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Journal of Chromatography A, 803 (1998) 111–119

JOURNAL OF CHROMATOGRAPHY A

Analysis of phosphorylated carbohydrates by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry utilising a β-cyclodextrin bonded stationary phase

J. Feurle[a], H. Jomaa[a], M. Wilhelm[a], B. Gutsche[b], M. Herderich[b,*]

[a]*Medizinische Poliklinik, Universität Würzburg, Klinikstrasse 6–8, 97070 Würzburg, Germany*
[b]*Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, 97074 Würzburg, Germany*

Received 11 July 1997; received in revised form 25 November 1997; accepted 26 November 1997

Abstract

Chromatographic separation of phosphorylated carbohydrates was achieved on a β-cyclodextrin (CD) bonded HPLC column. Applying acetonitrile–aqueous ammonium acetate gradient elution, the separation of sugar phosphates relied on the combination of anion-exchange and hydrophilic interaction properties of CD bonded phases in the presence of ammonium ions. Furthermore, this solvent system allowed on-line coupling to electrospray ionization mass spectrometry (ESI-MS) and direct structural characterisation of carbohydrate phosphates by tandem mass spectrometry (MS–MS). In addition to the analysis of various pentose and hexose phosphates, the HPLC–ESI-MS–MS method was successfully applied to demonstrate the enzymatic formation of D-1-deoxyxylulose 5-phosphate from pyruvate and glyceraldehyde phosphate catalysed by yeast transketolase. © 1998 Elsevier Science B.V.

Keywords: Carbohydrates; D-1-Deoxyxylulose-5-phosphate; Sugar phosphates

1. Introduction

Phosphorylated carbohydrates and related phosphate esters are key compounds in a variety of biological processes such as biosynthesis of oligosaccharides, nucleotides and isoprenoids and are involved in signal transduction and catabolite regulation [1–3]. Due to their polarity, structural variety, instability and their non-characteristic UV absorption phosphorylated carbohydrates are difficult to analyse by means of conventional liquid chromatography. Established methods for the analysis of small anionic compounds rely on ion-exchange or ion-pair chromatography [4,5]. However, the high salt content necessary for elution and the presence of ion-pairing reagents limit the successful application of electrospray ionisation for on-line coupling of these chromatographic techniques with mass spectrometry. In order to overcome problems related to high electrolyte concentrations, anion-exchange based high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis using micromembrane ion suppression has been described [6,7]. Nevertheless, limitations of the micromembrane ion suppression technique due to the loss of amino sugars as well as sensitivity problems resulting from void volume have been discussed in a recent publication [7].

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01214-4

Cyclodextrin (CD) bonded phases in combination with polar organic solvents have been applied for HPLC analysis of neutral mono- and oligosaccharides [8], for investigation of sugar mutarotation [9] and separation of enantiomeric amino acids [10] and various drugs [11]. Under reversed-phase conditions separations of phenolic compounds [12], aromatic acids [13], oligopeptides [14], sulfonated aromatics [15] and oligogalacturonic acids [16] have been accomplished. CD bonded phases exhibit a unique combination of properties affecting analyte–stationary phase interactions: resembling a firmly packed diol phase cyclodextrins are able to form enantioselective complexes with guest molecules [10]. In addition, it has been proposed that by inclusion of ammonium ions in the cyclodextrin cavities, CD bonded phases function as weak anion-exchange resins [15–17].

Amongst the phosphorylated carbohydrates included in this study D-1-deoxyxylulose 5-phosphate (dxyl 5-P) represents a key metabolite involved in the non-mevalonate depending isoprenoid biosynthesis. While labelling experiments clearly have demonstrated the incorporation of the precursors pyruvate and glyceraldehyde phosphate as well as of deoxyxylulose itself into isoprenoids in *E. coli*, intermediates and enzymes involved in this novel biosynthetic pathway leading to isopentenyl pyrophosphate in various bacteria and plant organelles are still ill-defined [18–21].

