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Improved *N*-Ethylcarbazole Determination of Carbohydrates with Emphasis on Sea Water Samples

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► A modified method for determination of carbohydrates in sea water with *N*-ethylcarbazole will determine as little as 1 γ of carbohydrate per ml. of sample. There is no salt interference and, with the exception of xylose and fructose, pentoses,

hexoses, and their polymers yield the same amount of color per unit weight.

THE USE of *N*-ethylcarbazole as a reagent for the determination of carbohydrates in sea water was de-

veloped originally by Erdman (?). It was used extensively by Collier *et al.* in various experimental studies (1-3), and as a routine method for the measurement of the carbohydrate content of sea water in this laboratory from 1951 to 1956. The technique is ad-

vantageous in sea water studies because it measures total carbohydrate content rather than that of specific classes of carbohydrates. This method often gives a very low slope and is extremely sensitive to changes in temperature, to light, and to various types of contaminants. In an effort to improve this method for routine sea water analysis, the technique has been re-examined with regard to heating time, method of reagent addition, reagent concentration, and precision. The reaction of this reagent with various sugars has been studied under these revised standardized conditions.

METHOD

Purification of *N*-Ethylcarbazole.

N-Ethylcarbazole obtained in crude form from the Reilly Tar & Chemical Corp. is dissolved in hot alcohol and allowed to crystallize. The mother liquor from this crystallization is discarded because it contains most of the impurities. The crystals are then redissolved in hot alcohol and allowed to recrystallize, and the mother liquor is treated with an equal volume of distilled water to precipitate the remaining *N*-ethylcarbazole. This process of recrystallization from warm alcohol followed by water precipitation is continued until pure white needle-shaped crystals are obtained. The adsorbed alcohol is removed by drying the crystals at a temperature no higher than 55° C. (*N*-ethylcarbazole decomposes at 69° C.). The dried purified compound dissolved in acid and treated as a routine blank sample as described below, should give a scale reading less than 4.5 in the 23-ml. cuvette of a Fisher Electrophotometer; this is equivalent to an absorbance reading on the Beckman DU spectrophotometer in a 1-cm. cuvette at 560 $m\mu$, of no more than 0.020. Starting with approximately 1 pound of crude *N*-ethylcarbazole dissolved in 2 liters of hot alcohol yields pure compound sufficient for 8000 determinations. Best results are obtained if the alcohol is heated first and the crude compound or crystals added so slowly that they dissolve readily. Heat that is too strong or too direct causes the *N*-ethylcarbazole to melt and decompose, rather than to dissolve.

Acid. Du Pont c.p. sulfuric acid, full strength.

REAGENT. One gram of purified *N*-ethylcarbazole per liter of concentrated sulfuric acid. Usually 2.2 liters are prepared directly in the acid bottle, mixed well, and divided into equal parts in two amber glass bottles fitted with amber glass automatic acid burets. This reagent may be stored in the cold, but the storage period should not exceed 48 hours because the standard slope decreases both with age and chilling. Results are more satisfactory if the reagent is used immediately after preparation. If amber glass equipment is not used, additional precautions must be taken against light—e.g., the reagent must never be left in open sunlight or exposed to light containing considerable

ultraviolet. All glassware used in the preparation and transfer of the reagent should be rinsed with Du Pont c.p. sulfuric acid before use.

Procedure. Samples of 3 ml. each are pipetted in duplicate into 50-ml. Erlenmeyer flasks and covered with 30-ml. beakers. To groups of 12 samples (six duplicates), reagent is added as follows. (Times indicated are counted from the initial addition of reagent to the first sample.) At time 0, 5 ml. of reagent are added to sample I from buret I. After 30 seconds, 5 ml. of reagent are added to sample II from buret II. At 60 seconds, 22 ml. more of reagent are added to sample I from buret I, and at 90 seconds, 22 ml. more of reagent are added to sample II from buret II: the burets are refilled with reagent and a second pair of samples treated in the same way. This procedure is followed until reagent has been added to all 12 samples. The samples are then placed in a water bath at 70° C. for 30 minutes, after which they are brought to room temperature in a refrigerator. They are then read against distilled water, either in a Fisher Electrophotometer fitted with a G.A.B. 560 $m\mu$ interference filter (Photovolt Corp., New York, N. Y.), or the 525 $m\mu$ B filter. Samples may also be read in a Beckman DU spectrophotometer at 560 $m\mu$, but this is not recommended for the routine handling of large numbers of samples. When this instrument is used, a 5-cm. cell should be used for concentrations below 4 or 5 mg. of carbohydrate per liter. In the experiments described, readings with the Electrophotometer have been made using a single 23-ml. cuvette nulled with distilled water. Spectrophotometer readings have been made using four 1.00-cm. Corex cells balanced within 2% at 544 and 560 $m\mu$; a single cell has been used for a null and the remaining three have been used as sample cells. The spectrophotometer slit width was held at 0.04 mm.

Blanks and a set of standards in triple distilled water must be run with each batch of reagent prepared. D(-)-arabinose was used in concentrations of 1.0, 2.0, 3.0, 5.0, and 10.0 mg. per liter (0.67×10^{-5} , 1.33×10^{-5} , 2.00×10^{-5} , 3.33×10^{-5} , and 6.67×10^{-5} mole per liter). These standards are run in duplicate, the best straight line is calculated through the points, and the sample concentrations are obtained from the slope of this line.

