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A Colorimetric Formaldehyde Assay

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ABSTRACT

A room-temperature assay of formaldehyde is described. The assay uses few reagents and is colorimetric, read at a wavelength of 649 nm. Tryptophan and tryptamine were noted as interfering with the assay, probably by binding with the formaldehyde. High levels of sugar show smaller effects on final absorbance. Glyceraldehyde also reacts in the assay, but six other aldehyde compounds do not, although they do reduce the absorbance of added formaldehyde.

Index Entries: Formaldehyde; glyceraldehyde; colorimetric assay.

INTRODUCTION

Methods for the colorimetric assay of formaldehyde (HCHO) have included reacting the chemical with acetylacetone (1–3), with 4-amino-5-hydrazino-3-mercapto-1,2,4-triazole (AHMT) *(4)*, 3-methyl-2-benzothiazolone hydrazone *(5)*, and with tryptophan *(6,7)*.

During work on an assay for uronic acids (Taylor and Buchanan-Smith, in preparation), a color reaction with HCHO was noticed. This reaction was investigated and a simple assay of HCHO was developed.

MATERIALS AND METHODS

Chemicals Used

Concentrated H_2SO_4 was from Fischer Scientific. All other chemicals were from Sigma (Mississauga, Ontario).

Standard Method

From 0 to 60 μg of in 100 formaldehyde μL water was placed in 16 × 150 mm borosilicate test tubes. Concentrated sulfuric acid (3mL) was added, and then 100 μL of 0.1% carbazole in absolute ethanol. The tubes were well-mixed and then allowed to remain at room temperature until read in a spectrophotometer at 649 nm.

Uronic Acid Reagent

The Bitter and Muir *(8)* reagent (7.947 mg/mL sodium tetraborate decahydrate · $10H_2O$ in sulfuric acid:water, mixed 5:1) was investigated first. The necessity of borate ion was checked.

Optimum Carbazole Concentration

Maximum absorbance value at a 1:5, water:conc. acid ratio was determined.

Optimum Acid Strength

Using the optimal carbazole concentration, acid strength giving maximum absorbance value was determined.

Absorbance Curve of Chromagen

The absorbance curve of the chromagen was plotted and the best wavelength for use was determined.

Optimum Carbazole Concentration in Optimum Acid Strength Reagent

Carbazole concentration was again investigated to determine the maximum absorbance. The relationship of carbazole concentration to HCHO was then investigated.

Optimum Time-Course

The time to maximum absorbance was calculated using a room temperature assay.

Optimum Temperature

The effect of incubating at elevated temperature on the assay was investigated.

Interference by Other Compounds

The effect of several other compounds on absorbance with and without added HCHO was investigated.

RESULTS AND DISCUSSION

Uronic Acid Reagent

The sulfuric acid–borate, uronic acid reagent, as described by Bitter and Muir *(9)* was used as the starting point for the assay. When HCHO was reacted in this reagent, the peak absorption occurred at 649 nm. This reaction, carried out at room temperature for 90 min, was within 90% of the absorbance achieved at 5.5 h. Unfortunately, when a standard curve was constructed, it was found to be sigmoidal, probably because of precipitation of the chromagen. If the reaction mixture remained at room temperature for 16 h, those containing more than 37 μg of HCHO showed complete precipitation of the chromagen. Carbazole is not soluble in water, and if it was mixed with the aqueous HCHO sample before the acid was added, it would precipitate. This caused highly variable results.

An attempt was made to obtain a linear standard curve by optimizing the reaction mixture. When the amounts of water and acid were held constant at a ratio of 1:5, and varying amounts of sodium tetraborate were added to the reaction, no effect was noted, either on the rate of chromagen formation or on the final absorption. The borate ion, which is included in the Bitter and Muir reagent, was not needed in this reaction and was omitted.

Optimum Carbazole Concentration

The effect of the amount of carbazole in the reaction mixture using a ratio of 1:5 water to concentrated acid was examined, 100 μg carbazole (100 μL of the 0.1% ethanolic mixture per sample) was optimal for low amounts of HCHO. For the highest amount of HCHO tested (74 μg), the absorbance continued to rise with increasing amounts of carbazole.

