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# Carbohydrate analysis by a phenol–sulfuric acid method in microplate format

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**Abstract**

Among many colorimetric methods for carbohydrate analysis, the phenol–sulfuric acid method is the easiest and most reliable method. It has been used for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids. This method is used widely because of its sensitivity and simplicity. In its original form, it required 50–450 nmol of monosaccharides or equivalent for analysis and thus is inadequate for precious samples. A scaled-down version requiring only 10–80 nmol of sugars was reported previously. We have now modified and optimized this method to use 96-well microplates for high throughput, to gain greater sensitivity, and to economize the reagents. This modified and optimized method allows longer linear range (1–150 nmol for Man) and excellent sensitivity. Moreover, our method is more convenient, requiring neither shaking nor covering, and takes less than 15 min to complete. The speed and simplicity of this method would make it most suitable for analyses of large numbers of samples such as chromatographic fractions.


*Keywords:* Carbohydrate; Analysis; Phenol; Sulfuric acid; Microplate

Measurement of carbohydrate contents in a variety of samples is a basic analytical operation in many phases of biosciences. Among many colorimetric methods for carbohydrate determination, the phenol–sulfuric acid method [1,2] is the easiest and most reliable method for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids. The phenol–sulfuric acid method is used widely because of its sensitivity and simplicity. Other methods using anthrone [3], orcinol [4], or resorcinol [5] may be as sensitive but are not as convenient. In its original form [2], it required 50–450 nmol of monosaccharides or equivalent for analysis and thus is inadequate for precious samples. A scaled-down version of it requiring only 10–80 nmol of sugars was developed before [6]. We have now adapted this method for 96-well microplates for higher throughput to gain greater sensitivity and to economize the reagents. Monsigny et al. [5] reported a resorcinol–sulfuric acid assay in microplate format, but it needed covering the reaction mixture with a layer of pristane, vortexing, and heating at 90 °C in an oven for 30 min. Recently, Laurentin and Edwards [3] reported a microplate version of the anthrone method which also required covering of the wells with clingfilm and acetate tape, vortexing twice, incubating at 92 °C in a nonshaking water bath, and additional drying in an oven at

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45 °C for 15 min. In this paper, we report a simpler and more sensitive phenol–sulfuric acid assay using a 96-well microplate, without additional shaking or drying of the plate.

## Materials and methods

### *Materials*

Concentrated sulfuric acid and phenol were from J.T. Baker (Phillipsburg, NJ). D-Mannose (Man), D-xylose (Xyl), L-fucose (Fuc), D-galactose (Gal), and D-glucosamine hydrochloride (GlcN) were from Pfanstiehl Laboratories, Inc. (Waukegan, IL). L-Glucose (Glc), D-galacturonic acid (GalUA), and *N*-acetylneuraminic acid (NeuAc) were from Sigma Chemical Co. (St. Louis, MO). A 96-well, flat-bottomed, polystyrene miocroplate (Nunc, Cat. No. 269620) was from VWR (Bridgeport, NJ). A microplate shaker (Model Titer plate shaker) was from Lab-Line Instruments Inc. (Melrose Park, IL). A spectrophotometer (Model UV 160) was from Shimadzu Scientific Instruments, Inc. (Columbia, MD). A microplate reader (Model Benchmark) and its accompanying software, Microplate Manager, version 5.1, were from Bio-Rad Laboratories (Richmond, CA). All regression analyses were performed with a software Prism (GraphPad Software, San Diego, CA).

### *Optimization of microplate-based method*

Rao and Pattabiraman [7] reported that, in the phenol–sulfuric acid reaction, phenol underwent sulfonation in situ and the phenol–sulfonic acid formed decreased the color intensity for many hexoses and pentoses. Similarly, our preliminary experiments indicated that the addition of concentrated sulfuric acid to the sample followed by phenol (abbreviated sulfuric acid–phenol) yielded the best results. This protocol was optimized for shaking time and volumes of sulfuric acid and phenol as described below.

*Shaking time*. To 50 μl of Man in a well (100 nmol/well) of a 96-well microplate was added rapidly 150 μl of concentrated sulfuric acid and the mixture was shaken for 0–30 min. Then, 30 μl of 5% phenol in water was added and the mixture was heated for 5 min at 90 °C in a static water bath (the microplate was carefully floated). After cooling to room temperature for 5 min in another water bath, the microplate was wiped dry and $A_{490\ nm}$ was measured by microplate reader.

*Volume of concentrated sulfuric acid*. To 50 μl of Man in a well (100 nmol/well) of a 96-well microplate was added 0–150 μl of concentrated sulfuric acid rapidly. Immediately thereafter, 30 μl of 5% phenol was added and the plate was kept in a static water bath for 5 min at 90 °C. After cooling to room temperature for 5 min in another water bath, it was wiped dry and $A_{490\ nm}$ was measured by microplate reader.

