Derivatization Reaction of Carbohydrates with Urea as the Reagent and Fluorimetric Determination of Carbohydrates

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It is found that in the presence of sulfuric acid carbohydrates condense with urea to afford the condensation products, which emit fluorescence. Under optimum conditions, the fluorescence intensities of system are proportional to the concentrations of carbohydrates. Based on this linear relationship, quantitative determination of kinds of carbohydrates has been made. Among all the carbohydrates tested, the sensitivity of α-rhamnose is the highest and its limits of detection reaches 3.5 × 10^{-8} mol/L. So α-rhamnose can be selectively determined in the presence of other carbohydrates. A interaction mechanism is also discussed.

Keywords carbohydrate, urea, derivatization reaction, fluorimetric determination

Introduction

Carbohydrates play an essential role in nature and almost all the life processes. They are not only central to the generation and storage of energy but also useful in the diagnosis of diseases and dynamic adjustment for the functions of protein. So establishing high sensitive and effective methods for the quantitative analysis of carbohydrates is important for understanding the role of carbohydrates in organisms.

Since carbohydrates have neither chromophores nor the fluorophore group that can be sensitively detected using the usual methods, it is important to derive them into detectable compounds with conventional methods. Several derivatizing reagents have been reported, such as 1,2-di(4-methoxyphenyl)ethylenediamine (DME),[1] 1,2-phenylenediamine (PDM),[2] malonamide,[3] guanidine,[4] arginine,[5] benzamidine,[6] 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS),[7] 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC),[8] 2-metthyl-3-oxo-4-phenyl-2,3-dihydrofunan-2-yl acetate (PDFAc)[9] and 2,3-diaminonaphthalene.[10] However, when they are applied to the determination of carbohydrates, they all have some drawbacks such as long reaction time, harsh reaction conditions and/or low sensitivity and selectivity.

Our research focuses on finding new derivatizing reagents with the emphasis on high selectivity and low cost. In this paper, a new sensitive and selective fluorimetric method for the determination of carbohydrates is described. The method is based on the condensation reaction of carbohydrates with urea in the presence of sulfuric acid. Under optimum conditions, an excellent linear relationship is obtained between the fluorescence intensity of the condensation products and the concentration of carbohydrates. The proposed method has the highest sensitivity and selectivity for α-rhamnose, which can be selectively determined in the presence of other carbohydrates. The interaction mechanism discussed in this paper shows that it is the methyl group in the terminal of α-rhamnose that is responsible for the high selectivity.

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Experimental

Apparatus

The fluorescence spectra and all fluorescence intensities were measured with a Hitachi Model 850 spectrofluorimeter.

Reagents and solutions

The urea solution (1.00 mol/L) was prepared by dissolving 6.000 g of urea in 100 mL of deionized water. Glucose, fructose, galactose, glucosamine hydrochloride and *N*-acetylglucosamine were all obtained from Detecting Department of Medicines and Bioproduct of China. Trehalose and xylose were purchased from Medicine Company of China. α-Rhamnose was obtained from Chemical Reagents Company of Beijing. *L*-Sorbose was obtained from Beijing Fangcao Medicine and Chemotechnology Research Company. *D*-Arobinose was purchased from Medicine Store of Military Medical College of China. The solutions of carbohydrates were all prepared by dissolving them in deionized water to make a concentration of 1.00×10^{-2} mol/L. These solutions should be stored at 0—4 ℃.

Sulfuric acid solution (1:1, V:V) is prepared by dissolving 50 mL of sulfuric acids in 50 mL of distilled water.

All the other were of analytical reagent grade and deionized water was used in this study.

Procedure

To a 25 mL of test tube, solutions were added in the following order: 1.5 mL of urea solution (1.00 mol/L), 0.5 mL of carbohydrate standard solutions (1.00×10^{-2} mol/L) and 5 mL of H_2SO_4 : H_2O (1:1, V:V). The mixture was diluted to 10 mL with water and then heated in a boiling water bath for 180 min. After the mixture was cooled to room temperature, its fluorescence intensity was measured in a 1-cm-quartz cell with excitation and emission wavelengths of 322 nm and 405 nm, respectively. The excitation and emission band-pass widths were both 10 nm. The wavelength scanning speed was set at 300 nm/min.

