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Qualitative Profiling of Polyglucose Degradation Products in Peritoneal Dialysis Fluids

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Abstract

Heat sterilization of peritoneal dialysis (PD) fluids leads to partial degradation of the osmotic agent to form reactive carbonyl structures, which significantly reduce the biocompatibility of PD fluids and impair long-term PD therapy. Hence, it is important to know the exact composition of the degradation products to improve biocompatibility of PD fluids. Our study conducted targeted screening for degradation products in polyglucose (icodextrin)-containing PD fluids (pGDPs) by applying *o*-phenylenediamine (OPD) to form stable derivatives, which were analyzed by ultrahigh-performance liquid chromatography with hyphenated diode array tandem mass spectrometry (UHPLC–DAD–MS/MS). For the first time, specific degradation products of polyglucose, namely 4-deoxyglucosone (4-DG) and 3,4-dideoxypentosone (3,4-DDPS), could be identified in PD fluids. Further, a reaction product of 5-hydroxymethylfurfural (5-HMF) and OPD could be characterized to be (5-(1*H*-benzo[*d*]imidazol-2-yl)furan-2-yl)methanol. Additionally, 3-deoxyglucosone (3-DG) and 3-deoxygalactosone (3-DGal), both known to be present in glucose-based PD fluids, were also detected in polyglucose-containing fluids. Trapping a hitherto unknown degradation product with OPD yielded 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one, which was present in heat- as well as in filter-sterilized PD fluids.

Introduction

Peritoneal dialysis (PD) is a common alternative to hemodialysis for blood purification in case of renal insufficiency. A highly-osmotic PD fluid is administered into the abdominal cavity so that the peritoneum as semipermeable membrane allows excessive water and uremic waste to diffuse from the patient's blood into the PD fluid.[1] To enable ultrafiltration and ensure biocompatibility, PD fluids contain water, electrolytes like calcium or sodium, a buffer substance and, in most cases, glucose as osmotic agent. In some PD fluids glucose is replaced by other osmotic agents, such as icodextrin, a high-molecular, water-soluble glucose polymer derived from starch.[2] During production, PD fluids are heat-sterilized to assure microbiological safety. It is well-known that heat treatment leads to the formation of reactive glucose degradation products (GDPs).[3, 4] GDPs, however, reduce the biocompatibility of PD fluids and, thus, limit long-term therapy, for example by enhanced formation of advanced glycation endproducts (AGEs).[5-8] These modifications may impair protein biofunctionality and eventually lead to a decline of membrane permeability.[9-11] Furthermore, GDPs are cytotoxic against human peritoneal mesothelial cells as well as fibroblasts and may interfere with cell signaling.[9, 12-14] Therefore, minimizing the GDP content by technological measures, changes in the product composition or the use of additives is crucial to provide maximum biocompatibility,[15-17] an objective that requires detailed knowledge about GDP profiles in PD fluids.

GDP profiles of glucose-based PD fluids have already been extensively investigated. Thus, several GDPs with monocarbonyl structure, like 5-hydroxymethylfurfural (5-HMF), acetaldehyde, formaldehyde, and 2-furaldehyde have been identified and quantified in PD fluids.[6, 7, 18, 19] Additionally, the α-dicarbonyls glucosone, 3-deoxyglucosone (3-DG), 3-deoxygalactosone (3-DGal), 3,4-dideoxyglucosone-3-ene (3,4-DGE), glyoxal, and

methylglyoxal have been identified as the major GDPs and quantified in different commercial products .[12, 19-22] Thus, it was shown that glucose-based PD fluids contain GDPs in concentrations up to 450 µM.[23] In contrast, the GDP content of polyglucose-based solutions has hardly been investigated. Exemplarily, selected typical GDPs were analyzed in few samples of icodextrin-containing fluids.[19, 24] However, icodextrin is an oligomer, where glucose monomers are linked by 1,4-α glycosidic bonds. Compared to glucose, 1,4-α glycosidically linked di- and oligosaccharides undergo different degradation reactions.[25-28] It can be assumed that the degradation products formed in icodextrin-based PD fluids differ from those formed in glucose-based products. Consequently, the content of process contaminants may be underestimated when only GDPs are quantified.

Therefore, we conducted targeted screening for the most important polyglucose degradation products (pGDPs) in PD fluids with reactive carbonyl structures. Thus, it was possible to identify 5-HMF, 3-DG, 3-DGal, 4-deoxyglucosone (4-DG), and 3,4-dideoxypentosone (3,4-DDPS) in polyglucose-containing PD fluids. The latter two compounds have not been detected before in glucose-containing products. Additionally, trapping with OPD identified a raw material contaminant which forms 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one.

Experimental Section

Reagents and Samples. All chemicals and reagents were obtained from Acros (Geel, Belgium), AppliChem (Darmstadt, Germany), Fluka (Steinheim, Germany), or Sigma-Aldrich (Steinheim, Germany) and were at least of analytical grade. 3-DG (purity > 95%) was purchased from Chemos (Regenstauf, Germany) and 5-HMF from SAFC (St. Louis, MO, USA). Water for all

experiments was taken from a labwater system (Synergi-185, Millipore, Schwalbach, Germany). Typical commercial PD fluids with 7.5% icodextrin were examined. Unheated and sterile-filtered PD fluids were prepared containing the buffer and electrolytes of a commercial product with 7.5% icodextrin.

Semipreparative HPLC System, UHPLC–DAD–MS/MS System, HR-MS, and NMR Spectroscopy. Semipreparative high-performance liquid chromatography (HPLC) was carried out on a Jasco Plus system (Groß-Umstadt, Germany) equipped with type 2057 autosampler, two 2087 pumps, 980-50 degasser, and 2077 ultraviolet–visible (UV/Vis)-detector. The system was controlled by Chrompass 1.7 software. Separation of compounds was achieved on a Nucleodur C18ec column (250 x 10 mm Macherey-Nagel, Dueren, Germany), which was connected to a precolumn of the same material (8 x 10 mm, Macherey-Nagel). Fractions were collected by Advantec fraction collector CHF 122 SB.

