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Novel RP-HPLC based assay for selective and sensitive endotoxin quantification

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The paper presents a novel instrumental analytical endotoxin quantification assay. It uses common analytical laboratory equipment (HPLC-FLD) and allows quantifying endotoxins (ETs) in different matrices from about 10^9 EU per mL down to about 40 EU per mL (RSE based). Test results are obtained in concentration units (*e.g.* ng ET per mL), which can then be converted to commonly used endotoxin units (EU per mL) in case of known pyrogenic activity. During endotoxin hydrolysis, the endotoxin specific rare sugar acid KDO is obtained quantitatively. After that, KDO is stoichiometrically reacted with DMB, which results in a highly fluorescent derivative. The mixture is separated using RP-HPLC followed by KDO-DMB quantification with a fluorescence detector. Based on the KDO content, the endotoxin content in the sample is calculated. The developed assay is economic and has a small error. Its applicability was demonstrated in applied research. ETs were quantified in purified bacterial biopolymers, which were produced by Gram-negative bacteria. Results were compared to LAL results obtained for the same samples. A high correlation was found between the results of both methods. Further, the new assay was utilized with high success during the development of novel endotoxin specific depth filters, which allow efficient, economic and sustainable ET removal during DSP. Those examples demonstrate that the new assay has the potential to complement the animal-based biological LAL pyrogenic quantification tests, which are accepted today by the major health authorities worldwide for the release of commercial pharmaceutical products.

Introduction

Endotoxins (ETs, LPS) are found all-over the world; they are non-covalently attached to the outer cell wall of all Gram-negative bacteria. The appearance of ETs is a serious problem in a variety of industries. In biological studies, biomedical and health diagnostics, ETs interfere with experiments and can cause false results and misinterpretation of studies. In healthcare, ETs cause serious health problems. On entering the blood stream, they induce a systemic inflammatory reaction. Individuals suffer from fever or get endotoxemia, which can lead to septic shock and death. ETs have even been linked to health issues such as allergies, asthma and obesity also in relation to work place circumstances.[1] Therefore, health authorities all-over the world have set maximum content levels for ETs in pharmaceutical products, usually expressed in EU per mL or EU per mg.[2,3] 10 EU is the biological pyrogenic activity of 1 ng of the FDA reference standard endotoxin (RSE), EC-6 obtained from *Escherichia coli O113:H10:K negative*.[2,4–6] The USP lists specific maximum ET contents for different pharmaceutical products, ranging from 0.07 to 90 EU per mg[3], *e.g.* for devices in contact with cerebrospinal fluid, the limit is 0.06 EU per mL or 2.15 EU per device. Maximum limits for the ET content in rural environments or working places have not been defined so far due to missing collection and reliable quantification tools.[1] On the other hand, ET levels in cell suspensions of Gram-negative bacteria are very high. An *E. coli* cell contains up to 10^6 ET molecules,[7] what corresponds to about 10^9 EU per mL (RSE). Thus, the concentration range for ET content measurements is vast, from about 10^{-2} EU per mL to 10^9 EU per mL. Medical products obtained from Gram-negative bacteria or non-sterile bioreactor processes must be purified from ETs from this high concentration down to the maximal levels allowed by health authorities. During DSP, respective ET removal procedures have to be developed. This requires intensive analytical testing.

ETs have a very complex and heterogeneous chemical structure.[8,9] They are composed of three distinct domains: the

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hydrophobic lipid A, a hydrophilic connecting inner and outer core and a hydrophilic O-antigen part (*O*-polysaccharide). Lipid A consists of a glucosamine disaccharide part to which a variable number of different fatty acyl chains is attached.[9] Its molecular mass is about 2000 Da in all ET types. The core is a non-repetitive oligosaccharide consisting of various monosaccharides, *e.g.* in case of ETs from *E. coli* strains: L-rhamnose, D-galactose, D-glucose, L-*glycero*-D-manno-heptose, *N*-acetylglucosamine, glucosamine and 3-deoxy-D-manno-oct-2-ulosonic acid (KDO).[10] The core is the most conserved part of all ETs[8] and has a MW of 600–2000 Da.[11] In all ETs the core contains at least one molecule of the rare and almost ET unique sugar acid KDO; maximal three KDO units (very seldom four)[12] were found in the ET core.[8] In some rare occasions the occurrence of KDO has also been reported in the outer core and even in the O-antigen, *e.g.* in *Cronobacter sakazikii* ET.[12] In these cases, the usual α-ketosidic bound of KDO to the heptose in the inner core can be found sometimes in the β-conformation. In addition, recent research shows that Gram-negative pathogenic bacteria possessing K-antigens may have capsular polysaccharides (CPS) that contain a β-poly-KDO linker. This linker, connecting a lipid moiety and repeating sugar moieties similar to LPS, is built up of five to nine KDO units.[13,14] The CPS can be synthetized by bacteria strains like *Pseudomonas* as a reaction to environmental stress.[15] One α-KDO sugar unit is also found in the primary cell wall of higher plants, *e.g.* lycophytes, bryophytes and green algae.[8,12,16,17] Depending on the ET kind, preparation, growth conditions *etc.*, the core region as well as the lipid A part of ETs may further show many different substitutions *e.g.* with phosphate; phosphoethanolamine; pyrophosphoethanolamine; galactose or α-4-amino-4-deoxy-L-arabinose.[18]

The O-antigen contains a distal repeating oligosaccharide chain of five defined oligosaccharides. Its oligosaccharide composition differs for ETs obtained from different bacteria strains.[7] ETs with zero (R-type) up to forty (S-type) repeating oligosaccharide chains were found.[19] Depending on the bacterial strain, growth conditions but also on the extraction method used to prepare *e.g.* ET standards, ET preparations have usually a heterogenic composition with a large molecular weight distribution. The ET molecular weight distribution of a preparation may range from about 2000 Da (R-type) to about 40 000 Da (S-type).[19] Most ETs found have a monomer average MW of about 10 000–20 000 Da [20] and one to two KDOs.[10,12] Detailed structural drawings of the complex and heterogeneous ETs are shown in the ET (LPS) structure reviews of Caroff *et al.* in 2003 [18] and Silipo *et al.* in 2010.[10] In the review of Lodowska *et al.* in 2013 [12] several different KDO containing core structures of ETs obtained from different bacterial species are shown.

ETs stick strongly to surfaces and they aggregate to micelles or vesicles in dependence on *e.g.* actual composition, concentration, temperature and solvent.[21] ETs lack chromophores what prohibits sensitive UV-detection. All these factors make their identification, quantification, purification and the final product purification from ETs during DSP tedious and usually expensive.

Several chemical analytical methods and kits to identify and quantify ETs have been developed in the past and are based on intact ET analysis or on detection of carbohydrate moieties, lipid A and core parts.[22–24] Recently, an interesting and sensitive approach has been published tackling the entire ET molecule.[25] To obtain high sensitivity, the free amino groups *e.g.* ethanolamine and 4-amino-4-deoxy-L-arabinose residues in the core and lipid A regions of an ET molecule, are reacted with fluorescein iso-thiocyanate (FITC). However, the derivatisation time is 12 h, what is long for a routine analytical assay. In the following, the FITC derivatized ETs are separated by CZE. Quantification is obtained by LIF detection. A LOQ of 50 ng mL^{-1} (about 500 EU per mL for RSE) was estimated. Further sensitivity is reached by sample concentration using solid phase extraction (SPE), which binds mainly the distal repeating oligosaccharide chain ET moieties. As a result, the ET LOQ is lowered to 5 ng mL^{-1}. It is obvious that this concentration strategy does not work for R-type ETs. ET quantification for non-characterized ET preparations will show a large error range, since the number of ET amine groups strongly varies between different bacterial serotypes.[26] Further, it will not provide a general ET identification/quantification strategy, since also free amino groups of other common matrix components, *e.g.* glycopeptides or glycoproteins react.[27,28] Another approach for ET quantification is based on the ET unique sugar acid KDO. It is present in the strongly conserved core of all ETs. Ketosidic linkages such as this α-ketosidic linkage between KDO and the di-glucosamine backbone of lipid A are susceptible to cleavage by acidic hydrolysis.[23,29,30] Afterwards, the released non-chromogen KDO is quantified, *e.g.* commonly after reaction with the chromogen 2-thiobarbituric acid (TBA).[23,31] The TBA test for KDO is based on the formation of 3-formyl pyruvic acid upon periodate oxidation. That reacts at elevated temperature with thiobarbituric acid, which results in a chromogen product. This assay allows the quantification of KDO in the nmol mL^{-1} range[23,31] and is often used during ET removal DSP development. However, TBA produces also coloured derivatisation products with other constituents of the LPS *e.g.* abequose and colitose.[32,33] Further, TBA reacts also with amino acids, oxidized fatty acids and sialic acids which might be present in the sample matrix[34,35] leading to false positive ET content results. To conclude, this assay is not ET specific and thus not suitable for ET quantification in complex, *e.g.* protein or glycoprotein containing mixtures as bioreactor cultures or protein APIs.[36] A more selective and sensitive approach was reported by Kiang *et al.*[37] After quantitative ET hydrolysis, they used anion exchange chromatography to separate KDO selectively from other matrix ingredients *e.g.* sialic acids found in *Salmonella Minnesota mutant R595* LPS preparations. Coupled to an amperometric detector, KDO could be detected without pre- or post-column derivatisation with a LOQ of 20 pmol. This value corresponds to about 8 000 EU assuming an ET with one KDO, a MW of 40 000 Da and the biological activity of the RSE EC-6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) based techniques can be used to analyse both ET aggregates[38] and monomeric ETs.[39] In general, SDS-PAGE allows a rough estimation of the average molecular weight, but it is tedious and quantification is not possible.[40] Relative quantification of different endotoxin species based on their fatty acid distribution was accomplished by the analysis of fatty acid