PRECAUTIONS. All glassware must be scrupulously clean. Flasks and beakers used in this determination are soaked overnight in Du Pont c.p. sulfuric acid, rinsed repeatedly in triple distilled water, and allowed to drain dry. The amber bottles and burets from which the reagent is dispensed are rinsed with triple distilled water after each use, and with fresh acid before the next use.

In the presence of high concentrations of nitrate the reagent will turn green, masking the pink color formed by the reaction of the carbohydrates and the reagents. A few samples have been found to turn orange when reagent was

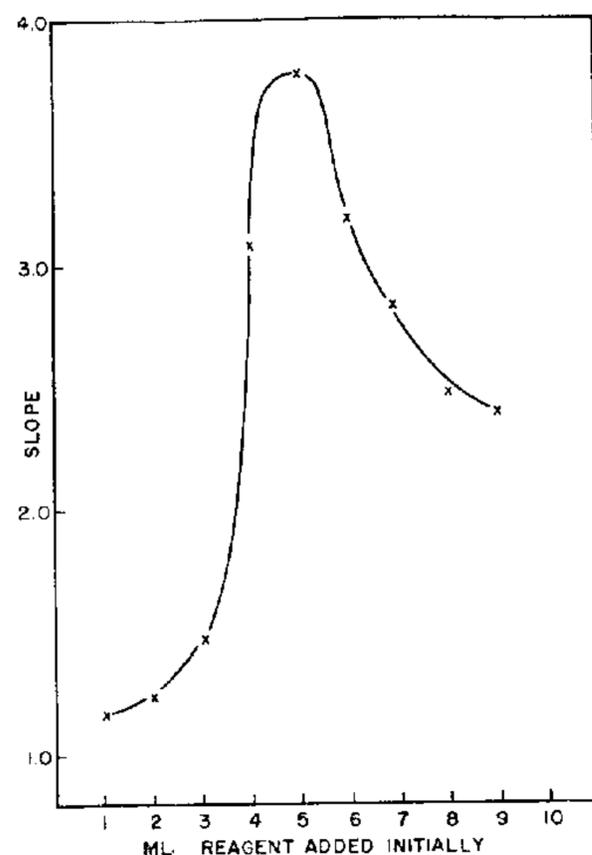


Figure 1. Slope vs. milliliters of *N*-ethylcarbazole added initially

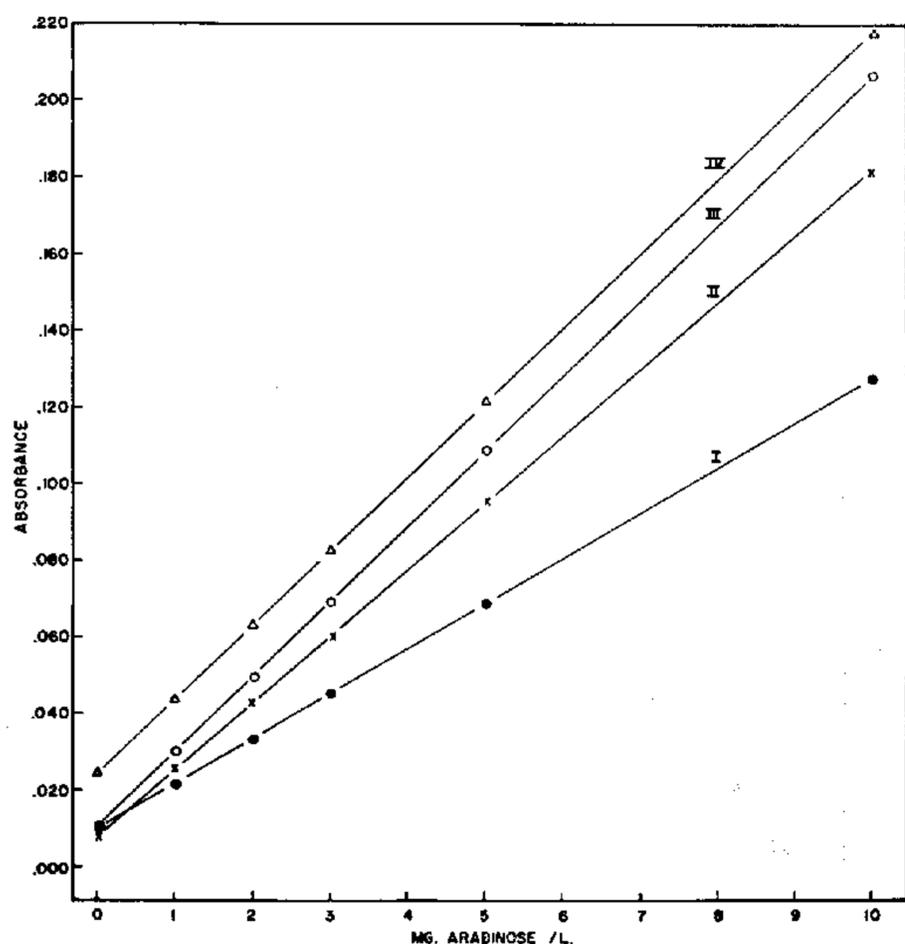
added but this contaminant is as yet unidentified.

