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Practicing IEF-PAGE of EPO: The impact of detergents and sample application methods on analytical performance in doping control

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Electrophoretic techniques, namely isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are key techniques used for confirming the doping-related abuse of recombinant erythropoietins and analogs. IEF-PAGE is performed on horizontal slab-gels with samples applied to the surface of the gel. Different sample application techniques can be employed, but application pieces and applicator strips are most frequently used. However, defective application pieces cause lane streaking during IEF of erythropoietin (EPO), which is especially pronounced in the acidic region of the gel. The effect is due to an incompatibility of the substance used for enhancing the wettability of the cellulose-based commercial product and is batch-dependant. A detailed mass spectrometric study was performed, which revealed that defective sample application pieces (bought between 2007 and 2010) contained a complex mixture of alcohol ethoxylates, alcohol ethoxysulfates, and alkyl sulfates (e.g. SDS). Anionic detergents, like the sulfates contained in these application pieces, are in general incompatible with IEF. Alternative application techniques proved partly useful. While homemade pieces made of blotting paper are a good alternative, the usage of applicator strips or shims is hampered by the risk of leaking wells, which lead to laterally diffused samples. Casting IEF-gels with wells appears to be the best solution, since sustained release of retained proteins from the application pieces can be avoided. Edge effects do not occur if wells are correctly filled with the samples. The evaluation of EPO-profiles with defects is prohibited by the technical document on EPO-analytics (TD2009EPO) of the World Anti-Doping Agency (WADA).

Keywords: erythropoietin (EPO); isoelectric focusing (IEF); sample application; detergents; doping control

Introduction

Isolectric focusing in polyacrylamide gel electrophoresis (IEF-PAGE) is one of the main methods for detecting doping with recombinant erythropoietins and its analogs.[1–3] Application pieces and applicator strips are mainly used for applying the samples (typically urinary retentates after ultrafiltration or eluates after immunoaffinity purification) to the gel.[4–5] Detergents of the polyoxyethylene sorbitan group (Tween-80, Tween-20) are added in order to inhibit hydrophobic interactions between amino acid residues in the 7 M urea containing gel. Lane streaking during electrophoretic separations usually indicates solubility problems, i.e. either the amount of detergent was too low, the capacity of the gel was exceeded, or a detergent with poor solubilizing power for the specific protein was used.[6] However, lane streaking may also be caused by badly impregnated application pieces. In that respect, different batches of pieces showed different performance characteristics. In order to completely avoid running the risk of using defective application pieces, gels with wells were introduced, and the performance characteristics of these gels compared with the standard application techniques for EPO IEF-PAGE.[7]

Experimental

Materials

Application pieces (AP) and applicator strips were purchased from two companies, Serva (Heidelberg, Germany; AP lot #?8631 (first number unreadable), #080125, #090898) and GE Healthcare (Uppsala, Sweden; AP lot #4421916, #4445978, #4454670, #4524724). According to one manufacturer (GE Healthcare, Uppsala, Sweden) the material of the pieces is Paratex, a cotton/cellulose-based material.[7–9] The silicon shims (inner diameter *c.* 3 and 5 mm, respectively) and electric isolation tape (polyvinylchloride (PVC); *c.* 15 mm width) were bought at a local shop. N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium peroxidisulfate (APS), acrylamide/bisacrylamide solution (40% T, 3% C; PlusOne ReadySol IEF), tris(hydroxymethyl)aminomethane (Tris; PlusOne), glycine (PlusOne), urea (PlusOne), sodium dodecyl sulfate (SDS; PlusOne), the gel casting moulds including clamps (FlexiClamps), gel support film (GelBond PAGfilm), and blotting papers (NovaBlot, Amersham Hybond, and Blotting Paper 21 × 26 cm) were from GE Healthcare. Servalytes 2–4, 4–6, and 6–8 were obtained from Serva. Methanol (LiChrosolv, gradient grade), glacial acetic acid (p.a.), formic acid (98–100%, p.a.), phosphoric acid (85%, p.a.), acetonitrile (ACN; LiChrosolv, hyper grade), and water (LiChrosolv) were bought from Merck (Darmstadt, Germany). Whatman 3 MM blotting paper was obtained from VWR (Vienna, Austria). ASB-14, C7BzO, Igepal CA-720, phosphate-buffered saline (PBS) tablets, dithiothreitol (DTT), sodium chloride (NaCl), and polyethylene glycols (PEG-200, PEG-1000) were from Sigma-Aldrich (St Louis, MO, USA). The detergents Tween-20, Tween-80, Triton

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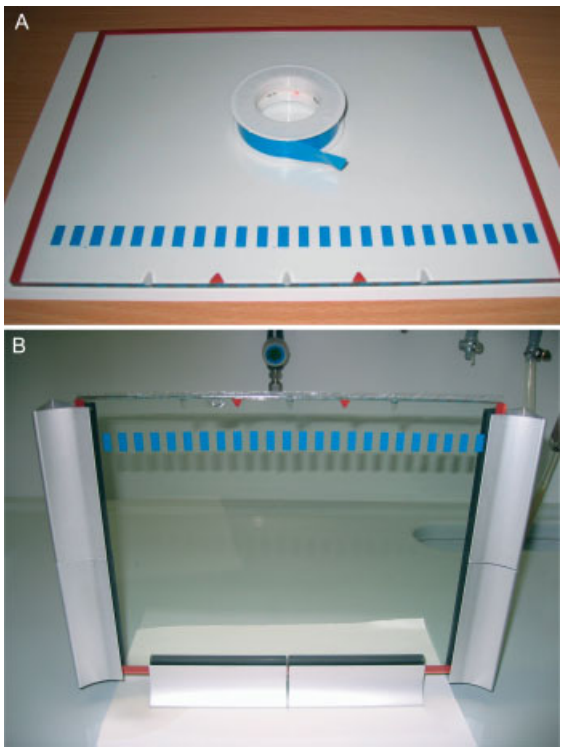

Figure 1. Casting polyacrylamide IEF-gels with wells. A slot-former is cut out of several layers of electric isolation tape (A) and the degassed gel solution is filled into the mould (B). Pictured is a double-sized gel but the technique works equally well for regular-sized gels.

X-100, and NP-40 (Surfact-Amps), and the biotinylated secondary antibody (ImmunoPure goat anti-mouse IgG (H+L)) as well as the chemiluminescence substrate (West Pico) were received from Pierce (Rockford, IL, USA). The anti-EPO antibody (clone AE7A5) was from R&D Systems (Minneapolis, MN, USA) and the streptavidin horseradish peroxidase complex from Biospa (Milano, Italy). Erythropoietin (EPO) standards were either reference materials (human urinary erythropoietin (uhEPO; second international reference preparation; National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK), human recombinant erythropoietin (rhEPO; BRP-EPO batch 3; European Directorate for the Quality of Medicines; Strasbourg, France) or an original pharmaceutical formulation (Aranesp (NESP); Amgen; Thousand Oaks, CA, USA). Micro- (Steriflip; 0.2 µm) and ultrafilters (Amicon Ultra-15, Amicon Ultra-0.5; nominal molecular weight limit (NMWL) 30 kDa) and blotting membranes (Durapore, Immobilon-P) were from Millipore (Billerica, MA, USA). Images were acquired on a LAS-4000 CCD-camera (Fujifilm, Tokyo, Japan) and analyzed with GASepo version 1.3b2 (ARC; Seibersdorf, Austria).[10] A semi-dry blotter (Trans-Blot SD; BioRad, Hercules, CA, USA) was used for all Western transfers. The flat-bed electrophoresis system (Multiphor II) was also from GE Healthcare (Uppsala, Sweden).

Methods

Preparation of the gel-casting mould and samples

Anonymous urine samples were received from healthy individuals and with written consent. The samples (20 mL) were centrifuged, micro- and ultrafiltrated as described elsewhere.[4] A gel-casting mould with slot-former was prepared by fixing three layers of electric isolation tape (each *c.* 25 cm in length) above each other

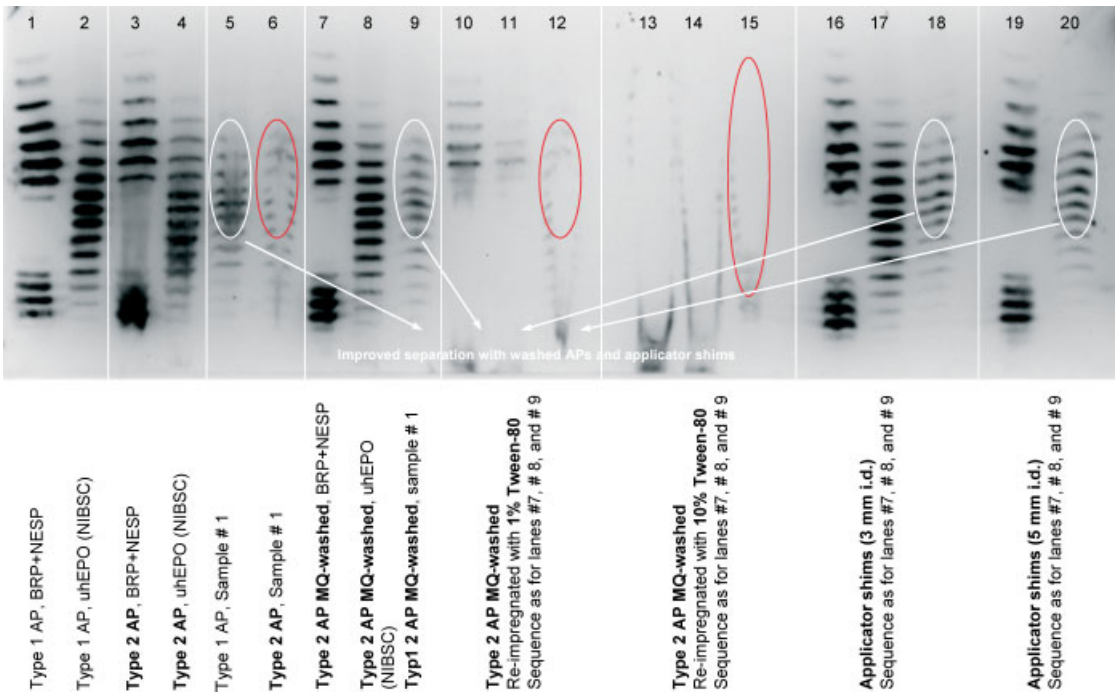

Figure 2. Comparison of the performance characteristics of type 1 and type 2 application pieces (lanes 1–6), and washed (lanes 7–9), and washed and re-impregnated (lanes 10–15) pieces. Lanes 16–20 were obtained with silicone applicator shims with 3 and 5 mm (inner diameter, i.d.), respectively. An improved separation was obtained for the urinary retentate using washed type 2 AP and applicator shims (marked in white). Re-impregantion with Tween-80 led to results comparable (1%) or enhanced (5%) to original type 2 AP (marked in red).

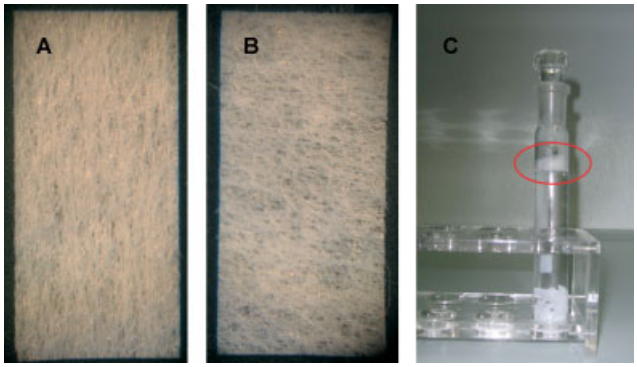

Figure 3. Batch-to-batch variability of commercial application pieces. Aside from structural differences (type 1 AP (A); and type 2 (B)) surface active substances were detectable (C).

on a rubber U-framed glass plate (cleaned with ethanol and deionized water) and in *c.* 25–30 mm horizontal distance from the upper (open) edge.[8,11–13] Then the stack of three layers was cut vertically with a scalpel every 5 mm starting *c.* 7 mm from one side of the rubber frame and successively moving to the other side. The width of the stack was reduced to 10 mm (again by cutting) and every second rectangular-shaped tape-stack was removed by using tweezers. Finally, the gel-casting mould consisting of the U-framed glass plate with well-former and a non-U-framed glass plate with one sheet of GelBond PAG film was assembled as usual. After degassing, the acrylamide gel solution (*vide infra*) was filled into the mould using a glass pipette and without introducing air bubbles. Thus, after polymerization a slab-gel was obtained which contained preformed wells in the size of application pieces (Figure 1).

