Hes·so VALAIS WALLIS
Haute Ecole d'Ingénierie
Hochschule für Ingenieurwissenschaften

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Vidjay Christinat

Investigation of Kdo derivates in endotoxin standards

Professor
Franka Kalman

Expert
Agnes Dienes Nagy

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Titre / *Titel*

Investigation of KDO-derivates in endotoxin standards

Description

Endotoxins (ETs) are frequent and dangerous contaminants in pharmaceutical and biotechnological products. They originate from the surface of Gram-negative bacteria where they are non-covalently attached. ETs cause severe health issues as fever, organ failures or deaths in humans when they enter the blood stream.
Today ET analysis is mainly done with biological assays as the rabbit pyrogen test (RPT), the monocyte activation test (MAT) or the Limulus Amoebocyte Lysate (LAL) assay. All these tests show serious setbacks e.g., they either do not convey to animal-welfare guidelines or they suffer from severe matrix effects commonly known as low endotoxin recovery (LER). That can lead to a 100 % masking of ETs in pharmaceutical preparations and with that give false negative test results what can endanger human health.
At HES-SO Valais-Wallis, a chemical quantitative ET assay (KDO-DMB-LC assay) was developed that overcomes the substantial drawbacks of the today commonly used tests. It is based on an ET specific sugar acid (KDO). One to four KDOs are present in the inner core of each ET molecule. In the KDO-DMB-LC assay KDO is liberated by mild acidic hydrolysis and subsequently derivatized with a fluorescent dye (DMB), allowing its sensitive detection. After LC separation, the KDO-DMB analyte is detected via a fluorescence detector.
The ET specific sugar acid KDO can bear several functional groups such as pyrophosphates, phosphates or phosphoethanolamines. Different ET types show different modification patterns. The different, until now known KDO derivates are separated in the current isocratic KDO-DMB assay. Until now only the free, not functionalized KDO is used to calculate the ET content of a sample.

Objectifs

— Optimization of the KDO-DMB-LC method to detect and quantify different KDO species in different R- and S-type ET standards (isocratic and flat gradient mode)
— Investigation of KDO-derivates stability in respect to underivatized KDO during hydrolysis and in stored hydrolyzed and derivatized samples
— Characterization of R- and S-type ET standards in terms of total KDO content and content of each KDO derivate present
— Determination of the total KDO recovery in the different ET standards
— Investigate KDO content (ET content) of a sample *e.g.,* after dephosphorylation
— Identification of the different KDO-derivates by LC-MS
— Challenge the concept whether it is sufficient to use only the not derivatized KDO to calculate the ET content of a sample or all KDO species in a sample must be used to calculate the ET content of a sample

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Investigation of Kdo-derivates in endotoxin standards

Graduate Vidjay Christinat

Bachelor's Thesis
| 2022 |

Degree programme
Technologie du vivant

Field of application
Chimie analytique

Supervising professor
Kalman Franka
franka.kalman@hevs.ch

Objective

At HES-SO Valais-Wallis, based on the ET unique sugar acid Kdo a novel quantitative ET assay (Kdo-DMB-LC assay) was developed. Kdo can be modified.

- Challenge the concept whether it is sufficient to use only the not derivatized Kdo to calculate the ET content of a sample.

Methods | Experiences | Results

Endotoxins (ETs) are frequent and dangerous contaminants in (bio)pharmaceutical products. In this work, the Kdo-DMB-LC fluorescence assay was optimized to detect all Kdo species of five ET standards (isocratic and flat gradient mode). Identification of the different Kdo-derivates was performed by LC-MS. Separation conditions were adapted accordingly. Four masses were assigned to Kdo-derivates as phosphoethanolamine-Kdo, galactose-Kdo, rhamnose-Kdo and heptose-Kdo. Results indicate that the ionization of Kdo and its derivates may lead to a partial loss of a carbonyl group. The three R-type ET standards investigated contain a substantial amount of phosphoethanolamine-Kdo and one neutral sugar derivates. The two S-types have only Kdo, one standard has a tiny amount of phosphoethanolamine-Kdo. The Kdo hydrolysis kinetics for all ET standards in dependence on the hydrolysis time is the same for the same Kdo-derivate found in different ET standards but differs for differently modified Kdo-derivates. To answer the question whether the quantification of Kdo alone is sufficient to calculate the ET content, more investigations about the stability and degradation of the Kdo-derivates are needed.

Scheme of the Kdo-DMB-LC assay workflow and picture of endotoxin structure; E. *coli* O111:B4, adapted from D. Petsch, F. Anspach, J. of Biotechnology, 2000,76, 79. (Hep) L-glycerol-D-manno-heptose; (Gal) galactose; (Glc) glucose; (Kdo) 2-keto-3-deoxyoctonic acid; (NGa) N-acetyl-galactosamine; (NGc) N-acetyl-glucosamine, 1,2-diamino-4,5-methylenedioxybenzene 2 HCl (DMB).

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I. Abbreviation

Ara4n	4-amino-4-deoxy-L-arabinose
BET	Bacterial endotoxin test
DAD	Diode-Array Detection
DMB	1,2-diamino-4,5-methylenedioxybenzene 2 HCl
ET	Endotoxin
FLD	Fluorescence Light detection
Gal	Galactose
GlcN	Glucosamine
Hep	L-glycero-alpha-D-manno-heptopyranose
HOA	Analysis made by Anika Hoffmann
Kdn	Deamino-alpha-neuraminic acid
Kdo	3-Deoxy-D-manno-oct-2-ulosonic acid
LAL	Limulus amoebocytes lysate
LPS	Lipopolysaccharide
MS	Mass spectrometry detector
MSDiQ	Mass selective detection
mQ	MiliQ Water
NANA	N-Acetylneuraminic acid
NGNA	N-Glycolyneuraminic acid
PEtN	Phosphoethanolamine
Rha	Rhamnose
RP-HPLC-FLD	Reverse phase high pressure liquid chromatography with fluorescence light detection
RP-HPLC-MS	Reverse phase high pressure liquid chromatography with mass spectrometry detection.
RSD	Residual Standard Deviation
TIC	Total ion chromatography
VIC	Analysis made by Vidjay Christinat

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1 Introduction

Endotoxins are complex molecules found on the outer membrane of Gram-negative bacteria; They are composed of three different parts (O-Antigen, core, Lipid A). The hydrophilic O-Antigen is made of a polysaccharide chain. Its length varies depending on the bacteria strain. Lipopolysaccharides (LPSs) having the O-Antigen are called S-type which stands for smooth and the LPSs lacking the O-Antigen are called R-type for rough [1], [2]. The core is divided into an outer and an inner part and is a non-repeating chain of sugar units attached to the Lipid A. It contains one to three 3-deoxy-D-manno-oct-2-ulosonic acids (Kdos) to which other sugars like heptose and hexoses such as glucose or galactose are linked [3]. Moreover, different molecules like phosphate, carbonyl groups and phosphoethanolamine (PEtN) may be found on Kdo. Lipid A is integrated in the outer membrane. It is responsible for the toxicity of the endotoxin molecule. This hydrophobic part is constituted of acyl chains attached to two glucosamines, to which the core is bonded[4].

Bacteria release LPS during their growth, but the release is more important during bacterial lysis or in contact with antibiotics[5]. If endotoxins enter the human blood stream, they can cause fever, hypotension, inflammation and in the worst-case septic shock leading to death [6], [7]. Even in low concentrations, endotoxin can be life-threatening. According to the FDA the concentration threshold can vary depending on the intended use, but also where on the body the devices or the injectable solution is used. For instance, the limit is lower for devices in contact with cerebrospinal fluid, the concentration of endotoxin cannot exceed 0.06 endotoxin units (EU)/ml or 6 pg/ml [8]. EU is the unit of measurement for endotoxins activity.

To quantify endotoxins different biological assays have been developed. The rabbit pyrogen test (RPT), which measures the body temperature increase in rabbits after an intravenous injection of a potentially contaminated product. This worldwide applied test for endotoxin detection, shows a lack of sensitivity [9], robustness and high false-positive/negative rates [6]. Moreover, this method kilies hundreds of thousands of rabbits every year and conflicts with animal-welfare [10]. An alternative to the use of rabbits for bacterial endotoxin testing (BET) was developed in the 1980s to increase sensitivity. This method, called Limulus Amoebocyte Lysate (LAL) assay uses the amoebocytes from the horseshoe crab blood [6]. In contact with endotoxins these cells form an agglutination which can be quantified by e.g., turbidimetric measurement. Although this method is with a limit of detection of 0.005 EU/ml the most sensitive endotoxin assay, it has a lack of repeatability and is subject to interference [11].

A chemical method was recently developed during the Innosuisse ET_Join project at the HES-SO Valais-Wallis using RP-HPLC-FLD for the quantification of endotoxins based on Kdo. This endotoxin specific sugar acid is found in the inner core of the LPS and is linked to the Lipid A by a ketosidic linkage that can be cleaved under acidic condition. Unlike other sugars, Kdo is always present in the endotoxin molecule proving that it is crucial for the viability of the bacteria[12] [13]. In the chemical method, Kdo is released from the lipopolysaccharide core by hydrolysis and is then stoichiometrically derivatized with the fluorophore 1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (DMB). Then the molecule is separated by RP-HPLC from the matrix and the Kdo-DMB is quantified using a fluorescence detector [4].

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The chromatogram obtained from this method shows beside the known Kdo-DMB peak, which was identified with a Kdo standard, additional peaks. These were assigned to different Kdo species e.g., Kdo-PEtN by previous MS experiments. Need all of these Kdo species to be considered when quantifying the endotoxin content or is the consideration of the originally only known Kdo-DMB peak sufficient for quantifying? To answer this question, the RP-HPLC-FLD method used for the endotoxin quantification procedure was first optimised and transferred to RP-HPLC-MSDiQ to confirm the previously found Kdo species and to identify new ones if present. Secondly, analyses were performed using RP-HPLC-FLD to quantify the Kdo species in different commercially available endotoxin standards. Finally, the impact of the hydrolysis time to better understand the kinetic of Kdo derivates release under chosen condition were examined

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2 Materials and Method

2.1 Instrumentation

HPLC : Agilent, LC1200

Instrument modules :

Degasser:	G1322A, Degasser, 1200 Series,	serial No: JP60653581
Pump:	G1311A, QuatPump, 1100 Series,	serial No: DE33225066
Sampler:	G1329A, ALS, 1200 Series,	serial No: DE60556326
Thermostat:	G1330A, ALSTherm, 1100 Series,	serial No: DE82207029
Oven:	G1316A, TCC, 1200 Series,	serial No: DE60556896
Detector:	G1321A, FLD, 1200 Series,	serial No: DE60555395

HPLC : Agilent, LC/MSD iQ

Instrument modules :

Pump:	G7120A, High Speed Pump, 1290 Infinity II,	serial No: DEBA200250
Sampler:	G7167B, Multisampler, 1290 Infinity II,	serial No: DEBAQ03434
Oven:	G7116B, MCT, 1290 Infinity II,	serial No: DEBA405606
Detector:	G6160A, InfinityLab LC/MSD iQ,	serial No: SG2036R005
Turbopump:	G6011B, Quiet Cover MS	serial No: US2040B228

Software: OpenLab Version 3.5

Column HPLC:

YMC Triart C18 ExRS /S-3um/ 8nm, size: 150 x 2.1 mmI.D, P.N. TAR08S03-15Q1PTH, S.N.108YB10030, Lot: 18799.

Column HPLC: (use for MS analysis only)

YMC Triart C18 ExRS /S-3um/ 8nm, size: 150 x 2.1 mmI.D, P.N. TAR08S03-15Q1PTH, S.N. 103HB00299, Lot: 17674.

Balance:

METTLER TOLEDO XPR205 (± 0,01mg)
METTLER TOLEDO XPR206 (± 0,005mg)
Refrigerated benchtop centrifuge Sigma 3-16KL
Casual laboratory material
Vial: Ecoline 1.5ml Crimp Neck Vial 32 x 11.6mm (amber)
Insert: 300ul Conical Glass Insert (3 x 6mm)

Micropipettes:

0.5 -10µl	Discovery Confort (A4042A062) (HES-SO, DxS-05, DxS LAB)
2-20µl	Discovery Confort (A404A078) (HES-SO, DxS-05, DxS LAB)
20-200µl	Discovery Confort (A4045A079) (HES-SO, DxS-05, DxS LAB)
100-1000µl	Discovery Confort (A4046A113) (HES-SO, DxS-05, DxS LAB)

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2.2 Products and Solvent

Table 1: Product and solvent used

Name	Abbreviation	Purity	Supplier	Product No.	Lot
3-Deoxy-D-manno-oct-2-ulosonic acid	Kdo	≥97%	Sigma	K2755	SLCD6533
1,2-diamino-4,5-methylenedioxybenzene.2HCl	DMB	≥98%	Sigma	66807	BCBR7805V
1,2-diamino-4,5-methylenedioxybenzene.2HCl	DMB	≥98%	Apollo	OR3723	AS452384
Acetic acid	HOAc	≥99.0%	Fluka	A6283	45731
2-Mercaptoethanol	BME	≥99.0%	Fluka	637000	STBH7709
Sodium hydrosulfite	$Na_2S_2O_4$	85%	Sigma	157953	#STBH8698
Acetonitrile	ACN	HPLC grade	Macron	6712-25	NA
Methanol	MeOH	HPLC grade	Macron	28-56-25	NA

2.3 Endotoxin standards

Table 2: Different purified endotoxin standards used for the analysis.

Name of lipopolysaccharide	Abbreviation	Supplier	Product No.	Lot
Escherichia coli EH100	*E. coli* EH100	Sigma	L9641	#025M4093
Escherichia coli O55B5	*E. coli* O55B5	Sigma	L2637	#0000102731
Escherichia coli F583	*E. coli* F583	Sigma	L6893	#128M4131V
Escherichia coli K12	*E. coli* K12	Sigma	NA	#5973-42-01
Salmonella Enterica	S. E	Sigma	L6261	#059M4137V
Pseudomonas Aeruginosa	P. A	Sigma	L9143	#075M4089V

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2.4 Analytical method

The analytical method as described in the SOP "Analytical procedure for quantification of the endotoxins content for filter development and in biological samples" see Annex 14 was used. The blank preparation of the SOP was modified by adding 2 µL of acetic acid to the blank solution before hydrolysis and derivatization.

2.5 Instrumental method

In this section all instrumental methods used for operating HPLC, and LCMS-IQ instruments are given Table 3. Details of the methods can be found in section Annex: or in the software OpenLab under the Path: /Students_Perso/2022/Vidjay/Methods.

Table 3: Table of method by HPLC-DAD, HPLC-FLD- HPLC-MS

Method name	Comment
Method 1: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC.amx	Initial method
Method 2: 2022_05_16_KDO_YMC_GC_12%_031mL_HPLC_iso	Run Optimisation
Method 3: 2022_05_17_optimisation_gradient_95%_78.6	Run Optimisation
Method 4: 2022_05_23_optimisation_gradient_20min_Plateau_95%_76min	Run Optimisation
Method 5: 2022_05_24_optimisation_gradient_plateau_8%in5min	Run Optimisation
Method 6: 2022_05_24_optimisation_gradient_plateau_8%in10min	Run Optimisation
Method 7: 2022_05_24_optimisation_gradient_plateau_8%in20min	Run Optimisation
Method 8: 2022_05_25_optimisation_gradient_plateau_8%in9min.amx	Run Optimisation
Method 9: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC_MS.amx:	MS analysis
Method 10: 2021_10_14_KDO_YMC_GC_12%_031mL_20ul_HPLC_MS.amx	MS analysis
Method 11: 2021_10_14_KDO_YMC_GC_12%_031m_20uL_HPLC_01%FA_MS.amx Eluent A: mQ water + 0.1% FA, Eluent B: ACN/MeOH (36/64) + 0.1% FA.	MS analysis
Method 12: 2022_05_25_optimisation_gradient_plateau_8%in9min_20ul_MS.amx A: mQ water + 0.1% FA, Eluent B: ACN/MeOH (36/64) + 0.1% FA.	MS analysis

2.6 Column Performance test

Before using the YMC columns Triart C18 ExRS SN: 108YB10030, 103HB00299 the column performance was tested. Tests according to the supplier instructions were performed. The retention time, the theoretical plate number, the capacity factor, and the tailing factor were checked. To verify these parameters, a test solution with the three compounds uracil (0.02 mg/mL), methyl benzoate (0.7 µL/mL) and naphthalene (0.24 mg/mL) was prepared (Raw data in Annex 1).

Remark:

For statistical purposes, a sequence of six injections of a Kdo standard with 5 ng/ml concentration was used. The value obtained show a relative standard deviation (RSD) of < 1 % for the retention time, the peak height, and the peak area (). Moreover, the resolution factor has an average of 2.9 for $n_{injections}$=6, which is higher than the recommended value 1.5 (see Annex 5 and Annex 6).

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3 Results

3.1 Run Optimisation

To identify the chromatographic profiles and retention times of each Kdo species, different endotoxins standards and four sugar acids were injected. For this experiment the initial method RP-HPLC was used (Method 1). This method shows an elution of the main Kdo species in 15 minutes. The results are shown in Figure 1. Here, in total seven different sugar acids are depicted in an overlay of different endotoxin standards.

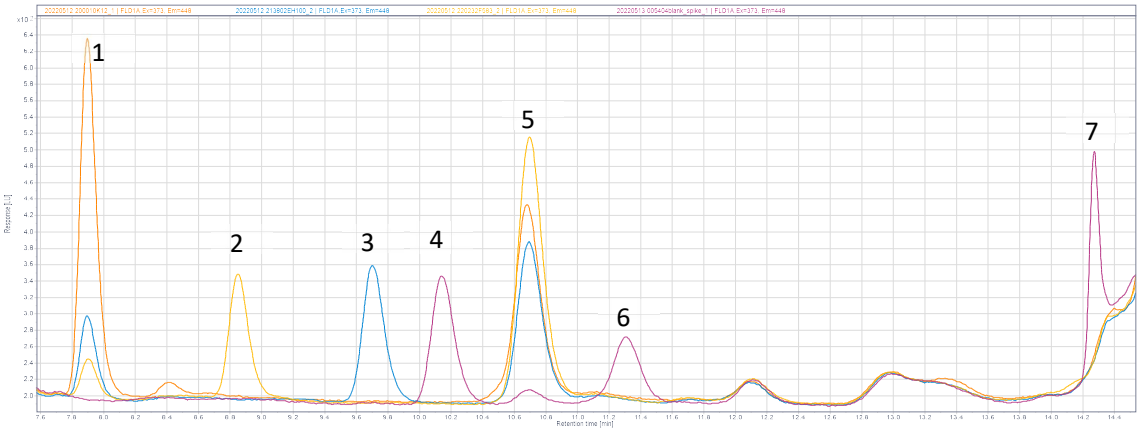

Figure 1: Overlay chromatograms of different endotoxin standards showing different sugar acids. Orange: LPS from E. coli K12 (500 ng/ml), Yellow: LPS from F583 (250 ng/ml), Blue: LPS from E. coli EH100 (250 ng/ml), Purple: sialic acids standard with Kdn, NANA, NGNA each at (5 ng/ml) 1 PEtN-Kdo-DMB, 2 Hep-Kdo-DMB, 3 Gal-Kdo-DMB, 4 Kdn-DMB, 5 Kdo-DMB, 6 NGNA-DMB, 7 NANA-DMB. Method used: Method 1

Molecules that react specifically with the DMB includes sialic acids which are not found in endotoxins molecules. To distinguish the sialic acids from the Kdo-species their retention times (see molecules 5, 6, and 7) were determined with commercial standards. To quantify the totality of Kdo species, it is necessary to verify if further Kdo modifications are observed that may elute after the isocratic separation condition of the routine analysis (Method 1). To investigate this statement, a method using a longer isocratic step and a gradient to a higher organic percentage was developed. The following section describes the procedure for the method development. The principal aspect of this research was a meticulous comparison of the chromatographic profile of the blank and endotoxin standards.

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3.1.1 Method optimisation

Before starting the method optimisation, it was decided to keep the first ten minutes of the initial Method 1. The analysis starts with 100 % of eluent A, mQ water, and changes after 0.1 seconds to 88 % eluent A and 12 % eluent B, methanol/ acetonitrile (64 %/36 %), for ten minutes. As these parameters have already been optimized, they were not modified. This allows to compare the first minutes during the optimization period.

Isocratic test method 2: To begin with, an isocratic method with 12 % of eluent B was used to see peak differences between a blank and an endotoxin standard.

Observation Method 2: Four different peaks were observed in Figure 2 respectively at t_R= 26.5, 38.9, 77.1, 85.8 minutes. In the chromatograms of endotoxin standards peaks were observed that were absent in the mQ water blank were as visible in Figure 2 e.g., peak n° 2. The result obtained with the isocratic method gave an indication on which elution areas the focus needed to be set.

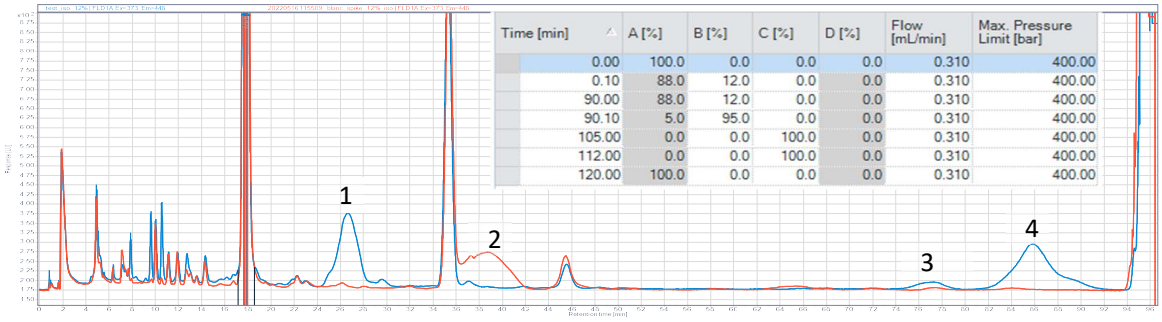

Figure 2: Overlay of chromatogram showing in red the mQ water blank spiked with 5ng/ml of sialic acid and in blue E. coli EH100 (250 ng/ml) + spike of sialic acids (Kdn, NANA, NGNA each at 5ng/ml). Method 2 used for analyses

Method 3: A first method RP-HPLC was created by HOA and used to observe peak differences between a blank and an endotoxin standard injection with an extended organic gradient (Method 3). The isocratic part with 12 % eluent B was kept until 30 minutes. For 45 minutes, the organic percentage was raised to 95 % eluent B. This percentage was kept for 3.6 minutes to finally return in the start condition. See below the timetable.

Table 4: Timetable for Method 3

Time [min]	A [%]	B [%]	C [%]	D [%]	Flow [mL/min]	Pressure [bar]
0.0	100.0	0.0	0.0	0.0	0.310	400
0.1	88.0	12.0	0.0	0.0	0.310	400
30.0	88.0	12.0	0.0	0.0	0.310	400
75.0	5.0	95.0	0.0	0.0	0.310	400
78.6	5.0	95.0	0.0	0.0	0.310	400
79.0	100.0	0.0	0.0	0.0	0.310	400

The results obtained were compared with the existing data from HOA and are shown in Figure 3. Despite higher signal intensities in chromatogram A, the two chromatographic profiles showed similarities. As the analysis was done on two different instruments with different FL detectors, this may explain the variation. The chromatogram A is annotated with red question marks to show the differences observed between the two injections. These differences are not found in the analysis done by VIC. This may be due to the difference in sensitivity of the detector.

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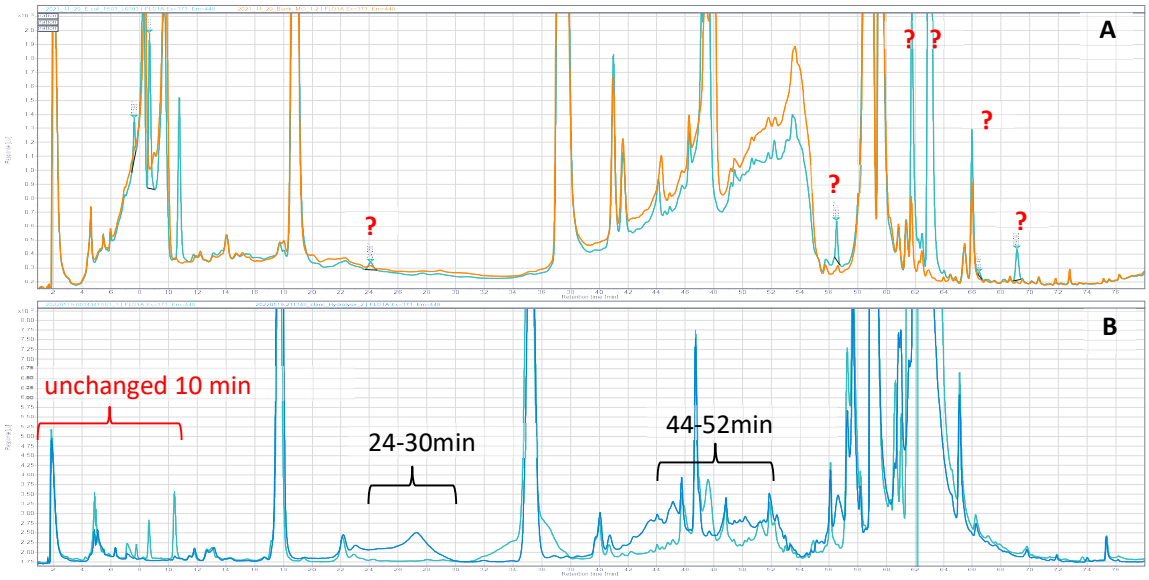

Figure 3: Overlay Chromatograms. A: chromatogram from HOA, Blue: E. coli F583 (500 ng/ml). Orange: Blank (mQ water). B: chromatogram from VIC, Light blue: E. coli F583 (500 ng/ml). Dark blue: blank (mQ water). Method 3 used for analyses.

Conclusion method 3: The differences of peaks appearing on chromatogram A are not found in analysis B using the same RP-HPLC method. On B: around 26 minutes a major peak for the *E. coli* F583 can be observed that is not present in the blank. Around 46 minutes signals show differences between blank and *E. coli* F583 but is not well resolved.

Method 4: The improvement consisted of reducing the peak width around 26 minutes and increasing the peak separation and resolutions around 46 minutes. The isocratic part was reduced to have a faster elution. A new gradient was added starting at 20 to 56 minutes from 12 % to 55 % eluent B and kept stable. Finally, to elute the components, the eluent B is increased up to 95 % in 10 minutes. See the details of Method 4

Figure 4: Chromatogram analysed by (Method 4), A: first injection F583 (250 ng/ml) F583 B: second injection F583 (250 ng/ml), C: Blank (mQ water).

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Conclusion Method 4: The peak around 26 minutes is sharper, and the separation around 46 minutes is better. The endotoxin standard *E. coli* F583 and the blank injection show similar chromatographic profiles (B and C on Figure 4) but differ slightly around 22 minutes and 60 minutes (see Figure 4).

Optimisation method 5, 6, 7:

The following experiments were focused on the first 30 minutes of the separation time, to improve the separation of the large peak at about 27 minutes. Furthermore, wanted compounds should contain sugars like galactose, heptose, rhamnose. The retention time of these compounds on a reverse phase column is not very strong, which results in a low retention time. It is not surprising to see on the right side of the chromatogram a large number of peaks especially at the end of the gradient when the proportion of eluent B is predominant. Endotoxins contain various types of sugar but also fatty acids, which are desorbed under apolar conditions.

Different optimizations were tested on the same basis. A slow gradient was applied in 20 minutes from 12 % to 20 % eluent B. In order to observe the behaviour of the chromatogram, two other methods with a five and a ten minutes gradient were tested (see Method 5, Method 6, Method 7).

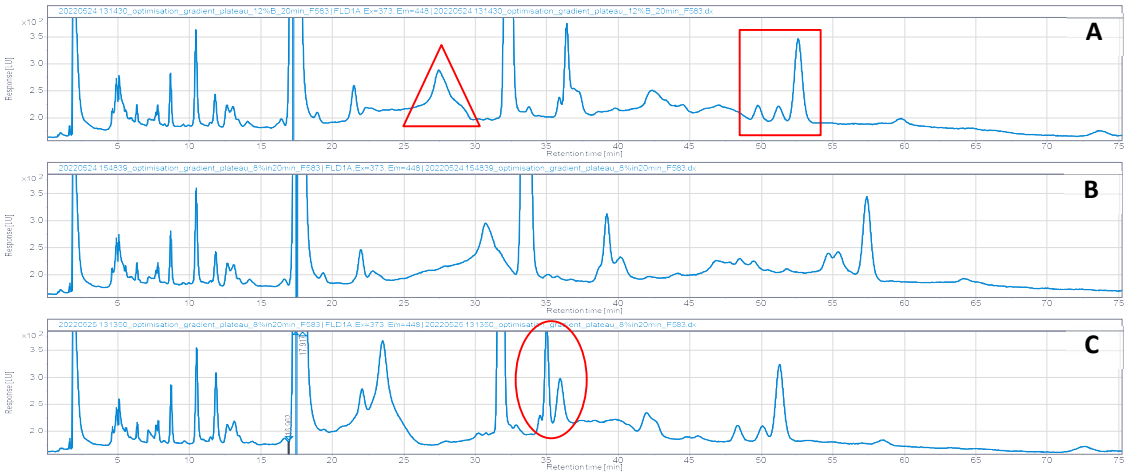

Figure 5: chromatograms from E. coli F583 (250 ng/ml):
A: Method 6: 2022_05_24_optimisation_gradient_plateau_8%in10min
B: Method 7: 2022_05_24_optimisation_gradient_plateau_8%in20min
C: Method 5: 2022_05_24_optimisation_gradient_plateau_8%in5min

Conclusion method 6, 7, 8: method 6 with a ten-minute gradient is the most suitable for the peak intensity and separation. The large peak in the triangle does not affect the small one before, peaks in the square are well resolved. But the circle shows better resolved peaks at 35 minutes separation time with Method 5. The gradient at ten minutes should be shortened slightly to achieve better separation at 35 minutes (circled area).

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Optimisation with Method 8: The final method used for the long gradient is *Method 8: 2022_05_25_optimisation_gradient_plateau_8%in9min.amx*. In this method the gradient is done in nine minutes instead of ten minutes.

Table 5: Timetable of Method 8: 2022_05_25_optimisation_gradient_plateau_8%in9min.amx

Time [min]	A [%]	B [%]	C [%]	D [%]	Flow [ml/min]	Pressure [Bar]
0 .0	100.0	0.0	0.0	0.0	0.310 mL/min	400
0.1	88.0	12.0	0.0	0.0	0.310 mL/min	400
22.0	88.0	12.0	0.0	0.0	0.310 mL/min	400
31.0	80.0	20.0	0.0	0.0	0.310 mL/min	400
80.0	80.0	20.0	0.0	0.0	0.310 mL/min	400
100.0	5.0	95.0	0.0	0.0	0.310 mL/min	400
104.1	5.0	95.0	0.0	0.0	0.310 mL/min	400
105.0	100.0	0.0	0.0	0.0	0.310 mL/min	400

Observation Method 8*:* The large peak at t_R= 21 minutes seems to shift to lower retention times during consecutive injections, and it is found with the same intensity in the blank and the endotoxin standard (Figure 6). Therefore, the large peak is not a specific endotoxin compound but something that is found in the matrix. Endotoxin standards in Figure 6 labelled with the letter A and C show different peak intensities for the encircled areas than the blank (Figure 6B).

Repeatability test on Method 8: Because of the observed shifts in retention times during the runs, a repeatability test was done on five different endotoxin standards. *E. coli* EH100, *E. coli* F583, *E. coli*

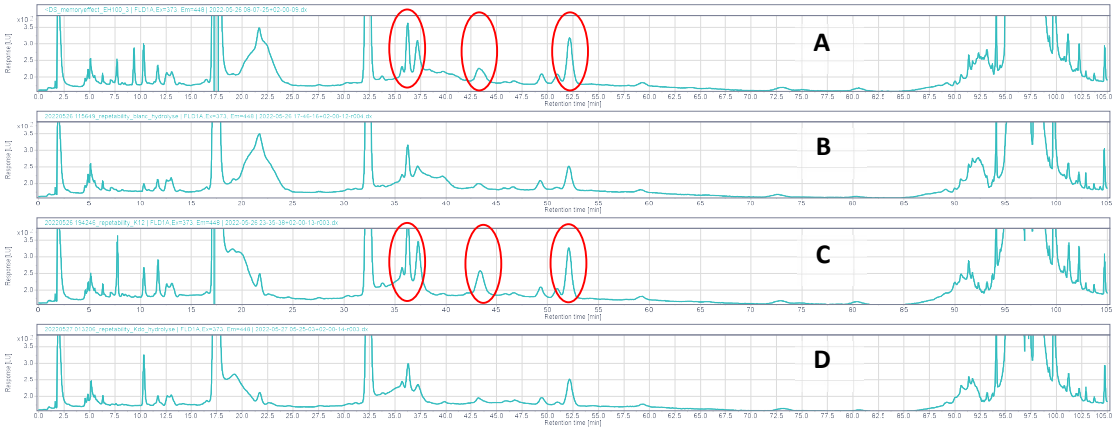

Figure 6: chromatograms measured with Method 8. A: Endotoxin standard E. coli EH100 (250 ng/ml), B: blank (mQ water), C: Endotoxin standard E. coli K12 (250 ng/ml), D: Kdo standard (5 ng/ml).

K12, *E. coli* O55:B5 and P. aeruginosa. The comparisons were made on the chromatogram's profiles.

Observation: The result is shown in Figure 7 below for the *E. coli* K12. *E. coli* K12 shows repeatable chromatographic profiles but no obvious differences are visible between the blank and the endotoxin standard. The absence of variation is also observed for the other endotoxins (See Annex 7, Annex 8, Annex 9).

Conclusion: Endotoxin standards and the blank show no differences after the Kdo-DMB peak at t_R= 10 minutes. The prolongated separation runs endotoxin standard are repeatable.

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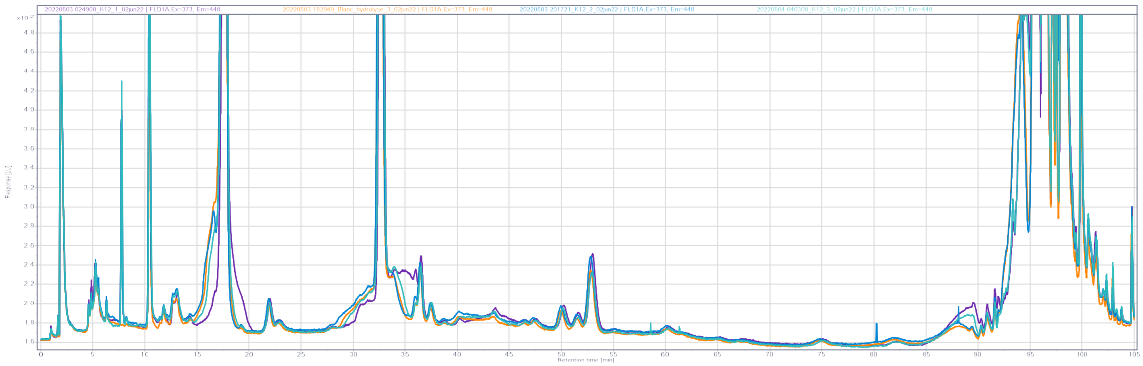

Figure 7: Overlay chromatograms from Method 8 repeatability test $n_{injections}$ =3, E. coli K12 (250 ng/ml), Purple: injection n°1, Dark blue: injection n°2, Light blue: injection n°3. Orange: blank (mQ water).

3.1.2 Discussion:

The goal was to improve the resolution between the peaks to see differences between endotoxin standards and mQ water blank injections. During the optimisation, many configurations were tested, adapting gradient time and steepness and the duration of the isocratic plateau to find a maximum of differences in peak number and intensity. A repeatability analysis showed similar chromatographic profiles. The objective is moderately met, many peaks could be separated, and some peaks show different intensities between the blank and the endotoxin standard injection. But this observation was not repeatable from one analysis to another, comparing Figure 6 and Figure 7. During the method optimisation period, observations were made of forward or backward peak shifts (see Figure 6). This shift may be due to a difference of matrix between blank and endotoxin, but this peak does not seem to be specifically present in endotoxins. Other reasons could be the inhomogeneity of the endotoxin powder or the purity of it. Comparing the two analyses from different operators and HPLC instruments, an important difference in intensity was noticed. It is important to mention that the FL detector used for the sample analysis for the bachelor thesis is less sensitive than the FLD used by HOA. In Figure 3A, the signal is significantly higher than on the chromatogram 3B. In chromatogram 3A six peaks were observed that differentiate from the blank to the measured endotoxin standard, but none of them were visible on chromatogram 3B. Overall, method 8 met the criteria for method improvement best, i.e., peak separation and resolution. Although the weaknesses such as the lack of difference observed on the chromatograms between the endotoxin standards and the mQ water blank, and the shift of the peaks could not be resolved. Nevertheless, it was decided to transfer this method to mass spectrometry measurements. These measurements are needed to scan the separated and nonidentified peaks to find masses that belong to Kdo-DMB fragments or different Kdo-species. The following retention time, where peaks were seen in higher intensity in endotoxin standard than in blank, were retained to investigate their masses (Table 6).

Table 6: Retention time of peaks that show different intensity in endotoxin standard than in mQ water blank.

Retention Time [min]	Remark
22.62	Large pics showing peak shift
36.27 and 37.21	Showing higher intensity for *E. coli* K12 and *E. coli* EH100 than blank
43.16	Showing higher intensity for *E. coli* K12 and *E. coli* EH100 than blank
51.02 and 52.24	Showing higher intensity for *E. coli* K12 and *E. coli* EH100 than blank

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3.2 Mass spectrometry analysis

Once the RP-HPLC-FLD run was optimized, the goal was to identify the observed peaks by mass spectrometry. Previous mass spectrometry analyses done by Blanka Bucsella revealed different Kdo species besides the known Kdo-DMB compound. They are grouped in Table 7. In addition, investigations on Kdo-DMB linked to other sugar types were conducted. In this bachelor's thesis a InfinityLab LC/MSD iQ instrument was used for sample analysis.

Table 7: Previous masses found in different endotoxins standard by Blanka Bucsella. Measurements were done on a HR-ESI-MS instrument.

Name	Chemical formula	[M+H] m/Z	Detected In
KDO-DMB	$C_{15}H_{18}N_2O_8$	355.11359	All
KDO-P-DMB	$C_{15}H_{19}N_2O_{11}P$	435.07992	All
KDO-PEtN-DMB	$C_{17}H_{24}N_3O_{11}P$	478.12212	All
KDO-PPEtN-DMB	$C_{17}H_{25}N_3O_{14}P_2$	558.08845	All
alpha-LD-Hep (1-5)-KDO	$C_{22}H_{30}N_2O_{14}$	547.17750	*E. coli* F583
alpha-D-Gal (1-7) KDO	$C_{21}H_{28}N_2O_{13}$	517.16444	*E. coli* EH100
alpha-L-Rha-(1-5)-KDO	$C_{21}H_{28}N_2O_{12}$	501.16998	*E. coli* K12

3.2.1 Method transfer

When transferring the method from one instrument to another, some parameters had to be adapted to have a sufficient signal height for analyte detection, as the injection volume or the eluent. A first test was performed with a ten µl injection of 5000 ng/ml of Kdo standard (Method 9). The intensity was low with about 35'000 Counts Height. The injection volume was adapted to 20 µl (Method 10), which gave a signal height of 61'000 counts. Simultaneously, this proved the linearity of the mass detector response as the number of counts approx. doubled with twice the injection volume. Working in positive ionization mode, another decision was to acidify the mobile phase with 0.1 % (v/v) of formic acid which allows a better protonation of the Kdo-DMB analyte. On one hand, the signal's response was almost four-times higher with 0.1 % of formic acid added to the eluents (Figure 8). On the other hand, the analytes elute with a different retention time, which changes the chromatographic profile.

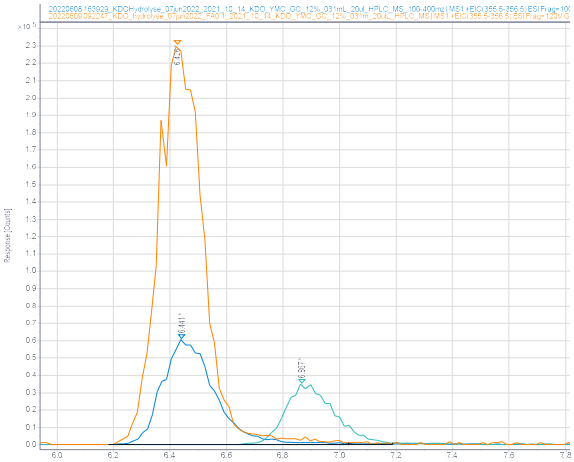

Figure 8: Mass Spectrum (extracted mass 355.5 to 356.5 m/Z) from Kdo hydrolysed + derivatized (5000ng/ml)
In light blue: Method 9 (10µl injection)
In dark Blue: Method 10 (20µl injection)
In orange: Method 11 (20µl injection, Eluent + 0.1% FA)

Conclusion: The decision was made to use the eluent containing formic acid to have a maximum of height and increase the sensitivity. To find different masses in endotoxins, analyses were made with the scan mode from 100 to 600 m/z using the Method 12. This range covers the vast majority of molecule searched as the Kdo-DMB mass is expected at 355.1 m/z. To be sensitive enough endotoxins were prepared at a concentration of 0.5 mg/ml. The sample preparation stayed the same as described in [14].

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3.2.2 Mass spectra signal and specific peaks

The first step was to identify differences between the blank and the Kdo standard injection followed by a search for the Kdo-DMB mass in the TIC signal. In Figure 9, the TIC signals from blank (n°2) versus Kdo (n° 3) show the same profile. Any differences could be distinguished in the TIC signal, due to an important noise. The Kdo-DMB ion is found with a mass of 355.8 m/z under the noise with S/N of about 15 (n°1).

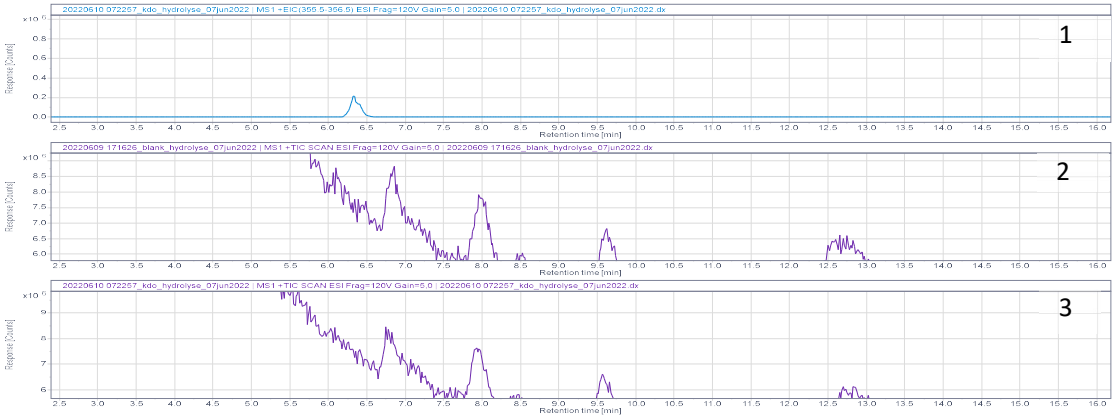

Figure 9: mass spectrometry Chromatograms: 1) mass 355.8 m/z extracted from TIC (5000 ng/ml Kdo). 2) TIC from blank 3) TIC from Kdo (5000 ng/ml)

The noise is characterised by an important contamination with the mass 153.4 m/z and 136.3 m/z, that were seen along the spectra. The background could be removed but the amount of contamination had a negative impact on the sensitivity. On Figure 10, on the right, the mass of Kdo-DMB is observed with a low intensity (6 %) in comparison with the mass 153.4 (100 %).

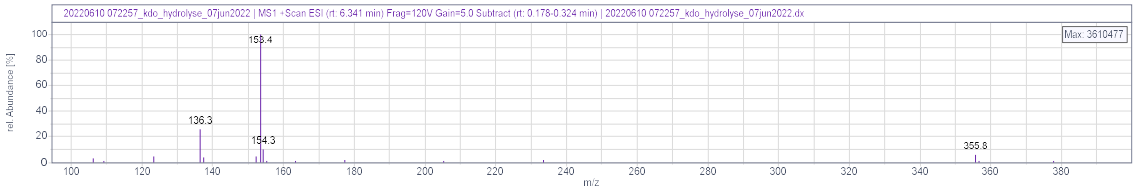

Figure 10: Mass extracted at 6.314min. Injection of Kdo hydrolysed (5000ng/ml) without background reduction.

Not knowing the masses of potential functional groups such as sugars that might be bound to Kdo, a search for differences in the chromatographic profile between the blank and endotoxins standards was performed. To reduce the noise, the spectra were reduced between 300 m/z to 600 m/z. Then the masses of the isolated peaks could be extracted and compared to Table 7. Figure 11 shows the major difference between the TIC and the extracted masses. The endotoxin standard from *E. coli* F583 (light blue) is compared with the blank (purple), no significant differences are observed on both TIC. In contrast, with the reduction of the noise, differences are observed for the first 20 minutes of the analysis.

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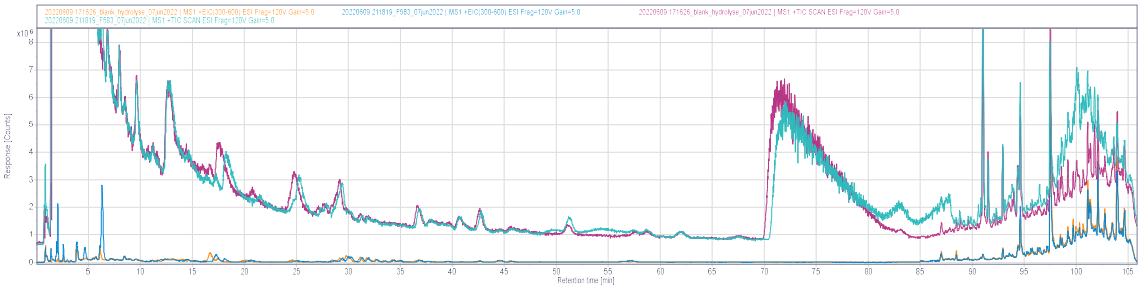

Figure 11: Overlays of mass chromatograms: A) Purple: TIC of blank (mQ water), Light blue: TIC of E. coli F583 standard (0.5 mg/ml), Dark blue: extracted mass 300 to 600 m/z from E. coli F583 standard (0.5 mg/mL), Orange: extracted mass from 300 to 600 m/z from blank (mQ Water).

For the method transfer, formic acid was added to the eluent, which led to a shift of the retention time. Using the mass spectrometry, molecules can be found by their mass and therefore their retention time. Using the already known masses in Table 7, it was possible to identify the compound usually seen with fluorescence detection. Figure 12, compares the masses found in mass spectrometry for the peak known in HPLC-FLD and the masses found with HPLC-MS. The four main molecules found with fluorescence detection for different endotoxin standards are respectively PEtN-Kdo-DMB, Hep-Kdo-DMB, Gal-Kdo-DMB, Kdo-DMB.

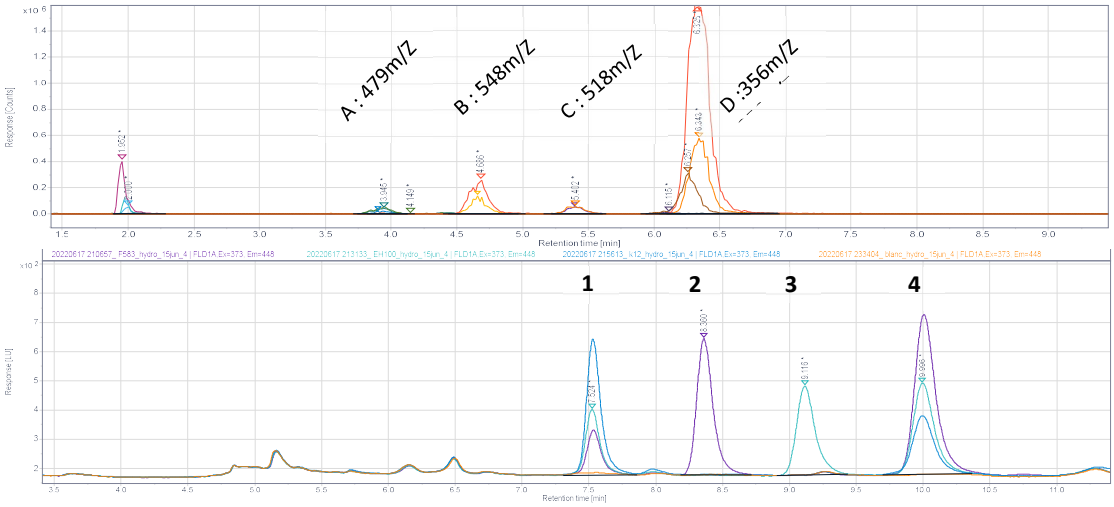

Figure 12: At the top: overlay of chromatogram from different endotoxin standards (0.5 mg/ml using method 11). At the bottom: overlay of chromatogram by FLD from different ET standards (250 ng/ml using method 8). A and 1: PEtN-Kdo-DMB. B and 2) Hep-Kdo-DMB 3) Gal-Kdo-DMB 4) Kdo-DMB

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3.2.3 Detection of unidentified masses

To find other masses and unidentified molecules, it was necessary to compare the chromatographic profile from endotoxin standards and a blank. This section describes the masses related to Kdo species found for each endotoxin. The Annex 13 show the chromatogram of the masses observed for the mQ water blank as a proof of no contamination.

E. coli EH100: Measuring three different peaks with the FLD detector, it was expected to find bonded to the fluorophore, PEtN-Kdo, Gal-Kdo and Kdo. Figure 13 shows masses found in *E. coli* EH100 that are not observed in the blank.

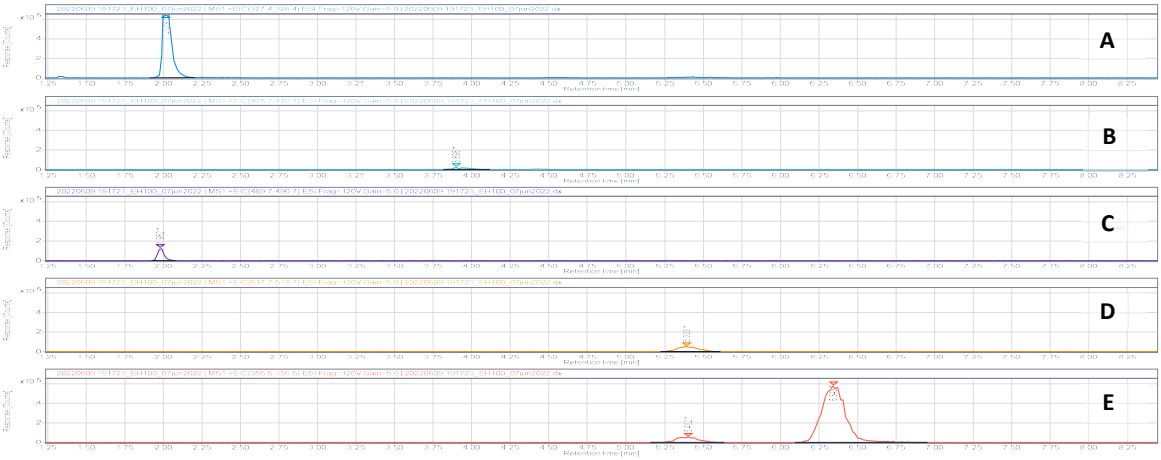

Figure 13: Chromatogram of E. coli EH 100 showing specific masses around 300 to 600 m/z. A) 327.7 m/z at 2.014 min B) 479.0 m/z at 3.898 min c) 490.0 m/z at 1.982 min d) 518.1 m/z at 5.393 min e) 355.8 m/z at 5.402 min and at 6.343 min.

Observation: Three of five masses found are identified as Kdo species. The profile B is PEtN-Kdo-DMB, profile D is Gal-Kdo-DMB, and profile E is Kdo-DMB. However, two other masses are not identified but might be Kdo-species A with a mass of 327.7 m/z and C with a mass of 490.0 m/z.

E. coli F583: With the FLD detector, three different peaks were observed. It was expected to find bound to the fluorophore, PEtN-Kdo, Hep-Kdo and Kdo. Figure 14 shows masses found in *E. coli* F583 that are not observed in the blank.

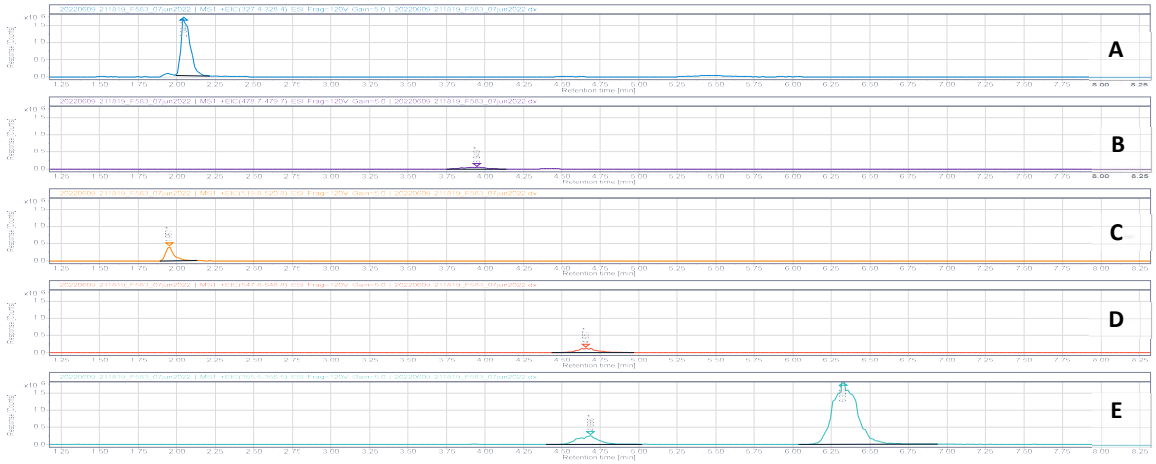

Figure 14: Chromatogram of E. coli F583 showing specific masses around 300 to 600 m/z. A)327.7 m/z at 2.044 min B) 479.0 m/z at 3.949 min c) 520.1 m/z at 1.952 min d) 548.1 m/z at 4.657 min e) 355.8 m/z at 4.686 min and at 6.325min

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Observation: The three masses observed in FLD were found in mass spectrometry. The profile B is PEtN-Kdo-DMB, profile D is Gal-Kdo-DMB, and profile E is Kdo-DMB. Other masses were found with a shorter retention time as the mass 327.7 m/z observed at 2.044 min and 520.1 m/z at 1.952 min. Those peaks were not identified.

E. coli K12: With the FLD detector, only two different peaks were observed. It was expected to find bound to the fluorophore, PEtN-Kdo and Kdo. Figure 15 shows masses found in *E. coli* K12 that are not observed in the blank.

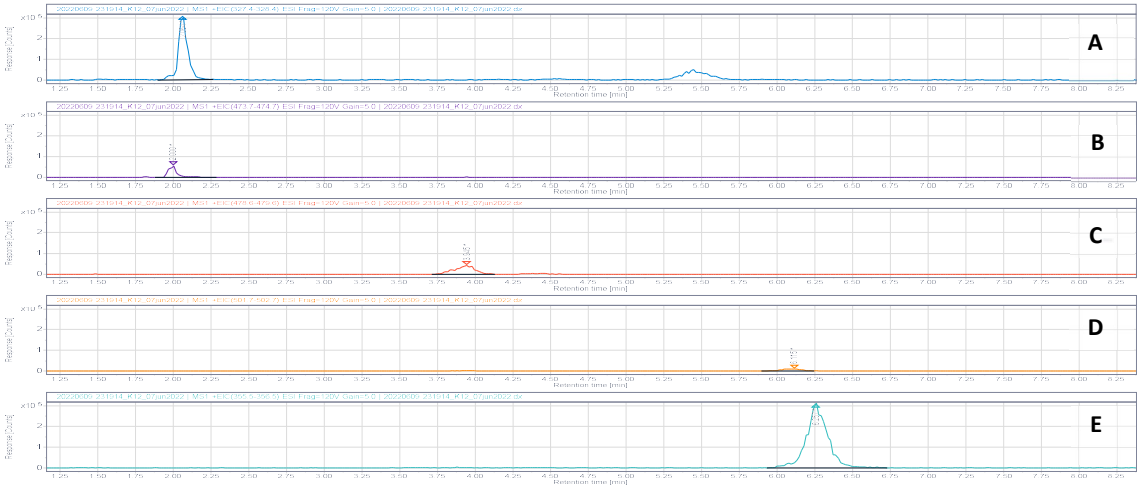

Figure 15: Chromatogram of E. coli K12 showing specific masses around 300 to 600 m/z. A) 327.7 m/z at 2.064 min B) 474.0 m/z at 2.000 min C) 479.0 m/z at 3.945 min D) 502.1 m/z at 6.115 min E) 355.8 m/z at 6.257 min.

Observation: The mass 327.7 m/z is again found in the endotoxin standard at 2.06 min with a high intensity. Another mass of 474.0 m/z is observed at 2.00 min. A peak at 6.12 min shows the mass 502.0 m/z which represents the sugar Rhamnose bound to Kdo-DMB (D). Its intensity is very low in comparison to the Kdo peak. The two expected masses are C: PEtN-Kdo-DMB and E: Kdo-DMB. The peaks from D and E on Figure 15 have a very close retention time and may coelute in fluorescence detection. Those observations can explain why the rhamnose is not seen in the FLD signal.

E. coli O55:B5: Only one peak is observed in fluorescence, the once corresponding to the Kdo bounded to the DMB.

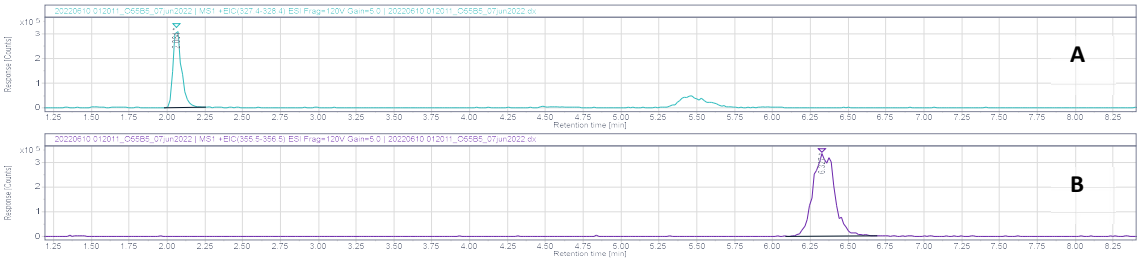

Figure 16: Chromatogram of E. coli O55:B5 showing specific masses around 300 to 600 m/z. A) 327.7 m/z at 2.064 min B) 355.6 m/z at 6.327 min.

Observation: The mass 327.7 m/z is observed at 2.06 min at the same retention time as for other endotoxin standards. The Kdo-DMB peak is seen at 6.33 min (profile B). In contrast to the fluorescence detection no PEtN-KDO was observed on mass spectrometry.

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Pseudomonas aeruginosa shows a similar profile as the *E. coli* O55:B5 endotoxin standard, only the Kdo bound to the fluorophore is observed in fluorescence detection.

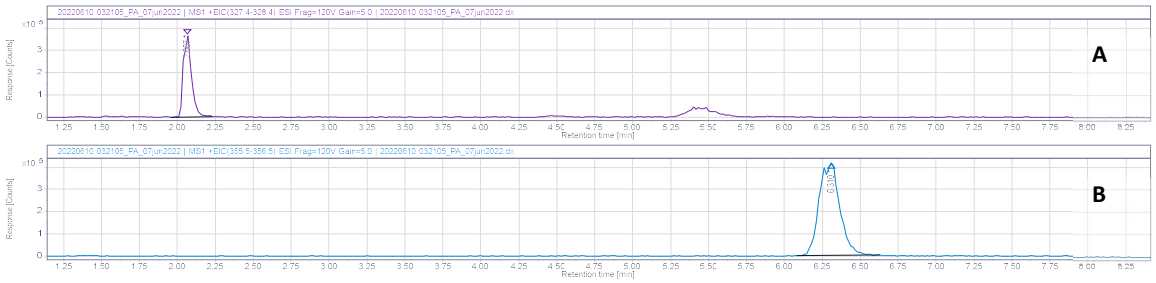

Figure 17: Chromatogram of P. aeruginosa showing specific masses around 300 to 600 m/z. A) 327.7 m/z at 2.067 min B) 355.6 m/z at 6.310 min.

Observation: On the spectra below, the mass 327.7 m/z is observed at 2.07 min (A) and the Kdo-DMB peak at 6.31 min (B). The intensities of the two peaks are almost the same.

3.2.4 Discussion

Despite the modifications made to the RP-HPLC-MS method, such as the addition of formic acid in the eluent, the order of elution of the four peaks observed in fluorescence was identical. However, the peak intensities for each Kdo species do not show the same trend than in FLD. The Kdo peak with 355.8 m/z seems to have a better response than the other Kdo species (see Figure 12), while the intensity of some peaks is very low, which is probably due to the contamination of the ionization source. Other unknown peaks were found as the one around 2.06 min (327.7 m/z) that appear to be the same mass for all endotoxin standards. This mass is about 28 m/z lower than the Kdo-DMB one. The decrease in the observed mass is probably caused by a loss of a carbonyl group. Other masses were discovered around two minutes specific for each endotoxin, those masses are seen in low intensity in three different endotoxins *E. coli* EH100, *E. coli* F583 and *E. coli* K12 respectively 490.0, 520.1, 474.0 m/z. By applying the same mass difference, 28 m/z, observed earlier on the mass 327.7 m/z. It is possible to find the masses of galactose, heptose, rhamnose bounded to Kdo-DMB. The four different masses found at relatively short retention times correspond to the peaks already identified but having a lower mass of 28. As mentioned earlier, this mass certainly comes from the carbonyl of Kdo, the sugar being open-ended when bound to DMB, which gives the possibility that it is ionized during the MS analysis. The different masses mentioned are summarized in the Table 8 Table 8.

Endotoxins	***E. coli* EH100**	***E. coli* F583**	***E. coli* K12**	**All of them**
Observed unknown masses [m/z]	490.0	520.1	474.0	327.7
Identified masses [m/z]	518.0	548.1	501.1	355.8
Compounds name	Kdo-DMB-Gal	Kdo-DMB-Hep	Kdo-DMB-Rha	Kdo-DMB

Table 8: Masses observed in mass spectrometry for three different endotoxins.

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3.2.5 Conclusion

Four different molecules were identified as the PEtN-Kdo-DMB, Gal-Kdo-DMB, Hep-Kdo-DMB, Rha-Kdo-DMB. Those molecules were also observed in Blanka Bucsella measurements. The final method 12 used in mass spectrometry with formic acid showed a good separation. But the signal sensitivity was limited by the mass detector that was a single quadrupole. For more sensitive measurements a different mass detector as e.g., a Triple quadrupole should be used.

3.3 Endotoxin standard analysis with Fluorescence light detection

To quantify endotoxins with the chemical assay based on Kdo, it is necessary to know the total amount of Kdo molecules that are present in each quantity of endotoxins and how much of it were measured. To know this recovery, named here total Kdo recovery, in different endotoxin standards and to quantify the content of Kdo derivates in R- and S-type endotoxins, analyses were made on three different days on five different endotoxins standards. The endotoxin standards were prepared in triplicates, following the procedure for quantification of the endotoxins as described in [14]. The Kdo standards were hydrolysed as well as non-hydrolysed. Details for the preparations of the used solutions and of the endotoxin standards are shown in Annex 11: Table of the date and concentration for the ET standard preparation.

3.3.1 Reproducibility of measurements

The aim was to control the repeatability of the three days of analysis, to have a relevant data set. Further, differences in peak heights were analysed and outliers were removed from the data set. To do so, an evaluation according to the student t test at a confidence level of 95 % was made. The procedure for quantification of the endotoxins specifies that the residual standard deviation value for the peak height should be less than 10 %. In case of higher values, it should be investigated, at 20 % action is required if the deviation persists. In Table 9 the residual standard deviation value from the peak height value of the four different Kdo species calculated on nine values are shown. The entire data can be seen in Annex 10.

Table 9: Statistics of compiled values over three different days-analysis (n=9), Based on the peak height. The concentration is 500 ng/ml for E. coli O55B5 and P. aeruginosa, 250 ng/ml for the other E. coli and 5 ng/ml for the Kdo standard. Two injection results were removed for being outliers (injection n°1 on day 2 and injection n°3 on day 3)

Residual standard deviation [%]				
Compounds	Kdo	**PEtN-Kdo**	**Hep-Kdo**	**Gal-Kdo**
Kdo hydrolysed (n=9)	13	-	-	-
Kdo derivatised (n=9)	5	-	-	-
E. coli EH 100 (n=9)	12	11	-	9
E. coli F 583 (n=7)	5 (18)	6 (29)	17 (39)	-
E. coli K12 (n=9)	5	6	-	-
E. coli O55B5 (n=9)	6	9	-	-
P. Aeruginosa (n=9)	4	-	-	-

In average, the values obtain are slightly high, but the *E. coli* F583 shows significant higher residual standard deviation values, also the residual value of the Kdo hydrolysed standard at 13 % is abnormally high. After checking the outliers, two injections were removed from the data set of *E. coli* F583, but the residual standard deviation remains high for the Hep-Kdo. During analysis air bubbles were detected in the measurement system which might have contributed to the increased deviation values. The air bubbles could be removed from the system. Another observation showed that the recovery is often higher for Kdo bearing substituents than the pure Kdo.

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3.3.2 Ratio of Kdo species for each endotoxin standard

The graphic below shows the ratio of Kdo species for each endotoxin standard. Looking at the rough type endotoxins, *E coli* EH100, *E coli* F583 and *E coli* K12, the pure Kdo represents no more than about 56 % of the total amount of Kdo. Other Kdo species such as the PEtN-Kdo are present in equal or even higher quantities than the unsubstituted Kdo. For the smooth type the unsubstituted Kdo is the main or the only peak as observed for *E.* coli O55B5 and *P. aeruginosa.*

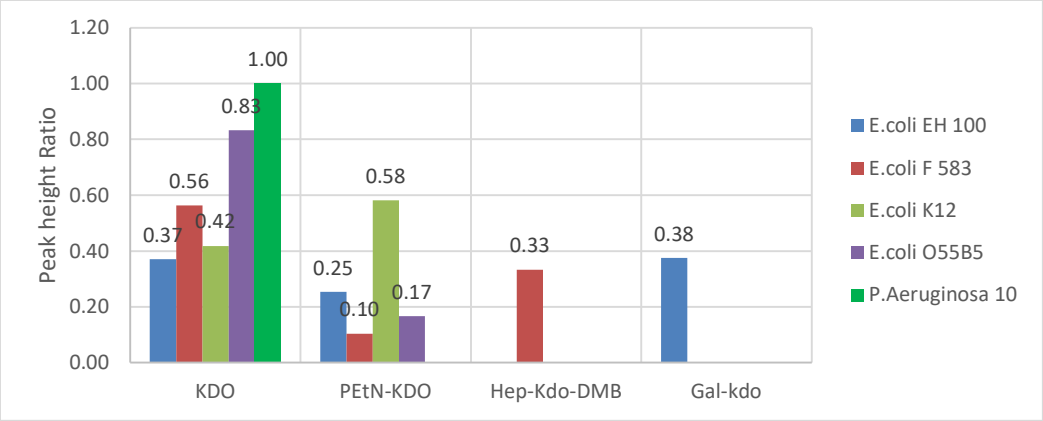

Figure 18: Graphic of Kdo-species Ratio over total amount of Kdo based on the peak height

The graph shows that the derived Kdos represent a significant proportion of the total amount of Kdos for an endotoxin. For some of them this may represent a majority, however the largest proportion remains the Kdo. In the next section, the difference between the amount of Kdo and the total amount of Kdo will be compared to see the impact of considering only the unsubstituted Kdo sugar acid.

3.3.3 Expected amount of Kdo

To calculate the expected amount of total Kdo, reference values were taken from the table in Annex 12. The raw data are taken from the data set of the three days of analysis. The peak height of the Kdo standard (5 ng/ml) was used as the external standard, and the concentration was calculated relative to the weighted mass. For the first hypothesis, the total heights of the Kdo species are summed and then quantified in relation to the standard. For the second hypothesis, only the Kdo-DMB peak is quantified.

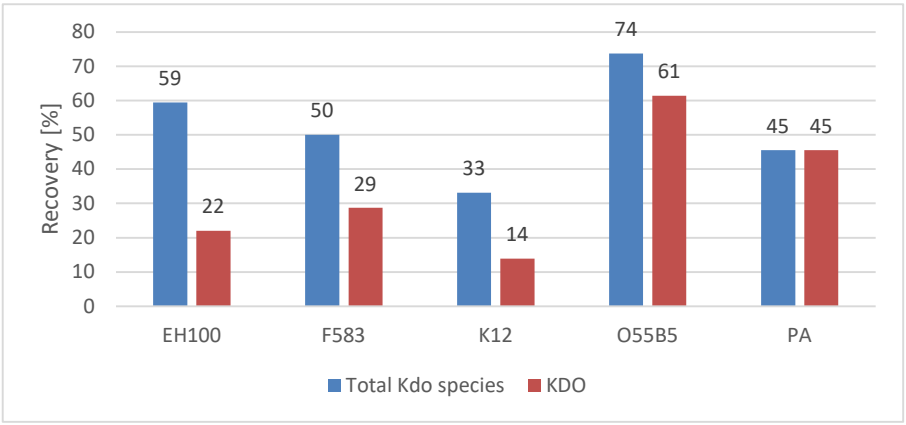

Figure 19:Graph showing the percentage of recovery per endotoxin standard based on the concentration. In blue the sum of the total Kdo species, in red the Kdo-DMB peak only.

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The recovery value for the total amount of Kdo species are close to half of what is expected. These percentages fluctuate from an endotoxin to another, which mean that the Kdo species release is not the same for all endotoxins. Looking at Figure 19, The difference between the calculated recovery with Kdo simple form and the calculated recovery with total Kdo species is greater for R-type than S-type endotoxins. Moreover R-type show different Kdo species while S-type seems to release mostly Kdo.

3.3.4 Discussion

After eliminating outliers from the data set, the residual standard deviation calculations were made. The values obtained are rather high and exceed the tolerance for *E. coli* F583. An interesting observation was made on the residual standard deviation values being higher for the Kdo species than for the unmodified Kdo. Assuming that there is more fluctuation for the Kdo derivatives than the Kdos, this could mean that the rate of hydrolysis may not be the same or that hydrolysis is not complete for these derivatives. This hypothesis must be confirmed by hydrolysis tests e.g., by prolongation the hydrolysis time. The proportion of Kdo species in relation to Kdo is not negligible, representing up to more than half of the total Kdo content. It is also noted that the overall proportion of Kdo is in the majority. If the recovery rates of total Kdo species and Kdo are compared there is a significant difference, which is more present in the R-type endotoxins. Observations have shown that S-type endotoxins release more Kdo in its simple form.

3.4 Variation of hydrolysis time for different endotoxin standards

To investigate the hydrolysis kinetic of Kdo species during hydrolysis, different times were tested. For this experiment six different endotoxins were prepared. The hydrolysis was done from 0 to 150 minutes with endotoxin standard aliquots being taken every 30 minutes. The samples were taken in decreasing order, meaning that every 30 minutes new aliquots were placed in the heating block. The derivatization is made as described in the protocol [14]. Acetic acid is also added for the 0-minute preparation and directly put on ice. The values at 0 minute represent the quantity of free Kdo in an endotoxin standard, this offset-value is subtracted from original values. As free Kdo, the amount of measured Kdo-DMB in sample is called that is present without hydrolysing it. To compare the data, it was necessary to rationalize the different injection and different endotoxins using the same parameters. For this reason, calculation was made using ratio of the peak height. The results are shown in the following Figure 20.

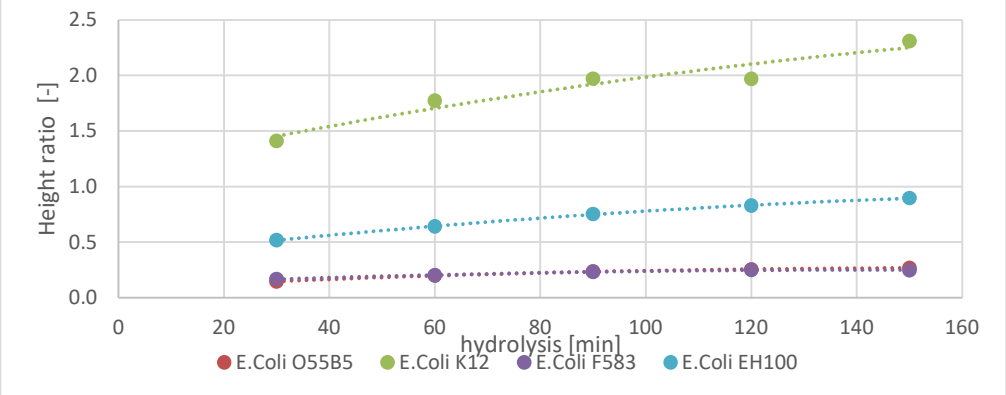

Figure 20: Graph showing the peak height ratio from Kdo-PEtN on Kdo for different ET standard over hydrolysis time. Endotoxins concentration (500 ng/ml), using Method 1 RP-HPLC. The equations of the curve are given in Table 9 below.

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Using the ratio between those two peaks, the tendency of different ET standards for the same Kdo-species is shown in Figure 20. The PEtN-Kdo-DMB peak from the four different endotoxins seems to react the same way, by increase faster their height over the hydrolysis time in comparison to the Kdo-DMB. The ratio of *E. coli* F583 and *E. coli* O55B5 over the time shows a perfect overlay. This observation is confirmed by Table 10, where the slopes from the derivative of each ETs trendline are compared.

Table 10: function reported from Figure 20.

ET standard	Polynomial	Slope of Derivative	Coefficient of determination (R^2)
E. coli K12	$-2x10^{-05}x^2 + 0.0101x + 1.1696$	$-4x10^{-05}x$	0.9294
E. coli EH 100	$-1\ x10^{-05}x^2 + 0.0053x + 0.3696$	$-2x10^{-05}x$	0.9997
E. coli F583	$-7\ x10^{-06}x^2 + 0.0019x + 0.116$	$-1.4x10^{-05}x$	0.9986
E. coli O55B5	$-7\ x10^{-06}x^2 + 0.0023x + 0.0849$	$-1.4x10^{-05}x$	0.9924

The four slopes show high similarities while *E. coli* K12 is slightly different. The slope from the derivative polynomial of *E. coli* K12 is higher. Observation on the Kdo-peak height decreased over 120 minutes faster than the PEtN-peak height which led to a bigger peak ratio. Comparing the ratios, a flat line on the graphic would means that both compounds will have the same kinetic.

Do different Kdo species from a same endotoxin standard react the same way during hydrolysis? To answer to this question, the peak height of Kdo is used to rationalize injection from different ET standard. As it could be seen in Figure 20, the profiles of the curve do not look planar, ratio increase which means that Kdo and Kdo-PEtN don't behave in the same way during the time of hydrolysis. This observation can also be made in Figure 21. The same approach of height ratio is used to compare the different kinetics of two different Kdo species from *E. coli* EH100. Here, the comparison is made between Kdo-PEtN and Gal-Kdo both rationalized on the Kdo.

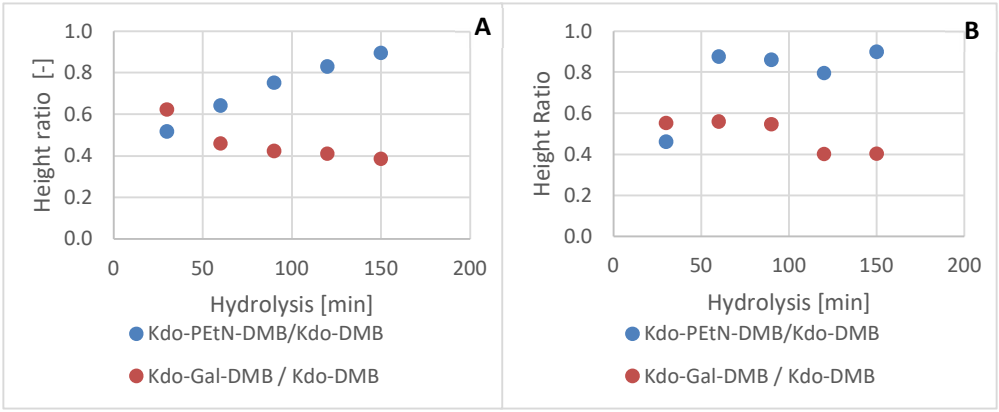

Figure 21: graphic showing peak height ratio from two different Kdo species, E. coli EH100 500ng/ml. Analysis made by VIC graphic A and HOA for graphic B.

The graphic shows the kinetic during the hydrolysis. For graphic A, when the ratio of Kdo-PEtN-DMB/Kdo-DMB decreases the ratio of Kdo-Gal-DMB/Kdo-DMB increase. The data from HOA, in Figure 21B the curves do not show a clear decrease or increase, but the tendency seems to be comparable.

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In Figure 22, the comparison is made with the Kdo species of the *E. coli* F583. The ratio with Kdo-Hep shows a faster increase while Kdo-PEtN seems to make a plateau. This observation is made in both graphic independently of the data set.

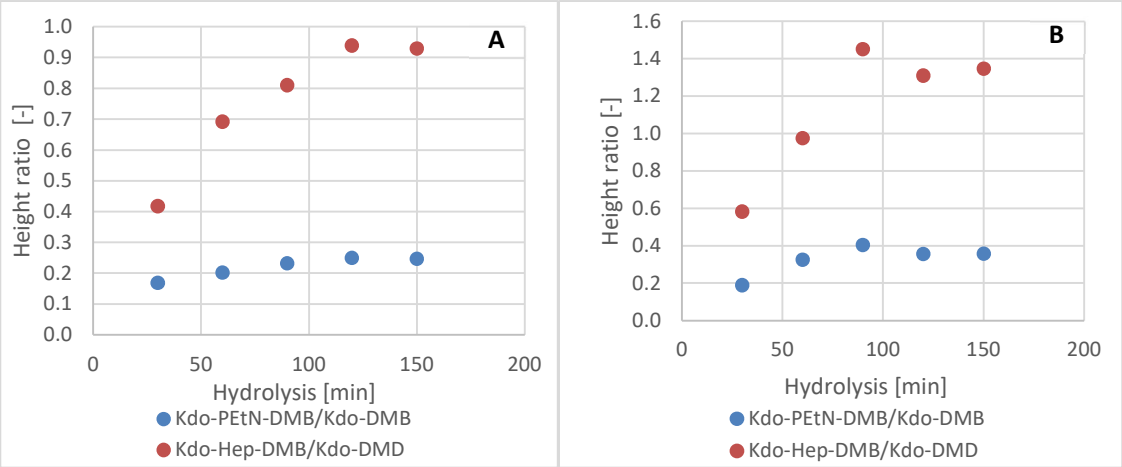

Figure 22: graphic showing peak height ratio from two different Kdo species, E. coli F583 500 ng/ml. Analysis made by VIC graphic A and HOA for graphic B.

3.4.1 Discussion

To strictly compare the kinetic of Kdo species, a smoothing was applied to reduce the differences between endotoxin standards. A ratio was calculated using the Kdo-DMB peak-height, present in each endotoxin. The results show for the compound PEtN-Kdo high kinetic similarities for four types of *E. coli*. However, it is not possible with the current data set to say that all kinetics from Kdo species for different endotoxin standards work in the same way. For instance, Hep-Kdo or Gal-Kdo cannot be compared with other endotoxins in this thesis. Other test of comparison should be made to know the kinetic behaviour of those compounds.

The other question was: Are different Kdo-species kinetic identic? The results obtained showed no similarities in the kinetic for different Kdo species. This observation is shown in Figure 21 and Figure 22 comparing ratio of Kdo-PEtN/Kdo, Kdo-Gal/Kdo and Kdo-Hep/Kdo.

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4 Conclusion

In this work, the initial method used for the endotoxin quantification procedure was optimised to see the maximum peak difference between the mQ water blank and the endotoxin standards. The final optimisation using the Method 8 met the criteria of method optimisation, numerous peaks where separated. But little difference was seen between the mQ water injections and the endotoxin standards. For a future work, it would be advantageous to further improve the gradient, changing the percentage of eluent to have sharper peaks, and to have a better separation for the first ten minutes or and after sixty minutes. Nevertheless, this method was transferred to RP-HPLC with a mass spectrum detector to identify the separated and nonidentified peaks.

The identification of the masses proved to be challenging due to an ionization source contamination that has considerably decreased the sensitivity. Nevertheless, five masses were identified, four of which are also visible in fluorescence detection (PEtN-Kdo-DMB, Gal-Kdo-DMB, Hep-Kdo-DMB, Kdo-DMB). The fifth one being identified as rhamnose but not visible in fluorescence detection. In addition, four other masses were found that had lost 28 mass units in comparison to the beforehand mentioned molecules. This indicates that during ionization a carbonyl group is lost. The experiences gained throughout the experiments would now allow a more targeted-oriented data analysis and a better understanding of the sample requirements such as sample concentration and preparation of the mobile phases. It is necessary to continue the research in mass spectrometry in order to identify potential other Kdo derivates using a more sensitive instrument.

After the mass identification, analyses were conducted using the initial method on both R- and S-type endotoxins to measure the ratio of Kdo species. The results showed that the R-type releases a bigger diversity of Kdo species than the S-type which releases mostly non-derived Kdo. This observation needs to be confirmed by further analysis using a wider diversification of endotoxin types from different bacteria strains. The variation of the hydrolysis time allowed the understanding of the release kinetics of the different Kdo derivatives. The same Kdo species e.g., PEtN-Kdo behaves in a similar way when present in different endotoxins. On the contrary, Kdo derivatives present in the same endotoxin show different hydrolysis kinetics. These results, together with those of the initial analyses, helped to answer the main question: is it sufficient to use Kdo-DMB for endotoxin quantification or should the totality of the Kdo species be considered? Here a difference between the R- and the S-type needs to be made. The proportion of Kdo derivative for S-type endotoxin being significantly lower compared to non-derivatized Kdo, it would be possible to calculate the endotoxin content only with Kdo in its simple form. Contrary to the R-type endotoxins, for which it is necessary to use the totality of the Kdo species. Furthermore, if the share of S-type endotoxin, is greater than that of R-type, the Kdo derivatives could be ignored in the calculation of the endotoxin amount. This statement assumes that in the environment bacteria with S-type endotoxin molecules are predominant and therefore outcompete present R-type endotoxin. With that the Kdo modifications as seen for R-type endotoxins are present in minor concentrations.

The working hypothesises of this thesis could be answered although all the parameters were not tested. Having observed the tendency for the Kdo derivatives release for both endotoxin types. It would be interesting for a future work to answer the following question. What would be the ratio of endotoxin S- and R-type in a representative sample as contaminated solution form hospital containing different types of bacteria? With that the impact of the Kdo derivatives a real world sample could be evaluated.

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Moreover, the data collected must be completed by other analyses to strengthen them statistically and to confirm the above discussed statement. For future work, the instruments made available should be checked more meticulously before their use to guarantee high quality data and in this case more sensitive results.

5 Acknowledgement

This work based entirely on analytical measurements from chemically purified endotoxin standards allowed me to realize the complexity of the biological matrix. The knowledge acquired during the short period of this work offered me a better understanding of different steps required for the development of an analytical method as well as the type of problems that could be encountered.

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7 Annex:

7.1 Method

Method 1: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC.amx
Method 2: 2022_05_16_KDO_YMC_GC_12%_031mL_HPLC_iso
Method 3: 2022_05_17_optimisation_gradient_95%_78.6
Method 4: 2022_05_23_optimisation_gradient_20min_Plateau_95%_76min
Method 5: 2022_05_24_optimisation_gradient_plateau_8%in5min
Method 6: 2022_05_24_optimisation_gradient_plateau_8%in10min
Method 7: 2022_05_24_optimisation_gradient_plateau_8%in20min
Method 8: 2022_05_25_optimisation_gradient_plateau_8%in9min.amx
Method 9: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC_MS.amx:
Method 10: 2021_10_14_KDO_YMC_GC_12%_031mL_20ul_HPLC_MS.amx
Method 11: 2021_10_14_KDO_YMC_GC_12%_031m_20uL_HPLC_01%FA_MS.amx Eluent A: mQ water + 0.1% FA, Eluent B: ACN/MeOH (36/64) + 0.1% FA.
Method 12: 2022_05_25_optimisation_gradient_plateau_8%in9min_20ul_MS.amx A: mQ water + 0.1% FA, Eluent B: ACN/MeOH (36/64) + 0.1% FA.

7.2 Annex

Annex 1: Table : Raw data from the column test, test solution injected: uracil (0.02mg/ml), Methyl benzoate (0.7uL/mL), Naphthalene (0.24mg/mL), Flow: 0.2ml/min, Detection: 254nm, injection volume: 2µl, 155bar, Eluent: ACN/Water (60/40)
Annex 2: Column inspection report from supplier YMC
Annex 3: Chromatogram showing DAD and pressure profile of four injection of a test solution: uracil (0.02mg/ml), Methyl benzoate (0.7uL/mL), Naphthalene (0.24mg/mL). Flow: 0.2ml/min, Detection: 254nm, injection volume: 2µl, 155bar, Eluent: ACN/Water (60/40)
Annex 4: Table: Raw Data Repeatability on Kdo standard 5ng/ml to check the instrument repeatability.
(Method: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC.amx)
Annex 5: Data Repeatability on Kdo standard 5ng/ml to check the peak resolution.
Annex 6: Overlay of chromatograms showing the six injections of Kdo standard 5ng/ml for test repeatability. The peak at 11.830min was taken to do the calculation for resolution factor
Annex 9: Overlay chromatograms from Method 8 repeatability test n=3, E. coli F583 (250ng/ml): in light blue injection N°1, in dark blue injection n°2, in violet injection n°3. In orange: mQ water Blank
Annex 7: Overlay chromatograms from Method 8 repeatability test n=3, E. coli F583 (250ng/ml): in light blue injection N°1, in dark blue injection n°2, in violet injection n°3. In orange:
Annex 8: Overlay chromatograms from Method 8 repeatability test n=3, E. coli EH100 (250ng/ml): in dark blue injection N°1, in light blue injection n°2, in yellow injection n°3. In orange:
Annex 10: Table: statistic of compilated value over three different days-analysis (n=9), Based on the peak height. The concentration is 500ng/ml for E. coli O55B5 and P. Aeruginosa, 250ng/ml for the other E. coli and 5ng/ml for KDO standard. *Bold values correspond to values with a correction due to an outlier
Annex 11: Table of the date and concentration for the ET standard preparation.
Annex 12:PowerPoint from ET_Join meeting 12.01.21, LPS standard characterization, hydrolysis and KDO degradation, Slide N°9 from PPT 2021_01_12_ET_std_KDO_LAL. Original value for the KDO proportion in Endotoxin standard.
Annex 13: Chromatogram of mQ water blank showing no peak for the selected masses. A)501.1 m/z, B) 518.0 m/z, C) 548.1 m/z, D) 355.8 m/z, E) 479.0 m/z, E) 327.7 m/z.
Annex 14:Standard procedure of "Analytical procedure for quantification of the endotoxin content for filter development and in biological sample"

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Method 1: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC.amx

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	10.00	A: 88 B: 12
	10.60	A: 5 B: 95
	14.60	A: 5 B: 95
	15.00	A:100 B: 0
Post time [min]	8.00	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Excitation wavelenght [nm]	373	
Emission Wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

Method 2: 2022_05_16_KDO_YMC_GC_12%_031mL_HPLC_iso

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	90.00	A: 88 B: 12
	90.10	A: 5 B: 95
	105.00	A: 0 B: 0 C: 100
	112.00	A: 0 B: 0 C: 100
	120.00	A:100 B: 0
Post time [min]	NA	NA
Oven Temperature[°C]	40	
Sampler Temperature [°C]	4	
Excitation wavelenght [nm]	373	
Emission wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

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Method 3: 2022_05_17_optimisation_gradient_95%_78.6

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	30.00	A: 80 B: 20
	75.00	A: 80 B: 20
	78.60	A: 5 B: 95
	79.00	A: 5 B: 95
Post time [min]	15	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Excitation Wavelenght [nm]	373	
Emission Wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

Method 4: 2022_05_23_optimisation_gradient_20min_Plateau_95%_76min

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0 .00	A: 100 B: 0
	0.10	A: 88 B: 12
	20.00	A: 88 B: 12
	56.00	A: 45 B: 55
	66.00	A: 45 B: 55
	76.00	A: 5 B: 95
	76.10	A: 0 B: 0 C: 100
	79.10	A: 0 B: 0 C: 100
	80.00	A: 100 B: 0
Post time [min]	8	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Excitation Wavelenght [nm]	373	
Emission Wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

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Method 5: 2022_05_24_optimisation_gradient_plateau_8%in5min

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0 .000	A: 100 B: 0
	0.100	A: 88 B: 12
	22.00	A: 80 B: 12
	27.00	A: 80 B: 20
	85.00	A: 80 B: 20
	105.0	A: 5 B: 95
	109.1	A: 5 B: 95
	110.0	A: 100 B: 0
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Excitation Wavelenght [nm]	373	
Emission Wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

Method 6: 2022_05_24_optimisation_gradient_plateau_8%in10min

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0 .000	A: 100 B: 0
	0.100	A: 88 B: 12
	22.00	A: 80 B: 12
	32.00	A: 80 B: 20
	85.00	A: 80 B: 20
	105.0	A: 5 B: 95
	109.1	A: 5 B: 95
	110.0	A: 100 B: 0
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Excitation Wavelenght [nm]	373	
Emission Wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

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Method 7: 2022_05_24_optimisation_gradient_plateau_8%in20min

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0 .000	A: 100 B: 0
	0.100	A: 88 B: 12
	22.00	A: 80 B: 12
	42.00	A: 80 B: 20
	85.00	A: 80 B: 20
	105.0	A: 5 B: 95
	109.1	A: 5 B: 95
	110.0	A: 100 B: 0
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Excitation Wavelenght [nm]	373	
Emission Wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

Method 8: 2022_05_25_optimisation_gradient_plateau_8%in9min.amx

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	22	A: 80 B: 20
	31	A: 80 B: 20
	80	A: 5 B: 95
	100	A: 5 B: 95
	104.10	A: 100 B: 0
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Excitation Wavelenght [nm]	373	
Emission Wavelenght [nm]	448	
Peak width	>0.05 in (1s resp. time) (9.26Hz)	
Gain	18	

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Method 9: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC_MS.amx:

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	10	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	10.00	A: 88 B: 12
	10.60	A: 5 B: 95
	14.60	A: 5 B: 95
	15.00	A:100 B: 0
Post time [min]	8.00	
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Ionisation mode	positive	
Scan type [m/z]	Scan 100 - 400	
Detector Gain Factor	5	
Fragmentor [V]	120	

Method 10: 2021_10_14_KDO_YMC_GC_12%_031mL_20ul_HPLC_MS.amx

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	20	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	10.0	A: 88 B: 12
	10.6	A: 5 B: 95
	14.6	A: 5 B: 95
	15.0	A:100 B: 0
Post time [min]	8.0	
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Ionisation mode	positive	
Scan type [m/z]	Scan 100 - 600	
Detector Gain Factor	5	
Fragmentor [V]	120	

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Method 11: 2021_10_14_KDO_YMC_GC_12%_031m_20uL_HPLC_01%FA_MS.amx Eluent A: mQ water + 0.1% FA, Eluent B: ACN/MeOH (36/64) + 0.1% FA.

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	20	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	10.00	A: 88 B: 12
	10.60	A: 5 B: 95
	14.60	A: 5 B: 95
	15.00	A:100 B: 0
Post time [min]	8.00	
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Ionisation mode	positive	
Scan type [m/z]	Scan 100 - 600	
Detector Gain Factor	5	
Fragmentor [V]	120	

Method 12: 2022_05_25_optimisation_gradient_plateau_8%in9min_20ul_MS.amx A: mQ water + 0.1% FA, Eluent B: ACN/MeOH (36/64) + 0.1% FA.

Parameters	Value	Eluant [%]
Flow rate [ml/min]	0.310	-
Injection volume [µl]	20	-
Timetable [min]	0	A: 100 B: 0
	0.1	A: 88 B: 12
	22	A: 80 B: 20
	31	A: 80 B: 20
	80	A: 5 B: 95
	100	A: 5 B: 95
	104.10	A: 100 B: 0
	15.00	A:100 B: 0
Post time [min]	10	
Oven Temperature[°C]	40	-
Sampler Temperature [°C]	4	
Ionisation mode	positive	
Scan type [m/z]	Scan 100 - 600	
Detector Gain Factor	5	
Fragmentor [V]	120	

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Annex 1: Table : Raw data from the column test, test solution injected: uracil (0.02mg/ml), Methyl benzoate (0.7uL/mL), Naphthalene (0.24mg/mL), Flow: 0.2ml/min, Detection: 254nm, injection volume: 2µl, 155bar, Eluent: ACN/Water (60/40)

Replicate	Name	RT (min)	Width 50% (min)	K'=(tRn-tR1)/tR1	N=5.54x(tR/W0.5) ^2	Tailing factor
	Uracil	1.276	0.07	NA	1841	0.94
	Methyl benzoate	4.681	0.106	2.67	10804	1.066
#1	Naphthalene	12.282	0.213	8.63	18420	1.437
	Uracil	1.278	0.072	NA	1745	0.954
	Methyl benzoate	4.701	0.108	2.68	10496	0.927
#2	Naphthalene	12.348	0.216	8.66	18105	1.389
	Uracil	1.28	0.071	NA	1801	0.867
	Methyl benzoate	4.727	0.108	2.69	10613	0.826
#3	Naphthalene	12.446	0.217	8.72	18224	1.31
	Uracil	1.281	0.071	NA	1803	0.885
	Methyl benzoate	4.747	0.109	2.71	10507	1.147
#4	Naphthalene	12.5	0.218	8.76	18214	1.34
	Uracil	1.28	0.07	NA	1798	0.91
	Methyl benzoate	4.71	0.11	2.69	10605	0.99
Average	Naphthalene	12.39	0.22	8.69	18241	1.37
	Uracil	1.15	NA	NA	3247	1.37
	Methyl benzoate	4.51	NA	2.93	16615	1.16
Specification	Naphthalene	11.82	NA	9.28	20492	1.06
	Uracil	***111***	NA	***NA***	***55***	***67***
	Methyl benzoate	***105***	NA	***92***	***64***	***85***
Deviation (RSD%)	***Naphthalene***	***105***	NA	***94***	***89***	***129***

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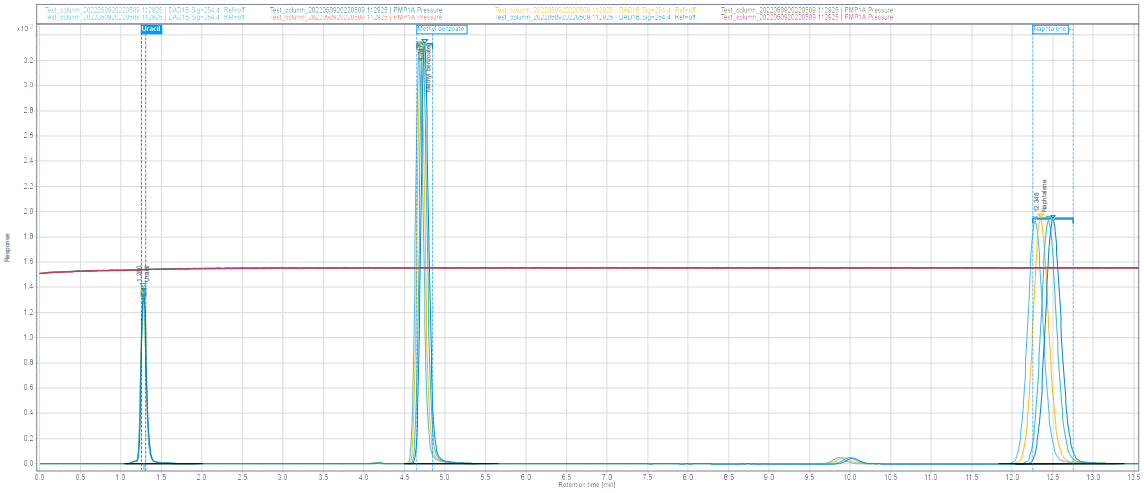
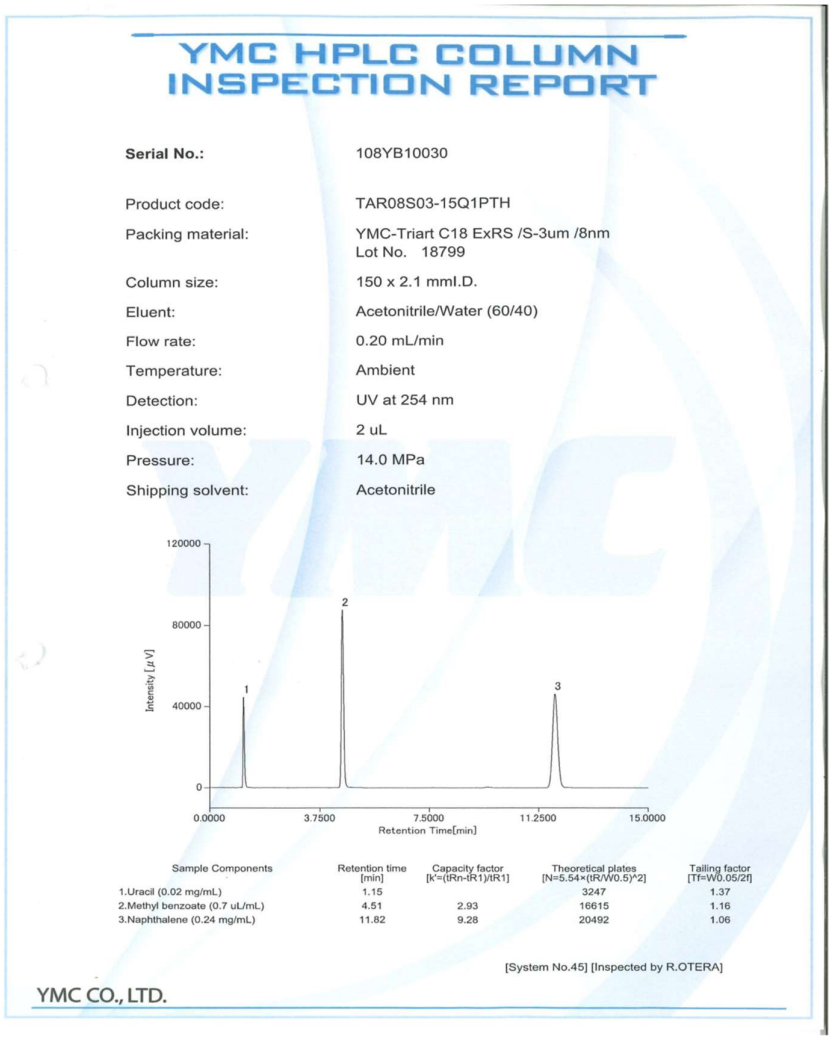


Annex 3: Chromatogram showing DAD and pressure profile of four injection of a test solution: uracil (0.02mg/ml), Methyl benzoate (0.7uL/mL), Naphthalene (0.24mg/mL). Flow: 0.2ml/min, Detection: 254nm, injection volume: 2µl, 155bar, Eluent: ACN/Water (60/40)



YMC HPLC COLUMN INSPECTION REPORT

Serial No.:	108YB10030
Product code:	TAR08S03-15Q1PTH
Packing material:	YMC-Triart C18 ExRS /S-3um /8nm Lot No. 18799
Column size:	150 x 2.1 mmI.D.
Eluent:	Acetonitrile/Water (60/40)
Flow rate:	0.20 mL/min
Temperature:	Ambient
Detection:	UV at 254 nm
Injection volume:	2 uL
Pressure:	14.0 MPa
Shipping solvent:	Acetonitrile

Sample Components	Retention time [min]	Capacity factor [k'=(tRn-tR1)/tR1]	Theoretical plates [N=5.54×(tR/W0.5)^2]	Tailing factor [Tf=W0.05/2f]
1.Uracil (0.02 mg/mL)	1.15		3247	1.37
2.Methyl benzoate (0.7 uL/mL)	4.51	2.93	16615	1.16
3.Naphthalene (0.24 mg/mL)	11.82	9.28	20492	1.06

[System No.45] [Inspected by R.OTERA]

YMC CO., LTD.

Annex 2: Column inspection report from supplier YMC

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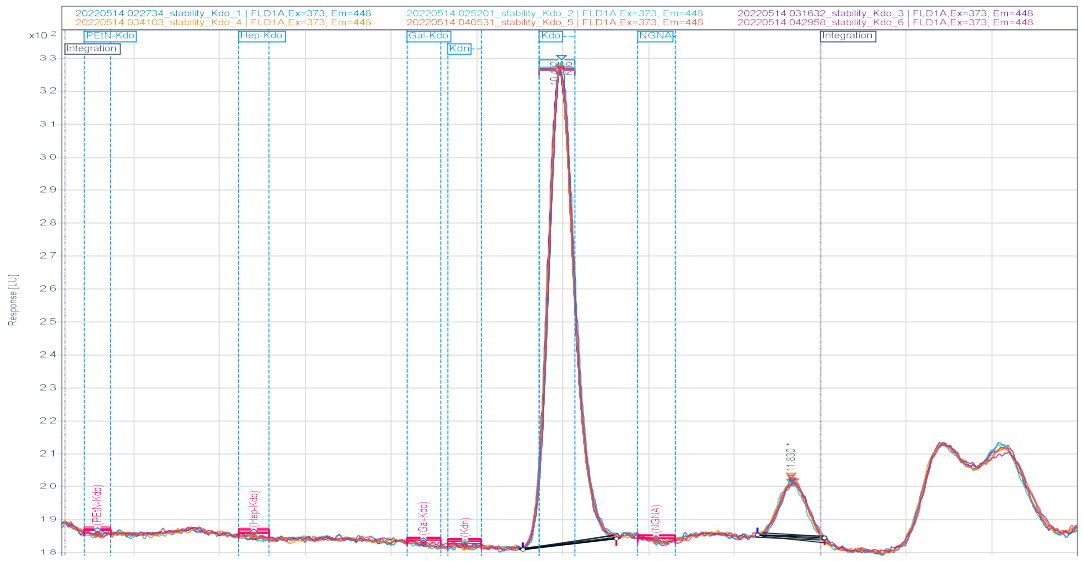
Name	RT (min)	Height (LU)	Area (LU·s)	Width (min)	Tailing
Kdo_5ng/ml	10.492	146.35	1569.249	0.534	1.24331
Kdo_5ng/ml	10.481	143.837	1551.303	0.502	1.23427
Kdo_5ng/ml	10.478	144.008	1570.236	0.568	1.27202
Kdo_5ng/ml	10.484	144.12	1566.935	0.583	1.26756
Kdo_5ng/ml	10.481	143.467	1566.165	0.574	1.26036
Kdo_5ng/ml	10.484	143.763	1563.791	0.541	1.2934
Average	10.5	144.3	1564.6	0.6	1.3
STDEV	0.0	1.0	6.9	0.0	0.0
RSD [%]	**0.0**	**0.7**	**0.4**	**5.5**	**1.7**

Annex 4: Table: Raw Data Repeatability on Kdo standard 5ng/ml to check the instrument repeatability. (Method: 2021_10_14_KDO_YMC_GC_12%_031mL_HPLC.amx)

Replicat	RT Kdo	Width Kdo	RT peak N°2	Width peak N°2	$R = \frac{T_{r2} - T_{r1}}{0.5 * (W_1 + W_2)}$
N°	[min]	[min]	[min]	[min]	Resolution factor [-]
1	10.492	0.534	11.834	0.355	3.02
2	10.481	0.502	11.823	0.389	3.01
3	10.478	0.568	11.827	0.371	2.87
4	10.484	0.583	11.83	0.341	2.91
5	10.481	0.574	11.854	0.403	2.81
6	10.484	0.541	11.837	0.389	2.91

Annex 5: Data Repeatability on Kdo standard 5ng/ml to check the peak resolution.



Annex 6: Overlay of chromatograms showing the six injections of Kdo standard 5ng/ml for test repeatability. The peak at 11.830min was taken to do the calculation for resolution factor

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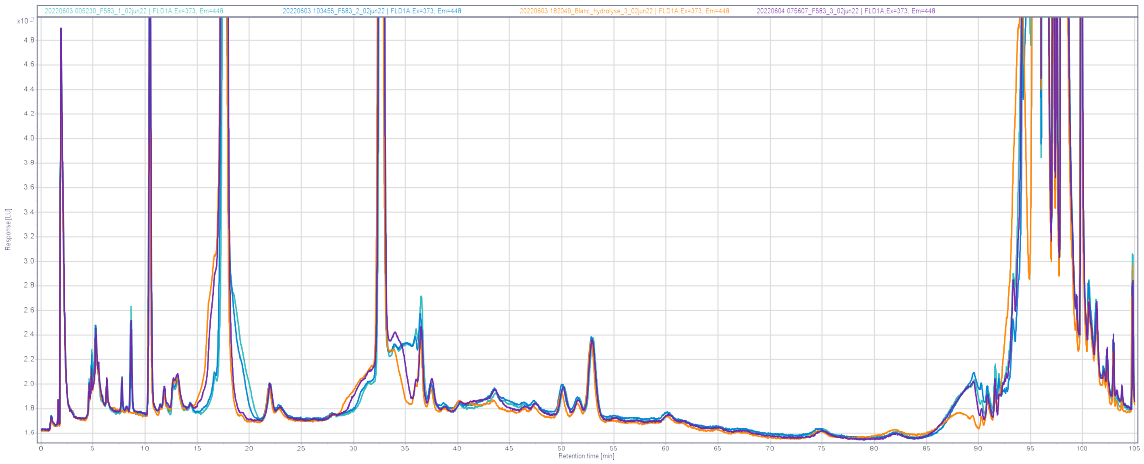
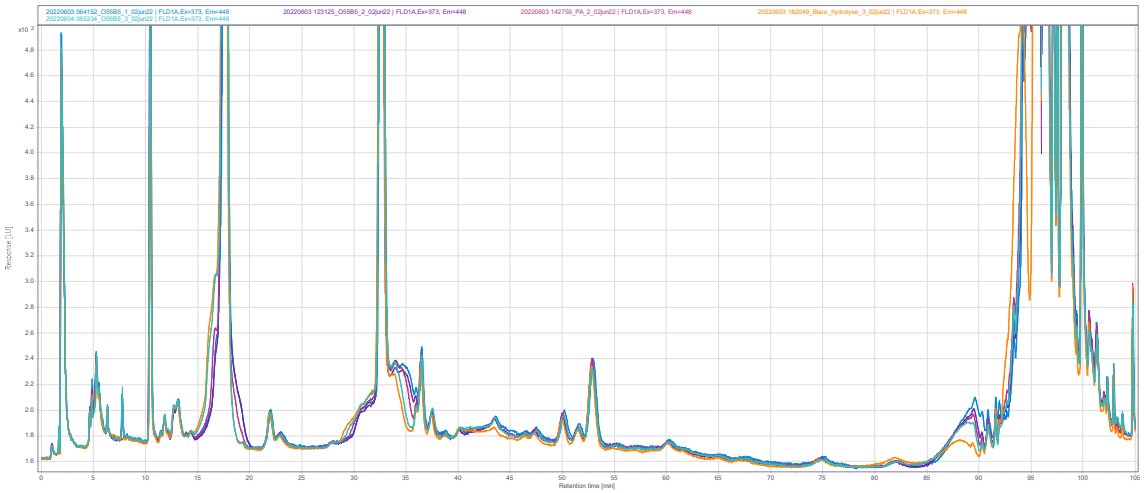
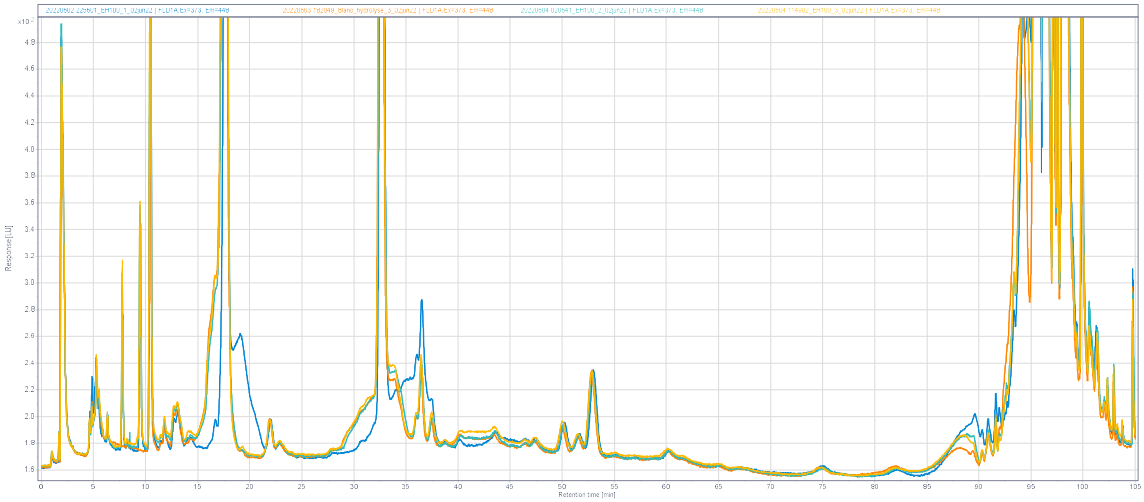


Annex 9: Overlay chromatograms from Method 8 repeatability test n=3, E. coli F583 (250ng/ml): in light blue injection N°1, in dark blue injection n°2, in violet injection n°3. In orange: mQ water Blank



Annex 7: Overlay chromatograms from Method 8 repeatability test n=3, E. coli F583 (250ng/ml): in light blue injection N°1, in dark blue injection n°2, in violet injection n°3. In orange: mQ water Blank



Annex 8: Overlay chromatograms from Method 8 repeatability test n=3, E. coli EH100 (250ng/ml): in dark blue injection N°1, in light blue injection n°2, in yellow injection n°3. In orange: mQ water Blank

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Compounds		Kdo	PEtN-Kdo	Hep-Kdo	Gal-Kdo
	Average	89			
	STDEV	11			
Kdo Hydrolysed (n=9)	**RSD%**	**13**			
	Average	140			
	STDEV	7			
Kdo Derivatised (n=9)	RSD%	5			
	Average	129	88		130
	STDEV	15	9		12
E. coli EH 100 (n=9)	RSD%	12	11		9
	Average	260	48	153	
	STDEV	13	3	26	
E. coli F583 (n=7)	RSD%	**5** *(18)	**6** *(29)	**17** *(39)	
	Average	96	133		
	STDEV	5	8		
E. coli K12 (n=9)	RSD%	5	6		
	Average	139	28		
	STDEV	8	3		
E. coli O55B5 (n=9)	RSD%	6	9		
	Average	150			
	STDEV	5			
P.Aeruginosa 10 (n=9)	RSD%	4			

*Annex 10: Table: statistic of compilated value over three different days-analysis (n=9), Based on the peak height. The concentration is 500ng/ml for E. coli O55B5 and P. Aeruginosa, 250ng/ml for the other E. coli and 5ng/ml for KDO standard. *Bold values correspond to values with a correction due to an outlier*

Standard	CAS °N	Batch	Stock solution preparation date	Aliquot solution preparation date	Aliquot concentration	Volume injection
Kdo Hydrolysed	K2755	SLCD6533	24-Jun-22	24-Jun-22	5ng/ml	10ul
Kdo Derivatised	K2755	SLCD6533	24-Jun-22	24-Jun-22	5ng/ml	10ul
E. coli EH 100	L9641	#025M4093	7-Jun-22	24-Jun-22	500 ng/ml	5ul
E. coli F 583	L6893	#128M4131V	26-Apr-22	24-Jun-22	500 ng/ml	5ul
E. coli K12	NA	#5973-42-01	12-Nov-21	29-Jun-22	500 ng/ml	5ul
E. coli O55B5	L2637	#0000102731	7-Jun-22	7-Jun-22	500 ng/ml	10ul
P. Aeruginosa 10	L9143	#075M4089V	7-Jun-22	7-Jun-22	500 ng/ml	10ul

Annex 11: Table of the date and concentration for the ET standard preparation.

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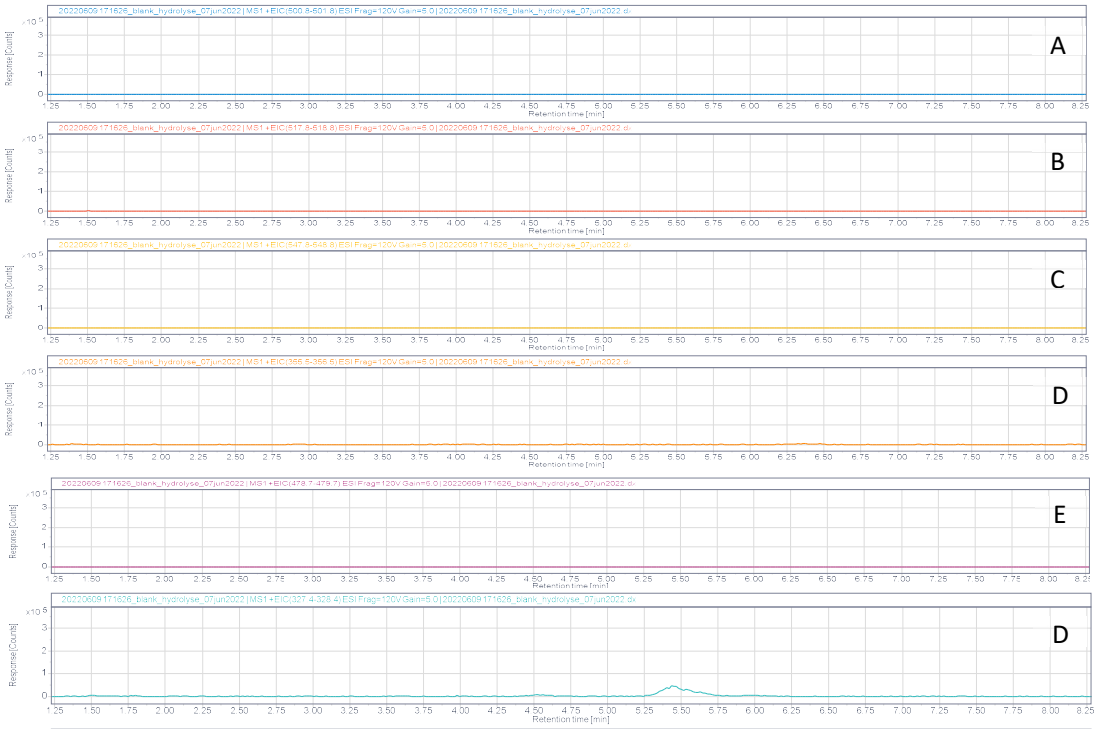
1. Overview of ET standards (state: 06.01.21)

LPS	Average molecular weight [Da]	Number KDO	Average KDO content [ng] in 500 ng LPS	% KDO in LPS	LAL conversion	Verified (yes/no)
E. coli F583	2429.49	2	98.0	19.6	?	Mass (MALDI, Qtof); half
E. coli EH100	3870.017	2	61.5	12.3	?	no
E. coli O55:B5	20051.066 (19 O-ag)	2	11.9	2.38	Approx. 4.7	no
E. coli K12	3587.369 (I) 3599.369 (IV)	2-3	72.9	14.58	? 29.7?	no
P. aeruginosa 10	5581.217 (4 O-ag)	2	42.7	8.54	?	No (based on MALDI Blanka)
P. aeruginosa 10	13698.39 (70 sugar units)	2	17.4	3.48	?	No (based on lit. King et al.)

9

Annex 12:PowerPoint from ET_Join meeting 12.01.21, LPS standard characterization, hydrolysis and KDO degradation, Slide N°9 from PPT 2021_01_12_ET_std_KDO_LAL. Original value for the KDO proportion in Endotoxin standard.



Annex 13: Chromatogram of mQ water blank showing no peak for the selected masses. A)501.1 m/z, B) 518.0 m/z, C) 548.1 m/z, D) 355.8 m/z, E) 479.0 m/z, E) 327.7 m/z.

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HES.SO	Analytical procedure for quantification of the endotoxin content for filter development and in biological samples	Version No.: KDO_Quant_Sial_2020.06.08
ISO 17025	Domain : CA	Edition :

1 Formula

N/A.

2 Principle of separation

Determination of endotoxin (ET) content and pyrogenity of biological samples is expensive. The recently mainly used method is the biological LAL test which, besides other disadvantages, also has a large quantification error.
Bacterial endotoxins contain the ET specific, rare sugar acid KDO. One ET molecule contains 1-3 KDOs per molecule. E.g. *E.coli O55:B5* contains usually 2 KDO molecules. For endotoxin filter development in the presence of e.g. proteins the *E.coli O55:B5* endotoxin standard is used. Until now, filtration was performed with the more expensive *E.coli O55:B5* L2637 from Sigma. Depending on the number of filtration experiments and the quantity per filtration a substitution with the cheaper L2880 should be envisaged. In one test, *E.coli O55:B5* L2880 was successfully used for filtration.
Quantification is based on the quantitative hydrolysis of ETs to obtain the ET specific, rare sugar acid KDO. The KDO containing solution is quantitatively labeled with the fluorophore DMB (DMB: 1,2-diamino-4,5-methylenedioxybenzene) and the strongly fluorescent quinoxaline product is formed. The reaction mixture is separated by RP-HPLC and the KDO-DMB peak is detected with a fluorescence detector. Relative quantification is based on external calibration with KDO-DMB, calibration based on biologic activity is performed using certified ET standards.
This SOP can be used for ET samples with strong matrix load, e.g. in case of ET standards containing 1 mg/mL protein used for filtration experiments. It was found that e.g. other alpha-beta-keto-acids attached to proteins may interfere with KDO-DMB in case of higher matrix load which requires optimized separation conditions at the cost of increased analysis time. Depending on matrix composition, further adaptation of the separation method may be required. For simple solutions without significant matrix load, **SOP KDO_Quant_Fast** is to be used.

From the KDO content determined (ng/ mL) the ET content (ng/ml) is calculated based on the known ET molecular mass and number of KDO in the particular ET. In case of unknown ET composition it is assumed that 1 ng endotoxin contains 0.6% KDO in the worst case (1 KDO, MW ET 40 000 Da) situation and in best case situation 36 % KDO (3 KDO, MW 2000 Da). Usually the worst case scenario is used, which may overestimate the total ET content in case of lower molecular weight or higher number of KDO per ng ET than assumed. Once the ET concentration in ng/mL is obtained it can be converted to EU/mL in case of known biological activity of the particular ET. Method evaluation in terms of LOQ in EU/mL may be performed by the use of ET standards. E,g. for the certified ET standard EC-6 1 ng ET is equal to ca. 10 EU/mL.

3 Safety and precautions

This method requires the handling of hazardous substances. It is recommended to use various regulations for potentially hazardous chemicals. Organizational, technical and personal safety has to be ensured. Eppendorf tubes, pipette tips and other consumables contaminated with ET are stored in

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Annex 14:Standard procedure of "Analytical procedure for quantification of the endotoxin content for filter development and in biological sample"

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the waste in the fume hood. The labeling procedure is carried out under the fume hood without the usage of a lamp (to avoid direct light which can cause photo degradation in the samples).

4 Area of application

The presented method with the settings below is appropriate for the quantification (LOQ) of endotoxins in ET standard solutions with protein matrix in the range of ca. 100 - 50,000 EU/mL (~10 – 5000 ng ET/mL; 0.1 – 50 ng KDO/mL). Known interferences (KDN, NGNA) can be well separated, in case of other contaminants method optimization may be necessary.

5 Materials

The method can be applied for 150-mm C18 columns with 2.1 or 4,6 mm diameter. **IMPORTANT:** In the following, column-specific parameters are given in the text for the 4.6 mm column and in "()" for the 2.1 mm column.

Standard columns used are
CC 150/4.6 Nucleodur C18 Gravity 3mm
YMC-TriartC18 ExRS
If e.g. shorter columns are to be used, the time table has to be adapted accordingly

HPLC Agilent 1100/Agilent 1200 equipped with FLD (8 µL cell)
Vortex mixer, e.g. Reax 2000, Heidolph
Heatblock, e.g. Thermo Shaker TS-100 from Biosan
Microcentrifuge, e.g. Glaxy Mini form VWR
Eppendorf tubes (1.5 mL) with screw caps
Sonicator Sonorex RK 100

Name	Abbreviation	Purity	Supplier	Product No.
keto-deoxy-octulonic acid	KDO	≥97%	Sigma	K2755
1,2-diamino-4,5-methylenedioxybenzene.2HCl	DMB	≥98%	Sigma	66807
Or 1,2-diamino-4,5-methylenedioxybenzene.2HCl (cheaper)	DMB	≥98%	Apollo	OR3723
Acetic acid	HOAc	≥99.0%	Fluka	A6283
2-Mercaptoethanol	BME	≥99.0%	Fluka	637000
Sodium hydrosulfite	$Na_2S_2O_4$	85%	Sigma	157953
Sodium phosphate dibasic dihydrate	$Na_2HPO_4.2H_2O$	≥98.5%	Sigma	30435
Potassium phosphate monobasic	KH_2PO_4	≥99.5%	Fluka	60220
Sodium chloride	NaCl	99.5%	Acros	207790010
Potassium chloride	KCl	≥99.5%	Fluka	60130
Acetonitrile	MeCN	HPLC grade	Macron	
Methanol	MeOH	HPLC grade	Macron	
water		MiliQ		

HES.SO	Analytical procedure for quantification of the endotoxin content for filter development and in biological samples	Version No.: KDO_Quant_Si al_2020.06.08
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Phosphate-buffered Saline 1x (pH=7.4)	PBS 1x		Thermo Fisher	
E. coli O55 :B5 lipopolysaccharide (TCA extracted, gel filtered)			Sigma	26
Or for Filter Tests: E. coli O55 :B5 lipopolysaccharide (purified with phenol extraction)			Sigma	28
For calibration with respect to reference standard of known biological activity: E-toxate 10 000 EU/vial *E.coli O55:B5* Or for calibration with respect to reference standard of known biological activity: 10 000 EU/vial *E.coli O55:B5*			Sigma Charles River	E8(LVE

6 Reagents

PBS 1x buffer (pH=7.4): Weigh 8.00g NaCl, 0.20g KCl, 1.78g $Na_2HPO_4.2H_2O$ and 0.24g KH_2PO_4 and dissolve them in 800 mL MilliQ water. Adjust the pH to 7.4 with HCL or NaOH and fill up to 1000 mL in a volumetric flask.
Note: This in-house fabricated PBS buffer will prospectively only be used to prepare endotoxin standards of 5,000 EU/mL. For the 100 µL PBS blanks a commercially purchased PBS 1x buffer of a higher quality has to be used as the ET contamination of the in-house PBS 1x negatively affects the measurements.

Preparation of 1 mL of 250 mM Sodium Hydrosulfite: 51.21 mg of sodium hydrosulfite are weighed and dissolved in 1 mL of Milli-Q water (alternatively, 20-30 mg of sodium hydrosulfite are dissolved in the respective volume of Mili-Q water to yield the same concentration). The solution is vortexed until the sodium hydrosulfite is completely dissolved.
Sodium hydrosulfite can be weighed 30 min before the labeling solution is prepared; the water should be added during the centrifugation step after endotoxin hydrolysis (Section 7.1). The solution has to be prepared freshly every day.

10,000 EU/mL endotoxin standards (Sigma or Charles River): Note: It is only necessary to prepare the 10 000 EU/mL ET standard (or any other ET standard with certified biological activity) if the measurement results are e.g. to be referenced against LAL test.
1 mL PBS (pH = 7.4) buffer is added to the vial (2 mL for 5000 EU/mL solution). The vial is thoroughly vortexed and shaken for 3 min. Then, the vial is centrifuged at 5000 rpm for 2 min to collect the liquid from the vial wall and the vial cap.

5,000 EU/mL endotoxin standard E.coli O55:B5 L2637 or L2880 (Sigma): 5,000 EU/mL endotoxin samples are prepared in pH=7.4 PBS buffer. A 0.5 mg/mL ET stock solution is prepared by weighing 0.5-1 mg endotoxin and adding the respective amount of PBS buffer to obtain a 0.5 mg/mL stock solution. The solution is thoroughly ultrasonicated for 45 min and visually inspected to ensure that all ET is completely dissolved. If necessary, repeat the process. In case solid ET in the vial is observed outside of the solution (e.g. at the inner side of the vial cap), the sample has to be discarded and remade. The solution is then vortexed for a minute. To prepare 5000 EU/mL solutions, 100 µL of

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the 0.5 mg/mL endotoxin solution is diluted to 100 mL with PBS buffer (500 ng/mL, approx. 5000 EU/mL). If other ET concentration are required, the dilution step can be adapted accordingly. 50 aliquots (100 µL each) of the obtained 5000 EU/mL solution can stored at -20°C in screw cap vials. The solutions can be stored for several months. The remaining solution is discarded.
Note 1: Endotoxins don't easily dissolve and form stable aggregates. It is important to verify that the endotoxins are completely dissolved (adjust ultrasonication time if required).
Note 2: Endotoxin storage is very challenging. The use of 100 µL aliquots which are directly taken for hydrolysis was shown to provide stable peak areas over at least 8 months. KDO peak height obtained from frozen ET solutions can be monitored over time to access long-term storage stability (in case of decreasing peak height, the stored aliquots are discarded and freshly prepared aliquots are used instead).
Note 3: In case of e.g. calibration experiments, the required ET solutions are freshly prepared by adjusting the respective dilution steps. The long-term storage stability has only been investigated for 500 ng/mL ET solutions (5 000 EU/mL).
Note 4: It was observed that different preparations of a 5000 EU/mL ET solution from the same batch (solid ET standard) showed significant differences (up to 50% deviation) in KDO peak height. In addition to weighing errors these deviations are assumed to be related to differences in the composition of the solid ET standard and the water in the hygroscopic solid ET. Accordingly, data interpretation in case of unexpected peak height of new ET standard solutions has to be performed carefully in order to avoid false conclusions.

5 ng/mL KDO solution: 0.8-1 mg KDO is dissolved in the respective amount of PBS to yield a 1 mg/mL KDO stock solution. The solution is vortexed, then 500 µL of the solution are transferred to a 100 mL volumetric flask and filled up to 100 mL with PBS (KDO concentration 5 µg/mL, solution 1). 100 µL of solution 1 are transferred to a 100 mL volumetric flask and filled up to 100 mL with PBS (KDO concentration 5 ng/mL, solution 2). 50 aliquots (100 µL each) of solution 2 are stored at -20 °C in screw cap vials. The solution can be stored for several months. It is possible to adjust the dilution step and final KDO concentrations if required.
Note 1: The use of 100 µL aliquots which are directly taken for labeling was shown to provide stable peak areas over at least 5 months. KDO peak height obtained from frozen 5 ng/mL KDO standards solutions can be monitored over time to access long-term storage stability (in case of decreasing peak height, the stored aliquots are discarded and freshly prepared aliquots are used instead).
Note 2: If necessary (e.g. in case of large method optimization/ validation measurement series), the number of aliquots can be increased.
Note 3: KDO is very expensive. Thus, for experiments which only require relative quantification, the aliquots stored at -20 °C can be used to prepare the required solutions. Accurate quantification would require to weigh at least 2 mg KDO with the current equipment.

ET samples from filtration experiments or other sources: In each case, 100 µL-aliquots are directly taken and stored in screw cap vials. The solutions can be stored at 2-8 °C overnight if the labeling is performed at the next day, otherwise they are stored at –20 °C. In order to reduce DMB consumption, the use of smaller aliquots (down to 70 µL sample and DMB reagent (each) is possible. In this case, the respective volume for hydrolysis and labeling are adjusted accordingly.
Note 1: For columns with 2.1 mm outer diameter, it can be tested if the labeling works reproducible with 50 µL-aliquots,
Note 2: Based on our experiences, the cheaper L2880 endotoxin can be used for filtration experiments. In order to ensure comparability of the data, the same ET type should be used for all measurements which are to be compared.

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DMB reagent preparation:
The DMB reagent is prepared according to the table. The ingredients are added in the order as they are listed below. The solution is vortexed after each step and used immediately after preparation. The calculated quantities based on the use of 100 µL samples.

DMB reagent preparation	# of Samples							
Number of prepared samples	3	4	5	6	7	8	9	10
DMB (mg)	1.05	1.4	1.75	2.1	2.45	2.8	3.15	3.5
MiliQ water (µL)	195	260	325	390	455	520	585	650
HOAc (µL)	53	71	89	107	124	142	160	178
2-Mercaptoethanol (µL)	35	46	58	70	82	93	105	117
0.25 M $Na_2S_2O_4$ (µL)	47	63	79	96	112	128	144	160
Total volume (µL)	330	440	551	663	773	883	994	1105

Note 1: The DMB can be weighed 15 min before the labeling solution is prepared. The weighed DMB can be stored in the dark until the labeling solution is prepared. DMB and DMB-labeled sugar samples are not UV stable. Direct light should be avoided if possible, the light in the fume hood has to be switched off.

Note 2: It is difficult to accurately weigh the respective DMB quantities and it should be avoided to keep the vials open for a long time period to avoid oxidation of the solid DMB or water adsorption. Instead, the volume of the respective solutions is calculated with a template based on the DMB weight (e.g. 3.62 mg for 10 samples requires 650 mL*3.62/3.52= 668 µL). The template is found in 09_SOP/4_Supplementary documents internal "Calculation tool DMB solution"

MeCN:MeOH 36:64 360 mL MeCN and 640 mL MeOH or 180:320 mL are mixed in a glass bottle (mobile phase B). Especially in case of the 2.1 mm column, 500 mL total volume is usually sufficient.

MilliQ Water : mobile phase A
Note: the method was developed without TFA in the mobile phase. In the absence of TFA, a very smooth baseline is obtained. Stability of the method without TFA has been confirmed.

7 Analytical procedure

7.1 Endotoxin hydrolysis with 2% HOAC:

2µL HOAc (>98%) is added to each 100 µL aliquot, the aliquots are gently mixed. Perform a short centrifugation step to ensure that no droplets stick to the inner wall of the screwcap. Hydrolysis is performed in a thermoshaker for 90 min at 80°C to release the KDO molecules. The tubes are fully covered with aluminum foil. After hydrolysis the samples are cooled on ice for 5 min and centrifuged @ 10000 rpm 4°C, 5 min with the microcentrifuge. The hydrolyzed samples are labeled immediately (the samples are left in the microcentrifuge until the labeling solution was prepared).
Note: Until 01-02.2018, hydrolysis was performed for 45 min. However, it was shown that endotoxin hydrolysis is incomplete after 45 min. In ET standards, hydrolysis appeared to be complete after 60-90 min, in samples which contain e.g. BSA hydrolysis kinetics are slightly slower. Based on the current data basis, 90 min hydrolysis time is selected as compromise.

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7.2 DMB labeling:

100 µL of DMB reagent is added to the 100 µL sample aliquots. Blank, KDO standard and samples are labled with the same conditions. Make sure that no liquid droplets stick to the inner wall of the screw cap after DMB addition (perform short centrifugation if observed). The tubes are placed into the thermoshaker at 60°C for 1.5 h fully covered with aluminum foil to avoid direct light. After the reaction, samples are cooled on ice, centrifuged @ 10000 rpm 4°C, 5 min with the microcentrifuge (to collect the liquid from the cap) and stored in freezer @ (-15 ± 3)°C with UV protection until further analysis.

2 sample preparations/sample and 2 injections/vial are usually performed (The data conclusively shows that repetitive measurements of the same sample are highly reproducible. Accordingly, it is sufficient to perform 2 measurements per sample vial if only relative quantification is required.

Note: Due to the high surface tension and ionic strength of the solution the pipetting procedure is crucial; The operator has to ensure that a reproducible pipetting procedure is performed (e.g. reversed pipetting) to ensure accurate data.

7.2.1 Blank

A blank is prepared in each labeling session. The blank is prepared from 100 µL PBS (ThermoFisher) and 100 µL DMB reagent with the same procedure mentioned above. The blank must not contain signals within the KDO-DMB elution time range (6.5 min for Nucleodur column, 7.8 min for YMC column) to ensure accurate quantification at low concentrations. If interfering blank signals are observed, method accuracy is compromised.

Note 1: In 2016 and 2017, blank subtraction was considered as suitable tool to improve method LOD. However, slight changes of the blank profiles were frequently observed within a measurement sequence, which indicates that blank subtraction requires the user to ensure that blank subtraction does not negatively affect data. Thus, baseline correction is not performed if not stated otherwise.

Note2: In some rare cases (ca. 5% of sample preparations) unexpected peak profiles are observed (KDO peak stable at the expected elution time, additional interfering signals are observed in the sample and the blank). It is thus assumed that differences during the labeling procedure or improper column conditioning are responsible for the observed deviations. Data evaluation at peak height >50 can be considered to be not affected by these deviations in most cases, while accurate quantification of smaller peaks will not be possible.

7.3 External standards

Endotoxin with 5000 EU/mL (corresponds to 500 ng/mL ET, aliquots stored in the freezer) can be used as reference to monitor the hydrolysis procedure.

7.4 Sample preparation

Prior analysis the DMB labeled samples are centrifuged @ 10000 rpm 4°C, 5 min and the supernatant is transferred to brown glass HPLC vials with insert. Make sure that no air remains in the lower part of the glass insert. Glass inserts from BGB have to be checked with an HPLC injection needle: in some cases, the inner diameter of the lower part of the glass insert is too small, leading to broken inserts during sample injection.

7.5 HPLC Separation

4.6*150 mm Nucleodur Gravity and a 2.1*150 mm YMC Triart ExRS column have been used successfully with flow rates of 1.5 mL/min (0.31 mL/min) with identical linear flow velocity. Time

tables are adjusted accordingly due to the different equilibration time at different flow rates (dwell volume).

7.5.1 Initial conditions

Column temperature: 40°C
Flow rate: 1.5 mL/min (0.31 mL/min)

7.5.2 Column equilibration

The column is rinsed with 80% solvent B at 40°C for at least 45min at 0.8 mL/min (0.15 mL/min). Next, the column is rinsed at 1.5 mL/min (0.31) with 89% mobile phase A for 30 min.

7.5.3 Solvents

A: MilliQ water
B: MeCN:MeOH 36:64
C: MeCN
D: MEOH/ MilliQ water

7.5.4 Control settings

Flow rate: 1.5 mL/min (0.31 mL/min)
Pressure limit: 360 bar for Nucleodur gravity columns, 350 bar for YMC columns(recommended values to increase column lifetime)
Column temperature: 40°C
Stoptime: 15 min
Posttime: 4 min (8 min)

7.5.5 FLD detector settings

Excitation wave length: 373nm
Emission wave length: 448nm
Peakwidth >0.2 min
PMT-Gain: 18

7.5.6 Injection settings

Injection volume: 50 µL (10 µL)
Wash needle in water: Vial 91
. In case of problematic samples, DMSO can be tested instead. The needle position is set to 1 mm to avoid the destruction of the glass inserts with the injection needle.

7.5.7 Elution timetable

Table 1 Method gradient for the Nucleodur column

Time [min]	Solv.A [%]	Solv.B [%]	Flow [mL/min]
0.00	89	11	1.5
8.5	89	11	1.5
9.0	50	50	1.5
14.00	50	50	1.5
15.00	89	11	1.5

Table 2 Gradient for « KDO_SEPOP_YMC_water_guard column"

Time [min]	Solv.A [%]	Solv.B [%]	Flow [mL/min]
0.00	89	11	1.5
8.5	89	11	1.5
9.0	50	50	1.5
14.00	50	50	1.5
15.00	89	11	1.5

Note 1: For the YMC column, a washing step with 80% mobile phase B has been applied successfully. This has to be confirmed and implemented for the Nucleodur column.

Note 2: It has to be tested if the isocratic separation can be shortened to 7 min, especially if NGNA signal quantification is not of interest. Furthermore, washing-solvent and –time have to be optimized to reduce solvent consumption, which also accounts for the post time.

Note 3: The same accounts for the 2.1 mm YMC column (flow rate 0.31 mL/min). Here, the dwell volume has a stronger impact due to the lower column volume. It is important to note that post time has to be longer for the YMC column. This SOP has to be updated when the results on method shortening are available.

7.6 Shutdown: cleaning and storage

After each session the column must be cleaned and stored properly. The 4.6 mm Nucleodur column is washed with 40% water 60% ACN for 45 min at 1.5 mL/min "Shutdown method". In case of severe contamination of the column, additional cleaning steps e.g. using methanol can be performed. The Nucleodur column is stored in 60% MeCN
The 2.1 mm YMC column is rinsed at 0.31 mL/min for 60 min with 70% MeCN, followed by 30 min with 100% MeCN. The column is stored in 100% MeCN.

7.7 System Suitability test

To check the performance of the Agilent 1100 or 1200 system, endotoxin hydrolysis and the labeling procedure, perform a separation with 5 ng/mL KDO solution and with a 500 ng/mL endotoxin solution.

1) For KDO, 100-140 LU peak height are usually observed with the 1100 system and the corresponding detector (DE92001512). At the DE60555395 detector (1200 system), signal response is about 30% lower.

2) For the ET standards, peak height of 80-100 LU are usually observed with the 1100 system and the corresponding detector (DE92001512). At the DE60555395 detector (1200 system), signal response is about 30% lower.

Note 1: Due to the low quantities which are commonly used, significant weighing errors are likely to affect peak height for KDO and ET standards.

Note 2: KDO and ET standards are hygroscopic. Furthermore, ET standards are very inhomogeneous, which increases potential deviations.

Note 3: For each system (2.1 vs 4.6 mm column diameter coupled with each detector, using 10% MeCN or 11% MeOH:MeCN at 64:36 ratio as mobile phase B) = 2*2*2= 8 combinations) peak height will be different. It is thus crucial to ensure that the correct parameters are compared.

In order to allow reliable relative quantification, e.g. 100* 100 µL-aliquots are prepared from the same solution and stored at -20 °C (commonly referred to as "long-term storage"/ "LT storage"). At identical conditions, RSD between these measurements performed at different days should be below 10% (10% warning; 20% action required if deviations persist), this can be monitored by a control chart. If new solutions for LT storage have to be prepared, 3 aliquots of the old and new LT solution are labeled and compared in one experiment. Potential deviations in the observed peak height have to be documented.

If higher deviations/ outliers are obtained for a known LT storage solution, measurements have to be repeated. If the deviations persist, deviations have to be reported to the supervisor. Potential reasons for the deviations (degradation of the stored solutions, functionality of the instrument and the derivatization procedure and) have to be tested and eliminated to ensure reliability of the method.

3) Retention time: If column conditioning has been performed properly, retention time has been observed to be extremely stable e.g. at consecutive measurement days. Retention time is approx.. 1 min different between both columns. Retention time stability has to be monitored by a control chart. Outliers/ a steady retention time shift have to be reported to the supervisor and column performance has to be verified.

4) Column back pressure: Commonly, back pressure between 160-220 (200-260) bar is observed during measurements. If pressure values outside of this range (20 bar difference) are observed, column performance has to be verified. Increased pressure may be caused by clogging of tubing or the pre-column.

8 Evaluation of the results

The KDO content is determined on the peak height basis due to potential noise in the signal close to the KDO peak, which prevents evaluation based on peak area. The high separation performance of the method allows the analysis of samples with high matrix load such as 1 mg/mL BSA (Fig 1 B). In this case NGNA (N-Glycolylneuraminic acid) from BSA is well separated from KDO (big peak in the blue trace in Figure 3B at 7.5 min behind KDO). Further optimization of the resolution can be achieved via gradient elution.

The baseline frequently shows some drift with the KDO_Quant_Sial analytical method. An example is given below in Figure 1. In case of near-coeluting peaks integration based on the baseline shape can be

performed (Figure 1 A). Otherwise, integration according to Figure 1 B is performed (in absence of matrix signals both methods lead to identical peak height). The "Baseline-method" is especially useful if e.g. a small peak elutes closely after KDO to reduce integration inaccuracy. The operator has to ensure that the integration procedure allows accurate quantification (report to supervisor in case of doubt/unexpected baseline behavior).

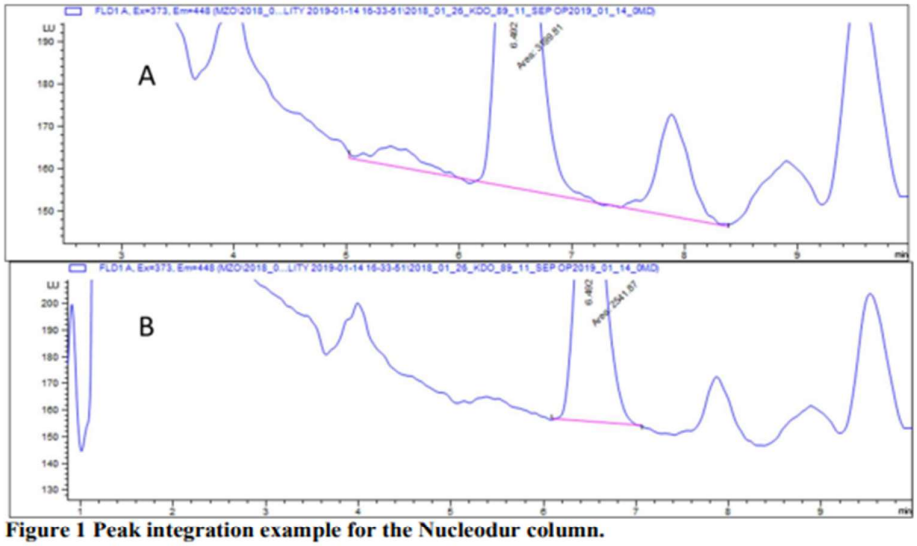

Figure 1 Peak integration example for the Nucleodur column.

Elution profile repeatability is exemplarily shown in Figure 2

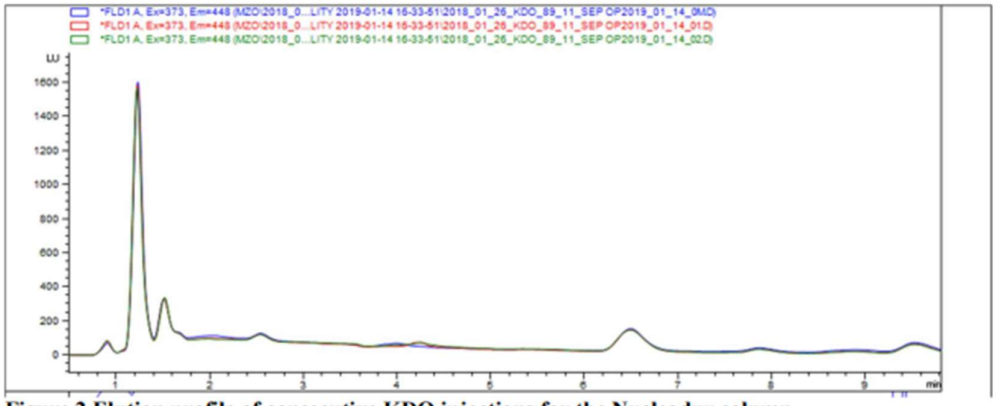

Figure 2 Elution profile of consecutive KDO injections for the Nucleodur column.

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Blank profile comparison as shown in Figure 3A. A very smooth baseline is obtained with the current method for e.g. ET standards. The method allows good resolution between KDO-DMB and a challenging interfering analyte (NGNA) at > 10-fold excess of NGNA (Figure 3 B). If better resolution is required, gradient elution may be performed instead.

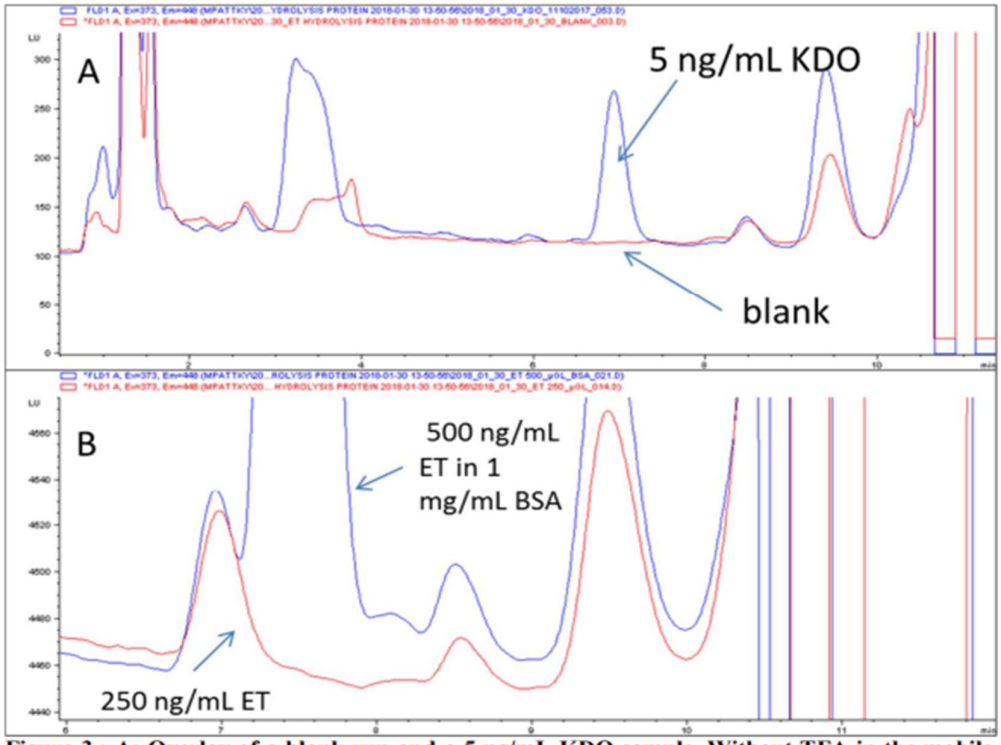

Figure 3 : A: Overlay of a blank run and a 5 ng/mL KDO sample. Without TFA in the mobile phase, very low noise in the elution time range of KDO is observed in the blank. B: The method allows good separation of KDO from a large excess of NGNA originating from BSA (big blue signal in Figure 3 B after KDO).

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