1) Name of Contractor : Professor R. Belcher

2) Type and Number of report : Final Technical Report. 1963.

3) Subject of the research : Submicro Methods for the Analysis of Organic Compounds.

(a) Determination of Organic Functional Groups.

4) Contract Number : DA-91-591-EUC-~~1009~~ 2387

5) Period covered by report : Sept. 62 - Sept. 63.

The research reported in this document has been made possible through the support and sponsorship of the U.S. Department of Army, through its European Research Office. This report, not necessarily in final form, is intended only for the internal management use of the Contractor and the U.S. Department of Army.

Abstract

The determination of functional groups on the microgram scale (ca. 50μg samples) which was initiated as described in the previous annual report, has been continued.

The present report describes methods which have been developed for the determination of :

I Sulfhydryl groups.
II Nitroso and other nitrogen-containing groups.
III Vicinal glycols (Malaprade reaction).
IV Olefinic unsaturation.

Sulfhydryl Groups.

Macro or micro determinations of mercapto groups are generally based on iodimetric or argentometric titrations. Argentometric methods usually require an amperometric or potentiometric method of end-point detection - a technique which is awkward in manipulation on the submicro scale. Accordingly, attention was given first to the iodimetric methods. The actual titration of iodine with thiosulfate to starch indicator was very accurate on the scale of working and up to 30% of ethanol could be present in the solution without affecting the end-point significantly - an important point in view of the difficulty of dissolving many mercaptans in water. The proposed method was to react the mercaptan with excess iodine, which would then be back-titrated with thiosulfate. It was shown that reduced glutathione could be determined accurately (± 2% relative) with a reaction time of 1 minute. However, other compounds tested gave very inaccurate and not readily explicable results, and it was thought unprofitable to pursue the iodimetric studies farther.

Attention was then turned to mercurimetric methods, of which the best seemed to be that of Fritz and Palmer (Anal.Chem., 1961, 33, 98). In this method, the mercaptan

in acetonic-pyridine medium is titrated with mercuric perchlorate solution to thio-Michler's ketone indicator. It was shown that the method could be scaled down to deal with 50µg samples without too much trouble. However, an 80% ethanolic medium tended to give more reliable end-points than an acetonic medium on the submicro scale, particularly in the case of blank determinations. A wide variety of samples were analysed and typical results are given in Table I.

TABLE I

Compound	Weight taken in ug.	Volume of mercuric perchlorate consumed in ul.	% SH		% Error	% Recovery
			found	expected		
L-cysteine-HCl	52.401	32.0	20.77	20.94	-0.17	99.19
	55.702	34.0	20.76		-0.18	99.14
	83.329	50.6	20.64		-0.30	98.57
Thionalide	92.684	40.0	14.68	14.54	+0.14	100.96
	72.863	31.0	14.47		+0.07	99.52
o-mercapto-benzoic acid	55.355	35.0	21.50	21.45	+0.05	100.23
	68.953	42.4	20.91		-0.54	97.48
2-mercapto-imidazole	76.260	73.0	32.55	33.02	-0.47	98.58
	81.235	80.0	33.49		+0.47	101.42
2-mercapto-benzimida-zole.	64.564	41.0	21.59	22.02	-0.43	99.05
	72.127	46.0	21.69		-0.33	98.50
	75.071	49.0	22.20		+0.18	100.82
2-mercapto-benzothia-zole.	80.906	46.0	19.33	19.77	-0.44	97.77
	61.061	36.0	20.05		+0.28	101.42
	59.488	35.0	20.01		+0.24	101.21

In general the overall accuracy of the method was ±0.5% absolute. For pure glutathione which was used as a standard compound, the average recovery on 12 determinations was 99.3% ± 3%. Several semi-solid and liquid samples were analysed with excellent results; in such cases, a solution was prepared from which aliquots were taken to contain ca 50µg of substance. Moderate amounts of sodium chloride and bromide interfered slightly, while iodide and cyanide interfered seriously.

