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Mass spectrometric analysis of EPO IEF-PAGE interfering substances in nitrile examination gloves

Christian Reichel*

Direct detection of doping with recombinant erythropoietins (rhEPO) is accomplished by isoelectric focusing (IEF) or sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). In a recent publication, Lasne *et al*. (Electrophoresis 2011, 32, 1444) showed that improper use of nitrile examination gloves during sample collection, sample preparation, and IEF-PAGE may lead to distorted or absent EPO IEF-profiles. In order to clarify which substances are responsible for this observation, a mass spectrometric study on water extractable compounds found in nitrile gloves was performed. Several substance classes were shown to be present, among them polyethylene glycols (PEG), anionic and nonionic surfactants, as well as alcohol ethoxylates and plasticizers. It could be demonstrated that alkylbenzenesulfonates, the main category of detectable anionic detergents, and among them sodium dodecylbenzenesulfonate (SDBS) and its homologs, are the prime reason for the interference of nitrile gloves with EPO IEF-PAGE. Copyright © 2012 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: erythropoietin; nitrile gloves; isoelectric focusing; doping control; IEF-PAGE; gloves

Introduction

During the past 12 years, since the development of a polyacrylamide gel electrophoresis (PAGE) based isoelectric focusing (IEF) method for the direct detection of doping with recombinant erythropoietins (rhEPO),[1] the IEF-PAGE method has been widely employed by doping control laboratories in routine testing. Several years later, sodium dodecylsulfate gel electrophoresis (SDS)-PAGE was introduced as a second direct detection method for the abuse of rhEPO.[2,3] Today, both methods are frequently employed for screening and confirmation purposes in anti-doping control.[4,5]

However, IEF-PAGE is prone to disturbances by ionic substances (e.g. salts), which have to be reduced to a minimum in order to generate well-resolved bands. The negative effect of unfractionated (high molecular weight) heparins[6] and anionic detergents[7] on the performance of EPO IEF-PAGE was demonstrated in 2010. Both groups of substances lead to streaked or in the worst case completely absent EPO profiles. In 2011, it was further shown that the usage of nitrile gloves may also lead to distortions or profile losses. It was speculated that anionic detergents might be the reason for this observation.[8] The production of nitrile butadiene rubber (NBR) involves emulsifiers, which are frequently anionic or non-ionic surfactants.[9–11] The latter group of substances may also lead to streaked EPO profiles if the hydrophobic tail contains linear alkyl groups instead of branched or cyclic ones.[12] Depending on the chain length, detergents with linear alkyls form inclusion compounds with the urea contained in IEF-gels and thus are no longer fully available for efficient protein solubilization.[12] In order to investigate which compounds might be the reason for the interference of nitrile examination gloves with EPO IEF-PAGE, a mass spectrometric study on water extractable substances found in nitrile as well as latex and vinyl gloves was performed.

Experimental

Materials

Disposable examination gloves were bought from SHIELD Scientific (*SHIELDskin Orange Nitrile*; Arnhem, the Netherlands), B. Braun (*Vasco Nitrile light*; Melsungen, Germany), Microflex Corporation (*MICROFLEX FreeForm SE Nitrile*; Reno, NV, USA), Carl Roth GmbH (*Rotiprotect Nitrile*; Karlsruhe, Germany), Best Manufacturing (*N-DEX PLUS Nitrile*; Wommelgem, Belgium), Süd-Laborbedarf GmbH (*SafeGrip Nitrile*; Gauting, Germany), VWR International (*VWR Nitrile*, *VWR Vinyl*; Leuven, Belgium), and Ansell Healthcare (*proFood Nitrile*, *Conform + Latex*; Brussels, Belgium). All gloves were powder free.

Phosphoric acid (85%, p.a.), glacial acetic acid (p.a.), and methanol (LiChrosolv, gradient grade) were from Merck (Darmstadt, Germany). Ultra liquid chromatography/mass spectrometry (ULC/MS) grade acetonitrile (ACN), formic acid (FA), and water were obtained from Biosolve (Valkenswaard, the Netherlands). Standards for surface active compounds and polymers were from Sigma-Aldrich (St. Louis, MO) (PEG-200, PEG-1000, Brij 30, Brij 52, Brij C10, Brij S10 and S20, Igepal CA-720, Igepal CO-720, Nonidet P40 substitute, sodium dodecylbenzenesulfonate (SDBS), sodium dodecyl sulfate, diisononyl phthalate (DINP)) and Thermo/Pierce (Rockford, IL, USA) (Surfact-Amps Triton X-100, NP-40, and Tween-80; 10% (v/v)). Materials for gel-casting and performing IEF-PAGE were bought from GE

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* *Correspondence to: Christian Reichel, Doping Control Laboratory, AIT Seibersdorf Laboratories, A-2444 Seibersdorf, Austria. E-mail: christian.reichel@seibersdorf-laboratories.at*

Doping Control Laboratory, AIT Seibersdorf Laboratories, A-2444, Seibersdorf, Austria

Healthcare (Uppsala, Sweden) (glass plates (200 x 260 x 4 mm, 200 x 260 mm with 1.0 mm rubber U-frame), GelBond PAG film, (203 x 260 mm), FlexiClamps, PlusOne ReadySol IEF acrylamide/ bisacrylamide solution (40% T, 3% C), PlusOne urea, ammonium peroxidisulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), electrode strips, Multiphor II slab-gel electrophoresis chamber, EPS 3500 XL power supply), Serva (Heidelberg, Germany) (Servalytes 2–4, 4–6, 6–8), and Sigma-Aldrich (sucrose (electrophoresis grade), methyl red, bovine serum albumin (BSA; ELISA grade)). For Western blots materials from Millipore (Billerica, MA) (Durapore, Immobilon-P membranes), GE Healthcare (tris(hydroxymethyl)aminomethane (Tris; PlusOne), glycine (PlusOne), NovaBlot blotting paper, and Blotting Paper 21 x 26 cm), BioRad (Hercules, CA, USA) (Trans-Blot SD blotter, non-fat dry milk (NFM)), Sigma-Aldrich (DL-dithiothreitol (DTT), phosphate buffered saline (PBS)), R&D Systems (Minneapolis, MN, USA) (cone AE7A5 anti-EPO antibody), Thermo/Pierce (ImmunoPure biotinylated goat anti-mouse IgG (H + L), West Femto), Biospa (Milano, Italy) (streptavidin horseradish peroxidase (HRP) complex) were employed. For image acquisition a CCD-camera from Fujifilm (LAS-4000; Tokyo, Japan) was used together with an image analysis software from Seibersdorf Laboratories Austria (GASepo version 2.1). Buffers and solutions were prepared with MilliQ (MQ) water (Millipore). Reference standards for recombinant human erythropoietin (rhEPO; BRP-EPO batch 3) and human urinary EPO (uhEPO; second international reference preparation) were received from the European Directorate for the Quality of Medicines (Strasbourg, France) and the National Institute for Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, UK). Darbepoetin alfa (Aranesp (NESP)) was from Amgen (Thousand Oaks, CA, USA).

All mass spectrometric analyses were performed on an LTQ-Orbitrap mass spectrometer (Thermo Electron; Bremen, Germany) equipped a Proxeon nano-electrospray ionisation (ESI) source (Odense, Denmark). Glass emitters for static (coated; 4 ± 1 μm tip opening) and dynamic (online) nano-ESI experiments (uncoated; 360 μm OD, 75 μm i.d., 15 μm tip i.d.) were from New Objective (Woburn, MA, USA). For online mode the ion source was coupled to an Ultimate 3000 nano-HPLC system (Dionex; Sunnyvale, CA, USA). Acclaim PepMap 100 columns (75 μm i.d.; C18, 3 μm, 100 Å) were used for pre-concentration (2 cm) and analyte separation (15 cm) and sodium chloride (NaCl) served as cationizing agent. The LTQ ESI positive ion calibration solution was from Pierce/Thermo.

Methods

Extraction of water-extractable substances from examination gloves

The index finger was cut off from each glove and put into a glass beaker containing 2 ml of ULC/MS-grade water. After 15 to 30 min without shaking, the finger was removed and the extract centrifuged for 15 min at 16100 *rcf*. For static nano-ESI experiments, the supernatant (80 μl) was mixed with 20 μl of aqueous 100 mM NaCl solution and 100 μl acetonitrile. Nano-LC experiments were performed with undiluted extracts. A negative control sample was generated by using the same protocol but without the glove finger. Kinetics of the extraction process was studied by incubating fingers for a defined period of time in water as described above (1 s, 2 s, 5 s, 15 s, 1 min, 2 min, 5 min, 10 min).

Preparation of surfactant and polymer standards for mass spectrometry

All standards (*vide supra*) were dissolved in a solution containing 10 mM NaCl and 50% (*v/v*) acetonitrile and at a concentration of 1 mg/ml or 0.1 % (*v/v*). Brand new glass flasks were used for the preparation of the standards in order to rule out cross-contamination from residues of rinsing agents.

