DETERMINATION OF HEPTOSES IN BACTERIAL LIPOPOLYSACCHARIDES

T. I. Burtseva and Yu. S. Ovodov

UDC 543.42.062.8

A method is proposed for the quantitative determination of heptoses in polysaccharide preparations of bacterial origin which eliminates the influence of all the other components present in the mixture on the results of the analysis. The method described is distinguished by high specificity, sensitivity, and reproducibility of the results, and also by simplicity of determination. It provides the possibility of determining low concentrations of heptoses in the presence of predominating amounts of other components. The standard deviation does not exceed 3.5%.

Higher monosaccharides, especially heptoses, have been detected in a whole series of biological materials [1]. They play an important role in various biochemical transformations [2]. Heptoses form components of the lipopolysaccharides (LPSs) of Gram-negative bacteria that determine the immunological specificity of the bacterial cell [3]. Consequently, the quantitative determination of heptoses, like that of other components present in LPSs, is an important, even if difficult, problem in chemical and immunochemical investigations.

Existing methods for the quantitative determination of heptoses are extremely imperfect. Dische's method [4] and a modification of it [5] have come into wide use, but they have proved inadequately specific and are unsuitable for the determination of heptoses in complex polysaccharide preparations of bacterial origin.

At the present time, Sinilova's method [6] is frequently used for determining heptoses in bacterial LPSs. According to this method, a heptose possesses a characteristic peak with a maximum at 395 nm. The method best fulfills the task set, but, unfortunately, it is not free from a number of defects. In the words of the author, it is not suitable for the analysis of preparations containing, together with a small amount of heptose, a predominating

TABLE 1. Influence of Potential Components of Mixtures Being Investigated on the Results of the Determination of Heptoses

Main components of the reaction mixture	Added to the glucoheptose, C, μg	Optical density, nm				Found: C, μg of heptose		
		395	430	470	395–470	according to Dische	according to Sinilova	according to the modification described
Glucoheptose	51,00	0,937	0,100	0,050	0,837	51,80	51,05	50,98
Glucose	201,25	0,940	0,105	0,045	0,835	63,52	50,93	51,14
Arabinose	198,75	0,935	0,100	0,050	0,835	65,20	50,93	50,87
Mannose	195,35	0,937	0,097	0,048	0,840	72,80	51,24	50,98
Xylose	200,01	0,935	0,098	0,055	0,837	58,93	51,05	50,87
Fucose	175,80	0,935	0,100	0,040	0,835	61,35	50,93	50,87
Galactose	187,95	0,938	0,101	0,050	0,837	72,32	51,05	51,03
N-Acetyl-D-glucosamine	197,85	0,935	0,097	0,045	0,838	58,30	51,11	50,87
Glucosamine·HCl	193,27	0,940	0,105	0,040	0,835	55,30	50,93	51,14
Galacturonic acid	205,32	0,939	0,103	0,050	0,836	57,30	50,99	51,09
Albumin	198,98	0,935	0,100	0,045	0,835	63,80	50,93	50,87
Lipid A	150,10	0,940	0,100	0,045	0,840	53,52	51,14	51,14
2-Keto-3-deoxy-D-mannooctonic acid	105,30	0,937	0,099	0,050	0,838	59,83	51,11	50,98
DNA	50,75	1,095	0,160	0,300	0,935	60,73	57,03	50,87
2-Deoxyribose	50,025	1,145	0,205	0,470	0,940	67,87	57,34	51,14
DNA + 2-deoxyribose	50,75+ 50,025	1,025	0,270	0,600	0,935	77,15	57,03	50,87

α-D-Glucoheptose (51,0 μg) +

Pacific Ocean Institute of Bioorganic Chemistry, Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostok. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 159-163, March-April, 1982. Original article submitted June 19, 1981.