*Volume of 5% phenol*. To 50 μl of Man in a well (100 nmol/well) of a 96-well microplate was added 150 μl of concentrated sulfuric acid as above, followed immediately with 0–100 μl of 5% phenol. The plate was heated for 5 min at 90 °C as above. After cooling to room temperature for 5 min in another water bath, it was wiped dry and $A_{490\ nm}$ was measured.

## Results

### *Optimization of reaction conditions*

For survey of optimal reaction conditions, we considered five factors, including sample size, sequence of addition, shaking time between additions of concentrated sulfuric acid and phenol, amount of concentrated sulfuric acid, and amount of 5% phenol. We selected the sample size of 50 μl to allow addition of sufficient amounts of sulfuric acid and the phenol solutions. Of the three sequences tested, sample–phenol–sulfuric acid and phenol–sulfuric acid–sample had lower absorbances than sample–sulfuric acid–phenol, and therefore the last sequence was adopted. Using this sequence, and when the 5% phenol was added immediately after the concentrated sulfuric acid, the maximal absorbance was obtained (Fig. 1).

It appears that the maximal absorbance was obtained when 150 μl of concentrated sulfuric acid and 30 μl of 5% phenol were added in rapid succession to 50 μl of Man (Figs. 2A and B).

### *Final optimized microplate phenol–sulfuric acid assay*

To 50 μl of Man in a well (100 nmol/well) of a 96-well microplate was added 150 μl of concentrated sulfuric

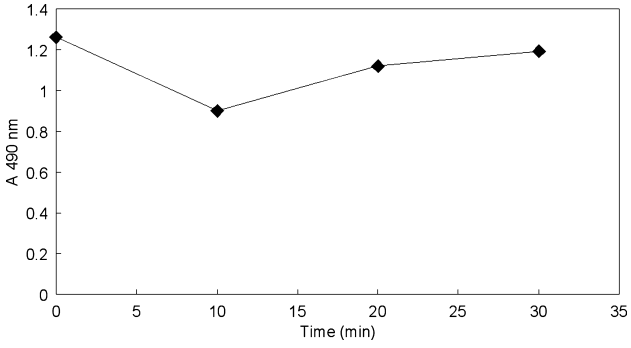


Fig. 1. Effect of the addition of 5% phenol at different time intervals after addition of concentrated sulfuric acid on the absorbance at 490 nm.

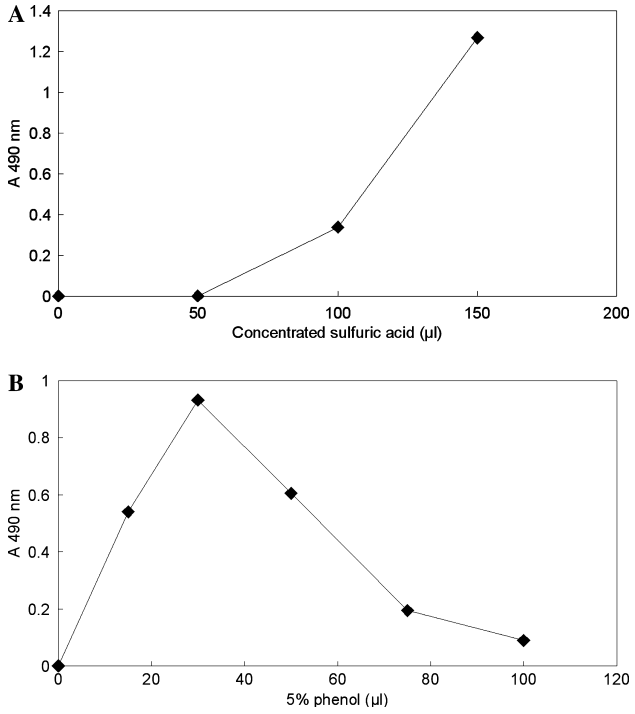


Fig. 2. (A) Influence of the volume of concentrated sulfuric acid on the absorbance at 490 nm. (B) Influence of the volume of 5% phenol on the absorbance at 490 nm.

acid rapidly to cause maximum mixing, followed immediately by 30 μl of 5% phenol in water. After incubating for 5 min at 90 °C in a static water bath by floating the microplate carefully, the plate was cooled to room temperature for 5 min in another water bath and wiped dry to measure $A_{490\ nm}$ by microplate reader. The whole assay takes <15 min.

### *Relative absorbance*

Eight sugars (Man, Xyl, Fuc, Gal, Glc, GlcN, GalUA, and NeuAc) were individually evaluated by this optimized microplate assay. Table 1 shows that each sugar (100 nmol/well) had different absorbance and relative absorbance at 490 nm. The absorbance of Xyl was the highest, but that of GlcN was negligibly low, as expected.

Table 1
Color responses of different sugars ($n = 5$)

| Sugar | Absorbance (at 490 nm) | Relative absorbance[a] |
|---|---|---|
| Man | 1.259 | 100 |
| Xyl | 2.098 | 167 |
| Fuc | 1.103 | 88 |
| Gal | 1.178 | 94 |
| Glc | 1.482 | 118 |
| GlcN | 0.039 | 3 |
| GalUA | 0.832 | 66 |
| NeuAc | 0.030 | <3 |

[a] Relative absorbance is the number based on the absorbance obtained with Man as 100.