Mass spectrometric analysis of water-extractable compounds in gloves

Before analysis, the mass spectrometer was calibrated with a ready-made calibration solution containing caffeine/MRFA tetrapeptide/ Ultramark 1621 according to the manufacturer's instructions. During online analysis, the instrument was additionally lock-mass recalibrated using the mass of the sodiated polysiloxane ion (*m/z* 467.101968).[13,14] All spectra we recorded in positive ion mode. Static (*offline*) nanospray experiments were performed both in the iontrap- and Fourier transform (FT)-part of the LTQ-Orbitrap instrument. Spray voltage and capillary temperature were 1.8 kV and 200 °C, the capillary and tube lens voltages were 140 V and 80 V, respectively. Automatic gain control target settings were $1{*}10^4$ / $2{*}10^5$ (iontrap / orbitrap) for full scan and $1{*}10^4$ / $1{*}10^5$ for MS^n spectra. The iontrap was operated at a maximum injection time of 10 ms and 3 microscans, the orbitrap analyzer at 100 ms maximum injection time, 1 microscan and a resolution setting of R = 60000 (@ *m/z* 400). Between 100 and 200 sub-spectra were recorded for each static nano-ESI experiment and then merged into one spectrum for further analysis. Typical mass ranges were from *m/z* 50 or 100 to 2000. Helium was used as damping and collision gas. For MS^n fragmentations, normalized collision energies (NCE) and activation times were optimized individually for each precursor ion and were typically between 20–60 and 30–75 ms, respectively. For each extract and standard a new emitter was used in order to rule out cross contaminations between experiments. Solvents for *online* nano-LC separations were 2% ACN / 10 mM NaCl / water (A) and 90% ACN / 10 mM NaCl / water (B). The system was operated in pre-concentration mode and with 2% ACN / 10 mM NaCl / water as loading solvent (2 μl/min). Ninteen μl of extract were loaded on the trap column (10 min) and then separated on the analytical column with a linear gradient from 2% B to 95% B in 45 min, followed by 25 min column regeneration at 100% B (300 nl/min, 40 °C column oven temperature). Spray voltage, capillary temperature, and capillary and tube lens voltages were 2.0 kV, 160 °C, 37 V and 175 V, respectively. AGC target settings were the same as for the offline nano-ESI experiments. Full scan spectra (*m/z* 50–1600) were acquired in the orbitrap analyzer at R = 60000, and MS/MS spectra in the iontrap in data-dependant acquisition (DDA) mode (one FT-scan followed by collision-induced dissociation (CID) of up to six of the most intense ions; default charge state +1, fixed NCE at 55, activation time 30 ms, dynamic exclusion 60 s).

IEF-PAGE and Western blot

Polyacrylamide gels (5% T / 3% C, pH 2–6) containing carrier ampholytes (Servalytes 2–4 and 4–6, 2% (*w/v*) each), 7 M urea and sample application wells were prepared as described elsewhere.[7,15] Gels were prefocused at constant voltage (250 V, 30 min; catholyte: 2% (*w/v*) Servalyte 6–8 in MQ water plus 10 μl methanolic methylred solution (1 mg/ml), anolyte: 0.5 M phosphoric acid) and then samples and standards were applied and focused for 3600 Vh at constant wattage (25 W; 10 °C). Samples were prepared by spiking 2 and/or 4 μl of each glove-extract into 0.2 ng of BRP-, NESP- and NIBSC-EPO standards. In order to study the effect of the extracts under 'regular' EPO IEF-PAGE conditions, both the standards and samples were supplemented with 1% (*v/v*) Tween-80.[4] For specifically investigating the effect of SDBS on IEF-PAGE and in particular in combination with Tween-80, different concentrations of SDBS (0.0, 0.02, 0.2%) and Tween-80 (1 and 5%) were spiked into EPO standards

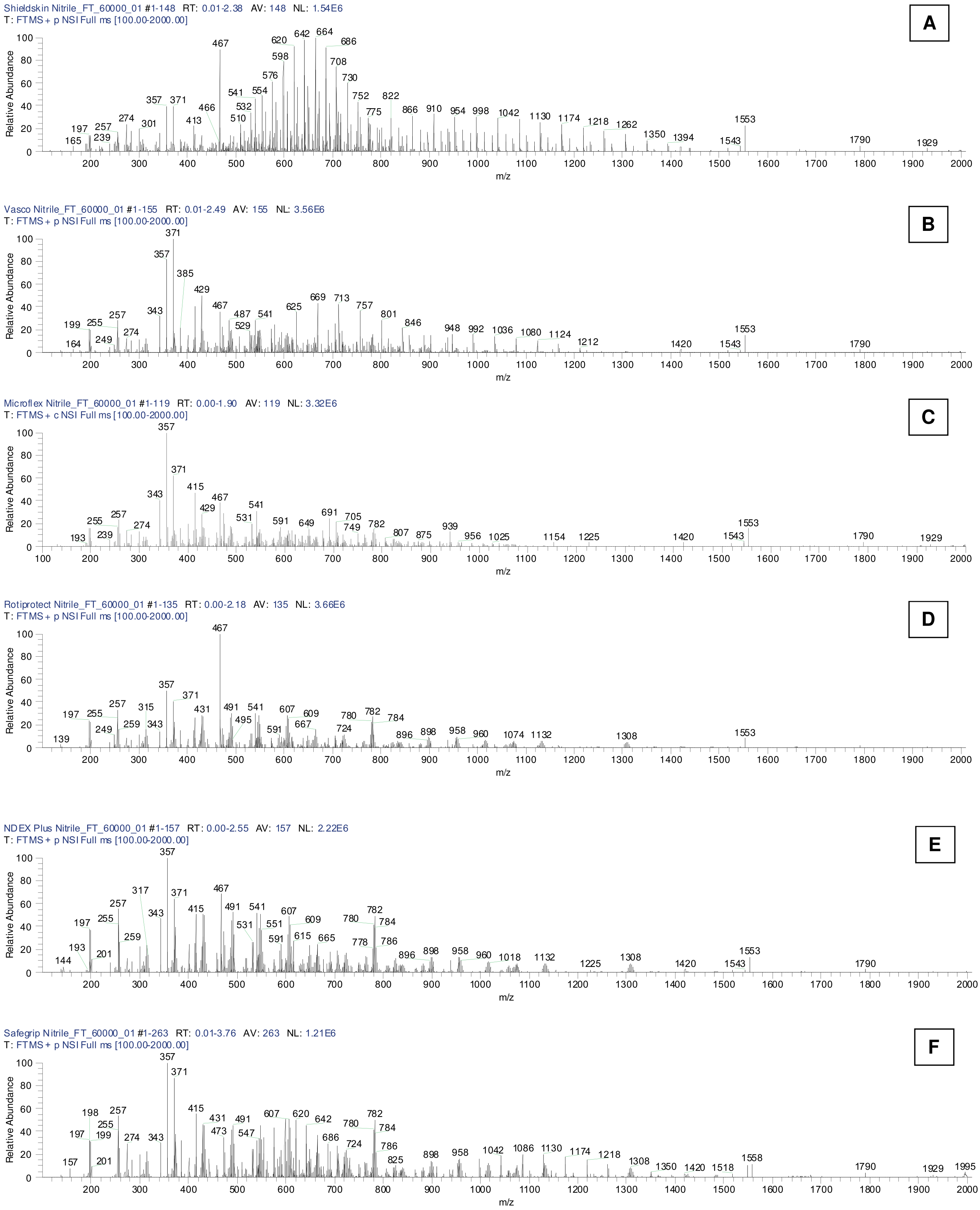

Figure 1. Results of the static nano-ESI high resolution (R = 60000) high accuracy experiments for SHIELDskin Orange Nitrile (A), Vasco Nitrile light (B), MICROFLEX FreeForm SE Nitrile (C), Rotiprotect Nitrile (D), N-DEX PLUS Nitrile (E), SafeGrip Nitrile (F), VWR Nitrile (G), proFood Nitrile (H), Conform + Latex (I), VWR Vinyl (J) gloves and the solvent blank (K). In addition to ethoxy-group containing polymers (series of peaks spaced in intervals of *m/z* 44 ($z = 1$), 22 ($z = 2$), etc.) (A, B, F), a group of ions with *m/z* 343, 357, 371, 385 was detectable in all nitrile gloves (A-H). The spectrum of the blank (10 mM NaCl / 50% ACN) shows mainly ions of the $[Na + (NaCl)_n]^+$ cluster type.