Results and discussion

Fluorescence spectra

The fluorescence spectra of the urea-H_2SO_4 (1), glucose-H_2SO_4 (2) and glucose-urea-H_2SO_4 (3) systems are shown in Fig. 1. It can be seen from Fig. 1 that both glucose and urea have no fluorescence in the strongly acidic medium. While under the same condition, when urea and glucose were added together, they formed a complex with a strong excitation peak ($\lambda_{ex}^{max} = 338$ nm) and a emission peak ($\lambda_{em}^{max} = 405$ nm).

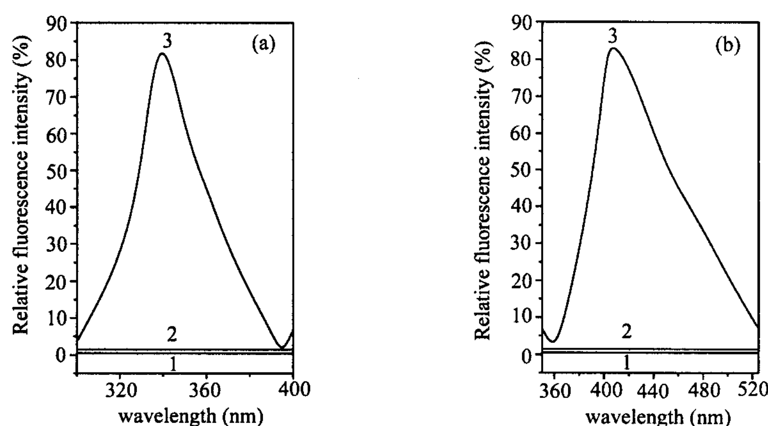


Fig. 1 Fluorescence spectra. (a) Excitation spectra; (b) emission spectra. (1) urea-H_2SO_4; (2) glucose-H_2SO_4; (3) glucose-urea-H_2SO_4. Conditions: glucose, 5.00×10^{-4} mol/L; urea, 0.15 mol/L; 1:1(V:V) H_2SO_4, 5 mL; heating time, 3 h.

Under the same conditions, the reactions of urea with other different carbohydrates was investigated. The results are shown in Table 1. From Table 1, it can be seen that except *N*-acetylglucosamine and HCl-glucosamine, all the other carbohydrates reacted with urea to give fluorescent compounds, and the fluorescence spectra of which are similar to that of glucose-urea-H_2SO_4 system, but their intensities are different. Among them, the intensity of the reaction product of α-rhamnose is the highest.

Table 1 Fluorescence spectra of different carbohydrates

Carbohydrates	Maximum excitation wavelength (nm)	Maximum emission wavelength (nm)	Relative intensity (%)
Glucose	338	405	11.8
Fructose	362	414.6	14.2
D-Xylose	354	442	12.0
D-Galactose	335	400	12.0
Trehalose	338	403	19.2
α-Rhamnose	373	415	100
L-Sorbose	349	413	11.0
D-Arabinose	360	448	7.0
HCl-glucosamine	—	—	—
N-Acetylglucosamine	—	—	—

Effect of the concentration of urea on the fluorescence intensity

The effect of the concentration of urea was studied. The results are shown in Fig. 2. From it, it can be seen that a final concentration of 0.13—0.25 mol/L gives the maximum fluorescence under the given conditions.

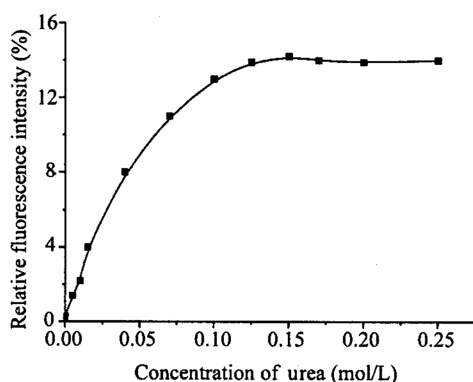


Fig. 2 Effect of the concentration of urea. Conditions: glucose, 5.00×10^{-4} mol/L; 1:1 (*V*: *V*) H_2SO_4, 5 mL; heating time, 3 h.

