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Interference of Azide in Assays of Carbohydrates*

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ABSTRACT

The effect of sodium azide at concentrations of 0·02% on three carbohydrate assay methods was investigated: it reduced the sensitivity of the phenol–sulfuric acid method for aldoses by 30–35%; the m-*hydroxydiphenyl and orcinol methods for galacturonic acid became insensitive. At this level of azide the sensitivity of the orcinol method for neutral sugar, but not for galacturonic acid, could be partially restored by increasing the amount of orcinol in the reaction mixture. The antimicrobial agent thimerosal did not interfere with the phenol–sulfuric acid or* m-*hydroxybiphenyl methods; with the orcinol method it interfered with the determination of galacturonic acid. This latter problem can be overcome by measuring the absorbance at longer wavelengths or correcting the measurements by an appropriate blank value.*

INTRODUCTION

The formation of colored complexes between phenols and degradation products of carbohydrates in concentrated sulfuric acid is the basis of several useful colorimetric assays of carbohydrates. The phenol–sulfuric

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acid method (Montgomery, 1961; Dubois *et al.*, 1956) is used as a general assay for carbohydrates. The *m*-hydroxydiphenyl colorimetric method (Blumenkrantz & Asboe-Hansen, 1973) is specific for uronic acids. By application of both methods for the analysis of fractions collected in column chromatographic procedures, it is possible to determine the elution profile of carbohydrates in general and to locate the fractions which are rich in uronide carbohydrates.

The orcinol–sulfuric acid reaction (Almog & Shirley, 1978; Navarro *et al.*, 1982) produces different colors with neutral and uronide carbohydrates. The orcinol method can therefore be used to determine the elution profile of these carbohydrates (Navarro *et al.*, 1982) when a complex mixture of polysaccharides is separated by column chromatography. In gel permeation chromatography, sodium azide is often added to the elution medium as an antimicrobial agent. During an investigation carried out in this laboratory on polysaccharides in tomato juice (P. Lindner, N. Ben Shalom and I. Shomer, in preparation), it appeared that azide also interferes with all of the above-mentioned methods. The effect of azide on the carbohydrate assays carried out by these methods was therefore studied and compared with the effect of thimerosal, another antimicrobial agent used in column chromatography. The errors caused by the presence of azide in the analysis of carbohydrate mixtures containing pectic material are discussed in this paper. Different mechanisms for the interference of azide in carbohydrate assays are suggested for assays conducted at different acid strength.

Interference of azide with carbohydrate assays was previously reported for the assay by the anthrone method (Tong *et al.*, 1973) and for the assay of galacturonic acid by an automated carbazole method described by Sarris *et al.* (1975).

MATERIALS AND METHODS

Materials

D-Glucose, D-galactose, D-arabinose, D-galacturonic acid and thimerosal ($C_2H_5HgC_6H_4COONa$) were obtained from Sigma; *m*-hydroxydiphenyl from Eastman Organic Chemicals; sodium tetraborate and concentrated sulfuric acid (94–96%) from Merck; sodium azide and orcinol from BDH and phenol (88%) from Malinkrodt.

Methods

All the reactions were carried out in test tubes with an internal diameter of 22 mm.

Phenol–sulfuric acid method
To a sample containing up to 90 μg of carbohydrate and 0–400 μg sodium azide in a volume of 2 ml, 1 ml of 5% phenol in distilled water is added. This is followed by 5 ml of conc. H_2SO_4 vigorously injected into the centre of the test tube. The mixture is then stirred for a few seconds on a Vortex, allowed to cool to room temperature for about 20 min, and the absorbance is measured at 485 nm.

m-*Hydroxydiphenyl method*
The procedure of Ahmed & Labavitch (1977) was followed. A solution of 0·0125M sodium tetraborate in conc. H_2SO_4 was prepared and chilled on ice before use. To test tubes placed in an ice bucket and containing 0–75 μg galacturonic acid and 0–200 μg sodium azide in a total volume of 0·6 ml, 3·6 ml of cold tetraborate reagent was added. After cooling, the test tubes were placed for 5 min in boiling water and allowed to cool in a water bath at room temperature. To the cooled tubes, 60 μl of 0·15% *m*-hydroxydiphenyl in 0·5% NaOH was added and the absorbance at 520 nm was measured. If the sample is kept cool while mixing with the tetraborate reagent, the color which develops is stable for many hours.