Optimum Acid Strength

Figure 1 demonstrates the effect of varying acid strength on the absorbance of 37 and 74 μg HCHO. Below an acid:water ratio of 2:1, the carbazole precipitated; at 1.5:1, the precipitate was blue. Although the highest absorbance occurred at 2.5 mL acid, the best relationship of absorbance to HCHO occurred when no water was added. This concentration showed no precipitation after several days; all others precipitated overnight. On this basis, it was decided to use concentrated acid for the reagent.

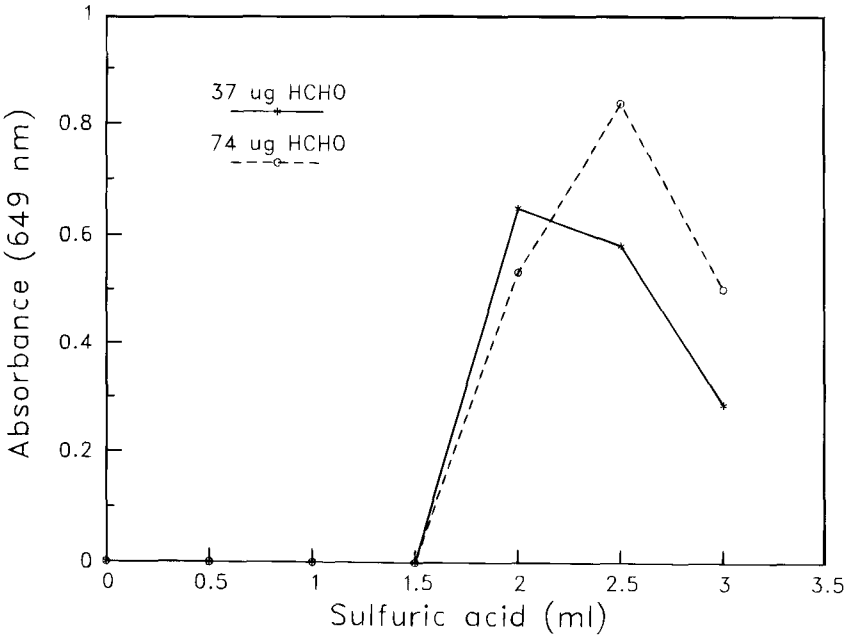

Fig. 1. Effect of acid concentration on absorbance. Acid and water were mixed to a final volume of 3 mL and allowed to cool to room temperature. HCHO (37 and 74 μg in 100 μL water) and 100 μL 0.1% carbazole in ethanol were added and mixed. The assay was allowed to stand at room temperature for 1.5 h.

Absorbance Curve of Chromagen

Figure 2 shows the absorbance curve of the chromagen against a reagent blank containing no HCHO. A peak was found between 620 and 640 nm, slightly lower than that found for the uronic acid reagent. Since other substances that showed a color reaction in the assay absorbed maximally at wavelengths of 523 nm (galacturonic acid) and at 456 nm (fructose), the wavelength of 649 nm was retained for the assay, to minimize interference.

Optimum Carbazole Concentration in Optimum Acid Strength Reagent

The amount of carbazole was varied in the concentrated acid reagent, to determine optimum values. Figure 3 shows that, for 7.4 and 37 μg HCHO, the optimum value was 100 μg (100 μL of 0.1%). As in the previous study of acid strength, this amount gave the best relationship between the amount of HCHO and absorbance. At 1.5 h, the absorbance was 80–100% of that at 4.5 h.

