where A = mg. of calcium per ml. of EDTA solution [this is equal to 2.004 divided by milliliters of EDTA used in the standardization of the titrant]. B = ml. of EDTA solution in the calcium titration. C = weight of the dry sample in grams. D = mg. of magnesium per ml. of EDTA solution [this is equal to 1.216 divided by milliliters of EDTA used in the standardization of the titrant]. E = ml. of EDTA solution used in the total calcium and magnesium titration.

Results and Discussion

Four standard fruit tree leaf samples of different origin—apple, cherry, citrus, peach—and a standard alfalfa sample were analyzed to establish the validity of the method. The results obtained show the precision and accuracy of the procedure (Table I). Obtained values for calcium and magnesium were within the range given for the standard sample values, except for the calcium content of the peach sample. However, the reported values obtained by chemical, flame, and spectrographic methods for the calcium content of this sample are 1.94, 2.02, and 2.04, respectively (*5*).

To check the reproducibility of the proposed complete procedure—after the wet ashing—separations and automatic titrations were carried out for 10 equal aliquots of a synthetic sample (Table II).

The delivery rate of the titrant is adjusted at about 2.5 to 3 ml. per minute —both for the standardization of the titrant and for the determination of calcium and magnesium—to provide a signal from the Spectro titrator which is sufficient to terminate the titrations automatically at their end points. This can be done with either a suitable capillary delivery tip or by adjustment of the Teflon stopcock and pinch-off valve. At this delivery rate no correction is necessary for calcium, but a blank of 0.03 ml. is applied when Eriochrome Black T is used as indicator, because of a slight but reproducible overshooting of the equivalence point.

The phosphate (PO_4) in plant material ranges from about 0.4 to 1.3%. Five drops of a 1% zirconium nitrate solution were best suited for the removal of phosphates, within the above range, from the recommended 10-ml. aliquot solution containing approximately 0.2 gram of the original dry sample.

Larger amounts of zirconium nitrate should be avoided—except in samples much richer in phosphate—because the remaining free zirconium interferes with the sharp color change at the end point in both titrations, particularly in calcium determination, and thus may impede the automatic termination of the titration.

Although a double extraction with carbon tetrachloride is recommended in the procedure, the first extraction was usually sufficient for removal of iron and manganese. The addition of triethanolamine prior to the titration masks traces of iron, manganese, copper, and aluminum which may remain in solution to block the indicator. However, in most cases, the removal of the interfering ions was complete and the addition of triethanolamine was not necessary.

A 1% freshly prepared aqueous solution of sodium diethyldithiocarbamate is used instead of the solid salt to avoid precipitation of calcium and magnesium with the probable excess of the salt. Larger aliquots of the sample can be taken for titration in larger beakers. A 10-ml. aliquot—corresponding to about 0.08 gram of the dry sample—is recommended for each titration, because of the smaller quantities of reagents and titrant consumed and the shorter titration time. The accurate results obtained justify this consideration.

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Received for review October 27, 1958. Accepted March 12, 1959. Research supported in part by the United States Air Force under Contract No. AF 18(603)-137, monitored by the Air Force Office of Scientific Research, Air Research and Development Command.

GIBBERELLINS ANALYSIS

Infrared Determination of Gibberellins

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A simple infrared method designed for the determination of mixtures of gibberellic acid and of gibberellin A is described. The method is based on the determination of the absorbances at 12.86 and at 10.85 microns of a 15% solution of the sample in pyridine. These bands are characteristic of gibberellic acid and gibberellin A, respectively.

THE GREAT INTEREST in the plant hormones of the gibberellin group (*8*) justifies efforts to develop analytical methods for determination of the components in mixtures—at least four have been described. The Japanese workers have named these gibberellins A_1, A_2, A_3, and A_4. Gibberellic acid is considered equivalent to gibberellin A_3 and gibberellin A and A_1 are considered equivalent. The terms gibberellic acid (or gibberellin X) and gibberellin A are more commonly used in this country. This paper is concerned primarily with determination of gibberellic acid (gibberellin X, gibberellin A_3) and gibberellin A (A_1) in mixtures. Samples of A_2 and A_4 have not been available for analysis by this method.

Gibberellic acid and gibberellin A have been separated by paper chromatography by Bird and Pugh (*4*) and by Varner, Hargie, and Schenck (*10*). These methods are suitable for qualitative analysis, but cannot detect small amounts of one in the presence of large amounts of the other component. The radioisotopic method of Baumgartner *et al.* (*3*) has been used for quantitative analysis.

