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SEPARATION AND QUANTITATIVE DETERMINATION OF THE RADIOLYSIS PRODUCTS OF D-FRUCTOSE AS THEIR O-BENZYLOXIMES

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SUMMARY

High-performance liquid chromatography of O-benzyloxime derivatives is shown to be suitable for the analysis of carbohydrates as well as for the detection of glucosone (the major product of fructose irradiated in the presence of oxygen), but the method is not ideally suited to the determination of minor radiolysis products. Gas–liquid chromatography (GLC) of the silylated O-benzyloximes gives excellent separation of the various radiolysis products. These compounds are also suitable for determination GLC–mass spectrometry owing to their characteristic fragmentation patterns. The good separation obtained with these derivatives, as well as the possibility of removing most of the unchanged starting material with no disturbance of product composition, makes this method a viable alternative to previously employed methods for the analysis of irradiated sugar solutions.

INTRODUCTION

In the course of an ongoing research programme on the radiation products of sugar solutions[1–3], with particular reference to the extrapolation of the results to wholesomeness evaluations of irradiated whole fruit (such as the Kent mango), a rapid, inexpensive and simple method was required for the identification of radiolysis products of sugars, in particular D-*arabino*-hexos-2-ulose (D-glucosone), a known mutagenic substance[4].

Methods previously employed for the analysis of irradiated sugar solutions generally involved reduction of the irradiation products to the corresponding alcohols, followed by silylation or acetylation and detection by gas–liquid chromatography–mass spectrometry (GLC–MS)[5–8]. This approach is subject to a number of limitations:

(1) The requirement of enrichment of radiolysis products by column chromatography[9,10] before GLC analysis to prevent column overloading and the obscuring of product peaks by unchanged starting material. This is due to the fact that,

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even at high doses (20 kGy), radiolysis products constitute no more than 1% of the total mixture, unchanged starting material being the main component. Enrichment is time-consuming and can result in decomposition of some radiolysis products. In addition, similarity in the affinities of starting material and products can result in changes in product composition of fractions from which starting material has been partially removed.

(2) Loss of information and the difficulty of quantisation owing to the reduction step, which yields the same alcohol for different irradiation products (*e.g.* D-gluconic acid, D-glucuronic acid and D-*gluco*-hexodialdose are all reduced to D-glucitol[11]).

Other methods used involved methoximation[6,7,11–13] or oximation[14,15] of the radiolysis mixture, followed by silylation or acetylation and GLC analysis. This method still gives a huge peak, due to unchanged sugar, that complicates the chromatogram. In addition, methoximation yields a rather complex mixture, since up to four isomers were found for compounds such as D-*gluco*-hexodialdose.

Attention was, therefore, given to the development of a more suitable method, particularly for the rapid detection and quantisation of glucosone. A method based on benzyloximation was successfully applied to the analysis by high-performance liquid chromatography (HPLC) and GLC of the products of D-fructose solutions (the main sugar component of ripe Kent mangoes) irradiated under oxygenated conditions. This method not only results in the efficient separation of radiolysis products, but overcomes the complications due to the presence of large amounts of unchanged starting material. The O-benzyloximes are readily detected by UV detection, and it was therefore decided to analyse these compounds directly, rather than as the corresponding perbenzoates[16], which have found some application in the analysis of mono- and disaccharides.

EXPERIMENTAL

Materials

Hexamethyldisilazane (HMDS), chlorotrimethylsilane (TMCS), pyridine and benzylhydroxylamine hydrochloride were purchased from Merck (Darmstadt, F.R.G.). Monosaccharides were also purchased from Merck, whereas D-glucosone (I)[17], D-*threo*-hexo-2,5-diulose (II)[18], D-*ribo*-hexos-3-ulose (IV)[17], D-*erythro*-hexo-2,3-diulose (V)[17] and 6-deoxy-D-*threo*-hexo-2,5-diulose (III)[19] were synthesised for use as standards in analyses (see Fig. 1 for structures).

Apparatus

GLC analyses were performed on a Varian 3700 gas chromatograph equipped with a flame-ionization detector. A glass capillary column (50 m × 0.5 mm I.D., coated with SE-30), purchased from Alltech, was used for all of the GC analyses.