IEF-PAGE and Western double-blot

The polyacrylamide gel mixture for isoelectric focusing in a pH 2–6 gradient was prepared as already described (5% T/3% C acrylamide-bisacrylamide solution containing 7 M urea and a 1:1 blend of Servalytes 2–4 and 4–6 carrier ampholytes at a final concentration of 2% (*w/v*) each).[4] Either standard gels or gels with wells were cast (25 × 11.5 cm, 1 mm). Focusing conditions were identical for all runs (prefocusing at constant voltage (250 V, 30 min; 10 °C), focusing at constant current (25 mA, maximum 25 W, 3600 Vh; 10 °C), interelectrode distance 10 cm). Urinary retentates (after heat-inactivation of proteases at 80 °C, 3 min) and EPO-standards were routinely supplemented with 1% Tween-80[4] and for test purposes with 1% Tween-20, ASB-14, C7BzO, or NP-40, respectively. Semi-dry Western double-blots were performed on all gels (Towbin buffer; 1 mA/cm^2, 30 min) using a polyvinylidene fluoride (PVDF) membrane (Immobilon-P) and a separator-membrane (Durapore) in between.[4] After the first blot the proteins on the Immobilon-P membrane were reduced (5 mM DTT/PBS; 37 °C, 60 min) and the membrane was blocked (5% non-fat milk (NFM)/PBS; 60 min), incubated overnight in primary antibody (clone AE7A5; 1:1000 (*v/v*) in 1% NFM/PBS, 4–8 °C), washed (0.5% NFM/PBS; 3 × 10 min), double-blotted (0.7% acetic acid; 0.8 mA/cm^2, 10 min) to a second Immobilon-P membrane,[4,14] this membrane was blocked (5% NFM/PBS; 60 min), incubated in biotinylated secondary antibody (goat anti-mouse IgG; 1:2000 (v/v), 1% NFM/PBS, 60 min),

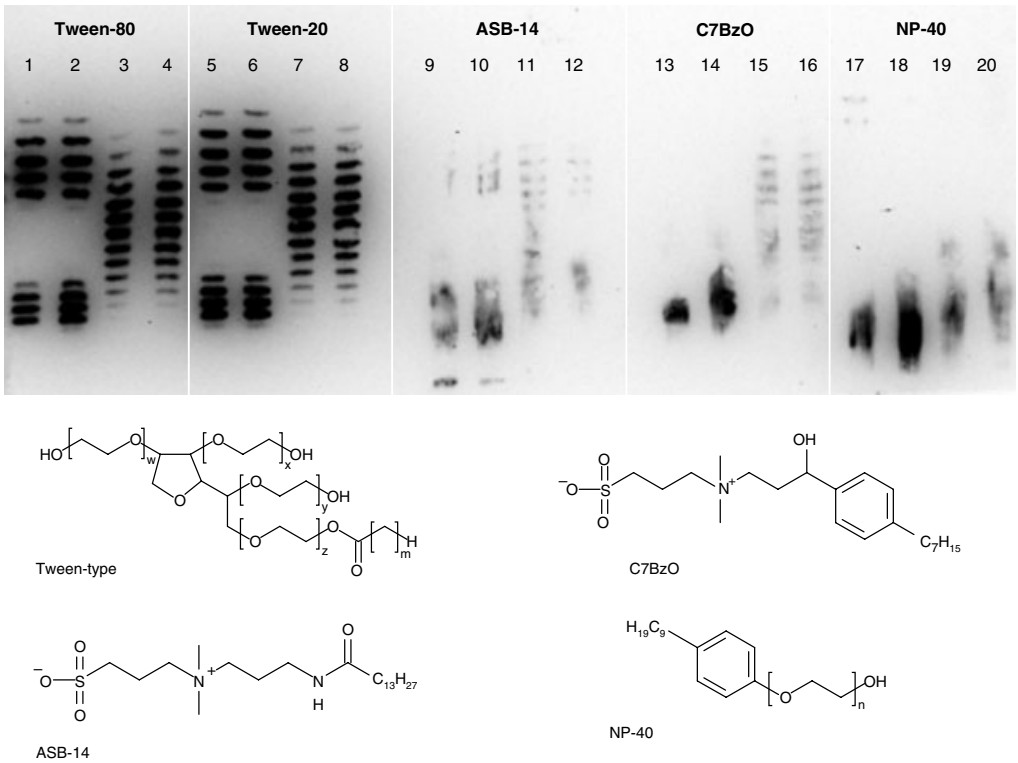

Figure 4. Study on the performance characteristics of different detergents on IEF-PAGE of EPO. The final detergent concentration was 1% (*v/v*). Out of five tested detergents (Tween-80, Tween-20, ASB-14, C7BzO, NP-40) only Tween-80 (lanes 1–4) and Tween-20 (lanes 5–8) generated EPO-profiles which fulfilled the criteria of TD2009EPO. Sequence on gel: BRP + NESP (lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18), NIBSC uhEPO (lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20). The chemical structures of the detergents used are shown below the Western blot.

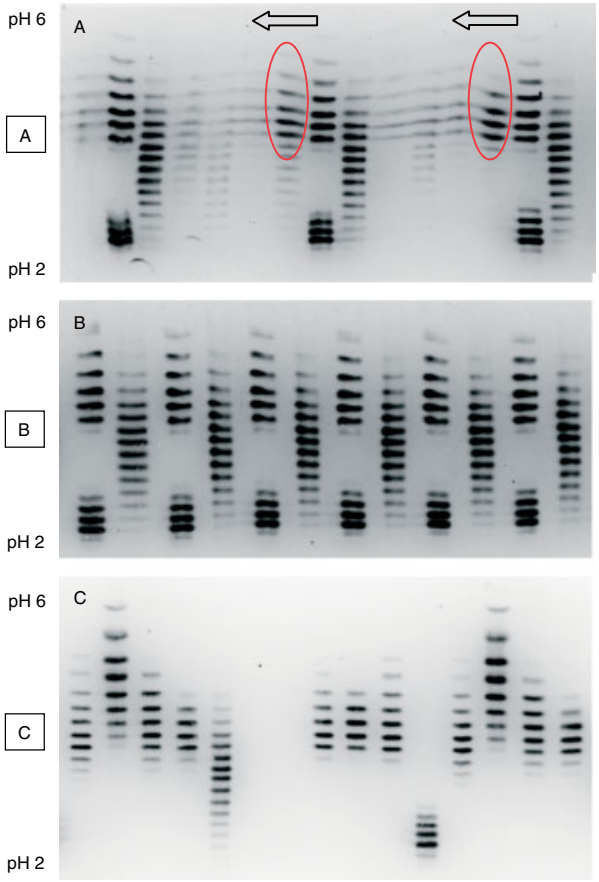

Figure 5. Lateral diffusion of a leaking applicator strip invalidates the EPO-profile (A). If the well volume has not been optimized edge effects might occur on gels with preformed wells (B). (C) shows the results obtained after correct well filling.

washed (0.5% NFM/PBS; 3 × 10 min), incubated in streptavidin horseradish peroxidase (1 : 2000 (*v*/*v*), 1% NFM/PBS), washed again (PBS; 3 × 10 min), and finally developed in chemiluminescent substrate (West Pico) for image acquisition (LAS-4000). Additional details about sample preparation, isoelectric focusing, and the immunoblotting steps can be found in the referenced literature.

Mass spectrometry of application pieces eluates

Application pieces were eluted with either a solution of 50% ACN/water containing 2% formic acid, 50% methanol/water containing 10 mM sodium chloride, or 50% ACN/10 mM sodium chloride/water (5 application pieces and 400 µL eluent were used for each elution; the extraction power of these solvents was tested by re-extracting the water-washed application pieces used for IEF-PAGE. A subsequent MS-analysis showed that these extracts still contained compounds which could be not extracted with water alone). All mass spectrometry-related sample preparation steps were performed in glass devices (pipetting of liquids, elution of application pieces) to exclude possible contamination with polymers from plastic materials. Standard solutions of detergents (Triton X-100, Igepal CA-720, NP-40) and polymers (PEG-200, PEG-1000) were prepared by diluting or dissolving (PEG-1000) the original substances in the eluents and at a concentration of 0.1%. The electrospay (ESI) mass spectra were recorded on an LTQ-Orbitrap (Thermo Electron; Bremen, Germany) and in positive ion mode. The instrument was equipped with a nano-ESI source (Proxeon; Odense, Denmark) and run either in offline (static) nanospray or online nano-LC mode. Coated glass emitters with a tip opening of 4 ± 1 µm were used for static experiments (New Objective; Woburn, MA, USA). In order to exclude cross contaminations, each sample was run on a separate emitter and the source was cleaned between runs. The source was operated with a spray voltage of 2 kV, a capillary temperature of 200 °C, a capillary voltage of 27 V, and a tube lens voltage of 100 V (offline experiments). Collision gas was helium. Normalized collision energy and activation time were optimized according to the fragmentation characteristics of the polymer precursors and typically ranged from 20 to 55 and 30 to 75 ms, respectively. Between 50 and 150 sub-spectra were recorded for each mass spectrum (MS, MS/MS). All full-scan spectra were recorded within the mass range of *m/z* 50 to 2000; high resolution mass spectra were only recorded for accurate mass measurements and charge state determinations, typically at resolving powers (R) of 15 000, 30 000, or 60 000. The instrument was externally calibrated with a mixture of caffeine, MRFA (tetrapeptide), and Ultramark 1621 (as provided by the manufacturer). For online LC separations an Ultimate 3000 nano-HPLC system (Dionex; Sunnyvale, CA, USA) in preconcentration mode was used. The eluate (19 µL) was first loaded on a trap column (5 min; 10 µL/min; 2% ACN in 10 mM NaCl water; C18 PepMap 100; 300 µm × 5 mm, 5 µm particle size, 100 Å pore size) and then separated on an analytical column (52 or 72 min; 300 nL/min; C18 PepMap100; 75 µm × 15 cm, 3 µm particle size, 100 Å pore size). A linear gradient from 100% A to 95% B was used (solvent A: 2% ACN, 10 mM NaCl/water; solvent B: 90% ACN, 10 mM NaCl/water). The column oven temperature was 40 °C. The column was connected with a MicroCross (Upchurch Scientific; Oak Harbor, WA, USA) to the glass emitter (360 µm OD, 75 µm i.d., 15 µm tip i.d.; New Objective) and via liquid junction to the source. Positive ion mode with a spray voltage of 2 kV and a capillary temperature of 160 °C was used. The mass spectrometer was run either in full-scan FT-mode (at R=15 000, 30 000, or 60 000) or in data-dependant mode (one full-MS scan (*m/z* 50–1600) in the Orbitrap at a R=15 000 followed by up to seven MS/MS scans of the most intense ions in the LTQ or alternatively the Orbitrap at R=7500 in case accurate mass information was required for fragment ion identification – a good compromise between the increase in cycle time and the decrease in sensitivity at increased resolving power of the Orbitrap-analyzer) and at a fixed normalized collision energy of 55. Only charge state 1+ ions were chosen for data-dependant fragmentations.

