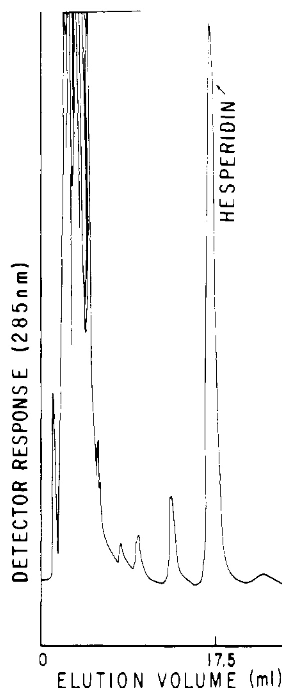

Figure 1. Separation of hesperidin in orange juice. For experimental details see text.

authentic hesperidin. The identity was also supported by peak enrichment. The hesperidin used in all the above standardization and identification work was purified by three crystallizations from dimethylformamide–water and drying in vacuo at 110 °C, mp 259–261 °C.

RESULTS AND DISCUSSION

This procedure can be carried out directly on filtered orange juice without extractions. The determination is quantitative, strictly objective, simpler, and faster than prevalent hesperidin determinations. The time required for a complete analysis was 25 min. The fresh hand-squeezed, processed single-strength orange juice or reconstituted concentrate all gave the same general chromatographic pattern (Figure 1). The samples were filtered to remove particulate material which may clog the system. The hesperidin was eluted isocratically after approximately 17.5 mL (11.5 min) (Figure 1). The number of theoretical plates for the column, using hesperidin as the reference peak, was 2675, equivalent to a plate height of 0.11 mm. The column capacity factor, k', was 5.0.

The base sample of fresh Hamlin orange juice used in the reliability test was collected under conditions designed to minimize its hesperidin content. The amount of native hesperidin in this base sample was 50 ppm. The recoveries of hesperidin from the five fortified base samples were all within ±8% of the hesperidin added. In order to fortify the base orange juice samples with hesperidin, it was necessary to add the appropriate aliquot of a solution of hesperidin dissolved in dimethylformamide to each base sample. The ultrasonic bath was helpful in effecting dissolution. The range of hesperidin found in the five repeatability experiments was 210 to 225 ppm, with a mean of 216 and a standard deviation of ±5.6 ppm.

A plot of peak areas vs. μg of hesperidin showed linearity over the range of 1.0 to 10.0 μg (r = 0.993).

An example of the use of this procedure is seen in Table I which shows the increasing amount of hesperidin found in Valencia and Hamlin orange juice obtained with increasing extractor pressure.

Table I. Hesperidin[a] in Orange Juice Obtained at Different Extractor Pressures

juice	soft squeeze, ppm[b]	medium squeeze, ppm[b]	hard squeeze, ppm[b]
Valencia	98	107	120
Hamlin	54	73	100

[a] Calculated from the linear regression equation.
[b] Average of five samples.

In the author's opinion, this procedure improves on previous hesperidin analyses in time of sample preparation and detection, as well as precision and reliability.

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Gas Chromatographic Determination of Diquat Residues in Potato Tubers

A gas chromatographic method is described for the determination of diquat residues in potato tubers by reduction of diquat with sodium borohydride to a volatile diamine derivative. It is demonstrated that reduction with sodium borohydride effectively displaces diquat from both soil and potato tubers without prior acid hydrolysis. After extractive cleanup the diquat derivative could be detected at levels down to 0.01 ppm in potatoes with a nitrogen–phosphorus specific detector. Recoveries of diquat from potatoes at fortification levels of 0.05 to 1.00 ppm averaged 87.4 ± 4.1%.

The herbicide diquat (1,1′-ethylene-2,2′-bipyridylium dibromide) is an extremely effective chemical dessicant, widely used for destruction of the potato foliage prior to harvest. This treatment occasionally causes damage to the

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tubers (Headford and Douglas, 1967) and also increases the possibility of storage rot (Logan et al., 1976). In order to provide information with regard to the relationship between the diquat residue levels in tubers and the extent of damage we required a sensitive and efficient method for analysis of diquat.

The major obstacle involved in analysis of diquat relates primarily to its cationic nature. To date, the most effective means discovered for displacing the herbicide from soils and crops when it is present in relatively low concentrations is to reflux the sample with strong acids, e.g. 18 N sulfuric acid. Boiling with strong sulfuric acid represents more than elution. The structure of the adsorbent is partially destroyed, and the binding sites are thus eliminated. The most widely used technique for the detection of diquat is colorimetry after concentration by cation-exchange chromatography (Calderbank and Yuen, 1965, 1967; Tucker et al., 1967). Khan (1974) was able to by-pass the time-consuming ion-exchange step by direct catalytic hydrogenation of the acid extracts. This procedure furnished two volatile derivatives which could be directly determined by gas chromatography using a flame ionization detector (Soderquist and Crosby, 1972).