Organically bound chlorine did not interfere, nor did sulphides and disulphides. The interferences thus seem to be about the same as on the larger scales of working used by Fritz and Palmer.

The use of diphenylcarbazone as indicator instead of Thio-Michler's ketone was also examined, since the former was known to be a good mercury(II) indicator on the submicro scale. A micro method has been described by Gregg et al. (Anal.Chem., 1961, 33, 269), which appeared to be quite straightforward. When the procedure was scaled down, the end-point was found to be rather sharper than that obtained with Thio-Michler's ketone, and excellent results were obtained with a good range of compounds. However, in a few cases, the stoichiometry of the reaction was different, the complex formed in the titration having a structure containing 1Hg : 1SH, instead of 1 Hg : 2 SH as happened in all cases when Thio-Michler's ketone was used as indicator, and in the great majority of cases when diphenylcarbazone was used as indicator. An attempt was made to use dithizone as indicator, being intermed_ate in structure between diphenylcarbazone and Thio-Michler's ketone, but this was completely unsuccessful because dithizone itself was titrated quantitatively. The explanation of the anomalous results obtained with diphenylcarbazone indicator for reduced glutathione, cysteine hydrochloride and 2-mercaptopyrimidine appears to be somewhat esoteric and need not be detailed here.

The diphenylcarbazone procedure appears to be preferable where it can be applied with certainty, but the Thio-Michler's ketone procedure is the more reliable when the sample is unknown.

EXPERIMENTAL

Thio-Michler's ketone procedure. The weighed sample (40 - 80 μg) was dissolved in 0.2 ml distilled water and 0.8 ml ethanol, both air-free and introduced from hypodermic syringes;

the order of addition of the two solvents depends on the solubility of the sulphhydryl compound. The test tube was then clamped over the white tile on the titration platform and the stirring bar was introduced. To the sample solution, 5 micro drops (ca. 0.08 ml) of pyridine buffer (pH6) and 3 micro drops (ca. 0.04 ml) of Thio-Michler's ketone indicator (0.01% in acetone) were added. The solution was left stirring for 30 seconds and the burette tip of the mercuric perchlorate solution was introduced. The bright yellow colour was then titrated under the "daylight" lamp until it almost vanished. Towards the end-point small increments of 0.2 µl of the mercuric solution was added till the appearance of the first blue colour facilitated by the white background.

Notes.

(i) For mercaptans which are insoluble in the ethanol-water mixture, use a similar volume of acetone where the starting colour is yellowish vivid-green and goes to a blue colour with slight greenish tint at the end-point.

(ii) Acidic mercaptans must be neutralised in non-acetone media (e.g. in ethanol or water solutions) with 0.02N sodium hydroxide added from an Agla syrings to just light pink colour of phenol-phthalein (one micro drop (ca. 0.01 ml) of 0.1% ethanol solution).

(iii) Blank determinations must be run in exactly the same way using the same amounts of reagents, but omitting the sample; the blank values found in the present work were of the order of 2 - 4 µl of 0.005 M. mercuric perchlorate solution.

Diphenylcarbazone procedure. The weighed sample (40 - 80µg) was dissolved in 0.2 ml distilled water and 0.8 ml ethanol, both air-free and introduced from hypodermic syringes; either the water or the ethanol was added first depending on

the solubility of the mercaptan. To the sample solution, 1 micro drop (ca. 0.015 ml) of bromophenol blue (0.03% aqueous), 1 micro drop (ca. 0.02 ml) nitric acid (0.05N) and 3 micro drops (ca. 0.03 ml) diphenyl-carbazone (0.5% ethanolic) were added while the test tube was standing clamped on the white tile and the solution was being stirred. The burette tip of the mercuric perchlorate solution was then inserted and the solution was then titrated under the illumination of a "daylight" electric lamp till the first sign of a reddish tinge, i.e. till the original bright yellow colour was just not restored. Increments of 0.2 µl were used towards the end-point and white sheet of filter paper standing behind the test tube helped in detection of the excellent end-point.