Results and Discussion

Application pieces and defects

Different batches (lot numbers) of application pieces (type 1 and type 2, *vide infra*) were investigated. Typically, the pieces were placed in 5–10 mm distance from the cathode and were either kept on the gel during the entire run or were removed 30 min after the main focusing step was started. Usually, it is recommended to remove application pieces from the surface of the gel, since they might retain proteins.[9,15] These proteins get successively released during the run and cause lane streaking. However, no difference was seen in that respect between EPO-standards and urinary retentates (data not shown). Streaking usually occurred when the protein content of the retentates was high (e.g. 40 µg/µL) and too much of the retentate was applied – resulting in protein overloading of the gels (Figure 2, lane 5).[16] Nevertheless, some

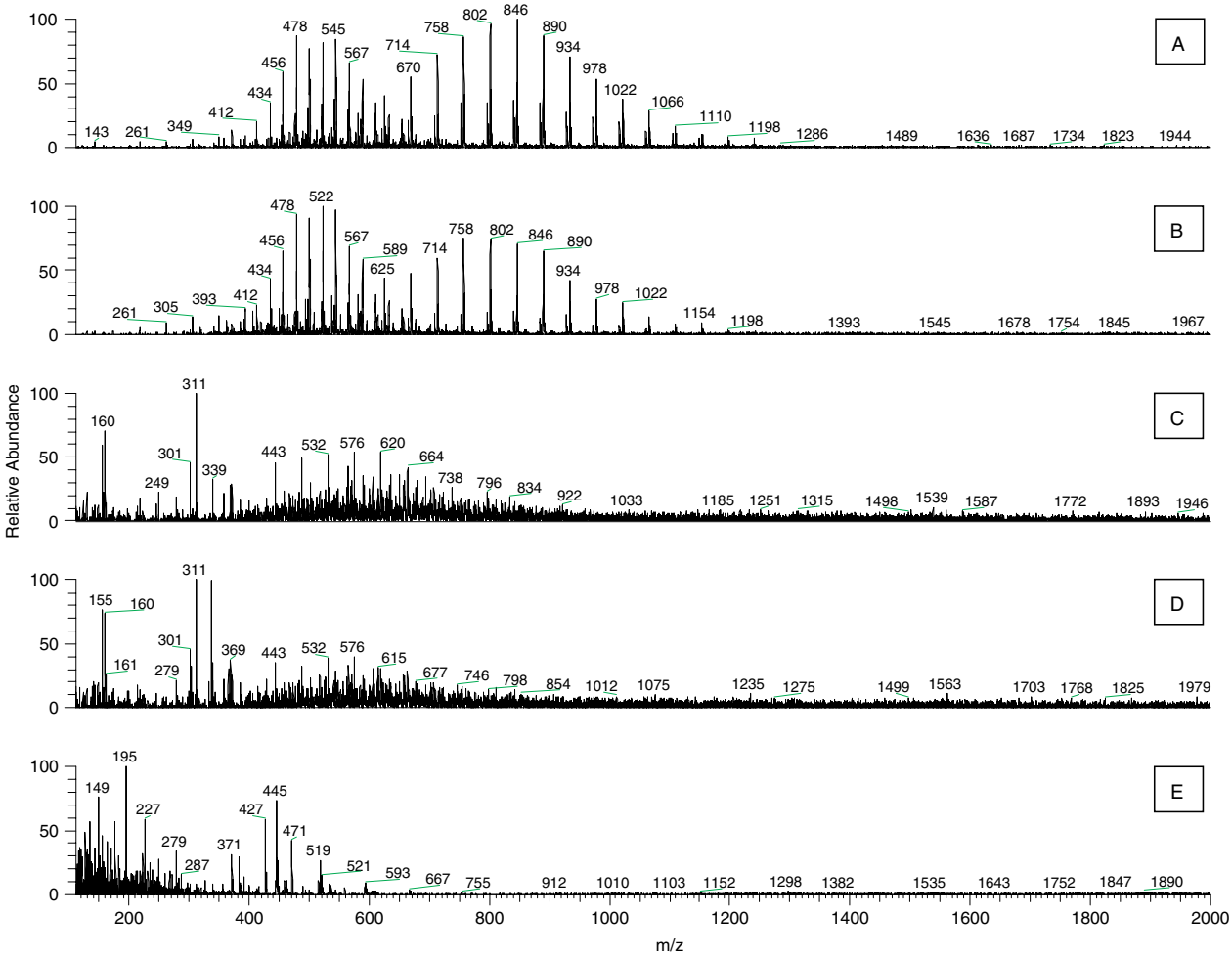

Figure 6. Full scan ion trap mass spectra (*m/z* 150–2000) of eluates of three different types of application pieces (50% methanol/10 mM sodium chloride/water). Static nano-ESI and positive ion mode were employed. The spectra of type 1 (A) and (B) and type 2 (C) and (D) application pieces greatly differed from each other and revealed the presence of PEG-based polymers. No such polymers were detectable in the homemade blotting paper (type 3) application pieces (E).

batches of application pieces (type 2) showed poor performance characteristics even with standards (Figure 2, lanes 3–4).

Microscopic inspection revealed slight structural differences between the working (type 1; GE lot number #4421916, Serva #?8631; both obtained between 2006 and 2008) and defective (type 2; GE #4445978, #4454670, #4524724, Serva #080125, #090898 – all received between 2007 and 2010) application pieces (Figures 3A and 3B) – hence, different materials were indeed used for manufacturing the pieces. In order to clarify whether the streaking behavior of type 2 was due to the structure or an impregnation of the material, the pieces were washed in MQ-water and on a horizontal shaker (overnight) and were then dried overnight at 37 °C. Foam was forming during the washing process, thus indicating the presence of a surface active substance or substance mixture (Figure 3C, circled in red).

The washed type 2 application pieces performed well, even better than the working (type 1) ones (Figure 2, lanes 7–8). Additionally, retentates which were slightly streaking on the working pieces (Figure 2, lane 5) were no longer streaking on the washed ones (Figure 2, lane 9). But there was one major drawback: the washed application pieces were poorly wettable – during application a drop of liquid (sample, standard) formed on the surface of the piece and stayed there for several seconds, then rapidly passed through the piece on the surface of the gel, and was then slowly adsorbed by the piece. Consequently, it became difficult to apply samples and standards without running the risk of mixing them together during the application step. Hence, samples were first individually adsorbed on the pieces and then the pieces were put on the gel surface. In order to regain wettability the washed and dried application pieces were re-impregnated with Tween-80 – a detergent with proven compatibility with EPO and IEF-PAGE (Figure 4; 5 detergents were tested, but only Tween-80 and Tween-20 were EPO compatible). Typically, isoelectric focusing in urea-containing gels has to be performed in the presence of detergents.[16] Urea breaks hydrophobic and hydrogen bonds within proteins, thus exposing the hydrophobic parts of the molecules to the solvent. Detergents are necessary for preventing hydrophobic interactions between these newly exposed residues. However, since urea forms inclusion compounds with many non-ionic and zwitterionic detergents (especially those with long linear alkyl tails)[17] only a limited number of detergents can be used. A 1% and 10% (*v/v*) solution were used for re-impregnation and the pieces were dried again at 37 °C after the detergent was adsorbed. On IEF-PAGE the re-impregnated pieces behaved similar to the defective (type 2) pieces, i.e. parts of the bands predominantly got lost in the center region of each band (Figure 2, lanes 10–15;

Table 1. Main ion series obtained after eluting type 1 application pieces with 50% methanol/water containing 10 mM sodium chloride. Aside from singly and doubly charged octylphenol ethoxylates sodiated polyethylene glycol was observable. Masses were recorded in the Orbitrap analyzer (static nanospray) and at a resolving power of R=60 000 (at *m/z* 400). Typical mass accuracies for monoisotopic ions were below ±5 ppm. The number of ethylene oxide repeating units is *n*, masses of tabulated ions are *m/z* values

Series	$[C_{14}H_{21}(OCH_2CH_2)_nOH + Na]^+$			$[C_{14}H_{21}(OCH_2CH_2)_nOH + 2Na]^{2+}$			$[H(OCH_2CH_2)_nOH + Na]^+$		
n	measured	calculated	Δ (ppm)	measured	calculated	Δ (ppm)	measured	calculated	Δ (ppm)
1	273.182	273.182	0.0	–	148.086	–	–	85.026	–
2	317.208	317.209	−3.2	–	170.099	–	129.052	129.052	0.0
3	361.235	361.235	0.0	–	192.112	–	173.078	173.078	0.0
4	405.261	405.261	0.0	–	214.125	–	217.104	217.105	−4.6
5	449.287	449.287	0.0	–	236.138	–	261.131	261.131	0.0
6	493.313	493.314	−2.0	–	258.151	–	305.157	305.157	0.0
7	537.339	537.340	−1.9	–	280.165	–	349.183	349.183	0.0
8	581.366	581.366	0.0	–	302.178	–	393.209	393.210	−2.5
9	625.392	625.392	0.0	–	324.191	–	437.235	437.236	−2.3
10	669.418	669.418	0.0	–	346.204	–	481.261	481.262	−2.1
11	713.444	713.445	−1.4	368.217	368.217	0.0	525.288	525.288	0.0
12	757.471	757.471	0.0	390.230	390.230	0.0	569.314	569.314	0.0
13	801.497	801.497	0.0	412.243	412.243	0.0	613.340	613.341	−1.6
14	845.523	845.523	0.0	434.256	434.256	0.0	657.367	657.367	0.0
15	889.550	889.550	0.0	456.269	456.269	0.0	701.393	701.393	0.0
16	933.576	933.576	0.0	478.282	478.282	0.0	745.419	745.419	0.0
17	977.602	977.602	0.0	500.295	500.296	−2.0	789.446	789.445	1.3
18	1021.629	1021.628	1.0	522.308	522.309	−1.9	833.472	833.472	0.0
19	1065.655	1065.654	0.9	544.321	544.322	−1.8	877.498	877.498	0.0
20	1109.681	1109.681	0.0	566.334	566.335	−1.8	921.525	921.524	1.1
21	1153.708	1153.707	0.9	588.348	588.348	0.0	965.551	965.550	1.0
22	1197.734	1197.733	0.8	610.361	610.361	0.0	1009.577	1009.577	0.0
23	1241.760	1241.759	0.8	632.374	632.374	0.0	1053.605	1053.603	1.9
24	1285.787	1285.785	1.6	654.387	654.387	0.0	1097.630	1097.629	0.9
25	1329.813	1329.812	0.8	676.400	676.400	0.0	1141.656	1141.655	0.9
26	1373.839	1373.838	0.7	698.414	698.414	0.0	–	1185.681	–
27	1417.864	1417.864	0.0	720.427	720.427	0.0	–	1229.708	–
28	–	1461.890	–	742.441	742.440	1.3	–	1273.734	–
29	–	1505.917	–	–	764.453	–	–	1317.760	–
30	–	1549.943	–	–	786.466	–	–	1361.786	–

especially compare lane 12 with lane 6). However, the effect was more pronounced (1% Tween-80 solution) than on the original type 2 pieces or led to an almost complete deletion of the isoforms (10% Tween-80 solution) – indicating that the concentration of the impregnation solution was too high. Consequently, the altered performance characteristics of defective application pieces were either due to a badly done impregnation of the pieces and/or the usage of an EPO-incompatible detergent during their production. This result was additionally confirmed when homemade application pieces were used.[18–21] The pieces were cut out of various types of blotting papers (NovaBlot, Amersham Hybond, Whatman 3 MM) in the size of commercial application pieces (5 × 10 mm). The homemade pieces performed excellent (data not shown), although some (NovaBlot) showed limited (*c.* 10 µL) sample capacity (hence two pieces above each other had to be used) or – due to the thickness of the paper – unusual stiffness (Hybond, Whatman 3 MM). Pieces made of Hybond paper had a sample capacity of *c.* 20 µL, which was comparable to the capacity of the commercial pieces (GE Healthcare, Serva). However, some authors do not recommend using blotting paper for sample application purposes since irreversible adhesion of proteins might occur.[7,22]

It must be stressed that none of the EPO-profiles generated by original type 2 and the re-impregnated type 2 pieces fulfilled the acceptance criteria of the technical document on the *Harmonization of the method for the identification of recombinant erythropoietins and analogues* (TD2009EPO) of the World Anti-Doping Agency (WADA).[3] Hence, the defective application pieces made a doping-relevant evaluation of the profiles impossible.

