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ANALYSIS OF PHOSPHORYLATED ALDOSES BASED ON GROUP SEPARATION AND REDUCTIVE TRYPTAMINE DERIVATISATION PRIOR TO HPCE REVEALING INITIAL PRODUCTS OF THE MAILLARD REACTIONS

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Key words: high performance capillary electrophoresis, sugar phosphates, phosphorylated monosaccharides, tryptamine derivatisation, meat, Maillard reactions

A High Performance Capillary Electrophoresis method for the detection and quantification of phosphorylated aldoses has been developed using D-thyminose (2-deoxy-D-ribose) as internal standard. The addition of an indolyl chromophor system to the carbonyl groups in the carbohydrates by reductive tryptamination resulted in high sensitivity by UV detection at 220 nm, and opportunities for identification based on the diode array spectrum with specific peaks at 220 and 280 nm. D-glucose 6-phosphate, D-ribose 5-phosphate, and the glycolytic intermediate DL-glyceraldehyde 3-phosphate were detected within 25 min, the analytes showing linearity in the concentration range from 2.5 to 100 mmol/L with correlation coefficients between 0.9290 and 0.9887. Detection and quantification limits were found to be from 210 to 450 μmol/L and from 360 to 750 μmol/L, respectively. The occurrence of two quantitatively dominating products from reductive tryptamination of D-ribose 5-phosphate and DL-glyceraldehyde 3-phosphate are believed to be a result of initial Maillard reactions. The two reaction products were separated and characterised using liquid chromatography and spectroscopy including NMR. The method proved to be an efficient tool to study the concentration of phosphorylated aldoses in pork revealing differences in concentration of the dominating aldose glucose-6-phosphate in different breeds of pig.

ABREVIATIONS USED: α, slope; C, concentration; FPLC, Fast Polymer Liquid Chromatography; HPCE, High Performance Capillary Electrophoresis; NA, normalised area; NMR, Nuclear magnetic resonance; LOD, limit of detection; LOQ, limit of quantification; MECC, Micellar electrokinetic capillary chromatography; MT, migration time; RNA, relative normalised area; RRF, relative response factor.

INTRODUCTION

Carbohydrates are the most widely distributed and abundant organic compounds on earth and play a central role in the animal and plant metabolism as storage and structural compounds and as glycoside part in various metabolites [Bjergegaard *et al*., 2001]. The analysis of carbohydrates is of great interest in a wide range of scientific fields including basic biochemistry and applied food science. Knowledge on distribution and concentration of different carbohydrates in a large variety of products both of plant and animal origin can thus provide useful information, making reliable, effective and sensitive analytical tools for carbohydrates of general interest.

The present study focuses on the group of phosphorylated aldoses, being important metabolic intermediates (*e.g.* of pentose- and glycolytic pathways), but also calling for special attention *e.g.* with respect to flavour development in meat. Phosphorylated carbohydrates pose a challenge with respect to analysis due to their polarity, structural similarity, and low concentration in biological samples [Buchholz *et al*., 2001]. Moreover, carbohydrates have a general lack of chromophoric and fluorophoric moieties limiting the possibilities for detection by UV-vis and fluorescence.

Much effort has been applied to solve the problems with low sensitivity in carbohydrate analysis, and the coupling of liquid chromatographic methods to mass spectrometry (MS) has proven useful. An increasing interest has been evoked with respect to combining High Performance Capillary Electrophoresis (HPCE) which is a powerful separation technique, with Mass Spectrometry (MS) [Campa *et al*., 2006], and capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) was *e.g.* used for the determination of anionic intermediates including glucose 1-phosphate, fructose 6-phosphate, glucose 6-phosphate, and fruc-

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tose 1,6-bisphosphate from *Bacillus subtilis* cells [Soga *et al*., 2002]. The MS gives a clear advantage in the identification process of unknown compounds. However, the MS is an expensive investment and the interface between MS and *e.g.* capillary electrophoresis is known to cause problems, and this underlines the need for more simple methods.

Analysis of carbohydrates by HPCE has received much attention over the last years [Campa *et al*., 2006; Sørensen *et al*., 1999; Ciringh & Lindsey, 1998]. The analytical procedures developed include a range of methods based on different kinds of derivatisation procedures performed in order to increase sensitivity of the methods [Andersen *et al*., 2003; Yamamoto *et al*., 2003; Taga *et al*., 2001; Lige *et al*., 2000; Noe & Freissmuth, 1995; Vorndran *et al*., 1992]. Very few reports are, however, devoted to the investigation of phosphorylated carbohydrates or other phosphorylated compounds and development of suitable analytical methods for these negatively charged compounds therefore calls for attention.

A method for the analysis of neutral reducing carbohydrates by reductive tryptamine derivatisation prior to micellar electrokinetic capillary chromatography (MECC) has previously been developed in our laboratory [Andersen *et al*., 2003]. The identification and quantification of reducing carbohydrates worked well in this system, and it seemed reasonable to further develop the system to also include the analysis of phosphorylated compounds. The method development has included optimization of the existing method with buffer and instrumental adjustments, and measurement of concentration linearity of all reference compounds. Moreover, the initial Maillard products obtained by the derivatisation procedure were studied in details, using NMR. The optimized method was finally used for detection and quantification of phosphorylated carbohydrates in meat samples.