In an extension of a method developed for the determination of diquat and paraquat in aqueous preparations (Ukai et al., 1973) we have established that reaction with sodium borohydride effectively displaces diquat from both soil and potato tubers without prior acid hydrolysis. The consequent reduction product after extractive cleanup can readily be determined at the 0.01 ppm level with a nitrogen–phosphorus specific detector.

EXPERIMENTAL SECTION

Chemicals. Sodium borohydride was purchased from Matheson, Coleman and Bell, Norwood, Ohio. Diquat dibromide was supplied by Chevron Chemical Co., Richmond, Calif., and was added in aqueous solution to the soil and plant materials in 1.0-mL volumes/5 g.

Equipment. Mass spectra were determined by a Finnigan 3100 GC/MS coupled to a D600 data aquisition system.

Gas–Liquid Chromatography. A Tracor 222 gas chromatograph equipped with a Perkin Elmer nitrogen–phosphorus detector and 1.8 m by 4 mm i.d. glass columns packed with 3% OV-17, 3% OV-101, and 3% Carbowax 20M + 1% KOH on 80–100 mesh H. P. Chromosorb W were used. The operating parameters were: injection port, 210 °C; column, 170–180 °C as indicated; and helium carrier gas flow rate, 60 mL/min.

Reduction of Diquat. A reference sample of the reduced herbicide was prepared by stirring diquat dibromide (0.5 g) in 95% ethanol (30 mL) with 5% $NaBH_4$ in H_2O (5 mL) for 30 min. The solution was acidified with 2 N HCl and the ethanol removed under vacuum. The aqueous residue was diluted with water (30 mL), transferred to a separatory funnel, and extracted once with chloroform (40 mL). The water layer was made alkaline with 3 N NaOH and extracted with chloroform (3 × 40 mL). The chloroform extracts were dried over anhydrous sodium sulfate, and the chloroform was removed under vacuum at room temperature. The colorless oil-like material (approximately 250 mg) obtained was relatively volatile and became gradually brownish-colored on prolonged exposure to air. GLC–MS indicated a mass spectral parent peak of m/e 190 for the major component (>90%).

ANALYTICAL PROCEDURES

Samples of diced potato tubers (5 g) were homogenized with 95% ethanol (50 mL) in a Waring blender for 3 min.

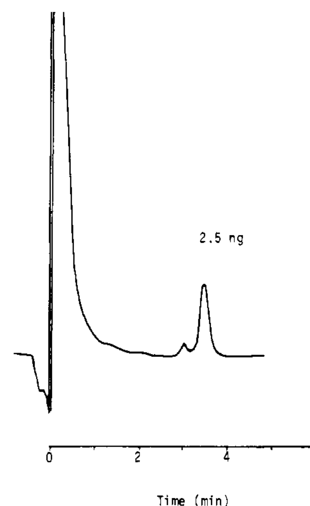

Figure 1. Gas chromatogram of the $NaBH_4$ reduction products derived from diquat (3% OV-101 with column temperature of 170 °C).

The macerated material was transferred to a 100-mL round-bottomed flask. The blender jar was rinsed with 95% ethanol (10 mL) and this was added to the flask. Sodium borohydride (50 mg or 1 mL of a 5% aqueous solution) was added to the flask and the contents stirred for 30 min. The reaction mixture was acidified with 2 N HCl and then filtered through Whatman No. 1 filter paper. Removal of the ethanol on a rotary evaporator (water bath at 35 °C) left an aqueous residue which was diluted to approximately 15 mL. This, along with an additional 5-mL water rinse, was transferred to a 125-mL separatory funnel. Chloroform (30 mL) was added to the contents of the separatory funnel, it was shaken, and the chloroform layer was discarded. The water layer remaining was made alkaline with 3 N NaOH and then extracted with chloroform (3 × 25 mL). A portion of the chloroform extracts was added to a 50-mL round-bottomed flask containing 2 N HCl (2 drops) and shaken, and the chloroform was removed under vacuum. The remaining chloroform extracts were added to the same flask, shaken, and then removed under vacuum. Exactly 2 mL of hexane and 3 drops of 3 N NaOH were added to the flask and the contents were shaken vigorously. Subsequently 10-μL aliquots of the hexane layer were drawn off in a chromatographic syringe for injection into the GLC.