Notes.

(i) Ethanol alone can be used as solvent with which a sharp end-point was also available; while with acetone the end-point was not satisfactory.

(ii) There is no need for the neutralisation of acidic mercaptans as acetone is not involved at all in the above procedure.

(iii) Blank determinations were carried out following exactly the same procedure but without the sample. The usual blank value obtained was 1 - 2 µl of 0.005M mercuric perchlorate solution.

II. Nitroso and other nitrogen-containing linkages.

Nitroso groups : The method described in the last report for the determination of nitro groups based on reduction with titanous solution was shown to be directly applicable to the determination of nitroso groups on the microgram scale. This was expected.

Azoxy groups : Little information seems available in the literature on determination or reduction of azoxy groups with titanous solution. Only 2 pure samples were available for the present work : azoxybenzene and p-azoxyanisole. The p-azoxyanisole was analysed by the procedure for nitro-groups and gave excellent results based on a consumption of 4 Ti^{3+} per mole of compound, i.e. a straightforward reduction. However, results for azoxybenzene were extremely variable depending on the time of reduction; reasonably good results were obtained after a 20-min. reaction time, but obviously the reduction takes complicated pathways and the procedure can scarcely be recommended as generally applicable.

Azo groups. On the macro and micro scales, azo groups have often been determined either by direct titration with titanous solution or by reaction with titanous ion followed by titration of its excess. In the present work, several alkyl azoates were analysed by the general nitro procedure on the microgram scale without difficulty, excellent results being obtained on the basis of 2 equivalents of titanous consumed, which argues a diphenyline or semidine re-arrangement. Azo linkages are usually reduced in highly acidic media, but in the present work it was found that azobenzene was reduced much more rapidly in the citrate-buffered solution used in the general procedure for nitrogroups. Excellent results were obtained after a 5-min. reaction time, but results were very high and did not appear to correspond to any stoichiometric reaction after 10-min. reduction or longer. After 5 min. reduction with benzidine re-arrangement had occurred, 2 equivalents of titanous solution being required. It was found that m-azotoluene and bis(trifluoromethyl) azobenzene could not be reduced at all, even on prolonged reaction in acidic or buffered media. Such behaviour can be

explained on the basis of the substituents hindering protonation and thus electron transfer (cf. Hinshelwood et.al., J.Chem.Soc., 1953, 3384; 1954, 2736; 1956, 620).

Taken as a whole, the results showed that no general technique for determination of azo groups can be based on reduction with titanous solution, although most compounds can be determined with reasonable accuracy if the structure is known. Obviously, the method would have no value for diagnostic purposes.

Hydrazo groups : Possible reduction of several compounds with titanous solution under a variety of conditions was examined. Under no circumstances did the reduction proceed quantitatively in a stoichiometric reaction. Several hydrazine compounds were also studied. It was found, in confirmation of results on other scales of working, that the hydrazine group alone was not reduced, but that when nitro groups were also present, the nitro group was reduced to the corresponding amine while the hydrazine linkage was reduced to the amine and ammonia. Thus, very good results could be obtained for compounds such as 2,4-dinitrophenylhydrazine and cyclohexanone-2,4-dinitrophenylhydrazine.

Oxime groups: A few oximes were examined to see if they could be reduced, since there is no report of such reduction in the literature. With some compounds, such as pyridine-2-aldoxime, reduction to the amine was quantitative but with other compounds, there was no reduction.

Conclusion : These studies on the reduction of nitrogen-containing linkages by titanous solution indicate that general procedures are possible for the determination of nitro and nitroso groups, excellent results being obtained even with 30-70 μg samples. For the other linkages examined (azo, azoxy, hydrazine and oxime), certain compounds can be

determined accurately, but with unknown materials there can be no certainty about the results.

Experimental detail for these determinations was given in the previous Annual Report.