Bubbling of the samples after pouring into the cuvette is reduced by the use of flasks with 30-ml. beakers as caps; loose fit of the beakers allows many hydrochloric acid bubbles to escape during the heating period. Careful attention must also be paid to the cooling period. Samples must be at room temperature and not below, to prevent condensation, but samples that are still warm will read less than those at room temperature. Samples should be poured carefully to prevent the formation of hydrochloric acid from salt sometimes deposited around the neck of the flask.

RESULTS

Effect of Two-Stage Addition of Reagent. The intensity of color produced was greater when the reagent was added as a two-stage delivery rather than as a single stage as suggested by Erdman. To find the optimum initial addition of reagent, ten sets of standards were prepared as described above and the Erdman procedure, which adds 9 parts of reagent to 1 part of sample (27 ml. to 3 ml.), was modified.

One milliliter of reagent was added to each of the first set of samples. The amount of reagent was then increased by a 1-ml. increment per set, so that each sample of the last series received 10 ml. as a first-stage addition. Each sample was allowed to stand for 1 minute and the remainder of the 27 ml. added (26 ml. for the first set, etc.). Samples were heated and cooled as usual, and read in the electrophotometer. The slope for each set of standards was plotted against the amount of reagent added initially (Figure 1).



◀ Figure 2. Variation of slope with *N*-ethylcarbazole concentration

Grams *N*-ethylcarbazole per liter

- I. 0.5
- II. 1.0
- III. 2.0
- IV. 3.0

The initial addition of 5 ml. of reagent gave the highest slope. With less reagent, the *N*-ethylcarbazole tended to precipitate in the water phase; this reaction may be a contributing cause of the lower slope. An error in delivery of ± 0.5 ml. of reagent is not significant. Although some *N*-ethylcarbazole may precipitate on the low side of 5.0 ml. and the color initially developed may be noticeably decreased, total color develops normally with heating. This may indicate that the increased color in the two-stage procedure results from the accelerated formation of colored reaction products by the heat arising from dilution of the sulfuric acid. Longer standing periods between reagent additions were also tested but the increase in slope when standing time was changed from 1 to 2 minutes was not large enough to justify the use of the longer period on a routine basis.

Effect of *N*-Ethylcarbazole Concentration. Although Erdman suggested the use of 1.0 gram of *N*-ethylcarbazole per liter of sulfuric acid, several concentrations of reagent were checked to determine whether the slope obtained with the two-stage method could be further improved. Four batches of reagent were prepared using the same lot of purified *N*-ethylcarbazole and acid from a single bottle. The *N*-ethylcarbazole concentration ranged from 0.5 to 3.0 grams per liter of acid. A set of standards was run by the method described using each reagent concentration, and the samples were read on the spectrophotometer at 544 and 560 $m\mu$. The slope was plotted for each concentration of reagent as shown in Figure 2. Doubling the concentration between 0.5 and 1.0 gram per liter increased the slope by 50%, while doubling between 1.0 and 2.0 grams per

liter raised the slope only 15%, and the further increase to 3.0 grams per liter caused no significant increase above that of 2.0 grams per liter. For practical reasons (expense of crude reagent and the time involved in purification), increasing the concentration above 1.0 gram per liter seemed undesirable. Therefore all further experiments were performed using 1.0 gram of *N*-ethylcarbazole per liter of acid.

Effect of Temperature and Heating Time. In Erdman's original technique, samples were heated at 70° C. It was thought that raising the temperature might enable the heating time to be reduced. However, the length of time samples remained in the water bath was far more critical at higher temperatures. There was an apparent tendency for the *N*-ethylcarbazole to react with the acid to form a green compound which gradually darkened and appeared black. This side reaction was accelerated by increased heat, and apparently varied both with the particular bottle of acid and the lot of purified *N*-ethylcarbazole. The magnitude of the masking effect of the reagent-acid compound upon the carbohydrate-reagent compound is dependent to some extent upon the individual sugar. High carbohydrate concentrations (5 to 10 mg. of carbohydrate per liter) also are less affected than lower concentrations. This suggests a competition between the acid and the carbohydrate for the reagent molecules.

Figure 3 shows the influence of temperature on the time of formation of the green reagent-acid compound. The color change may be reversed by shaking and cooling the samples within 1 or 2 minutes following development of the green color, but after this time the

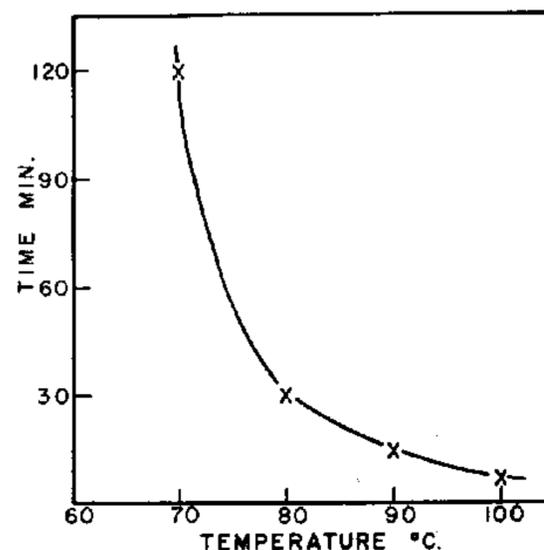


Figure 3. Influence of temperature on time of formation of green reagent-acid compound

change becomes irreversible. Because of this increased instability of the reagent at higher temperatures, further time checks were made at 70° C.