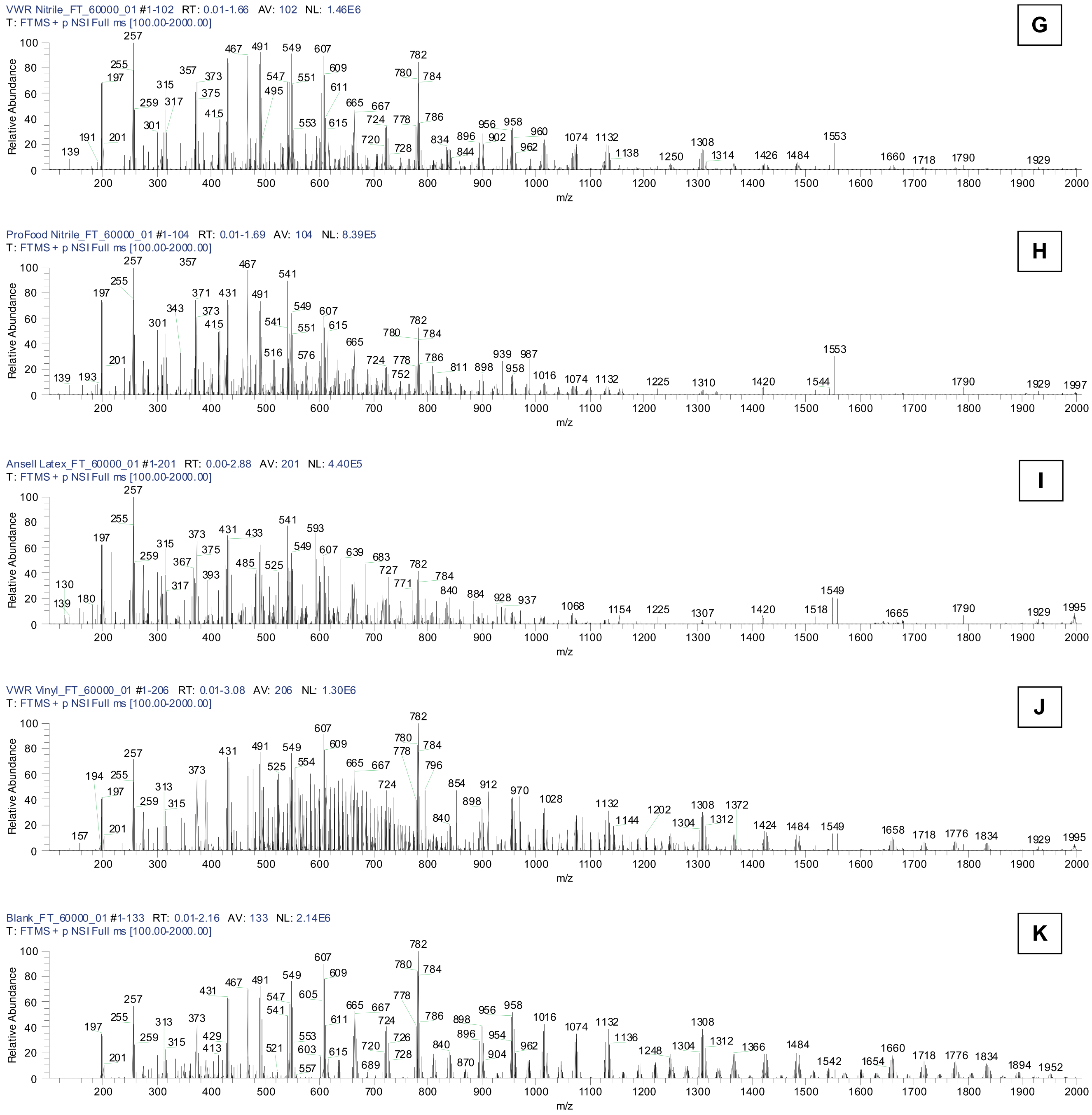

Figure 1. Continued

(ratios SDBS:Tween-80 of 1:5, 1:25, 1:50, 1:250). The influence of Triton X-100 and NP-40 on EPO standards was tested by combining different concentrations of these detergents (0.01, 0.05, 0.1, 0.5, 1.0, 5.0% (*v/v*)) with a fixed concentration of Tween-80 (1% (*v/v*)).

Western double-blots were performed as previously published.[16] Briefly, gels were equilibrated in blotting buffer (25 mM Tris / 192 mM glycin), released from the GelBond and semi-dry blotted to an Immobilon-P membrane (1 mA/cm^2, 30 min). A Durapore membrane served as separator membrane. After a reduction step (5 mM DTT / PBS; 45 min) the membrane was blocked (5% (*w/v*) NFM / PBS; 60 min) and incubated in primary antibody solution overnight (clone AE7A5, 1:1000 (*v/v*) in 1% NFM / PBS, coldroom, 4-8 °C). The second blot used 0.7% acetic acid as transfer buffer (0.8 mA/cm^2, 10 min) and the same blocking buffer as the first blot. Incubations in secondary antibody (biotinylated goat anti-mouse antibody, 1:2000 (*v/v*)) and streptavidin HRP (1:2000 (*v/v*)) were for 60 min at room temperature. All washing steps were done with 0.5% NFM / PBS and PBS (3 x 7 min). For the development step, the membrane was put on a clean glass plate and overlaid with chemiluminescent substrate (West femto). Images were acquired with a CCD-camera (LAS-4000) and typical exposure times between 30 and 120 s. All blots were performed with the same blotter (Trans-Blot SD unit).

Results and discussion

The majority of the glove extracts showed slight to moderate foam formation after manual shaking. Hence, it was concluded that surface active compounds might be present in the extract. A similar observation was already made for sample application pieces, which have been frequently used for isoelectric focusing with slab-gels.[7] The compounds present in these application pieces were successfully identified using nano-electrospray ionization (nano-ESI) of the sodiated species in combination with a high resolution high accuracy mass spectrometer (LTQ-Orbitrap). Many non-ionic emulsifiers used in the production of nitrile gloves are polyethylene glycol (PEG) based polymers.[10,11] Protonated PEG species are frequently instable in the gas-phase and undergo in-source fragmentation reactions.[7,17] Contrary to that, cationization of polyglycols with alkali ions (Na^+, K^+, Li^+) leads to formation of stable adduct ions, which easily fragment under low energy CID conditions.[18–20] Hence, MS/MS spectra of these ions have been preferably used for determining the polymerization degree as well as the type of the hydrophobic head-group of the detergent (e.g. for alkyl- or alkyl phenol-group containing PEG-polymers). It was further shown that anionic surfactants are also accessible to structural identification under the selected cationizing conditions. Hence, positive ion mode was used for studying the composition of glove extracts.

Table 1. Polyethylene glycol oligomers and their chain length distributions in the investigated glove extracts

Brand	Typical PEG chain length (n)
SHIELDskin Orange Nitrile	7-66
Vasco Nitrile light	7-60
MICROFLEX FreeForm SE Nitrile	16-68
Rotiprotect Nitrile	7-48
N-DEX PLUS Nitrile	7-43
SafeGrip Nitrile	7-43
VWR Nitrile	5-43
proFood Nitrile	5-54
Conform + Latex	6-48
VWR Vinyl	6-24

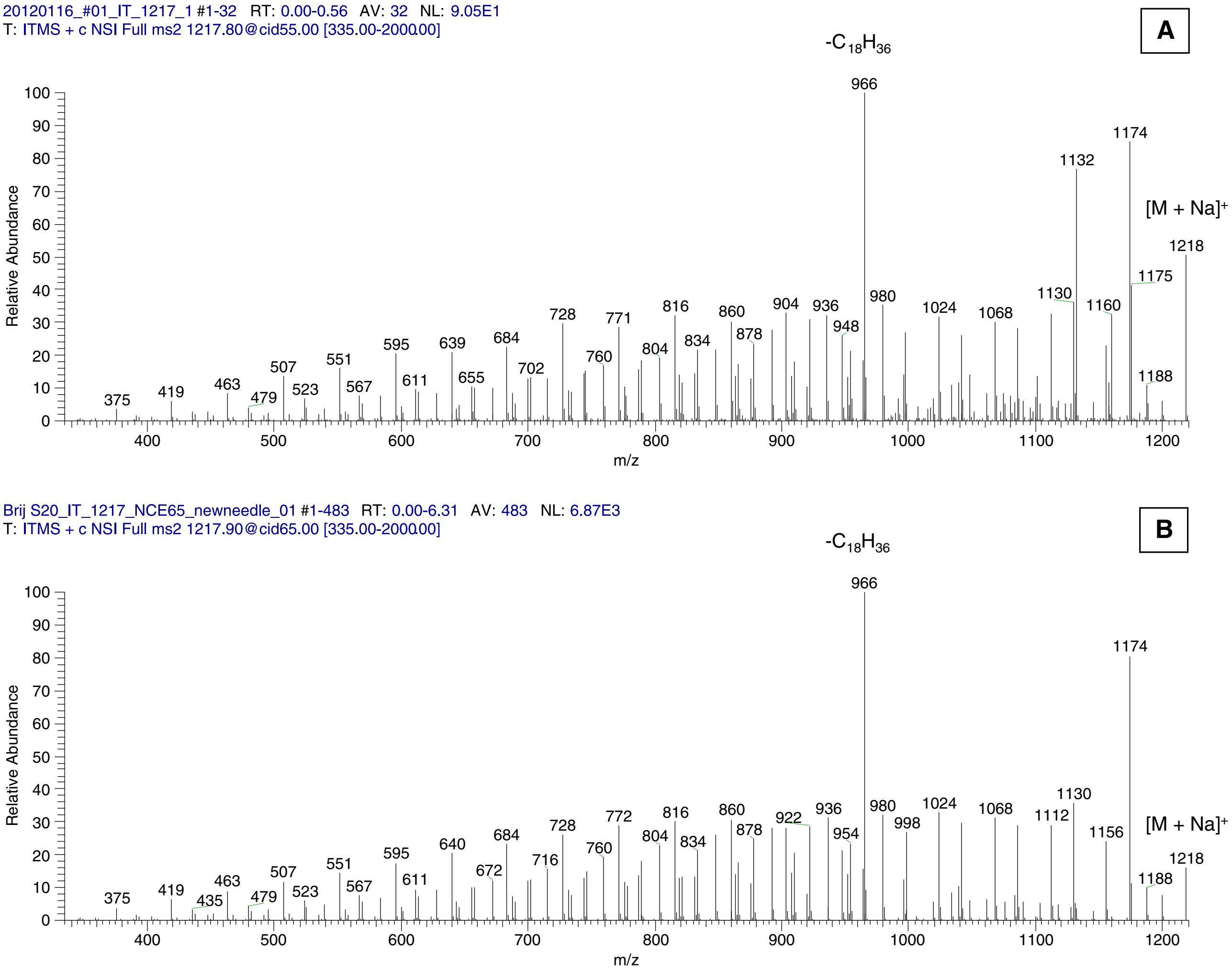

Figure 2. MS/MS spectrum of the C_{18} alcohol ethoxylate ion *m/z* 1217.8320 as found e.g. in SHIELDskin nitrile gloves (A) and the reference substance Brij S20 (B). A neutral loss of $C_{18}H_{36}$ is observed together with the characteristic ion series of PEGylated compounds (b_n, c_n, x_n, z_n, c_n'', z_n''). Refer to Jackson[23] for additional details.