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methyl esters after quantitative methanolysis. The LOD with FID is about 0.2 μg mL^{-1} for the fatty acids,[41,42] what corresponds to about 500 EU per mL for *E. coli*. With GC-MS, a LOD of 15 EU per mL was reported.[22] However, the fatty acid population and type differ for each ET and derivatisation efficiency varies for different fatty acids. Thus, reliable ET quantification based on fatty acid analysis is not possible and their analysis is tedious.

In general, it can be concluded that the current chemical analytical methods do not provide sufficient selectivity or sensitivity to detect ETs in complex mixtures or to the health authorities' relevant requirements.

Due to their strong pyrogenic properties, ETs are in general well suited for identification/quantification approaches based on their biological response. Consequently, ET analysis is performed today mainly with biological pyrogenic assays, which are not directly affected by the high degree of heterogeneity of ET preparations. The first routinely used endotoxin test is the so-called Rabbit Pyrogen Test (RPT).[43] It is based on the inflammatory response (fever) of rabbits after injection of pyrogenic substances. The test is included in the USP since 1942.[44] The RPT provides a LOD of 0.3–5 EU per mL.[45] It does not allow any quantitative determination of the ET content being therefore not useful for *e.g.* DSP ET removal optimisation. In 1991, the FDA approved the biological reagent “LAL” (Limulus Amebocyte Lysate) for quantitative ET testing in pharmaceutical formulations. Today, it is still the main ET identification and quantification test and included in all major pharmacopeia as USP <85>,[46] EP 2.6.14 (from 2019 also the recombinant version of the LAL test 2.6.32) or JP 4.01. The commercialized LAL assays work at very low ET concentrations. The Endosafe® product range produced by Charles River Laboratories has a LOQ of 0.001 EU per mL. Recombinant versions of the LAL test are available today, avoiding the use of animal material. Recently, Lonza Ltd (Basel, CH) commercialized the Pyrogene™ rFC kit and instrument. It uses recombinant factor C (rFC) instead of horseshoe crab blood,[47] the working range is similar to the conventional LAL tests 0.005–50 EU per mL. The EndoLISA assay from Hyglos Ltd (Bernried, Germany) uses a recombinant bacteriophage protein in combination with rFC; the working range is 0.05–500 EU per mL. Recently, the monocyte activation test (MAT) is considered as very promising and versatile tool for ET detection (LOD 0.03 EU per mL), since it uses human cells or *in vitro* human cell lines (*e.g.* MonoMac 6)[45] for ET detection. It is probably a better projection to measure human biological response on ET invasion than the ones using a different species as the rabbit or the horseshoe crab.[48] However, those tests are expensive and take very long; they are not applicable for process and product development.

Further, latest publications show that the EndoLISA assay and MAT suffer from the same potential matrix interference *e.g.* in monoclonal antibody formulations as the LAL or RPT tests.[49,50] This phenomenon is commonly known in the pharmaceutical industry as Low Endotoxin Recovery (LER).[49] It may lead to false negative results with respect to the ET content what endangers patient safety.[49,50] It follows that the LER effect is one of the most discussed issues in the area of pharmaceutical industry quality control today.[51]

Due to the various drawbacks of the current mainly applied biological ET tests, there is a very high demand to develop accurate methods for quantifying ETs; *e.g.* during DSP development and in-process control, in pharmaceutical preparations but also in biomedical and health diagnostics. They should be ET specific, sensitive, reproducible, solvent and matrix independent, applicable to a large scope, exhibit a large dynamic ET concentration range and work fast in a cost-efficient manner. Furthermore, for the sake of obtaining meaningful and comparable results between different ET sources/preparations/samples, it is desirable to quantify ETs in weight or mole units. Then, in a second step, one can determine how this quantity relates to a specific pyrogenic activity; since depending on the nature of the ET it is likely that not all ETs have the same activity.[52] In order to encourage the development of new ET assays, the FDA issued a Guidance for industry[2] in June 2012, which states that an alternative assay to those in the USP might be used after validation in respect to the official methods.

In this paper, we report our first results of a novel ET specific chemical analytical assay. It uses quantitative ET hydrolysis at elevated temperatures to release KDO. Then KDO is derivatised with DMB resulting in the highly fluorescent product KDO-DMB. After its separation by RP-HPLC from other matrix components KDO-DMB is detected with high sensitivity by a fluorescence detector. The KDO content is then converted into ET content.

Results and discussion

Hydrolysis and analysis of ET standards

In each ET lipid A is substituted at its non-reducing terminus by one KDO (in rare cases KO)[12] *via* an α-ketosidic linkage. In the core region of an ET, this KDO residue may be further substituted also *via* α-(2-4)-ketosidic linkages by more KDO residues (commonly one to two units, rarely three units) or by different sugars (*e.g.* heptose, L-rhmnosyl, or *N*-acetyl-D-glucosaminyl).[53] The glycosidic bonds of KDO are much more acid labile than common sugar bonds. ET hydrolysis at elevated temperatures and under acidic conditions is commonly used for cleavage.[54] The published conditions for KDO release from LPS vary strongly. Exemplary conditions like 1 to 2 M HCl at 100 °C, 0.125 M H_2SO_4 for 8 min in boiling water,[8] 0.2 M acetate buffer pH 4.5 from 70 °C to 100 °C for 1–2 h, 0.025 M HCl for 1 h at 80 °C or 1% acetic acid from 70 °C–100 °C for about 60 min are reported.[37,54,55] As mentioned earlier, in different ET types and ET preparations the KDO units in the core can be attached to different moieties and show different modifications *e.g.* alkylation or phosphorylation.[8,26] That might lead to differences in the accessibility of the α-ketosidic linkages and with that hydrolysis efficiency of the different KDOs. Thus, Chaby *et al.*[54] and Caroff *et al.*[55] postulate that the optimal quantitative hydrolysis conditions *e.g.* acid concentration, temperature, detergent use *etc.* could be different for different ET variants and preparations. That fact would inhibit a general ET quantification strategy based on acidic ET hydrolysis at elevated

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temperatures to obtain KDO quantitatively. Further, several investigations show that depending on the conditions of the acidic hydrolysis the KDO content decreases over time.[37,53] It follows, a certain mildness of hydrolytic conditions is required to minimize destruction of KDO. Kiang *et al.*[37] measured the KDO release rate for R-type ET isolated from *Salmonella Minnesota mutant R595*, named Re-LPS. It contains three connected KDOs, two glucosamines (substituted by phosphor and ethanolamine) and lipid A,[55] their preparation showed a molar ratio of GlcN : KDO : organic phosphate of 2.0 : 2.9 : 3.5. They also investigated the de-*O*-acetylated version of the same Re-LPS named DeOA-LPS; there the molar ratio GlcN : KDO : organic phosphate was 2.0 : 2.4 : 2.4, indicating some loss of phosphate and KDO during the hydrazinolysis what was used to prepare the de-*O*-acetylated version of the Re-LPS. They further investigated the small synthetic 4-methylunbelliferyl α-ketoside of one KDO unit (KDO-MU) as well as the breakdown rate of a KDO standard under various acidic hydrolysis conditions. Hydrolysis in 1 % acetic acid at 70 °C and acetate buffer pH 4.5 at 100 °C showed substantially lower KDO degradation in comparison to stronger commonly used hydrolysis conditions as 1% acetic acid at 90 °C and 100 °C. In the following, they corrected the KDO-MU and ET released KDO content with the degradation rate of the KDO standard at different hydrolysis time points. They observed that for KDO-MU as well as for both more complex ET preparations the released KDO content reached a constant maximum plateau after 60 min of hydrolysis using 1% acetic acid at 70 °C. That shows for all three different species the same KDO release rate, despite their miscellaneous structural complexity. Further, Ren and co-workers[56] used 0.025 M HCl to hydrolyse active sludge at 80 °C for 1 h. The complex active sludge consists of microorganisms, inorganic particles, organic fibres, filamentous bacteria, extracellular polymer substances (EPS, biopolymers, exopolymers), ions and water. They determined the KDO content of the sludge (after hydrolysis and reaction with DMB) and obtained a linear correlation between the amounts of mixed liquor suspended solid/quantity of bacteria (consisting in its majority of Gram-negative bacteria) and the KDO content. KDO is an integral part of all ETs that are bound to all Gram-negative bacteria. This correlation[56] indicates, that hydrolysis is globally quantitative, despite the probable very large ET complexity in active sludge, *e.g.* in respect to KDO number, position, substitution as well as ET kind and molecular weight heterogeneity. It is obvious at this point that in the future, a systematic investigation of many characterized ET standards and preparations with respect to their obtained KDO content under different hydrolysis conditions is needed.