The Dionex UltiMate 3000RS ultrahigh-performance liquid chromatography with hyphenated diode array tandem mass spectrometry (UHPLC–DAD–MS/MS) system (Thermo Fisher Scientific, Dreieich, Germany) consisted of a pump with degasser, autosampler, column compartment, and diode array detector, controlled by Chromeleon 6.80 software. Chromatographic separation was performed on an ACQUITY UPLC® BEH phenyl column (1.7 µm particle size; 2.1 x 100 mm, Waters, Eschborn, Germany), which was equipped with a VanGuard ACQUITY UPLC® BEH phenyl precolumn (1.7 µm particle size; 2.1 x 5 mm, Waters). For UHPLC–MS/MS analysis, an API 4000 QTrap mass spectrometer (AB Sciex, Foster City, CA, USA) fitted with an electrospray ionization (ESI) source (Applied Biosystems, Foster City, CA, USA) was connected to the UHPLC system. The ESI–MS was run in positive mode, operated at 700 °C with a voltage of +2500 V. Nitrogen was used for solvent evaporation

as well as for collision-induced dissociation. Product ion spectra were obtained at collision energy of 30 V and collision energy spread of 10 V in QTrap-enhanced mode. For data acquisition and processing, Analyst 1.5.1 software was used.

High-resolution (HR) ESI–MS spectra were obtained using a maXis™ time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). NMR spectra were recorded by an Avance 600 system (Bruker Biospin, Rheinstetten, Germany). Signals were assigned based on $^{1}H,^{1}H$-correlated spectroscopy (COSY), hetero nuclear single quantum coherence (HSQC), and hetero nuclear multiple bond correlation (HMBC). For analysis of the raw material contaminant, additional hydrogen–deuterium (H/D) exchange experiments were performed.

Synthesis of the Reference Compounds

3-DGal was prepared as previously reported.[29]

4-DG was synthesized according to Broberg et al. with some minor changes.[30] Briefly, maltose (60 mM) was dissolved in degassed aqueous potassium hydroxide (50 mM). The reaction mixture was kept light-protected under nitrogen and was stirred constantly at room temperature (RT) for 24 h. Afterward the solution was neutralized by adding Dowex 50W (H^+) ion exchanger, followed by filtration and evaporation of the solvent to yield a brown raw product. 4-DG was then isolated by column chromatography. For this purpose, methanol (100 mL) and silica gel (5 g) were added to the raw product. Subsequently, the solvent was removed under reduced pressure. The resulting dry powder was loaded onto a column (5 x 17 cm, silica gel). 4-DG was eluted with chloroform/methanol 17:3, controlled by thin-layer chromatography (TLC) (R_f 0.36, visualization with thymol/ethanol/sulfuric acid). When TLC indicated the presence of 4-DG, the respective fractions were concentrated under reduced pressure and lyophilized to yield

39 mg of 4-DG.

The product was characterized by NMR (atom numbering refers to **Scheme 1A**). NMR spectra showed two intramolecular hemiacetals of 4-DG, both formed by ring closure: form 1 ^{1}H-NMR (DMSO-d_6): δ 2.28 (1H, H-4a); 2.52 (1H, H-4b); 3.35 (1H H-1a); 3.43 (1H H-1b); 3.63 (1H H-6a); 3.65 (1H H-6b); 4.38 (1H H-5). ^{13}C-NMR (DMSO- d_6): δ 37.8 (C-4); 63.2 (C-1); 64.5 (C-6); 74.8 (C-5); 99.4 (C-2); 212.4 (C-3), form 2 ^{1}H-NMR (DMSO-d_6): δ 2.41 (1H H-4a); 2.51 (1H H-4b); 3.36 (1H H-1a); 3.48 (1H H-1b); 3.51 (1H H-6a); 3.53 (1H H-6b); 4.41 (1H H-5), ^{13}C-NMR (DMSO- d_6): δ 37.7 (C-4); 62.9 (C-6); 63.9 (C-1); 74.1 (C-5); 99.8 (C-2); 213.4 (C-3).

(*S*)-3-(3-(Hydroxymethyl)quinoxalin-2-yl)propane-1,2-diol (4-DG$_{qx}$). For the preparation of the quinoxaline derivative of 4-DG, maltose was treated as described above. After neutralization with Dowex 50W and subsequent filtration, the reaction mixture was concentrated under reduced pressure to about 100 mL. Then 20 mL of OPD (0.2% in water) was added to convert 4-DG into 4-DG$_{qx}$. The reaction mixture was stirred at RT for about 20 h prior to isolation of 4-DG$_{qx}$ by semipreparative HPLC with the following parameters: eluent A formic acid (0.1 %), eluent B methanol; flow rate 2.0 mL/min; (time (min)/%B) 0/10, 15/90, 20/90, 20.1/10, 25/10. Aliquots of 400 µL of the reaction mixture were injected and chromatograms were recorded at 316 nm. Fractions 17.1–17.8 min were collected, combined, concentrated under reduced pressure, and lyophilized yielding 50 mg of 4-DG$_{qx}$.

The product was characterized by NMR (atom numbering refers to **Scheme 1A**) and by UHPLC–DAD–MS/MS. A representative product ion spectrum is shown in **Figure 1B**. MS/MS: 235.0 Da $[M+H]^+$, 171.1 Da, and 217.0 Da; ^{1}H-NMR (DMSO-d_6): δ 3.11 (dd, J = 14.2, J = 5.4 1H, H-4a); 3.18 (dd, J = 14.2 J = 4.3 1H, H-4b); 3.45 (m, 2H, H-6); 4.07 (m, 1H, H-5); 4.69 (t, J = 5.7 1H, OH-6); 4.78 (d, J = 5.0 1H, OH-5); 4.84 (dd, J = 13.4 J = 5.2 2H H-1a); 4.92 (dd, J =

13.4 J = 6.1 2H H-1b); 5.40 (t, J = 5.7 1H, OH-1); 7.78 (m, 2H, Ar-H); 8.03 (m, 2H, Ar-H), ^{13}C-NMR (DMSO-d_6): δ 38.3 (C-4); 63.3 (C-1); 65.9 (C-6); 71.6 (C-5); 128.1; 128.3; 129.1; 129.4; 139.5; 140.9; 155.3; 155.7 (Ar-C), UV absorption maxima: 199 nm, 238 nm, 316 nm. Because NMR spectra of 4-DG$_{qx}$ have not been published before, ^{1}H-NMR- and ^{13}C-NMR spectra are shown in the **Supporting Information, Figures S-1** and **S-2**.