Effect of the concentration of H_2SO_4 *on the fluorescence intensity*

Experiments indicate that media have an important effect on the fluorescence intensity of the carbohydrate-urea-H_2SO_4 system. Since both HCl and HNO_3 are volatile, they will escape and produce a smaller concentration of acids when the system is heated. Meanwhile, using H_2SO_4 as the reaction medium, the fluorescence intensity of the system is higher than that using HCl and HNO_3. The effect of H_2SO_4 concentration is shown in Fig. 3. From Fig. 3, it can be seen that the fluorescence intensity increases with the increasing of the concentration of H_2SO_4. The fluorescence intensity of the system reaches the maximum and retains constant when the volume of H_2SO_4 solution added is 4.5—8.0 mL. Therefore, 5 mL of H_2SO_4 solution was selected for future study.

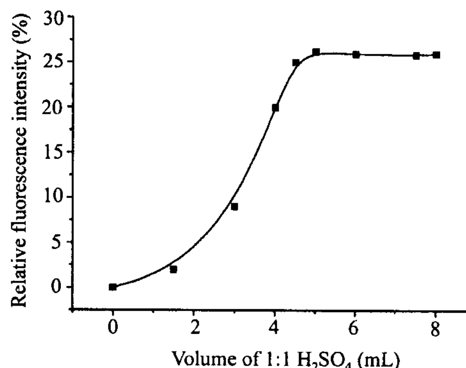


Fig. 3 Effect of the concentration of H_2SO_4. Conditions: glucose, 5.00×10^{-4} mol/L; urea, 0.15 mol/L; heating time, 3 h.

Effects of reaction temperature and heating time on the fluorescence intensity

The effects of different reaction temperatures were studied. The results are given in Fig. 4. It shows that at lower temperature the fluorescence intensities are very weak and the time to reach the balance is very long, but at 100 ℃ the fluorescence intensities are quite strong and the balance time is very short. So the temperature was chosen at 100 ℃ in this study. The results also show that the fluorescence intensities increase with the increasing of the heating time. The fluorescence intensity reached the maximum after the system was heated in a boiling water bath for 170—270 min (Fig. 4). Other carbohydrates are similar to glucose. Here, a heating time of 180 min was selected.

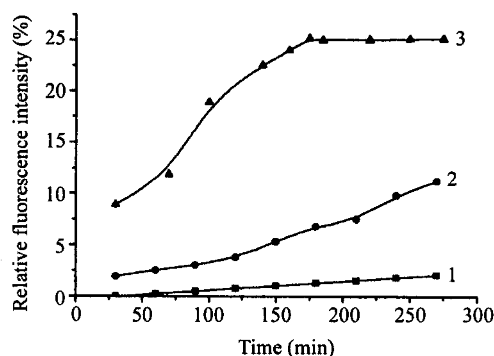


Fig. 4 Effects of heating time and temperatures. (1) 80 ℃; (2) 90 ℃; (3)100 ℃. Conditions: glucose, 5.00×10^{-4} mol/L; urea, 0.15 mol/L; 1:1(V: V) H_2SO_4, 5 mL.

In addition, it is discovered that the fluorescence intensity of this system remained stable for three days after it reached the maximum.

Interference test

It can be seen from Table 1 that the fluorescence intensity of α-rhamnose is much higher than that of other carbohydrates. The interference of various other carbohydrates with the α-rhamnose determination was tested according to the standard procedure (see Table 2). It was found that other carbohydrates at a level of 5—12 times higher than α-rhamnose concentration had little effects on the determination of α-rhamnose under the permission of 5% error.