Using a single batch of reagent, sets of standards were run as usual varying the heating time from 10 to 30 minutes. Samples were cooled and read in the spectrophotometer. Figure 4 shows the relationship of time of heating and slope for such an experiment. There is a significant increase in slope between 10 and 15 minutes, and between 15 and 20 minutes of heating. However, the increase between 20 and 30 minutes is slight. Further testing of these two heating times indicated that the increase in slope between 20 and 30 minutes was statistically significant, with a *t* value for the comparison of slopes significant at the 2% confidence level. The 30-minute time interval was selected for routine use.

Dische, in his work with carbazole (4) emphasized that all samples should be chilled before and during the addition of reagent to increase the precision, and to control the heat generated by dilution of the reagent. This cooling procedure, as suggested by Dische, was tried with the Erdman method.

The reagent was prepared as usual and precooled in the refrigerator. One set of standards was pipetted and left at room temperature and the entire 27 ml. of reagent were added to the warm samples without any standing period. Cooled reagent was added to the flasks prepared for the second set of standards, the samples were pipetted carefully into the flasks which were in an ice

Figure 4. Variation of slope with time of heating at 70° C.

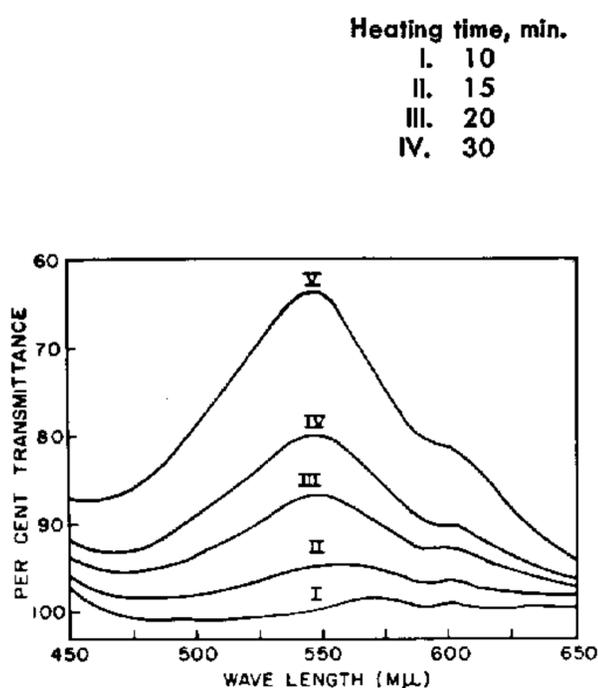


Figure 5. Absorption spectra of arabinose-N-ethylcarbazole compounds

Mg. arabinose per liter
 I. Blank
 II. 1
 III. 3
 IV. 5
 V. 10

bath, and the flasks were swirled to mix reagent with sample. Both sets of standards were then placed in the hot water bath and heated. The complete range of temperatures, 70° to 100° C. was tested as described.

At no temperature did the chilled samples produce as much color as the samples treated at room temperature when both were heated the same length of time. Most chilled samples showed only half the color of the samples at room temperature as indicated by absorbance readings. Precision with the two methods appeared to be the same and the advantage of using room temperature was such that there was no further testing of this procedure using the modified method.

A better slope results when the reagent is freshly prepared in acid kept at room temperature than when it is made in quantity, stored in the refrigerator, and used cold.

Effect of Salt Concentration. Because several of the well-known carbohydrate methods show a definite salt

effect—e.g., anthrone—the method was tested on standards made up both in distilled water and in Brujevicz artificial sea water. No statistically significant difference was found either in slope or in precision between the two sets of standards, although the usual precautions to prevent bubbling are necessary when the salt water standards are used.

Effect of Brand of Acid. The brand of acid used was most important. Of five brands tested, Du Pont c.p. sulfuric acid gave the most satisfactory blanks. Three of the five acids were unusable. One gave a deep pink color upon the addition of the *N*-ethylcarbazole to the acid while the other two became green. The Du Pont acid, as well as the fifth brand, showed only a pale straw color after *N*-ethylcarbazole was added.

Stability of Color of Carbohydrate-Reagent Compound. The stability of the colored compound to light was also checked. Standards of 1, 5, and 10 mg. of arabinose per liter were prepared and run in duplicate by the usual method, except that samples were brought to room temperature by slow cooling in a dark cabinet. When room temperature was reached, one set of

duplicates was placed under a fluorescent lamp, while the other set was left in the cabinet. Readings were made with the spectrophotometer at 544 mμ at 10-minute intervals to determine changes in absorbance with time. Representative data are given in Table I. Thus samples kept in the dark will maintain constant color for at least 4 hours, while illuminated samples show increasing color, the proportion of increase being much greater for these samples of lower concentration.

