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# Determination of KDO from bacterial lipopolysaccharides


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## Keywords

Dionex CarboPac PA20 column, sugar acid, gram-negative bacteria, HPAE-PAD, LPS


## Goal

To develop, optimize, and validate an HPAE-PAD method for the determination of KDO as a marker for the presence of a lipopolysaccharide (LPS)

## Introduction

Endotoxins are lipopolysaccharides found in the cell wall of gram-negative bacteria. Lipopolysaccharides areD large molecules consisting of a lipid and a polysaccharide, composed of O-antigen, outer core, and inner core, joined by a covalent bond. The carbohydrate part of the LPS contains the sugar acid, 3-deoxy-D-manno-oct-2-ulosonic acid (ketodeoxyoctonic acid, KDO), in addition to several other monosaccharides such as L-glycero-D-manno-heptose, galactose, glucose, mannose, galactosamine, glucosamine, and rhamnose. KDO is an eight-carbon sugar acid mostly confined to gram-negative bacteria and is a ubiquitous component of bacterial LPSs (endotoxin). It provides a bridge between lipid A and the core oligosaccharide in all bacterial LPSs. KDO is often involved in attaching surface polysaccharides to their lipid anchors.

All parenteral and medical device products need to be tested for pyrogen/endotoxin activity prior to their release for human use as required by the drug regulatory agencies. The commonly used test for determining endotoxin levels in pharmaceutical products uses the white cell lysate from horseshoe crabs and is described in the United States Pharmacopeia (USP) General Chapter <85>.[1]

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In addition to the LAL, there are other chemical methods for endotoxin determinations using GC-MS[2] and LC-MS.[3-5] LC-MS/MS methods provide direct analysis of the 3-hydroxy fatty acids after hydrolysis of the LPS; however, they are complex methods and require expensive instrumentation. Chemical methods using GC-MS require a long sample preparation time as these polar acids require derivatization to obtain a volatile compound for the analysis.

High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for the determination of monosaccharides and oligosaccharides obtained from glycoproteins, polysaccharides, and other glycoconjugates.[6-11] The sugar acid KDO is a characteristic component of bacterial LPS. Thus, it can be used as a marker for the presence of a LPS, and if the identity of the LPS is known, quantify the amount of the LPS. Acid hydrolysis conditions were optimized for the release of KDO from the LPS, and KDO was quantified with a HPAE-PAD method.[12] The HPAE-PAD method has the advantage of resolving compounds that interfere with colorimetric methods. In this application note, we develop, optimize, and validate a HPAE-PAD method for the determination of KDO as a marker for the presence of a LPS.

## Experimental

### Equipment

- High-pressure Thermo Scientific™ Dionex™ ICS-5000+ HPIC system* including:
  - Thermo Scientific™ Dionex™ ICS-5000+ DP Dual Pump with degas option (P/N 079975)
  - Thermo Scientific™ Dionex™ ICS-5000+ DC Detector Chromatography Compartment with dual temperature zones, two injection valves (P/N 075943)
  - Thermo Scientific™ Dionex™ ICS-5000+ ED Electrochemical Detector (P/N 072042) and Thermo Scientific™ Dionex™ ICS-5000+ ED Electrochemical Detector Cell (P/N 072044)
- Thermo Scientific™ Dionex™ AS-AP Autosampler (P/N 074926) with tray cooling option (recommended)
- Thermo Scientific™ Chromeleon™ Chromatography Data System Software, Version 7.2.9
- Thermo Scientific™ Savant™ SpeedVac™ Medium Capacity Vacuum Concentrator (P/N SPD140DDA-230)

*This application can be performed on a Dionex ICS-6000 system, and a SP single pump module can be used instead of a DP dual pump module.

### Consumables

- Thermo Scientific™ Dionex™ ICS-5000+ ED Electrochemical Detector Ag/AgCl pH Reference Electrode (P/N 061879)
- Thermo Scientific™ Dionex™ ICS-5000+ ED Electrochemical Detector Gold on PTFE Disposable Electrodes, pack of 6 (two 2.0 mil gaskets included) (P/N 066480) (1 mil = 25.4 µm)
- Thermo Scientific™ Dionex™ Vial Kit, 10 mL Polystyrene with Caps and Blue Septa (P/N 074228)
- Thermo Scientific™ Nalgene™ Syringe Filters, PES, 0.2 µm (Fisher Scientific, P/N 09-740-61A)
- Air-Tite™ All-Plastic Norm-Ject™ Syringes, 5 mL, sterile (Fisher Scientific, P/N 14-817-28)
- Vial Kit, 10 mL Polypropylene with Caps and Septa (P/N 055058)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane (1 L 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- MilliporeSigma™ Amicon™ Ultra Centrifugal Filters UFC900324, 15 mL, 3K NMWL

