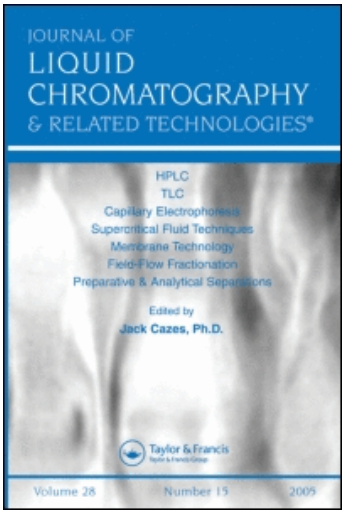


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Isolation and Determination of Trace Levels of D-Arabino-2-Hexosulose (D-Glucosone) by Microcolumn, Thin Layer and High Performance Liquid Chromatography

Albert K. Athnasios[a]

[a] Corporate Technology, Nabisco Brands, Inc., Wilton, Connecticut

To cite this Article Athnasios, Albert K.(1984) 'Isolation and Determination of Trace Levels of D-Arabino-2-Hexosulose (D-Glucosone) by Microcolumn, Thin Layer and High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 7: 10, 1991 — 2001

To link to this Article: DOI: 10.1080/01483918408068851

URL: http://dx.doi.org/10.1080/01483918408068851

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 1991-2001 (1984)

ISOLATION AND DETERMINATION OF TRACE LEVELS OF D-ARABINO-2-HEXOSULOSE (D-GLUCOSONE) BY MICROCOLUMN, THIN LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Albert K. Athnasios
Corporate Technology, Nabisco Brands, Inc.
15 River Road, Wilton, Connecticut 06897

ABSTRACT

Liquid chromatographic methods have been developed for the isolation and determination of D-glucosone at nanogram and sub-nanogram levels. D-glucosone is separated from complex matrices by reverse phase column chromatography (RPCC) and reacted with 2,4-dinitrophenylhydrazine to yield D-glucosone-bis-2,4-dinitrophenylhydrazone (bis-DNP). The latter compound is analyzed by normal phase thin layer chromatography (TLC) and by normal phase high performance liquid chromatography (HPLC). Visualization of bis-DNP on thin layer plates is enhanced by spraying with an alcoholic alkali reagent whereby concentrations as low as 50 ng D-glucosone can be detected. HPLC offers a highly sensitive and specific method for the quantitation of bis-DNP and concentrations as low as 500 pg D-glucosone are detected at a wavelength of 436 nm. The HPLC analysis follows the Beer-Lambert law at 436 nm with a precision (relative standard deviation) of 5.1%, 4.5%, 4.2%, 3.9% and 2.4% at 5, 10, 15, 20 and 25 ng respectively of standard D-glucosone.

INTRODUCTION

In spite of the increasing interest in D-glucosone, the key intermediate in the process of conversion of D-glucose to D-fructose, there is at present no method for its determination at trace levels. A colorimetric method involving the reaction of D-glucosone with triphenyltetrazolium chloride has been

0148-3919/84/0710-1991$3.50/0

reported. Such method suffers from interference of D-glucose, D-gluconic acid and other reducing compounds (1). A gas chromatographic method involving the analysis of D-glucosone trimethylsilyl derivative has also been reported, but it is time consuming since rigorous removal of water from the samples is required, and qualitative at best owing to the heat lability of the formed derivative (1). A thin layer chromatographic method has been reported for the separation of hydroxycarbonyl compounds as their 2,4-dinitrophenylhydrazone derivatives from complex mixtures (2). However, such study was purely qualitative.

A high performance liquid chromatographic method has been reported for the analysis of D-glucosone (1). The authors used a u-Bondapak carbohydrate analysis column (Waters Associates, Milford, MA, U.S.A.) and a mobile phase of aqueous acetonitrile buffered with potassium phosphate (pH 6.0). Detection is achieved using both a refractive index detector (RI) and a U.V. absorbance detector at 192 nm. The minimum detectable quantity of D-glucosone is 20 ug using RI detection, and 0.1 ug using U.V. detection at 192 nm. However, the use of lower UV detectors to monitor the absorption due to the carbohydrate carbonyl groups requires that great care be taken to ensure the purity of solvents used as the mobile phase and the chance of interference from trace levels of contaminants is increased (3).

The present study was undertaken to develop rapid procedures for the routine determination of trace levels of D-glucosone in complex biological matrices. D-glucosone is isolated and purified by RPCC and converted to its bis-DNP derivative which is subsequently analyzed by TLC and HPLC. The application of both techniques allows the rapid analysis of a large number of samples for D-glucosone content.