(5-(1*H*-Benzo[*d*]imidazol-2-yl)furan-2-yl)methanol (5-HMF$_{bfm}$). To prepare this benzimidazole derivative of 5-HMF, 45 mL of a solution containing 8 mM 5-HMF was mixed with 5 mL of OPD solution (2% in water). The reaction mixture was stirred in the dark at RT overnight and then subjected to semipreparative HPLC using the following gradient: eluent A formic acid (0.075%), eluent B methanol; flow rate: 2.0 mL/min; (time (min)/%B) 0/30, 12/50, 12.1/70, 15/70, 15.1/30, 20/30. Aliquots of 500 µL were injected, chromatograms were recorded at 316 nm and fractions 12.9–14.1 min were collected and combined. The organic solvent was evaporated and the product was lyophilized. The product was analyzed by NMR (atom numbering refers to **Scheme 1B**), HR-ESI–MS, and UHPLC–DAD–MS/MS. **Figure 1D** shows a representative product ion spectrum. MS/MS: 169.0 Da, 215.0 Da $[M+H]^+$, 197.0 Da; ^{1}H-NMR (DMSO-d_6):): δ 4.54 (s, 2H, H-6); 6.50 (d, J = 3.2 1H, H-4); 7.10 (d, J = 3.3 1H, H-3); 7.12 (m, 2H, Ar-H); 7.54 (m, 2H, Ar-H); 12.90 (s, 1H, N-H). ^{13}C-NMR (DMSO-d_6): δ 55.8 (C-6); 109.3; 110.1 (C-3, C-4); 122.1; 143.7; 144.7; 157.1 (Ar-C). HR-ESI–MS: 215.0817 Da $[M+H]^+$ measured, 215.0815 Da $[M+H]^+$ calculated. UV absorption maxima: 194 nm, 256 nm, 318 nm. Because NMR spectra of 5-HMF$_{bfm}$ have not been published before, ^{1}H-NMR and ^{13}C-NMR spectra are shown in the **Supporting Information, Figures S-3** and **S-4**.

3-(Quinoxalin-2'-yl)propane-1-diol (3,4-DDPS$_{qx}$). The quinoxaline derivative of 3,4-DDPS was synthesized according to Mavric and Henle with some modifications.[26] Maltose solution (50

mL, 0.2 M) in phosphate buffer (0.5 M; pH 7.0) was mixed with 25 mL of an OPD solution (0.2% in identical phosphate buffer). The reaction mixture was refluxed for 2 h. After cooling to RT and membrane filtration, the sample was purified by semipreparative HPLC as follows: eluent A formic acid (0.1%), eluent B methanol; flow rate 2.0 mL/min; (time (min)/%B) 0/48, 20/80, 25/80, 25.1/48, 30/48. Injection volume was set to 500 µL and chromatograms were recorded at 316 nm. Fractions 15.8–16.8 min were collected and combined, the organic solvent was evaporated and the residue was freeze-dried to yield 3.3 mg of 3,4-$DDPS_{qx}$.

The product was characterized by NMR (atom numbering refers to **Scheme 1C**), HR-ESI–MS, and UHPLC–DAD–MS/MS. **Figure 1F** depicts a representative product ion spectrum. MS/MS: 171.1 Da, 143.1 Da, 189.1 Da $[M+H]^+$. ^{1}H-NMR (DMSO-d_6): δ 1.96 (m, 2H, H-4); 3.05 (t, J = 8.0 2H, H-5); 3.51 (t, J = 6.3 2H, H-3); 4.55 (s, 1H, OH); 7.81 (m, 2H, Ar-H); 8.04 (m, 2H, Ar-H); 8.88 (s, 1H, H-1). ^{13}C-NMR (DMSO-d_6): δ 31.7 (C-4); 32.1 (C-3); 60.1 (C-5); 128.5; 128.8; 129.0; 130.0; 140.5; 141.4 (Ar-C); 146.5 (C-1); 157.7 (C-2).). HR-ESI–MS: 189.0988 Da $[M+H]^+$ measured, 189.1022 Da $[M+H]^+$ calculated. UV absorption maxima: 195 nm, 237 nm, 317 nm.

3-Methylquinoxalin-2-ol was synthesized by the reaction of OPD with pyruvic acid. OPD (452 mg) was dissolved in 20.0 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (1 M pH 7) and 145 µL of pyruvic acid was added. This reaction mixture was stirred in the dark at RT for 14 h. Then the reaction mixture was fractionated by semipreparative HPLC with the following parameters: eluent A formic acid (0.1%), eluent B methanol; flow rate 2.0 mL/min; (time (min)/%B) 0/10, 20/90, 25/90, 25.1/10, 30/10. Detection was carried out at 316 nm and fractions 22.0–23.0 min were collected. The organic solvent was removed under reduced pressure and the product was freeze-dried. The isolated compound was characterized by

UHPLC–DAD–MS/MS and by NMR (atom numbering refers to **Scheme 1D**): MS/MS: 161.0 Da $[M+H]^+$, 133.0 Da, 92.0 Da; ^{1}H-NMR (DMSO- d_6): δ 2.40 (s, 3H, H-3); 7.26 (m, 2H, Ar-H); 7.46 (m, 1H, Ar-H); 7.69 (m, 1H, Ar-H), 12.29 (s, 1H, OH), ^{13}C-NMR (DMSO- d_6): δ 20.4 (C-3); 115.1; 122.9; 127.8; 129.1; 131.6; 131.8; 154.8; 159.1 (Ar-C). UV absorption maxima: 198 nm, 227 nm, 339 nm.

1-(1*H*-Benzo[*d*]imidazol-2-yl)ethane-1,2-diol (1-BIME) was synthesized according to Voigt et al.[31] Briefly, 189 mg of glyceraldehyde was dissolved in 20.0 mL of phosphate buffer (0.1 M pH 7.4) containing 208 mM OPD. After 16 h in the dark at RT, 20 mL of water was added. The reaction mixture was extracted three times with 50 mL of ethyl acetate. The organic phases were combined and dried over sodium sulfate. The remaining solvent was evaporated to dryness and the residue purified using a LiChroprep RP-18 column (20 cm x 3 cm, Merck-Millipore, Schwalbach, Germany). Elution was performed with water/methanol (90:10). Fractions containing the target substance were combined and further purified by semipreparative HPLC using the same parameters as for 3-methylquinoxalin-2-ol (see above). Injection volume was set to 500 µL, chromatograms were recorded at 272 nm and fractions 10.1–11.1 min were collected. The organic solvent was evaporated and the product was freeze-dried. The product was analyzed by NMR (atom numbering refers to **Scheme 1E**) and UHPLC–MS/MS: ^{1}H-NMR (DMSO-d_6): δ 3.66 (dd, J = 11.0 J = 6.4 1H, H-3a); 3.82 (dd, J = 11.1 J = 4.2 1H, H-3b); 4.78 (t, J = 5.6 1H, H-2); 7.12 (m, 2H, Ar-H); 7.50 (m, 2H, Ar-H), ^{13}C-NMR (DMSO-d_6): δ 65.2 (C-2); 69.3 (C-3); 114.8; 121.0; 138.5; 155.8 (Ar-C). UV absorption maxima: 199 nm, 213 nm, 270 nm.