### *Absorption spectra*

Five sugars (Man, Xyl, Fuc, Gal, and Glc) for spectral measurement were generated by this optimized assay. Five wells (100 nmol/well) were used for each sugar. After incubating and cooling to room temperature, the products in five wells were put together and applied to one cuvette to measure by spectrophotometer. Fig. 3 shows that at 490 nm most sugars can be measured at or near their absorption maxima and that the absorption spectra of Man, Xyl, Fuc, Gal, and Glc have peaks at 491, 486, 482, 491, and 493 nm, respectively.

### *Linearity of color responses*

A linear relationship between the absorbance and the sugar quantity was observed within the range of 1–150 nmol/well of Man. Figs. 4A–C show the regression

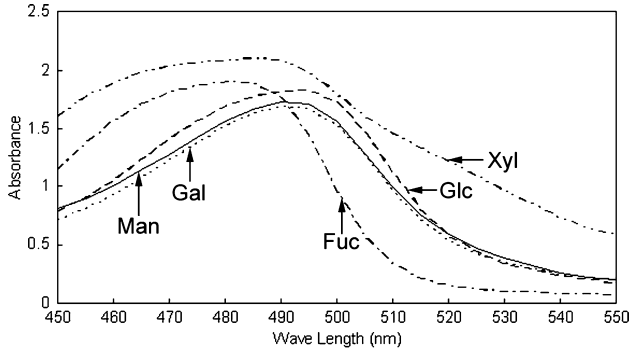


Fig. 3. Absorption spectra of color products from of Man, Xyl, Fuc, Gal, and Glc. Peaks are at 491, 486, 482, 491, and 493 nm, respectively.

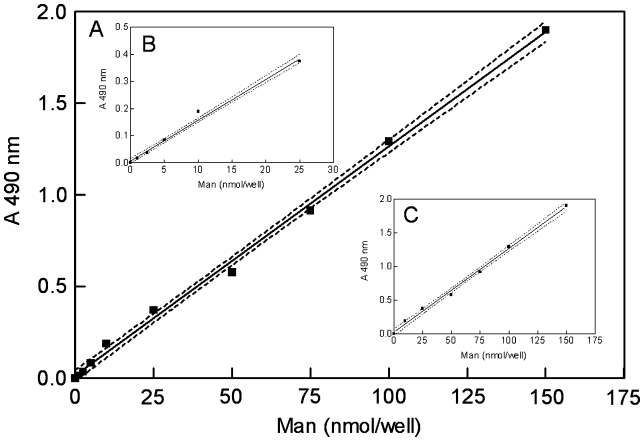


Fig. 4. Lines of Man within 1–150 nmol/well (A), 1–25 nmol/well (B), and 10–150 nmol/well (C) on the absorbance at 490 nm.

Table 2
Linearity of color responses ($n = 4$)

| Range (nmol/well) | Regression equation | Coefficient of determination $(r^2)^2$ | Standard deviation |
|---|---|---|---|
| 1–150 | $\hat{Y} = 0.01251X + 0.01390$ | 0.9880 | 0.07016 |
| 1–25 | $\hat{Y} = 0.01511X + 0.005961$ | 0.9868 | 0.01638 |
| 10–150 | $\hat{Y} = 0.01247X + 0.01823$ | 0.9834 | 0.08382 |

Table 3
Comparison of microplate versions of phenol–sulfuric acid method

| Authors | Coloring reagent | Detectable range (nmol/well) | Shake times | Requirements |
|---|---|---|---|---|
| Monsigny et al. [5] | Resorcinol | 1–20 (at 430 nm)<br>10–100 (at 480 nm) | Once | Pristane |
| Laurentin and Edwards [3] | Anthrone | 11.1–88.8 | Twice | Clingfilm<br>Acetate tape |
| This report | Phenol | 1–150 | No time | None |

lines within 1–150 nmol/well, 1–25 nmol/well, and 10–150 nmol/well, respectively. Table 2 shows their constants, coefficient of determinations $(r^2)^2$, and estimated standard deviations ($s$).

## Discussion

Table 3 shows comparison of our method with two previous reports using microplates. Monsigny et al. [5] and Laurentin and Edwards [3] reported microplate assays using resorcinol and anthrone, respectively, but both requiring shaking and adding of pristane to avoid any projection or covering of the plate or plate wells.

In addition to not requiring the cumbersome covering and shaking, our method has many advantages: (1) it requires less than 15 min (2) it requires only small amounts of phenol and sulfuric acid and (3) it is quite sensitive with a workable range of 1–150 nmol/well.

The speed and simplicity of this method would make it most suitable for high-throughput analyses of large number of samples such as chromatographic fractions with little consumption of materials.

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