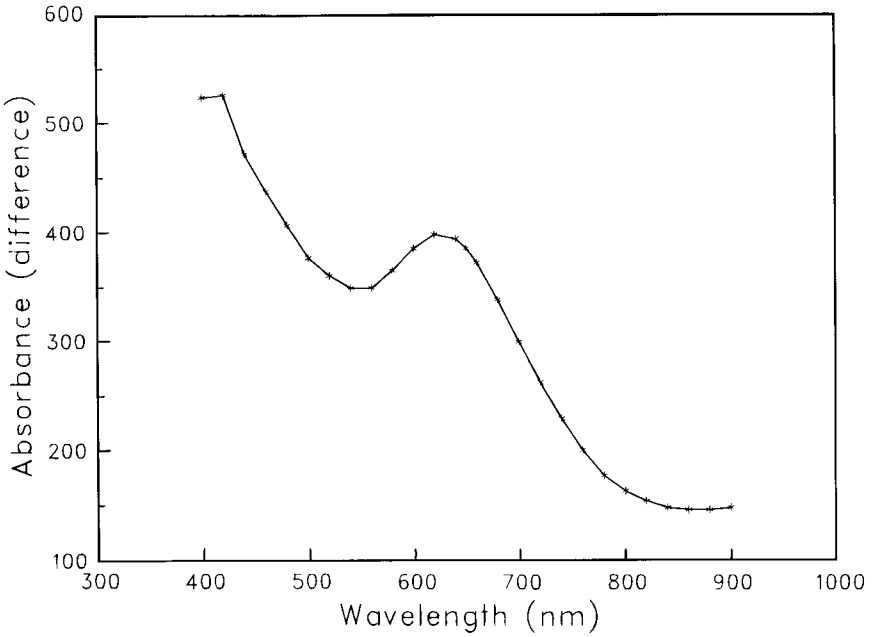

Fig. 2. Absorption spectrum of the chromagen produced, using 44.4 µg of HCHO. A 2-h incubation at room temperature was used.

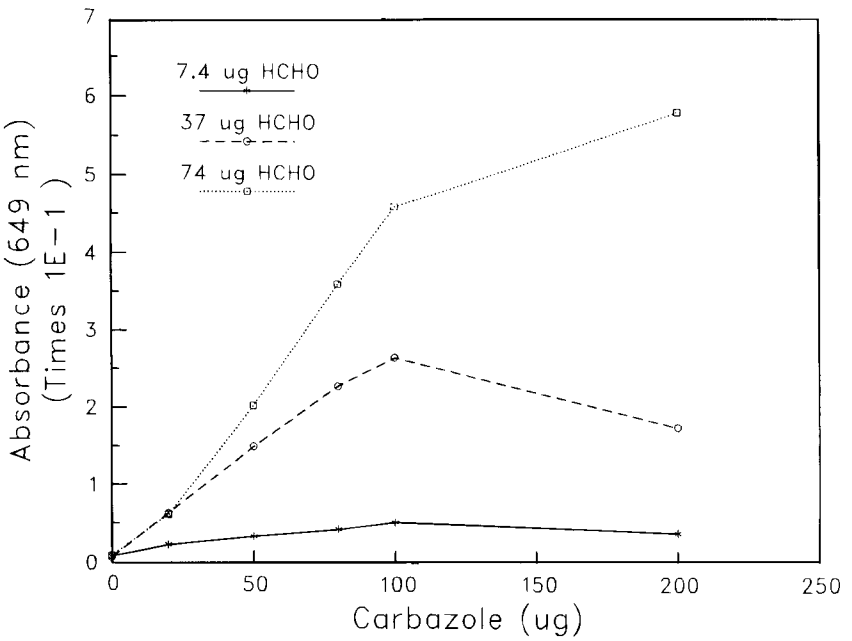

Fig. 3. The relationship of carbazole to absorbance for three levels of HCHO (7.4 µg, dotted line; 37 µg, dashed line; and 74 µg, solid line). The incubation period was 1.5 h at room temperature.