Isolation of the Raw Material Contamination 1,4-Bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one. Icodextrin (15.0 g) was dissolved in 100.0 mL of an aqueous solution containing 5.38 mg/mL sodium chloride, 4.5 mg/mL sodium lactate, 0.36 mg/mL calcium

chloride dihydrate, and 0.12 mg/mL magnesium chloride hexahydrate. Twenty-seven microliters of a 2% HEPES-buffered (1 M, pH 7) OPD solution was added to 75.0 mL of this icodextrin-containing solution. The reaction mixture was stirred for 3 h in the dark. Afterward, the solution was divided into three aliquots, which were separately applied to a Strata-X solid phase extraction (SPE) column (Phenomenex, Aschaffenburg, Germany, 1 g/20 mL). Each column was washed with 70 mL of methanol/water (5:95) and 70 mL of methanol/water (50:50). The product was eluted with 100 mL methanol/water (50:50). The solvents were evaporated to dryness and the residue was dissolved in 20 mL of water. Then the mixture was fractionated by semipreparative HPLC as follows: eluent A formic acid (0.1%), eluent B methanol; flow rate 2.0 mL/min; (time (min)/%B) 0/10, 20/60, 20.1/90, 25/90, 25.1/10 30/10. Injection volume was set to 500 µL, chromatograms were recorded at 316 nm and fractions 15.1–16.1 min were collected. The organic solvent was removed under reduced pressure and the product was freeze-dried. The product was analyzed by NMR (signal assignment based on H/D exchange, COSY, HSQC, and HMBC, atom numbering refers to **Scheme 1F**), HR-ESI–MS, and UHPLC–DAD–MS/MS. A representative product ion spectrum is shown in **Figure 2**: 337.0 Da $[M+H]^+$, 318.9 Da, 301.0 Da. ^{1}H-NMR (DMSO-d6): δ 3.28 (dd, *J* = 16.0 *J* = 3.9 1H, H-2a); 3.50 (dd, *J* = 16.0 *J* = 9.1 1H, H-2b); 4.60 (m, 1H, H-3); 4.83 (t, *J* = 5.0 1H, H-4); 5.10 (d, *J* = 6.2 1H, OH-4); 5.94 (d, *J* = 5.6 1H, OH-3); 7.10-7.81 (m, 8H, Ar-H) 12.26 (s, 1H, NH); 13.27 (s, 1H, NH), UV absorption maxima: 198 nm, 238 nm, 277 nm, 311 nm.

Separation of the Derivatives of 5-HMF, 3-DG, 3-DGal, and 4-DG was achieved by UHPLC–DAD–MS/MS applying the following parameters: eluent A 1% methanol and formic acid (0.1%), eluent B methanol; flow rate: 0.4 mL/min; (time (min)/%B) –5.0/0, 10.0/0, 30.0/80, 30.1/95, 35.0/95; column temperature was set to 55°C. Aliquots of 10 µL were injected. Data

acquisition started at 0.0 min and chromatograms were monitored at 316 nm. DAD spectra were recorded from 190 nm to 490 nm.

Separation of 3,4-DDPS$_{qx}$ and 1,4-Bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxylbutan-1-one was achieved chromatographically by a different UHPLC method: eluent A acetic acid (0.075%), eluent B methanol; flow rate: 0.3 mL/min; (time (min)/%B) –5.0/5, 15.0/20, 25.0/50, 25.1/80, 30.0/80; column temperature: 55°C; injection volume: 10 µL. Data acquisition started at 0.0 min. Chromatograms were obtained at 316 nm, DAD spectra from 190 to 490 nm.

Qualitative Profiling of PD Samples. Twenty microliters of a 1:1 mixture of OPD (4% in HEPES buffer, 1 M, pH7) and diacetylquinoxaline (internal standard, 25 µg/mL in water) was added to 80 µL of each PD sample. The solutions were mixed thoroughly, filtered through a PVDF membrane (0.22 µm, Roth, Karlsruhe, Germany) and rested light-protected for at least 9 h before UHPLC–DAD–MS/MS analysis. Full DAD spectra were recorded to differentiate between quinoxaline and non-quinoxaline derivatives, followed by full mass scans and MS/MS experiments.

As shown in previous studies, the applied derivatization conditions effectively suppressed de novo formation of various α-dicarbonyl compounds.[21, 23]

Results and Discussion

Screening for Reactive Icodextrin Degradation Products. The present study intended to investigate reactive degradation products of the polyglucose icodextrin (pGDPs) in PD fluids. Because degradation of polyglucose under sterilization conditions was expected to form novel, as yet unidentified products, we opted for a targeted screening for degradation products. To this

end, OPD was used as derivatizing reagent prior to UHPLC–DAD–MS/MS analysis. In contrast to the parent compounds, the derivatization products are stable, UV-active and easily ionized by ESI. Particularly α-dicarbonyl structures are often trapped by OPD to form quinoxaline derivatives (**Schemes 1A and 1C**), which can be analyzed by reversed-phase UHPLC. Because the concentrations of carbohydrate degradation products in PD fluids are higher than in biological samples, no derivatization agent leading to fluorescent products was necessary.[32] After derivatization with OPD, UHPLC analysis was performed following a method of Mittelmaier et al.,[23] which had been developed to separate GDPs in glucose-based PD fluids. In the present study, however, the coelution of several pGDPs was observed indicating that polyglucose-based PD fluids contain additional and/or different degradation products compared to glucose-based products. For identification, UHPLC separation of all pGDPs was attempted. Because the baseline separation of all peaks could not be achieved in one single run, two complementary UHPLC methods were applied. The first method was optimized to separate the peaks in a retention time window of 8.5–13.5 min, leading to a separation of four pGDPs at 9.8, 11.2, 12.2, and 12.8 min (**Figure 3**). The co-elution of two peaks at a retention time of 17.5 min (**Figure 3A,** signal marked with an asterisk), however, could not be avoided. In order to separate both pGDPs, a second UHPLC method was applied using acetic acid instead of formic acid as eluent buffer and adjusting the gradient (**Figure 3B**). Because the concentration of one of the compounds (later identified as 3,4-DDPS$_{qx}$) was below the detection limit of the UHPLC-DAD method, analysis was performed by UHPLC–MS/MS.