For soils, the samples were filtered and air-dried (after fortification by stirring with aqueous solutions of diquat for 30 min) at room temperature. Subsamples (2 g) were used for the extraction of residues and with the exception of the blender homogenization step analyzed by the procedure described for potato tubers.

All samples were analyzed in duplicate and average values were reported.

RESULTS AND DISCUSSION

Sodium borohydride ($NaBH_4$) reduction of diquat in contrast to catalytic hydrogenation furnishes only one major product (Figure 1). This product has previously been identified as the *N,N'*-ethylene-1,1',2,2',3,3',6,6'-octahydro-6,6'-bipyridyl analogue (Ukai et al., 1973). Variation of solvent, reaction time, or quantity of excess $NaBH_4$ used did not noticeably affect the derivative mixture or yield. , Most important, however, was the unexpected discovery that high recoveries of diquat (in the reduced state) from potato tubers could be achieved without recourse to an initial acid hydrolysis (Table I).

Table I. Recovery of Diquat from Fortified Samples

sample	diquat added, ppm	recovery %
soil	0.1	60.2
	0.5	63.4
	1.0	66.7
	5.0	65.2
potatoes	0.05	85.3
	0.10	88.4
	0.50	87.1
	1.00	88.9

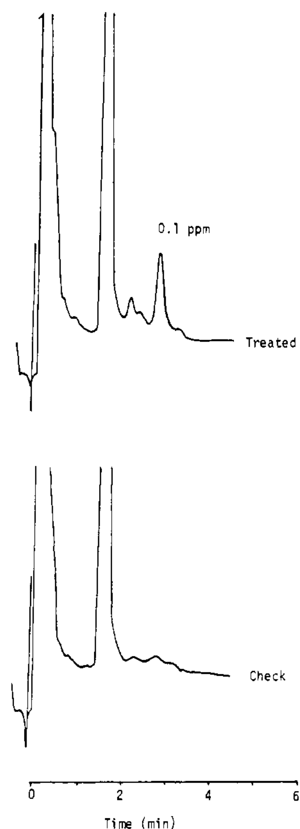

Figure 2. Gas chromatograms from determination of diquat in a clay loam soil (3% OV-17 with column temperature of 180 °C).

These findings suggest the possibility that either diquat is not bound by the tuber or if so it is still accessible to attack by the reducing agent.

To help clarify the situation, samples of a clay loam (which are known to tightly bind *N*-alkylpyridinium salts) were spiked with varying amounts of diquat dibromide (Table I). Subsequent $NaBH_4$ reduction and workup still gave only one major product but the yield of minor byproducts increased (Figure 2). The slight variation in the derivative mixture from reduction of diquat in soil is possibly a consequence of its bound nature therein. Of interest in the preceding context are the recent studies by Burdon et al. (1977) which indicate that in pyridinium and bipyridylium compounds (bound or unbound) the positive charges in the organocations are distributed around the molecule and not just on the nitrogen atom as previously assumed. This may explain the apparent accessibility of the nitrogen moiety in bound diquat for reaction with $NaBH_4$.

Recoveries of diquat from soil (based upon computation of the predominant derivative) were somewhat lower than from potato tubers but still averaged a respectable 64.1 ± 5.3% in the 0.1 to 5 ppm range.

The use of ethanol as the reaction solvent was preferable to methanol or water since methanol reacts quite rapidly

Figure 3. Gas chromatograms from determination of diquat residues in potato tubers (3% OV-101 with column temperature of 170 °C).

with $NaBH_4$ and a troublesome frothing problem is encountered with water. The subsequent extractive workup circumvents a potential loss of the volatile diquat derivative, a reference sample of which was consistently recovered in yields greater than 95% by this procedure.

Of the three columns tested, the Carbowax 20M + 1% KOH column produced sharper peaks and was the most sensitive. However, for the determination of diquat residues in potatoes and soils either 3% OV-101 or 3% OV-17 (Figures 2 and 3) were preferred due to their superior separation of the co-extractives.

The recovery of diquat added to potato samples in the 0.05 to 1.0 ppm range averaged 87.4 ± 4.1%. At each level, extracts equivalent to 5 g of sample were used. The lower limit of sensitivity was approximately 0.01 ppm of diquat using a nitrogen–phosphorus specific detector.

Samples of potato tubers taken from plants that had been top-killed with diquat were found to contain levels of diquat ranging no higher than 0.15 ppm. GLC–mass spectrometry was used to confirm that the peak occurring on GLC was due to the diquat derivative.