Applicator strip and lateral diffusion

In order to avoid the possible harmful influence of defective application pieces on EPO IEF-PAGE, direct application of samples and standards to the surface of the gel was evaluated. Since only small sample volumes can be directly pipetted on the gel surface (e.g. 2 µL)[23–24] silicon shims were first placed on the gel and then filled with *c.* 10–20 µL of the samples and standards.[7] While excellent results were obtained with both the 3 and 5 mm i.d. (inner diameter) shims (Figure 2, lanes 16–20), the 3 mm i.d. shims occasionally showed bands with drop-like shape (Figure 2, lane 16) which was presumably due to the small inner diameter. However, the main drawback was that the shims could not be

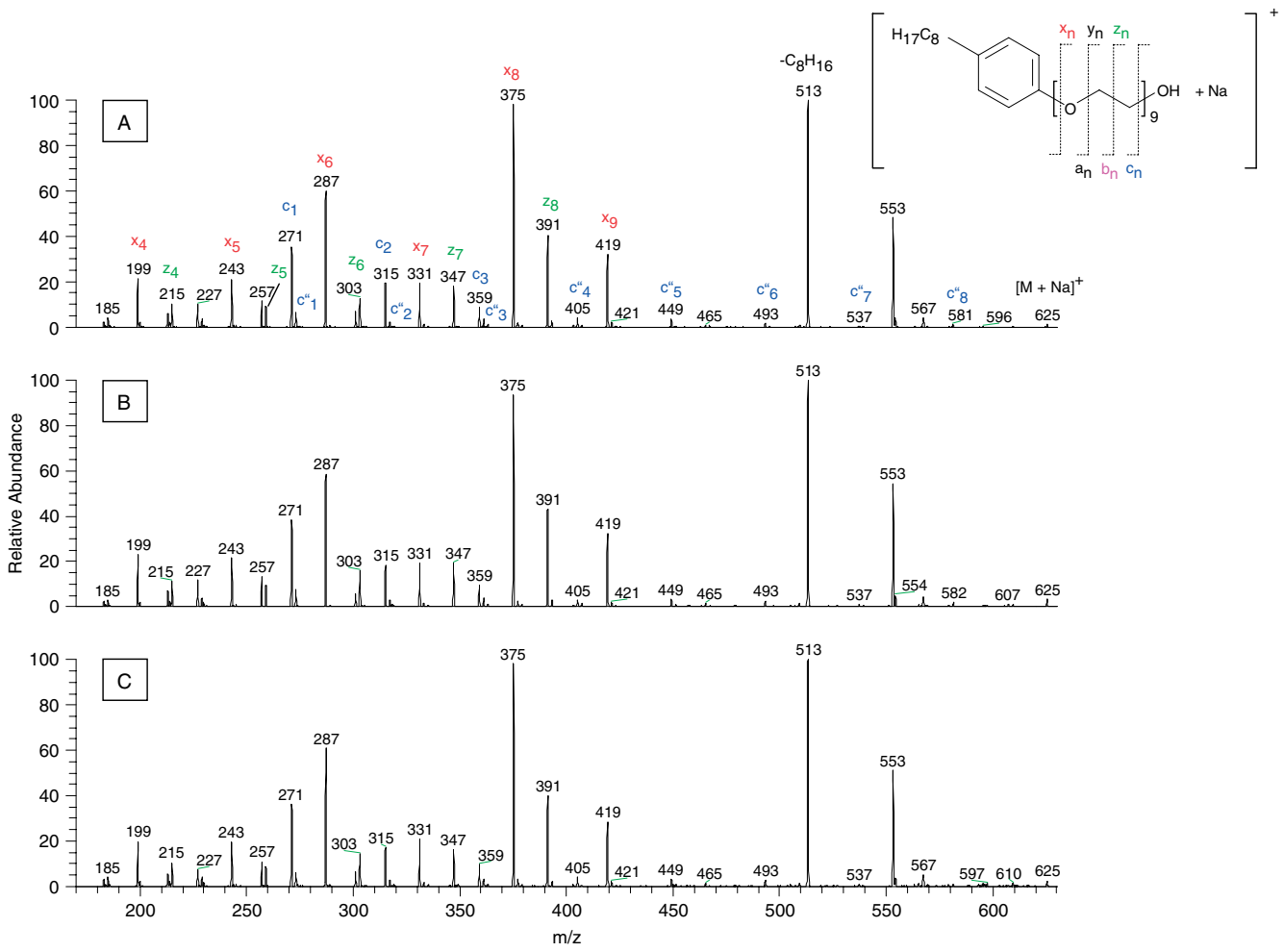

Figure 7. Nano-ESI-MS/MS spectrum of *m/z* 625.4 ($z = 1$) of the sodiated Triton X-100 oligomer ($n = 9$; (A)). The corresponding spectra of GE (B) and Serva (C) type 1 application pieces were identical to the Triton X-100 spectrum, confirming the presence of octylphenol ethoxylates. The main observed fragment ion series were of the x_n ($[H_2C{=}CH\text{-}[OCH_2CH_2]_{n-1}\text{-}OH + Na]^+$), z_n ($[O{=}CHCH_2\text{-}[OCH_2CH_2]_{n-1}\text{-}OH + Na]^+$), c_n ($[H_{17}C_8PhO\text{-}[CH_2CH_2O]_{n-1}\text{-}CH_2CH{=}O + Na]^+$), and c_n'' ($[H_{17}C_8PhO\text{-}[CH_2CH_2O]_n\text{-}H + Na]^+$) type. The Fragment ions were labeled according to the nomenclature proposed by A. T. Jackson (2010).[32]

placed completely leak-free on the gel surface. While no mixing occurred, the potential risk existed and silicon shims were no longer considered an alternative to defective application pieces.

Applicator strips (GE Healtchcare, Serva) were tested next.[22] As expected from the previous results it was quite difficult to apply strips completely leak-free on the gel surface and over the entire length of the gel. In extreme cases lateral diffusion between samples and standards occurred (Figure 5A) – thus making an evaluation of the EPO-profiles impossible according to the technical document of WADA (TD2009EPO).[3] Filling only every second or third well of the strip with liquid solved the problem and also allowed to prove that no mixing between samples and standards occurred. However, a drastic decrease in the maximum number of samples which could be run on one gel was the result. While applicator strips with twice the number of wells (i.e. 52 instead of 26) were available, the well-capacity (5–20 µL instead of 40 µL)[8] and width of the lanes was reduced accordingly.

Using gels with wells

The gels obtained with the homemade slot-former casting mould had wells with a typical sample capacity of *c.* 30 µL – which turned out to be ideally suited for most applications (i.e. sample volumes between *c.* 20 and 30 µL). In cases where the wells were filled with lower sample volumes 'edge effects' occurred. Due to capillary forces, the liquid was drawn up the well edges resulting in a slight increase in EPO-concentration at the edges and bone-shaped bands on the Western blots (Figure 5B).[25] The edge effect was less pronounced in the acidic region of the IEF-gel (probably due to the increased migration distance) and could be easily avoided by correctly filling (i.e. not underfilling) the wells (Figure 5C). A possible disadvantage of gels with wells, namely the distortion of the electric field in the region of the wells (due to the lower gel thickness),[12,22] had no effect on the separation of the EPO-isoforms and the overall performance characteristics of the gels. The addition of a dilute solution of carrier ampholytes into the wells during prefocusing[20] was not necessary. Careful treatment of the slot-former during cleaning of the mould (U-framed glass plate) allowed repeated usage, typically for several years. For this reason it is important to choose an electric isolation tape with smooth surface characteristics and high adhesive strength.

Table 2. Monoisotopic masses (*m/z*) of sodiated polyethylene glycol (PEG) oligomers (first column from left; theoretical masses for $n = 6–20$) and measured monoisotopic masses of exemplary four unknown sodiated ion series (A–D) as found in type 2 application pieces. Series A and B showed mass defects, which were - within comparable *m/z* values – above the mass defect of PEG, series C and D mass shifts, which were below that of PEG (e.g. in the example shaded in grey for masses around *m/z* 600–640). The mass defect criterion allowed a classification of the measured ion series of ethoxylated compounds in two groups

$PEG_{(n=6-20)}$ *m/z*	*Mass defect*	Unknown series A	*Mass defect*	Unknown series B	*Mass defect*	Unknown series C	*Mass defect*	Unknown series D	*Mass defect*
305.1571	*0.1571*	299.2199	*0.2199*	–	–	–	–	–	–
349.1833	*0.1833*	343.2462	*0.2462*	–	–		–	–	–
393.2095	*0.2095*	387.2727	*0.2727*	–	–	–	–	–	–
437.2357	*0.2357*	431.2992	*0.2992*	–	–	–	–	–	–
481.2619	*0.2619*	475.3256	*0.3256*	489.3410	*0.3410*	–	–	–	–
525.2881	*0.2881*	519.3518	*0.3518*	533.3673	*0.3673*	547.2521	*0.2521*	–	–
569.3144	*0.3144*	563.3784	*0.3784*	577.3937	*0.3937*	591.2790	*0.2790*	575.2853	*0.2853*
613.3406	***0.3406***	607.4050	***0.4050***	621.4199	***0.4199***	635.3056	***0.3056***	619.3116	***0.3116***
657.3668	*0.3668*	651.4312	*0.4312*	665.4467	*0.4467*	679.3316	*0.3316*	663.3380	*0.3380*
701.3930	*0.3930*	695.4578	*0.4578*	709.4731	*0.4731*	723.3596	*0.3596*	707.3647	*0.3647*
745.4192	*0.4192*	739.4833	*0.4833*	–		–	–	751.3911	*0.3911*
789.4454	*0.4454*	–	–	–		–	–	795.4176	*0.4176*
833.4717	*0.4717*	–	–	–		–	–	839.4445	*0.4445*
877.4979	*0.4979*	–	–	–		–	–	883.4711	*0.4711*
921.5241	*0.5241*	–	–	–		–	–	–	–

Mass spectrometric analysis of application pieces

Three different solvent mixtures were used for eluting application pieces for subsequent mass spectrometric investigations. The protonating mixture (50% ACN/2% FA in water) generated full-scan spectra in static nanospray mode which revealed that both types of application pieces contained PEG-based polymers (i.e. homologous series of masses at intervals of 44 ($z = 1$) and/or 22 ($z = 2$) were observable indicating the presence of polyethylene oxide polymers) and that the polymers were different for type 1 and type 2 pieces, but identical within each type. Aside from polyethylene glycol many non-ionic detergents contain polymeric ethylene oxide (e.g. detergents of the Tween- and Triton-type, Nonidet P-40 (''NP-40''), and the Igepal CA- and CO-series). However, the obtained mass spectra of the protonated eluates allowed no direct clarification whether one of these detergents was present or not. Since protonated PEG and PEG-derivatives easily undergo in-source fragmentation reactions under ESI conditions[26] all further studies were carried out using Na^+ as cationizing ions. In general, alkali adduct ions of polyglycols are more stable in the gas phase than the corresponding protonated ions and also promote collision induced dissociation (CID) reactions. Hence, ions of this type (Li^+, Na^+, K^+) are preferentially used for studying PEG-based polymers with MALDI- and ESI-MS and -MS/MS.[27–31] Figure 6 shows the results obtained after the elution of application pieces with a 50% methanol/10 mM sodium chloride/water mixture. While type 1 pieces generated rather clean spectra in static nanospray mode, which allowed interpretation without further analyte separation, the spectra of the second type of pieces were too complex for direct interpretation – hence, eluates were first separated on nano-LC (*vide infra*). For these separations, eluates based on 50% methanol/10 mM sodium chloride/water and 50% ACN/10 mM sodium chloride/water were used.

Type 1 ('working', 'old') application pieces

Presence of octylphenol ethoxylates

Type 1 pieces (called 'old' type because they were bought between 2006 and spring 2008) generated a relatively simple mass spectrum, which was characteristic of a polymer containing a single PEG-chain (Figures 6A and 6B; GE and Serva, respectively). By comparing the measured high accuracy *m/z* values of the two main ion series ($[C_{14}H_{21}(OCH_2CH_2)_nOH + Na]^+$ and $[C_{14}H_{21}(OCH_2CH_2)_nOH + 2Na]^{2+}$; Table 1) with the calculated *m/z* values of the sodiated ion series of octylphenol ethoxylates (i.e. the non-ionic surfactants Triton-X100 and Igepal CA-720) and nonylphenol ethoxylates (i.e. Nonidet P-40), it became evident that the polymer used for impregnating type 1 application pieces belonged to the octylphenol ethoxylate group of surfactants. This was confirmed by comparing the MS/MS spectra of the eluates with the corresponding MS/MS spectra of Triton X-100 and Igepal CA-720. The spectra showed identical fragment ions (Figure 7). Observed numbers of ethylene oxide (EO) repeating units ranged from 2 to 24 (median $n = 13$) for the singly and 11 to 28 (median $n = 17$) for the doubly charged ion series.