Identification of 3-DG, 3-DGal, 4-DG, 5-HMF, and 3,4-DDPS. At a retention time of 11.2 min (**Figure 3A**) a signal with the characteristic UV/Vis spectrum of a quinoxaline derivative was detected. By comparison of retention times, UV/Vis spectra, mass spectra (*m/z* 235), and

product ion spectra of the analyte and the reference compound, the signal could be assigned to 3-DG_{qx}, which is already known to be present in glucose as well as in icodextrin-containing PD fluids.[17, 19, 22, 23]

The peak at 12.2 min (**Figure 3A**) also showed a specific quinoxaline UV/Vis spectrum with maxima at 198 nm, 237 nm, and 317 nm. UHPLC–MS/MS analysis revealed identical mass and product ion spectra as 3-DG_{qx} indicating that this signal could be caused by 3-$DGal_{qx}$. 3-DGal is a diastereomer of 3-DG, which is already known to be present in glucose-containing PD fluids.[22] By comparison of its chromatographic and spectroscopic behavior with the independently synthesized reference compound after derivatization with OPD, the structure could be unequivocally assigned to the quinoxaline derivative of 3-DGal. To date, 3-DGal had not been detected in icodextrin-based PD fluids and was consequently identified as a novel pGDP.

Another compound, which could not been assigned to any known GDP, eluted at 12.8 min (**Figure 3A**). This structure also showed a characteristic UV spectrum for quinoxalines (**Figure S-5A**) indicating an α-dicarbonyl structure as parent compound. UHPLC–MS/MS analysis revealed an apparent molecular weight of *m/z* 235, equal to 3-DG_{qx} and 3-$DGal_{qx}$. Therefore, it was hypothesized that the mass of 235 Da $[M+H]^+$ indicated the presence of 4-DG_{qx} (**Scheme 1A**), a diastereomer of 3-DG_{qx} and 3-$DGal_{qx}$. To verify the identity of the unknown GDP, 4-DG was synthesized as a reference compound.[30] Complete consistency of retention times, UV/Vis-, mass-, and product ion spectra (**Figures 1A/B**) proved the identity of the compound. 4-DG had not been detected in PD fluids so far and could be identified in the present study as a novel pGDP. Because 4-DG has not been reported for glucose-containing PD fluids yet, it can be assumed that the compound is specific for icodextrin-based products. 4-DG has been detected before in reaction mixtures of maltose and lysine or as a degradation product of cellobiose or

maltose in alkaline solution.[33-35]

The UV/Vis spectrum of the signal at a retention time of 9.8 min did not indicate the presence of a quinoxaline derivative (**Figure S-5B**). Consequently, the parent compound of this structure is probably not an α-dicarbonyl, but must possess another entity that reacts with OPD. To obtain more information on the structure, HR-MS experiments were performed, resulting in an apparent molecular mass of *m/z* 215.0817, which is consistent with the elemental formula of $C_{12}H_{10}N_2O_2$. Based on this knowledge, it was hypothesized that the compound was a reaction product of 5-HMF with OPD. In order to test this hypothesis, 5-HMF was reacted with OPD, which led to a derivative showing the same retention time, UV/Vis spectrum and product ion spectrum as the unknown compound when analyzed by UHPLC–DAD–MS/MS (**Figures 1C/D**). For detailed characterization of the reaction product of 5-HMF with OPD, the substance was isolated by semipreparative HPLC. NMR experiments revealed the presence of the benzimidazolyl derivative of 5-HMF (5-HMF_{bfm}, **Scheme 1B**). Although 5-HMF is a well-known GDP in PD fluids,[19] 5-HMF_{bfm} has not been covered yet by targeted screening of sugar degradation products in PD fluids using OPD.[23] Instead it has been generally analyzed separately by HPLC–UV without derivatization.[36] A possible derivatization mechanism is displayed in **Scheme S-1**.

Further, a second chromatographic method was developed to analyze two unknown compounds that co-eluted under the applied conditions (marked by an asterisk in **Figure 3A**). Under the optimized separation conditions, baseline separation of both signals could be achieved. The compound eluting at 15.1 min (**Figure 3B)** showed the UV/Vis spectrum of a quinoxaline structure (**Figure S-5C**) indicating that the parent compound was an α-dicarbonyl. HR-MS experiments revealed an apparent exact mass of *m/z* 189.0988, which is in accordance with the elemental formula of $C_{11}H_{12}N_2O$. Therefore, 3,4-DDPS (**Scheme 1C**), which has been reported

by Mavric and Henle as a degradation product of 1,4-linked disaccharides,[26] was suggested as putative structure. To confirm this hypothesis, 3,4-DDPS$_{qx}$ was synthesized and characterized by UHPLC–DAD–MS/MS. The reference compound 3,4-DDPS$_{qx}$ and the analyte had identical retention times, UV/Vis-, mass-, and product ion spectra (**Figures 1E/F**). Thus, the unknown pGDP could be identified as 3,4-DDPS, which was detected for the first time in PD fluids.

To evaluate if the products were indeed formed during thermal processing, unheated, sterile-filtered icodextrin-containing PD fluids were analyzed in the same way. In the unheated controls, 3-DG, 3-DGal, 4-DG, 5-HMF and 3,4-DDPS were below the detection limit indicating that the compounds were neither present in the raw materials nor formed from the raw materials during sample workup. Further derivatization experiments were performed for different periods of time between 9 and 24 h. The signals of the five analytes remained stable during prolonged derivatization, suggesting that artificial formation of those products during derivatization was very unlikely.

Identification of 1,4-Bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one. Another unknown product eluted at the retention time of 14.4 min (**Figure 3B**). The respective UV/Vis spectrum had four maxima at 198 nm, 238 nm, 277 nm, and 311 nm (**Figure S-5D**). Consequently, the precursor could not be assigned to an α-dicarbonyl, but rather contained other functional groups reacting with OPD. To investigate the source of this product, unheated, sterile-filtered icodextrin-containing PD fluids were analyzed, where the compound was detected in similar concentrations (data not shown). Its formation could not be forced by additional heating of icodextrin, suggesting that it was rather a contamination of raw material formed during

icodextrin production than a thermal degradation product. HR-MS analysis revealed a doubly charged ion with *m/z* 169.0697 $[M+2H]^{2+}$ indicating the elemental formula $C_{18}H_{16}N_4O_3$. The four nitrogen atoms could be inserted by the reaction of 2 moles of OPD with 1 mole of the degradation product leading to a parent compound with 6 carbon atoms equivalent to the glucose backbone. Product ion spectra clearly showed a mass difference of 18 Da and of 36 Da compared to the precursor ion indicating a loss of 2 moles of water (**Figure 2**). Therefore, the unknown structure must have at least two aliphatic hydroxyl groups, which eliminates fragmentation induced during dissociation.