Orcinol–sulfuric acid method
To a 1-ml sample containing up to 300 μg neutral carbohydrates or up to 80 μg galacturonic acid and 0–200 μg sodium azide, 0·5 ml of 0·1% orcinol solution in 70% H_2SO_4 was added. After addition of 6 ml of conc. H_2SO_4, the mixture was heated to 95 °C for 5 min and then cooled to room temperature. Absorbance was measured at 475 nm for neutral carbohydrates and at 443 nm for galacturonic acid. To study the effect of increasing the amount of orcinol in the reaction mixture, a solution of 0·4% orcinol in 70% H_2SO_4 was used.

To study the effect of thimerosal on these methods, the material was added to the carbohydrate samples instead of sodium azide. In the orcinol method the absorbance of a series of blanks containing various amounts of thimerosal was measured.

To measure the spectra of the degradation products of carbohydrates

under the conditions of the three assay methods, the same procedures were carried out, replacing phenolic reagents by water to obtain the correct final concentration of acid.

RESULTS AND DISCUSSION

The effect of azide on the assay of various carbohydrates by the phenol–sulfuric acid method is shown in Table 1. The presence of azide in the reaction mixture reduces the sensitivity of the assay. In Fig. 1, the effect of various concentrations of sodium azide on the assays of glucose

TABLE 1
Effect of Sodium Azide on the Assay of Carbohydrates by the Phenol–Sulfuric Acid Method

Carbohydrate (*μg*)		*Sodium azide* (*μg*)	*Absorbance* (*485 nm*)
Glucose	90	—	0·560
	90	240	0·300
Galacturonic acid	165	—	0·280
	165	240	0·180
Arabinose	90	—	0·330
	90	240	0·230
	90	480	0·210
Xylose	90	—	0·510
	90	240	0·370
	90	480	0·270
Fructose	90	—	1·220
	90	240	0·340

and fructose is shown. From Table 1 and Fig. 1 it is seen that the assay by the phenol–sulfuric acid method is more affected by azide in the case of fructose than of glucose. The assay is less sensitive to galacturonic acid than to neutral sugars. The concentration of sodium azide which is recommended for elution media in gel permeation chromatography is 0·02 % and, with a sample of 2 ml, 400 μg sodium azide will be present in the reaction mixture. As shown in Fig. 2, a calibration curve for glucose by the phenol–sulfuric acid method can still be obtained in the presence of this amount of sodium azide.

With the *m*-hydroxydiphenyl method for uronic acids, the interference

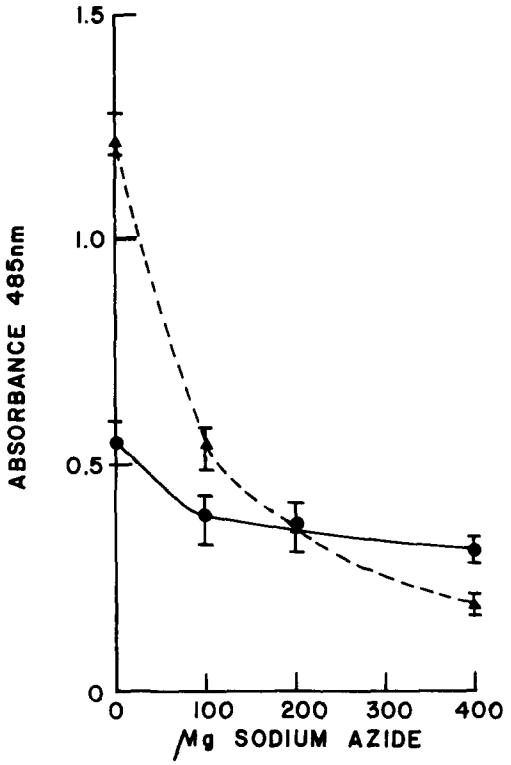

Fig. 1. Effect of sodium azide on the assay of glucose and fructose by the phenol-sulfuric acid method. —●—●— 105 μg glucose; —▲—▲— 105 μg fructose.

