5 C. ILLE, *Lucraville Inst. Cercetari Aliment.*, 8 (1967) 359.
6 M. H. KLOUWEN AND R. TER HEIDE, *J. Chromatog.*, 7 (1962) 297.
7 H. J. WOBBEN, R. TER HEIDE AND R. TIMMER, *Soap, Perfumery Cosmetics*, 42 (1969) 739.
8 I. OTTO, *Miltitzer Ber.*, (1965/66) 154.
9 I. OGNYANOV, R. VLAHOV, S. TCHORBADJIEV AND D. IVANOV, *Perfumery Essent. Oil Record*, 57 (1966) 87.
10 R. VLAHOV, D. IVANOV, I. OGNYANOV AND S. TCHORBADJIEV, *Riechstoffe Aromen*, 19 (1969) 293.
11 H. FARNOW AND F. PORSCH, *Dragoco Ber.*, 6 (1959) 163.
12 F. PORSCH AND H. FARNOW, *Dragoco Ber.*, 7 (1960) 115.
13 Y. R. NAVES AND P. TULLEN, *Bull. Soc. Chim. France*, (1960) 2123.
14 J. SFIRAS AND L. VANDERSTREEK, *Parfumerie*, 1 (1943) 235.

Received March 26th, 1970

J. Chromatog., 50 (1970) 127–131

CHROM. 4771

Gas chromatographic determination of some maleimides produced by the oxidation of heme and chlorophyll *a*

Our studies of chlorophyll biosynthesis (*e.g.*, see ref. 1), which involve the feeding of specifically labeled ^{14}C compounds to plants and the determination of the ^{14}C incorporation into the various portions of the chlorophyll molecule, have been hindered because of the lack of a method for the determination of microgram quantities of maleimides produced upon chromic acid oxidation of milligram quantities (usually available for these studies) of chlorophyll*. Only very recently have we established that the technique of chromate oxidation can be used with less than 10 mg quantities of porphyrin[2].

MORLEY AND HOLT[3] have shown that gas phase chromatography is an excellent method for non-destructively purifying milligram quantities of maleimides. Based on their report, a study was made of the feasibility of gas phase chromatography for the quantitative estimation of microgram quantities of maleimides. This report describes a gas chromatographic procedure found to be suitable for the determination of some maleimides, derived from the oxidation of heme and chlorophyll, in concentrations as low as 0.050 $\mu g/\mu l$ to within 3% experimental error.

Methods and materials

Preparation of maleimides. Maleimide, succinimide, citraconimide, methylethylmaleimide, hematinic acid methyl ester, and dihydrohematinic acid methyl ester were used for the sensitivity and reproducibility tests described herein. These compounds were prepared and characterized during an earlier study[2].

Gas phase chromatography. Maleimides were separated and estimated by gas phase chromatography on a 10 ft. × $\frac{1}{8}$ in. stainless-steel column packed with 20%

* The maleimides obtainable from the chromic acid oxidation of chlorophyll *a*, which are demonstrated in Fig. 1, include methylvinylmaleimide (ring I) which has been isolated only recently[4], methylethylmaleimide (ring II), methylmaleimide (ring III—citraconimide) when the oxidation is performed after degradation of pheophorbide *a* to pyrroporphyrin, and dihydrohematinic acid (ring IV).