For structure identification, the raw material contaminant was isolated from the polyglucose matrix after derivatization with OPD by semipreparative HPLC. ^{1}H-NMR as well as COSY spectra revealed two separated aromatic rings with four protons each. Additionally, the signals of one of these aromatic rings were split up into four single signals at chemical shifts of 7.30 ppm, 7.37 ppm, 7.54 ppm, and 7.81 ppm, indicating that a substituent exerts a negative mesomeric effect on the aromatic system. Furthermore, a carbonyl group was indicated by coupling of protons of a CH_2-group to a carbon atom with a chemical shift of 192.4 ppm in the HMBC spectra. Based on ^{1}H, COSY, HMBC, and HSQC spectra as well as H/D exchange, the contamination of the raw material was identified to be 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one (**Scheme 1F**), which could be derived from 2 moles of OPD and 1 mole of a contaminant with two reactive functional groups.

Because benzimidazole derivatives may arise from the reaction of OPD with aldehydes or carboxylic acids, respectively, the parent compound was investigated in more detail. Voigt and Glomb described the formation of 1-BIME from OPD and glyceraldehyde, which corresponds to the green part of the molecule in **Scheme 1E**.[31] To confirm the presence of this partial structure

in the contamination product, OPD was reacted with glyceraldeyde, the reaction product was isolated and NMR spectra (^{1}H, COSY, HMBC, and HSQC) were recorded. The aromatic protons of 1-BIME induced signals at 7.12 ppm and 7.50 ppm, which could also be observed for 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one. The signal for H-2 at 4.78 ppm was also comparable to the corresponding signal of the isolated raw material contamination. Thus, 1-BIME induced the same signals as the green part of 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one (**Scheme 1E**), proving the presence of a glyceraldehyde moiety in the raw material contamination.

It is unlikely that the second part of 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one (the orange section of the molecule in **Scheme 1F**) was formed by the reaction of OPD and an aldehyde group as part of an α-dicarbonyl function. In this case, derivatization with OPD would rather lead to a quinoxaline instead of the observed benzimidazole derivative.[23, 29, 37-40] In order to confirm the structure of the orange part of 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one, the reference 1-(1*H*-benzo[*d*]imidazol-2-yl)ethan-1-one was analyzed by NMR. This compound showed very characteristic signals for the aromatic protons. Due to the mesomeric effect of the carbonyl group, the signals of the four aromatic protons appear at 7.31 ppm, 7.38 ppm, 7.56 ppm, and 7.82 ppm. Comparison of NMR spectra (^{1}H-NMR, COSY, HMBC, and HSQC) of the reference and the isolated compound added up to equal signals for these protons. Chemical shifts as well as multiplicity were the same for the protons bound to the carbon atoms numbered with 3'–6'. Additionally, coupling of protons bound to C-2 and the carbon atom 1 were observed in HMBC spectra for both molecules (**Scheme 1F**). Thus, these atoms must be arranged in the same way and, therefore, the partial structure of the contaminant must be 1-(1*H*-benzo[*d*]imidazol-2-yl)ethan-1-one.

A possible precursor of 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one could contain a carboxylic group at C-1' (**Scheme 1F**). To test this hypothesis, OPD was reacted with pyruvic acid followed by isolation and characterization of the reaction product. The NMR spectra clearly revealed 3-methylquinoxalin-2-ol as the product of this reaction (**Scheme 1D**), which was confirmed by NMR analysis of commercial 3-methylquinoxalin-2-ol. Both substances resulted in identical NMR spectra, which, in contrast, were not included in the spectra of 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one. Therefore, a partial quinoxalinol structure of the raw material contamination could be excluded. Consequently, it is unlikely that C-1' (**Scheme 1F**) is derived from a carboxylic group. Because it could be excluded that the second benzoimidazole derived from an α-keto acid or an α-dicarbonyl, further studies are required to elucidate the full structure of the parent compound of 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one. A possible explanation could be, for example, that the carbonyl group is released from a precursor structure after/during the derivatization step.

The compounds 4-DG, 3-DGal, and 3,4-DDPS have not been analyzed in icodextrin-containing products before. 4-DG and 3,4-DDPS seem to be typical degradation products of icodextrin, because they have not been detected so far in glucose-containing PD fluids. Mavric and Henle reported 3,4-DDPS as a degradation product of lactose.[26] Recently, Smuda and Glomb described 3,4-DDPS in Maillard model reaction mixtures of maltose and lysine.[33] The observation that 3,4-DDPS has been detected so far only as degradation product of lactose, maltose, and icodextrin indicates that a 1,4-glycosidic link is required for its formation and that it is, therefore, not present in glucose-based PD fluids. Smuda and Glomb suggested a pathway of formation via 3-deoxypentosone, whereas Mavric and Henle proposed formation via 4-DG.[26] Since no 3-deoxypentosone, but 4-DG was detected in the present study, the reaction mechanism described

by Mavric and Henle seems more likely to occur during sterilization of icodextrin-based PD fluids. 3,4-DDPS formation via 3-deoxypentosone, however, cannot be excluded, because the concentration levels of 3,4-DDPS were very low and, thus, many intermediates like 3-deoxypentosone could be present in quantities below the limit of detection.

Conclusion

The present study identified 4-DG, 3-DGal, and 3,4-DDPS as novel degradation products in polyglucose-containing PD fluids. Additionally, a hitherto unknown raw material contaminant, which is converted to 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one during derivatization, has been detected in the samples indicating that contamination of the icodextrin raw material may be an important issue. Furthermore, 3-DG and 5-HMF were analyzed in icodextrin-based PD fluids and it was shown that 5-HMF can by covered in parallel with the α-dicarbonyl degradation products after derivatization with OPD yielding 5-HMF_{bfm}.

Knowledge on the structures of the predominant reactive carbonyl compounds in polyglucose-based PD fluids is a prerequisite for structure-specific risk assessment as previously carried out for GDPs.[9] Furthermore, measures to reduce the load of process contaminants can only be developed if the nature and consequently the formation mechanisms of those contaminants are known. In the past, for example, the content of glucose-derived degradation products could be effectively reduced by changing the pH value during sterilization,[17, 41] by alternative heating methods [42] or by removal of GDPs after production using affinity absorption.[43]

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Supporting Information Available including NMR spectra of 4-DG_{qx} and 5-HMF_{bfm}, UV/Vis spectra of 4-DG_{qx}, 5-HMF_{bfm}, 3,4-$DPPS_{qx}$, and 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one, and a scheme with the proposed formation of 5-HMF_{bfm}. This material is available free of charge via the Internet at http://pubs.acs.org.