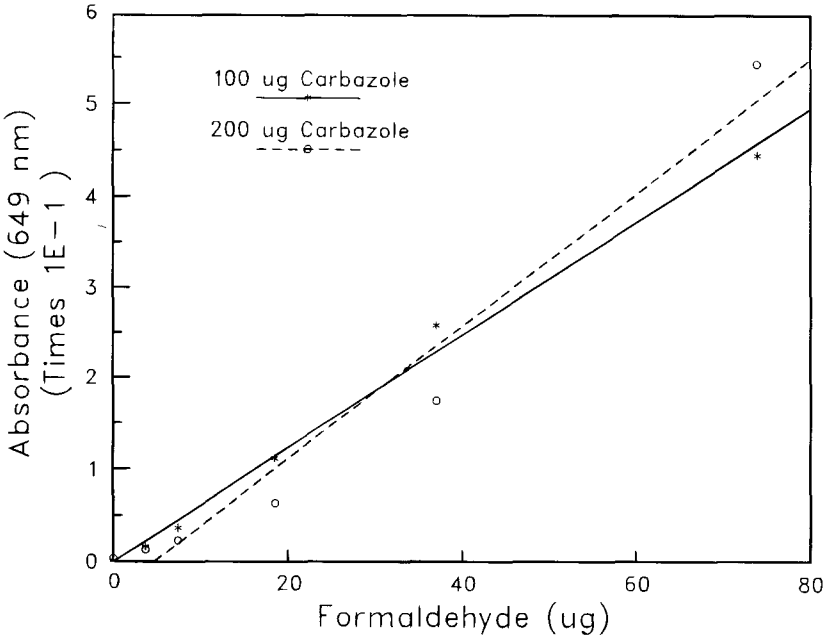

Fig. 4. Curves showing the amount of HCHO against absorbance, using 100 μg, solid line, and 200 μg, dashed line.

Standard curves at 100 and 200 μg carbazole are shown in Fig. 4. At 100 μg, the curves were linear to at least 60 μg HCHO with a small drop at 74 μg. This pattern was the same for incubation periods of 1.5 and 4.5 h. When 200 μg carbazole was used, the curve lost its linearity. When larger amounts of HCHO were assayed, the absorbance stopped increasing at 150 μg HCHO, using 100 μg carbazole. This would represent a molar ratio of approx 8:1. With added carbazole, a rise in absorbance is again seen: Figure 5 illustrates this effect. These experiments indicate that the ratio of carbazole to HCHO in the assay is critical.

Optimum Time-Course

Figure 6 shows the time-course of a room-temperature assay on 44.4 μg HCHO with 100 μg carbazole. At 1.5 h, the value was 83% of that at 5 h. A method of stopping the assay completely at a specific time was not sought, since it was found that a reasonable number of assays could be read without excessive change in their absorbance. Dagani and Archer *(10)* mention the use of 2,4-pentanedione to remove HCHO from samples to be tested for acetaldehyde. This chemical might have some value in the current assay as a method to eliminate free HCHO, and to stop the color development. The resulting compound is reported to be yellow in color, which should not interfere at 649 nm. This compound was not tested.