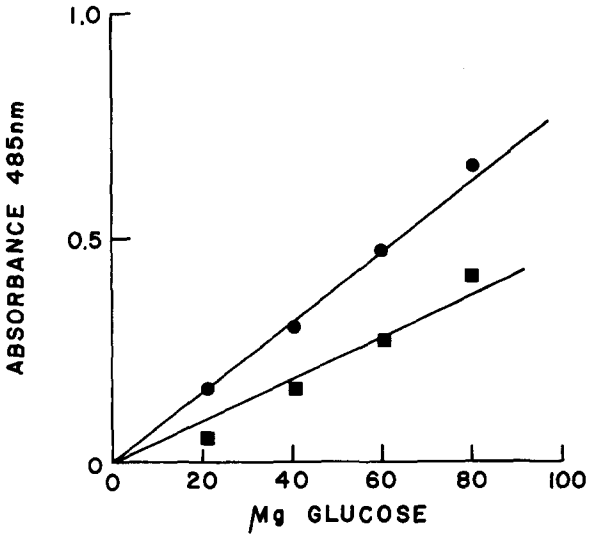

Fig. 2. Calibration curves for glucose with the phenol-sulfuric acid method in the absence and presence of sodium azide. —●—●— no sodium azide; —■—■— 200 μg of sodium azide.

of azide is more serious. From Fig. 3 we see that no color develops with 45 μg of galacturonic acid in the presence of as little as 25 μg of sodium azide. The assay is linear up to 75 μg of galacturonic acid. Therefore, in the case of samples containing 0·02 % sodium azide, serious interference is expected if the content of galacturonic acid is less than 750 μg/ml, and almost no color is expected to develop if the content of galacturonic acid is less than 450 μg/ml when samples of about 0·1 ml are to be used for the assay.

In the orcinol–sulfuric acid reaction, neutral sugars yield brown-colored products with an absorption maximum at 470–480 nm, while for uronide carbohydrates a yellow color with a maximum at 443 nm is obtained. The interference of azide with the orcinol–sulfuric acid method is demonstrated in Table 2. For neutral sugars the absorbance depends on the

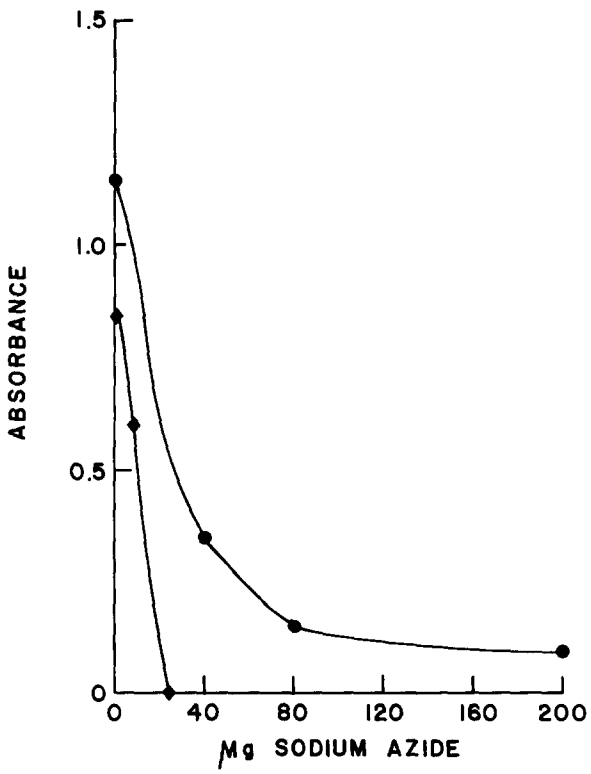

Fig. 3. Effect of sodium azide on the assay of galacturonic acid by the *m*-hydroxydiphenyl and orcinol methods. —◆—◆— *m*-hydroxydiphenyl method, 45 μg galacturonic acid; —●—●— orcinol method, 80 μg galacturonic acid.

TABLE 2
Effect of Sodium Azide on the Assay of Carbohydrates by the Orcinol Method

Carbohydrate (μg)		*Sodium azide* (μg)	*Orcinol* (μg)	*Absorbance*	λ (*nm*)
Glucose	225	—	500	0·855	475
	225	200	500	0·480	475
Arabinose	225	—	500	0·445	475
	225	—	2000	1·140	475
	225	200	500	0·120	475
	225	200	2000	0·445	475
Galactose	225	—	500	0·660	475
	225	—	2000	0·990	475
	225	200	500	0·125	475
	225	200	2000	0·300	475
Galacturonic acid	80	—	500	1·160	443
	80	—	2000	1·055	443
	80	200	500	0·100	443
	80	200	2000	0·135	443

amount of orcinol in the reaction mixture. Azide always reduces the sensitivity of the orcinol–sulfuric acid method. The assay is more sensitive to galacturonic acid than neutral sugar, but the interference of azide is more prominent in the former case (Table 2). The concentration dependence of the interference of azide with the determination of galacturonic acid by the orcinol–sulfuric acid method is shown in Fig. 3. Serious interference with this assay is already observed with 40 μg azide in the reaction mixture.