Table 2. Typical water extractable alcohol ethoxylates as observed in glove extracts

Brand	Alcohol ethoxylates									
	C_2 to C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	C_{19} to C_{23}
SHIELDskin	-	-	-	-	+	-	-	+	-	+	-
Vasco	-	-	-	-	+	-	-	+	-	+	-
MICROFLEX	-	+	+	+	-	+	-	+	-	-	-
Rotiprotect	-	-	-	-	-	-	-	+	-	-	-
N-DEX	-	+	+	+	+	-	-	-	-	-	-
SafeGrip	-	-	-	-	-	-	-	+	-	-	-
VWR Nitrile	-	-	-	-	-	-	-	+	-	-	-
proFood Nitrile	-	-	-	+	-	+	-	-	-	-	-
Conform + Latex	-	-	-	-	-	-	-	-	-	-	-
VWR Vinyl	+	+	+	+	+	+	+	+	+	+	+

Results of offline nano-ESI experiments

Due to its high ionization efficiency and rapid analytical set-up, static nano-ESI experiments provide a quick overview about specific compounds and compound groups present in detergent containing specimens.[7] The full scan MS spectra obtained under these conditions from aqueous glove-extracts are shown in Figure 1. Ion-series typical for PEG-containing polymers were immediately observable for *SHIELDskin Orange Nitrile*, *Vasco Nitrile light* and *SafeGrip Nitrile* gloves (homologous series of peaks spaced in mass intervals of 44.0262 ($z = 1$), 22.0131 ($z = 2$), and 14.6754 ($z = 3$); e.g. Figures 1A, 1B, 1F, 1I). Detailed inspection of the remaining nano-ESI spectra revealed that PEG-polymers were present in all of the ten tested gloves including those, which showed no interference with EPO IEF-PAGE (*vide infra*).

Depending on the amount of available ionisable compounds, the formation of NaCl cluster ions is more or less pronounced. In case that no other ions than Na^+ and Cl^- are present, the spectrum only consists of a series of cluster ions of the $[(NaCl)_n + Na]^+$ type. Additional complexity is added by chlorine isotopes ^{35}Cl and ^{37}Cl. Hence, the $[(NaCl)_n + Na]^+$ clusters are actually composed of $Na^{35}Cl$ and $Na^{37}Cl$, i.e. all combinations of $[(Na^{35}Cl)_{n\text{-}x} + (Na^{37}Cl)_x + Na]^+$ with $0 \leq x \leq n$ (Figure 1K). Clusters started with accurate masses of 80.9478 and 82.9449 for the adduct ions $[(Na^{35}Cl)_1 + Na]^+$ and $[(Na^{37}Cl)_1 + Na]^+$ and were observable over the entire mass range up to *m/z* 2000. With increasing concentration of additional ionisable compounds the formation of these clusters was gradually suppressed. Hence, it was concluded that gloves like *N-DEX PLUS Nitrile*, *VWR Nitrile* or *proFood Nitrile* contained less interfering substances than e.g. *SHIELDskin Orange Nitrile* or *Vasco Nitrile light*.

Except for the NaCl cluster ions, several additional masses were present in all offline nano-ESI spectra. Based on the accurate masses these ions were identified as the sodiated counterparts of commonly present nano-ESI background ions, i.e. ions *m/z* 393.083, 467.102, 541.120, 615.139, 689.158, 763.177 were attributed to sodiated polysiloxanes and ions *m/z* 301.141 and 413.266 to sodiated dibutyl- and dioctylphthalates, respectively. Due to its high intensity *m/z* 467.101968 was used as lock mass for online mass recalibration in static and also nano-LC-MS mode (under protonating conditions, *m/z* 445.120025 is a frequently used lock mass, i.e. *m/z* 445.120025 – H^+ + Na^+ = *m/z* 467.101968).

Among the prominent ions, which were only found in the spectra of nitrile gloves, were *m/z* 343.131, 357.147, 371.163, and 385.178. From the mass difference of 14.016 between these ions it was obvious that they represented homologs of a single compound differing only in the number of methyl-groups. The mass *m/z* 371.163 was identified as the mass of sodium dodecylbenzenesulfonate, an anionic emulsifier frequently used in nitrile production (*vide infra*). Studies on the kinetics of the extraction process revealed that already after two seconds incubation time, SDBS (including homologs) and alcohol ethoxylates were detectable in the aqueous extract of the glove-outside (*SHIELDskin Orange Nitrile* gloves; Supplementary Figure 1). Contrary to that, the inside of the fingers showed significantly different kinetics, which resulted in lower amounts of alcohol ethoxylates but increased quantities of SDBS and homologs in the extract (*SHIELDskin Orange Nitrile*; Supplementary Figure 2). Since the outside of gloves primarily gets into contact with samples and electrophoretic materials, only extracts of this side were used for further investigation.

In summary, static nano-ESI experiments demonstrated the presence of ethoxylates and sodium dodecylbenzenesulfonate as possible IEF-PAGE interfering substances in nitrile gloves.

Results of online nano-LC-ESI-MS and MS/MS experiments

The offline experiments showed that some of the extracts (e.g. *SHIELDskin Orange Nitrile, VWR Vinyl*) contained complex mixtures of polyethylene glycol derived compounds, which required LC-separation for structural identification in addition to acquisition of high resolution high accuracy MS and MS/MS spectra. For spectra interpretation, the mass defect of these polymers relative to the non-derivatized PEG-polymer was used. Since ethoxy-groups only contain elements C, H, and O, which

Table 3. Presence of octyl- and nonylphenol ethoxylates in examination gloves

Brand	Octylphenol ethoxylates	Nonylphenol ethoxylates
SHIELDskin	+	-
Vasco	+	+
MICROFLEX	+	-
Rotiprotect	-	+
N-DEX	-	-
SafeGrip	-	-
VWR Nitrile	-	+
proFood Nitrile	+	-
Conform + Latex	-	-
VWR Vinyl	-	-

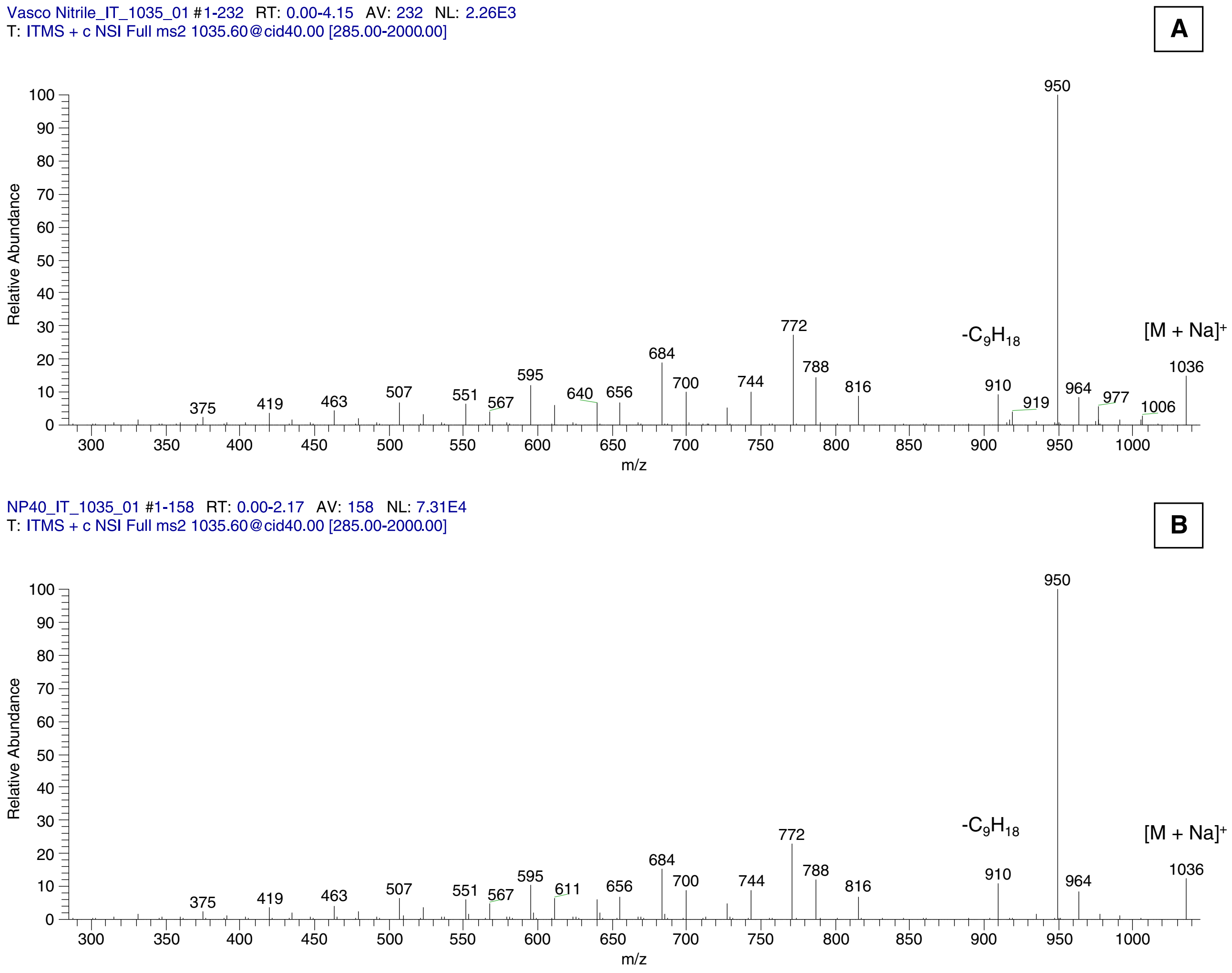

Figure 3. CID spectrum of one oligomer of nonylphenol ethoxylate (*m/z* 1035.6438). Aside from the typical fragmentation reactions of the ethoxy-group (x_n, z_n, c_n, c_n''),[23] cleavage of the nonylphenol unit is observed. (A) Vasco nitrile gloves, (B) NP-40 standard.