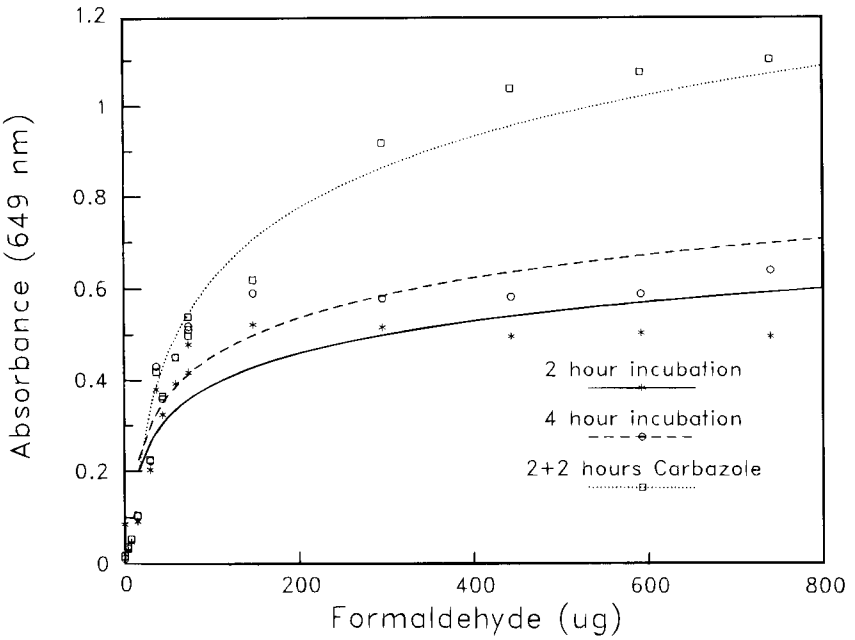

Fig. 5. Standard curves showing the effect of high HCHO concentrations in the assay. The solid line shows 100 µg carbazole at 2 h incubation; the dashed line shows the same amount after 4 h incubation. The dotted line shows the effect of an additional 100 µg carbazole added, at 2 h and allowed to incubate a further 2 h.

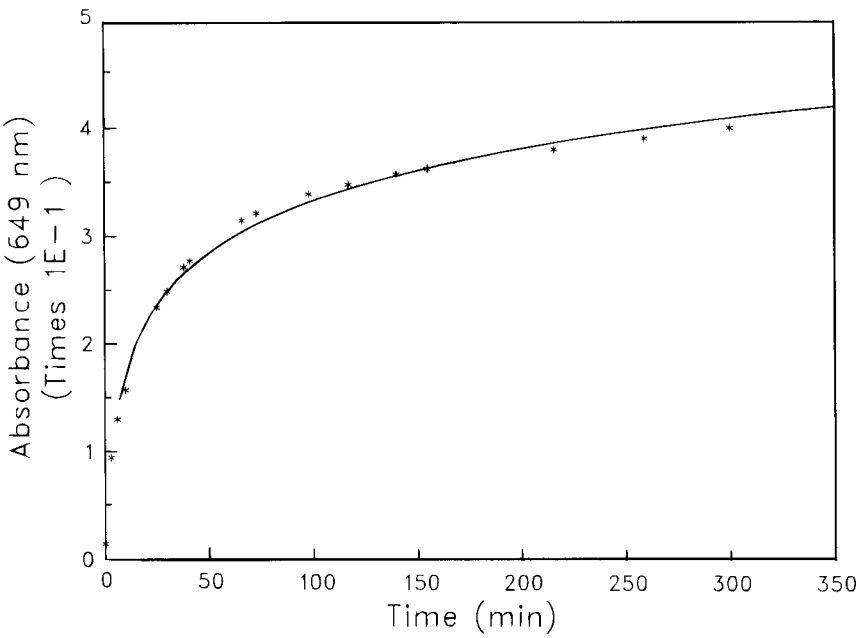

Fig. 6. The increase in absorbance over time at room temperature. Three mL of concentrated sulfuric acid was added to 44.4 µg of HCHO in 100 µL water. One hundred µg of carbazole in 100 µL of ethanol was then added and the tubes read at various times.

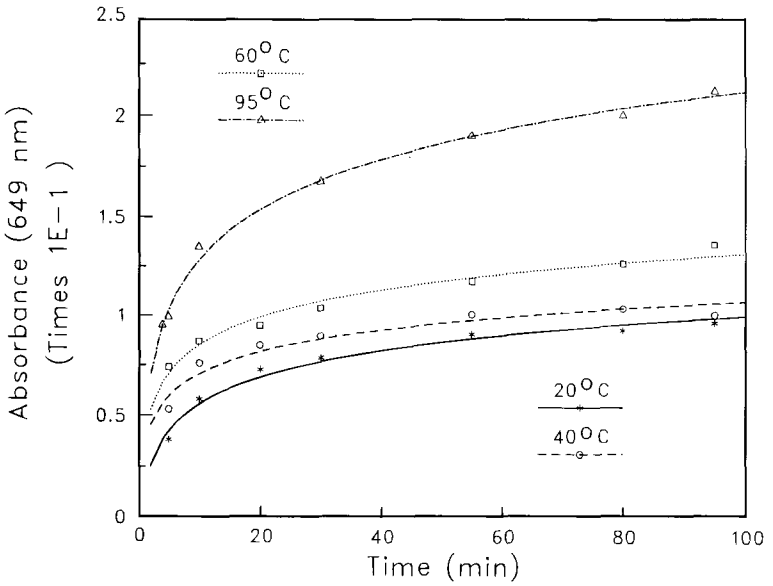

Fig. 7. The increase in absorbance over time at room temperature (solid line), 40° (dashed line), 60° (dotted line) and 95° (dots and dashes). Three mL of concentrated sulfuric acid was added to 14.8 µg of HCHO in 100 µL water. One hundred µg of carbazole in 100 µL of ethanol was then added and the tubes read at various times.