### Materials and standards

- LPS standards (Sigma-Aldrich)
  - Product # L6136 – LPS from *Serratia marcescens* (S#1)
  - Product # L9143 – LPS from *Pseudomonas aeruginosa* (S#2)
  - Product # L4268 – LPS from *Klebsiella pneumoniae* (S#3)
  - Product # L2018 – LPS from *E. coli* K-235 (S#4)
  - Product # L1887 – LPS from LPS from *Salmonella enterica* (S#5)
- KDO (Sigma-Aldrich, P/N K2755)
- Glacial acetic acid (Fisher Scientific)
- Sodium hydroxide, 50% *w/w* (Fisher Scientific, P/N SS254-500)
- Sodium acetate, anhydrous, electrochemical grade (P/N 059326)

### Preparation of solutions and reagents

#### Eluent solutions

Brief instructions are listed below, but for detailed information on eluent preparation for HPAE-PAD, please see Thermo Scientific Technical Note 71.[13]

### 100 mM sodium hydroxide

To make 0.1 M NaOH, add 5.2 mL of 50% *(w/w)* NaOH to 1 L of degassed DI water by removing the NaOH aliquot from the middle of the 50% solution where sodium carbonate is least likely to have formed. Do not pipet from the bottom where there may be sodium carbonate precipitate, and prepare eluent only from a bottle of 50% sodium hydroxide that still contains at least a third of its original volume. Place the tip of the pipette containing the aliquot of NaOH ~1 in. (2.54 cm) below the surface of the DI water and dispense. After the sodium hydroxide transfer is complete, put the container on the system under helium or nitrogen and swirl to mix. Replace the cap on the 50% hydroxide bottle. Always keep the eluent blanketed under helium or nitrogen at 34 to 55 kPa (5–8 psi), and store for no more than one week.

### 1 M sodium acetate/100 mM sodium hydroxide

To make 1 L of 100 mM sodium hydroxide containing 1.0 M sodium acetate, dispense approximately 800 mL of DI water into a 1 L volumetric flask. Vacuum degas for approximately 5 min. Add a stir bar and begin stirring. Weigh 82.0 g anhydrous, crystalline sodium acetate. Add the solid acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt has dissolved, remove the stir bar with a magnetic retriever. Add DI water to the flask to bring the volume to the 1 L mark. Vacuum filter the solution through a 0.2 µm nylon filter. This can take some time because the filter may clog with insoluble material from the sodium acetate. Using a plastic volumetric pipette, measure 5.2 mL of 50% (*w/w*) sodium hydroxide solution from the middle of the bottle. Dispense the sodium hydroxide solution into the acetate solution ~1 in. (2.54 cm) under the surface of the acetate solution and then mix in the same manner as the 100 mM NaOH above. Keep the eluent blanketed under helium or nitrogen at 34 to 55 kPa (5–8 psi) at all times and store for no more than one week.

### Standard solutions

Prepare 100 µM KDO stock standard solution by dissolving 2.55 mg in 100 mL DI water. Maintain the stock solution at -20 °C until needed.

## Sample preparation

### Hydrolysis of LPS standards

We started with the same hydrolysis conditions as in reference 12 and modified them by adding a sample clean-up step (Step 5) to remove lipids. It is important to have this clean-up step as it prevents rapid column overloading and column contamination.

**Step 1:** Weigh approximately 2.5 mg of sample and transfer it to a 5 mL volumetric flask.

**Step 2:** Add 1% acetic acid solution to the flask up to the mark and mix thoroughly.

**Step 3:** Heat the sample at 70 °C for 60 min.

**Step 4:** Evaporate the samples to dryness (5–6 h) using a SpeedVac equipped with an acid trap. Then, resuspend the dried residue in 10 mL DI water.

**Step 5:** Filter the samples using centrifugal filter units (3,000 MWCO).

- 5a. Add 10 mL of 0.1 M NaOH to the filter unit and centrifuge for 30 min at 5,000 rpm, discard the filtrate.
- 5b. Add 10 mL DI water to the filter unit and centrifuge for 30 min at 5,000 rpm, discard the filtrate.
- 5c. Add 10 mL of sample (from Step 4) to the filter unit and centrifuge for 30 min at 5,000 rpm, collect the filtrate and analyze using HPAE-PAD.