To obtain sensitive KDO detection, the KDO content after hydrolysis is historically estimated using different colourimetric reaction assays *e.g.* the TBA test, the semicarbazide reaction, the diphenylamine test and lately DMB.[54,56,57] It is obvious that separation after derivatisation improves specificity and reproducibility. It has been shown that at least some of these assays detect different KDO derivatives or break down products with different selectivity[58] and they are also influenced by matrix compounds as amino acids, sialic acids, *etc.*, resulting in false positive numbers.[54] All these factors made and still make it difficult to achieve accurate KDO quantification and to optimize hydrolysis or to find generic hydrolysis conditions for different ET preparations and matrices independently from the derivatisation reaction. In order to eliminate the above described problems, we were initially choosing, similar to Kiang *et al.*,[37] a direct approach to develop and optimize hydrolysis conditions. Liberated KDO units were quantified by CZE-DAD. We used a pH 12.6 buffer system, where carbohydrates are charged and separated with high efficiency. Employing a diode array detector, the separated carbohydrates undergo a quantitative on-capillary reaction in the capillary detection window resulting in UV active species. Those are then directly detected at 273 nm.[59] Typical electropherograms of 0.05 mg mL^{-1} to 1 mg mL^{-1} KDO standards containing glucose as internal standard dissolved in PBS are shown in Fig. 1.

As seen in Fig. 1, the signals of KDO and glucose are well separated. By plotting the peak area of KDO *versus* its concentration a linear correlation was obtained between the tested 1 mg mL^{-1} and 0.05 mg mL^{-1} KDO showing a correlation coefficient R^2 of 0.9996. Results are the means obtained from two hydrolysis of one sample, each injected three times. The LOD for KDO is estimated to be about 0.005 mg KDO per mL, which corresponds to about 8×10^5 ng ET per mL and 8×10^6 EU per mL (worst-case scenario and RSE pyrogenic activity, explanation see material and methods). It is obvious, that the CZE-DAD method is not very sensitive for KDO detection and with that not for ET quantification. It does not work in the by health authority required low ET concentration ranges. On the other hand, it allowed us the optimisation of the hydrolysis independently from the later introduced derivatisation reaction of liberated KDO with DMB, which is used to reach much higher sensitivity.

To test the quantitative release of KDO units from ETs, hydrolyses of 3 mg mL^{-1} *E. coli O55:B5* and 3 mg mL^{-1} *P. aeruginosa 10* LPS standards were performed. 2 M TFA was used

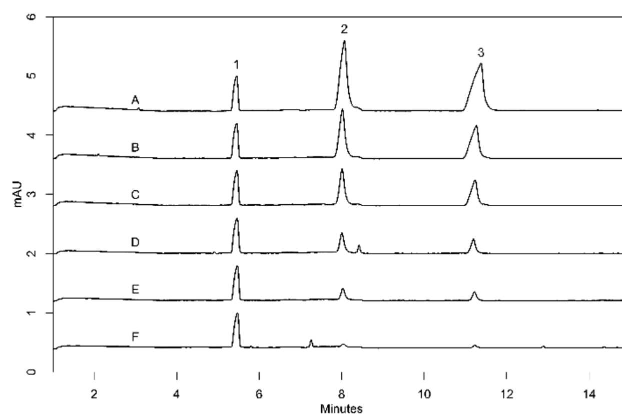

Fig. 1 CZE-DAD electropherograms of a KDO standard (peak 3) and glucose (peak 2, internal standard, same concentration as KDO) dissolved in PBS obtained with pH 12.6 background electrolyte; UV detection at 273 nm. KDO concentration (A) 1 mg mL^{-1}; (B) 0.5 mg mL^{-1}; (C) 0.3 mg mL^{-1}; (D) 0.1 mg mL^{-1}; (E) 0.05 mg mL^{-1}; (F) PBS, peak (1) represents the electroosmotic flow; electrophoretic conditions see material and methods.

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and samples were kept at 80 °C between 0 and 240 min. These conditions have often been applied in polysaccharide hydrolysis.[60] An advantage of TFA is its compatibility with later used RP-HPLC eluents and in addition, it can be easily and fast evaporated after hydrolysis, leading to improved KDO stability.

As shown in Fig. 2, maximal KDO response appeared for both ET standards after 30 min hydrolysis time. Afterwards, the KDO content decreased due to KDO degradation.[37] In agreement with Kiang[37] a correction was applied for the KDO content at each time point with the % peak area of degraded KDO under the same conditions. Now, from 30 min to 240 min a constant maximum plateau for the KDO peak area occurred for both ETs (data not shown). Fig. 2 shows that the response of the *E. coli O55:B5* ET is bigger than the one obtained for *P. aeruginosa 10*. On the other hand, the difference in their responses is constant after 30 min, indicating a finished and probably quantitative hydrolysis. It is impossible to conclude at this stage of development, whether all different KDOs of one ET were hydrolysed since the average monomer MW, number of KDOs and purity of the ET standards are unknown and there are no easy-to-use tools available today to get those values.

For all follow-up experiments, the hydrolysis time was set to 30 min. The linearity of the hydrolysed ET KDO response in relation to the ET concentration was tested. *P. aeruginosa 10* LPS standard solutions with 0.5, 2, 3 mg mL^{-1} ET dissolved in PBS were hydrolysed and analysed by the CZE-DAD method (Fig. 3). The lowest ET concentration of 0.5 mg mL^{-1} tested corresponds to about 0.03 mg mL^{-1} KDO (worst-case scenario), what is close to the estimated LOQ of the CZE-DAD method (0.02 mg KDO per mL). With this method, lower ET concentrations cannot be analysed.

Like in the case of the KDO standards, a linear correlation between the ET concentration and the KDO peak area with a correlation coefficient R^2 of 0.995 was obtained over the investigated ET concentration range.

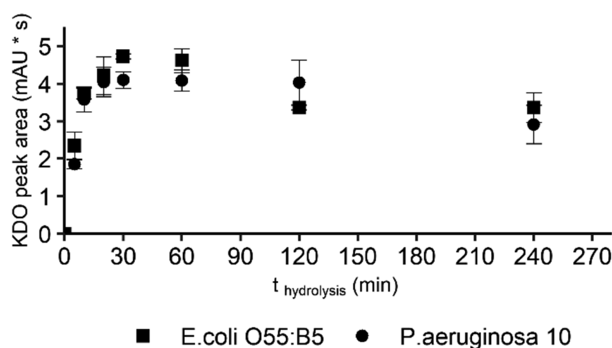

Fig. 2 Peak area of KDO detected after 0, 5, 10, 20, 30, 60, 120 and 240 min hydrolysis of 3 mg mL^{-1} *E. coli O55:B5* and *P. aeruginosa 10* ET standards. Hydrolysis was performed for two aliquots for each ET standard with 2 M TFA at 80 °C. CZE-DAD separation was performed at pH 12.6 and UV detection at 273 nm. For each time point, two injections were performed for each aliquot. Each data point represents the average of the four peak areas per time point and error bars are their standard deviation (SD = 0.1–0.5). Peak areas were not corrected with the KDO degradation rate.[37]

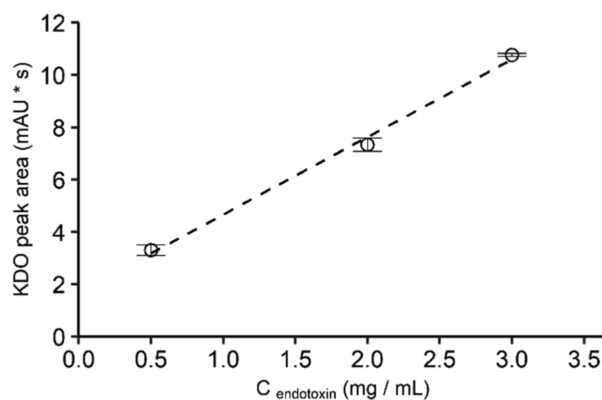

Fig. 3 Linear plot of the KDO peak area obtained after *P. aeruginosa 10* LPS standard hydrolysis *versus P. aeruginosa 10* LPS standard concentration, CZE-DAD analysis at pH 12.6, UV detection at 273 nm; each concentration: $n_{\text{sample hydrolysis}} = 2$, $n_{\text{injection}} = 3$; each data point represents the average of the KDO peak area obtained from the six injections at each concentration. Error bars represent the standard deviation of KDO peak area of the six injections at each concentration.