Of the three assay methods tested, the phenol–sulfuric acid method is the least affected, and the *m*-hydroxydiphenyl method the most affected, by the presence of azide. The orcinol–sulfuric acid method will also lose its sensitivity in the presence of azide. If the amount of orcinol in the reaction mixture is increased in order to restore sensitivity, the assay will then be sensitive to neutral sugars but not to galacturonic acid (Table 2). Also, when analysis of samples containing azide is carried out by both the phenol–sulfuric acid and *m*-hydroxydiphenyl methods, the sensitivity toward neutral sugars is only moderately affected whereas the sensitivity to galacturonic acid is greatly reduced. In the case of analysis of samples containing pectic material, the amount of uronic acids will thus be underestimated relative to the amount of neutral sugars.

From Table 3, it is seen that thimerosal, a mercurial antimicrobial

TABLE 3
Effect of Thimerosal on Carbohydrate Assay Methods

Carbohydrate (*μg*)		*Thimerosal* (*μg*)	*Absorbance*	*λ* (*nm*)
			Phenol–sulfuric acid method	
Glucose	75	—	0·550	485
	75	200	0·590	485
Fructose	75	—	0·790	485
	75	200	0·810	485
Arabinose	75	—	0·450	485
	75	200	0·410	485
Galacturonic acid	70	—	0·162	485
	70	200	0·176	
			m-*Hydroxydiphenyl method*	
Galacturonic acid	70	—	0·990	520
	70	60	0·980	520
	70	140	1·060	520
			Orcinol method	
Glucose	150	—	0·650	475
	150	100	0·630	475
Fructose	150	—	0·790	475
	150	100	0·750	475
Arabinose	150	—	0·320	475
	150	100	0·320	475
	150	200	0·320	475
Galacturonic acid	70	—	0·740	443
	70	100	0·980	443
	70	200	1·060	443

agent, does not interfere with the phenol–sulfuric acid or *m*-hydroxydiphenyl methods. Similar results were obtained by Tong *et al.* (1973) for the anthrone method. With the orcinol method, thimerosal does not interfere with the measurement of netural sugars at 475 nm. Under the conditions of the orcinol method, thimerosal was found to yield a yellow colour with absorbance at 443 nm (see Fig. 4), which can be corrected by a suitable blank. Whenever possible, thimerosal at the recommended level of 0·005% is, therefore, preferable to azide as an antimicrobial agent.

The reaction between azide and carbonyl compounds and carboxylic

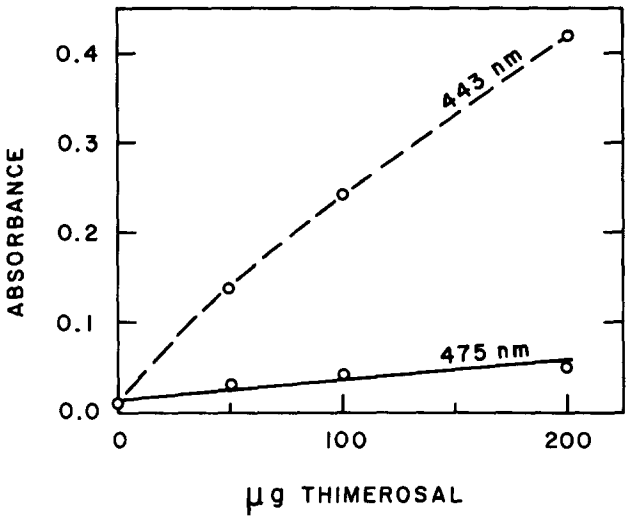

Fig. 4. Absorbance produced by thimerosal blanks in the orcinol method.

acids in concentrated sulfuric acid is known as the Schmidt reaction (Cram & Hammond, 1964). In this reaction, carboxylic acids yield amines, aldehydes yield formylamides or nitriles and ketones yield substituted amides (Tucker, 1964).

The effect of azide on the UV spectra of the degradation products of galacturonic acid in concentrated H_2SO_4 is shown in Figs 5 and 6 for the conditions of the *m*-hydroxydiphenyl (88 % H_2SO_4) and phenol–sulfuric acid (72 % H_2SO_4) methods, respectively. The contribution of the free azide in concentrated H_2SO_4 to the spectra is negligible at wavelengths longer than 225 nm. The corresponding spectra of furfural are shown in Figs 7 and 8.