## System preparation

The Dionex ICS 5000+ HPIC system is configured for electrochemical detection, operating under high pressure conditions up to 5,000 psi. To install this application, connect the Dionex AS-AP autosampler and Dionex ICS-5000+ system modules. Please refer to the Technical Note 73348 for details on the preparation of the electrochemical cell and installation of the Ag/AgCl reference electrode.[14]

**Chromatographic conditions**

| System | Dionex ICS-5000+ HPIC System | |
|---|---|---|
| Columns | Thermo Scientific™ Dionex™ CarboPac™ PA20-Fast-4µm Analytical, 2 × 100 mm (**P/N 302749**)<br>Dionex CarboPac PA20-Fast-4µm, Guard, 2 × 30 mm (**P/N 302750**) | |
| Column temp. | 30 °C | |
| Compartment temp. | 20 °C | |
| Eluent A | 100 mM NaOH | |
| Eluent B | 1 M Sodium acetate/100 mM NaOH | |
| Gradient | 0–4 min: 50 mM sodium acetate/ 100 mM NaOH<br>4–9 min: 500 mM sodium acetate/ 100 mM NaOH<br>9–20 min: 500 mM sodium acetate/ 100 mM NaOH | |
| Flow rate | 0.25 mL/min | |
| Injection volume | 10 µL | |
| Inject mode | Push full | |
| Loop overfill factor | 5 | |
| Detection | Pulsed amperometry | |
| Working electrode | Gold on PTFE Disposable Electrode, 2 mil gasket (P/N 066480) | |
| Reference electrode | Ag/AgCl* | |
| | **Time (s)** | **Potential (V) integration** |
| Waveform | 0.00<br>0.20<br>0.40<br>0.41<br>0.42<br>0.43<br>0.44<br>0.50 | +0.1<br>+0.1 Begin<br>+0.1 End<br>–2.0<br>–2.0<br>+0.6<br>–0.1<br>–0.1 |
| System backpressure | ~3,700–4,500 psi (100 psi = 0.689 MPa) | |
| Background | 25–35 nC | |
| Noise | ~35 pC/min peak-to-peak | |
| Run time | 20 min | |

* A PdH reference electrode can also be used for this work. If using the PdH reference electrode, the waveform will have different potential values. See reference 14 for details.

## Results and discussion

### Separation

The published HPAE-PAD method[12] is based on the separation of KDO using a Dionex CarboPac PA1 column. Although this is a good column choice for a KDO determination, we chose a newer column, the Dionex CarboPac PA20-Fast-4µm column. This column[15] uses a smaller resin particle that produces more efficient peaks, resulting in greater sensitivity, more accurate integration, and thus, more reliable results. The smaller resin particles combined with reduced column length provide about 50% shorter runtimes than a standard size Dionex CarboPac PA20 column (3 × 150 mm) without sacrificing performance. KDO also elutes faster than the published separation[12] though it is unclear if our overall injection to injection time is shorter than the published method as our method employs a column wash and subsequent equilibration to keep retention time stable and the published method does not discuss a column wash.

We experimented with isocratic separations, using a fixed concentration of sodium hydroxide with different sodium acetate concentrations. We found 50 mM NaOAc/100 mM NaOH as the best eluent condition to separate KDO from interfering peaks. The method starts with 50 mM NaOAc/100 mM NaOH from 0 to 4 min and finishes with a short column wash with 500 mM NaOAc/100 mM NaOH and 10 min of column equilibration to starting conditions. Each chromatographic run must have a 5 min wash step and a 10 min equilibration step to ensure retention time reproducibility. Figure 1 displays chromatograms of DI water and a 1 µM KDO standard analyzed using these mobile phase conditions and the remaining chromatographic conditions listed in the Experimental section.

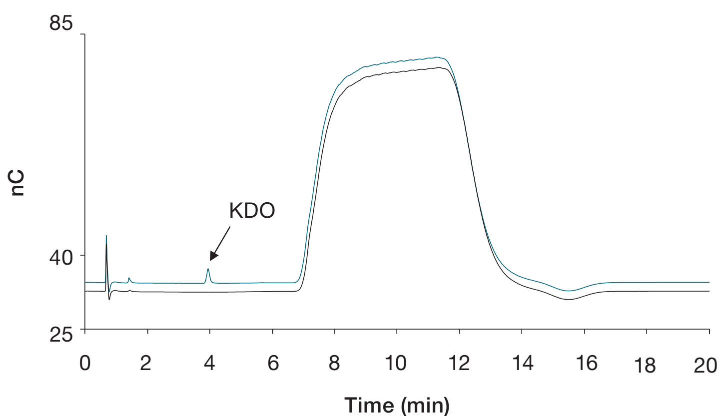


**Figure 1. Chromatograms of DI water and a 1 µM KDO standard**

## Evaluation of LOD, LOQ, and linearity

We used the KDO standard to evaluate LOD, LOQ, and linearity. Figure 2 displays the chromatograms of six calibration standards ranging from 0.25 µM to 10 µM KDO. Figure 3 displays the KDO calibration curve. Figure 3 and the coefficient of determination ($r^2$) of 0.999 indicate a linear relationship between peak area and detector response in the specified concentration range.