Precision within Batches of Reagent. A batch of reagent was made up as usual and divided between two bottles. Six sets of standards were set up using the same volumetric pipet for each sample. The 12 blanks to be used in all six sets were pipetted successively, followed by the 12 1-mg. standards. This was repeated for each group, ending with the highest concentration, which was 10 mg. of arabinose per liter. The usual procedure was followed, using sets of standards consisting of the usual concentrations (blanks, 1, 2, 3, 5, and 10 mg. of arabinose per liter) in duplicate. The samples were read simultaneously, one operator reading the Beckman DU spectrophotometer at 544 and 560 mμ, and a second operator reading the Fisher Electrophotometer using the special filter described. Three cells were used in reading the spectrophotometer, while a single cell was used with all Electrophotometer readings. Statistics for this experiment are given in Tables II and III.

Standard deviations between duplicates were 0.21 mg. per liter of arabinose for Beckman readings at 544 mμ, 0.24 mg. per liter for Beckman readings at 560 mμ, and 0.16 mg. per liter for Fisher readings. These figures compare well

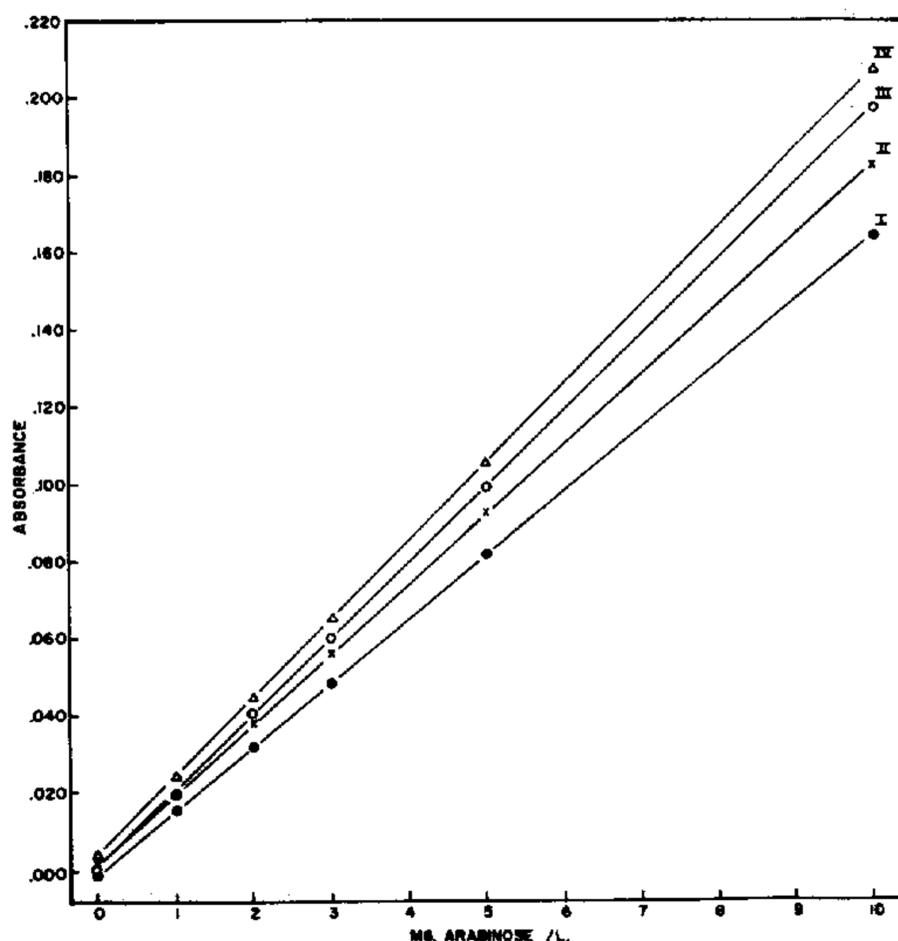


Table I. Absorbance Changes with Time

Time, Min. after Reaching Room Temperature	Absorbance					
	Dark			Illuminated		
	1 ^a	5	10	1	5	10
0	0.037	0.097	0.190	0.034	0.103	0.186
30	0.037	0.097	0.190	0.041	0.108	0.187
60	0.037	0.098	0.191	0.044	0.117	0.190
100	0.037	0.098	0.191	0.042	0.118	0.188
180	0.037	0.098	0.192			
240	0.037	0.098	0.192			

^a Concn. of arabinose in mg. per liter.

with values determined during routine analyses involving thousands of samples. They are, however, almost three times the values reported by Lewis and Rakestraw (8) using sucrose as a standard. For this reason results were routinely carried only to tenths of a milligram, in contrast to the values of Lewis and Rakestraw, which are reported to hundredths of a milligram.

A *t* test, comparing the values determined with the Fisher with those of the Beckman at 544 $m\mu$, showed that the differences were not significant at the 5% confidence level. Differences are just significant at the 5% level when the concentrations calculated from the Fisher data are compared with those calculated from the Beckman data at 560 $m\mu$. This may be partly due to the fact that the Beckman readings at 560 $m\mu$ are taken at a point where the slope of the absorbance curve is changing rapidly. Moreover the interference filter does pass light of wave length 544 $m\mu$, so that Fisher readings are a result of absorption over this entire region. This, and the inability of the operator to distinguish small differences in readings, are undoubtedly factors which account for the greater precision between duplicate readings when the Fisher Electrophotometer is used.