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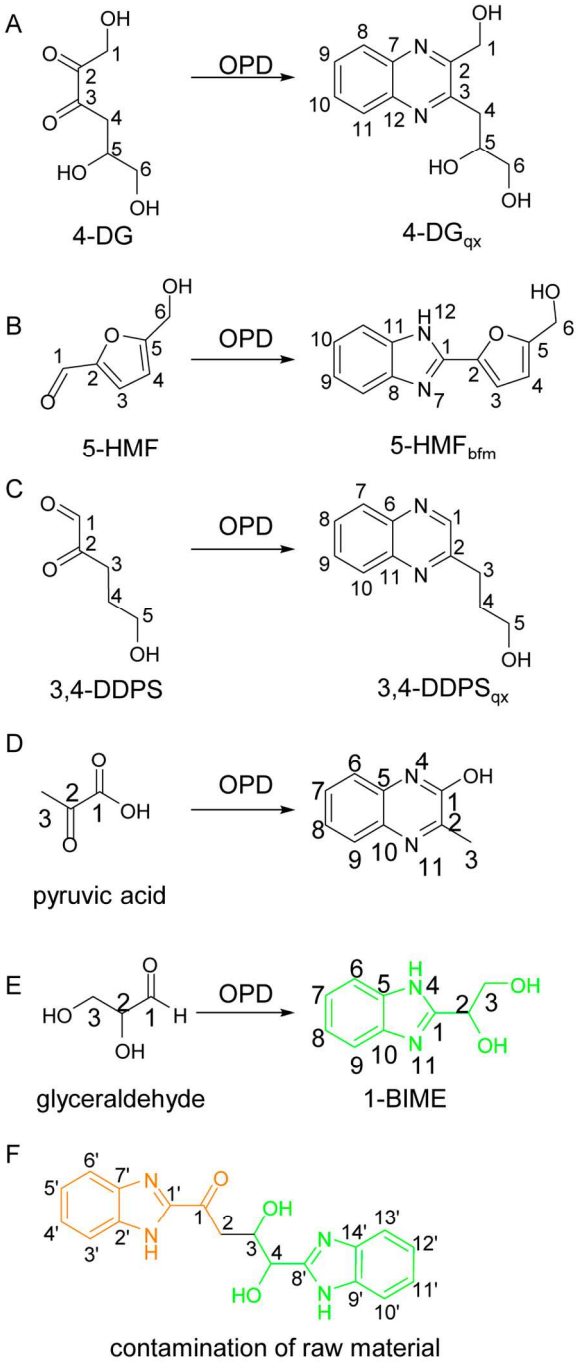
Figure Captions

Scheme 1. Structures of the degradation products **(A)** 4-DG, **(B)** 5-HMF, **(C)** 3,4-DDPS and their corresponding OPD derivatives; formation of **(D)** 3-methylquinoxalin-2-ol and **(E)** 1-BIME by the reaction of OPD with pyruvic acid and glyceraldehyde, respectively; **(F)** structure of the isolated raw material contamination 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one after derivatization with OPD.

Figure 1. Product ion spectra of compounds after conversion by OPD in PD fluids (a, c, e) and the respective synthesized references (b, d, f): **(A/B)** 4-DG_{qx}; **(C/D)** 5-HMF_{bfm} and **(E/F)** 3,4-$DDPS_{qx}$.

Figure 2. Product ion spectrum of the raw material contamination in icodextrin-based PD solutions after conversion to 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one by OPD.

Figure 3. (A) UHPLC-DAD chromatogram of a typical icodextrin-containing PD fluid after adding diacetylquinoxaline as internal standard and derivatization with OPD, recorded at 316 nm. The chromatographic parameters were optimized for the separation of 5-HMF_{bfm} and 3-DG_{qx}, 3-$DGal_{qx}$, and 4-DG_{qx}. Signals within the grey rectangle are caused by OPD degradation, the asterisk indicates two coeluting products 3,4-$DDPS_{qx}$ and the contamination of the raw material 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one ; **(B)** UHPLC–MS/MS chromatogram of a typical icodextrin-containing PD fluid after trapping the degradation products with OPD. Mass transitions *m/z* 189–171 (grey) and *m/z* 337–319 (black) are shown. Chromatographic separation was optimized for 3,4-$DDPS_{qx}$ and the contamination of the raw material 1,4-bis(1*H*-benzo[*d*]imidazol-2-yl)-3,4-dihydroxybutan-1-one.


A
OPD
4-DG
4-DG$_{qx}$
B
OPD
5-HMF
5-HMF$_{bfm}$
C
OPD
3,4-DDPS
3,4-DDPS$_{qx}$
D
OPD
pyruvic acid
E
OPD
glyceraldehyde
1-BIME
F
contamination of raw material