MATERIALS AND METHODS

APPARATUS

Sample clarification kits, aqueous and organic. (Waters Associates, Milford, MA, U.S.A.)

Solvent clarification kit. (Waters Associates)

Millipore filters (organic), type FH with pore size 0.45 um. (Millipore Corporation, Bedford, MA, U.S.A.)

Reverse phase C_{18} SEP-PAK Cartridges. (Waters Associates)

U.V. - Visible Spectrophotometry - Hewlett Packard Model 8450A UV/Vis spectrophotometer moduled with Hewlett Packard Model 7225B Plotter.

TLC apparatus - 9-1/8" x 11-1/2" x 3-7/8" developing chamber with cover.

High resolution precoated TLC plates - SilG-25HR, 20 x 20 cm. (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.)

Aeresol spray unit and aspirator. (Brinkmann Instruments, Inc.)

High performance liquid chromatograph (Waters Associates) equipped with Model 6000A solvent delivery system, U6K septumless injector, dual channel Model 440 absorbance detector with filters for 436 nm and 405 nm, analytical 5u Partisil Silica Column - 4.6mm ID x 25 cm - (Whatman, Inc., Clifton, NJ, U.S.A.) and Omniscribe dual pen, 10", 10 mv recorder (Houston Instruments, Bellair, TX, U.S.A.)

For completely automated routine analysis the liquid chromatograph is equipped with a Waters WISP Model 710B automated sample injector, a Waters Model 720 System Controller and a Waters Model 730 Data Module.

REAGENTS

All reagents and solvents were of analytical reagent (AR) grade. Unless otherwise specified, the water used was double deionized.

Dichloromethane, anhydrous methanol, distilled in glass (Burdick & Jackson, Muskegon, MI, U.S.A.).

Ethyl acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.)

Ethyl alcohol, 95%.

Sodium sulfate, anhydrous.

Sodium hydroxide, pellets.

Methanolic sodium hydroxide soln. - prepared by dissolving 2 g sodium hydroxide in 10 ml water and diluting the resulting solution to a volume of 100 ml with anhydrous methanol, followed by thorough mixing.

Sulfuric acid, conc.

2,4-Dinitrophenylhydrazine-DNPH (Eastman Kodak Co., Rochester, NY, U.S.A.).

DNPH derivatizing reagent - prepared by adding 2 ml conc. sulfuric acid to 0.4 g DNPH in a 25 ml Erlenmeyer flask with swirling or stirring until solution is complete. To the resulting warm solution 10 ml of 95% ethyl alcohol are added down the sides of the flask, mixed and kept tightly covered when not in use.

D-glucosone - Synthesized by the Biochemistry Department, Nabisco Brands, Inc., Wilton, CT, U.S.A., purified by micro-column reverse phase chromatography of its aqueous solution on C_{18} SEP-PAK cartridges, dried under vacuum and kept frozen when not in use.

Authentic standard D-glucosone - bis-2,4-dinitrophenylhydrazone (bis-DNP) - is prepared by reacting purified D-glucosone with an excess of DNPH derivatizing reagent at room temperature for one hour. The resulting orange precipitate is filtered, washed with water and recrystallized from 95% ethyl alcohol to give yellow-orange crystals, m.p. 258°C (literature 257-258°C). Analytical microanalysis of the bis-DNP was performed by Galbraith Laboratories, Knoxville, TN, U.S.A. Found: C 40.22%, H 3.39%, N 20.77%; calculated for $C_{18}H_{18}N_8O_{12}$: C 40.16%, H 3.37%, N 20.81%.

Purity of the bis-DNP derivative is additionally checked by thin layer and high performance liquid chromatography of its dilute methanolic solution. The bis-configuration is confirmed by treating its dilute solution or spraying TLC chromatograms with methanolic sodium hydroxide solution whereby a violet color is obtained (4). High performance liquid chromatography using dual absorbance detection at 436 nm and 405 nm give peaks having

the absorbance ratio A_{436}/A_{405} = 1.05. This value is used in addition to retention times to confirm the identity and purity of the glucosone-bis-DNP peak in the analysis of natural isolates (5).

Standard-bis-DNP solutions - Standard solutions of bis-DNP are prepared in methanol or ethyl acetate to have a D-glucosone equivalent concentration of 1, 2, 3, 4, and 5 ug/ml for HPLC analysis and 10 ug/ml for TLC analysis.