Periodate Oxidations (Malaprade Reaction)

Periodate oxidations are very useful in carbohydrate analysis, giving a good deal of information on structure from a very simple determination. Virtually all the work done on this area has been on the macro-scale, apart from a few biochemical studies. It was, however, considered valuable to develop a suitable procedure for microgram samples.

In the macro methods, it is usual to react the carbohydrate with excess of periodate, and to determine the excess of periodate, as well as one or more of the reaction products, usually a simple aldehyde or organic acid. With the simpler carbohydrates at least, the products usually include formaldehyde or acetaldehyde and/or formic or acetic acid.

In the present work concerned with scaling the methods down to the 50µg range, it was quickly shown that the reactions proceed essentially as on larger scales of working. No difficulty was found in titrating the excess of periodate used, a direct titration with arsenite solution to a Thyodene end-point proving most satisfactory. This method was preferred to a thiosulphate titration, because periodate was reduced only to iodate in the bicarbonate medium employed, thus improving the blank : sample ratio.

It was found that for simple carbohydrates such as mannitol and sorbitol, a reaction time of 15 min. is sufficient; as on larger scales, more complex materials, and particularly cyclic compounds, take a longer time for degradation. In general, the time of reaction and, where appropriate, incomplete reaction to a defined product, were found to follow closely the data available for macro analysis.

On the macro-scale it is normal practise to take aliquots of the periodate-treated solution for determination of excess periodate. Aliquotting presents some difficulties in submicro-scale analysis and, accordingly, the methods were designed so that as many as possible of the required steps could be carried out consecutively on the same solution.

The common acid formed in the oxidations is formic acid. It was found possible to titrate this acid before the titration of excess periodate, when methyl red was used as indicator, and suitable precautions were taken. An attempt to use an iodimetric titration of the acid failed because of incomplete reaction at the dilution involved, no matter which modification was applied.

The aldehyde formed in the reaction is generally determined by a spectrophotometric method involving chromotropic on a separate aliquot, or by an iodimetric bisulphite procedure after separation by distillation. Attempts to apply distillation techniques were a complete failure on the submicro scale. Accordingly, the hypoiodite oxidation method of Romijn for formaldehyde was examined, for it was thought that this method could be applied to the solution after the titration of excess of periodate. It was shown that under controlled conditions, the hypoiodite method could give quite good results for microgram quantities of formaldehyde (and even acetaldehyde when appropriate corrections were made).

It was finally shown that it was quite feasible to run 3 consecutive determinations of acid, excess periodate and aldehyde on the same solution by the following procedure.

Procedure.

30 - 80 µg of the sample were weighed into the reaction vessel, and 150 µl of periodate solution was added, the tip of the burette being washed with 2 micro drops of water. The tubes were then stoppered and stored in a cool dark place until reaction was complete.

50 µl of 0.01% methyl red indicator were then added either from a dropper or an Agla burette, followed by a rotor and 100.0 µl of standard sodium hydroxide (0.025N). The tip of the burette was raised above the liquid surface and washed with 1 drop of water. The hydrochloric acid burette was then introduced, the tip also dipping about 1 mm. under the liquid surface. The excess of alkali was titrated to the disappearance of the yellow colour of methyl red (red-brown end-point).

The burette tip was raised above the liquid surface and washed with 1 drop of water. Then approx. 0.1g of sodium bicarbonate was added, followed by 1 drop of potassium iodide solution, and the liberated iodine was titrated with standard arsenite solution the "thyodene" indicator being added soon after the start of the titration, and the end-point being taken when the brown-purple colour completely disappeared leaving a clear yellow solution.

Sufficient 4N hydrochloric acid to destroy the bicarbonate and iodate was then added (preliminary tests showed that 1 drop of the acid solution used was sufficient for this purpose; this amount left only a very small excess). The solution was then carefully rebuffered with sodium bicarbonate (approx. 0.015g.), and the liberated iodine was titrated with arsenite solution until the solution was completely clear yellow. Quickly 6 drops of approx. 4N sodium hydroxide and 50 µl of approx. 0.1N iodine were added, in that order, the solution being vigorously stirred during the addition. The resulting solution was then placed in a cool dark place after the tube had been stoppered.