Two additional lower intensity ion series were also observed, of which one was identified as sodiated polyethylene glycol ($n = 2$ to 20). PEG is an undesired byproduct of the chemical synthesis of ethoxylated alkylphenoles, which utilizes ethylene oxide for the polymerization reaction. Both ion series were present in the Triton X-100 standard, too (data not shown).

Type 2 ('Defective', 'New') Application Pieces

Due to the complexity of the full scan spectra (Figure 6C and 6D) only a few global characteristics were derivable from the static nano-ESI data of type 2 application pieces, namely (1) several PEG-based polymers had to be present, (2) the majority of the

Table 3. Alcohol ethoxylates typically found in type 2 application pieces. Ion series generated by C_8 to C_{16} alcohol-initiated PEGs were found. All masses represent *m/z* values of sodiated monoisotopic species and the general formula $[C_mH_{2m+1}(OCH_2CH_2)_nOH + Na]^+$. Theoretical monoisotopic masses are in brackets

Ion series		$[R\text{-}(OCH_2CH_2)_nOH + Na]^+$							
n	R-	C_8H_{17}-	C_9H_{19}-	$C_{10}H_{21}$-	$C_{11}H_{23}$-	$C_{12}H_{25}$-	$C_{13}H_{27}$-	$C_{14}H_{29}$-	$C_{16}H_{33}$-
1		–	–	–	–	–	–	(281.245)	–
		(197.151)	(211.167)	(225.182)	(239.198)	(253.214)	(267.229)		(309.276)
2		–	–	269.209	–	–	311.256	325.272	353.303
		(241.177)	(255.193)	(269.209)	(283.224)	(297.240)	(311.256)	(325.271)	(353.303)
3		–	–	313.236	327.251	341.266	355.282	369.298	397.329
		(285.204)	(299.219)	(313.235)	(327.251)	(341.266)	(355.282)	(369.298)	(397.329)
4		–	343.246	357.262	371.277	385.293	399.308	413.324	441.356
		(329.230)	(343.245)	(357.261)	(371.277)	(385.292)	(399.308)	(413.324)	(441.355)
5		373.257	387.273	401.288	415.304	429.320	443.335	457.351	485.382
		(373.256)	(387.272)	(401.287)	(415.303)	(429.319)	(443.334)	(457.350)	(485.381)
6		417.283	431.299	445.315	459.330	473.346	487.361	501.377	529.408
		(417.282)	(431.298)	(445.314)	(459.329)	(473.345)	(487.361)	(501.376)	(529.407)
7		461.310	475.326	489.342	503.356	517.372	531.388	545.403	573.435
		(461.308)	(475.324)	(489.340)	(503.355)	(517.371)	(531.387)	(545.402)	(573.434)
8		505.336	519.353	533.368	547.383	561.398	575.414	589.429	617.462
		(505.335)	(519.350)	(533.366)	(547.382)	(561.397)	(575.413)	(589.429)	(617.460)
9		549.363	563.371	577.394	591.407	605.425	619.440	633.456	661.488
		(549.361)	(563,377)	(577.392)	(591.408)	(605.424)	(619.439)	(633.455)	(661.486)
10		593.389	607.398	621.420	635.433	649.451	663.467	677.482	705.515
		(593.387)	(607.403)	(621.418)	(635.434)	(649.450)	(663.465)	(677.481)	(705.512)
11		637.414	651.426	665.444	679.460	693.477	707.494	721.508	749.541
		(637.413)	(651.429)	(665.445)	(679.460)	(693.476)	(707.492)	(721.507)	(749.539)
12		681.442	695.451	709.469	723.486	737.503	751.520	765.534	793.566
		(681.440)	(695.455)	(709.471)	(723.487)	(737.502)	(751.518)	(765.533)	(793.565)
13		725.462	739.478	753.493	767.510	781.530	795.546	809.561	837.587
		(725.466)	(739.481)	(753.497)	(767.513)	(781.528)	(795.544)	(809.560)	(837.591)
14		769.488	783.504	797.520	811.537	825.556	839.573	853.588	881.613
		(769.492)	(783.508)	(797.523)	(811.539)	(825.555)	(839.570)	(853.586)	(881.617)
15		813.514	827.530	841.544	855.564	869.581	883.599	897.614	925.640
		(813.518)	(827.534)	(841.550)	(855.565)	(869.581)	(883.596)	(897.612)	(925.643)
16		857.540	871.557	885.571	899.590	913.607	927.626	941.641	969.667
		(857.544)	(871.560)	(885.576)	(899.591)	(913.607)	(927.623)	(941.638)	(969.670)
17		–	915.583	929.597	943.616	957.633	971.652	985.667	1013.693
		(901.571)	(915.586)	(929.602)	(943.618)	(957.633)	(971.649)	(985.665)	(1013.696)
18		–	959.610	973.624	987,642	1001.660	1015.678	–	1057.719
		(945.597)	(959.613)	(973.628)	(987,644)	(1001.659)	(1015.675)	(1029.691)	(1057.722)
19		–	1003.636	1017.651	1031,670	1045.688	1059.704	–	1101.746
		(989.623)	(1003.639)	(1017.654)	(1031,670)	(1045.686)	(1059.701)	(1073.717)	(1101.748)
20		–	1047.662	1061.677	1075,694	1089.713	–	–	1145.771
		(1033.649)	(1047.665)	(1061,681)	(1075,696)	(1089.712)	(1103.728)	(1117.743)	(1145.774)
21		–	1091.690	1105.704	1119,726	–	–	–	–
		(1077.675)	(1091.691)	(1105.707)	(1119.722)	(1133.738)	(1147.754)	(1161.769)	(1189.801)
22		–	1135.713	1149.730	–	–	–	–	–
		(1121.702)	(1135.717)	(1149.733)	(1163.749)	(1177.764)	(1191.780)	(1205.796)	(1233.827)
23		–	1179.745	1193.757	–	–	–	–	–
		(1165.728)	(1179.744)	(1193.759)	(1207.775)	(1221.791)	(1235.806)	(1249.822)	(1277.853)
24		–	1223.770	1237.783	–	–	–	–	–
		(1209.754)	(1223.770)	(1237.785)	(1251.801)	(1265.817)	(1279.832)	(1293.848)	(1321.879)
25		–	–	1281.811	–	–	–	–	–
		(1253.780)	(1267.796)	(1281.812)	(1295.827)	(1309.843)	(1323.859)	(1337.874)	(1365.906)
26		–	–	1325.837	–	–	–	–	–
		(1297.807)	(1311.822)	(1325.838)	(1339.854)	(1353.869)	(1367.885)	(1381.900)	(1409.932)
27		–	–	1369.863	–	–	–	–	–

Table 3. (*Continued*)

Ion series		$[R\text{-}(OCH_2CH_2)_nOH + Na]^+$							
n	R-	C_8H_{17}-	C_9H_{19}-	$C_{10}H_{21}$-	$C_{11}H_{23}$-	$C_{12}H_{25}$-	$C_{13}H_{27}$-	$C_{14}H_{29}$-	$C_{16}H_{33}$-
		(1341.833)	(1355.848)	(1369.864)	(1383.880)	(1397.895)	(1411.911)	(1425.927)	(1453.958)
28		–	–	1413.893	–	–	–	–	–
		(1385.859)	(1399.875)	(1413.890)	(1427.906)	(1441.922)	(1455.937)	(1469.953)	(1497.984)
29		–	–	1457.913	–	–	–	–	–
		(1429.885)	(1443.901)	(1457.917)	(1471.932)	(1485.948)	(1499.963)	(1513.979)	(1542.010)
30		–	–	–	–	–	–	–	–
		(1473.911)	(1487.927)	(1501.943)	(1515.958)	(1529.974)	(1543.990)	(1558.005)	(1586.037)

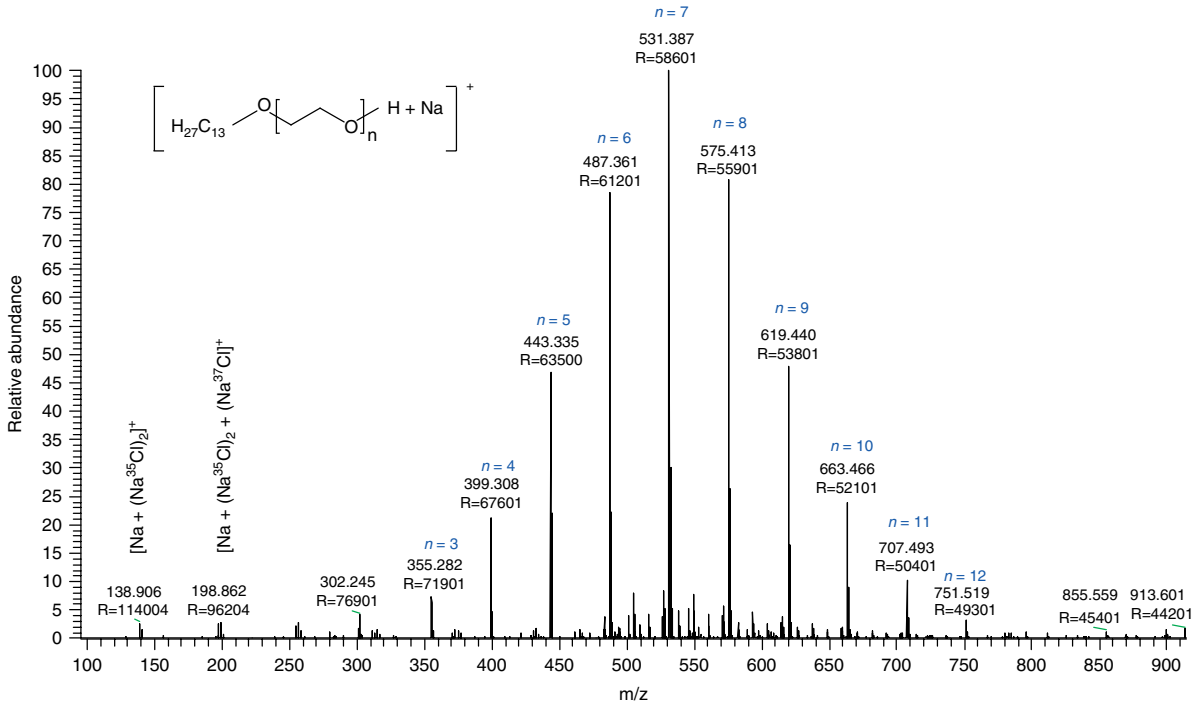

Figure 8. Presence of alcohol ethoxylates in type 2 application pieces. A high accuracy MS spectrum of sodiated C_{13} alcohol ethoxylates (n = 3–12) obtained after nano-LC separation and online nano-ESI-MS is shown. Similar spectra were obtained for the C_8-C_{16} alcohol ethoxylates. Also note the appearance of $[Na + (NaCl)_n]^+$ cluster ions containing the ^{35}Cl and ^{37}Cl isotopes.

ions had *m/z* values below 1000 and charge states of 1+ (i.e. several ion series spaced in 44-u intervals existed), (3) the most prominent ion series had a median of *m/z* 575.413 in static nanospray (Orbitrap high accuracy data at R=60 000; data not shown), and (4) several high abundant ions were present which appeared to belong to no ion series (e.g. the ion at *m/z* 311). Hence, the eluates were first separated by liquid chromatography (nano-LC) and then analyzed online in an LTQ-Orbitrap.