contribute with mass defects of 0, +0.00783, and −0.00509 to the monoisotopic mass of the PEG-polymer, each deviation from this mass must be caused by additional elements and/or additional groups with different C/H/O ratios. Hence, increases in mass relative to the mass of the corresponding PEG can be explained by the presence of additional elements with positive (e.g. N (+0.00307)) and/or zero (C) mass defects, decreases in mass on the other hand by additional elements with negative mass shifts (e.g. O, P (−0.02624), S (−0.02793)). This strategy has already been used for the identification IEF-PAGE interfering substances in sample application pieces.[7,21,22]

Presence of polyethylene glycols

Non-derivatized PEG ($H(OCH_2CH_2)_nOH$) was detectable in all of the tested gloves, including those brands based on latex and vinyl polymers. Table 1 summarizes typical PEG chain lengths found in the ten tested brands and within the mass range of *m/z* 50–1600. Charge states up to +4, i.e. ions of the $[H(OCH_2CH_2)_nOH + 4\ Na]^{4+}$ type, and chain lengths between n = 5–68 were observed (data not shown). Polyethylene glycols are used as non-aqueous solvents in the nitrile emulsion polymerization process.[10]

Presence of alcohol ethoxylates

Successful polymerization of nitrile rubber monomers (2-propenenitrile and butadiene monomers (e.g. 1,2-butadiene, 1,3-butadiene)) requires surfactants, which act as emulsifiers during polymerization. Typically, anionic detergents (*vide infra*) or a combination of anionic

Table 4. Presence of SDBS and homologs in glove extracts

Brand	Sodium alkylbenzene sulfonates				
	C_9	C_{10}	C_{11}	C_{12}	C_{13}
SHIELDskin	+	+	+	+	+
Vasco	+	+	+	+	+
MICROFLEX	+	+	+	+	+
Rotiprotect	+	+	+	+	+
N-DEX	+	+	+	+	+
SafeGrip	+	+	+	+	+
VWR Nitrile	+	+	+	+	+
proFood Nitrile	+	+	+	+	+
Conform + Latex	-	-	-	-	-
VWR Vinyl	-	-	-	-	-

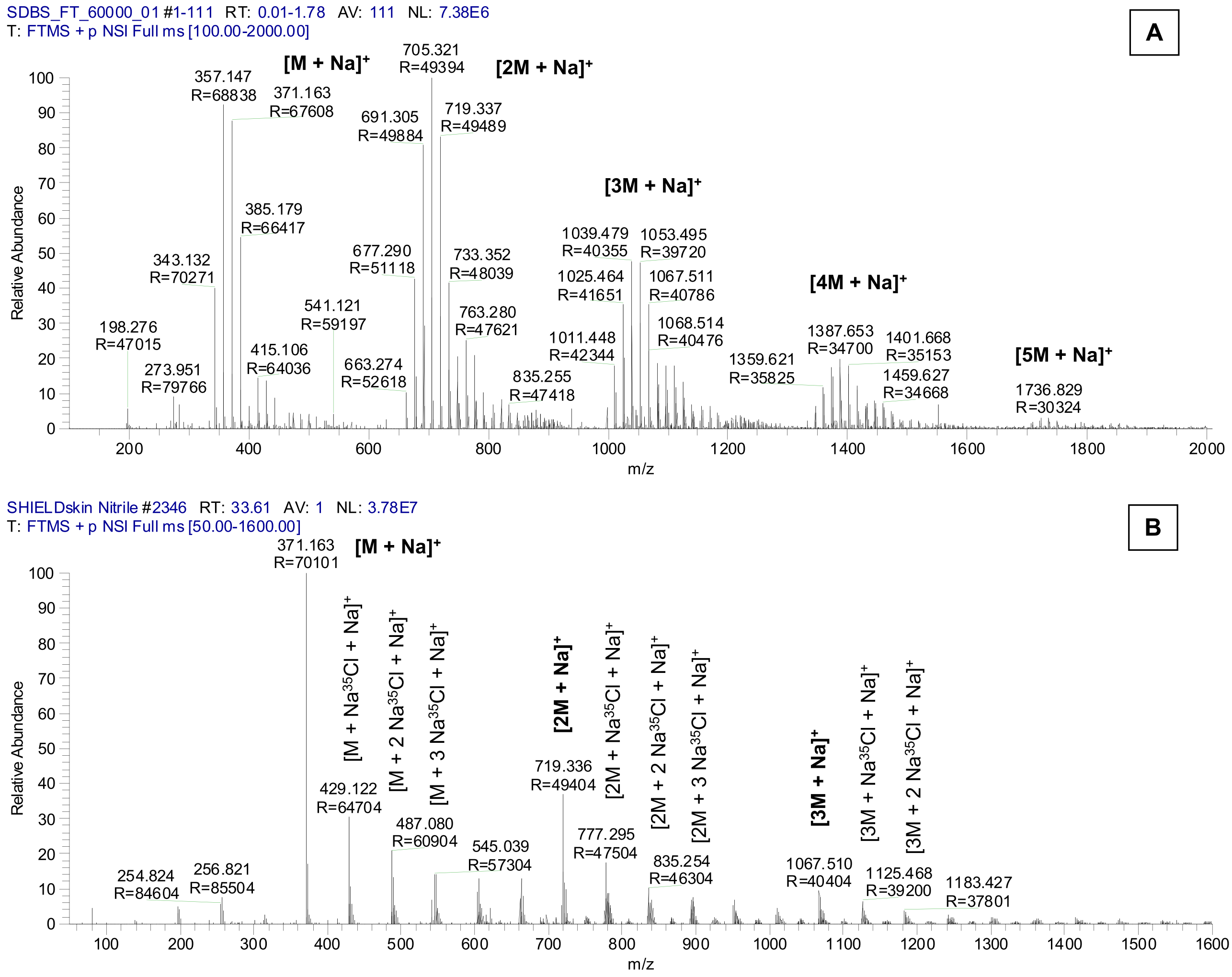

Figure 4. High resolution (R = 60000) high accuracy spectra of SDBS (*m/z* 371.1627) and homologs (*m/z* 343.1314, 357.1471, 385.1784) found in all nitrile gloves (shown here for SHIELDskin) (A) and the SDBS reference (B). Note the characteristic formation of di-, tri- and oligomeric adduct ions with Na^+ and NaCl cluster ions of the $[M + (Na^{35/37}Cl)_n + Na]^+$ type. Only $Na^{35}Cl$ clusters are labelled (B).

and nonionic detergents is used. Among the many suitable nonionic surfactants, alcohol ethoxylates are frequently applied. Alcohol ethoxylates (AEO) were found in nearly all of the investigated nitrile but also latex and vinyl gloves. Alkyl-group chain-lengths between C2 and C23 were observed. Under the employed low-energy CID-conditions, all of the characteristic fragment ion series of this compound class were generated, i.e. $[C_mH_{2m+1}(OCH_2CH_2)_n\text{-}OH + Na]^+$ fragmented into ions of the b_n ($[H_{2m+1}C_mO\text{-}[CH_2CH_2O]_{n-1}\text{-}CH = CH_2 + Na]^+$), c_n ($[H_{2m+1}C_mO\text{-}[CH_2CH_2O]_{n-1}\text{-}CH_2CH = O + Na]^+$), x_n ($[H_2C = CH\text{-}[OCH_2CH_2]_{n-1}\text{-}OH + Na]^+$), z_n ($[O = CHCH_2\text{-}[OCH_2CH_2O]_{n-1}\text{-}OH + Na]^+$), c_n'' ($[H_{2m+1}C_mO\text{-}[CH_2CH_2O]_n\text{-}H + Na]^+$) and z_n'' ($[H\text{-}[OCH_2CH_2]_n\text{-}OH + Na]^+$) type.[7,23–27] A typical MS/MS spectrum is shown in Figure 2. Table 2 summarizes the main AEO found in glove-extracts.