TLC developing solvent - Dichloromethane and methanol (95 + 5) V/V. Prepare fresh daily.

HPLC mobile phase - Dichloromethane and methanol (95 + 5) V/V. Filter the dichloromethane solvent using the solvent clarification kit fitted with an organic filter. Degas the filtrate thoroughly by swirling for about 5 minutes while under vacuum. Filter the methanol solvent the same way. Using the degassed solvents, prepare a 5% solution V/V of methanol in dichloromethane. Filter and degas the resulting solution for about 2 minutes.

PROCEDURE

Isolation of D-glucosone:

Solid Samples - Weigh 1 g sample into a 100 ml glass stoppered Erlenmeyer flask. Add 50 ml methanol, secure stopper with masking tape, agitate 30 minutes on a wrist action shaker at room temperature. Filter through Whatman No. 4 filter paper, rinse the flask with 3 x 10 ml portions methanol, passing the methanol washings through filter paper. Collect methanol extract and washings in a 100 ml volumetric flask, make up to volume with methanol, stopper and mix thoroughly. Evaporate 50 ml of methanol extract to dryness under vacuum at room temperature. Redissolve residue in 1 ml water and subject to reverse phase column chromatography (RPCC).

Biological Solutions - Filter aqueous solutions to remove any undissolved constituents. Subject clear aqueous filtrates to RPCC.

RPCC - Pipette an appropriate aliquot (1-2 ml) of aqueous solution containing D-glucosone into the barrel of a 10 ml hypodermic syringe to which is attached a single or a series of C_{18} SEP-PAK cartridge(s) which are already activated by passing methanol followed by water through them. Filter the solution through the cartridge or cartridge train. Wash with additional 30-35 ml water. Any coloring material will be tightly held as a narrow band at the top of the cartridge bed. The clear solution eluting from the cartridge(s) is collected into a 50 ml volumetric flask, adjusted to volume with water and thoroughly mixed. Evaporate 25 ml to dryness under vacuum at 30°C. Redissolve the residue in 1 ml of 50% aqueous methanol. Derivatize the resulting solution by reacting it with DNPH.

Derivatization of D-glucosone - Add excess (0.5 ml) of derivatizing DNPH reagent to the aqueous methanol solution in a screw cap 4 dram vial, mix by swirling and let stand for one hour at room temperature. At the end of the reaction and depending on the level of D-glucosone in the aliquot derivatized, the reaction mixture will acquire an orange color or an orange precipitate will be formed.

Depending on the method of analysis to be applied the reaction products are treated as follows:

For TLC - Add 5 ml methanol to the reaction vial, dissolve by swirling, quantitatively transfer into a 10 ml volumetric flask, make up to volume with methanol, stopper and mix thoroughly. Subject to TLC analysis.

For HPLC - Dissolve the contents of the sample reaction vial in the minimum volume of anhydrous methanol (1-5 ml), quantitatively transfer the resulting solution into a 125 ml separatory funnel. Add 50 ml water and extract with 3 x 20 ml portions of ethyl acetate. Filter ethyl acetate extract through a 2 g bed of anhydrous sodium sulfate contained in a filtering funnel fitted with a glass wool plug. Collect the dried filtrate into a 100 ml volumetric flask. Wash the separatory funnel and the sodium sulfate bed with fresh ethyl acetate until

complete removal of yellow color. Adjust to volume with ethyl acetate, stopper and mix thoroughly. Evaporate 50 ml - under a stream of N_2 - to dryness and redissolve in 5 ml ethyl acetate, filter through a 0.45 um FH Millipore filter. Subject concentrated solution to HPLC.

Thin Layer Chromatography - Spot 50 ul of each sample bis-DNP solution alongside 5, 10 and 20 ul of standard bis-DNP solution (10 ug D-glucosone/ml), having 50, 100 and 200 ng D-glucosone respectively, on a high resolution thin layer plate. Develop the plate in an unlined unequilibrated developing chamber containing 200 ml freshly prepared TLC developing solvent. After 20 minutes the solvent will travel about 15 cm, remove plate and air dry in a hood. Spray the dried plate with methanolic sodium hydroxide solution. A violet color will develop for both standard and sample spots having an R_f value of 0.15. Compare the intensity of the violet color of sample spots with those of standard spots and estimate the level of D-glucosone in the samples.