KDO release repeatability was tested by hydrolysing six aliquots of a 3 mg mL^{-1} *P. aeruginosa 10* LPS standard dissolved in PBS. Each hydrolysate was injected six times and measured by CZE-DAD. The variability of the KDO peak areas among the multiple sample preparations and analyses were analysed by the ANOVA Tukey HSD test. The statistical analysis showed no significant difference ($p < 0.05$) between the released KDO quantities. A RSD of 1.2% was obtained for the average peak area among the six sample preparations, proving a high repeatability of the ET standard hydrolysis.

The applicability of the procedure for biological samples was tested using aliquots of different *P. putida* cultivation supernatants. Two samples were taken at two different cultivation time points. After centrifugation and hydrolysis of the collected supernatant, samples were analysed by CZE-DAD at pH 12.6 (Fig. 4).

In case of the sample with an OD_{600} of 14, a 0.55 mg mL^{-1} KDO content was obtained, which corresponds to about 9×10^8 EU per mL (worst-case scenario, RSE pyrogenic activity assumptions). That value is in the order of magnitude of the maximum ET levels published for Gram-negative bacteria cell suspensions (about 10^9 EU per mL [7,61]). In the sample with the lower OD_{600} of 1.2 KDO was not quantifiable. This is probably due to the low cell density and therefore low ET content of the culture.

Assessment of KDO solution stability after resuspension following hydrolysis and TFA removal

Stability of KDO in the analysis solution (auto sampler solution) is important to obtain a quantitative ET assay and establish quantitative fluorescence labelling conditions. Solutions of 0.5 mg mL^{-1} KDO standard and 3 mg mL^{-1} *E. coli O55:B5* ET standard (about 0.18 mg mL^{-1} KDO; worst-case scenario), were kept in 2 M TFA for 30 min at 80 °C. TFA was removed immediately by nitrogen evaporation and samples were reconstituted

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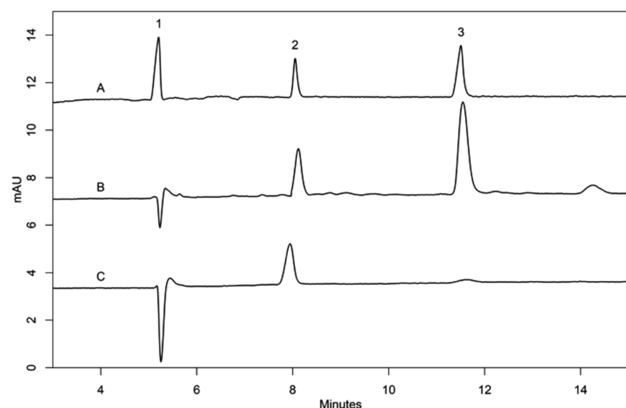

Fig. 4 CZE-DAD electropherograms of two samples of *P.putida* cultivation supernatants taken at different cultivation time points; (A) 0.5 mg mL^{-1} KDO standard (peak 3) dissolved in PBS; (B) sample with OD_{600} = 14; (C) sample with OD_{600} = 1.2. To each sample 0.25 mg mL^{-1} glucose (peak 2) was added as internal standard; pH 12.6 background electrolyte was used for CZE separation; UV detection at 273 nm; electrophoretic conditions see material and methods. Peak 1 represents the electroosmotic flow.

in distilled water or PBS buffer (pH 7). The KDO peak area was monitored for 25 h. As shown in Fig. 5, the KDO peak area of the hydrolysed KDO and ET standards reconstituted in PBS was constant for at least 25 h. The recovery was in comparison to the initial time point peak area at least 98% (4% RSD) and 97% (5% RSD), respectively. In contradiction, the KDO content of samples reconstituted in water decreased substantially after 5 h storage at room temperature. These results support earlier findings that KDO stability in solution is critical to obtain a quantitative method.[37,54]

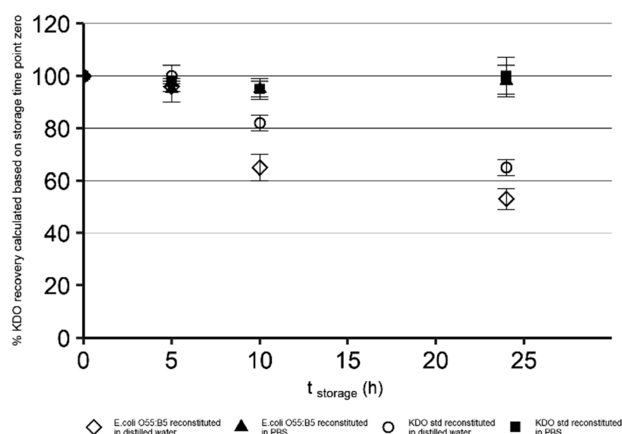

Fig. 5 KDO recovery of a 0.5 mg mL^{-1} KDO standard and 3 mg mL^{-1} *E. coli O55:B5* standard at different storage time points and stored in different solvents at room temperature. Before storage, both samples were hydrolysed in 2 M TFA at 80 °C for 30 min, followed by immediate TFA removal by fast nitrogen evaporation. The samples were reconstituted in distilled water or PBS (pH 7). Analysis was carried out by CZE-DAD pH 12.6 at 273 nm; $n_{sample\ hydrolysis}$ = 2, $n_{injection}$ = 3 each sample and solvent, error bars represent the standard deviation. Recovery was calculated based on the peak area of KDO at each time point investigated divided by the KDO peak area at time point zero in the respective solvent.

Quantification of DMB-labelled KDO by FLD after separation by RP-HPLC

After quantitative release of KDO, its sensitive detection must be solved to obtain a low LOQ for ETs. KDO has a similar molecular structure as sialic acid. Therefore, we concluded that reactivity should be similar for both. Both are alpha-keto-acids, which can selectively react with vicinal amino groups attached to a benzene ring, *e.g.* as present in the DMB molecule. The different sialic acids can be detected with very high sensitivity by fluorescence detection after derivatisation with DMB,[62] *e.g.* in glycoprotein samples.[29,63] Several commercial kits using this reaction are available. The application note of the Takara Bio labelling kit[64] states a LOQ of 57 fmol for DMB–sialic acid, representing the high sensitivity of its fluorescence detection. KDO was derivatized with DMB before.[17,56,57] Identical to common sialic acid–DMB assays,[29] after RP-HPLC separation of the reaction mixture, fluorescence detection of KDO-DMB at 373/448 nm was performed. However, from the data provided in those studies it is difficult to estimate a LOQ for KDO-DMB. Further, those publications provide only a rough estimation of the KDO content in the samples investigated. In addition, no data were shown proving a linear response between the KDO - DMB signal and its concentration.

For our initial tests, a KDO standard dissolved in PBS was reacted with DMB as described in the literature for sialic acids.[29,65] After labelling, the reaction mixture was separated *via* RP-HPLC followed by fluorescence detection. We used low elution force isocratic conditions.[66]

The chromatogram in Fig. 6A shows a good separation of the KDO signal from other reaction products, KDN, NANA and NGNA as well as from excess labelling reagent. No interfering peaks appeared at the KDO-DMB elution time (6.8 min).