Presence of alkylphenol ethoxylates

Alkylphenol ethoxylates (also known as alkoxylated alkylphenols) are another group of nonionic emulsifiers frequently described in patents of nitrile gloves.[10,11] In particular, ethoxylated octylphenol, nonylphenol, diisooctylphenol, and dinonylphenol surfactans are mentioned. Octyl and nonylphenol ethoxylates (OPEO, NPEO) are also used as detergents in protein biochemistry and are known under trade names like Triton X-100, Igepal CA-series (e.g. CA-520, 630, 720) (octylphenols) and Nonidet P-40, Igepal CO-series (e.g. CO-630, 720, 890) (nonylphenols), respectively. Both surfactants were found in some of the tested nitrile but not in the latex and

Table 5. Results of the targeted MS-search for frequently used phthalates

Brand	Phthalates				
	DBP	DOP	DINP	DIDP	BBP
SHIELDskin	(+)	-	-	-	-
Vasco	(+)	-	-	-	-
MICROFLEX	+	+	+	-	-
Rotiprotect	(+)	-	-	-	-
N-DEX	(+)	-	-	-	-
SafeGrip	(+)	-	-	-	-
VWR Nitrile	(+)	-	-	-	-
proFood Nitrile	+	-	-	-	-
Conform + Latex	(+)	-	-	-	-
VWR Vinyl	+	-	+	-	-

vinyl gloves (Table 3). Only N-DEX and SafeGrip nitrile gloves contained none of these substances, while Vasco gloves contained both.

Typically, ion series of the singly and doubly charged species were observed in mass spectra (i.e. ($[C_{14}H_{21}(OCH_2CH_2)_nOH + Na]^+$, $[C_{14}H_{21}(OCH_2CH_2)_nOH + 2Na]^{2+}$ and ($[C_{15}H_{23}(OCH_2CH_2)_nOH + Na]^+$and $[C_{15}H_{23}(OCH_2CH_2)_nOH + 2Na]^{2+}$). Identification was accomplished by accurate mass measurement and the characteristics of the MS/MS spectra. Under the applied CID conditions, the alkyl-group was cleaved of, which resulted in an intense peak in the higher mass region of the spectrum. The other fragment ions present were mainly of the x_n ($[H_2C = CH\text{-}[OCH_2CH_2]_{n-1}\text{-}OH + Na]^+$) and z_n ($[O = CHCH_2\text{-}[OCH_2CH_2]_{n-1}\text{-}OH + Na]^+$) type, but also and with lower intensity of the c_n ($[H_{17}C_8$ or $H_{19}C_9PhO\text{-}[CH_2CH_2O]_{n-1}\text{-}CH_2CH = O + Na]^+$), and c_n'' ($[H_{17}C_8$ or $H_{19}C_9PhO\text{-}[CH_2CH_2O]_n\text{-}H + Na]^+$) species (Figure 3).[7,23]

An additional series of oligomers, which started at *m/z* 381.188 ($z = 1$) and *m/z* 444.233 ($z = 2$), was traceable in *SHIELDskin* extracts. Based on the accurate mass measurements and fragmentation behaviours, the ion series could be attributed to oligomers of the non-alkylated phenol ethoxylate (data not shown).

Presence of alkyl-aryl and alkyl-naphthalene sulfonates

Rotiprotect Nitril gloves are manufactured based on the US patent 5,014,362 (issued 1991, reissued in 1997 as patent Re. 35,616).[9] The production protocol explicitly lists sodium dodecylbenzene sulfonate (SDBS) as the main anionic surfactant, which has to be used in the polymerization process. This alkyl-aryl sulfonate (a linear alkylbenzene sulfonate, LAS) is also specifically mentioned in other patents for manufacturing nitrile rubber gloves (e.g. US 7,329,442; US 6,566,435).[10,28,29] Mass spectrometric analysis of the extracts revealed that all of the eight tested nitrile gloves contained this compound as the main water extractable anionic surfactant. The dominance of this substance could be already seen in the offline experiments (Figure 1). Online LC-separation led to a cluster of typically 3 to 5 peaks within the retention time window between *ca* 30 and 35 min. Accurate mass analysis showed that not only the C_{12} homolog (SDBS; *m/z* 371.1633) was present, but also all of the C_9 (*m/z* 329.1164), C_{10} (*m/z* 343.1320), C_{11} (*m/z* 357.1477), and C_{13} (*m/z* 385.1790) homologs, which are byproducts of SDBS synthesis. SDBS and its homologs were undetectable in latex and vinyl gloves (Table 4).

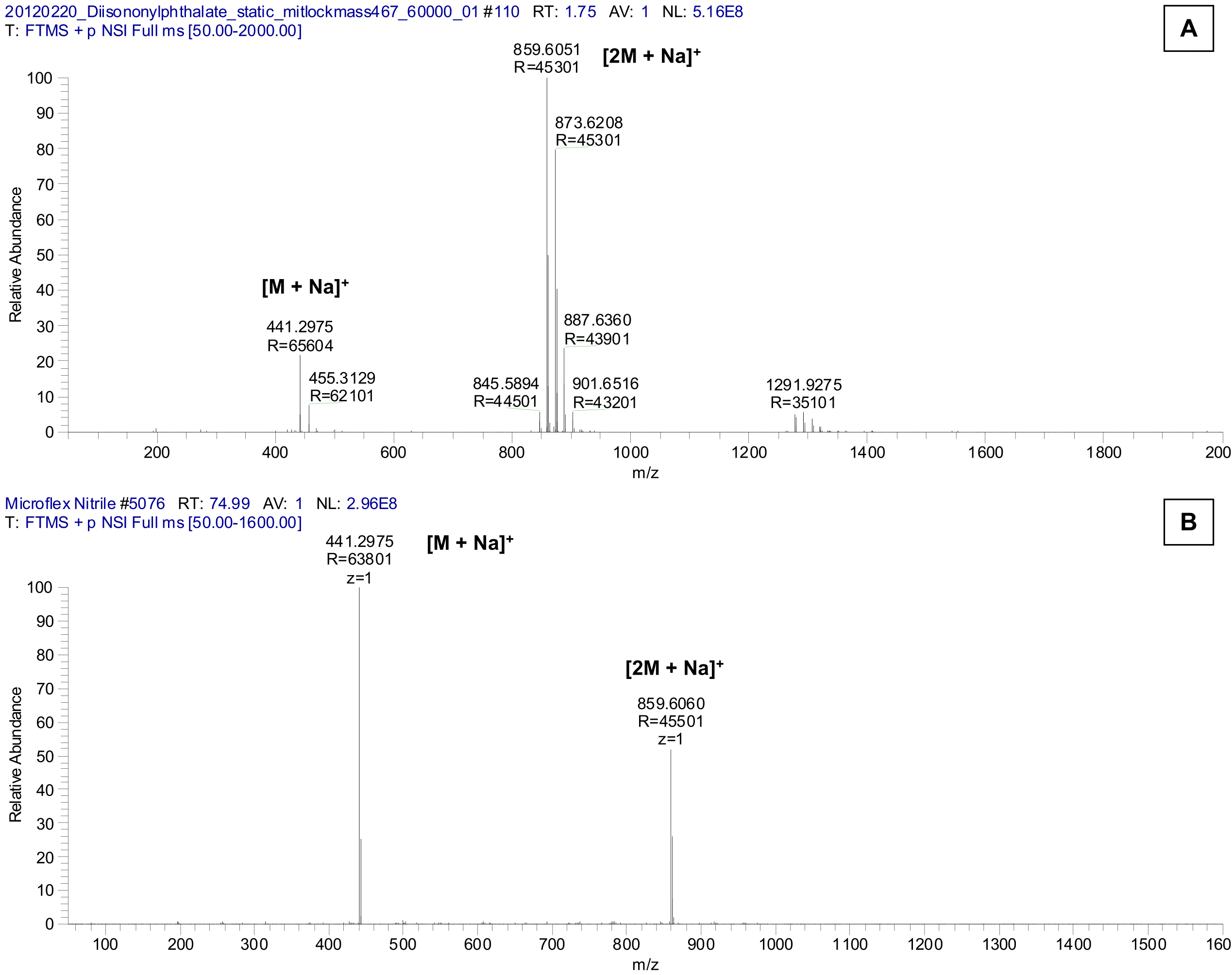

Figure 5. Presence of DINP plasticizer in some of the tested gloves. High resolution high accuracy MS spectra of (A) the reference substance and (B) as found in MICROFLEX nitrile gloves. In addition to DINP (*m/z* 441.2975) the reference also contains the *m/z* 455.3129 homolog with an extra methyl-group. Note the formation of dimeric sodium adduct ions in the gas-phase.