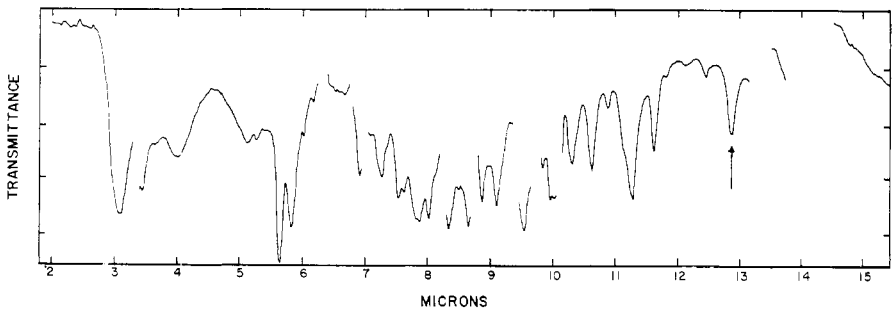

Figure 1. Gibberellic acid in pyridine

Breaks in the spectrum indicate regions of total absorption by pyridine

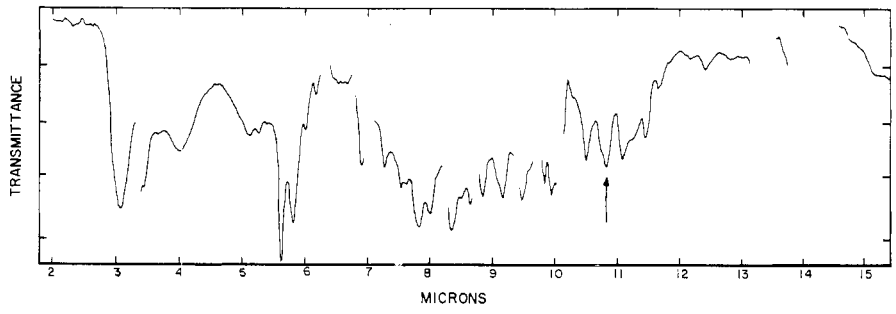

Figure 2. Gibberellin A in pyridine

Breaks in the spectrum indicate regions of total absorption by pyridine

Gibberellic acid has been measured by fluorometric methods (*1, 7, 9*), but gibberellenic acid or impurities associated with it also yield fluorescence under the conditions of the assay. Other impurities may interfere with the method (*6*). An isotope dilution assay has recently been reported (*2*).

Standards

The standard gibberellic acid (A_3) was prepared by recrystallization of a commercial lot. It contained about 1.4% gibberellenic acid as estimated from its ultraviolet absorption at 253 mμ (*5*). $[\alpha]_D$ = +86° (*C* = 1, 95% ethyl alcohol). Analysis calculated for $C_{19}H_{22}O_6$: C, 65.88; H, 6.40. Found: C, 65.49, 66.08; H, 6.64, 6.58.

The standard gibberellin A (A_1) was obtained from a special fermentation. A fluorometric method (*6*) indicated that it could have as much as 3.6% gibberellic acid. $[\alpha]_D$ = +38° (*C* = 1, 95% ethyl alcohol). Analysis calculated for $C_{19}H_{24}O_6$: C, 65.50; H, 6.94. Found: C, 65.55; H, 7.17.

All analytical figures have been calculated in terms of these standards, without correction, although they each may contain several per cent of impurity.

Experimental

All qualitative and quantitative measurements were made using a Perkin-Elmer Model 21 infrared spectrophotometer equipped with sodium chloride optics.

The spectra of the reference materials over the range of 2 to 15 microns were obtained from 10% pyridine solutions in cells of 0.10-mm. thickness (Figures 1 and 2). The absorption band at 12.86 microns was selected as the analytical band for the gibberellic acid content and the absorption at 10.85 microns was selected as the analytical band for the gibberellin A content.

A working curve for gibberellic acid was established in the range 50 to 100%. This was done by plotting absorbance against concentration at 12.86 microns for synthetic mixtures containing 50% acid plus 50% A, 75% acid plus 25% A, 90% acid plus 10% A, and 100% acid. The working curve is a straight line extending through the origin.

A working curve for gibberellin A was established in the range 0 to 50% by plotting absorbance against concentration at 10.85 microns using the same synthetic mixtures. This working curve was also found to be a straight line. It does not extend through the origin, however, because gibberellic acid itself has a weak absorption band at 10.87 microns.

Procedure

Transfer exactly 300 mg. of sample to a 25-ml. Erlenmeyer flask and dissolve in 2.0 ml. of pyridine (analytical reagent grade). Fill a 0.10-mm. cell with this solution and place it in position in the sample beam. Fill another 0.10-mm. cell with pyridine and place it in the reference beam. With slits fixed at 0.40 mm., scan the interval from 12.5 to 13.1 microns. Determine the gibberellic acid content measuring the absorbance from the left shoulder at 12.6 microns to the absorption peak at 12.86 microns.

With slits fixed at 0.26 mm., scan the interval from 10.65 to 11.0 microns. Determine the gibberellin A content by measuring the absorbance from the right shoulder at 10.96 microns to the absorption peak at 10.85 microns. Run a qualitative spectrum from 2 to 15 microns on this solution as a further check on identity and on purity.