In the following, the relation between the signal intensity and the KDO standard concentration was tested. KDO standards, dissolved in PBS, with concentrations ranging from 0.5–500 ng mL^{-1} were reacted with DMB and injected. Due to the wide concentration range, it was not possible to measure all concentrations with the same FLD photomultiplier gain. KDO-DMB standards from 100 ng mL^{-1} to 500 ng mL^{-1} were detected at a detector gain of 10; for 0.5–50 ng mL^{-1} a gain of 18 was used (Fig. 6B). For both concentration ranges, high correlation between KDO-DMB peak area and KDO concentration with regression coefficients R^2 of 0.992 for 0.5–50 ng mL^{-1} and 0.980 for 100–500 ng mL^{-1} were obtained. This proves constant labelling efficiency, needed for accurate and precise quantification. The lowest KDO concentration analysed in our preliminary tests was 0.5 ng mL^{-1}. Using the worst-case KDO average molecular weight scenario for an ET (0.6% (w/w) KDO per ET molecule) that would correspond to an ET concentration of 83 ng mL^{-1} and 830 EU per mL (assuming RSE pyrogenic activity). Using the published composition of the RSE EC-6 (average MW about 10 000 Da and 2 KDOs per ET molecule,[7,67] what equals 4.8 % (w/w) KDO per ET molecule), 0.5 ng mL^{-1} KDO result in an ET concentration of about 10 ng mL^{-1}, what corresponds to 100 EU per mL for the RSE EC-6. Under the conditions employed, a LOQ of 0.2 ng KDO per mL was estimated based on a signal-to-noise ratio of 10. That would

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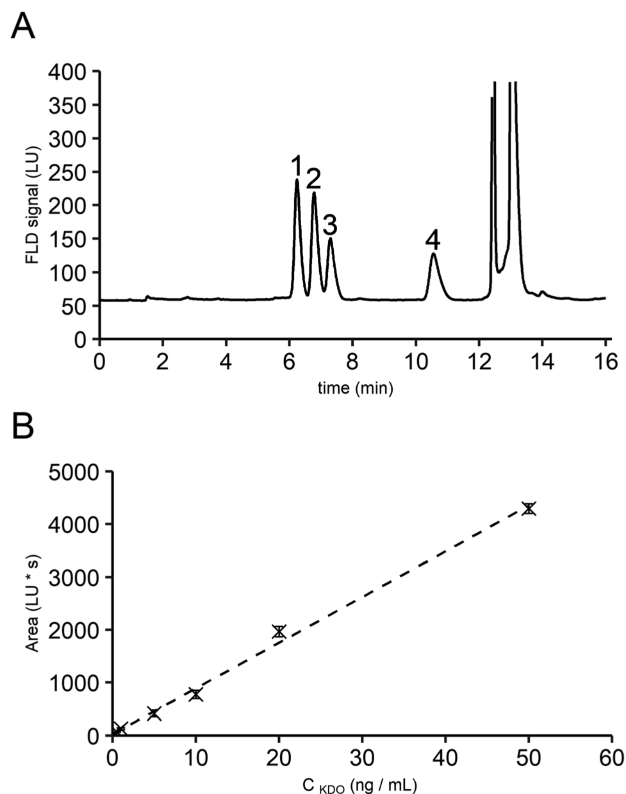

Fig. 6 (A) Chromatogram of a mixture of four sugar acid standards as (KDN (1), KDO (2), NGNA (3), NANA (4)) dissolved in 1× PBS (each 5 ng mL^{-1}) and labelled with DMB, FLD gain 18. Preparation see material and methods. (B) Plot of KDO-DMB peak area *versus* KDO standard dissolved in 1× PBS, concentrations were between 0.5–50 ng mL^{-1}; at each concentration two aliquots were labelled; each aliquot was injected twice.

result for the RSE EC-6 in a theoretical LOQ of about 4 ng ET per mL corresponding to 40 EU per mL. That makes the KDO-DMB-RP-HPLC fluorescence ET assay at the current stage 10^4 times more sensitive than the CZE-DAD ET assay. Further decrease of the ET quantification limits was reached by (1) increasing the injection volume; (2) optimizing labelling conditions, (3) using higher efficiency columns, (4) optimizing separation conditions and (5) applying sample pre-concentration techniques (data not shown due to confidentially reasons).

Comparison of the performance of the KDO-DMB assay and the LAL assay

As mentioned before, LAL assays (mainly in commercial kit form) are today the most commonly used ET identification and quantification tests, especially in the quality control laboratories, but also in DSP development of the pharmaceutical industry. Main advantages are their very high specificity but also their very high sensitivity. On the other hand, LAL testing has substantial drawbacks. Since LAL test results are based on biological response, they exhibit usually a large experimental error. The order of magnitude of the experimental error reflects the system suitability acceptance criteria of compendial LAL testing. The USP 38 and NF 33 [46] state: "Acceptance criteria for a valid assay consists of a positive product control (PPC), [*e.g.* characterized ET standard, CSE] recovery value of 50–200% [accuracy], and a coefficient of variation (CV%) [precision] of less than 25% on reaction times for both sample and PPC channels".[46] These acceptance criteria are applied for cassettes of the Endosafe® product range from Charles River Laboratories. Further, the value of the pyrogenic biological activity, which the LAL test result represents, is different for micelles, vesicles, aggregates and single ET molecules.[68,69] However, ET aggregation in a solution is very difficult to control and depends strongly on matrix, ET concentration and the solvent used.[21] Thus, in order to harmonize assay results for a large variety of samples and matrices, USP 38 and NF 33 [46] request: "Samples should be tested (following the USP BET recommendation) at a dilution (less than the MVD) necessary to consistently eliminate interfering factors such as pH, ionic strength, and high background endotoxin." It follows that common commercialized LAL assays work usually at very low ET concentrations *e.g.* the Endosafe® from 0.001 EU per mL to 50 EU per mL (EU value is RSE based) and samples are diluted to that level with pyrogen free water. To cover the whole concentration range above, one needs to purchase several different cartridges, each with a quite small dynamic concentration range. In contrast, and as mentioned before, the concentration range of common ET measurement is vast, from about 10^9 EU per mL in cell suspensions to about 10^{-2} EU per mL in pharmaceutical products. To fit in the pre-defined small concentration range of *e.g.* an Endosafe® kit cartridge one needs in general to try/perform numerous dilution steps. That makes testing of samples, especially with unknown ET concentration, tedious and quite expensive. In addition, concentration errors in highly concentrated ET samples like in cell suspensions will multiply up from the low dilution result to the original content in the order of magnitude of several million EUs, what makes *e.g.* process development very trial and error based. Further, in order to "standardize" aggregation, LAL testing sample dilutions and handling procedures *e.g.* provided by Charles River[70] have to be followed extremely carefully. Otherwise, results might be incorrect or show large variability. In this sense, a chemical test should be less laborious and still deliver results with small errors. To test this hypothesis, we compared the impact of simple sample handling steps as vortexing or usage of PBS buffer as sample solvent instead of water on targeted ET concentration.

Equally treated samples were measured with LAL as well as with the DMB-KDO RP-HPLC assay. Nominal 5000 EU per mL ET solutions were prepared from a 1 000 000 EU per vial indicator ET standard *E. coli O55:B5* (CSE) stock solution with PBS or with ET free water. The 5000 EU per mL samples were subject to different simple sample handlings; see Fig. 7.

The results in Fig. 7 show that LAL data have a large variability. For the certified CSE the ET content is between 2800 and 16 400 EU per mL, the average value is 8980 EU per mL (instead of the theoretical value of 5000 EU per mL). If one assumes, that the nominal 5000 EU per mL is the "true" value, this average result represents 180% recovery and is with that in the accuracy error limits of the LAL test. The RSD of the five ET contents measured by the LAL assay is with 65% quite large. In contrast,

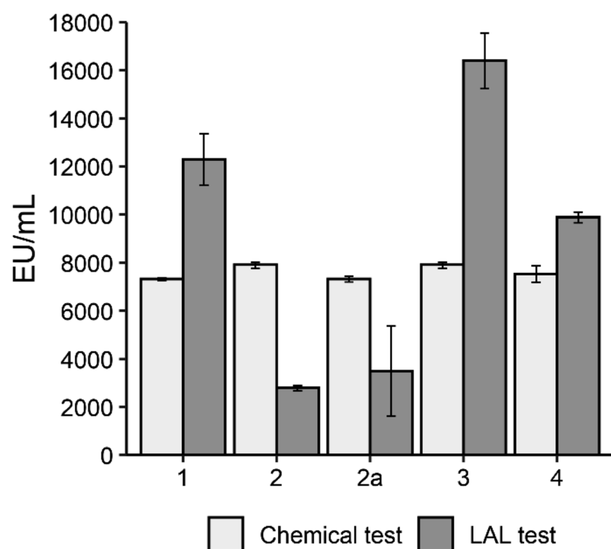

Fig. 7 Comparison of LAL and KDO-DMB assay ET content results for a CSE standard with a nominal ET content of 5000 EU per mL. After preparation of the 5000 EU per mL CSE standard solutions (see Experimental section), solutions were prior to analysis subjected to different simple sample handlings. (1) Samples were measured immediately after the ultrasonic bath (LAL result = 12 300 EU per mL), no extra vortex. (2) Samples were immediately vortexed for 2 min after the ultrasonic bath, and then left standing for 30 min. During those 30 min they were vortexed for 1 min every 10 min, then samples were immediately analysed (LAL result = 2800 EU per mL). (2a) Samples were immediately vortexed for 2 min after the ultrasonic bath, and then left standing for 30 min. During those 30 min they were vortexed for 1 min every 10 min, then samples were left for 60 min standing before being analysed (LAL result = 3500 EU per mL). (3) Samples were left standing for 30 min after the ultrasonic bath before analysis (LAL result = 16 400 EU per mL). (4) Sample handling procedure same as in (1) but the 5000 EU per mL ET solution was prepared with pyrogen free water instead of PBS (LAL result = 9900 EU per mL). For the DMB-KDO RP-HPLC-fluorescence assay the average peak height from three injections of one hydrolysis of each sample handling was used the calculate the EU per mL, error bars represent the standard deviation of the peak heights of the three injections. LAL test result average values and error bars are based on the Charles River testing protocol.[70] The error bars represent the standard deviation extracted from the coefficient of variation (CV%) provided by the LAL protocol.

the results of the chemical test are consistent and virtually unaffected by the different sample pre-treatments/solvent (in contradiction to the fact that sample handling/solvent affects ET aggregation and with that possibly ET hydrolysis kinetics). The assay shows the same KDO-DMB peak height (ET content) for the different simple sample handlings. The average value of the calculated ET content for all five settings was 7609 EU per mL (RSE) with a RSD of 3.9%. This value is in order of magnitude of common instrumental analytical assay precisions and proves the high reproducibility of the assay.