Furthermore, the light path of the Fisher 23-ml. cuvette is over twice that of the Beckman 1-cm. Corex cell. A slightly greater precision might also be expected because of the use of a single cell with this instrument. Experiments using a single cell with the Beckman have emphasized the necessity for rinsing the cells with the solution to be read; samples of higher concentrations (5 and 10 mg. of arabinose per liter) are particularly susceptible to dilution effects with readings varying as much as 10% in absorbance when the standard of the next higher concentration is read without rinsing out the previously read lower concentration.

Studies of Reaction Product Formed between Carbohydrates and *N*-Ethylcarbazole Reagent. Solutions of various carbohydrates of the best commercial grades available were prepared at concentrations equivalent to 1, 2, 3, 5, and 10 mg. of arabinose per liter (see molarities given above) in distilled water. Each compound was run in duplicate by the routine method, cooled, and read in the spectrophotometer against distilled water after a trace of the compound spectrum had been made with the Cary recording spectrophotometer, Model 10 M.

Two types of absorbance spectra were observed, the type depending upon the classification of the sugar. The pentoses, arabinose, xylose, ribose, and lyxose showed a peak of maximum absorption at 544 $m\mu$, with a secondary

Table II. Precision within Bottle of Reagent
(Fisher Electrophotometer)

Sample ^a	Concn. of Arabinose, Mg./L.					
	0	1	2	3	5	10
	$A \times 10^2$	$A \times 10^2$	$A \times 10^2$	$A \times 10^2$	$A \times 10^2$	$A \times 10^2$
I	5.3	9.2	12.4	16.2	23.3	41.3
	4.8	8.6	12.0	16.0	23.9	40.6
II	5.4	8.6	12.0	16.1	24.3	39.3
	5.6	8.6	12.0	15.6	23.6	39.4
III	5.0	8.5	12.0	16.0	23.0	40.9
	5.5	8.0	11.5	15.7	23.3	40.5
IV	4.2	7.9	11.5	15.5	23.5	41.0
	4.8	8.0	11.2	14.8	22.6	40.0
V	5.1	8.8	13.5	15.5	23.0	40.0
	4.2	7.3	11.9	14.6	22.5	40.0
VI	5.4	8.0	11.8	15.4	22.5	41.4
	4.7	8.0	11.8	15.5	22.0	39.1
Std. dev.	0.475	0.513	0.574	0.490	0.658	0.781
Regression coefficient ^b	3.551					
S_b	0.0218					
Deviation from regression	0.6120					
Variance between duplicates	0.2642					
Variance between samples	0.9516					
$F = 3.65^c$						

^a Samples refer to set of standards heated, cooled, and read as one group.

^b Computed using all observations individually.

^c $F = \frac{\text{between samples}}{\text{duplicates}}$; significant above 1% confidence level.

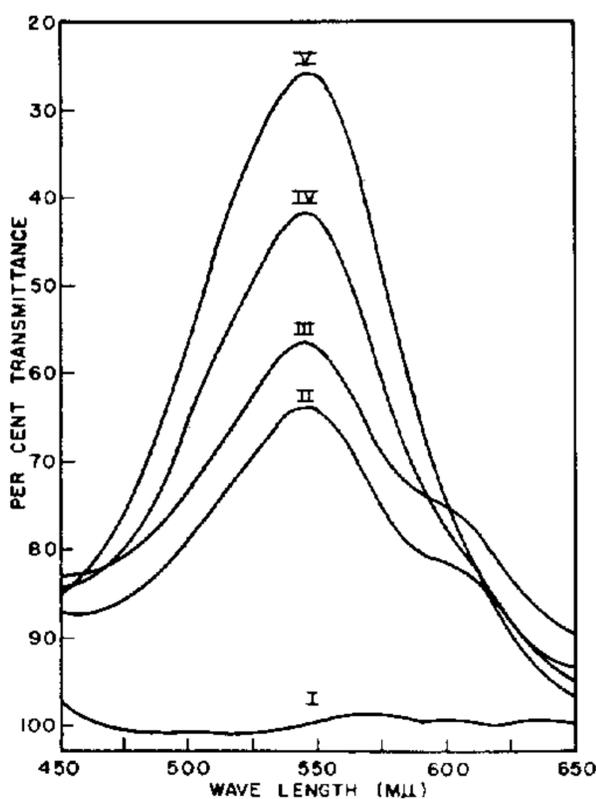


Figure 6. Absorption spectra of pentose-*N*-ethylcarbazole compounds

- I. Blank
- II. 10 mg. arabinose per liter
- III. 10 mg. lyxose per liter
- IV. 10 mg. xylose per liter
- V. 9.8 furfural per liter

peak at about 600 $m\mu$. The hexoses, glucose, fructose, galactose, mannose, and sorbose as well as their di- and oligosaccharides, maltose, lactose, sucrose, turanose, trehalose, melibiose, melezitose, and α -methyl glucoside showed a single maximum at 560 $m\mu$, while the methylated sugars (fucose and rhamnose) as well as ascorbic acid showed a single maximum at 548 to 550 $m\mu$. Figures 5, 6, and 7 give