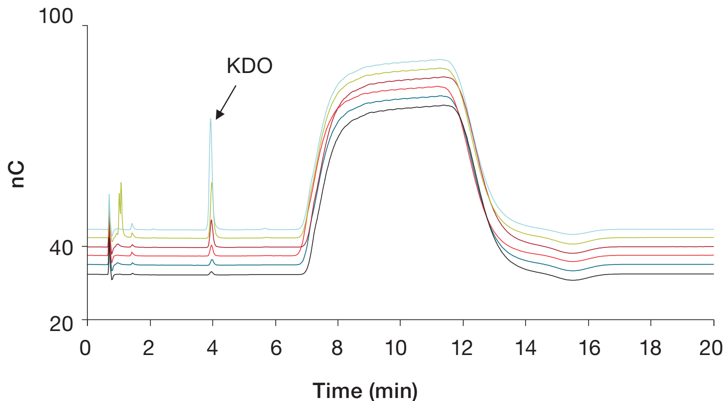


**Figure 2. Chromatograms of KDO calibration standards (0.25 µM to 10 µM)**

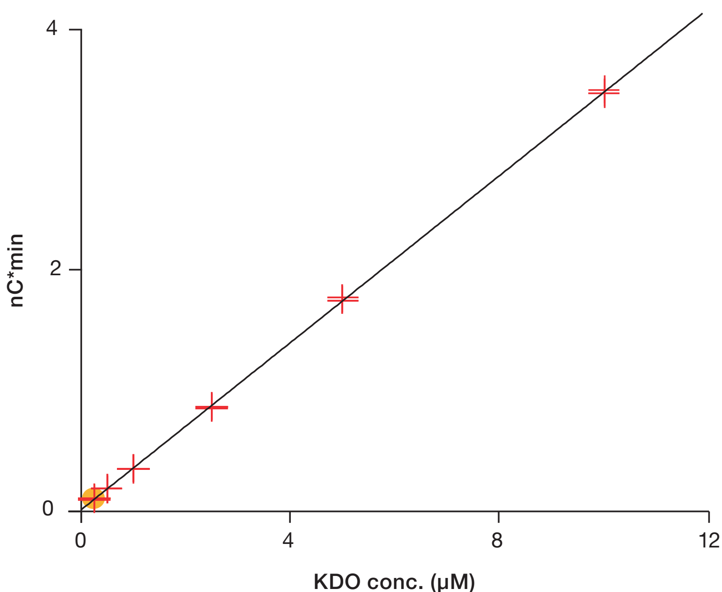


**Figure 3. KDO calibration curve**

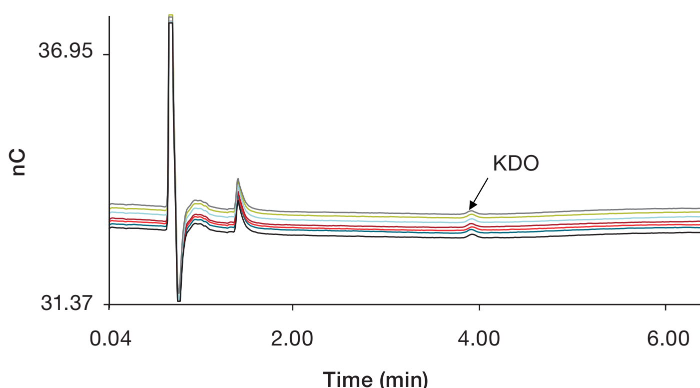


**Figure 4. Chromatograms of seven consecutive injections of 0.025 µM KDO standard**

To determine the LOD and LOQ, the baseline noise was first determined by measuring the peak-to-peak noise in a representative one-minute segment of the baseline where no peaks elute, but close to the peak of interest. The signal was determined from the average peak height of seven injections of 0.025 µM KDO standard. The LOD and LOQ were determined by 3 × and 10 × S/N, respectively. Figure 4 displays the chromatograms of seven consecutive injections of 0.025 µM KDO standard. The estimated LOD and LOQ for KDO are 0.01 and 0.04 µM, respectively.