The foregoing method developed for the determination of diquat in potatoes is rapid and straightforward. It should be readily applicable to other crops and for other bipyridylium herbicides such as paraquat and cyperquat.

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Alfalfa Trypsin Inhibitor

Trypsin inhibitor was isolated from defatted alfalfa by extracting with 0.2 M NaCl, adsorbing on a trypsin–Sepharose affinity column, and eluting with a glycine buffer. Subsequent CM-cellulose chromatography did not improve its purity appreciably. Its amino acid composition was compared with that of Bowman-Birk soybean trypsin inhibitor and with an average composition of other proteins. Inhibitor content increased as plants matured. The inhibitor is present in greatest concentration in leaves.

Trypsin inhibitors are distributed widely in plants. Although their nutritional effects on animals are poorly understood generally, a trypsin inhibitor of soybean seeds depresses rat growth (Rackis, 1965; Kakade et al., 1973), while isolated corn grain inhibitor does not (Mitchell et al., 1976). Alfalfa, a major forage crop, also contains a trypsin inhibitor (Chien and Mitchell, 1970), but its nutritional effects on animals are not known. To determine its nutritional effects, it will be necessary to isolate the inhibitor in pure form and in appreciable quantity. The feasibility of such an isolation by affinity chromatography is reported here.

EXPERIMENTAL SECTION

Analytical Methods. Protein concentration was measured by the method of Lowry et al. (1951). Trypsin inhibitory activity was determined by the benzoyl DL-arginine p-nitroanilide assay (method II) of Erlanger et al. (1961) to measure trypsin activity. Preliminary work with crude extracts revealed a linear relation between inhibitor concentration and percent inhibition up to about 75%. It was convenient, therefore, to define an inhibitor unit as the amount of inhibitor which inhibits 50% of trypsin activity as measured by the Erlanger Assay procedure.

Isolation of Inhibitor. Chopped, fresh alfalfa was dehydrated by two extractions with acetone. The tissue was ground and again extracted with acetone to remove lipids and chlorophyll. The meal (200 g) was stirred in 1 L of 0.2 M NaCl for 4 h. The liquid was collected by vacuum filtration, and the residue was extracted again for 4 h with 600 mL of the salt solution. The extracts were combined and centrifuged at 10 000*g* in a refrigerated centrifuge. The supernatant was adjusted to 0.05 M Tris–HCl, pH 8.2, 0.02 M $CaCl_2$, and 1 M NaCl and again centrifuged to remove an inactive precipitate that formed. The inhibitor was adsorbed from the extract onto a trypsin–Sepharose affinity column (Swartz et al., 1977) on which 1 g of bovine trypsin had been attached. The column was washed thoroughly with the starting buffer to remove inactive material, and 0.1 M glycine–HCl buffer, pH 1.5, was then applied. Inhibitor activity eluted in a single sharp peak of about 100 mL. Progress of the washing and elution was monitored by measuring absorbance at 220 nm of the column effluents. Exposure of the inhibitor to pH 1.5 was limited to about 12 h at 4 °C.

Table I. Purification of Alfalfa Trypsin Inhibitor

stage	inhibitor units	protein, mg	sp act.	recovery, %
crude extract	11600	17000	0.67	100
affinity column	9600	85	113	83
Sephadex G-10 column	8400	68	122	72
CM-cellulose column	6800	55	122	59

The inhibitor activity was found to be dialyzable. Therefore, to exchange buffers in preparation for ion-exchange chromatography, active material from the affinity column was applied to a 3.5 × 45 cm Sephadex G-10 column that had been equilibrated with 0.01 M Tris–HCl, pH 7.5. The column was developed with the same buffer. The inhibitor activity eluted as a single early peak, presumably in the exclusion volume. It was followed by two smaller peaks of absorbance at 220 nm. The inhibitory fraction was applied to a 2.5 × 25 cm CM-cellulose column, which was developed with a linear gradient from 0 to 0.5 M NaCl in 0.01 M Tris–HCl buffer, pH 7.5. Only one peak of absorbance at 220 nm was obtained, and it was inhibitory (Figure 1).

The progress of purification through the various stages is shown in Table I. Most of the inhibitor was removed from the crude extract by the affinity column and was recovered from that column (83%). The gel chromatography step resulted in only a slight increase in specific activity, which indicates that the last two peaks in the elution profiles probably were not protein. No further increase in specific activity was obtained by CM-cellulose chromatography.

The active fractions from the affinity and CM-cellulose columns were subjected to 15% polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol (Maizel, 1969). The protein bands were made visible by staining with amido black. Although the bands were rather diffuse, each gel contained only one band, which is consistent with little improvement in purity

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