Optimum Temperature

The effects of temperature on the assay were evaluated. As the temperature of incubation rose, the absorbance also rose. At 1.5 h incubation, there was a 45% rise in absorbance comparing room temperature to 95°C. Figure 7 shows the increase in absorbance over time for 14.8 µg HCHO at four different termperatures. The values at 95 min, when compared to those after the tubes remained overnight, were 71, 80, 93, and 99% for the room temperature, 40, 60, and 95° incubations, respectively. For the room-temperature incubation, this represented an increase of 0.003 absorbance units per h. Standard curves assayed at these four temperatures showed an increasing linearity, but a decreasing slope as the incubation temperature was lowered.

Higher temperature assays were more sensitive and faster, but may be subject to interference problems. Table 1 shows the apparent HCHO and percent of true HCHO on an equal weight basis of several common biological substances. The samples were in 100 µL water, 3 mL concentrated acid, and 100 µL 0.1% carbazole, and the incubation period was 1.5 h. All of the elevated temperatures showed a general increase in interference from the sugars and sugar acids. This is a common problem with assays that use strong acid as a reagent. Because of the wavelength used (649 nm) for reading the assays, all interferences were low. Fructose and

Table 1
Reactivity of Other Substances at Different Temperatures

Sample	Amount[a] (μg)	Apparent HCHO (μg)				% True HCHO w/w			
		RT[b]	40	60	95	RT	40	60	95
Glucose	200(1.1)	1.3	1.5	0	4.1	0.7	0.8	0	2.0
Xylose	200(1.3)	0	0	0	0	0	0	0	0
Fructose	100(0.6)	1.1	1.8	0	0.8	1.1	1.8	0	0.8
Sucrose	200(0.6)	1.6	2.3	0.2	4.7	0.8	1.2	0.1	2.3
Galacturonic acid	100(0.5)	1.6	9.5	6.7	0.6	1.6	9.5	6.7	0.6
Glucuronic acid	200(1.0)	0	0.6	0	0.4	0	0.3	0	0.2
Lactic acid	50(0.6)	0	0	0	0	0	0	0	0
Bovine serum albumin	1000	0	0.4	0	0	0	0.04	0	0
HCHO	74(2.5)	73	73	71	71	99	99	96	96

[a] Values in brackets are amounts in μmol.

[b] Samples incubated for 1.5 h at room temperature or elevated temperatures (°C).

sucrose were yellow in color, as was lactic acid. When boiled in strong sulfuric acid, lactic acid is broken down to acetaldehyde *(11,12)*. The uronic acids were pink; glucose and xylose tended toward orange.

Interference by Other Compounds

Table 2 shows some other substances treated in the same manner. In this case, the incubations were at room temperature for 2 h. After 2 h, 14.8 or 29.6 μg HCHO was added and a further 2-h incubation carried out. Only the coconut oil sample showed any reaction in the assay. Tryptophan, tryptamine, and the protein casein (which is high in tryptophan) interfered with the recovery of HCHO; the sugars increased the absorbance. Table 3 shows an analysis of HCHO recovery from several substances after being mixed prior to analysis. The samples were incubated at room temperature for 1.5 h. In this case, the recovery from a tryptophan-containing sample is even less than in the previous experiment. This would indicate that tryptophan and carbazole compete for the HCHO. In this table, the recovery percentages for the sugars are not exaggerated, as are those in Table 2.

Table 4 illustrates the effect of some solvents and other substances that might be included in a sample mixture. Acetone, methanol, and acetic acid had little effect on the assay. Methyl ethyl ketone (2-butanone) dramatically inhibited the chromagen formation, as did formic acid.