Global data interpretation using differential mass-based and mass defect-based considerations

The obtained high accuracy full-scan MS-spectra were manually analyzed in a retention-time-dependant manner and with respect to the combined appearance and disappearance of ion series. Subsequently, the obtained ion series themselves were analyzed regarding (1) the accurate mass difference between oligomers (e.g. 44.0262 u for the monoisotopic CH_2CH_2O group), and (2) differences in the mass defect of ethoxylated ions with similar *m/z* values but within different ion series and in comparison to the theoretical mass defect of sodiated polyethylene glycol ions (i.e. whether an increase or decrease in the mass defect relative to the mass defect of PEG occurred). Two main types of ion series were found: ion series resembling the accurate mass difference of ethoxylated polymers (44.0262 u), and series spaced in nominal mass intervals of 58 u, indicating the formation of NaCl cluster ions (i.e. accurate mass differences of 57.9586 and 59.9556 for the $Na^{35}Cl$ and $Na^{37}Cl$ clusters, respectively). With regard to changes in the mass defect relative to the mass defect of PEG, ion series with increased and decreased mass defects were found. PEG contains only three elements–C, H, and O–one causing a positive (H; +0.00783), one a negative (O; −0.00509), and one a zero (C) mass shift relative to the nominal masses.[33–34] Hence, in cases where an increase in the relative mass defect occurred, O must have been replaced by elements with positive and/or zero mass shifts – for example, H, C, and/or N (+0.00307). On the other hand, a decrease in the mass defect meant that H and/or C must

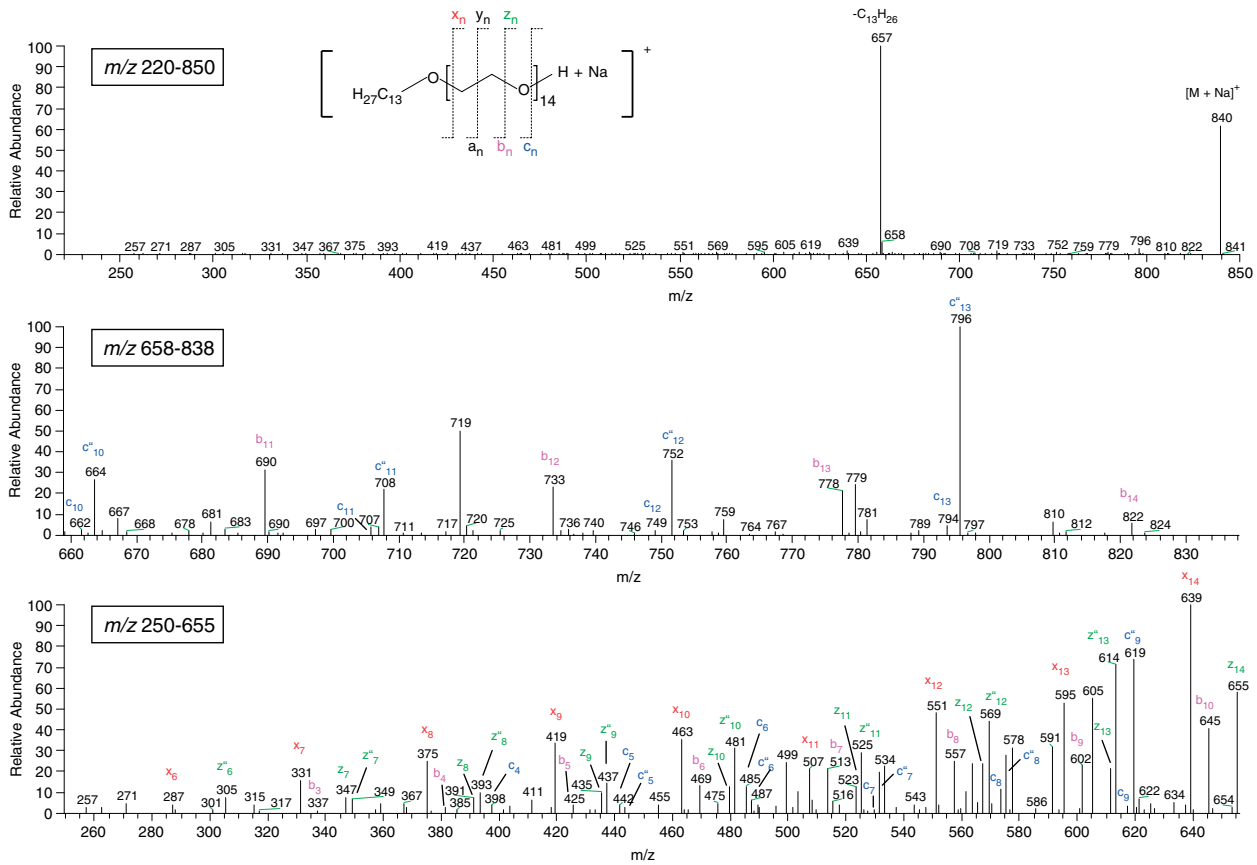

Figure 9. A representative MS/MS spectrum of the $C_{13}H_{27}(EO)_{n=14}OH$ oligomer (precursor mass *m/z* 839.57). The spectrum was recorded in the linear ion trap. Fragment ion series were mainly of the b_n ($[H_{27}C_{13}O\text{-}[CH_2CH_2O]_{n-1}\text{-}CH{=}CH_2 + Na]^+$), c_n ($[H_{27}C_{13}O\text{-}[CH_2CH_2O]_{n-1}\text{-}CH_2CH{=}O + Na]^+$), c_n'' ($[H_{27}C_{13}O\text{-}[CH_2CH_2O]_n\text{-}H + Na]^+$), x_n ($[H_2C{=}CH\text{-}[OCH_2CH_2]_{n-1}\text{-}OH + Na]^+$), z_n ($[O{=}CHCH_2\text{-}[OCH_2CH_2O]_{n-1}\text{-}OH + Na]^+$), and z_n'' ($[H\text{-}[OCH_2CH_2]_n\text{-}OH + Na]^+$) type. The first spectrum shows mass range *m/z* 220–850, the two additional spectra magnify *m/z* regions, which are rich in structural details.

Table 4. Fragment ion series in the MS/MS spectrum of the $C_{13}H_{27}(EO)_{n=14}OH$ oligomer. The series are mainly composed of b_n, c_n, c_n'', x_n, z_n, and z_n'' type of ions. Tabulated are *m/z* values of the sodiated species. The data were acquired in the linear ion trap

Ion Series *n*	b_n	c_n	c_n''	x_n	z_n	z_n''
	m/z measured (calculated)					
1	– (249.2)	– (265.3)	– (267.2)	– (67.0)	– (83.0)	– (85.0)
2	– (293.2)	– (309.3)	– (311.3)	– (111.0)	– (127.1)	– (129.1)
3	337 (337.3)	– (353.3)	– (355.3)	– (155.1)	– (171.1)	– (173.1)
4	381 (381.3)	397 (397.3)	– (399.3)	– (199.1)	– (215.1)	– (217.1)
5	425 (425.3)	441 (441.4)	443 (443.3)	– (243.1)	– (259.2)	– (261.1)
6	469 (469.3)	485 (485.4)	487 (487.4)	287 (287.1)	– (303.2)	305 (305.2)
7	513 (513.4)	529 (529.4)	531 (531.4)	331 (331.2)	347 (347.2)	349 (349.2)
8	557 (557.4)	573 (573.4)	575 (575.4)	375 (375.2)	391 (391.2)	393 (393.2)
9	601 (601.4)	617 (617.5)	619 (619.4)	419 (419.2)	435 (435.3)	437 (437.2)
10	645 (645.5)	661 (661.5)	663 (663.5)	463 (463.3)	479 (479.3)	481 (481.3)
11	689 (689.5)	705 (705.5)	707 (707.5)	507 (507.3)	523 (523.3)	525 (525.3)
12	733 (733.5)	749 (749.5)	751 (751.5)	551 (551.3)	567 (567.3)	569 (569.3)
13	777 (777.5)	793 (793.6)	795 (795.5)	595 (595.3)	611 (611.4)	613 (613.3)
14	821 (821.6)	– (837.6)	839 (839.6)	639 (639.4)	655 (655.4)	657 (657.4)

have been replaced by elements contributing negatively to the mass shift, for example, S (−0.02793), O, and/or P (−0.02624). The measured ion series were then classified in two groups according to the increase or decrease in the mass defect relative to PEG (Table 2).

Presence of alcohol ethoxylates

Formal exchange of O atoms in the chemical formula of PEG ($H(OCH_2CH_2)_nOH$) for C and H atoms in order to increase the mass defect (*vide supra*), lead to the formulas of 'alkylether-substituted

Table 5. Ion series of alcohol ethoxylsulfates frequently found in type 2 application pieces. Tabulated masses are *m/z* values of the sodiated monoisotopic species and the general formula $[C_mH_{2m+1}(OCH_2CH_2)_nOSO_3Na + Na]^+$

Ion series		$[R\text{-}(OCH_2CH_2)_nOSO_3Na + Na]^+$					
n	R-	C_8H_{17}-	C_9H_{19}-	$C_{10}H_{21}$-	$C_{12}H_{25}$-	$C_{13}H_{27}$-	$C_{14}H_{29}$-
1		–	–	–	–	–	–
		(299.090)	(313.106)	(327.121)	(355.153)	(369.168)	(383.184)
2		–	–	–	–	–	–
		(343.116)	(357.132)	(371.147)	(399.179)	(413.194)	(427.210)
3		–	–	–	–	–	471.238
		(387.142)	(401.158)	(415.174)	(443.205)	(457.221)	(471.236)
4		–	445.184	–	–	–	515.264
		(431.169)	(445.184)	(459.200)	(487.231)	(501.247)	(515.263)
5		475.198	489.211	503.226	–	545.275	559.291
		(475.195)	(489.210)	(503.226)	(531.257)	(545.273)	(559.289)
6		519.224	533.237	547.253	575.286	589.301	603.317
		(519.221)	(533.237)	(547.252)	(575.284)	(589.299)	(603.315)
7		563.250	577.263	591.280	619.311	633.327	647.344
		(563.247)	(577.263)	(591.279)	(619.310)	(633.325)	(647.341)
8		607.277	621.289	635.305	663.337	677.353	691.370
		(607.273)	(621.289)	(635.305)	(663.336)	(677.352)	(691.367)
9		651.304	665.316	679.332	707.364	721.380	735.396
		(651.300)	(665.315)	(679.331)	(707.362)	(721.378)	(735.394)
10		695.330	709.343	723.358	751.390	765.405	779.422
		(695.326)	(709.342)	(723.357)	(751.388)	(765.404)	(779.420)
11		739.354	753.368	767.385	795.417	809.432	823.449
		(739.352)	(753.368)	(767.383)	(795.415)	(809.430)	(823.446)
12		783.381	–	–	839.444	853.459	867.476
		(783.378)	(797.394)	(811.410)	(839.441)	(853.457)	(867.472)
13		827.409	–	–	883.471	897.486	911.503
		(827.405)	(841.420)	(855.436)	(883.467)	(897.483)	(911.498)
14		–	–	–	927.497	941.513	955.529
		(871.431)	(885.446)	(899.462)	(927.493)	(941.509)	(955.525)
15		–	–	–	971.524	985.539	999.556
		(915.457)	(929.473)	(943.488)	(971.520)	(985.535)	(999.551)
16		–	–	–	1015.550	1029.565	1043.582
		(959.483)	(973.499)	(987.514)	(1015.546)	(1029.561)	(1043.577)
17		–	–	–	1059.576	1073.591	–
		(1003.509)	(1017.525)	(1031.541)	(1059.572)	(1073.588)	(1087.603)
18		–	–	–	–	–	–
		(1047.536)	(1061.551)	(1075.567)	(1103.598)	(1117.614)	(1131.630)
19		–	–	–	–	–	–
		(1091.562)	(1105.577)	(1119.593)	(1147.624)	(1161.640)	(1175.656)
20		–	–	–	–	–	–
		(1135.588)	(1149.604)	(1163.619)	(1191.651)	(1205.666)	(1219.682)

PEGs' or 'alkyl ethoxylates', a group of non-ionic surfactants commonly known as alcohol ethoxylates (AEO; $R\text{-}(OCH_2CH_2)_nOH$ or $R\text{-}(EO)_nOH$). The technical synthesis of alcohol ethoxylates typically applies C_6 to C_{20} alcohols as initiating molecules for the ethoxylation reaction.[35–36] By comparing the measured accurate monoisotopic masses of the ion series with an increased mass defect compared to PEG with the calculated monoisotopic ion series of sodiated alcohol ethoxylates the majority of the ion series could be identified as ion series generated by C_8 to C_{16} AEO (Table 3).