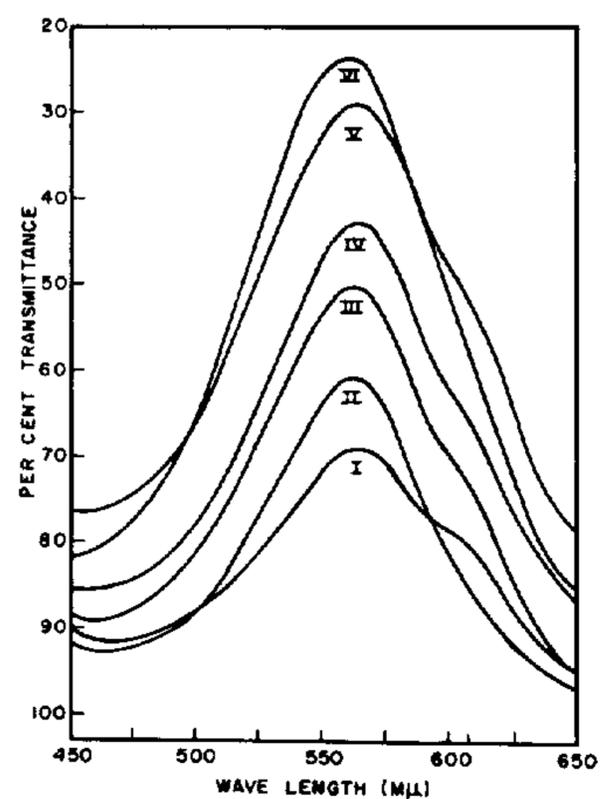


Figure 7. Absorption spectra of hexose-*N*-ethylcarbazole compounds

- I. 10 mg. galactose per liter
- II. 10 mg. glucose per liter
- III. 10 mg. fructose per liter
- IV. 24.4 mg. melibiose per liter
- V. 28.8 mg. sucrose per liter
- VI. 29.1 mg. melezitose per liter

representative Cary traces of the pentoses and hexoses as well as the family of curves of the arabinose standard.

To minimize errors in the calculation of absorptivity arising from the observations, individual values were determined for each concentration of each sugar using the formula

$$a = A/bc$$

Table III. Precision within Bottle of Reagent
(Beckman Spectrophotometer)

Sample ^a	Concn. of Arabinose, Mg./L.											
	0		1		2		3		5		10	
	<i>A</i> ₅₄₄ ^b	<i>A</i> ₅₆₀	<i>A</i> ₅₄₄	<i>A</i> ₅₆₀								
I	0.006	0.010	0.030	0.029	0.045	0.044	0.076	0.070	0.108	0.095	0.213	0.180
	0.013	0.016	0.034	0.034	0.049	0.046	0.067	0.060	0.118	0.103	0.208	0.177
II	0.016	0.020	0.032	0.032	0.050	0.050	0.076	0.069	0.113	0.099	0.205	0.170
	0.015	0.020	0.035	0.035	0.050	0.048	0.070	0.062	0.115	0.100	0.204	0.173
III	0.018	0.021	0.037	0.037	0.052	0.050	0.080	0.075	0.116	0.103	0.210	0.181
	0.016	0.020	0.035	0.034	0.050	0.047	0.071	0.065	0.110	0.100	0.211	0.184
IV	0.009	0.011	0.032	0.033	0.051	0.049	0.070	0.064	0.112	0.099	0.216	0.187
	0.016	0.020	0.035	0.035	0.048	0.045	0.075	0.066	0.115	0.101	0.206	0.176
V	0.009	0.011	0.035	0.034	0.061	0.056	0.071	0.065	0.116	0.103	0.215	0.184
	0.014	0.016	0.034	0.035	0.051	0.048	0.074	0.068	0.111	0.099	0.204	0.175
VI	0.012	0.015	0.035	0.035	0.050	0.049	0.076	0.070	0.114	0.101	0.213	0.185
	0.018	0.021	0.037	0.039	0.055	0.051	0.069	0.063	0.109	0.096	0.205	0.176
Std. dev.	0.0038	0.0042	0.0024	0.0025	0.0039	0.0032	0.0038	0.0042	0.0031	0.0026	0.0044	0.0053
Regression coefficient ^c	0.01959 ^d			0.01623 ^e								
<i>S</i> _i	0.000131			0.000135								
Deviation from regression	0.00366			0.00379								
Variance												
Within samples	0.00000628			0.00001506								
Between samples	0.000015			0.0000284								
<i>F</i> ^f	2.39			1.886								

^a Samples refer to set of standards heated, cooled, and read as one group.

^b *A* = absorbance.

^c Computed using all observations individually.

^d All statistics below refer to data at 544 mμ.

^e All statistics below refer to data at 560 mμ.

^f Not significant at 5% level.

Table IV. Absorptivity and Slopes of Various Carbohydrate-N-Ethylcarbazole Compounds