High amounts of acetaldehyde (394 μg) and rhamnose (1.143 mg) inhibited the color formation by 74 and 33%, respectively.

Table 2
Reactivity of Other Substances and Recovery of HCHO Added Later

Sample	Amount[a] (μg)	A649nm 0 HCHO	% of HCHO absorbance 14.8 μg HCHO + 2h[b]	29.6 μg HCHO + 2h
Tryptophan	204(1.0)	.009	58	49
Tryptamine	197(1.0)	.010	71	76
Casein	10000	.020	34	13
Mannitol	200(1.1)	.011	126	143
Mannose	200(1.1)	.012	138	154
Rhamnose	200(1.2)	.003	141	151
Arabinose	200(1.3)	.008	127	142
Fucose	200(1.2)	.005	121	139
myoInositol	200(1.1)	.016	124	133
Coconut oil	1250	.038	130	76
HCHO[3][c] A64 nm		0.020	.091	.202

[a] Values in brackets are amounts in μmol.

[b] Samples incubated for 2 h, after which time HCHO was added and the tubes incubated a further 2h.

[c] Absorbance values for HCHO alone (100% recovery).

Table 3
Reactivity of Other Substances and Recovery of Added HCHO

Sample	Amount[a] (μg)	A649 nm 0 HCHO	% of HCHO absorbance 14.8 μg HCHO	29.6 μg HCHO
Tryptophan	204(1.0)	.014	6	49
Glucose	200(1.1)	.014	81	92
Xylose	200(1.3)	.015	86	93
Arabinose	200(1.3)	.017	75	88
Rhamnose	200(1.2)	.010	85	89
Fructose	200(1.1)	.027	116	105
Galacturonic acid	100(0.5)	.034	95	119
Mannitol	200(1.1)	.016	98	92
myoInositol	200(1.1)	.020	79	94
HCHO A649 nm		.020	.112	.332

[a] Values in brackets are amounts in μmol.

Table 4
Reactivity of Other Substances and Recovery of Added HCHO

Sample	Amount[a]	A649 nm 0 HCHO	% HCHO detected (37 µg added)
Acetone	50 µL(681)	0.007	90[b]
Methyl ethyl ketone	50 µL(558)	0.0052	
Methanol	50 µL(1234)	0.024	93
Formic acid	50 µL(1325)	0.010	0
Acetic acid	50 µL(809)	0.034	84
2-heptanone	82 µg(0.7)	nd[c]	95
Na-Periodate	640 µg(3.0)	0.250	83
K-Iodate	100 µg(3.8)	1.540	0
Rhamnose	1143 µg(6.9)	0.013	67
Acetaldehyde	394 µg(8.9)	0.007	26
	78.8 µg(1.8)	nd	73
Benzaldehyde	105 µg(1.0)	nd	96
Formaldehyde	37 µg(1.2)	0.300	100
Glutaraldehyde	100 µg(1.0)	nd	49
Glyceraldehyde	50 µg(0.6)	0.515	0
Hexanal	83 µg(0.8)	nd	73
Octanal	82 µg(0.6)	nd	72
Propionaldehyde	81 µg(1.4)	nd	72

[a] Values in brackets are amounts in µmol.

[b] Percent of absorbance (649 nm) caused by 37 µg HCHO alone.

[c] nd = not detected.

Glyceraldehyde showed a color reaction in the assay, but six other aldehyde compounds show none. These aldehyde compounds all show a reduction in the absorbance of added HCHO.

In the analysis of sugars by periodation, HCHO, acetaldehyde, or both can be liberated (*4*). In many cases the excess periodate in the assay mixture interferes with the subsequent analysis of HCHO, and several methods have been used to eliminate it. Vaskovsky and Isay (*2*) showed that rhamnose can be used to eliminate periodate, liberating iodate and acetaldehyde in the process.