Both methods show higher EU per mL than the theoretically prepared 5000 EU per mL, the KDO-DMB method about 30%, the LAL test about 50%. To speculate for the reason is not very meaningful and out of the scope of this study, since *e.g.* the actual composition, sample heterogeneity and with that the real activity of the preparation are unknown.

Application of the new assay in applied research

Quantification of ETs in bacterial biopolymers. The chemical assay presented in this study was used to analyse the ET content of purified biopolymers, namely poly(3-hydroxyalkanoates) (PHAs) synthetized by bacterial fermentation. Samples were obtained during biopolymer purification.[71,72] These PHA biopolymers are synthesized and intracellular accumulated by *Pseudomonas putida*. They serve naturally as intracellular energy and carbon source. PHAs can be extracted from the cell culture using different organic solvents. These polymers are intended for industrial (*e.g.* packaging) but also for medical applications (*e.g.* for implants and drug delivery systems in humans). *Pseudomonas putida* is a Gram-negative strain and therefore the pyrogenic toxicity of purified PHA must be assessed prior further use, especially, for biomedical applications. In this study, four different extracts obtained during PHA purification were measured with the LAL test and the DMB-KDO RP-HPLC assay, in parallel; results are presented in Table 1.

By plotting the LAL ET content results *versus* the KDO-DMB peak areas of the corresponding samples a linear relation with high correlation ($R^2 = 0.996$) between both assays is obtained. This fact supports our assumption that the chemical-based assay is a suitable tool to be used for process optimisation with respect to ET removal during DSP development. It has the advantages of working over a wide concentration range (without tedious dilutions) with small error. Further, it uses common laboratory equipment, no special LAL device and no LAL cartridges are needed. Using the KDO-DMB HPLC assay, all those facts should contribute to make ET purification development more efficient and economic.

As seen in Table 1, the calculated KDO-DMB based ET content is higher by a factor of about two than the LAL content for each sample. Both, the composition (with respect to KDO number and average molecular weight) and specific pyrogenic activity (in respect to the horseshoe crab) of the *P. putida* ET are unknown so far. For that reason, the KDO-DMB based ET content in Table 1 was calculated with the worst-case scenario

Table 1 Comparison of LAL and KDO-DMB assay's content results obtained for four differently purified *Pseudomonas putida* KT2440 bioreactor samples. LAL assays were performed according to the LAL test protocol from Charles River.[70] DMB-KDO RP-HPLC assay: 2 hydrolysis and 3 injections each, peak area was used for KDO-DMB quantification with external KDO standard calibration, for experimental details refer to Experimental section

LAL assay based ET content [EU per mL]	KDO-DMB HPLC assay based ET content[a] [EU per mL]	RSD chemical test [%]
3550	5314	1
882	1054	3
141	283	6
35	68	3

[a] The KDO-DMB based ET content was calculated using the worst-case scenario and the assumption that the *Pseudomonas putida* ETs have the same biological activity as the RSE, since pyrogenic activity, molecular weight and KDO content of the *Pseudomonas putida* ETs in the bioreactor are unknown.

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(one KDO and an average molecular weight of 40 000 Da was taken for *Pseudomonas putida* ET for calculation) and assuming the biological activity of the RSE. This overestimation supports our assumption, that in case of unknown ET structure and pyrogenic activity, one can use for regulatory questions the worst-case scenario for the conversion of the KDO content to EU per mL. It overestimates the ET content and thus ensures patient safety. It is clear, to strength this assumption, more correlation data with a large array of purified and characterized ET standards/preparations need to be generated in the future.

Testing of novel ET removal depth filters. We applied our analytical method to screen and develop novel depth filter materials applicable for ET removal, *e.g.* in early DSP processes. In Fig. 8, the results for two novel ET filter materials (commercialised in 2019) are shown. Their ET removal capacity (breakthrough) was investigated. Filtration was performed for 500 ng mL^{-1} *E. coli O55:B5* standard ET solutions (theoretical 5000 EU per mL; RSE pyrogenic activity assumption). The goal was to determine if and to what extent filtration efficiency is reduced in dependence on increasing filtration volume. Filtration was performed with a total sample volume of three litres. As seen in Fig. 8, for both PURAFIX® ET-R filters, the measured ET content in the successive fractions was constant over the total filtered volume. For neither of them breakthrough nor drop in filtration efficiency was observed. For the PURAFIX®ET-R1 prototype about 100% ET removal was found (calculation based on the estimated LOQ of 40 EU per mL (RSE)). For the PURAFIX®ET-R2 filter 76% average filtration efficiency was obtained. The RSD for the PURAFIX®ET-R2 efficiency was 5.6% ($n_{\text{fractions}} = 13$). This data demonstrates a stable and high filtration efficiency of both filters. In addition, the data also exemplify the excellent accuracy and precision of the chemical ET assay, which makes it a reliable and reproducible tool in filter development. Data interpretation would have been more difficult using LAL equipment due to the large error of the LAL test. In addition, testing would have been much more expensive because of many trials to find the right sample dilution for LAL testing. We estimate a factor of 10× higher only for LAL testing, what is a substantial number screening many different filter materials. Further, cellulose based depth filters usually contain a small percentage of β-glucans. Since the LAL test is not only responding to ETs but also to β-glucans this can lead to false positive LAL results for those filters. To dispose of this artefact, filtration samples have to be tested for their content of β-glucans with separate cartridges and LAL results have to be corrected respectively. This duplicates the cost of filter testing/screening. This problem is not present using the chemical ET assay, since common β-glucans do not react with DMB and are therefore not detectable with the fluorescence detector.

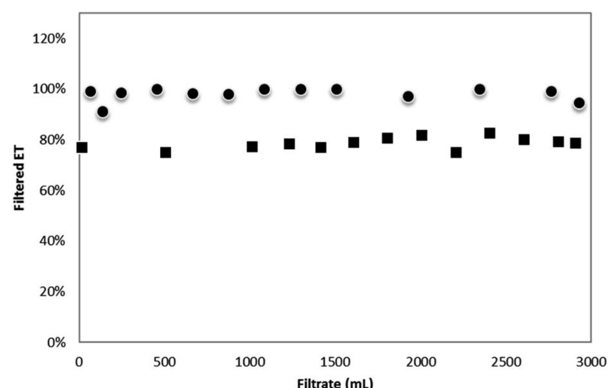

Fig. 8 Plot of percentage of the KDO-DMB peak height with respect to the peak height of the unfiltered ET solution (representing filtration efficiency) in different fractions of a 3 L 500 ng mL^{-1} *E. coli O55:B5* ET solution (Sigma-Aldrich) dissolved in PBS. Depth filter sheets PURAFIX®ET-R1 (13 fractions, circles) and PURAFIX®ET-R2 (13 fractions, squares) (FILTROX AG) were tested for filter breakthrough.

In conclusion, our data shows that the chemical analytical assay is an economic, reliable, robust and easy-to use-tool *e.g.* during product and/or DSP process development with respect to ET removal.

Experimental section

Chemicals

KDO (2-keto-3-deoxyoctonate ammonium salt), DMB (1,2-diamino-4,5-methylenedioxybenzene.2HCl), sodium hydrosulfite ($Na_2S_2O_4$), DMSO (dimethyl sulfoxide), *E. coli O55:B5* and *P. aeruginosa 10* lipopolysaccharide (TCA extracted, gel filtered L2880 and L7018, respectively), *E. coli O55:B5* (purified by gel filtration chromatography, L2637), *P. aeruginosa 10* (purified by phenol extraction, L9143) were purchased from Sigma-Aldrich Ltd (Darmstadt, Germany).

KDN (ketodeoxynonultosonic acid, order number 60714- 50 MG), NANA (*N*-acetylneuraminic acid, order number A0812- 100 MG), NGNA (*N*-glycolylneuraminic acid, order number 50644-10 MG) were purchased from Sigma Aldrich.