Table 2 Interference from other carbohydrates[a]

Carbohydrates	Concentration coexisting (mol/L)	Change of ΔI_f (%)
Glucosum	4.0×10^{-4}	4.8
Fructose	3.8×10^{-4}	4.5
D-Xylose	4.5×10^{-4}	4.2
D-Galactose	4.2×10^{-4}	4.6
Trehalose	2.5×10^{-4}	4.8
L-Sorbose	4.0×10^{-4}	4.9
D-Arabinose	6.0×10^{-4}	4.7

[a] Conditions: α-rhamnose, 5.00×10^{-5} mol/L; urea, 0.15 mol/L; 1:1 (V: V) H_2SO_4, 5 mL; heating time, 3 h.

Analytical application

Calibration curve and detection limit

Under the optimum conditions determined, a linear relationship was obtained between the fluorescence intensity and the concentration of carbohydrates such as glucose, fructose, galactose, xylose, *L*-sorbose and *D*-arabinose (Table 3). It can be seen from Table 3 that in the linear ranges all kinds of carbohydrates tested have excellent correlation coefficients, and their limits of detection could reach 10^{-7} mol/L ($S/N = 2$).

Table 3 Linear range and detection limit of different carbohydrates

Carbohydrates	Linear range (mol/L)	Correlation coefficient	Detection limit (mol/L)
Glucose	5×10^{-6}—8×10^{-4}	0.9981	7.8×10^{-7}
Fructose	7×10^{-6}—1×10^{-3}	0.9918	1.9×10^{-7}
D-Xylose	6×10^{-6}—8×10^{-4}	0.9972	2.9×10^{-7}
D-Galactose	5×10^{-6}—9×10^{-4}	0.9926	8.5×10^{-7}
Trehalose	3×10^{-6}—8×10^{-4}	0.9935	1.6×10^{-7}
α-Rhamnose	1×10^{-6}—4×10^{-4}	0.9983	3.5×10^{-8}
L-Sorbose	5×10^{-6}—8×10^{-4}	0.9919	6.5×10^{-7}
D-Arabinose	6×10^{-6}—1×10^{-3}	0.9990	9.3×10^{-7}

Recovery tests and sample determination

The standard addition method was used for determinations of glucose, fructose and xylose solutions. From Table 4, it can be seen that the results obtained are satisfactory.

Table 4 Recovery tests of carbohydrates

Samples	Added (10^{-5} mol/L)	Found (10^{-5} mol/L)	$\bar{x}+s$	Recovery (%)
Glucose	1.00	0.93, 0.95, 0.94, 0.96, 0.95	0.946 ± 0.011	94.6
Fructose	1.00	0.96, 0.94, 0.95, 0.93, 0.96	0.948 ± 0.013	94.8
Xylose	1.00	0.95, 0.94, 0.96, 0.94, 0.93	0.944 ± 0.011	94.4

The proposed method was also used to determine glucose in glucose injection solution (Jinan Mingshui Limin Pharmaceutical, China). The satisfied results are shown in Table 5.

Table 5 Determination of Glucosum in glucosum injection sample

	Marked (%)	Found (%)	$\bar{x} \pm s$	Relative error (%)
Glucose injection solution	50	48.0, 49.2, 49.5, 48.6, 48.8	48.8 ± 0.58	-2.4

A synthetic sample, prepared based on the interference of other carbohydrates with the determination of α-rhamnose (Table 2), was analyzed. As shown in Table 6, the results are reproducible and reliable.

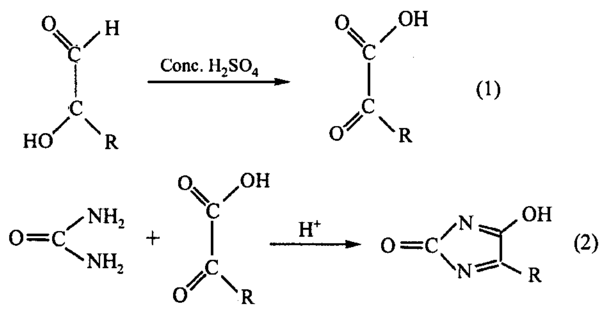
Table 6 Determination of α-rhamnose in synthetic sample[a]