The degree of ethoxylation ranged from $n = 2$ to 29 ethylene oxide units. A typical high accuracy mass spectrum of a series of sodiated alcohol ethoxylate oligomers is shown in Figure 8 ($C_{13}H_{27}(EO)_{n=3-12}OH$ series). Within one series, oligomers eluted in reverse order according to the number of ethylene oxide units, i.e. due to the decreased hydrophobicity oligomers with higher *m/z* values eluted earlier. Mass accuracies for the full scan FT-spectra were usually better than ±5 ppm.

Data-dependent MS/MS spectra showed the characteristics of low-energy CID spectra of alcohol ethoxylates,[37–40] i.e. a neutral loss of the terminal alkyl group and product ions mainly resulting from charge-induced fragmentations and to a lesser extent from charge-remote rearrangement reactions.[27–28,41–43] The main fragment ion series found in the CID-spectrum of the $C_{13}H_{27}(EO)_{n=14}OH$ oligomer are illustrated in Figure 9 and Table 4.

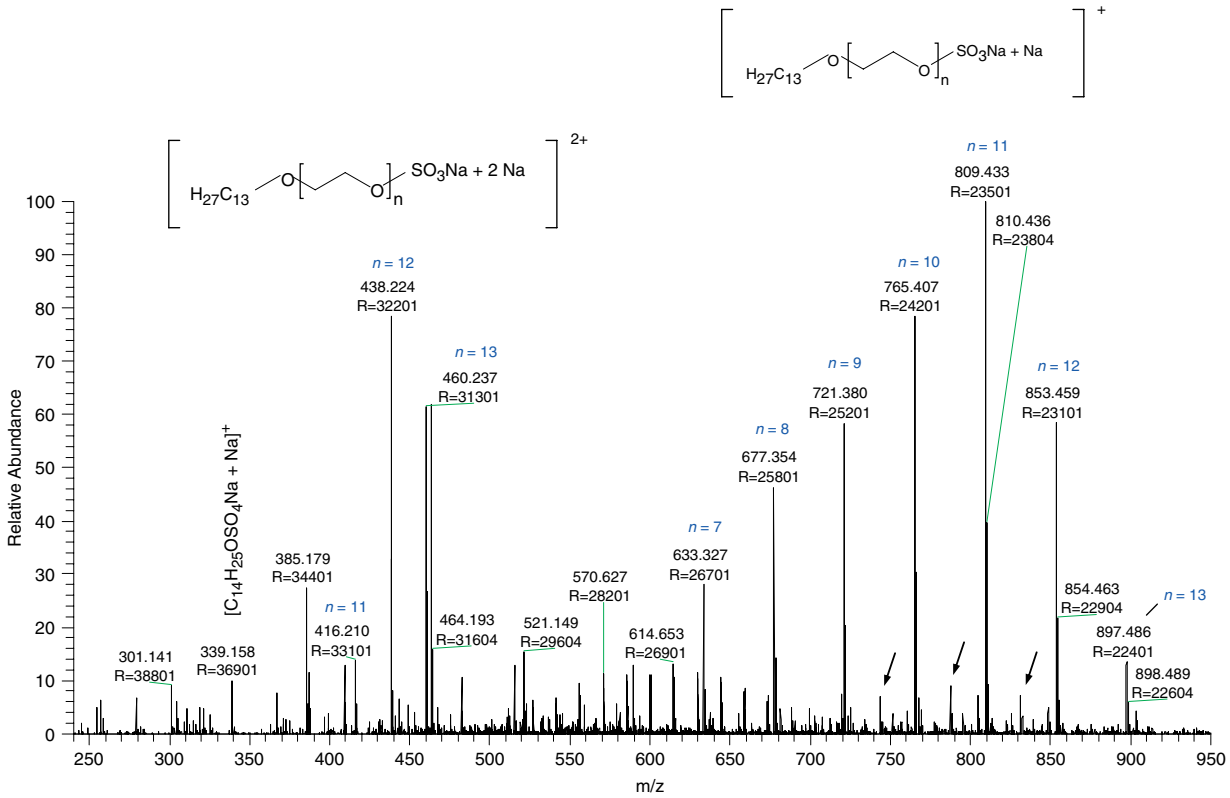

Figure 10. High accuracy mass spectrum of C_{13} alcohol ethoxysulfates (n = 7–13) found in type 2 application pieces. The spectrum represents oligomers found within the retention time window of 36.90–37.04 min. Note the appearance of the $[C_{13}H_{27}(EO)_nOSO_3Na + 2Na]^{2+}$ (labeled) and $[C_{13}H_{27}(EO)_nOSO_3H + Na]^+$ (arrows) species in addition to the $[C_{13}H_{27}(EO)_nOSO_3Na + Na]^+$ oligomers. The ion at m/z 339.158 is the sodiated form of tetradecyl sulfate, $[C_{14}H_{29}OSO_3Na + Na]^+$. Higher oligomers appear later in the chromatographic run. Data were recorded at a resolving power of R=30 000 at m/z 400.

Table 6. Main fragment ion series found in the MS/MS spectrum of the $[C_{12}H_{25}(EO)_{11}OSO_3Na + Na]^+$ oligomer. CID data were recorded online in the Orbitrap analyzer and at R=7500. Tabulated are m/z values of the sodiated species

Ion series n	$[M]^+$	$[M-SO_3]^+$	$[M-NaHSO_3]^+$	$[M-NaHSO_4]^+$	$[M-C_{12}H_{24}]^+$	$[M-C_{12}H_{24}-NaHSO_4]^+$	$[M-C_{12}H_{24}-SO_3]^+$	$[M-C_{12}H_{24}-H_2SO_4]^+$
	m/z measured (calculated)							
11	795.417 (795.415)	715.460 (715.458)	691.465 (691.460)	675.470 (675.465)	627.232 (627.227)	507.281 (507.278)	– (547.270)	– (529.260)
10	751.390 (751.388)	– (671.432)	– (647.434)	631.447 (631.439)	– (583.201)	463.254 (463.251)	– (503.244)	– (485.233)
9	707.369 (707.362)	– (627.405)	603.410 (603.408)	587.418 (587.413)	539.174 (539.174)	419.229 (419.225)	– (459.218)	441.210 (441.207)
8	– (663.336)	– (583.379)	559.383 (559.382)	543.392 (543.387)	– (495.148)	375.202 (375.199)	415.197 (415.191)	397.183 (397.181)
7	– (619.310)	– (539.353)	515.357 (515.355)	499.363 (499.361)	– (451.122)	331.176 (331.173)	371.169 (371.165)	353.158 (353.155)
6	– (575.284)	– (495.327)	– (471.329)	455.338 (455.334)	– (407.096)	287.147 (287.147)	327.142 (327.139)	309.130 (309.128)
5	– (531.257)	– (451.301)	427.306 (427.303)	411.309 (411.308)	– (363.070)	243.122 (243.120)	283.115 (283.113)	265.103 (265.102)
4	– (487.231)	– (407.274)	383.277 (383.277)	367.287 (367.282)	– (319.043)	– (199.094)	239.088 (239.087)	– (221.076)
3	– (443.205)	– (363.248)	– (339.251)	– (323.256)	– (275.017)	– (155.068)	– (195.060)	– (177.050)
2	– (399.179)	– (319.222)	– (295.224)	– (279.229)	– (230.991)	– (111.042)	– (151.034)	– (133.024)
1	– (355.153)	– (275.196)	– (251.198)	– (235.203)	– (186.965)	– (67.015)	– (107.008)	– (88.997)

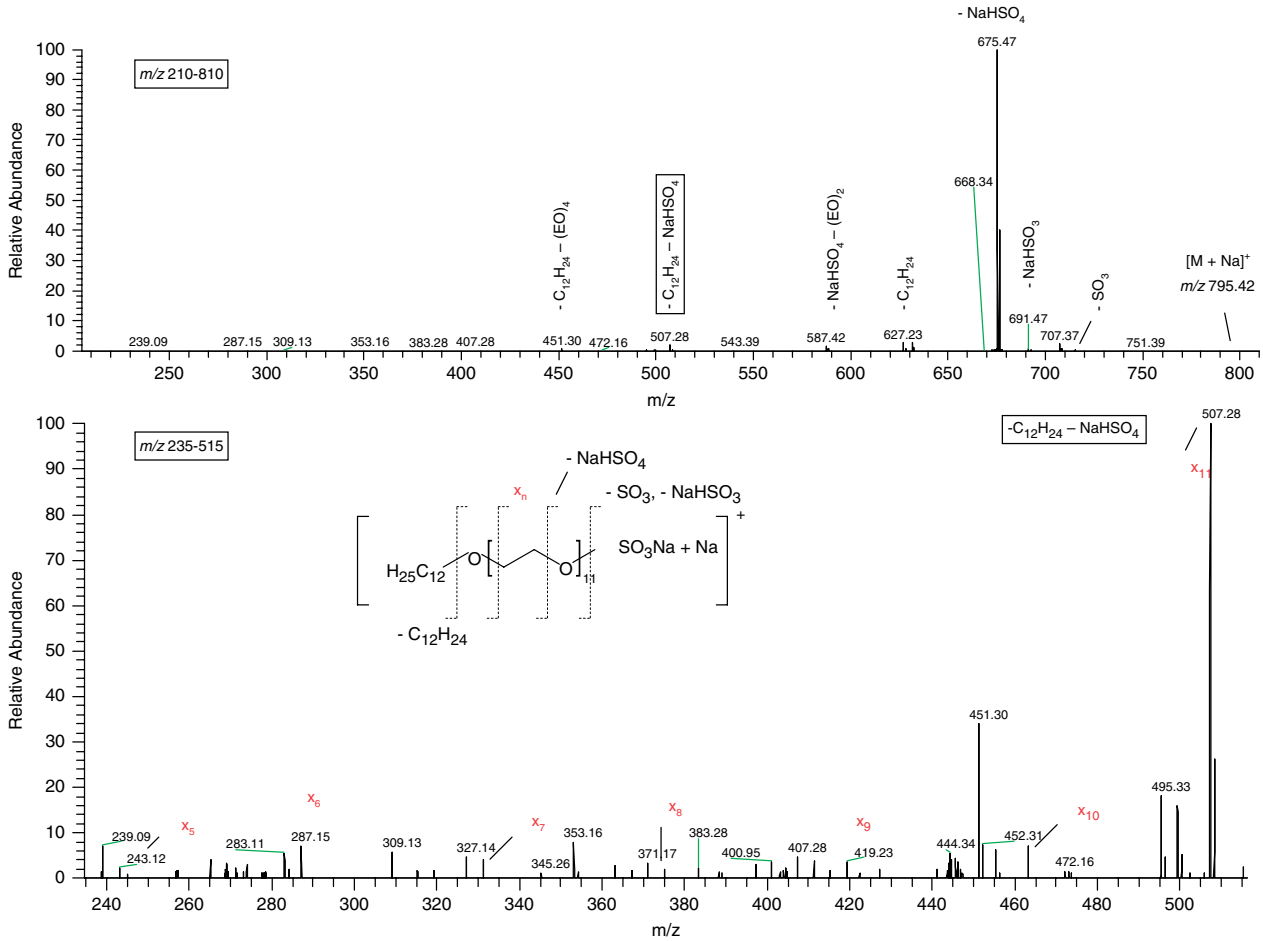

Figure 11. A representative low energy CID spectrum of a C_{12} alcohol ethoxysulfate, $[C_{12}H_{25}(EO)_{11}OSO_3Na + Na]^+$ (precursor mass *m/z* 795.42). The spectrum is dominated by a peak resulting from the neutral loss of $NaHSO_4$ (*m/z* 675.47), but is rich in structural details (only x_n ions of the $[H_2C{=}CH\text{-}[OCH_2CH_2]_{n-1}\text{-}OH + Na]^+$ type were labeled; also refer to Table 6 for additional information). The upper part of the figure shows mass range *m/z* 210–810 and the lower is an enlargement of the *m/z* 235–515 region of the spectrum. The MS/MS data were acquired online in the FT analyzer and with R=7500 at *m/z* 400.