Table I. Results of Analyses by the Proposed Method on Commercial Preparations

Sample	Gibberellic Acid, %	Gibberellin A, %
1	95	3
	94	4
2	87	8
	90	9
3	91	6
	92	7
4	94	1
	95	1
5	71	6
	76	8
6	90	2
	89	2
7	76	17
	75	18
8	95	0
	94	0
9	76	21
	75	20
10	79	9
	78	8

Results and Discussion

Table I shows analyses on a number of commercial preparations. These results indicate that the precision obtained is entirely satisfactory for adequate plant control. Analyses run by fluorometric methods (*6, 9*) and by a radioisotopic method (*3*) are in good agreement with the infrared determination of the gibberellic acid content. Preparations treated with acid according to the procedure of Gerzon, Bird, and Woolf (*5*) and which should be largely gibberellenic acid on the basis of the ultraviolet absorption do not show interfering infrared absorption in the regions involved in the analysis for gibberellin A or gibberellic acid.

Although the method has been used primarily for the estimation of small amounts of gibberellin A in the presence of large amounts of gibberellic acid, it is applicable for mixtures of gibberellic acid and gibberellin A in all proportions. A qualitative spectrum is run to ensure the absence of any significant amounts

of impurities which might interfere with the quantitative analyses, particularly of the minor component.

Acknowledgment

The authors thank E. F. Shelberg and associates for microanalyses.

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Received for review November 6, 1958. Accepted February 9, 1959.

GIBBERELLINS ANALYSIS

Determination of Gibberellins by Derivative Labeling with Diazomethane-C^{14} and by Isotopic Dilution Analysis with Tritium-Labeled Gibberellins

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In fermentation broths, the concentration of gibberellic acid and of gibberellin A_1 is determined by derivative labeling with diazomethane-C^{14}. In pilot plant studies, step by step recovery data are obtained by the addition of tritium-labeled gibberellic acid. Complex mixtures are analyzed by a combination of these two methods.

VARIOUS PROCEDURES have been worked out for determining the concentration of gibberellic acid and of gibberellin A_1 in various media (*4, 5*). During the same period of time, an isotopic dilution method using deuterium-labeled gibberellic acid was published (*1*). In the current paper, three radiochemical procedures are outlined which were used routinely for the screening of broth samples and for residue studies in this laboratory's gibberellin program.

Experimental

Materials and Equipment The gibberellic acid and gibberellin A_1, which were used for the purpose of standardization, were Abbott products with an isomeric purity of at least 95% and a chemical purity of at least 98% as determined by radiochemical assay and by infrared analysis (*5*).

Diazomethane-C^{14} was prepared following the method of Stoll (*3*) from *N*-methyl-C^{14}, *N*-nitroso-*p*-toluenesulfonylamide. This nitroso intermediate could be stored at room temperature for extended periods of time.

Tritium-labeled gibberellins were prepared by exposure of the isomerically pure gibberellins to tritium gas (*6*). This preparation will be discussed in detail.

For the assay procedure, a Forro radiochromatogram scanner with an NRD precision ratemeter and a Packard Tricarb liquid scintillation spectrometer were used.

Methods

Determination of Gibberellic Acid and of Gibberellin A_1 in Broths

Sample Preparation. Small volumes, 1 to 5 ml., of the broth samples were extracted three times at a pH of 2.5 with 10-ml. portions of ethyl acetate. The washed extracts were combined, and the volumes were adjusted to contain an estimated 0.01 to 0.1 mg. of gibberellin per ml. of solution. Samples, 1-ml., were then transferred to the 5-ml. round reaction flasks. The solvent was removed under reduced pressure and the residues were redissolved in 1 ml. of ethyl alcohol.

Reaction with Diazomethane-C^{14}. An ether solution of diazomethane-C^{14} was prepared from an amount of the intermediate nitrosomethyl-C^{14}-sulfonamide (specific activity, 1 μc. per mg.) equivalent to 0.5 mg. per sample. Volume equivalents of the chilled solution were then pipetted into the various sample flasks. After standing in ice water for a few minutes, the excess reagent and the solvent were removed under reduced pressure. Excess activity was trapped by reaction with an organic acid.

Parallel with each series of test samples, one to three solutions of known composition were treated identically for calibration purposes.

Chromatographic Separation. The labeled sample residues were applied to Whatman No. I paper strips. The strips were developed overnight by descending flow chromatography with the upper phase of a mixture of water, methanol, petroleum, petroleum ether, and toluene (7:3:10:20 volume ratios). The lower phase of this mixture was used to saturate the atmosphere in the cabinet.

Assay. The dried strips were scanned at a speed of 0.75 inch per minute—time constant selection at 5% error—and the counting rates were recorded. The areas under the various peaks were measured with a planimeter and compared with the areas of the standard samples. These planimetric data were usually precise enough for the routine evaluation of broth samples. If higher accuracy was desired, the spots were extracted in the presence of unlabeled ester. Final specimens could then be isolated and assayed by liquid scintillation counting. As the specific activity of the reagent was known, the results were not influenced by the purity of the standard samples.

Results. Figure 1 shows the position of the various peaks in a typical radiochromatogram. The distance traveled by the methyl-C^{14} ester of gibberellic acid was assigned the R_{GA} value of 1.00, the locations of the other spots were expressed correspondingly.