 0009-3130/82/1802-0144$07.50

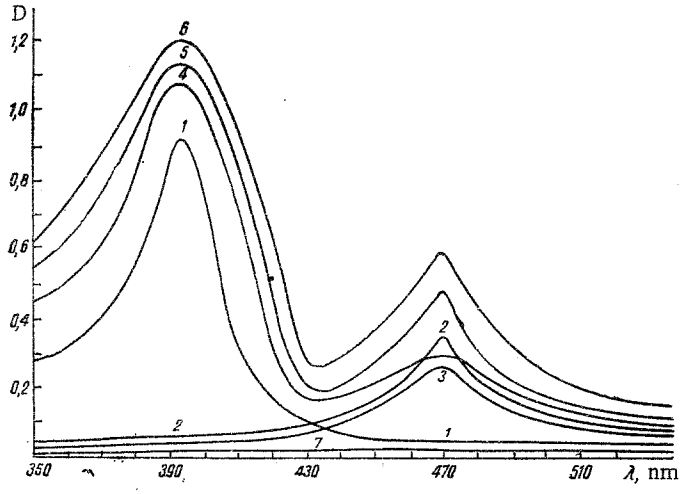

Fig. 1. Absorption spectra for glucoheptose and for various components of bacterial LPSs and their mixtures in 0.1 ml of solution obtained by Sinilova's analytical procedure [6]: 1) glucoheptose (51 μg); 2) 2-deoxyribose (50 μg); 3) DNA (50.75 μg); 4) glucoheptose + DNA (51 + 50.75 μg); 5) glucoheptose + 2-deoxyribose (51 + 50 μg); 6) glucoheptose + DNA + 2-deoxyribose (51 + 50.75 + 50 μg); 7) glucose, arabinose, mannose, xylose, fucose, galactose, N-acetyl-D-glucosamine, glucosamine·HCl, galacturonic acid, albumin, lipid A, and 2-keto-3-deoxo-D-mannooctonic acid in concentrations of 2 mg/ml.

amount of deoxyribose and other monosaccharides.

We propose to introduce into Sinilova's method [6] corrections to the calculations the essence of which is that the concentration of heptoses is found from the difference in the values of the optical densities at 395 and 430 nm when interfering components are present in the mixture but the value of the optical density at 395 nm is used when such components are absent. The presence or absence of interfering factors is shown by the absorption at 470 nm. Such an approach provides the possibility of eliminating the difficulties mentioned in the application of the method to bacterial LPSs.

According to Sinilova's method [6], in calculating the concentration of heptoses the difference in the values of the optical densities at 395 and 430 nm is used in all cases.

Since we have applied Sinilova's method of determining heptoses [6] to LPSs and LPS-protein complexes (LPSPs) of Gram-negative bacteria, we have ascertained, in the first place, the influence of the components usually present in these materials. It has been found that neutral monosaccharides and uronic acids, and also proteins, fats, and 2-keto-3-deoxyaldonic acids do not, under the conditions of the proposed procedure and when they are present in concentrations of up to 2 mg/ml, form colored compounds (Fig. 1) and do not inhibit the development of the coloration formed by a heptose (Table 1). A substantial error in the results of the analysis can be caused by the presence in the mixture of even small amounts of 2-deoxyribose and DNA, although the absorption spectrum of the colored derivatives formed by them (λ_{max} 470 nm) differs from the analogous spectrum for heptoses (Fig. 1).

If the solution under investigation does not contain components interfering with the determination, its spectrum consists of a single peak, while when interfering components are present (in the present case, DNA and 2-deoxyribose), the spectrum has the form of a curve with two peaks (λ_{max} 395 and 470 nm) (see Fig. 1). The error in the determination in the latter case is due to the appearance in the spectrum of an additional background which is observed at 430 nm.

In this connection, we consider it necessary: 1) to construct a calibration graph from solutions of pure heptose, measuring the optical density at 395 nm; and 2) in the analysis

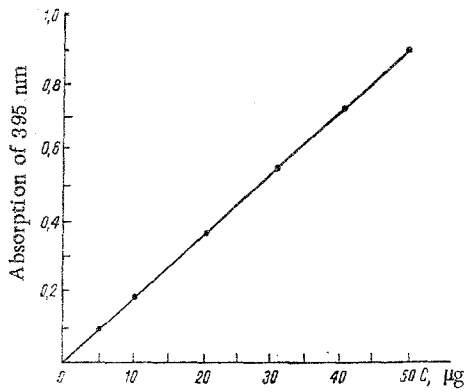

Fig. 2. Calibration curve for the determination of heptoses.

of solutions under investigation with an unknown composition to decide whether interfering components are present or absent. For this purpose, after the performance of the main analytical reaction the optical densities of the solutions under investigation must be measured at 395 and 470 nm, and the level of the background at 430 nm formed through the interfering components must also be determined (see Fig. 1 and Table 1). If it is found that $D_{470} > D_{430}$, the solution contains components interfering with the determination. Then in calculating the concentration of heptoses the value $D = D_{395} - D_{430}$ must be used to exclude the influence of the interfering factors on the results of the determination. If $D_{470} \leqslant D_{430}$, the value of the optical density at 395 nm must be used to calculate the concentration of heptoses in the systems under investigation, since in this case the impurities present in the solution have no influence on the results of the determination.

The method has been applied to the analysis of individual substances and standard mixtures containing definite amounts of glucoheptose, DNA, and 2-deoxyribose (see Table 1), and also to the analysis of various preparations of bacterial origin (Table 2).

As can be seen from Tables 1 and 2, the procedure described excludes the influence on the results of the determination of all the components present in the mixture, while Dische's method gives a positive result with almost all the components of the mixture and Sinilova's method [6] gives high results when DNA and 2-deoxyribose are present in the mixture.

Thus, the method described for determining heptoses is highly specific and provides the possibility of determining low concentrations of heptoses when overwhelming amounts of other components are present.

It has been found experimentally that 1 μg of glucoheptose corresponds to an optical density of 0.018. The molar absorption coefficient is $2 \cdot 10^4$. The standard deviation calculated in each case for ten determinations does not exceed 3.5%. The minimum amount of heptoses determinable by the proposed procedure, determined by Blank's method [7] is 0.5 μg, which shows the fairly high sensitivity of the procedure. The upper limit of sensitivity due to the increase in the error of the measurements themselves at $D > 1.0$ is 50 g.

EXPERIMENTAL

The investigations were performed on LPS preparations isolated from the microbe *Yersinia pseudotuberculosis* serotype 1B ($LPSP_{1B}$, LSP_{1B}, PS_{1B}) and of serotype IV (LPS_{IV}). In addition we have available a preparation of the blue-green alga *Spirulina platensis* (LPS_{SP}). The LPS preparations were used without additional purification; before analysis they were dried to constant weight in vacuum with phosphorus pentoxide at 50°C. A standard sample of α-D-glucoheptose was recrystallized from water and was then found to have a melting point of 210°C and an optical rotation of $[\alpha]_D^{20}$ −25° (c 2; water). These characteristics correspond to those given in the literature [8].

Procedure. To 0.1 ml of a solution under investigation was added 4.9 ml of concentrated sulfuric acid (ch.d.a. ["pure for analysis"], d 1.83) that had previously been cooled to 3-4°C. The mixture was carefully stirred with the avoidance of splashing and was heated in the boiling water bath for four minutes; then it was cooled and the optical densities were measured on a SF-26 spectrophotometer (l 1 cm) at wavelengths of 395, 430, and 470 nm. The amount of heptoses in the sample analyzed was calculated from a previously plotted calibration curve for which standard solutions of α-D-glucoheptose with concentrations of from 1 to 50 μg in 0.1 ml were used. The optical densities of the calibration solutions were measured at 395 nm. The calibration curve is shown in Fig. 2.

TABLE 2. Determination of Heptoses in Bacterial LPS Preparations by Various Methods

Preparation investigated*	Found, % of heptoses		
	according to Dische	according to Sinil-ova	according to the modification described
$LPSP_{1B}$	4,87	1,23	1,11
LPS_{1B}	8,52	2,38	2,12
PS_{1B}	4,56	0,81	0,71
LPS_{IV}	5,77	1,96	1,75
LPS_{SP}	3,81	0,28	0,25

*See the Experimental part.

CONCLUSION

1. A method is proposed for the quantitative determination of heptoses in polysaccharide preparations of bacterial origin which excludes the influence of all the other components present in the mixture from the results of the analysis.

2. The procedure described is distinguished by high specificity, sensitivity, and reproducibility of the results, and also by simplicity of the determination.

LITERATURE CITED

1. A. Nordal and A. A. Benson, J. Am. Chem. Soc., 76, 5054 (1954); V. V. Rending and E. A. McComb, Arch. Biochem. Biophys., 89, 323 (1960); A. J. Charison and N. K. Richtmyer, J. Am. Chem. Soc., 81, 1512 (1959); H. H. Shepton and N. K. Richtmyer, J. Org. Chem., 28, 2388 (1963); H. H. Sephton and N. K. Richtmyer, J. Org. Chem., 28, 1691 (1963).
2. V. Ginsberg, P. J. O'Brien, and C. Hall, J. Biol. Chem., 237, 497 (1962).
3. D. A. L. Davies, Adv. Carbohydr. Chem., 15, 271 (1960).
4. Z. Dische, J. Biol. Chem., 204, 983 (1953).
5. M. L. Osborn, Proc. Nat. Acad. Sci. USA, 50, 499 (1963).
6. N. G. Sinilova and K. K. Ivanov, Vopr. Med. Khim., 17, 19 (1971).
7. A. B. Blank, Zh. Anal. Khim., 17, 1040 (1962).
8. F. Micheel and A. Klemer, Chemie der Zucker und Polysaccharide, Akademie Verlag, Leipzig (1956), p. 401.