Acetic acid (≥99.0%), 2-mercaptoethanol (≥99.0%), $Na_2HPO_4 \cdot 2H_2O$ (≥99.0%) were from Fluka. Acetonitrile (HPLC grade), methanol (HPLC grade) were from Macron. Trifluoracetic acid (TFA, ≥99%) was from Acros. MilliQ-water and pyrogen free water (Charles River Lab.) were used. PBS buffer (1×; pH 7.4) was prepared as follows: 8.00 g NaCl, 0.20 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$ and 0.24 g K_2HPO_4 were weighed and dissolved in 800 mL ultrapure distilled pyrogen free water. The pH was adjusted to 7.4 and filled up to 1000 mL in a volumetric flask. 450 mM Na_2HPO_4 was prepared as follows: 2.0024 g $Na_2HPO_42H_2O$ was weighted and filled up with pyrogen free water to 25 mL in a volumetric flask followed by sonication. The solution was prepared freshly before usage. TFA 4 M was prepared by diluting 2.28 g TFA with MilliQ-water to 5 mL in a volumetric flask. The CZE background electrolyte consisting of 90 mM NaOH and 36 mM Na_2HPO_4 (pH 12.6) was prepared as follows: 18 mL 1 M NaOH and 16 mL 450 mM Na_2HPO_4 were filled up to 200 mL with distilled water.

An ultrasonic bath RK100H from Sonorex, Bandelin, Berlin, Germany was used.

Preparation of KDO standard solutions

A stock solution of 10 mg mL^{-1} KDO in distilled pyrogen free water was prepared and further diluted into 1000 ng mL^{-1}, 500

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ng mL^{-1}, 200 ng mL^{-1}, 100 ng mL^{-1}, 50 ng mL^{-1}, 20 ng mL^{-1}, 10 ng mL^{-1}, 5 ng mL^{-1}, 1 ng mL^{-1}, 0.5 ng mL^{-1} KDO solutions with pyrogen free water (two samples per concentration). Several aliquots of 100 μL were taken, dried under vacuum or immediately deep-frozen at −18 °C. Dried KDO samples were then resuspended in 100 μL PBS buffer before derivatisation and analysis.

A stock solution of each sugar acid as KDO, KDN, NANA, NGNA of 1 mg in 1 mL 1× PBS was prepared. Each of the single sugar acid solutions has been further diluted with 1× PBS to 100 μg mL^{-1}. 100 μL of each of the four sugar acid solutions were combined and filled up to 1 mL with 1× PBS to obtain an intermediate solution containing each sugar acid at 10 μg mL^{-1}. This solution was further diluted with 1× PBS to 5 ng mL^{-1} each sugar acid. The identification of the respective peaks was done by injecting the labelled solution of each sugar acid (KDN, NGNA, NANA) $c = 0.005$ μg mL^{-1}. The obtained chromatogram was compared to a labelled 1× PBS blank for the determination of the sugar acid peak (data not shown).

P. putida KT2440 cultivation samples

Two samples of *Pseudomonas putida KT2440* cultivations, one with an optical density $OD_{600\ nm} = 14$ and one with an $OD_{600\ nm} = 1.2$ were provided by the Biotechnology and Sustainable Chemistry Group (BSC), Institute of Life Sciences HES-SO Sion. They were obtained during the development of the downstream procedure to remove ET from purified poly(3-hydroxyalkanoate) polymers. Aliquots of 1 mL were centrifuged at 4 °C and 14 000 rpm (Centrifuge 5417 R, Eppendorf Ltd Hamburg, Germany) for 4 min. Supernatants were collected, hydrolysed and immediately used for CZE analysis.

LAL testing

LAL test was performed with an Endosafe®-MCS™ system (Charles River Laboratories) according to standard practice (Endosafe®-MCSTM system, Charles River Laboratories, manual).[70] Pyrogen free glassware/vials were always used. For the LAL test, all samples were analysed in duplicate (on one cartridge); the system suitability criteria as requested by the supplier were always fulfilled.

Comparison of the performance of the KDO-DMB assay and the LAL assay; Fig. 7

5000 EU per mL samples were prepared from the same 0.5 mg ET per mL stock solution (about 5 000 000 EU per mL RSE). 0.13 mg of CSE *E. coli O55:B5*, Lot EVV4133 (1 000 000 EU per mL vial, certificate of analysis Charles River Laboratories) were dissolved in 260 μL pyrogen free water. The stock solution was homogenized by slow vortex. 100 μL stock solution was diluted with 100 mL of PBS (experiment 1–3) or pyrogen free water (experiment 4) to theoretical 5000 EU per mL and homogenized in an ultrasonic bath for 15 min.

Samples were analysed with LAL cartridge batch number 5472153; range 1–0.01 EU per mL. In order to reach the LAL cassette measurement range, for all LAL experiments further dilution of the 5000 EU per mL samples was performed with pyrogen free water according to the LAL test protocol from Charles River.[70]

For the DMB-KDO RP-HPLC assay, the nominal 5000 EU per mL samples were subject to ET hydrolysis, DMB labelling, separation and quantification based on KDO-DMB peak height and external KDO-DMB calibration. For conversion of the KDO to EU content, it was assumed that the *E. coli O55:B5 Charles River Standard* (CSE) has like the RSE EC-6 *E. coli 055:B5* ET an average MW of about 10 000 Da and two KDOs[7,67] as well as RSE activity.

Endotoxin hydrolysis with 2 M TFA

3 mg mL^{-1} *E. coli O55:B5* and 3 mg mL^{-1} P. *aeruginosa* 10 ET (Sigma-Aldrich Ltd) standards were used. Standards were hydrolysed with 2 M TFA at 80 °C (100 μL of 4 M TFA added to 100 μL sample). Samples were collected after 5, 10, 20, 30, 60, 120 and 240 min, cooled down on ice, dried with nitrogen evaporation or speed vac and rehydrated before analysis with 100 μL PBS. Further, *P. aeruginosa* 10 ET standard solutions with 0.5, 2 mg mL^{-1} were prepared from 3 mg mL^{-1} stock solution by dilution with PBS.

Procedure of KDO and sialic acid DMB labelling

The labelling reaction of KDO with DMB is a 2-step process.[10,73] First, the pyranoside ring of KDO is equilibrated with its acyclic open ring form catalysed by acidic conditions. During this process, a reactive α-keto-acid is obtained. Second, a multi-step reaction occurs during which the primary amino group of DMB reacts with the carbonyl group of the α-keto-acid to form an imine. This intermediate product is stabilized by sodium dithionite before the second primary amino group reacts with the carboxyl group of KDO to form the final KDO-DMB di-imine.

The KDO (but also NANA, KDN and NGNA) labelling procedure was carried out according to Hara *et al.*[65] Briefly, 100 μL of DMB reagent is added to 100 μL of hydrolysed sample or 100 μL KDO standard solution or to the 5 ng mL^{-1} sugar acid as KDO, KDN, NANA, NGNA sialic acid standard or mix solution (Fig. 6A). The solutions were placed into the heating block at 50 °C for 2.5 h fully covered with aluminium foil to avoid direct light (when the samples are exposed to direct light, their colour becomes reddish). After the reaction, samples were immediately cooled on ice. To collect the liquid from the cap they were centrifuged 5 min at 10 000 rpm (4 °C) with a micro centrifuge (Eppendorf Ltd Hamburg, Germany). Then they were stored in a freezer at −18 °C until analysis. Two sample preparations per sample and two injections per vial were usually performed, if not stated differently. A blank was prepared in each labelling session. The blank was prepared from 100 μL PBS buffer and 100 μL DMB reagent.

RP-HPLC separation and fluorescent detection of labelled KDO

KDO-DMB was detected by fluorescence detection after separation on a RP-HPLC column (Nucleodur Gravity RP-C18, 4.6 × 150 mm, 3 μm). The separation was carried out with an Agilent HPLC Series 1200 containing a cooled auto sampler (4 °C); (Palo Alto, CA, USA). Solvents used were (A) ultra-pure distilled water with 0.1% TFA; (B)

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ACN; (C) MeOH. Isocratic conditions with 86% (A), 7% (B), and 7% (C) were applied; flow rate was 1.5 mL min^{-1}, column temperature was set to 25 °C. The injection volume was 10 μL and the needle was washed after each injection with DMSO. The FLD detector settings were: excitation wavelength: 373 nm, emission wavelength: 448 nm. Depending on the actual KDO concentration, the gain of the fluorescence detector varied between 10–18.

The limit of detection of the method is about 20 EU per mL and limit of quantitation about 40 EU per mL calculated for RSE. The LOD was estimated based on a signal-to-noise ratio S/N = 3 and LOQ on S/N = 10.