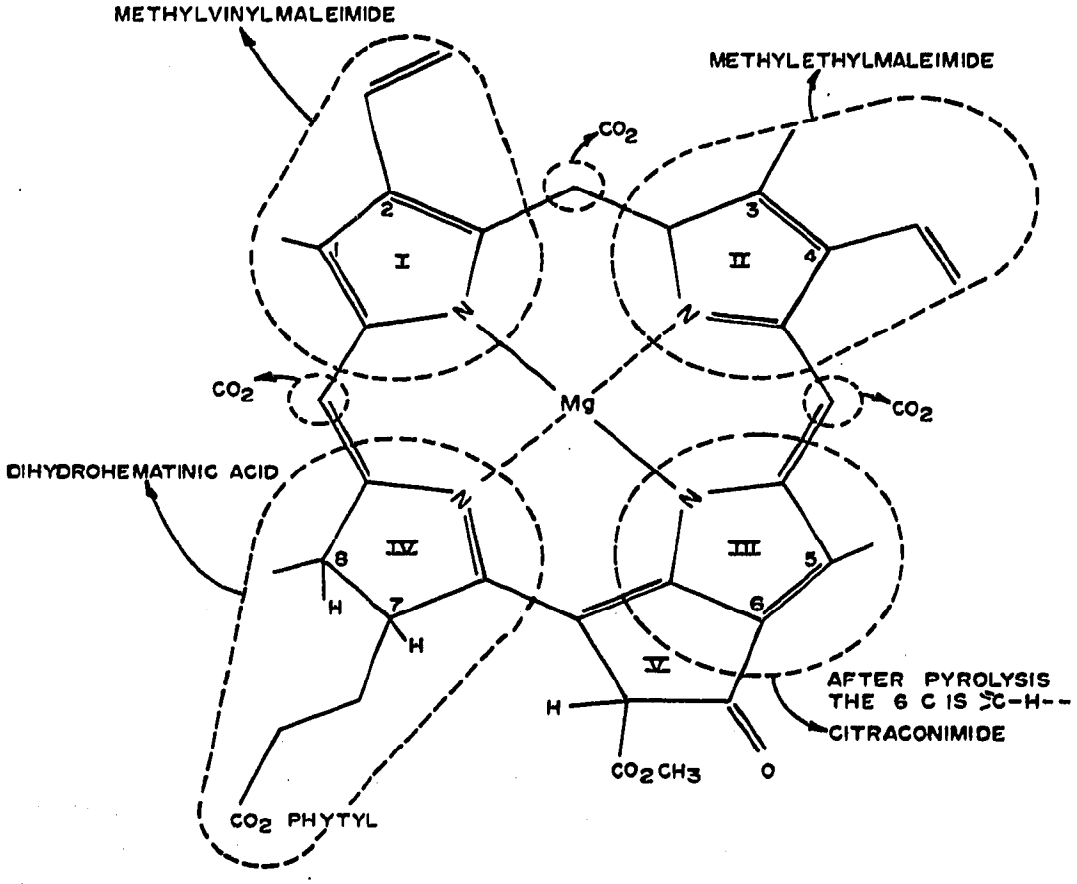

Fig. 1. Chromic acid oxidation products of chlorophyll *a*.

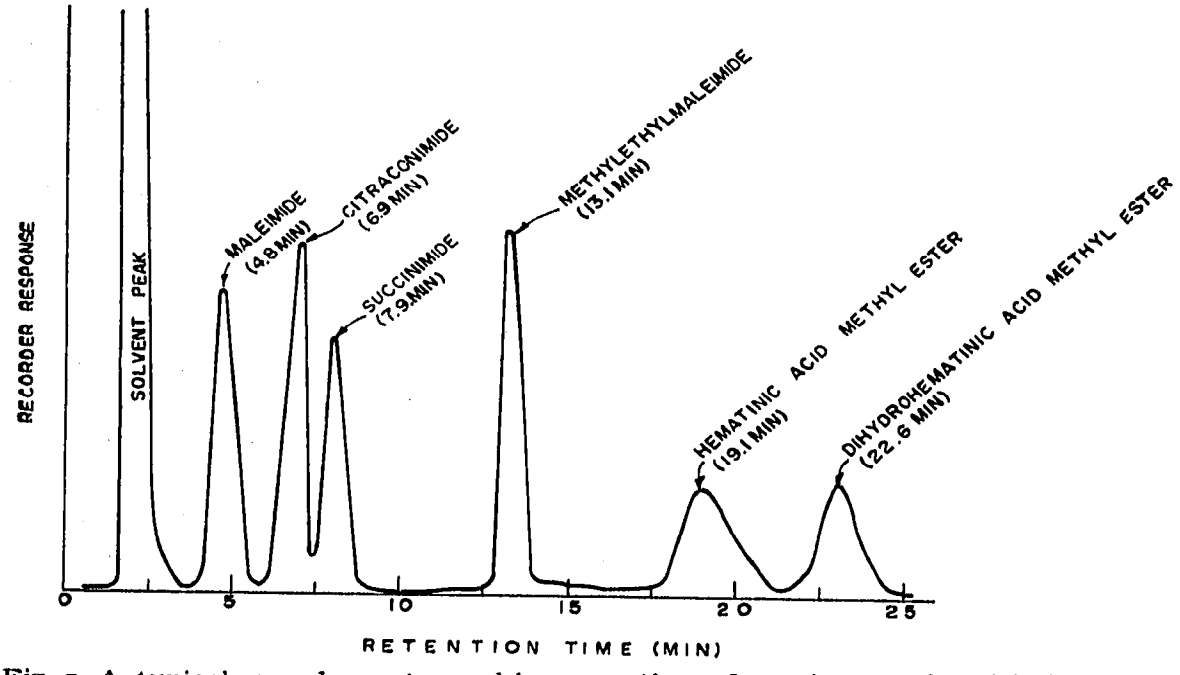

Fig. 2. A typical gas chromatographic separation of a mixture of maleimides (concentrations unequal).