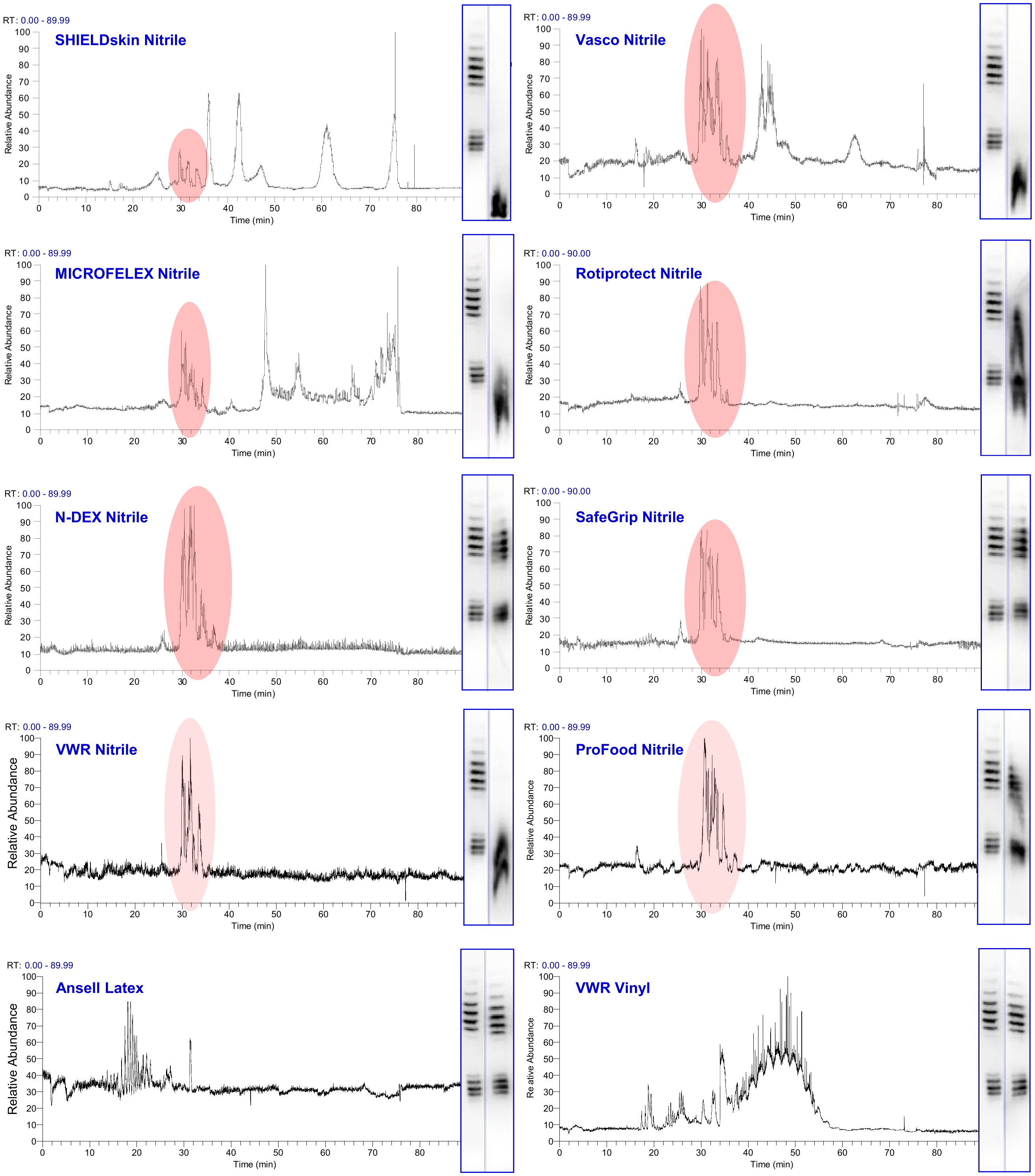

Figure 6. Summary of the online high resolution (R = 60000) high accuracy LC-MS experiments (total ion current (TIC) chromatograms) combined with the results obtained by IEF-PAGE of the BRP-EPO and NESP standards without (*left*) and with spiked-in glove-extracts (*right*). Note the presence of SDBS and its homologs in all nitrile gloves.

Sodium alkylbenzene sulfonates were observed in full MS spectra as Na$^+$ adduct ions, which formed NaCl clusters over the entire mass range up to *m/z* 1600 (*vide supra*). A typical MS spectrum is shown in Figure 4 together with the MS spectrum of the reference compound.

Analyses of nitrile gloves also yielded ions at *m/z* 327.1001, 341.1158, 355.1314, 369.1471, and 382.1627, which formed similar ion series as the C_9-C_{13} sodium alkylbenzene sulfonates. The ions were identified as sodiated dialkyltetralin sulfonates (DATS). C_9-C_{13}-DATS are main impurities found in commercial LAS mixtures.[30]

In some of the total ion chromatograms (TIC) of the LC-MS runs (e.g. *SHIELDskin*, *Vasco*, *Rotiprotect*, *proFood Nitrile*) one or two less intense peaks were observed at retention times between *ca* 15 and 20 min representing primarily mass pairs *m/z* 252.9906 / 482.9919 and *m/z* 494.9919 / 966.9947. Based on accurate mass as well as relative mass defect considerations and the fact that these ions behaved like anionic detergents with respect to the formation of NaCl cluster ion series, *m/z* 252.9906 could be identified as sodiated sodium naphthalene sulfonate ($[\text{Naph-SO}_3\text{Na} + \text{Na}]^+$) and *m/z* 482.9919 as its dimer ([2 Naph-

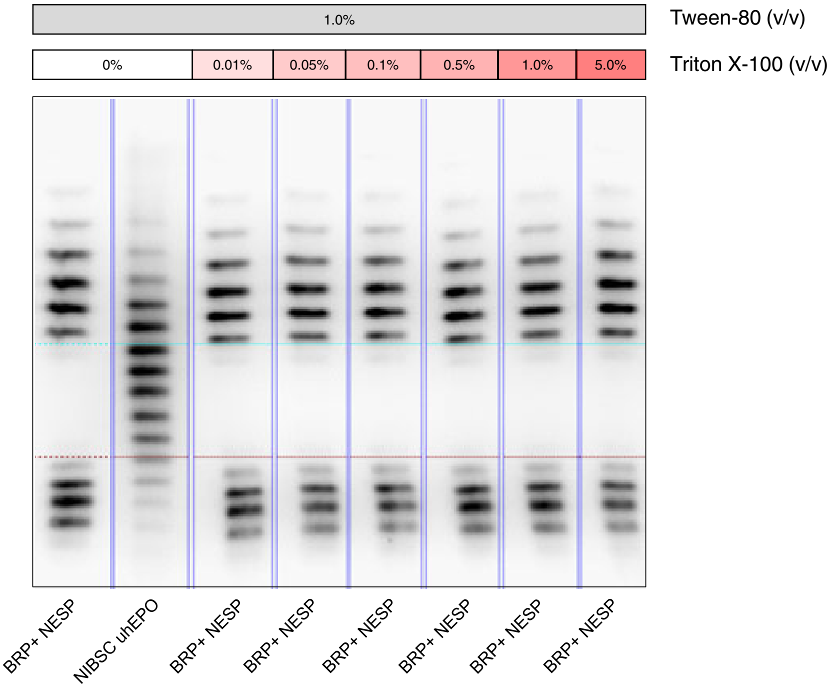

Figure 7. No interference of octylphenol ethoxylates (Triton X-100 in combination with 1% (*v/v*) Tween-80) with EPO IEF-PAGE was observed for the concentration range of 0.01–5.0% (*v/v*). Identical results were obtained for the NP-40 experiment (data no shown).

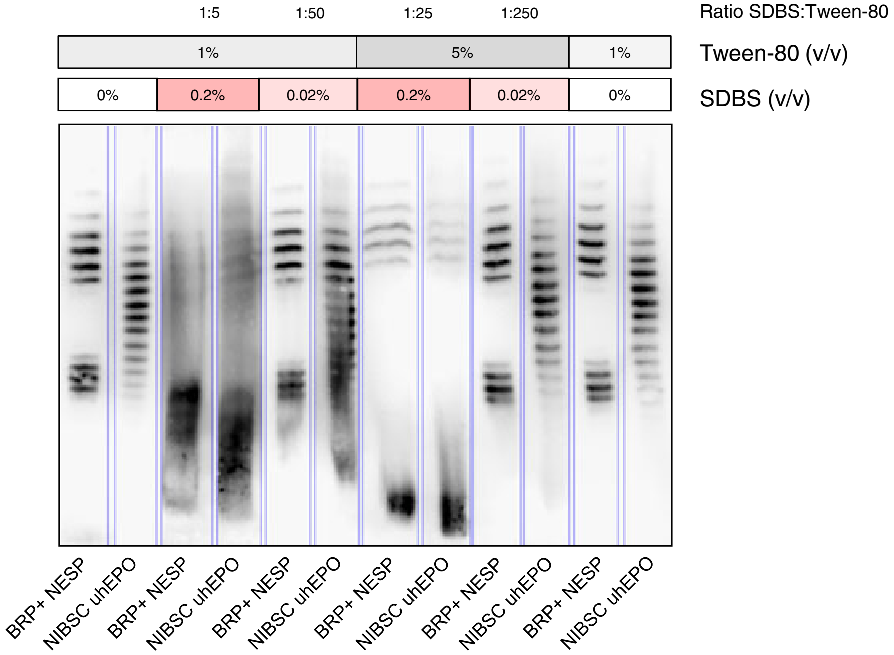

Figure 8. Interference of SDBS and its homologs as found in nitrile gloves. Note that the simultaneous presence of Tween-80 abolishes the negative effect of the anionic surfactant at an SDBS:Tween-80 ratio over 1:50. Tween-80 (or Tween-20) is usually added at a concentration of 1% (*v/v*) to samples and standards on EPO IEF-PAGE gels.