CZE-DAD at pH 12.6

High performance capillary electrophoresis (HP 3D CE, Agilent Technologies Inc. Palo Alto, CA) equipped with a DAD and an uncoated bare-fused silica capillary of 50 μm inner diameter; total length: 48 cm; effective length: 40 cm (BGB Analytics, Geneva, Switzerland) was used. The analysis was performed according to the method of Rovio *et al.*[59] The background electrolyte (BGE) contained 90 mM NaOH, 36 mM Na_2HPO_4 in MilliQ-water. BGE was filtered through a 0.45 μm filter and sonicated for 10 min before use. For separation 17 kV was applied in positive polarity mode. The capillary was thermostated at 15 °C. Current was between 110 and 155 μA. Sample injection was performed by pressure: 50 mbar for 12 s. Before each injection, the capillary was rinsed with BGE for 10 min. The detection was performed at 273 nm. Each sample was injected three times and the average peak area calculated.

Calculation of the endotoxin content

The ET content can be calculated based on the KDO content and the ET molecular weight present in a sample. The molecular mass of ETs varies between 2 and 40 kDa in function on the fermentation settings (substrates, phase of the bacterial growth cycle *etc.*), the species, environmental conditions and the purification strategy. Usually a sample is a mixture of ETs with different monomer molecular weights (high sample heterogeneity and complexity).[9,74] Depending on ET concentration, matrix and solvent properties, the amphiphilic properties of ETs let them form micelles/vesicles in water and inverse micelles/vesicles in organic solvents.[21] Due to these properties, no simple, accurate and reliable methods for ET (average) molecular weight determination are available today. To convert the KDO content to an ET content in an unknown ET sample one might establish several scenarios: in the "worst-case" scenario, one ET has a maximal monomeric molecular weight of 40 000 Da (LPS S-type) and contains one KDO. In the "best case", it has a MW of about 2000 Da for LPS R-type and contains three KDOs (in very rare cases four). In particular, the KDO to ET ratio is about 11.9 (w/w) % for one KDO per LPS R-type, about 23.8 (w/w)% for two KDO per LPS R-type, about 35.7 (w/w)% for three KDO per LPS R-type (assuming 2000 Da average monomeric molecular weight), ("best case"). It is about 0.6% for one KDO per LPS S-type (worst case), about 1.2% for two KDO per LPS S-type, about 1.8% for three KDO per LPS S-type (assuming 40 000 Da). The calculated KDO content in a sample can then be converted into ET concentration and the value is converted to EU per mL in case of known biological activity of the particular ET under investigation.

Testing of the ET removal capacity and breakthrough of novel depth filters

In the framework of the EUREKA project ETpure E! 9893 (2015–2018) the depth filter manufacturer FILTROX AG, St. Gallen, Switzerland developed novel ET removal materials in cooperation with our group. These filters can be used for efficient large-scale ET removal, *e.g.* during early stage DSP. They are marketed today as PURAFIX®ET-R. 6 cm diameter prototypes of the PURAFIX®ET-R 1 and PURAFIX®ET-R2 were installed in a metal filter sheet holder (FILTROX AG) and tested. All parts were sterilized before each filtration by NaOH and/or heat (200 °C). PURAFIX®ET-R 1 was tested with ET from *E. coli O55:B5* (Sigma-Aldrich Ltd L2637). The ET filtration solution was prepared as follows: 1.85 mg ET were dissolved in 3.7 mL ET free water, 3 mL of this solution was diluted with 3 L PBS, resulting in 500 ng ET per mL (about 5000 EU per mL). PURAFIX®ET-R2 was tested with the same ET standard. The ET solution was prepared as follows: 1.61 mg ET were dissolved in 3.2 mL ET free water, 3 mL of this solution was diluted with 3 L PBS, resulting in 500 ng ET per mL (about 5000 EU per mL). 100 μL of each fraction/start solution was hydrolysed once and labelled once with DMB, Apollo Scientific, OR3723. Then it was injected two times. Separation and detection were performed as described before. KDO-DMB quantification was based on the average peak height. External standard calibration was performed using two aliquots of a 5 ng mL^{-1} KDO standard (all from the same stock solution) dissolved in PBS and stored at −20 °C. Using a peristaltic pump each tested filter sheet was pre-rinsed with 220 mL PBS. The filtration speed was set to 10–11 mL min^{-1}. This corresponds to a total filtered volume of 1000 L m^{-2}. The 3 L vessel was stirred continuously with a magnetic stirrer at low speed. For PURAFIX®ET-R 1 13 fractions of 10 mL in intervals from 60 to 410 mL were collected successively in ET free (sterilized) volumetric cylinders. For PURAFIX®ET-R 2 13 fractions of 10 mL in intervals from 10 to 495 mL were collected. Filtration recoveries (efficiencies) were calculated in relation to the average KDO-DMB peak height of the unfiltered start solution. Filtration experiments were performed in a hood at room temperature.

Conclusions

After relatively mild acidic cleavage of the ETs, the ET unique and specific building block KDO is obtained quantitatively. KDO is then reacted with the fluorescence marker 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB), which specifically reacts with alpha-keto-acids and which is known to provide very sensitive detection of sialic acids. Derivatisation is followed by RP-HPLC separation of KDO-DMB from other reaction products, ET parts and matrix constituents. The assay uses common lab instrumentation and is, in contrast to LAL testing, independent of sample handling conditions. The assay is easy to perform and shows high accuracy and precision. It has a large dynamic range (10^9 EU per mL to currently about 40 EU

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per mL (LOQ estimated for FDA Reference Standard Endotoxin (RSE), EC-6)) which makes ET analysis of samples with unknown ET content substantially less expensive then LAL testing. In case of LAL testing of samples with unknown ET concentration (*e.g.* during endotoxin removal DSP development or ET filter material development) many of the quite expensive LAL cartridges have to be used to find the right dilution factor corresponding to the very small dynamic measurement range of the cartridges. Using the chemical assay, matrix effects are negligible (due to the specific derivatisation and separation steps). *E.g.* cellulose based ET removing depth filters as developed during our project usually contain a small percentage of β-glucans. Since the LAL test is not only responding to ETs but also to β-glucans one can obtain false positive LAL results for those filters. To circumvent this artefact, filtration samples have to be tested for their content of β-glucans with separate cartridges and LAL results have to be corrected. This duplicates the cost of filter testing/screening. This problem is disposed of using the chemical ET assay, since common β-glucans do not react with DMB, it follows they are not detectable in the chemical assay. In addition, false results due to strong sticking of the ET to matrix parts (LER phenomenon) and aggregation issues are not probable (due to hydrolysis). The test is relatively fast and simple, the workflow can be automated, making measurement of many samples *e.g.* during DSP process development feasible.

The assay has the potential to find its place in the field of ET quantification, especially in product and process development. The test gets the ET quantity in ng mL^{-1}, which can then be converted to EU per mL in case of known biological activity of the analysed ET type. If the biological activity and composition with respect to numbers of KDO and ET average molecular weight of an ET containing solution are unknown, one can work with the worst-case scenario and the pyrogenic activity of the FDA RSE (also used by LAL), what may overestimate the ET content. With that, it ensures patient safety in case of ET quantification for regulatory purposes. The new chemical assay can be a substantial add on to the current ET research, since information about the quantity/biological activity relation of ETs with different structures can be derived.

Abbreviations

ACN	Acetonitrile
BET	Bacterial endotoxin test
CSE	Control standard endotoxin
CZE-DAD	Capillary zone electrophoresis - diode array detection
DMB	1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride
DMSO	Dimethyl sulfoxide
DSP	Down stream processing
ET	Endotoxin
EU	Endotoxin unit
FDA	Food and drug administration
FLD	Fluorescence detection
HOAc	Acetic acid
KDN	Ketodeoxynonultosonic acid
KDO	3-Deoxy-D-manno-oct-2-ulosonic acid
KO	D-*glycero*-α-D-Talo-oct-2-ulopyranosidonic acid
LAL	Limulus amoebocyte lysate
LER	Low endotoxin recovery
LIF	Laser induced fluorescence
LOD	Limit of detection
LOQ	Limit of quantitation
LPS	Lipopolysaccharide
MDV	Maximum valid dilution
MeOH	Methanol
NANA	*N*-Acetylneuraminic acid
NGNA	*N*-Glycolylneuraminic acid
NF	National formulary
OD	Optical density
PBS	Phosphate-buffered saline
RP-HPLC	Reversed-phase high-performance liquid chromatography
RP-U-HPLC	Reversed-phase ultra-high-performance liquid chromatography
RSD	Relative standard deviation
RSE	Reference standard endotoxin
TFA	Trifluoroacetic acid
USP	United States pharmacopeia

Conflicts of interest

There are no conflicts to declare.

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