HPLC analyses were performed on a Varian 5000 liquid chromatograph coupled to a Partisil 5 column (25 cm × 4.6 mm I.D.) purchased from Whatman and a variable-wavelength UV detector from Varian, set at 254 nm.

Irradiated solutions

Solutions (2%) of D-fructose in triply distilled water were irradiated with doses

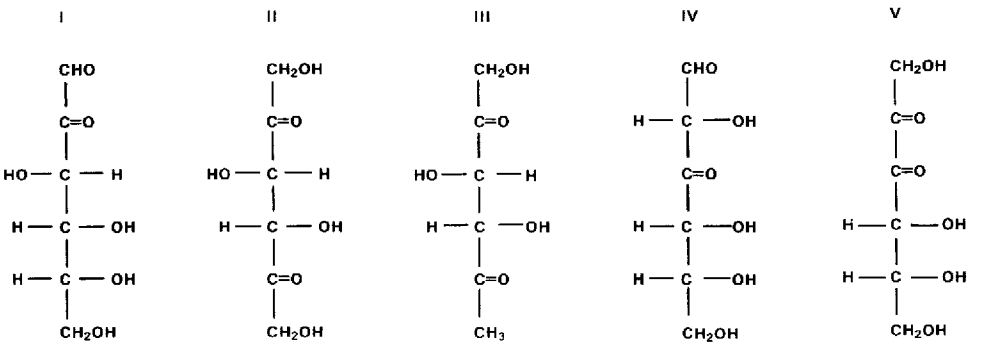

Fig. 1. Synthetic standards: I = D-*arabino*-hexos-2-ulose; II = D-*threo*-hexo-2,5-diulose; III = 6-deoxy-D-*threo*-hexo-2,5-diulose; IV = D-*ribo*-hexos-3-ulose; V = D-*erythro*-hexo-2,3-diulose.

of up to 20 kGy at 20°C by γ-rays from an AECL Gammabeam-650 facility at a dose rate of 16.64 kGy/h under continuous oxygenation. The solutions were freeze-dried before derivatisation.

Preparation of O-benzyloximes and trimethylsilylation

A method described by Devaux *et al.*[20] for the derivatisation of steroid ketones was used for the preparation of sugar oximes. To a sample containing 200 mg of D-fructose or irradiated D-fructose were added 400 mg of O-benzylhydroxylamine hydrochloride, and the mixture was dissolved in pyridine (2 ml). After 48 h at room temperature the solvent was removed at 60°C. The residue was dissolved in 10 ml of chloroform and the solution washed with two 5-ml portions of water. The chloroform phase was dried (sodium sulphate), filtered and evaporated to dryness. The residue was used directly for HPLC and after trimethylsilylation for GLC analyses. Trimethylsilylation was effected by dissolving 10 mg of the material in pyridine (1 ml) containing cellobiose (2 mg) as internal standard, and adding HMDS (0.2 ml) and TMCS (0.1 ml). After 1 h at room temperature the mixture was analysed directly.

Analyses

GLC analyses for the trimethylsilylated samples were carried out at an initial temperature of 180°C (5 min), followed by temperature programming (5°C/min) from 180°C to 240°C, with nitrogen as carrier gas (1 ml/min). The splitless injection mode of Grob and Grob[21] was used. After injection a period of 0.5 min was allowed before the splitter was activated. The split ratio was 1:100.

HPLC analyses for the benzyloximated samples were carried out at room temperature with 3% methanol in chloroform for 10 min, followed by 10% methanol in chloroform for 10 min at a flow-rate of 2 ml/min.

RESULTS AND DISCUSSION

A number of hexosuloses and hexodiuloses (possible irradiation products[17] of sugars) were synthesised and used as standards in the analyses (see Fig. 1).