Acetaldehyde added at 158, 315, and 394 µg to 37 µg HCHO in the assay gave absorbance values 61, 40, and 26% of that for HCHO alone. This is a linear relationship, and suggested that the acetaldehyde might be competing for the carbazole. At low concentrations, acetaldehyde should not be a problem in the assay of HCHO.

Acetaldehyde alone gives a yellow color in the assay, which shows an absorbance peak at 406 nm. This wavelength was used to test whether the carbazole assay could be used to quantitate this substance. Absorbance rose with added acetaldehyde to a plateau of 0.250 at 200 µg. Thirty-seven µg of HCHO showed an absorbance of 0.431 at 406 nm. It seemed unlikely that a useful acetaldehyde assay could be developed using these conditions.

Rhamnose up to 0.8 mg suppressed the absorbance less than 10%. For amounts up to 3 mg, the chromagen absorbance was still 70% of that for HCHO alone. As the rhamnose content in the assay was increased to 8 mg, the color caused by browning of the sugar by the acid began to interfere with the assay. Rhamnose did not present a problem unless present in very large excess.

Periodation mixtures typically contain sodium periodate in concentrations of 0.01–0.04 *M (2,4,5)*. Sodium periodate at 0.03 *M* (in 100 µL of sample) was added to a set of assay mixtures containing 0–74 µg HCHO. All of the mixtures were green, and all showed an absorbance of about 0.250. Any absorbance caused by HCHO was not detectable.

Periodate alone, added at 0.015–0.3 *M* had variable effects in the assay, giving colors from brown through yellow-green to deep blue-green. The absorbance values dropped and then rose again with increasing molarity, the lowest value being obtained at 0.06 *M*. When 37 µg of HCHO was added to a similar series of periodate concentrations, the differential absorbance caused by HCHO dropped rapidly, periodate at 0.015 *M* giving less than one-third the absorbance of 37 µg HCHO alone.

Sucrose (2mg/mL), D-fructose, L-rhamnose and D-glucose (all 1 mg/mL) were allowed to react with 0.03 *M* or 0.27 *M* sodium periodate overnight at room temperature in the dark. When 50 µL of this mixture was assayed, the 0.03 *M* iodate samples all showed an absorbance of over 1.2; the 0.27 *M* samples showed absorbances of about one-half that. Clearly, this assay cannot be used without removing excess periodate.

An attempt was made to neutralize the periodate by reacting it with rhamnose. Sodium periodate at 0.03 *M* in a 1 mL vol was treated with various concentrations of rhamnose (0.002–0.2 *M*) with or without 0.2% sodium bicarbonate *(5)*. These mixtures were incubated overnight, in the dark, at room temperature. Fifty µL of each mix was tested with and without 37 µg HCHO. Absorbances of the tubes with HCHO were determined and the absorbance of the corresponding HCHO minus tube was subtracted. For the mixtures with bicarbonate,the differential absorbance values for 0.04, 0.1, and 0.2 *M* rhamnose were 43, 56, and 30% of those for 37 µg HCHO alone. Sodium bicarbonate had no effect on the results. Mixtures containing rhamnose at less than 0.04 *M* showed no absorbances that could be attributed to HCHO. These results indicate that the carbazole assay for HCHO

might be useful for determining the HCHO released by periodation, if an efficient method of removing periodate, without releasing large amounts of acetaldehyde, is used.

The assay method presented here can be used for the quantitation of HCHO in the range of 2–60 μg (0.7 m*M* to 20 m*M* in the sample). This compares to a range of 20–300 μg for the Chrastil and Reinhardt *(7)* tryptophan assay. The Nash reagent is also sensitive to this range of HCHO. The MBTH method of Durand et al. *(5)* uses a standard curve of 0–3 mg HCHO. The AHMT method of Avigad *(4)* is more sensitive, with a standard curve of 0–1.5 ng HCHO, but this assay is not specific to that substance. The Nash reagent also suffers from low specificity *(6)*. The tryptophan method *(7)* is much more specific, interferance coming from acetaldehyde, oxidizing compounds, and tryptophan, or proteins high in tryptophan. The carbazole reaction also suffers from interferance from acetaldehyde and tryptophan.

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