In this contribution we report the analysis of various sugar phosphates by a new HPLC–electrospray ionization (ESI)-MS–MS method using a β-cyclodextrin bonded phase with 5 m*M* aqueous ammonium acetate–acetonitrile as solvent. In addition, the detection and structural characterisation of dxyl 5-P, a putative key metabolite involved in the novel non-mevalonate pathway of isoprenoid biosynthesis, in presence of other sugar phosphates is described.

2. Experimental

2.1. Chemicals

Sugar phosphates were purchased from Sigma (Deisenhofen, Germany), except mannose 1-phosphate, which was obtained from Serva (Heidelberg, Germany). Aqueous solutions containing 50 μg ml^{-1} of each sugar phosphate were prepared directly prior to HPLC–MS analysis. Rabbit muscle aldolase (RAMA; EC 4.1.2.13), transketolase (TK; EC 2.2.1.1) and alkaline phosphatase (AP; EC 3.1.3.1) were purchased from Sigma. All other chemicals used were of highest purity available. Solvents used for chromatography were of HPLC gradient grade.

2.2. HPLC–MS–MS conditions

Chromatography was performed on a Nucleodex β-OH HPLC column, 250×4 mm, 5 μm, from Macherey–Nagel (Düren, Germany), with a binary gradient by an Applied Biosystems 140b pump and a Rheodyne 7725 injection valve equipped with a 5 μl sample loop. Solvent A was 5 m*M* aqueous ammonium acetate pH 4.0, solvent B was acetonitrile. Injection volume was 5 μl, flow-rate was 0.7 ml min^{-1}. Using a post-column T-splitter 20 μl min^{-1} were directed into the mass spectrometer via the ESI interface.

Gradients applied for separation:

Time (min)	% B
Gradient I	
0	80
1	80
15	50
Gradient II	
0	80
1	80
10	60
15	60

HPLC–ESI-MS–MS analysis was performed with a triple stage quadrupole TSQ 7000 mass spectrometer with ESI interface (Finnigan MAT, Bremen, Germany). Data acquisition and evaluation were conducted on a personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). Nitrogen served both as sheath and auxiliary gas; argon served as collision gas.

The following ESI parameters were used: temperature of the heated capillary (220°C), electrospray capillary voltage (3.0 kV), sheath gas (50 psi; 1 psi=6894.76 Pa) and auxiliary gas (5 units). Negative ions were detected scanning from 20–300 u (for hexose and pentose monophosphates) and 20–500 u (for TK reaction mixture) with a total scan duration of 1.0 s. MS–MS experiments were performed at a collision gas pressure of 2 mTorr Ar and a collision energy of 15 eV (1 Torr=133.322 Pa).

2.3. Synthesis of D-1-deoxyxyluose 5-phosphate

The reaction was carried out as described by Mocali et al. [22]. In brief, a mixture of 3 m*M* fructose 1,6-diphosphate (FDP), 15 m*M* sodium pyruvate, 2.5 m*M* $MgCl_2$ and 0.2 m*M* thiamine pyrophosphate (TPP) in 25 m*M* Tris–HCl buffer, pH 7.7 (final volume 40 ml) was incubated with 0.7 U RAMA at 37°C. After 30 min 6 U TK were added and incubation carried on for 3.5 h. The reaction was terminated by addition of 2 g of wet Dowex 50WX4 (H^+-form; Serva). The suspension was filtered through a sintered-glass funnel, adjusted to pH 6 with NaOH and directly subjected to HPLC–MS analysis. Estimated from the MS signal intensities the yield obtained by this reaction was around 5%.

2.4. Derivatisation as methoxyoxime acetate

The TK reaction mixture (450 μl) was incubated for 60 min at 37°C with 50 μl 500 m*M* NH_4HCO_3, pH 9.0, and 10 U AP, lyophilised, derivatised as methoxy oxime acetate and subjected to GC–MS analysis as described earlier [23].

2.5. Synthesis of D-1-deoxyxylulose

Synthesis of D-1-deoxyxylulose was carried out according to Broers [19]. ^1H and ^{13}C NMR data were consistent with those already published [19].

3. Results and discussion

Phosphorylated carbohydrates and other small phosphate-bearing compounds such as inositol phosphates or isopentenyl pyrophosphate (IPP) play an important role in isoprenoid biosynthesis, protein prenylation, signal transduction or regulation of carbohydrate catabolism [1–3]. Metabolites involved in theses processes are difficult to analyse due to their structural variety, instability, non-characteristic UV absorption and low in vivo concentrations. Conventional methods for separation of small anionic compounds such as ion-exchange or ion-pair chromatography are likely to interfere with the electrospray process due to the presence of background electrolytes [25]. In order to overcome these problems, we applied a β-CD bonded HPLC-phase for the analysis of various sugar phosphates. Utilising the solvent system acetonitrile–5 m*M* aqueous ammonium acetate for gradient elution, on-line coupling of the HPLC to ESI–MS was accomplished. At pH 4.0 ESI of the sugar phosphate esters under study exclusively yielded the deprotonated ions $[M–H]^-$; no double charged anions could be detected. Subsequent collision activation of the respective molecular ions allowed direct structural characterisation of carbohydrate phosphates by means of MS–MS (Fig. 1).

The retention times and the major product ions of the sugar phosphates under study are summarised in Table 1. Using 5 m*M* aqueous ammonium acetate pH 4.0 and acetonitrile for gradient elution, reasonable retention times (between 7 and 11 min) and k' values (around 4) were achieved. Analysis time including reconditioning of the HPLC column was less than 30 min, thus allowing high sample throughput. Comparison of the retention times showed that polarity as well as structural properties of the analytes influenced chromatographic behaviour. The unique separation properties of CD bonded phases have been demonstrated to rely on hydrophilic (with the hydroxyl groups on the surface) and hydrophobic (with the apolar cavity of the CD molecule) interactions as well as on size-dependent and enantiospecific formation of inclusion complexes [11,13,26]. However, the inclusion of the polar and ionic sugar phosphates into the hydrophobic cavity of the CD molecule appeared to be less likely. In addition, an anion-exchange mechanism has been proposed to account for the chromatographic differentiation of negatively charged compounds. In the latter case, inclusion of cationic constituents of the solvent, e.g., ammonium ions, in the CD cavity might form a dynamic anion-

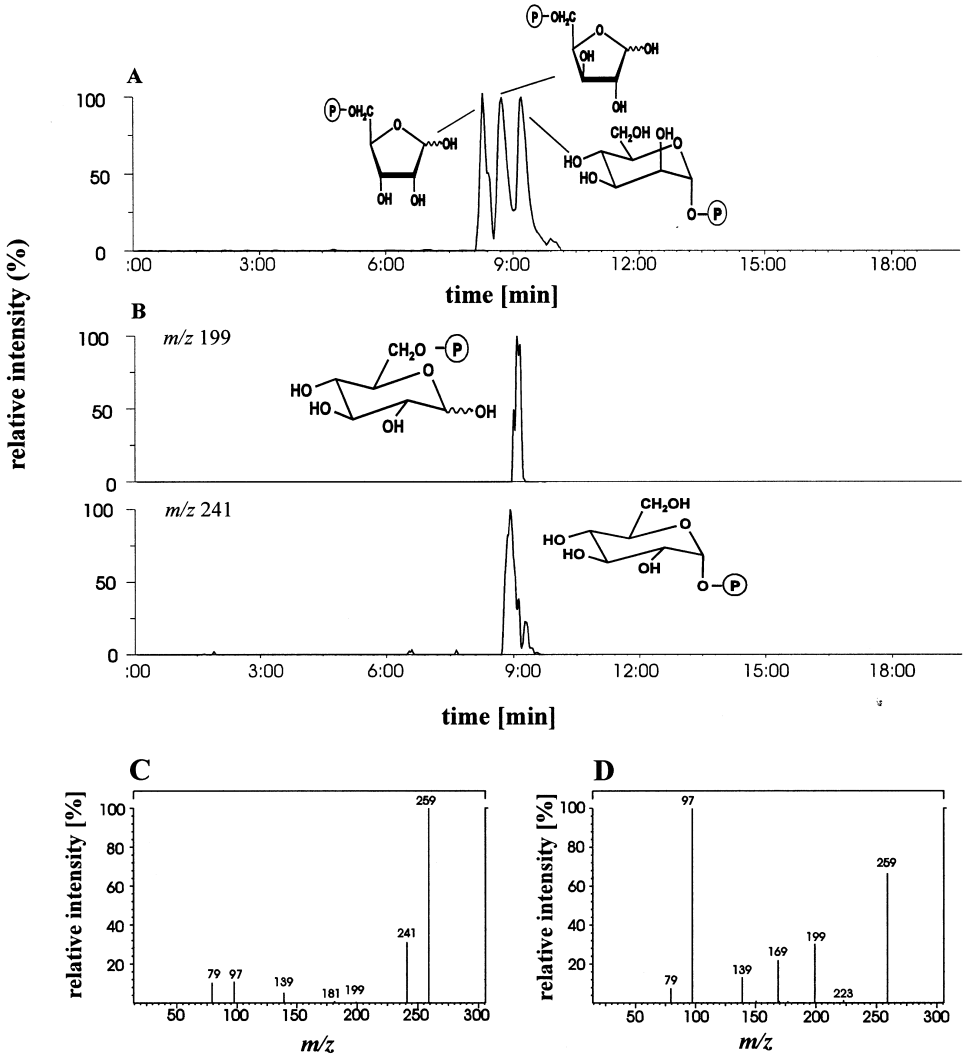

Fig. 1. Analysis of phosphorylated carbohydrates by HPLC–ESI-MS–MS. (A) HPLC separation of ribose 5-phosphate, xylose 5-phosphate and mannose 1-phosphate; product ion scan, precursor ions m/z 259 (man 1-P) and m/z 229 (pentose 1-P); Nucleodex β-OH, gradient I. P=HPO_3^-. (B) HPLC–ESI-MS–MS differentation of glucose 1-phosphate and glucose 6-phosphate by characteristic product ions; Nucleodex β-OH, gradient I. (C) Product ion spectrum of glucose 1-phosphate; precursor ion m/z 259; 15 eV, 2.0 mTorr Ar. (D) Product ion spectrum of glucose 6-phosphate; precursor ion m/z 259; 15 eV, 2.0 mTorr Ar.

exchange site [15–17]. In our hands, separation of phosphorylated carbohydrates such as hexose and pentose monophosphates or dihydroxyacetone phosphate (DHAP), glyceraldehyde phosphate (GAP) and fructose 1,6-diphosphate (FDP) relied on the proposed anion-exchange mechanism, as increasing polarity and ionic properties of the analytes resulted in increasing retention times. This conclusion was supported by the observation that retention of phosphates required thorough equilibration of the CD column with aqueous ammonium acetate prior to analysis. Otherwise, the sugar phosphates eluted within the dead volume of the column, apparently due to incomplete loading of the CD cavities with ammonium. In addition, other mechanisms did influence chromatographic behaviour. As demonstrated by the difference in retention times observed for xylose 5-phosphate and ribose 5-phosphate (Fig.

Table 1
HPLC–ESI-MS–MS separation and characterisation of various sugar phosphates: capacity factors (k') and spectroscopic data

Compound	k'	$[M-H]^-$	Main product ions m/z (relative intensity)
glc 6-P	3.58[a]	259	259 (68); 223 (2); 199 (30); 169 (24); 139 (12); 97 (100); 79 (8)
glc 1-P	3.54[a]	259	259 (100); 241 (35); 199 (1); 181 (2); 139 (6); 97 (12); 79 (10)
gal 1-P	3.56[a]	259	259 (100); 241 (8); 199 (2); 139 (8); 97 (10); 79 (14)
man 1-P	3.68[a]	259	259 (100); 241 (1); 199 (1); 169 (1); 139 (1); 97 (45); 79 (6)
xyl 5-P	3.29[a]	229	229 (100); 211 (38); 151 (1); 139 (8); 97 (12); 79 (22)
rib 5-P	3.21[a]	229	229 (36); 193 (2); 139 (17); 97 (100); 79 (10)
dxyl 5-P	3.17[b]	213	213 (32); 195 (100); 139 (46); 135 (48); 97 (64); 79 (28)
DHAP/GAP	3.42[b]	169	169 (8); 123 (1); 97 (100); 79 (19)
FDP	4.33[b]	339	339 (100); 321 (1); 241 (20); 177 (2); 151 (1); 97 (24); 79 (1)

Nucleodex β-OH 200×4 mm, 5 μm.
[a] Gradient I, [b] gradient II, 0.7 ml/min; MS–MS: ESI neg.; colllision gas 2 mTorr Ar.

1A), both exhibiting comparable polarity and ionic properties, stereospecific interactions between the epimeric hydroxy groups and the CD stationary phase have to be taken into consideration.

While some of the phosphorylated carbohydrates were difficult to differentiate by merely chromatographic means, application of on-line MS–MS allowed the reliable identification of compounds under study. Generally, all sugar phosphates were detected as molecular ions $[M-H]^-$; upon collision activation all of them showed characteristic cleavage at specific positions of the carbon backbone (Table 1). This

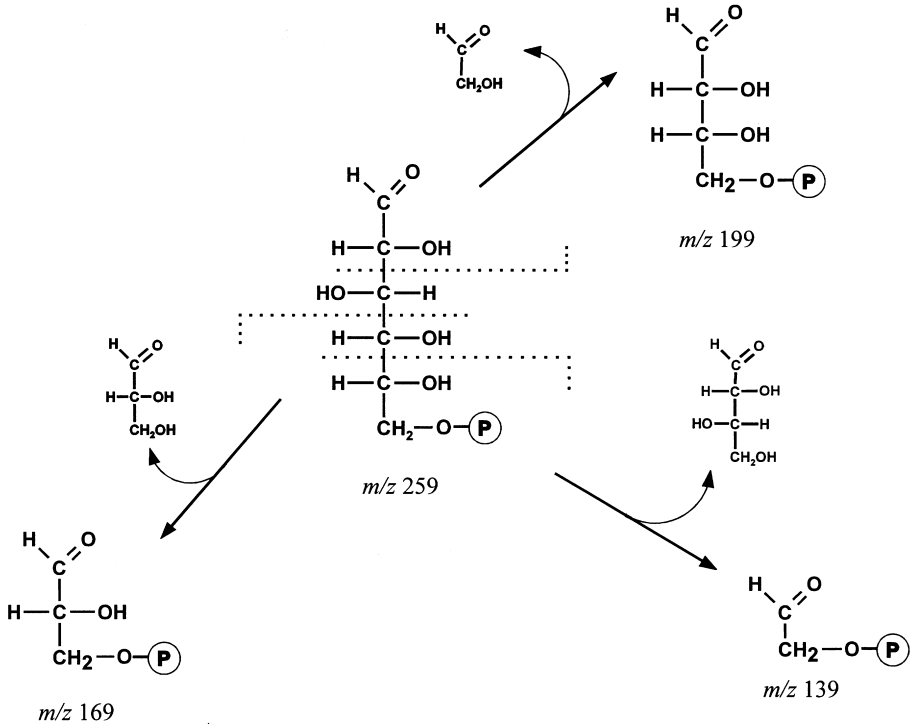

Fig. 2. Retroaldol-type backbone cleavage of glucose 6-phosphate leading to product ions differing by 30 u. P=HPO_3^-.

resulted in series of product ions differing by 30 u, e.g., ions *m/z* 199, *m/z* 169 and *m/z* 139 for glucose 6-phosphate (Fig. 1D, Fig. 2).

Additionally, most of the compounds under study were characterised by facile dehydration (−18 u and −36 u, respectively). Product ions *m/z* 79 $[PO_3]^-$ and *m/z* 97 $[H_2PO_4]^-$ were indicative for the phosphate moiety; as already demonstrated in the case of phosphopeptides, the respective precursor ion scans provided an efficient approach to substructure specific screening of phosphorylated carbohydrates [24]. Sugar phosphates that were difficult to differentiate by merely chromatographic means such as isomeric glucose 1-phosphate and glucose 6-phosphate could be identified by characteristic product ions (Fig. 1B–D). However, differentiation between glucose 1-phosphate, mannose 1-phosphate and galactose 1-phosphate was hampered by almost identical mass spectra differing only in relative signal intensities of the respective product ions (Table 1). Still, for these epimeric aldohexose 1-phosphates the ratio of relative intensities as calculated for product ions *m/z* 241 versus *m/z* 97 was indicative.

Having established the HPLC–ESI-MS–MS analysis of commercially available sugar phosphates we applied yeast transketolase for the biomimetic synthesis of D-1-deoxyxylulose 5-phosphate (dxyl 5-P). Transketolase, an enzyme primarily involved in pentose phosphate cycle, has been shown to catalyse the condensation of hydroxypyruvate with glyceraldehyde phosphate yielding stereochemically pure xylulose 5-phosphate [22]. Using pyruvate and D-glyceraldehyde phosphate, which was formed in situ from fructose diphosphate by RAMA, as substrates we obtained the corresponding 1-deoxy sugar dxyl 5-P, one putative key metabolite in the non-mevalonate pathway of isoprenoid biosynthesis. Chromatographic separation of the crude reaction mixture on the CD bonded HPLC column in combination with

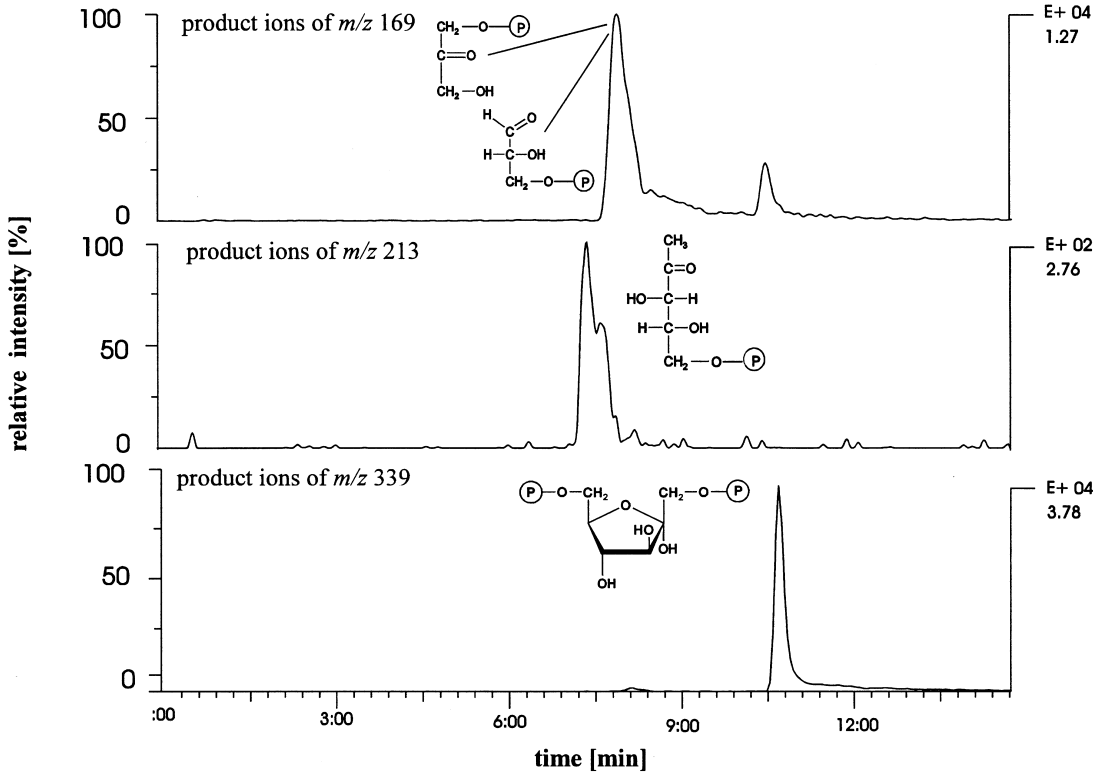

Fig. 3. HPLC–MS–MS chromatogram of the TK reaction mixture; product ion scan, precursor ions *m/z* 169 (DHAP, GAP), *m/z* 213 (dxyl 5-P) and *m/z* 339 (FDP). Nucleodex β-OH; gradient II, 15 eV, 2.0 mTorr Ar.

on-line ESI-MS–MS allowed detection and structural characterisation of dxyl 5-P even in an excess of fructose diphosphate, glyceraldehyde phosphate and dihydroxyacetone phosphate (Fig. 3). The ESI mass spectra of the compounds in the reaction mixture are depicted in Fig. 4. Upon collision activation deprotonated dxyl 5-P ($[M–H]^-$=213 u) revealed the product ions m/z 195 ($[M–H–H_2O]^-$) and m/z 139; the

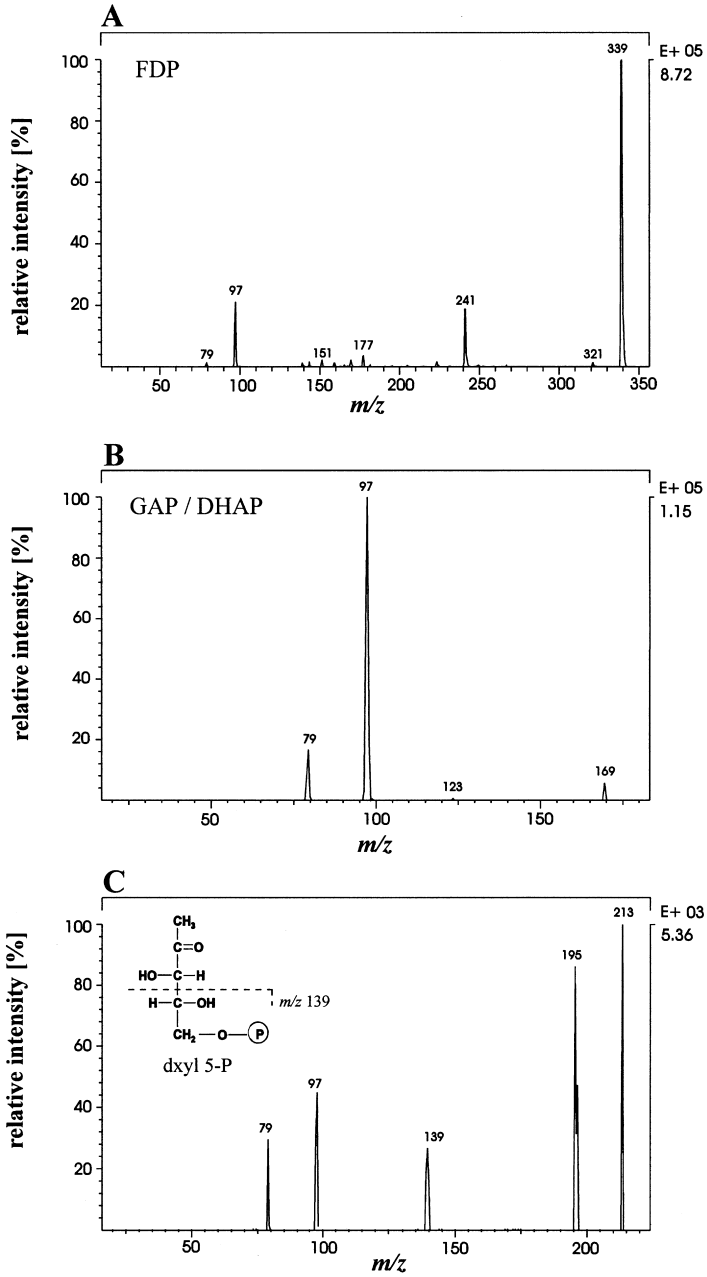

Fig. 4. Product ion spectra of RAMA/TK reaction products. (A) FDP, precursor ion m/z 339 $[M–H]^-$. (B) GAP and DHAP, precursor ion m/z 169 $[M–H]^-$. (C) dxyl 5-P, precursor ion m/z 213 $[M–H]^-$. Nucleodex β-OH, gradient II, 15 eV, 2.0 mTorr Ar.

latter fragment resulted from the retroaldol type backbone cleavage of the C_3–C_4 carbon-carbon bond (Fig. 4C), thus confirming the position of the phosphate ester on C_5. The structure of the sugar moiety originating from the TK catalysed reaction was unambiguously confirmed by GC–MS analysis after enzymatic dephosphorylation with alkaline phosphatase and derivatisation as methoxyoxime acetate [23]; retention time and electron impact ionization mass spectrum of the enzymatically formed sugar were identical to data obtained with chemically synthesised D-1-deoxyxylulose [19].

Dxyl 5-P has been postulated a key metabolite in terpenoid biosynthesis in *E. coli* and other bacteria as well as in plant organelles, where it is supposed to derive from a direct condensation of pyruvate and glyceraldehyde phosphate [18,21]. The enzymes and metabolites involved in deoxyxylulose-depending isoprenoid biosynthesis yet remain to be characterised. However, this novel non-mevalonate pathway and its specific metabolites may have a much more general relevance. For example, feeding of isotopically labelled mevalonate or acetate resulted in distinctively low incorporation rates during isoprenoid biosynthesis in numerous bacteria which could not be explained by classical isoprenoid biosynthesis [27–32]. As IPP, the common key metabolite in both the classical pathway via mevalonate and the novel non-mevalonate pathway, can also be analysed using our HPLC–MS–MS method (data not shown), HPLC utilising a CD bonded stationary phase in combination with ESI-MS provides a valuable approach to study early steps in isoprenoid biosynthesis.

4. Conclusions

The HPLC–ESI-MS–MS method utilising a β-cyclodextrin bonded stationary phase proved to be a successful approach towards separation and structural characterisation of phosphorylated carbohydrates. On-line combination of chromatographic separation with MS–MS yielded the structural information necessary for identification of sugar phosphates and allowed differentiation of co-eluting analytes. Especially with regard to the growing interest in sugar phosphates as key metabolites involved in biosynthetic pathways, signal transduction processes and regulative mechanisms, this HPLC–MS method will enable the direct analysis of these important compounds in biological samples. Furthermore, it may provide the analytical basis for biosynthetic studies utilising various precursors labelled with stable isotopes.

Acknowledgements

We thank Professor D. Arigoni for providing the Ph.D. thesis of S.T.J. Broers. This work was supported by program BMBF 2000 of the Bundesministerium für Bildung und Forschung (J.F.), the Graduiertenkolleg ''Infektiologie'' (H.J.) and by the Deutsche Forschungsgemeinschaft (M.H., B.G.).

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