After exactly 30 minutes sufficient 4N hydrochloric acid was added to liberate the iodine and make the solution slightly acidic (ca. 6 drops). The liberated iodine was titrated with standard 0.025N sodium thiosulphate to the first clear red end-point.

Blank titrations were carried out in exactly the same way.

About 10 relatively simple carbohydrates were tested using the above method. The results shown in the following table are fairly typical of the results obtained.

Dulcitol. M.W. 182.17.

a) Formic Acid Determination.

	Wt.Sample µg.	Alkali Titre Theory µl.	Alkali Titre Expt[1]. µl.	% Recovery
NaOH	55.04	47.64	46.09	96.8
0.02537N	72.94	63.13	61.74	97.8
	69.77	60.38	59.13	97.9
	44.36	38.39	38.09	99.2

b) Periodate Consumption.

	Wt.Sample µg.	Arsenite Titre µl.	Wt. Sample Expt[1]. µg.	% Recovery
Arsenite	55.04	58.3	53.39	97.0
0.05027N	72.94	77.9	71.33	97.8
	69.77	75.7	69.32	99.4
	44.36	47.3	43.31	97.6

c) Formaldehyde determination.

	Wt.Sample µg.	Wt.HCHO Theory (µg.)	Titre of Thio µl.	Wt.HCHO Expt[1].µg.	% Recovery
	55.04	18.13	44.0	17.00	93.8
Thio:	72.94	24.03	60.0	23.18	96.5
0.02576N	69.77	22.98	60.4	23.34	101.6
	44.36	14.61	34.0	13.14	90.0

A number of acid-sensitive materials required analysis, and it was shown that the submicro oxidation was also satisfactory in bicarbonate medium. Of course, no determination of acid formed was then possible, but the remainder of the procedure offered no difficulty.

As a general rule the maximum errors to be expected from the 3 steps are :

±2% relative for periodate consumption,

±2% relative for acid determination, and

±10% relative for formaldehyde determination.

These figures are of the same level as those generally found on the macro scale. The error in the aldehyde determination seems large, but the results are in fact quite accurate enough for diagnostic purposes. It is interesting to point out that these three consecutive analytical steps would be very difficult to achieve on larger scales of working.

An examination of more complex compounds, e.g. α-methyl-D-glucoside and 1,2,5,6-diisopropylidene-D-mannitol, by the above procedure, showed that while the acid and excess periodate determinations proceeded according to expectations, the determination of formaldehyde did not. Several compounds which produced no formaldehyde or acetaldehyde in the oxidation with periodate still consumed alkaline hypoiodite, the large aldehydic moeities formed being determined quite accurately. Thus, although all the results could be explained on a post mortem basis, and such experiments could yield data of a useful confirmatory nature, the hypoiodite method was obviously of little value for analysis of complicated unknown carbohydrates.

Accordingly, it was decided to examine the spectrophotometric method for the determination of formaldehyde, based on reaction with chromotropic acid - a reagent which is very selective for formaldehyde among the various aldehydic moeities which might be formed. A scale-down of the normal macro technique proved quite satisfactory and could be applied to the solution from reaction with periodate directly after the titration of excess periodate. Generally, the results for formaldehyde were better when the oxidation was done in a bicarbonate medium. The following method was found satisfactory.

Experimental

Reagent : Chromotropic acid solution. Dissolve 1.00 g. of chromotropic acid in distilled water and filter. Add 300 ml of concentrated sulfuric acid to 150 ml of distilled water, cool and add to the chromotropic acid solution, diluting to 500 ml. Store in a brown glass bottle and re-prepare every week.

Procedure: A suitable sample weighing between 30 and 80 µg was placed carefully in a clean dry 5 ml graduated flask, the sides of the flask being gently tapped to ensure that all the sample reached the bottom. Approximately 0.01 g sodium bicarbonate was added carefully by means of a moderately heaped micro spatula. The sides of the flask were then washed down with 0.5 ml of water, and the contents of the flask gently swirled to dissolve the bicarbonate and sample. Then 150µl, approximately 0.05N sodium periodate, was added, the tip of the burette being washed with 0.1 ml water. The flask was stoppered and placed in a cool dark place until reaction was complete.