Depending on conditions, the degradation products of galacturonic acid in acid media are reductic acid and the furan derivatives, furfural and 5-formyl-2-furoic acid (Scott *et al.*, 1967; Feather, 1982). Scott *et al.* (1967) measured the UV spectra of these materials in 89 % H_2SO_4. Using the spectra as reference spectra, they concluded that heating galacturonic acid for 30 min at 70 °C in 89 % H_2SO_4 yielded mainly 5-formyl-2-furoic acid, together with substantial amounts of furfural. Using the same reference spectra, it can be concluded from Fig. 5 that the same is true for the conditions prevailing in the *m*-hydroxydiphenyl method. The use of the same reference spectra is justified since the acid strength in the

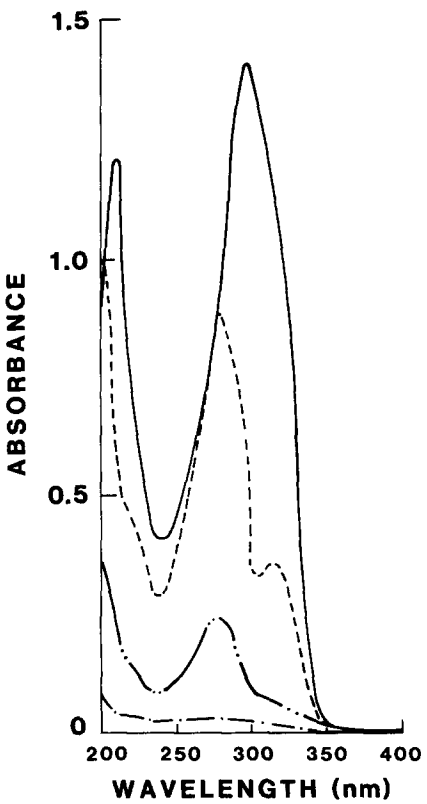

Fig. 5. Spectra of degradation products of galacturonic acid under the conditions of the *m*-hydroxydiphenyl method. ——— 105 μg galacturonic acid; – – – – 105 μg galacturonic acid and 40 μg sodium azide; —·—·— 105 μg galacturonic acid and 200 μg sodium azide; – · – · – 200 μg sodium azide.

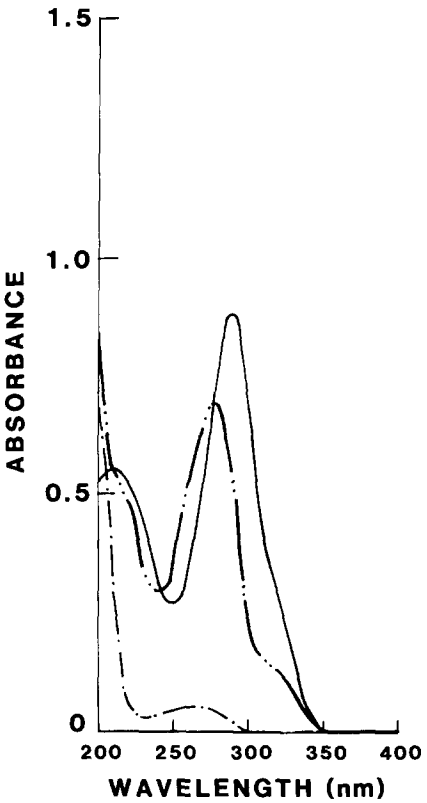

Fig. 6. Spectra of degradation products of galacturonic acid under the conditions of the phenol–sulfuric acid method. ——— 105 μg galacturonic acid; —·—·— 105 μg galacturonic acid and 200 μg sodium azide; – · – · – 200 μg sodium azide.

m-hydroxydiphenyl method (88 % H_2SO_4) is similar to the acid strength used by Scott *et al.* (1967). These authors showed that the absorption maxima of the degradation products of galacturonic acid undergo a red shift with increasing concentration of H_2SO_4. By adding water to a sample treated under the conditions of the *m*-hydroxydiphenyl method to obtain an acid strength corresponding to the phenol–sulfuric acid method, a spectrum similar to spectra obtained by treatment under the latter method is obtained. This means that the same products, probably in