$SO_3Na + Na]^+$). The fact that the mass difference between *m/z* 482.9919 and *m/z* 494.9919 corresponds exactly to the mass of one ^{12}C isotope (12.0000), allowed to identify the ion *m/z* 494.9919 as the sodiated form of a methylene-bridged sodium naphthalene sulfonate ($[(Naph\text{-}SO_3Na)_2CH_2 + Na]^+$). In analogy to *m/z* 482.9919 the mass *m/z* 966.9947 could be attributed to its dimer ($[2\ (Naph\text{-}SO_3Na)_2CH_2 + Na]^+$). US patent 7,923,518 explicitly mentions sodium salts of alkylated naphthalene sulfonates and methylene-bridged naphthalene sulfonates as suitable anionic emulsifiers for nitrile rubber production.[11]

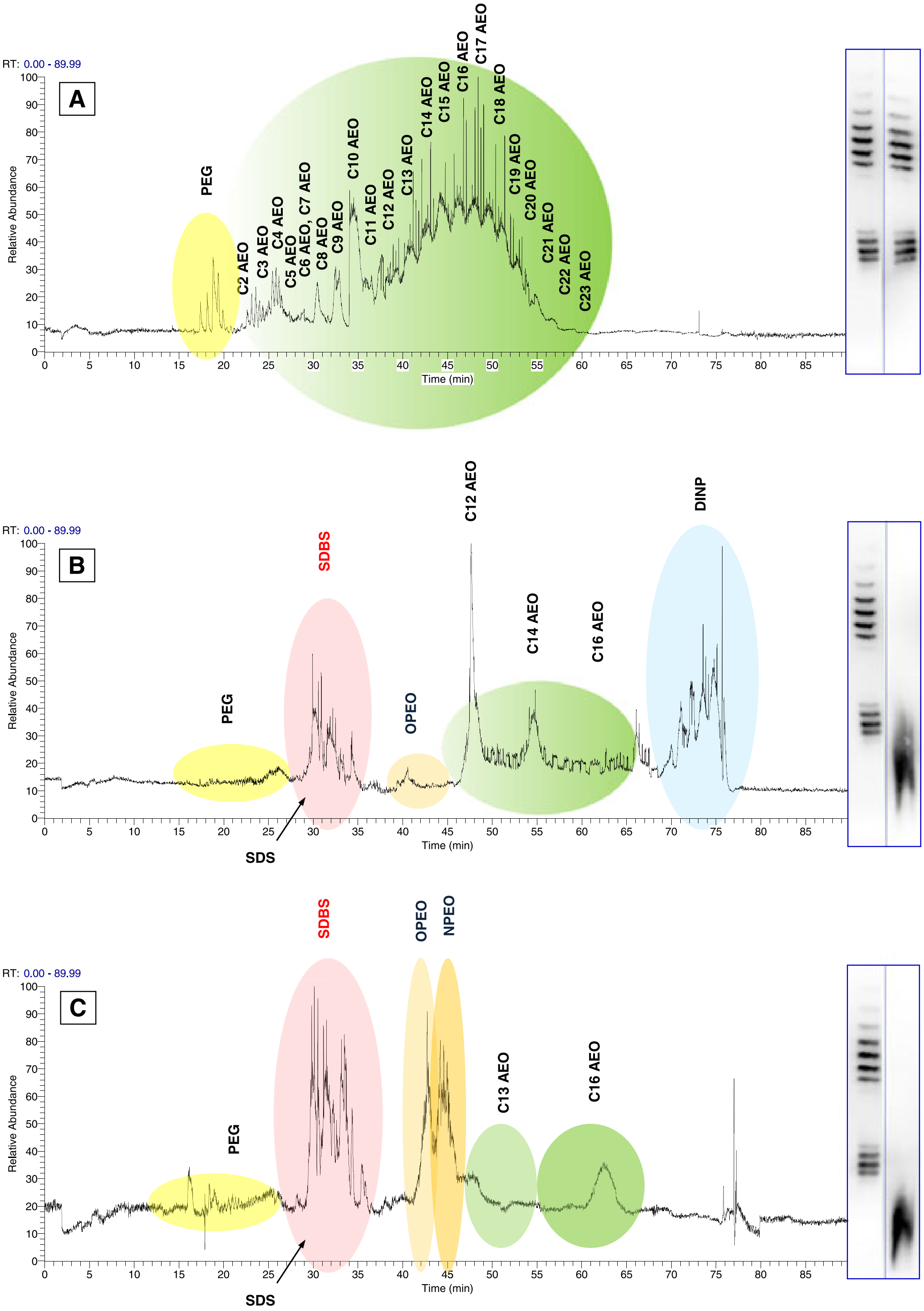

Figure 9. Summary of the main water extractable compounds found in the tested gloves. Examples for vinyl (A, VWR Vinyl) and nitrile (B, MICROFLEX FreeForm; C, Vasco light) gloves are shown. Despite highly present in vinyl gloves, alcohol ethoxylates (AEO) do not interfere with the EPO IEF-profile (BRP-EPO and NESP standard mixture without (*left*) and with (*right*) added glove extract; red arrows indicate streaking towards the anode).

Presence of phthalates

Phthalates are commonly used as plasticizers in polymer chemistry (e.g. dibutyl phthalate (DBP; *m/z* 301.1410), di-n-octyl phthalate (DnOP; *m/z* 413.2662), di-(2-ethylhexyl) phthalate (DEHP; *m/z* 413.2662), diisononyl phthalate (DINP; *m/z* 441.2975), diisodecyl phthalate (DIDP; *m/z* 469.3288), benzyl butyl phthalate (BBP, BzBP; *m/z* 335.1254)) (all *m/z* are for the $[M+Na]^+$ form). Due to their ubiquitous presence in ambient air, dibutyl and dioctyl phthalates are sometimes used as lock masses in ESI mass spectrometry. Targeted search for these phthalates showed that some of them (DINP, DOP, DBP) were indeed present in extracts of *MICROFLEX, proFood*, and *VWR Vinyl* gloves (Table 5). Trace amounts of DBP were detectable in all extracts.

Figure 5 shows the high resolution high accuracy MS spectrum of the DINP reference compound (*m/z* 441.2975) and a spectrum obtained from a nitrile glove extract after LC-separation. The DINP MS/MS spectrum consisted of only three peaks, and resembled the neutral loss of the two alky-groups (C_9H_{18}; 315.1567, 189.0158).

Influence of glove extracts on IEF-PAGE of EPO

In order to clarify which of the water extractable substances of gloves might interfere with the performance of IEF-PAGE, EPO standards (BRP rhEPO, NIBSC uhEPO, NESP; 0.2 ng) were spiked with 2 or 4 µl of each extract. Latex and vinyl gloves served as negative controls as they were already shown to not disturb the focusing process.[8,31] Figure 6 summarizes the IEF-PAGE results obtained for the ten glove extracts and combines these data with the data of the mass spectrometric analyses for SDBS. SDBS was present in all extracts of nitrile gloves and obviously interfered with IEF-PAGE, except in two of them (*N-DEX PLUS, SafeGrip Nitrile*), which showed only weak interference. The weak/non-interference behavior of *N-DEX PLUS* gloves was independently shown in a recent publication.[8] Additionally, trace amounts of SDS were also detectable in all nitrile and latex gloves

SHIELDskin gloves contained the highest amount (Figure 1A) and diversity of water extractable compounds other than SDBS, namely PEG, octylphenol ethoxylates, and alcohol ethoxylates. Since not all non-ionic detergents are compatible with IEF-PAGE of EPO,[7,12] these substance classes had to be also taken into account for potential interference. Alcohol ethoxylates were shown to not interfere with EPO IEF-PAGE even at higher concentration and various chain lengths of the alcohols (*VWR Vinyl gloves*; Figure 6). PEG could be also ruled out, as *Conform + Latex* gloves generated no interference behaviour. However, the main other extractable compound class present in, for example, *Vasco Nitrile* gloves next to SDBS was alkylphenol ethoxylates (Figure 1B). Both, octyl- and nonylphenol ethoxylates were detectable in the *Vasco* extract. Moreover, octylphenol or nonylphenol ethoxylates were also present in those gloves, which produced severe EPO-profile distortions (*MICROFLEX Nitrile, Rotiprotect Nitrile, VWR Nitrile, proFood Nitrile*; Figures 7C, 7D, 7G, 7H), but were missing in nitrile gloves, which hardly interfered (*N-DEX PLUS, SafeGrip Nitrile*). Hence, the influence of alkylphenol ethoxylates on IEF-PAGE was tested by adding different concentrations of Triton X-100 and NP-40 (0.01, 0.05, 0.1, 0.5, 1.0, 5.0% (*v/v*)) to EPO standards containing a fixed concentration of Tween-80 (1%). It could be demonstrated that for all of these concentrations neither the octyl- nor the nonylethoxylates were interfering with IEF-PAGE of EPO (Figure 7).

Hence, it was concluded that the negative effect, which SDBS and anionic surfactants in general normally have on isoelectric focusing, must have been – at least – partly compensated by the addition of Tween-80, which is usually added to the standards and samples at a concentration of 1%. In order to clarify whether Tween-80 is able to abolish the harmful influence of SDBS, different ratios of Tween-80 and SDBS were tested (Figure 8). It could be shown that demonstrated that above a ratio of 1:50 (Tween-80: SDBS) the nonionic detergent is indeed capable of abrogating the negative effect of the anionic detergent.[32,33]

Conclusion

All of the tested nitrile gloves contained sodium alkylbenzenesulfonates as main anionic water extractable compounds. One of them, sodium dodecylbenzenesulfonate (SDBS), is frequently used as emulsifier in nitrile emulsion polymerization. The commercial formulation of SDBS is a blend of different alkylbenzenesulfonates consisting mainly of the C_9 to C_{13} homologs. All of these homologs were found in the extracts of nitrile gloves, which explained their interference with IEF-PAGE of EPO. At a low concentration, their negative effect on isoelectric focusing can be partly overcome by the addition of an IEF-PAGE and EPO compatible nonionic detergent like Tween-80. Hence, different degrees of interference were seen for the different glove-extracts. Aside from polyethylene glycol (PEG), the majority of the other water extractable nonionic substances were alcohol and alkylphenyol ethoxylates, which showed no interference (Figure 9).

Supporting information

Supporting information may be found in the online version of this article.

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