After this time 1 drop of 10% potassium iodide was added with a magnetic stirrer, and the liberated iodine titrated with standard sodium arsenite solution, the tip of the burette dipping slightly below the liquid surface. When the iodine colour had almost completely disappeared, 1 micro drop of starch solution was added and the titration continued until the solution was completely clear. The burette tip was then raised above the liquid surface and washed with a few drops of distilled water.

The magnetic stirrer was removed from the liquid with a magnet outside the flask, being washed with about 1 ml of water when halfway up the neck of the flask.

From a pipette 2.0 ml of 0.05N arsenite solution were

then added and the resulting solution shaken occasionally for about 10-15 minutes. The solution was then diluted to 5 ml.

1 ml of the resulting solution was pipetted into a 25 ml graduated flask, or any other ~25 ml container of approximately the same dimensions.

10 ml of chromotropic reagent solution was then added, again from a pipette, and the resulting solution shaken gently to mix. The flask was then loosely stoppered and placed in a vigorously boiling water bath for 30 minutes. The water bath should preferably be in a dark corner where light or direct sunlight cannot fall on the reaction flasks. Blank determinations were carried out in exactly the same way.

Colour Measurement.

The colour was measured on a spectrophotometer at 570mμ using a 1 cm. cell. The optical density for a blank solution carried through the same procedure was also measured, although in most cases this was zero, i.e. 100% transmittance.

The weigh of formaldehyde was determined by reference to a previously prepared calibration curve using mannitol as the standard.

Conclusions.

1) The determination of formaldehyde produced during a Malaprade reaction by measuring the colour produced with chromotropic acid can be carried out with an accuracy of ±1% - 2% relative, provided the oxidation is carried out in bicarbonate medium. If the oxidation is carried out in a solution at reagent pH results are very variable and appear to be, on average, about 10% lower than those obtained in bicarbonate solution.

2) A simultaneous determination of periodate consumption and formaldehyde produced during a Malaprade reaction

showed that the former determination was very accurate and an average relative error of ± 1-2% was obtained. The determination of formaldehyde however, although generally within ±5%, occasionally fell to ± 20-25%. Although this accuracy is perfectly adequate for diagnosis of structure it is of little value for purely analytical purposes. The fall in accuracy of the formaldehyde determination appears to be due to the starch indicator solution.

3) The fact that the hypoiodite procedure, when used for the determination of formaldehyde after the determination of the acidity and periodate consumption, gave recoveries of between 90-95% generally, might be due to two facts :

   a) The formic acid determination involved addition of alkali, followed by back titration of the excess with acid. The presence of the alkali might have caused hydrolysis of any formate ester, which normally would cause low results, and would explain the slightly higher recoveries of formic acid and formaldehyde obtained by that method than by other procedures.

   b) The addition of bicarbonate to the oxidation mixture prior to the periodate titration might have a similar effect to (a).

Generally speaking all the results obtained on the submicro scale have echoed results obtained by other workers on other scales, but for the best results for both analytical and diagnostic purposes the following procedures are recommended:

1) The formic acid and periodate consumption can be determined with an accuracy of ~ ±2% relative using

a back-titration technique with methyl red indicator for the acid, and titration of the periodate with arsenite in buffered solution in the presence of iodide both on the same sample solution.

2) When a separate sample is used, and oxidation is done in a bicarbonate medium, the formaldehyde can be determined by measurement of its colour produced with chromotropic acid with a relative accuracy of ± 1-2%.

Such a procedure would involve 60-100 μg of sample or less, which is a reduction of ca. 10,000 times the sample normally used.