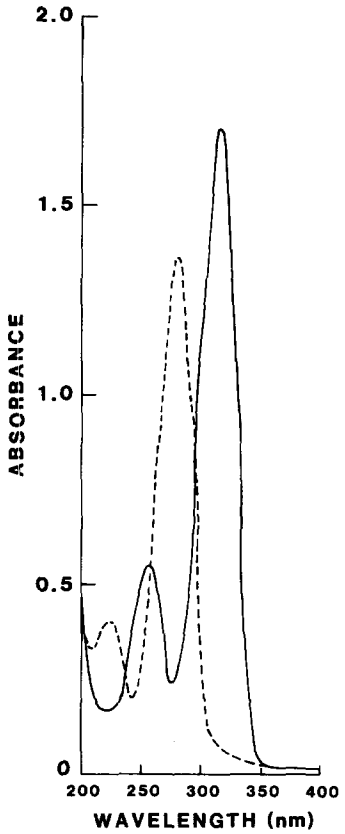

Fig. 7. Spectra of furfural and of furfural with sodium azide under the conditions of the *m*-hydroxydiphenyl method. ——— 23 μg furfural; – – – – – 23 μg furfural and 160 μg azide.

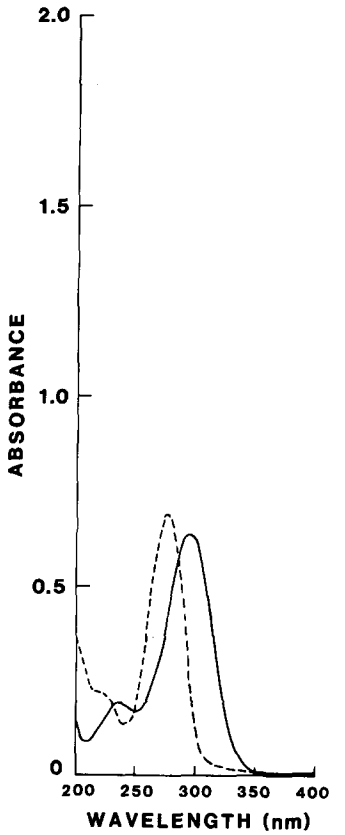

Fig. 8. Spectra of furfural and of furfural with sodium azide under the conditions of the phenol–sulfuric acid method. ——— 23 μg furfural; – – – – – 23 μg furfural and 160 μg azide.

somewhat different proportions, are obtained with both methods in the absence of azide.

In the presence of an excess of azide; the UV spectrum almost disappears under the conditions of the *m*-hydroxydiphenyl method (Fig. 5). Under the phenol–sulfuric acid method's conditions, it is mainly the reaction products between furfural and azide that are observed (Figs 6 and 8). Since the products of the interaction of furfural and azide under the conditions of the *m*-hydroxydiphenyl method give a UV spectrum (Fig. 7), it can be concluded that under these conditions the interference

of azide occurs mainly in the first stages of the dehydration of the galacturonic acid, before the formation of the furan derivatives. If this were not the case, we would at least observe a spectrum of the products of azide and furfural similar to that in Fig. 7. A possible explanation for the disappearance of the UV spectrum, in the presence of azide under the conditions of the *m*-hydroxydiphenyl method, might be inhibition of cyclization to form furan derivatives. This also explains the reduction in the sensitivity of the assay by this method.

At lower acid strength, under the conditions of the phenol–sulfuric acid method, cyclization to form furan derivatives is not inhibited; these derivatives then interact with azide to yield products whose absorption spectra are shown in Fig. 6. The reduction in sensitivity under the conditions of the phenol–sulfuric acid method can be ascribed to either a lower formation constant or lower molar absorptivity of the complex between the phenol and the product of the reaction between azide and the furan derivatives.

In conclusion, the data suggest that the interference of azide depends on the acid strength in the reaction mixture. In the case of galacturonic acid, the sensitivity of the *m*-hydroxydiphenyl and orcinol–sulfuric acid methods, which are carried out at high acid strengths of 88 % and 87 %, respectively, is greatly reduced by the presence of azide in amounts recommended for use as an antimicrobial agent. This probably occurs due to the inhibition of the formation of furan derivatives which yield colored complexes with the appropriate phenols. A similar explanation holds for the inhibition by sodium azide of the automatic carbazole method described by Sarris *et al.* (1975). At a lower acid strength, e.g. at 72 % acid as in the phenol–sulfuric acid method, the loss of sensitivity in the presence of azide is less drastic and depends on the properties of the complex formed with phenol in the presence of azide.

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