α-Rhamnose in sample (10^{-4} mol/L)	α-Rhamnose found (10^{-4} mol/L)	$\bar{x} \pm s$ (10^{-4} mol/L)	Relative error (%)
1.00	0.96, 0.94, 0.97 0.95, 1.02	0.97 ± 0.031	-3.0

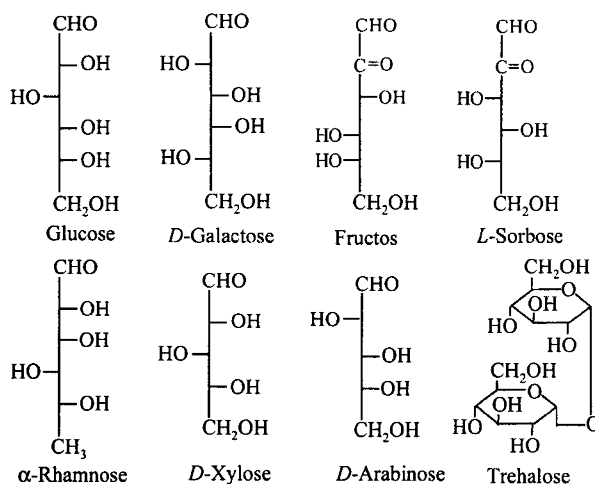
[a] Concentrations of co-existing substances in synthetic sample: glucose, 1.0×10^{-4} mol/L; *D*-xylose, 1.0×10^{-4} mol/L; *D*-arabinose, 2×10^{-4} mol/L.

Interaction mechanism

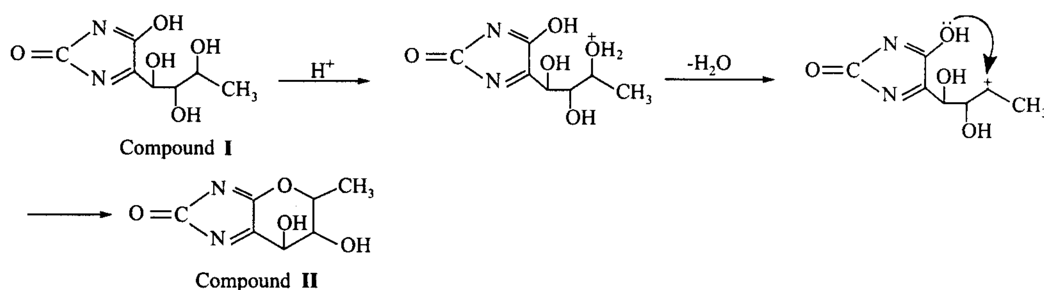
Experiments indicated that the fluorescence intensities of carbohydrates could be enhanced by the addition of urea under conc. H_2SO_4 conditions. In 1962, John E.[11] found that using the fluorescence complex formed by the reaction of 1, 2-phenylenediamine with α-ketone, trace α-ketone could be determined. Based on this principle, the following reaction may happen during the derivatizing process.



Under conc. H_2SO_4 conditions, carbohydrates were oxidized into α-keto acids. The latter then reacted with urea forming imidazole compounds which could emit fluorescence. Why is the fluorescence intensity of α-rhamnose system stronger than that of all the other carbohydrates systems tested (Table 1)? We think that this large difference may be related to their structures. The structures of different carbohydrates are listed below:



It can be seen that, besides CHO, another terminal group for α-rhamnose is CH_3, however, for all the other carbohytrates, it is CH_2OH. It is this structure difference that makes the compound **I** formed by reaction α-rhamnose with urea succeed the following reaction:



Methyl is an electron-donating group. Under strongly acidic medium it makes the protonation of OH adjacent to CH_3 easy. After dehydration, a centre with a positive charge is formed, which is subjected to the attack of OH, resulting in the formation of a six-member ring. This process is easier than that of other carbohydrates with electron-withdrawing CH_2OH group. The formed compound **II** has a pair of conjugate double-bond adjacent to the six-member ring that can enhance the electron absorption. So its fluorescence intensity is much higher than that of the reaction products between urea and other carbohydrates.

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