IV. The Determination of Olefinic Unsaturation.

Halogenation reagents are, from an analytical standpoint, the most convenient for the determination of olefinic/ethylenic unsaturation. The order of increasing reactivity of the various halogens in the addition to the double bond is as follows:

$$I_2 < IBr < Br_2 < ICl < BrCl.$$

The analytical convenience of the halogens is to some extent offset by three main disadvantages. Firstly the reagents are volatile, secondly substitutive side-reactions may occur, and thirdly, less reactive olefines do not undergo addition of halogens to the double bond.

However, because other possible methods appeared to offer even greater disadvantages, it was decided to attempt to develop a reasonably general method for the determination of olefinic unsaturation using a halogenation reagent, and to couple this with an ancillary method which would take care of the less reactive olefines.

It was decided to compare two halogenation reagents :-

1) Tribromide ion in methanol. This reagent originally studied by Kaufmann (Kaufmann,H.P., Z.Untersuch.Lebensm.,

1926, 51, 3) has the advantage that the volatility of bromine is decreased whilst the tribromide ion is only slightly less reactive than bromine itself.

2) Bromine monochloride. Schulek (Schulek,E., and Burger, K., Z. Analyt.Chem.) has applied this reagent to the analysis of a variety of unsaturated compounds with satisfactory results.

1) Tribromide ion:- The results obtained from the analysis of several α,β-unsaturated acids and esters using this reagent were unsatisfactory. The problem is that the -COOH, or -COOEt group deactivates the double bond to such an extent that the addition of bromine proceeds extremely slowly. In order to overcome this effect it is necessary to correct the -COOH or -COOEt to the carboxylate anion. The increase in volume when the neutralisation or hydrolysis was carried out on the submicro scale resulted in the bromine concentration being far too low. It was impossible to avoid this by increasing the bromine concentration, and it was essential to obtain a significant difference between sample and blank titrations. The study of this reagent was abandoned.

2) Bromine monochloride:- Bromine monochloride is a far more reactive reagent than bromine itself, and will add to the double bond in α,β-unsaturated acids, without the need for prior neutralisation. The disadvantage of this reagent is inherent in its extreme reactivity, i.e. the more reactive olefines tend to 'over-brominate' unless the excess of bromine monochloride is carefully controlled.

Acidification of a bromate/bromide mixture with hydrochloric acid proceeds as follows.

$$BrO_3^- + 2Br^- + 6H^+ + 3\ Cl^- \longrightarrow 3BrCl + 3\ H_2O.$$

Accordingly three types of procedure were examined.

i) Addition of a bromate/bromide solution to the olefinic compound, and then acidification with hydrochloric acid, i.e. BrCl liberated in situ.

ii) A similar procedure except that the reaction vessel was evacuated before addition of the hydrochloric acid through the funnel-shaped neck of the reaction flask by releasing the stopper slightly. The use of a slight vacuum lowered the volatility of the bromine chloride.

iii) Addition of a standard bromine chloride solution to the olefinic compound (at atmospheric pressure).

The most promising results were initially obtained with procedure (i) (procedure ii gave more reproducible blanks, but the slightly improved accuracy was not offset by the increased technical difficulty). However, when it was discovered that by carrying out the determination at a temperature of 0°C the losses of bromine monochloride from a standard bromine monochloride solution were greatly reduced, it was decided to adopt this method as a general practice.

General Method

i) Known Samples.

The sample is placed in the submicro flask (standard type) and dissolved in measured volume of methanol, (0.10 ml.). After cooling in ice an amount of 0.01N standard bromine chloride solution is added, and the sample allowed to react for between 5 minutes and 1 hour (depending on the nature of the sample). 0.5 ml of distilled water is then added, together with 1 drop of 15% potassium iodide solution, and the liberated iodine titrated with 0.01N standard thiosulphate solution. For some samples a mercuric sulphate catalyst is required (prepared from precipitated HgO), but this must be ascertained by experiment as some compounds give very high results when Hg(II) is present.

ii) Unknown Samples.