Compound	<i>a</i> ₅₄₄ ^a	<i>a</i> ₅₆₀	Slope ₅₄₄	Slope ₅₆₀	Slope	
					Compound 544	Compound 560
						Slope Arabinose
Arabinose	2600	2080	0.0187	0.0155		
Xylose	4980		0.0334	0.0288	1.79	1.86
Ribose	2690	2080	0.0178	0.0143	0.95	0.92
Glucose	2440	2840	0.0115	0.0137	0.61	0.88
Arabinose	3000	2520	0.0181	0.0148		
Fructose	4530	5320	0.0264	0.0309	1.46	2.09
Galactose	2100	2460	0.0125	0.0146	0.69	0.99
Mannose	1790	2150	0.0096	0.0116	0.53	0.78
Maltose	5330	6150	0.0169	0.0206	0.93	1.39
Arabinose	2440	2070	0.0170	0.0141		
Sorbose	4680	5450	0.0280	0.0321	1.65	2.28
Arabinose	2600	2050	0.0149	0.0122		
Lactose	3870	4500	0.0098	0.0118	0.66	0.97
Arabinose	2630	2140	0.0180	0.0146		
Rhamnose	1980	1910	0.0136	0.0119	0.76	0.82
Arabinose	2970	2610	0.0188	0.0159		
α-Methyl glucoside	1220	1500	0.0074	0.0086	0.39	0.54
Fucose	770	740	0.0054	0.0052	0.29	0.33
Arabinose	2980	2500	0.0198	0.0168		
Lyxose	3180	2620	0.0223	0.0189	1.13	1.12
Turanose	5340	6240	0.0150	0.0178	0.76	1.06
Sucrose	5210	6180	0.0149	0.0176	0.75	1.05
Melibiose	4120	4980	0.0113	0.0134	0.57	0.80
Arabinose	3180	2880	0.0252	0.0235		
Trehalose	4920	5760	0.0133	0.0161	0.53	0.69
Melezitose	8980	10790	0.0177	0.0211	0.70	0.90
Arabinose	2870	2370	0.0198	0.0164		
Furfural	5300	4110	0.0586	0.0456	2.96	2.78

^a Wave lengths are given in mμ.

where *a* is the absorptivity, *A* is the absorbance, *b* is the path length, and *c* is the concentration in moles per liter. The values were averaged to give the *a*_{av} found in Table IV. These values are not absolute, but indicate the order of magnitude because *a*_{av} for arabinose varied from 2440 to 3180 while the slope varied from 0.0149 to 0.0252 at 544 mμ.

The slope per milligram of carbohydrate was calculated using the formula

$$m = \frac{S_{xy} - n\bar{x}\bar{y}}{S_x^2 - n(\bar{x})^2}$$

where *x* is the concentration, *y* is the absorbance, *n* is the number of observations, \bar{x} is the mean value of *x*, and \bar{y} is the mean value of *y*. A set of arabinose standards was run from each bottle of reagent and both calculations made. This served to check variations in the reagent due to differences in lots of acid or in the *N*-ethylcarbazole. Thus calculations for each compound listed in Table IV should be compared with the values for arabinose which are given with the group.

Table IV indicates that routine readings using the spectrophotometer should be made at 560 mμ; at this wave length the majority of the compounds tested gave a slope ratio of approximately 1, compared with arab-

inose. Sorbose, xylose, and fructose are notable exceptions; each yields about twice as much color per milligram as the standard. The methyl sugars fucose and rhamnose as well as α -methyl glucoside yield substantially less color than might be expected on a milligram basis. Glucosamine hydrochloride, ascorbic acid, and malic acid showed no perceptible color at a concentration of 10 mg. per liter. The reactivity of the carbohydrates is dependent to a large extent upon their structure, which probably explains much of the variation in the data of Table IV.

A comparison of the color produced by arabinose with that yielded by its primary degradation product, furfural (9), would indicate about a 55% conversion of arabinose to furfural by this method. Because arabinose has been found to produce 76% of its theoretical yield of furfural (6) apparently only a two-thirds conversion is accomplished under the conditions of this method.

DISCUSSION

The method described has been used for several thousand determinations, both routine and experimental, and was found to be simple in operation and relatively rapid. The lack of salt effect makes it readily usable for sea water as well as for biological fluids. Lewis and Rakestraw (8) have used both the Erdman *N*-ethylcarbazole and anthrone methods for determining total

carbohydrates in sea water and concluded that the two methods were approximately equal in sensitivity to most sugars, but that anthrone was much less sensitive to arabinose and xylose. The sensitivity of the *N*-ethylcarbazole reagent has been increased at least three times by the modification described here; 3 γ of total carbohydrate per sample (1 mg. per liter) may be determined accurately. This compares favorably with other general methods of carbohydrate determination such as indole and sulfuric acid, 1-naphthol and sulfuric acid, or tryptophan and sulfuric acid, all of which require samples containing 10 γ of carbohydrate per ml. (4). Only the cysteine, carbazole, and sulfuric acid method of Dische and Borenfreund (5) is as sensitive, and in that method the color maximum is not developed for 18 hours.

Although most of the routine work was done with the Fisher Electrophotometer with the special interference filter, the method is equally adaptable to the use of the Beckman spectrophotometer without any decrease in speed of analysis. In the latter case it is recommended that readings be made at 560 $m\mu$ in accordance with Table IV, unless the sugars being determined are known to be pentoses. Furthermore, a 5-cm. cell should be used for concentrations below 4 mg. of carbohydrate per liter, to increase the accuracy of the readings.

With the precautions mentioned, this method is easily adaptable to routine use. Under ideal conditions, 96 samples can be run during the working day. Slight modifications in the time of heating may be made if the standards are run in the same manner. Variations in sample volume are also possible; the method is adaptable to biochemical or clinical work using 1 ml. of sample, if the ratio of 9 parts of reagent to 1 part of sample is maintained, and if about one fifth of the total volume of reagent is added before the standing period.

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