MATERIALS AND METHODS

Chemicals. Sodium cyanoborohydride ($NaBH_3CN$), cholic acid, disodium tetraborate decahydrate ($Na_2B_4O_7$-$10H_2O$), disodium hydrogenphosphate dihydrate (Na_2HPO_4-$2H_2O$), taurine, sodium dodecyl sulphate (SDS), D-glucose 6-phosphate (monosodium salt) were obtained from Sigma Co. (St. Louis, MO, USA). D-Ribose 5-phosphate (disodium salt) and DL-glyceraldehyde 3-phosphate (diethylacetal monobarium salt) were purchased from Fluka (Buchs, Switzerland). D-Thyminose (2-deoxy-D-ribose) was from Serva Finechemicals (New York, NY, USA), tryptamine hydrochloride and deuterium oxide (D_2O) was from Aldrich Chemical Co. (Milwaukee, WI, USA), 1-propanol and salt (NaCl) was purchased from Merck (Darmstadt, Germany), and sodium dihydrogen phosphate (NaH_2PO_4) was from Riedel-de Haën (Selze, Germany). Methanol was purchased from VWR International (Rødovre, Denmark), and ammonia from Applichem GmbH (Darmstadt, Germany). Column materials for ion-exchange chromatography (Sephadex CM-25 H^+, Dowex 50W×8 H^+ 200–400 mesh, and Dowex 1×8 acetate 200–400 mesh) were obtained from Sigma Co. (St. Louis, MO, USA). Water was purified (18.2 $\mu\Omega$) in a Millipore Milli-Q system (Bedford, MA, USA).

Meat. Meat (*longissimus dorsi*) from the pure breed of Hampshire, Black spotted, and the cross-breed of Duroc and Landrace × Yorkshire (DLY) were analysed for the content of phosphorylated carbohydrates after three days of ageing at 2°C. Furthermore, meat (*semimembranosus*) from the cross-breed of DLY and Hampshire and Landrace × Yorkshire (HLY) was previously analysed after 22 days at 2°C [Meinert *et al*., 2007]. Four pigs of each breed were analysed in triplets giving a total of 12 replications per breed. The meat was kindly provided by The Danish Meat Research Institute.

Sample preparation. The reference compounds were dissolved in Milli-Q water to a concentration of 0.1 mol/L before derivatisation with tryptamine, except DL-glyceraldehyde 3-phosphate (barium salt), which was dissolved in 0.2 mol/L HCl. The internal standard D-thyminose was dissolved in Milli-Q water to a concentration of 12.5 mmol/L.

The meat was trimmed for all visible fat and grounded (Bizerba-Werke, type FN70, Balingen, Germany) twice through a finger wheel of 2 mm. Two grams of meat was weighed into a 10 mL centrifuge tube and 4 mL of 70% methanol was added, and the mixture was homogenised (Ultra Turrax T 25, Janke & Kunkel, Staufen, Germany) for 1 min at moderate speed, followed by centrifugation at 2000 × *g* for 3 min. The supernatant was transferred to a clean tube. The procedure was repeated in a total of three times, and the pooled supernatants were evaporated to dryness by compressed air. The residues were redissolved in 4 mL Milli-Q water of which 2 mL was subjected to group separation by ion exchange chromatography as described by Andersen *et al*. [2003]. The anionic compounds were eluted from the anion exchanger with 4×4 mL 2 mol/L NH_3. The effluents containing the phosphates were evaporated to dryness and redissolved in 200 μL Milli-Q water before reductive amination and HPCE-analysis.

Reductive amination. The derivatisation of carbohydrates by reductive amination was performed as described by Andersen *et al*. [2003] with a minor modification. The internal standard D-thyminose (20 μL 12.5 mmol/L), which was not found present in meat, was added to 10 μL of the dissolved pure carbohydrates and to 100 μL of the redissolved purified elute from the anion exchanger. To these mixtures 12.5 μL of 0.15 mol/L tryptamine (dissolved in 10% propanol) was added, the tryptamine was in excess, and the mixtures were heated for 10 min at 90°C. Subsequently 4.5 μL of aqueous sodium cyanoborohydride solution (0.3 g/mL) was added and the mixtures were additionally heated at 90°C for 60 min. This last step should be performed in the fume cupboard owing to the toxic properties of cyanoborohydride.

HPCE analysis. The apparatus used was an ABI 270A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a UV detector. The capillary was a 500 mm × 0.05 mm I.D. coated fused silica capillary with a UV detection window placed on-column at a position of 270 mm from the injection end. The capillary was conditioned by flushing with 1.0 mol/L NaOH for 40 min before use. Before each analysis the capillary was flushed with 1.0 mol/L NaOH for 2 min followed by buffer for 5 min. The specific HPCE conditions varied during optimization of the method. The final separation parameter comprised analysis at 30°C and 14 kV, the buffer consisting of 100 mmol/L

borate (pH 9.7). Samples were introduced from the anodic end of the capillary by vacuum injection for 1 s at 5 kPa. For data processing, a Shimadzu (Kyoto, Japan) Chromatopac C-R3A integrator was used.