High Performance Liquid Chromatography - The liquid chromatograph is equilibrated using the following parameters:

Column	: Whatman PXS Partisil Column, 5u (4.6mm ID x 25 cm)
Mobile phase	: Dichloromethane and methanol (95 + 5) V/V
Mobile phase flow rate	: 2 ml/minute
Chart speed	: 1 cm/minute
Temperature	: Ambient
Detector	: 440 Absorbance detector at 436 nm, 0.005 AUFS or at both 436 nm and 405 nm, 0.005 AUFS

Inject 5 ul from each bis-DNP standard solution - D-glucosone level of 1, 2, 3, 4 and 5 ug/ml - into the liquid

chromatograph. This will be equivalent to 5, 10, 15, 20 and 25 ng D-glucosone respectively. The bis-DNP peak will elute with a retention time of about 3.8 minutes (Fig. 1). Measure peak height responses. Establish a calibration curve by plotting D-glucosone concentration in ng versus corresponding responses. Establish instrument linearity and thereafter monitor detector response using only one external standard having an equivalent

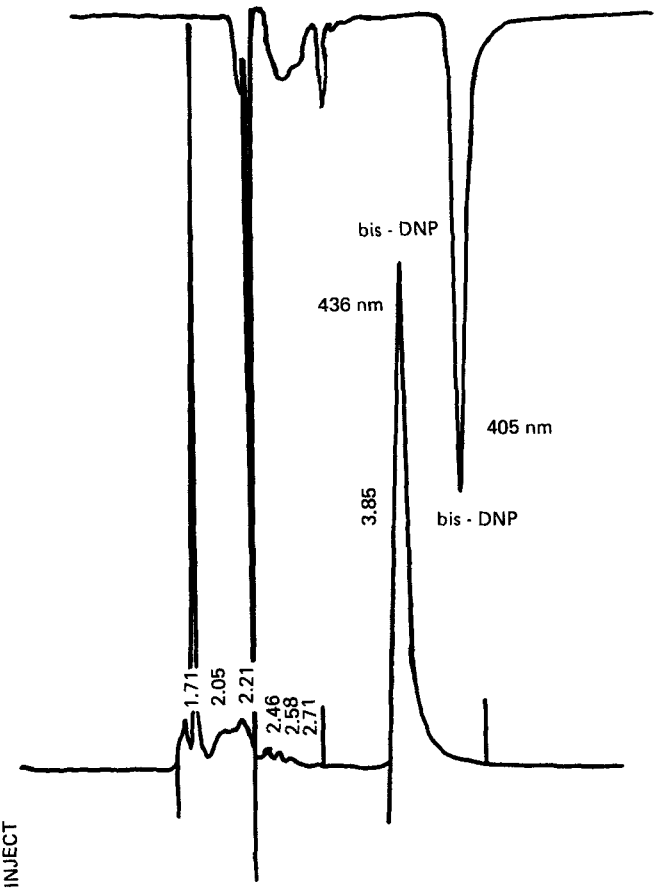


Figure 1. HPLC Chromatogram of 5 ul of bis-DNP solution (4 ug/ml) at 436 nm and 405 nm, 0.005 AUFS.

level of bis-DNP as in sample(s). Inject 5 ul from each sample bis-DNP solution in ethyl acetate into the liquid chromatograph. Measure bis-DNP peak height responses and estimate the level of D-glucosone in original samples by comparing sample peak responses with the responses of standards.

It is recommended that an aliquot of a standard solution be chromatographed between sample runs, e.g. an injection from a standard solution between 4-5 sample injections. This will monitor the chromatographic behavior and detector responses during the analysis of a large number of samples.

Aliquots of 1% aqueous solutions of potential interfering substances, e.g. D-glucose, D-fructose, galactose and a-ketogluconic acid when subjected to the specified D-glucosone-DNPH reaction conditions (1 hour at room temperature) do not yield any products that interfere with the TLC and HPLC of bis-DNP. The bis-DNP peak purity is confirmed by com-

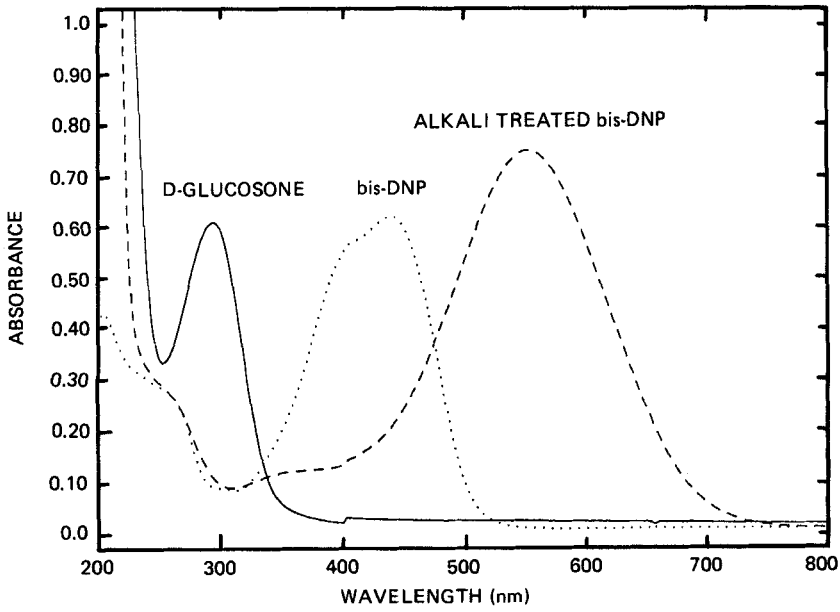
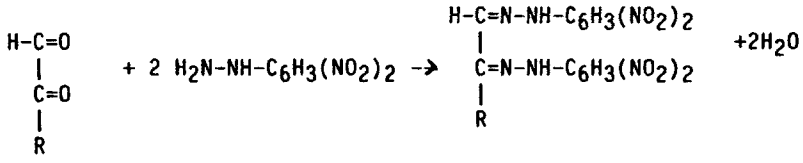


FIgure 2.

paring the peak absorbance ratio A_{436}/A_{405} for samples and authentic standard (5).

RESULTS AND DISCUSSION

D-glucosone is a 1,2-dicarbonyl polyhydroxy compound. Such 1,2-dicarbonyl compounds react with DNPH to give bis-DNP derivatives which have orange-yellow colors. When bis-DNP's are treated with solutions of alcoholic alkali, they are transformed to highly conjugated compounds that will undergo a bathochromic shift and as a result will absorb light at a higher wavelength acquiring purple-violet colors (4). Figure 2 illustrates the bathochromic shifts that take place when D-glucosone is derivatized to bis-DNP and when the latter compound is treated with alcoholic alkali.



1,2-dicarbonyl compound R=-(CHOH)$_3$ -CH_2OH in glucosone

DNPH

bis-DNP

The formation of bis-DNP from the reaction between D-glucosone and DNPH takes place at room temperature. Maximum bis-DNP yield is attained after one hour reaction time. Potential interfering compounds, e.g. D-glucose, D-fructose, galactose and a-ketogluconic acid - when subjected to the specified D-glucosone - DNPH reaction conditions - do not yield any products that interfere with the TLC and HPLC analyses of bis-DNP.

As low as 50 ng D-glucosone are detectable by TLC and 500 pg are detectable by HPLC using a 10 mv recorder. The HPLC sensitivity could be further increased to detect as low as 50 pg D-glucosone by using a 1 mv recorder.

The TLC methodology allows for the simultaneous analysis of a large number of samples in a short time. Such a cost effective technique will streamline the analysis of biological samples for D-glucosone.

An added advantage is that any further required HPLC analysis to quantitate D-glucosone could be performed on the same solutions subjected to TLC analysis.

ACKNOWLEDGEMENTS

The author thanks Mr. R. J. Carbonell for his permission to publish this work, Messrs. A. Loter, O. Ibrahim and R. O. Horwath for the synthesis of D-glucosone and Mr. A. Voyiaziakis for spectral measurements.

REFERENCES

1. Geigert, J., Hirano, D.S. and Neidelman, S.L., High Performance Liquid Chromatographic Method for the Analysis of D-arabino-2-hexosulose (D-glucosone), J. Chromatogr., 202, 319 (1980).

2. Anet, E.F.L.J., Thin Layer Chromatography of 2,4-Dinitrophenylhydrazine Derivatives of Hydroxycarbonyl Compounds, J. Chromatogr., 9, 291 (1962).

3. Lawson, M.A. and Rusell, G.F., Trace Level Analysis of Reducing Sugars by High Performance Liquid Chromatography, J. Food Science, 45, 1256 (1980).

4. Jones, L.A. and Hancock, C.K., Spectrophotometric Studies of the 2,4-Dinitrophenylhydrazones of Some Dicarbonyl Compounds, J. Amer. Chem. Soc., 82, 105 (1960).

5. Yost, R.W., Ettre, L.S. and Conlon, R.D., Practical Liquid Chromatography, An Introduction, The Perkin-Elmer Corp., 190 (1980).