Apiezon M on a support of 100–120 mesh dimethyldichlorosilane-treated and acid-washed Chromosorb W. The chromatograph used was an Aerograph Hi-Fy Model 600-D (Varian Aerograph, Walnut Creek, Calif.) equipped with a hydrogen flame detector (the hydrogen was generated by an Aerograph, Model 650, hydrogen generator). The Brown recorder (Minneapolis-Honeywell Regulator, Co., Brown Instrument Division, Philadelphia, Pa.) was equipped with a disc integrator (Disc Instruments, Inc., Santa Anita, Calif.). Prepurified nitrogen was used as the carrier gas. Injections of solutions of maleimides in reagent grade acetone into the chromatograph were made with a Hamilton (Hamilton Co., Whittier, Calif.) 10-μl syringe (701N-W/g). The chromatographic conditions used were as follows: nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; chromatograph oven temperature (injector port and chromatographic column), 200°.

Results and discussion

A typical chromatogram demonstrating the separation of maleimides achieved

TABLE I

MALEIMIDE SOLUTIONS DETERMINED BY GAS CHROMATOGRAPHY

Imide	*Lowest concentration achieved (μg/μl)*	% *error*[a]
Maleimide	1.00	± 3
Succinimide	0.50	± 3
Citraconimide	0.50	± 2
Methylethylmaleimide	0.050	± 2
Hematinic acid methyl ester	0.050	± 3
Dihydrohematinic acid methyl ester	0.050	± 3

[a] These values represent deviations (determined for ten 1-μl injections of each concentration indicated) of integrator counts at the concentration shown.

with this system is presented in Fig. 2. The retention time obtained for each of the maleimides did not vary from those values observed when a mixture of the maleimides was run. The retention times, however, did change appreciably with small changes in temperature (*e.g.*, 10°) and in flow rate (*e.g.*, 5 ml/min).

The data in Table I represent the sensitivity limits, and error at the limits shown, for this method of maleimide determination on the instrument used. When considerably higher concentrations of maleimide were used (about 100 times those indicated in Table I) errors of less than 2% were routinely observed.

The reliability of this method was tested when an independent worker prepared acetone solutions of the various maleimides. These unknowns were then given to a second worker to perform the analysis. In all cases the maleimide was identified and then quantitatively estimated to within 3% error when the various "unknown" maleimide solutions were run by the analyst. These error limits were well within the range desired of this procedure to fill our needs of maleimide determination.

Finally, the sensitivity of the entire procedure, for our previously stated use, was tested. A sample of approximately 5 mg pure chlorophyll *a* was oxidized with

chromic acid[2]. The residue, redissolved in ether, was treated with diazomethane[5] to esterify the dihydrohematinic acid produced during the oxidation. After evaporation of the ethereal diazomethane solution, the residue was redissolved in 0.5 ml acetone and run on the gas chromatograph. The methylethylmaleimide and dihydrohematinic acid methyl ester fractions were collected and then redissolved in 0.5 ml acetone. Their concentrations were found to be 0.21 and 0.15 μg/μl, respectively. This result indicates that the method is sensitive enough for our work with the [^{14}C]chlorophylls.

It should be noted that the limit of maleimide detection was at least an order of magnitude lower in maleimide concentration than reported herein, but the error in quantitative determination at those levels was very high (*i.e.*, 10–15%). The concentration limit for maleimide detection was about 1 ng/μl.

The author wishes to thank Mr. ROBERT BREDENBERG for his skilled technical assistance with parts of the experiments described herein and Dr. S. J. BAUM of the Department of Chemistry of this College for his critical review of the manuscript.

Department of Chemistry, State University of New York, College of Arts and Science, Plattsburgh, N.Y. 12901 (U.S.A.) R. K. ELLSWORTH

1 H. J. PERKINS AND D. W. A. ROBERTS, *Biochim. Biophys. Acta*, 58 (1962) 586.
2 R. K. ELLSWORTH AND S. ARONOFF, *Arch. Biochem. Biophys.*, 124 (1968) 358.
3 H. V. MORLEY AND A. S. HOLT, *Can. J. Chem.*, 29 (1961) 755.
4 R. BONNETT AND A. F. MCDONAGH, *Chem. Ind. (London)*, (1969) 107.
5 H. FISCHER AND H. ORTH, *Die Chemie des Pyrrols*, Vol. 2, Part 1, Akademie-Verlag, Leipzig, 1937.

Received April 9th, 1970