Relative Response Factors (RRF). To quantify the individual phosphorylated compounds, RRFs were calculated. RRF (relative to the internal standard D-thyminose) was determined as $\alpha_{\text{D-thyminose}}/\alpha_x$ where α is the slope of the calibration curve and x refers to the individual phosphorylated aldoses.

Purification and separation by FPLC. In order to study the derivatisation products, a ribose 5-phosphate solution was derivatised by reductive amination, as previously described, and filtered through a 0.2 μm filter (Sartorius, Goettingten, Germany) before applying the solution to FPLC separation. The FPLC was equipped with a UV-MII detector (214 nm) and two P-500 pumps, all from GE Health (Uppsala, Sweden). The column was a Superdex peptide HR 10/30 with I.D. of 10 mm, and a packed bed height of 30–31 cm (Amersham Biosciences Limited, Buckinghamshire, England). Prior to analysis, the column was equilibrated with the elution buffer consisting of 20 mmol/L NaH_2PO_4 and 50 mmol/L NaCl, pH 6.9. The collected fractions (1 mL volume) was analysed by HPCE, as previously described, and fractions with identical peaks based on migration times (MT) in the electropherograms were then pooled into two pools in total.

NMR. NMR analysis was conducted on: (i) non-derivatised ribose 5-phosphate, (ii) derivatised ribose 5-phosphate, and iii) derivatised, purified, and separated (FPLC) reaction products of ribose 5-phosphate. Pure ribose 5-phosphate (7.5 mg of the salt) was dissolved in 700 μL D_2O (i). Derivatised ribose 5-phosphate solutions was freeze-dried and redissolved in 600 μL D_2O (ii and iii). This procedure was conducted twice before NMR-analysis. The ^{1}H NMR spectra were recorded on a Bruker Avance 400 NMR instrument.

Quantification. The concentration of the carbohydrates (x) in meat was calculated as:

$$C_x\ (\mu\text{mol/g}) = (RNA_x * C_{\text{D-thyminose.}}(\mu\text{mol}) * RRF_x)/m_{meat}\ (\text{g})$$

where $RNA_x = NA_x / NA_{\text{D-thyminose}}$, and $NA_x = (A_x/MT_x)$.

RESULTS AND DISCUSSION

Analysis of the tryptamin derivatised phosphorylated aldoses in the existing MECC system for neutral reducing carbohydrates using cholate as a detergent [Andersen *et al*., 2003] showed that none of the compounds migrated within 90 min (max runtime for the HPCE used), and a higher voltage and increased temperature were unable to solve this problem. The slow migration was probably due to in the relatively high negative charge of the phosphate groups at buffer pH 9.7. The migration velocity of each compound is a combination of the electroosmotic flow (EOF) in the system and the individual electrophoretic mobility of the analytes. In the present system the EOF runs from the anode (injection site) to the cathode (detection site), and the anions are thus retarded due to their net charge. At pH 9.7 the approximate net charge of the phosphorylated carbohydrate derivatives are minus 1.5, and the anode therefore attracts the molecules. A lowering of buffer pH is expected to reduce this attraction and consequently increase the overall migration velocity.

The cholate based buffer system are unstable at pH lower than neutral [Bjergegaard *et al.*, 1993] and a SDS-based system (pH 7) was thus chosen in order to study whether this commonly used micellar system was able to promote the migration. The SDS-buffer consisted of 50 mmol/L SDS, 30 mmol/L disodium hydrogenphosphate, 18 mmol/L disodium tetraborate decahydrate, and 5% 1-propanol, adjusted to pH 7 [Bjergegaard *et al*., 1994]. The lowering of buffer pH from 9.7 to 7 is expected to result in a positively charged tryptamine group due to a pH value of approx. two units below the pK_a value for the amine. Moreover, one of the protolytic active phosphate groups will be partly pro-

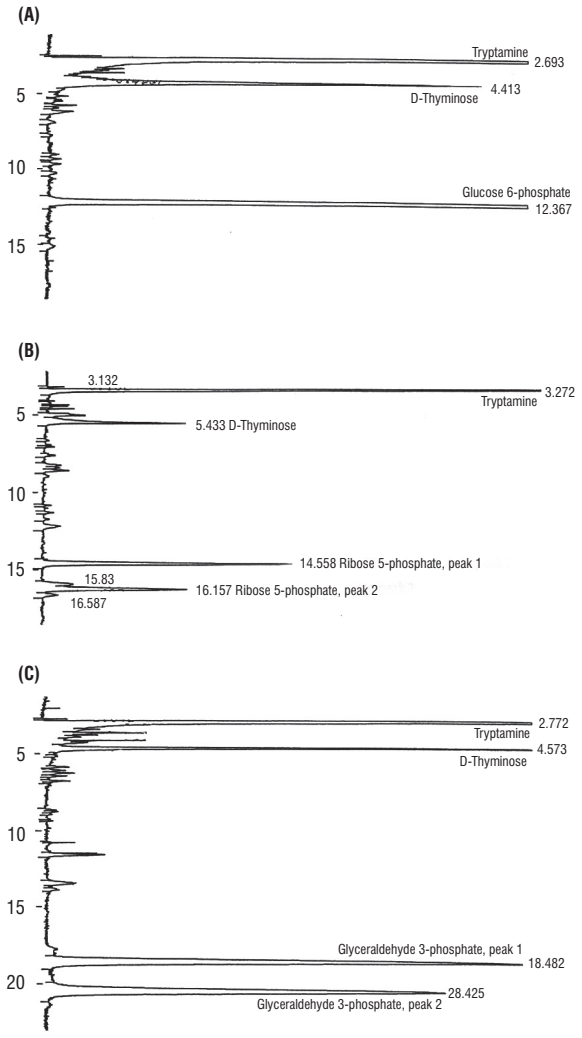

FIGURE 1. Electropherograms of the tryptaminated carbohydrates shown with migration times (min): (A) D-glucose 6-phosphate (0.04 mol/L), (B) D-ribose 5-phosphate (0.04 mol/L), and (C) DL-glyceraldehyde 3-phosphate (0.04 mol/L) using the following conditions: 100 mmol/L borate buffer (pH 9.7), 500 mm × 0.05 mm I.D. coated fused silica capillary, 30°C, 14 kV, and UV detection at 220 nm. When comparing migration times for tryptamine and D-thyminose in A, B, and C small shifts are noted. These are due to minor differences in buffer strength and capillary length, which is inevitable in the practical use of the method.

tonised resulting in a net charge for the tryptaminated sugar phosphate molecules of approx. -½. The derivatised aldoses were detected at 220 nm, and they all migrated with MT of less than 30 min as expected due to their reduced negative charge. A severe problem with the SDS-system was, however, a consistent high amount of noise and false peaks in the electropherograms. The noise increased the limit of detection (LOD, signal to noise ratio of three) and limit of quantification (LOQ, signal to noise ratio of five) to concentrations higher than 100 mmol/L, which are unwanted with regard to determination of phosphorylated carbohydrates in biological samples including meat.

An alternative and simple way to reduce MT of the phosphorylated carbohydrates in HPCE was to reduce the effective and/or total length of the capillary. A reduction in the length of the capillary, keeping the voltage unchanged at the same time, resulted in higher field strength per centimetre and thus a reduced MT of the compounds. A problem with this approach was a simultaneous increase in current, which may lead to excess heat generation. An acceptable current (90–120 μA) was obtained by decreasing the voltage from 20 to 14 kV simultaneously with a reduction of the total capillary length from 76 cm to 50 cm. The corresponding reduction in the effective length of the capillary was from 53 cm to 27 cm, resulting in MT for the analytes within 25 min (Figure 1) in a borate buffer. Cholic acid was left out of this system, as it did not improve the separation. The faster migration was due to the shorter effective length, as the voltage to total length ratio was almost the same in this system compared to the original system. Figure 1 shows the electropherograms of tryptaminated D-glucose 6-phosphate, D-ribose 5-phosphate, and DL-glyceraldehyde 3-phosphate, respectively, in the newly developed system.

As it can be seen in Figure 1, the electropherograms contain several minor peaks besides the peak(s) of the quantitatively dominating compounds. These minor peaks are assumed to represent reaction products caused by further transformation of the initial products of the reductive amination corresponding to Maillard transformation reactions. From Figure 1 it can furthermore be seen, that both glyceraldehyde 3-phosphate and ribose 5-phosphate resulted in two quantitatively dominating products. This was however not expected, as simple initial products of the derivatisation process of aldehydes [Andersen *et al*., 2003] do not give two products. The two peaks of glyceraldehyde 3-phosphate and ribose 5-phosphate, respectively, were confirmed by UV to be tryptamine derivatives of the two aldose phosphates.

The occurrence of two quantitatively dominating products in the reaction mixtures from both glyceraldehyde 3-phosphate and ribose 5-phosphate was investigated further in order to find a possible explanation. A hypothesis to be tested was the possibility of a simple formation of complexes, based on the zwitterionic nature of the derivatised compounds. The pH value of the buffer was 9.7, and at this pH it might be possible for the negatively charged phosphate group to combine with the positively charged tryptamine

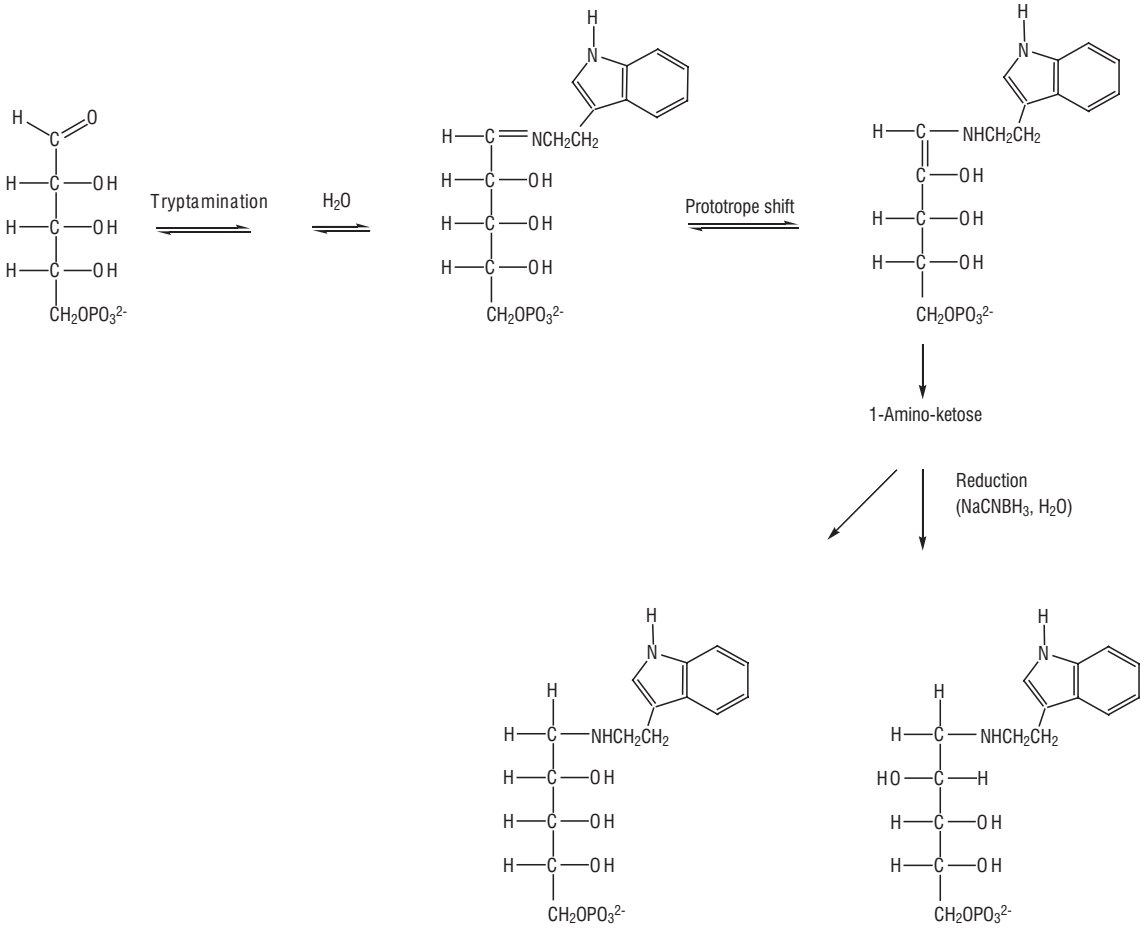

FIGURE 2. Proposed formation and nature of major D-ribose 5-phosphate products succeeding tryptamination and reduction.

group in a bridge formation. In order to change the charge of the tryptamine and phosphate groups and thus examine a possible bridge formation the borate buffer was adjusted to pH 8 and pH 10, respectively. The bridge formation was expected at pH 8 but only to a minor degree at pH 10 at which the positive charge of tryptamine is reduced. Furthermore, glyceraldehyde 3-phosphate was analysed in a phosphate buffer with pH 6 reducing the negative charge of the phosphate group. At all three pH values the glyceraldehyde 3-phosphate derivative migrated as two compounds with well separated peaks and the theory of zwitterionic complexes were therefore abandoned.

A second approach to reveal the reason for the appearance of two dominating peaks after tryptamination was testing of the possible influence of the buffer compounds borate and cholic acid. Glyceraldehyde 3-phosphate was analysed in a phosphate-cholic acid buffer adjusted to pH 8 to investigate the influence of borate, and in a borate buffer without cholic acid adjusted to pH 9.7 to investigate the role of cholic acid. In both of these buffer systems glyceraldehyde 3-phosphate migrated still as two compounds with well separated peaks.

A more likely explanation could be found in the nature of the derivatisation procedure including reductive amination of the aldose phosphates possibly leading to initial Maillard reactions and thus formation of more than one reaction product. ^{1}H-NMR analysis using ribose 5-phosphate as model compound was conducted. Starting up with analysis of an aqueous solution of non-derivatised ribose 5-phosphate showed no sign of other compounds than ribose 5-phosphate in this solution. The next approach was investigation of derivatised ribose 5-phosphate to test the hypothesis that more than one product were formed during the derivatisation process. A D_2O solution of tryptaminated ribose 5-phosphate was, therefore, analysed by ^{1}H-NMR. The obtained spectra showed that the solution consisted of derivatised ribose 5-phosphate. The final approach was then separation of the two ribose 5-phosphate derivatives including removal of the excessive amount of tryptamine by liquid chromatography. The ^{1}H-NMR spectra of the thereby separated compounds obtained from derivatisation of ribose-5--phosphate revealed that both compounds represented tryptaminated monosaccharides. The proposed reaction scheme is shown in Figure 2.

The presence of a partly protonated phosphate group makes protonisation of nitrogen in the tryptaminated intermediate possible, and it gives thus basis for prototropic shift – the Amadori rearrangement. The introduced positively charge functions as an electron sink leading to the illustrated change in position of the double bond, including formation of an 1-aminoketose. The carbonyl group of this product could theoretically react with another tryptamine as shown in Figure 3; however, this is not the case for the dominating products as revealed from the ^{1}H-NMR spectra showing a 1:1 proportion of tryptamin and monosaccharide in the two isolated ribose 5-phosphate derivatives. The two dominating peaks in the electropherograms thus more likely reflect two diasteroisomers as a result from the following reductive step (Figure 2).

Among the possible products corresponding to the minor peaks in the electropherograms of derivatised phosphorylated aldoses may be a reduced Heyns compound (2-amino-2--deoxyaldose) [Bjergegaard *et al*., 2005]. A proposed reaction scheme is shown in Figure 3. The minor peaks were not investigated by ^{1}H-NMR.

The developed method was investigated with respect to robustness, detection limit and RRF. Individual dilutions were made for all phosphorylated carbohydrates to reveal

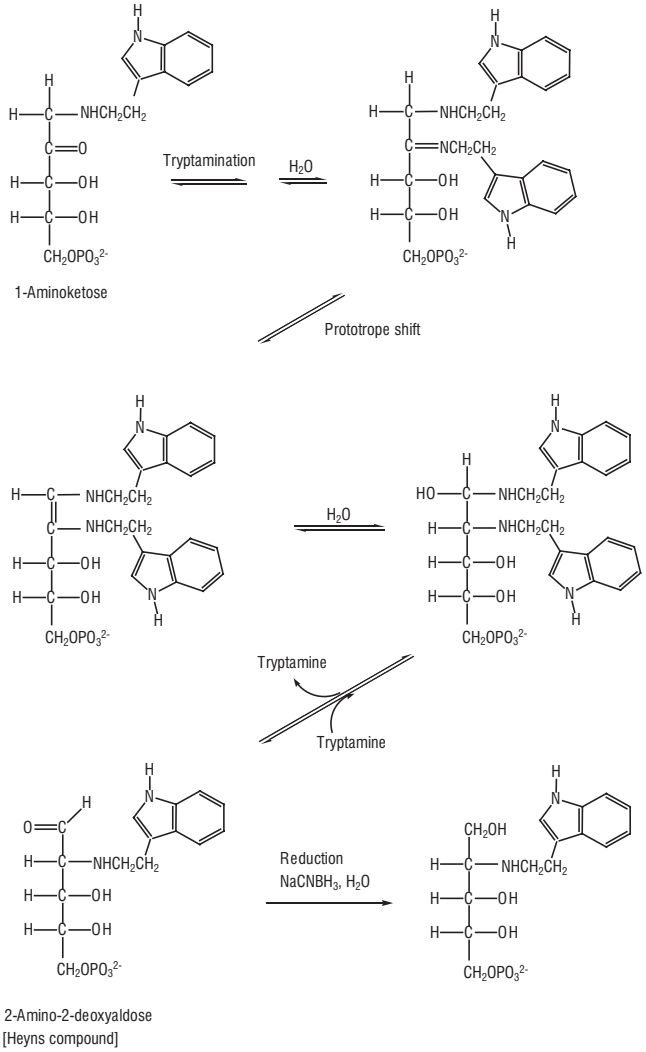

FIGURE 3. Proposed formation and nature of minor D-ribose 5-phosphate products succeeding tryptamination and reduction.

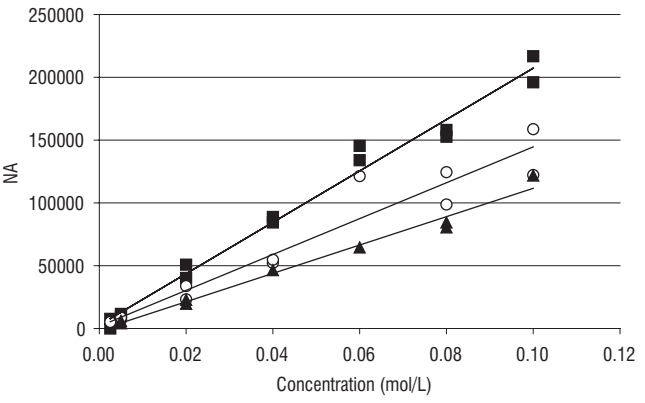

FIGURE 4. Linearity of D-glucose 6-phosphate (■), DL-glyceraldehyde 3-phosphate (○), and D-ribose 5-phosphate (▲), normalised area (NA) *vs*. concentration (mol/L). The correlation is for each compound based on two individual measurements in the concentration range from 0.0025 to 0.1 mol/L, and the run conditions were as described in Figure 1.

the concentration range, which resulted in linearity. Solutions with aldoses in concentrations from 0.0025 mol/L to 0.2 mol/L were analysed by HPCE showing acceptable linearity between concentration and normalised peak area in the tested concentration range (Figure 4).

The calculated RRF values are shown in Table 1 for D-glucose 6-phosphate, D-glyceraldehyde 3-phosphate, and D-ribose 5-phosphate. As seen, the RRF values are all higher than the RRF value found for D-thyminose meaning that the phosphorylated carbohydrates all have a lower molar extinction coefficient at 220 nm compared to that of D-thyminose. A possible explanation for this can be a higher degree of binding among the molecules, as *e.g.* seen in nucleic acids as a result of hydrogen binding. With the molecules now considered, it can be a result of salt binding in molecules both containing positively charged groups from the amine and negative charges from the phosphate group.

Detection limits found in this and other studies are listed in Table 2.

The limit of detection obtained in this study was higher than that found by Soga *et al*. [2002] and Buchholz *et al*. [2001] who used MS for detection. LOD for glucose 6-phosphate was thus 525 times more sensitive using the method by Soga *et al*. [2002] and 16 times more sensitive when using the method by Buchholz *et al.* [2001] (Table 2). Obviously, low LOD values are preferable; however, taking the simplicity of the optimised HPCE method using simple UV-detection into account, the LOD obtained in this study was acceptable. LOQ (signal to noise ratio of five) was found to be 204 μmol/L (D-thyminose), 360 μmol/L (D-glucose 6-phosphate), 430 μmol/L (D-ribose 5-phosphate), and 750 μmol/L (DL-glyceraldehyde 3-phosphate).

TABLE 1. Standard deviation (SD) of ten times repetition (calculated for NA) using the following concentrations; D-thyminose (0.06 mol/L), D-glucose 6-phosphate (0.0025 mol/L), DL-glyceraldehyde 3-phosphate (0.02 mol/L) and D-ribose 5-phosphate (0.01 mol/L), together with slope (α), linearity (R^2), and relative response factors (RRF) for D-thyminose, D-glucose 6-phosphate, D-ribose 5-phosphate, and DL-glyceraldehyde 3-phosphate determined in the concentration range from 0.0025 to 0.1 mol/L.

Compound	SD (%)	α	R^2	RRF
D-Thyminose	4.7	2575224	0.9909	1.00
D-Glucose 6-phosphate	2.2	2029468	0.9848	1.27
DL-Glyceraldehyde 3-phosphate	7.6	1420649	0.9290	1.81
D-Ribose 5-phosphate	9.9	1129457	0.9887	2.28

The results from the HPCE analysis of a mixture containing the three tryptaminated aldose phosphates are shown in Figure 5.

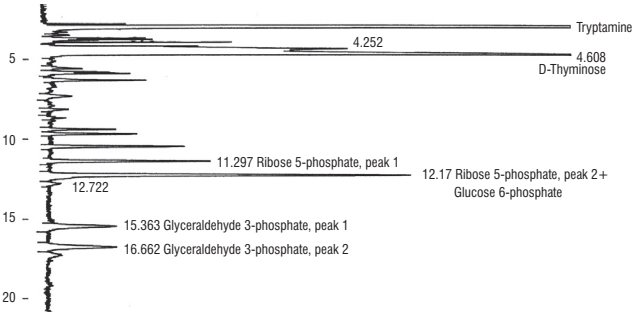

FIGURE 5. Electropherogram shown with migration times (min) of D-thyminose (internal standard), D-ribose 5-phosphate, D-glucose 6-phosphate, and DL-glyceraldehyde 3-phosphate, run conditions were as described in Figure 1.

As it can be seen from Figure 5, glucose 6-phosphate co-migrates with the second peak of ribose 5-phosphate, even though the MT obtained from analysis of the individual compounds could not predict this co-migration. This change of migration time is noteworthy; however, interaction of molecules in solutions is a well-known phenomenon that may lead to such effects.

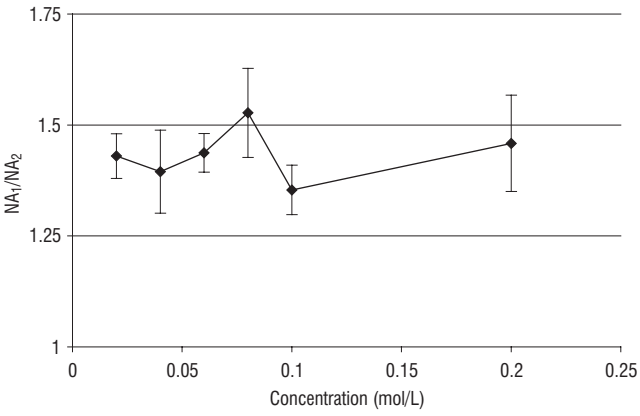

FIGURE 6. Relationship between the normalised areas of the two D-ribose 5-phosphate peaks.
($NA_{peak\ 1}$ /$NA_{peak\ 2}$) in the concentration range from 0.02 to 0.2 mol/L, each point is the average of three individual measurements, and the error bars represent standard deviation.

TABLE 2. Detection limits of glucose 6-phosphate, ribose 5-phosphate, and glyceraldehydes 3-phosphate analysed by capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS), Liquid chromatographic-electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS), and the now developed HPCE method.

Method	Definition of LOD	Aldose phosphate	LOD (μmol/L)	Reference
CE-ESI-MS	Signal to noise ratio of 3	Glucose 6-phosphate	0.4	Soga *et al*. [2002]
LC-ESI-MS-MS	Three times the standard error of the estimate for the regression of the linear regression plot	Glucose 6-phosphate Ribose 5-phosphate Glyceraldehyde 3-phosphate	13.2 8.8 19.6	Buchholz *et al*. [2001]
HPCE	Signal to noise ratio of 3	D-Thyminose D-Glucose 6-phosphate D-Ribose 5-phosphate DL-Glyceraldehyde 3-phosphate	122 210 260 450	This study

Reliable quantification of glucose 6-phosphate and ribose 5-phosphate in various matrix systems, *e.g.* meat, may be questionable due to the shown co-migration of the tryptaminated compounds. In order to unveil co-migration of peaks, the relationship between the two peaks of the tryptaminated ribose 5-phosphate was investigated further. Analyses of the pure compound showed that the normalised area of the first peak was consistently approx. 1.5 times higher than that of the second peak within the range from 0.02 to 0.2 mol/L (Figure 6). Possible co-migration can be discovered by changes in this ratio.

An example of an electropherogram from analysis of a DLY meat sample using the now developed HPCE method is shown in Figure 7.

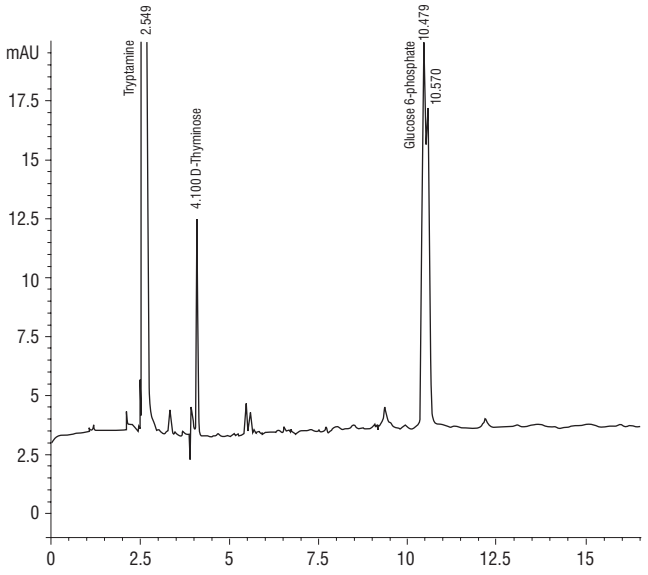

FIGURE 7. Electropherogram shown with migration time (min) of D-glucose 6-phosphate extracted from pork with run conditions as described in Figure 1. It is noteworthy, that the D-glucose 6-phosphate peak is split in two, as also seen for D-ribose 5-phosphate and for DL-glyceraldehyde 3-phosphate.

The aldose phosphates were isolated by group separation (ion chromatography) of the methanol extract obtained from the homogenised meat samples. It can be seen from Figure 7 that glucose 6-phosphate is the quantitatively dominating phosphorylated carbohydrate detected, accompanied by some yet unidentified compounds. The concentration of glucose 6-phosphate determined for the four breeds is shown in Table 3.

TABLE 3. Concentration of glucose 6-phosphate in the four pig breeds; Duroc and Landrace × Yorkshire (DLY), Hampshire and Landrace × Yorkshire (HLY), Hampshire, and Black spotted. The concentrations are the mean of 12 replicates.

Breed	Glucose 6-phosphate (μmol/g)
DLY	0.6 ± 0.2
DLY[a]	0.2 ± 0.1
HLY[a]	0.6 ± 0.1
Hampshire	0.4 ± 0.1
Black Spotted	0.4 ± 0.1

[a]: Meinert *et al*. [2007]

The concentration of glucose 6-phosphate varied in the five meat samples (Table 3). Müller [1994] determined the concentration of glucose 6-phosphate in three pure breeds and six corresponding cross-breeds using an enzyme assay and found concentrations from 0.85 to 2.32 μmol/g. Bee [2002] determined the concentration of glucose 6-phosphate in two muscles and two genders in the breed of Swiss Large Whites using a diagnostic kit and found concentrations from 5.1 to 13.1 μmol/g. The concentrations found in this study were lower than the concentrations reported in the literature. However, comparison of glucose 6-phosphate concentrations between studies is difficult, as natural variations caused by differences in *e.g.* breeds, muscles, and ageing must be taken into account along side with different methods of analysis.

The concentrations of ribose 5-phosphate and glyceraldehyde 3-phosphate were also studied in the pork samples, as these two aldoses are involved in metabolism pathways and their presence in meat thus could be expected. However, neither aldoses were detected in pork. Ribose 5-phosphate could likewise not be detected in beef [Koutsidis *et al*., 2003], and the presence of glyceraldehyde 3-phosphate in pork has to the best of our knowledge not previously been reported.

CONCLUSIONS

In conclusion, the HPCE method for the analysis of phosphorylated carbohydrates works acceptably. It is an easy and reliable method, though there is still room for improvement, and further work with identification of unknown compounds appearing in the electropherograms will thus add to the applicability of the method. The co-migration of ribose 5-phosphate and glucose 6-phosphate was, however, a minor problem in this study, as only glucose 6-phosphate was present in pork.

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