The first step in the analysis of an unknown olefinic compound consists of preparing a series of samples containing varying mole ratios of BrCl:sample, and after having allowed sufficient time for the addition to occur (1 hour is usually ample) the excess BrCl is determined by iodometry.

This will give an indication of the olefinic content of the molecule together with the nature and extent of any side-reactions. In some cases the consumption of BrCl is constant at mole ratios > 1.5:1 so that if a graph is plotted of bromine chloride consumed against mole ratio of BrCl:sample the curve becomes horizontal, whilst in others the over-bromination is so marked that the curve continues to rise only slightly less steeply than in the initial portion with a slight plateau around the 2.0 or 1.5:1 values.

This exploratory examination then enables a more accurate determination to be carried out. A further series of samples containing mole ratios of bromine chloride to sample corresponding to a point on the previous graph where the curve became horizontal (i.e. mole ratios between 1.25 and 1.75:1) are prepared and allowed to react for varying lengths of time. The graph of BrCl consumption as a function of reaction time is plotted and extrapolation of the two straight portions of the curve will give an accurate value for the olefinic content.

Results.

The following compounds have been analysed successfully. Maleic, fumaric, cinnamic, crotonic, itaconic, elaidic and sorbic acids; allyl thiourea; 3:6 methylene tetrahydrophthallic acid; 3:6 methylene tetrahydrophthalimide; citral; cinnamaldehyde; trans stilbene.

The results shown in the following are typical (calculated on basis of percentage of $\rangle C = C \langle$ group in molecule).

Table of Results

(All results are given in order of increasing reaction time)

1. Maleic acid. (Sample range 40 ⟶ 80 μg).
   Reaction time ½ ⟶ 1 hr. (Hg^{II} catalyst)
   % $\rangle C = C\langle$ (relative). 101.3, 104.0, 97.7, 99.1, 97.5, 97.0.

2. Cinnamic acid. (Sample range 40 ⟶ 100μg)
   Reaction time 15 min. ⟶ 1 hr.
   % $\rangle C = C\langle$ (relative). 97.3, 99.9, 97.5, 98.9, 98.3, 96.7.

3. 3:6 Methylene tetrahydrophthallic acid. (Sample range 40 ⟶ 100 μg) (Hg^{II} catalyst - but very little effect).
   % $\rangle C = C\langle$ (relative). 97.9, 100.4, 87.8, 100.7, 98.6, 93.2

4. Cinnamaldehyde. Samples from standard solution ≡ 66.08μg.
   Reaction times. 15 min. ⟶ ½ hr (-9 ⟶ -5°C)
   % $\rangle C = C\langle$ (relative). 10.17, 102.9, 97.1, 100.9, 106.0, 106.9.

Conclusions. The method as presented is capable of analysing a wide range of olefinic compounds, and with suitable modification can be even further extended. Very reactive olefines can be titrated directly, whilst α,β-unsaturated esters can be determined after hydrolysis.

Olefines which contain a strongly electron-attracting group, such as -C≡N, -COEt, conjugated to the double bond, cannot be determined with bromine chloride. Work is at present in progress to develop a method based on the use of morpholine, (Critchfield, F.E. Johnson, J.R., and Funk, G.L., Anal. Chem., 1956, 28, 76) a secondary amine, which adds to the double bond. Initial results have been promising, but further work is required to find an indicator more suitable for the submicro procedure.

Personnel utilized	Man-hrs.
B. Fleet and G. Dryhurst.	4,200
Mrs. M. Clarke, clerical and typing	360
Mr. R.W. Dackus, glassblower	100

Expenditure over one year	£	s	d
Salaries	1,000	0	0
Glassblower	40	0	0
Typist	75	0	0
Expendable materials	94	6	6
Overheads	175	0	0
	£1,384	6	6

Expenditure : £1,384. 6. 6d.
Deficit from last year : 26. 0. 0d.
£1,410. 6. 6d.

Permitted expenditure ... £1,410. 0.0d.
Actual expenditure ..... £1,410. 6.6d.
Deficit : 6.6d.