The product ion types are characteristic of polyethers produced from 1,2-epoxide-based monomers.

Presence of alcohol ethoxysulfates

In order to identify those ion series which showed a decrease in the mass defect compared to PEG, H, and/or C was formally substituted by S and O atoms in the chemical formula of polyethylene glycol (*vide supra*). This led to the assumption that also sulfated PEG derivatives might be present, in particular 'alkylether-substituted PEG-sulfates' – a group of anionic surfactants which is also known as alcohol ethoxysulfates or alkylether sulfates (AES; $R\text{-}(OCH_2CH_2)_nOSO_3^-\ X^+$) and which are frequently used in personal care, household, and industrial applications; for example, sodium laureth sulfate surfactants.[43–44] The chemical synthesis of alkylether sulfates starts from the same raw materials as the synthesis of alcohol ethoxylates. The ethoxylated alcohols are then sulfated, usually by reaction with sulfur trioxide (SO_3). A byproduct of this reaction is 1,4 dioxane, which is removed by stripping. However, since neither the ethoxylation nor the sulfatation reaction is complete the final product also contains significant amounts of alcohol ethoxylates. In order to clarify whether alkyl ethoxysulfates were present in type 2 application pieces the high accuracy experimental masses of ion series with a relative decrease in the mass defect were compared with the theoretical monoisotopic masses of sodiated C_2-C_{20} AES. By using this approach it could be demonstrated that for nearly all ion series of the detected ethoxylates corresponding ethoxysulfate ion series existed (Table 5 and Figure 10).

The MS/MS spectra showed the characteristics of positive ion mode spectra of alkyl ethoxysulfates, i.e. neutral losses of ethylene oxide ($\Delta m/z$ 44), SO_3 ($\Delta m/z$ 80), $NaHSO_3$ ($\Delta m/z$ 104), $NaHSO_4$ ($\Delta m/z$ 120), the alkyl chain (as alkene, $\Delta m/z$ R–H), and the alkyl chain minus $NaHSO_4$.[40,45–46] With exception of the $[M-SO_3]^+$ fragment ion each of the resulting product ions served as a starting point for an ion series based on the consecutive loss of ethylene oxide units. Figure 11 (lower spectrum) and Table 6 illustrate the fragmentation characteristics using the sodiated $[C_{12}H_{25}(EO)_{11}OSO_3Na + Na]^+$ oligomer ($[M]^+$) as example. Fragment ion data were recorded in the Orbitrap analyzer (R=7500) in order to calculate accurate mass differences for evidencing the formation of these ion series. Two additional ion series were found to be formed and based on the theoretically calculated product ions $[M-(R-H)-SO_3]^+$ and $[M-(R-H)-H_2SO_4]^+$.

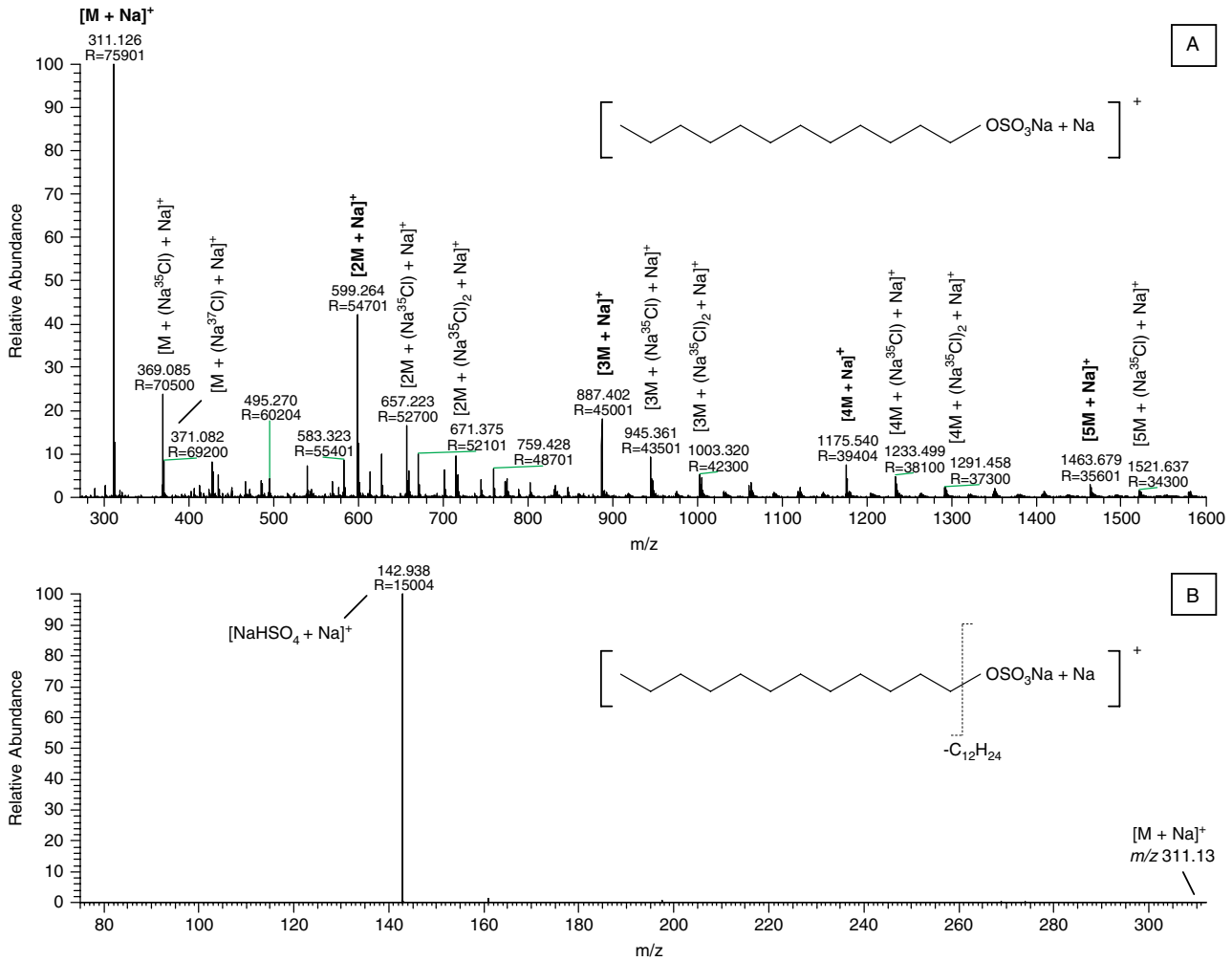

Figure 12. High accuracy mass spectrum of SDS in type 2 application pieces (2A). Under nano-ESI(+) conditions and in solvents containing 10 mM NaCl alkyl sulfates ionized as both Na^+ adduct and NaCl cluster ions. Also note the formation of dimers, trimers, tetrames, and pentamers and the presence of $Na^{35}Cl$ and $Na^{37}Cl$ clusters ($\Delta m/z$ of 1.997 between ^{35}Cl and ^{37}Cl). A representative MS/MS spectrum is shown in (B). In positive ion mode alkyl sulfates formed only one main product ion ($[NaHSO_4 + Na]^+$, *m/z* 142.938), resulting from the neutral loss of the alkyl chain. The CID spectrum shown was generated from the C_{12} sulfate precursor ion (*m/z* 311.126).

Presence of alkyl sulfates

In addition to alkyl ethoxysulfates with variable oligomerization degree, the non-ethoxylated species were also detectable in the eluate of the defective application pieces. They form another class of detergents which are commonly known as alkyl sulfates. Sodium dodecyl sulfate (SDS) – the C12 homolog – is the most prominent representative of this group. Their technical synthesis is similar to the synthesis of alcohol ethoxysulfates, i.e. fatty alcohols (typically from C_8 to C_{18}) are sulfonated by reaction with SO_3.[43] Since the starting material for the production of ethoxysulfates is ethoxylated alcohols (*vide supra*) and since these feedstocks frequently contain significant amounts of non-ethoxylated alcohols, the formation of alkyl sulfates as byproducts of the synthesis of alcohol ethoxysulfates is inevitable. This explains why these three classes of detergents were detectable in type 2 application pieces.

High accuracy full-scan MS spectra revealed the presence of at least C_{10} (*m/z* 283.095), C_{12} (*m/z* 311.126), C_{13} (*m/z* 325.142), C_{14} (*m/z* 339.158), and C_{16} (*m/z* 367.189) alkyl sulfates. Since they were analyzed in positive ion mode and under conditions favouring the formation of sodiated species (presence of 10 mM NaCl in the eluents) the alkyl sulfates were detected as $[C_mH_{2m+1}OSO_3Na + Na]^+$ ions. However, contrary to alcohol ethoxylates and alcohol ethoxysulfates they additionally formed series of NaCl cluster ions ($\Delta m/z$ 58) of the general type $[C_mH_{2m+1}OSO_3Na + (NaCl)_n + Na]^+$ as well as series of di-, tri- and oligomers, which themselves formed NaCl cluster ions, i.e. ions following the formula of $[(C_mH_{2m+1}OSO_3Na)_{x>1} + Na]^+$ and $[(C_mH_{2m+1}OSO_3Na)_{x>1} + (NaCl)_n + Na]^+$. Figure 12A shows a representative spectrum of an alykyl sulfate found in defective application pieces, which is the spectrum of SDS under sodiating nano-ESI(+) conditions. An additional study under ESI(-) conditions – the preferable mode for analyzing alkyl sulfates[40,46–47] – was not performed, since accurate mass information as well as NaCl cluster ion formation and CID fragmentation were completely identical to the spectra of the SDS standard under these conditions (data not shown). Accurate mass information was especially important since other detergents also generate ions with a nominal mass of *m/z* 311 in positive ion mode (e.g. the accurate mass of the C_{12} alcohol ethoxylate ion $[C_{13}H_{27}(OCH_2CH_2)_2OH + Na]^+$ is *m/z* 311.256, the mass of the monoethoxylated stearic acid ion $[C_{17}H_{35}CO-$

$OCH_2CH_2]^+$ – a product ion generated by the fragmentation of the non-ionic detergents Tween-20, 40, 60, and 80[48] – is *m/z* 311.294). The positive ion mode MS/MS spectra of all observed alkyl sulfates showed only one main fragment ion (*m/z* 142.938), which resulted from the neutral loss of the alkyl group and the formation of the $[NaHSO_4 + Na]^+$ ion (Figure 12B).

Type 3 ('homemade') application pieces

No polymers were detectable in the homemade application pieces (e.g. Hybond blotting paper; Figure 6E). Only common background ions characteristic of the ESI-process were observable (e.g. phthalic anhydride, *m/z* 149, dibutylphthalate, *m/z* 279, and polysiloxanes, *m/z* 371 and 445).[49]

Conclusion

Due to an incorrect (i.e. EPO-incompatible) impregnation some batches of commercial application pieces (bought from GE Healthcare and Serva between 2007 and 2010) might lead to lane streaking during IEF-PAGE of EPO, which is also accompanied by a loss in sensitivity and particular loss in resolution in the acidic region of the gel. A mass spectrometric investigation revealed that defective application pieces contained a complex mixture of alcohol ethoxylates, alcohol ethoxysulfates, alkyl sulfates (including SDS), and a few other non-identified ethoxylates. Mixtures like this are characteristic for technical surfactants. Ionic detergents in general (except zwitterionics) are incompatible with isoelectric focusing, anionic detergents as in the present case (i.e. sulfates) in particular with focusing in acidic pH-gradients and cathodic sample application. The presence of these surfactants explains the distortions observed when type 2 application pieces were used. An evaluation of EPO-profiles containing smears or partly absent signals for doping control purposes is prohibited according to the articles of the technical document on EPO-analytics (TD2009EPO) of the World Anti-Doping Agency (WADA). Casting gels with wells appears to be the best solution in order to completely avoid running the risk of using defective application pieces.

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