Scheme 1

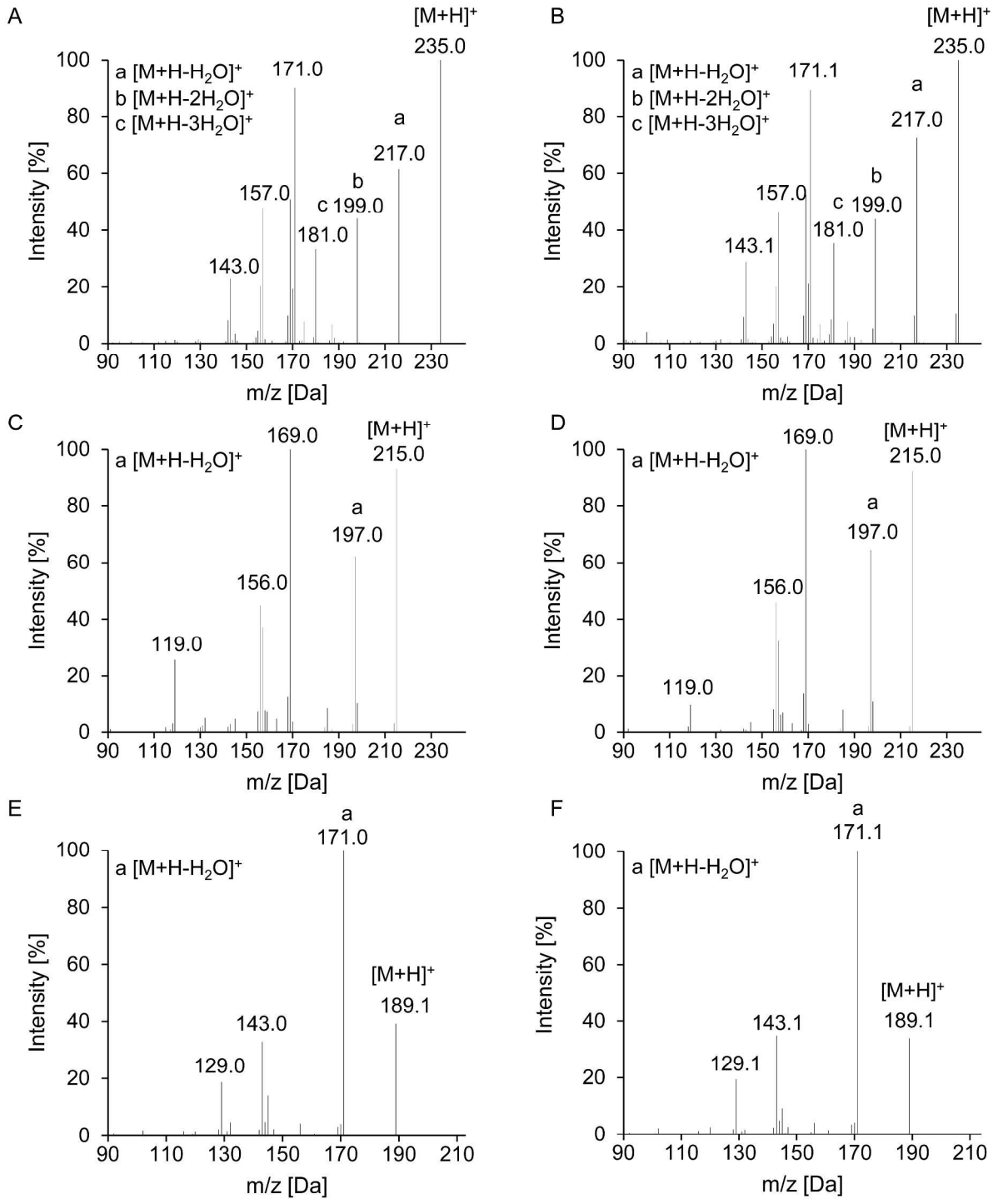

A
[M+H]+
235.0
a [M+H-H2O]+
b [M+H-2H2O]+
c [M+H-3H2O]+
171.0
a
217.0
b
157.0
c 199.0
181.0
143.0
Intensity [%]
m/z [Da]
B
[M+H]+
235.0
a [M+H-H2O]+
b [M+H-2H2O]+
c [M+H-3H2O]+
171.1
a
217.0
b
157.0
c 199.0
181.0
143.1
Intensity [%]
m/z [Da]
C
169.0
[M+H]+
215.0
a [M+H-H2O]+
a
197.0
156.0
119.0
Intensity [%]
m/z [Da]
D
169.0
[M+H]+
215.0
a [M+H-H2O]+
a
197.0
156.0
119.0
Intensity [%]
m/z [Da]
E
a
171.0
a [M+H-H2O]+
[M+H]+
189.1
143.0
129.0
Intensity [%]
m/z [Da]
F
a
171.1
a [M+H-H2O]+
[M+H]+
189.1
143.1
129.1
Intensity [%]
m/z [Da]

Figure 1

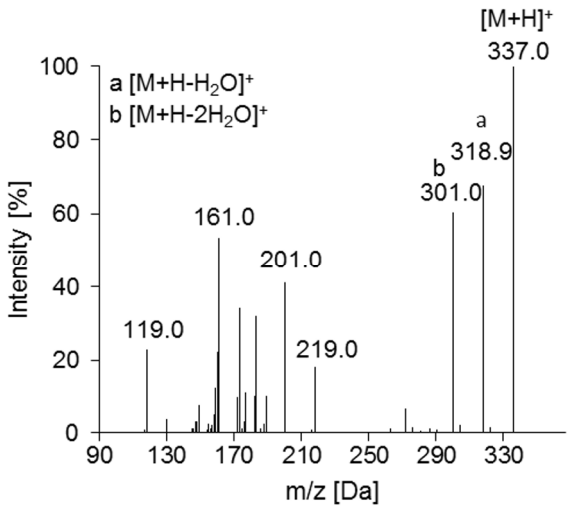

[M+H]+
337.0
100
a [M+H-H2O]+
b [M+H-2H2O]+
80
a
318.9
b
301.0
60
161.0
201.0
Intensity [%]
40
119.0
219.0
20
0
90
130
170
210
250
290
330
m/z [Da]

Figure 2

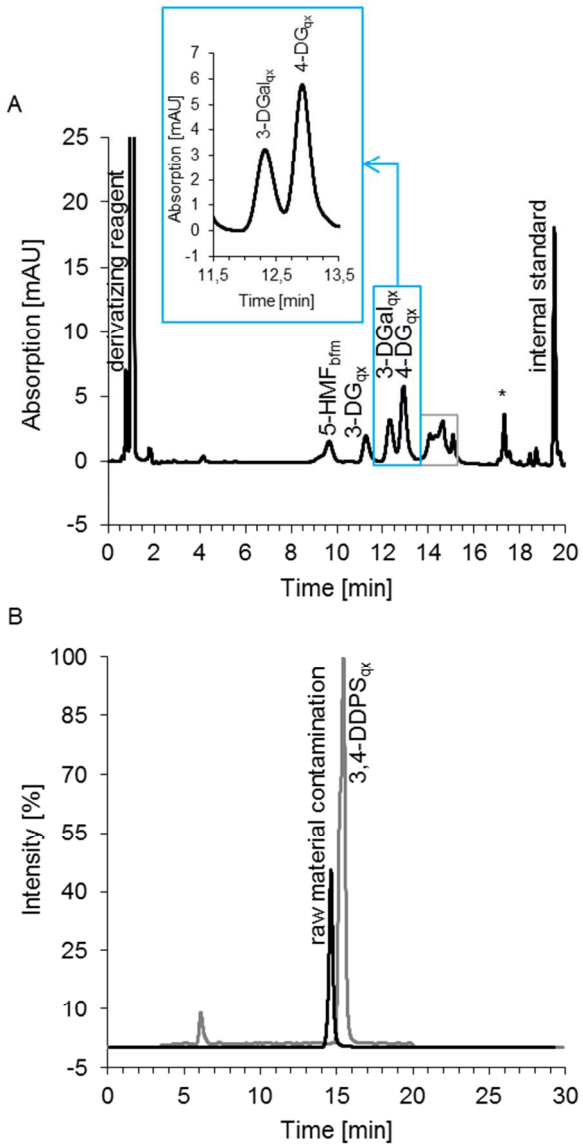

A
Absorption [mAU]
Time [min]
derivatizing reagent
5-HMF$_{bfm}$
3-DG$_{qx}$
3-DGal$_{qx}$
4-DG$_{qx}$
*
internal standard
Absorption [mAU]
3-DGal$_{qx}$
4-DG$_{qx}$
Time [min]
B
Intensity [%]
Time [min]
raw material contamination
3,4-DDPS$_{qx}$

Figure 3

For TOC only: