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AN IMPROVED METHOD FOR THE AUTOMATED DETERMINATION OF META- AND ISO-SACCHARINIC ACIDS

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ABSTRACT

Manual and automated spectrophotometric methods are described for the determination of 3-deoxyhexonic and 3-deoxy-2-*C*-hydroxymethylpentonic acids The method utilises the chromophores formed by condensation of barbituric acid with the products of oxidation with periodate This method obviates the need for solvent extraction required when using 2-thiobarbituric acid for chromophore production

INTRODUCTION

The types of saccharinic acid formed on alkaline degradation of oligosaccharides can reveal the position of substitution of the monomer units[1] A spectrophotometric method using 2-thiobarbituric acid has been described for the determination of 3-deoxyhexonic (metasaccharinic) and 3-deoxy-2-*C*-hydroxymethylpentonic (isosaccharinic) acids[2] In the automated version of this procedure[3], the solvent-extraction stage increases the complexity of the analysis This paper describes a method that may be readily automated and avoids solvent extraction

EXPERIMENTAL

Standardised, analytical procedure — (*a*) *Reagents* (*1*) 0 2M Sodium metaperiodate in 9M phosphoric acid, (*2*) 5% aqueous sodium arsenite, adjusted to pH 9 0 with phosphoric acid, (*3*) 1% aqueous barbituric acid, adjusted to pH 9 0 with sodium hydroxide

(*b*) *Manual procedure* Sodium metaperiodate reagent (0 1 ml) was added to solutions of 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid (50 μg/ml, 0 2 ml) After 20 min at room temperature, sodium arsenite reagent (1 0 ml) was added to terminate the oxidation. The addition of barbituric acid reagent (1 5 ml), followed by heating for 30 min at 100°, resulted in the development of a characteristic chromophore (λ_{max} 505 nm) After the solutions had been cooled to room temperature, the absorbance was determined at 505 nm The procedure was similarly applied to

3-deoxy-D-*arabino*-hexonic acid and 2-deoxy-D-*arabino*-hexose, when a chromophore of λ_{max} 486 nm was obtained Calibration graphs and absorption spectra are shown in Fig 1

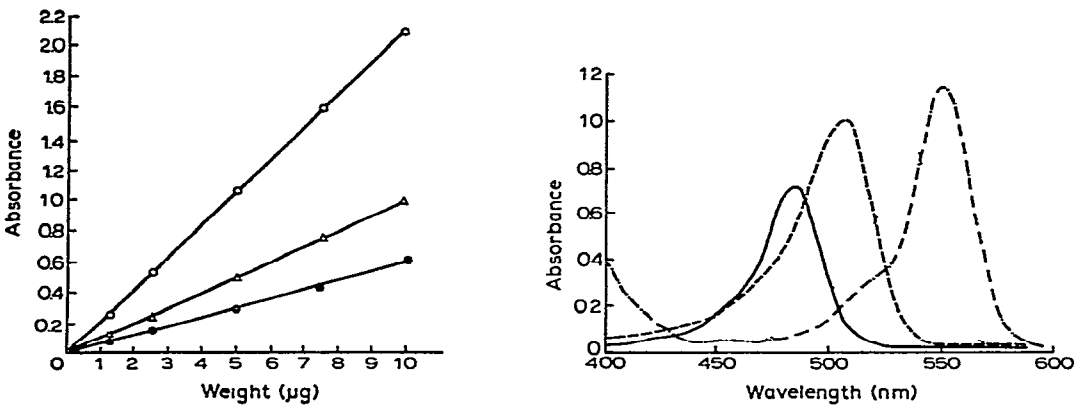
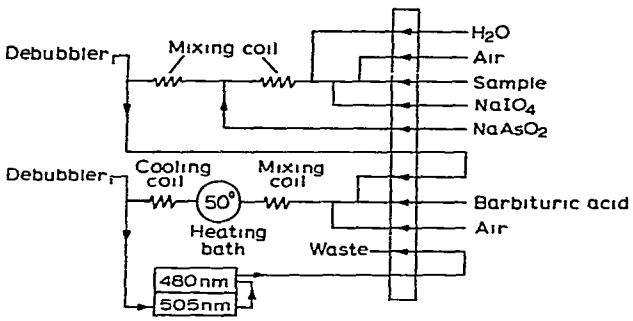


Fig 1 Calibration and chromophore absorption spectra for the barbituric acid analysis procedure —○—, 2-deoxy-D-*arabino*-hexose (485 nm), —●—, 3-deoxy-D-*arabino*-hexonic acid (485 nm), —△—, 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid (506 nm), ———, 2-deoxy-D-*arabino*-hexose (barbituric acid), , 2-deoxy-D-*arabino*-hexose (2-thiobarbituric acid), — — —, 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid (barbituric acid), —·—·—, 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid (2-thiobarbituric acid)

(*c*) *Automated procedure.* The reagent compositions were identical to those used in the manual procedure Technicon Autoanalyser modular equipment was employed throughout, and a schematic representation is presented in Fig 2 Solutions were sampled continuously (0 10 ml/min), and mixed with sodium metaperiodate reagent (0 10 ml/min) and water (0 10 ml/min) during 10 min Sodium arsenite reagent (1 06 ml/min) was then added during 5 min After removal of air, a proportion of the sample stream was pumped (1 20 ml/min) and mixed with barbituric acid reagent (1 37 ml/min) After heating at 95° for 8 min, the reaction stream was cooled and the absorption determined at 480 and 505 nm Calibration graphs are shown in Fig 3

Effect of heating time on colour development — Solutions containing 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid (50 µg/ml, 0 2 ml) and 2-deoxy-D-*arabino*-hexose (50 µg/ml, 0.2 ml) were analysed by the standard procedure, using heating times of 0 to 60 min at 100°, The rate of chromophore development is shown in Table I

Effect of concentration of barbituric acid on colour development. — The terminated, periodate-oxidation mixture of 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid (100 µg/ml) was sampled continuously and mixed with barbituric acid reagent, the concentration of which was steadily increased by a linear, concentration gradient The absorbance of the chromophore developed on heating was continuously recorded A similar procedure was employed for a solution of periodate-oxidised 2-deoxy-D-*arabino*-hexose The results are shown in Table II



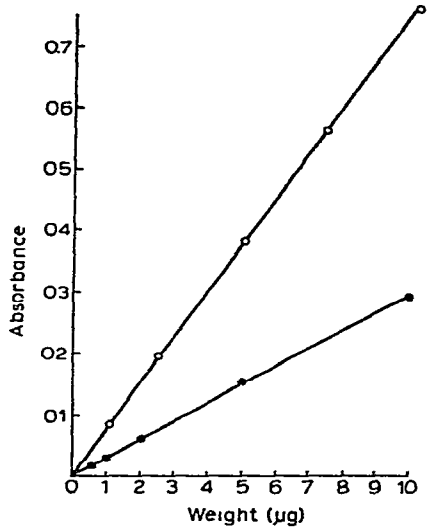


Fig 2 Schematic representation of the automated system for the determination of iso- and meta-saccharinic acids Reagent composition and flow rates air (0 32 ml/min), water (0 10 ml/min), $NaIO_4$ (0 2M, in 9M phosphoric acid, 0 10 ml/min), sample (0 10 ml/min), $NaAsO_2$ (5% w/v, adjusted to pH 9 0 with phosphoric acid, 1 06 ml/min), recycled sample (1 2 ml/min), barbituric acid (1% w/v, 1 37 ml/min), and air (1 32 ml/min)

Fig 3 Calibration of the automated barbituric acid procedure —○—, 2-Deoxy-D-*arabino*-hexose, —●—, 3-deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid

TABLE I

EFFECT OF TIME OF HEATING ON COLOUR DEVELOPMENT

Heating time (min)	2	4	7	10	20	30	60
Absorbance at 505 nm for *A*[a]	0 47	0 65	0 91	1 00	1 17	1 17	1 17
Absorbance at 486 nm for *B*	0 48	1 02	1 60	1 67	1 85	2 00	2 00

[a] *A*, 3-Deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid, *B*, 2-deoxy-D-*arabino*-hexose

TABLE II

EFFECT OF BARBITURIC ACID CONCENTRATION ON COLOUR DEVELOPMENT

Conc. (%)	0 1	0 2	0 3	0 4	0 5	0 6	0 7	0 8	0 9	1.0
Absorbance at 505 nm for *A*	0 153	0 225	0 268	0 308	0 337	0 362	0 382	0 397	0 397	0 397
Absorbance at 486 nm for *B*	0 248	0 560	0 760	0 890	1 000	1 06	1 12	1 14	1 17	1 17

A, 3-Deoxy-2-*C*-hydroxymethyl-D-*erythro*-pentonic acid, *B*. 2-deoxy-D-*arabino*-hexose

Determination of the effect of possible interfering compounds. — L-Rhamnose (which gives acetaldehyde on oxidation with periodate), D-mannitol (formaldehyde and formic acid), sodium pyruvate, and glyoxylic acid did not interfere in the analytical procedure or inhibit the production of chromophore

DISCUSSION

In the 2-thiobarbituric acid assay for the determination of iso- and metasaccharinic acids, precipitates and turbidity are encountered on cooling the assay solutions after colour development, and solvent extraction is therefore necessary Whereas this can be readily performed in the manual assay, problems arise in an automated procedure In the manual procedure, centrifugation is employed to clarify the two layers after solvent extraction, thus removing turbidity from the organic phase In the automated procedure, no centrifugation can be employed, and thus when sampling from certain solutions (*e g* , an alkaline degradation in 0 25M barium hydroxide) the turbid solutions may be carried into the optical flow-cell, giving rise to increasing baselines and/or false readings of absorbance It was therefore necessary to develop an assay in which turbidity did not develop and which, preferably, did not require a solvent-extraction stage

Analysis of the precipitates showed the presence of sulphur-containing compounds, suggesting that a non-sulphur analogue of 2-thiobarbituric acid would eliminate this source of interference. In the assay for *N*-acetylneuraminic acid, Warren[4] noted that if barbituric acid was substituted for 2-thiobarbituric acid a chromophore of different absorption maximum was obtained

When barbituric acid was used for the determination of isosaccharinic acid and 2-deoxy-D-*arabino*-hexose after periodate oxidation, characteristic chromophores with absorption peaks at 505 and 486 nm, respectively, were obtained (*cf* 2-thiobarbituric acid, 549 and 532 nm) In the systems under study, such as the alkaline degradation of oligosaccharides to saccharinic acids, no precipitates or turbidity were encountered

Optimal conditions for the development of chromophores in the present assay were established (Tables I and II) and found to be slightly different from those for 2-thiobarbituric acid Whereas the chromophores obtained with 2-thiobarbituric acid showed a marked increase in extinction coefficient when extracted into cyclohexanone, those obtained in the present system were not usually extracted by organic solvents Acidified 1-butanol partially extracted both chromophores Compounds derived from the oxidation of various carbohydrates with periodate caused no interferences in the procedure, although glyoxylic acid gave a chromophore (λ_{max} 410 nm) of low molar extinction coefficient, which did not interfere in the determination

Although the sensitivity of the present method is lower (~50%) than when using 2-thiobarbituric acid, the ease of operation, particularly with respect to the automated method, provides a distinct advantage

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