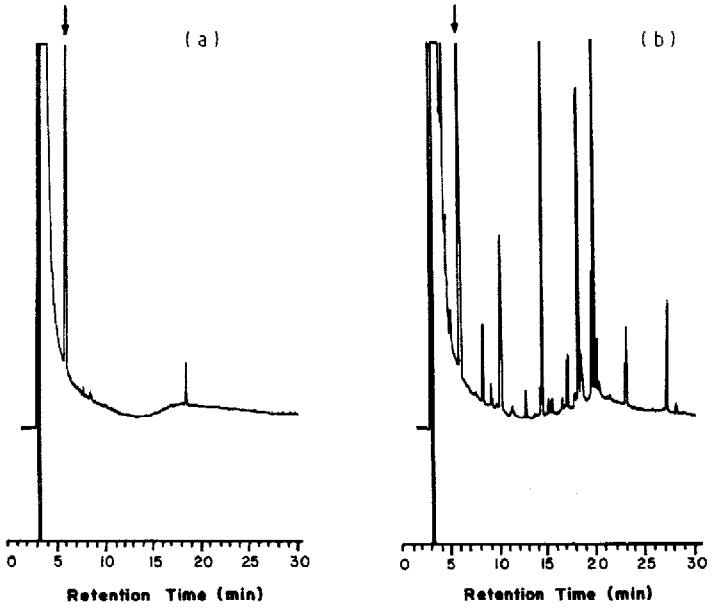

Fig. 2. Chromatograms of dimethyl oxalate standard (A) and derivatized cucumber leaf extract (B). The peaks indicated by the arrow correspond to dimethyl oxalate.

TABLE I

RECOVERY OF POTASSIUM OXALATE, OXALIC ACID AND DIETHYL OXALATE ADDED TO 3 *M* HYDROCHLORIC ACID, AQUEOUS EXTRACTS FROM CUCUMBER LEAVES AND FRESH CUCUMBER LEAF TISSUE

Potassium oxalate, oxalic acid and diethyl oxalate were added separately to three 60-ml aliquots of 3 *M* hydrochloric acid, 3 *M* hydrochloric acid extracts of 5 g (fresh weight) cucumber leaf samples and to three 5-g (fresh weight) freshly harvested cucumber leaf samples. The freshly harvested cucumber leaf samples were extracted with 60 ml of 3 *M* hydrochloric acid. Dimethyl oxalate was prepared with diazomethane and quantified by GC described in the text.

Sample	*Form of oxalate added*	*Amount added (mg)*	*Amount quantified by GC analysis (mg)*	*Recovery (%)*
3 *M* HCl	Potassium oxalate	168	162	96
(60 ml)	Oxalic acid	90	92	102
	Diethyl ester	146	144	99
Extract from	Potassium oxalate	168	168	100
cucumber leaves	Oxalic acid	90	90	100
(60 ml)	Diethyl ester	146	153	105
Fresh cucumber	Potassium oxalate	168	154	92
leaf tissue	Oxalic acid	90	87	97
(5 g fresh weight)	Diethyl ester	146	148	101

tion of oxalates. Repeated extraction of the remaining residue failed to yield detectable levels of oxalate.

Determination of oxalic acid content

The oxalate concentration was determined for different plant samples and the oxalic acid producing fungus *Endothia parasitica* (Table II). The amount of oxalic acid detected in rhubarb, *Castanea tentata* and *Endothia parasitica* was similar to that reported previously[4]. Oxalic acid was also found in cucumber leaves and the amount increased as a function of age (Table II).

DISCUSSION

A capillary GC method has been developed for the rapid and accurate determination of oxalic acid and oxalates. The procedure described in this report is useful for the determination of very low concentrations of these compounds by derivatization to dimethyl oxalate. This technique also does not require time consuming purification steps before analysis.

In order to develop a reliable technique for determination of oxalates, it was necessary to have an efficient method for derivatizing oxalic acid. Different methods of derivatizing oxalic acid were investigated. These methods included alcoholysis with methanol, ethanol, *n*-propanol and isopropanol as well as esterification with diazomethane. Monoesters of oxalic acid were found after alcoholysis, which indicated that the formation of the diesters was not complete. On the other hand, methyl esterification with diazomethane was complete, and therefore, it was the method of choice for quantitative analysis of oxalic acid.

The technique for determination of oxalates outlined in this paper is reliable as evidenced by the high levels of recovery we observed from samples of different origins as well as by the low determination limit for dimethyl oxalate compared to known GC and HPLC methods[4,9,10]. We found that the determination limit of our capillary

TABLE II

DETERMINATION OF OXALIC ACID AND OXALATE IN DIFFERENT PLANT MATERIAL

Sample	*Oxalic acid*[a] *(μg/g fresh weight)*
Rhubarb	980
Cucumber leaf No. 1[b] (2 weeks old)	ND[c]
Cucumber leaf No. 1 (3 weeks old)	57
Cucumber leaf No. 1 (4 weeks old)	217
Castanea tentata, infected with *Endothia parasitica*	5904
Endothia parasitica	4519[d]

[a] One determination.

[b] Refers to the first true leaf. Leaves were from two plants at each age.

[c] None detected.

[d] Data for one agar culture of the fungus representing the total oxalic acid content of agar and fungus.

GC for dimethyl oxalate to be approximately 500 pg, whereas the lowest limit of determination for potassium oxalate was about 200 mg (ref. 4).

Though oxalic acid has been reported previously in spinach, rhubarb, stem material of *Castanea tentata* infected with *E. parasitica* and a culture of *E. parasitica*[4,17], we are not aware of any reports of oxalic acid in cucumber. The data suggest that the oxalate content increases with age in cucumber leaves. Although the exogenous application of oxalate has been demonstrated to induce resistance to fungal diseases in cucumber[4], it is not known what role endogenous oxalates have in the expression of resistance in cucumber.

The procedure described in this paper for the determination of oxalates is simple, rapid, reliable and sensitive. This technique should be useful for the determination of oxalate from a variety of sources.

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