These compounds were separately converted into their corresponding O-benzyloxime derivatives, isolated by column chromatography and fully characterised[17]

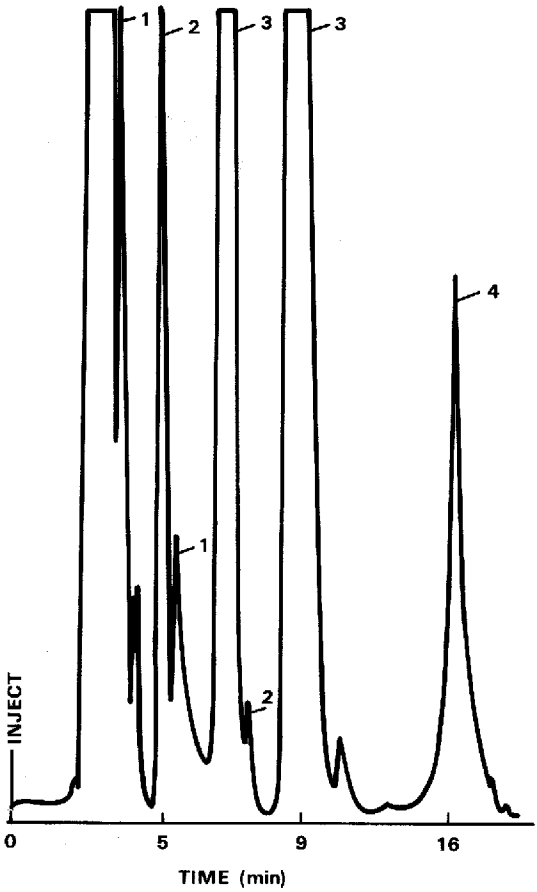

Fig. 2. HPLC analysis of O-benzyloxime derivatives of a 2% fructose solution irradiated (20 kGy) under oxygenated conditions. Peaks: 1 = D-*erythro*-hexo-2,3-diulose; 2 = D-*threo*-hexo-2,5-diulose; 3 = D-*arabino*-hexos-2-ulose; 4 = D-fructose.

by NMR spectroscopy and MS. Quantitative derivatisation, without the formation of rearrangement or other by-products, was obtained in each case. The method, as described in the Experimental section, was applied to an irradiated (20 kGy) 2% fructose solution. The extraction step resulted in a final concentration of unchanged

TABLE I

IDENTIFIED RADIOLYSIS PRODUCTS OF D-FRUCTOSE

Peak No.	*Sugar (as O-benzyloxime)*	*Retention times (min)*	*Relative peak areas*
1	D-*erythro*-hexo-2,3-diulose	4.95 6.20	7:1
2	D-*threo*-hexo-2,5-diulose	5.47 8.49	7:3
3	D-*arabino*-hexos-2-ulose	7.15 9.16	5:7
4	D-fructose	16.85	

starting material of *ca.* 8%, compared with 99% found on direct analysis. This simple and clean separation cannot be achieved by using the corresponding O-methyloximes, owing to their higher polarity. Fig. 2 shows the HPLC chromatogram obtained on analysis of an irradiated (20 kGy) 2% D-fructose solution. The results are summarised in Table I.

The peak for fructose is well separated from the other peaks. The small area of the peak clearly demonstrates the effectiveness of the method in removing the large amount of unchanged starting material. A number of components in the mixture were identified by means of spiking, using the synthetic standards. Glucosone was easily detected and well separated from the other compounds. Note that the glucosone derivative (with its two oxime groups in conjunction) shows a response about ten times greater than those of most of the other hexosuloses and hexodiuloses. The same increased response was found for D-*erythro*-hexo-2,3-diulose, a minor irradiation product of D-fructose. Even correcting for this enhancing effect, glucosone was still shown (method of standard addition) to be the main product (*G ca.* 2.1) of D-fructose irradiated in the presence of oxygen, confirming the results of Kito *et al.*[22]. In addition, the analysis unambiguously demonstrated that D-*erythro*-hexo-2,3-diulose is a radiolysis product of D-fructose solutions. In this case, as in others, the multiplicity of peaks for each component (corresponding to the geometric isomers of the oximes) is an advantage for the identification of individual compounds in complex mixtures by using, for example, spiking. Note that Kito *et al.*[22] have recently presented indirect evidence, based on GLC–MS data for alditol acetates, for the presence of D-*erythro*-hexo-2,3-diulose in irradiated D-fructose solutions.

The HPLC method is equally straightforward in the case of other sugars and confirmed, for example, the finding of Schuchmann and Von Sonntag[11] that irradiation of D-glucose in the presence of oxygen also yields glucosone in substantial amounts (*G ca.* 0.4), although not as the main product.

Although this HPLC method is extremely useful for glucosone determination, the different sensitivities towards different alduloses and diuloses impedes the quantitative determination of minor radiolysis products. This problem was overcome by GLC analysis. The O-benzyloxime derivatives of the synthesized standards were converted into their corresponding trimethylsilyl ethers and analysed on a 50-m glass capillary column coated with SE-30. (Fig. 3 shows a chromatogram of the combined standards. Results are summarised in Table II).

The analysis of an irradiated (20 kGy) 2% D-fructose solution after O-benzyloximation and trimethylsilylation was successfully performed on the same column (Fig. 4). The individual compounds were again identified by spiking, and the multiplicity of peaks was used advantageously for the unambiguous assignment of peaks. Amongst others, the presence of D-*erythro*-hexo-2,3-diulose is clearly demonstrated. This result is the first example of the identification of minor radiolysis products of D-fructose by direct comparison with synthesised standards.

The long retention times were not a disadvantage, because of good recoveries of these relatively thermally stable derivatives. [The use of a shorter (25 m) Varian SE-30 column (3100 plates/m) also afforded excellent separations, but with slightly shorter retention times.] Cellobiose, giving rise to one major and one minor peak (10:1), was used as an internal standard. Its high water solubility, however, required its addition after the oximation step. An alternative internal standard, L(+)-arabi-

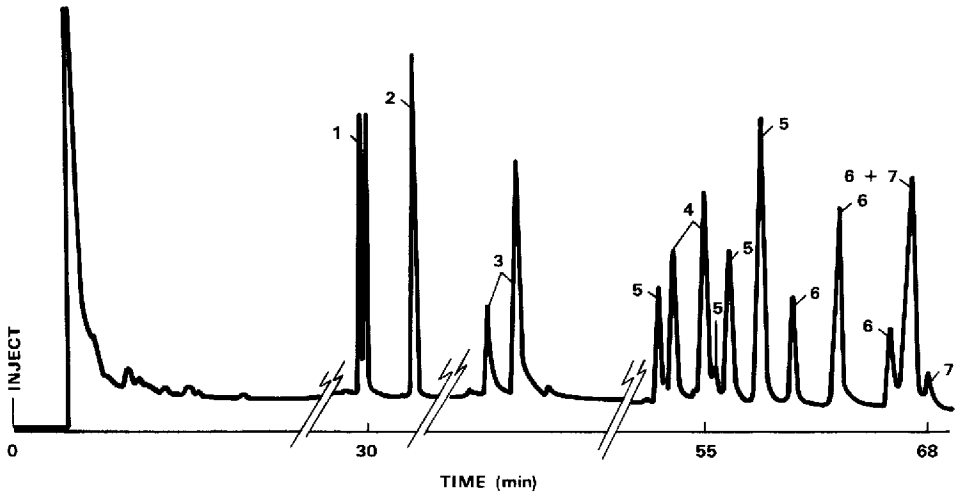

Fig. 3. Capillary GLC analysis of trimethylsilylated benzyloxime derivatives of synthesised standards. Peaks: 1 = D-fructose; 2 = D-glucose; 3 = 6-deoxy-D-*threo*-hexo-2,5-diulose; 4 = D-*threo*-hexo-2,5-diulose; 5 = D-*erythro*-hexo-2,3-diulose; 6 = D-*ribo*-hexos-3-ulose; 7 = D-*arabino*-hexos-2-ulose.

nose diphenyl dithioacetal, did not suffer from this disadvantage and could be added directly to the radiolysis mixture. It showed only one peak (after silylation) on GLC, and good linearity of the detector response was found for weight ratios of glucosone to L(+)-arabinose diphenyl dithioacetal from 0.3 to 3. Similar results were obtained for the other radiolysis products which also showed comparable detector responses. With the dithioacetal as internal standard, glucosone and D-*erythro*-hexo-2,3-diulose could be readily quantitated; they furnished *G* values of *ca.* 2.15 and 1.9, respectively.

The GLC method based on the analysis of benzyloximes offers several advantages over the previously used methyloximes. Our results with the corresponding O-methyloximes were essentially identical with those of Kito *et al.*[22], *viz.* poor separation between some alduloses and diuloses. Better separation is achieved with the corresponding benzyloximes, which would be advantageous for the analysis of com-

TABLE II

GC ANALYSIS OF MODEL COMPOUNDS

Peak No.	*Sugar (as trimethyl-sililated O-benzyloxime)*	*Retention times (min)*	*Relative peak areas*
1	Fructose	29.2, 29.9	1:1
2	Glucose	33.8	
3	6-Deoxy-D-*threo*-hexo-2,5-diulose	43.1, 44.7	1:20
4	D-*threo*-hexo-2,5-diulose	53.2, 54.9	1:1
5	D-*erythro*-hexo-2,3-diulose	52.8, 56.2 56.9, 58.7	7:1:8:13
6	D-*ribo*-Hexos-3-ulose	60.0, 62.6 65.8, 66.9	5:7:4:6
7	D-*arabino*-hexos-2-ulose	66.7, 67.8	2:1

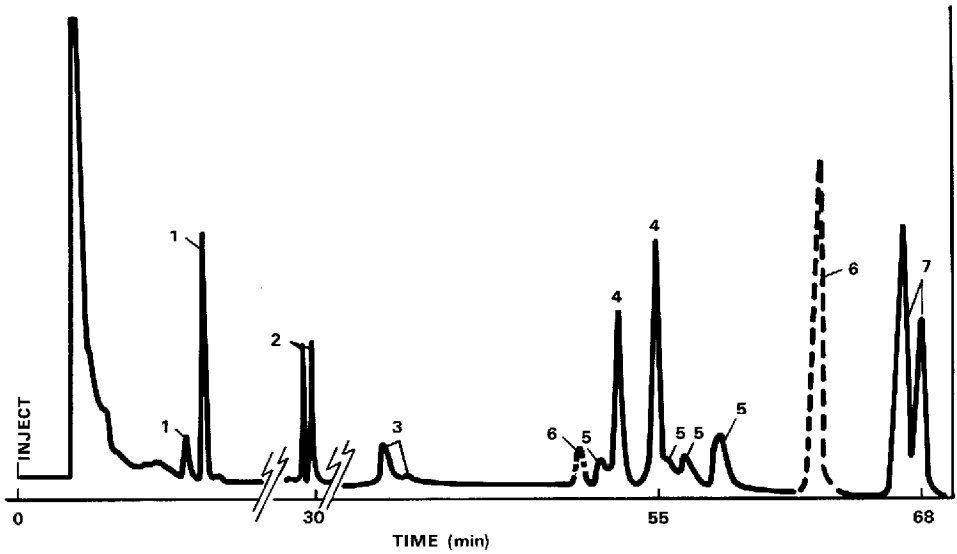

Fig. 4. Capillary GLC analysis of trimethylsilylated O-benzyloxime derivatives of products in a γ-irradiated (20 kGy), oxygenated aqueous solution of D-fructose. Peaks: 1 = unknown, probably chain products with fewer than six carbon atoms; 2 = D-fructose; 3 = unknown; 4 = D-*threo*-hexo-2,5-diulose; 5 = D-*erythro*-hexo-2,3-diulose; 6 = cellobiose; 7 = D-*arabino*-hexos-2-ulose.

plex mixtures. Furthermore, O-benzyloximation, rather than O-methyloximation, allows separation of the radiolysis products from unchanged starting material simply by partitioning between solvents. It was also found[16] that the silyl ethers of these compounds are suitable for identification and structure determination using MS.

The investigation of other oximating procedures has also received attention. Excellent results were also obtained with the O-(pentafluorobenzyl)oxime derivatives, which resulted in better separation with slightly shorter retention times (t_R) and, in some cases, reduced multiplicity [*e.g.* fructose, t_R 24.5; 26.0 min (1:1); glucosone t_R = 42.5; 42.9 min (2:3); D-*ribo*-hexos-3-ulose, t_R = 51.1; 53.6 min (1:2)]. The relatively high cost of this reagent, however, precluded its use in routine analysis. Although the results described above cannot be generalised for all carbohydrate analyses, the greater sensitivity and better peak separation obtained by GLC is, in the case of alduloses and diuloses, preferred to the corresponding HPLC method, particularly when applied to irradiated sugar solutions, owing to the extremely low concentration of the radiolysis products. The HPLC method, however, holds considerable promise for the analysis of mono- and disaccharides.

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