## Determination of KDO in LPS samples

For this study, we tested the four lipopolysaccharides listed in Table 1 and determined their KDO content using HPAE-PAD. Figure 5 displays the chromatograms of the LPS S#1 along with 1 µM KDO standard. Figure 6 displays the magnified chromatograms of LPS S#1 through 4 along with 1 µM KDO standard. We used the KDO standard calibration curve to determine the amount of KDO in LPS standards and then converted the amount of KDO (µM) to endotoxin unit (EU). We found this to be >15,000 EU in all five LPS samples tested. Table 1 lists the calculation for conversion of the amount of KDO to EU. For calculations, we used a molecular weight of 4,900 g/mol for all LPS standards.

**Table 1. Calculation of EU for LPS standards**

| LPS | KDO (µM) | KDO (nmol/mL) | Endotoxin* (nmol/mL) | Endotoxin* (ng/mL) | EU |
|---|---|---|---|---|---|
| S#1 | 6.15 | 6.15 | 3.08 | 15,100 | 151,000 |
| S#2 | 0.96 | 0.96 | 0.48 | 2,350 | 23,500 |
| S#3 | 0.78 | 0.78 | 0.39 | 1,910 | 19,100 |
| S#4 | 0.67 | 0.67 | 0.335 | 1,640 | 16,410 |

* Assuming 2 moles KDO per mole of endotoxin

## Calculation of conversion of EU/mL to KDO (µM)

1 endotoxin unit (EU) equals 0.1 to 0.2 ng endotoxin per mL

0.1 ng/4,900 ng/nmol = 0.02 pmol/mL (molecular weight of LPS: 4,900 g/mol)

1 EU/mL equal to 0.04 pmol/mL KDO (assuming there are two mol of KDO per mol LPS) = 0.04 µM KDO

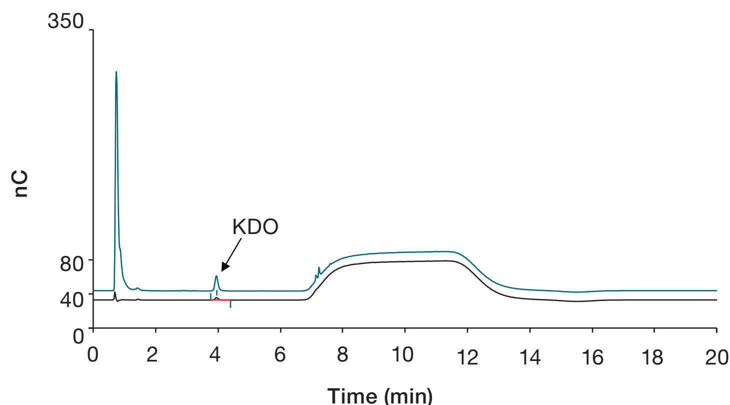


**Figure 5. Chromatograms of LPS (S#1, upper (blue) chromatogram) and a 1 µM KDO standard**

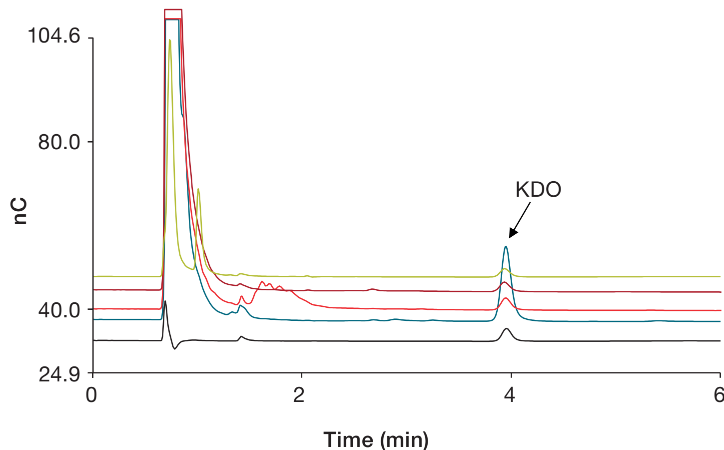


**Figure 6. Chromatograms of LPS samples 1 through 4 along with a 1 µM KDO standard**

## Recovery of KDO in LPS samples

Method accuracy was determined by spiking with different KDO concentrations (1 and 4 µM) depending on the concentration of KDO in the original sample. The recovery percentages were calculated using the formula shown below:

$$\text{Recovery \%} = \frac{C_{spiked\ sample} - C_{unspiked\ sample}}{C_{analyte\ added}} * 100$$

Figure 7 shows the representative chromatogram of LPS S#1 spiked with 4 µM KDO. The recovery percentages for KDO in all the LPS standards are in the range of 80 to 90% (Table 2), suggesting good method accuracy.

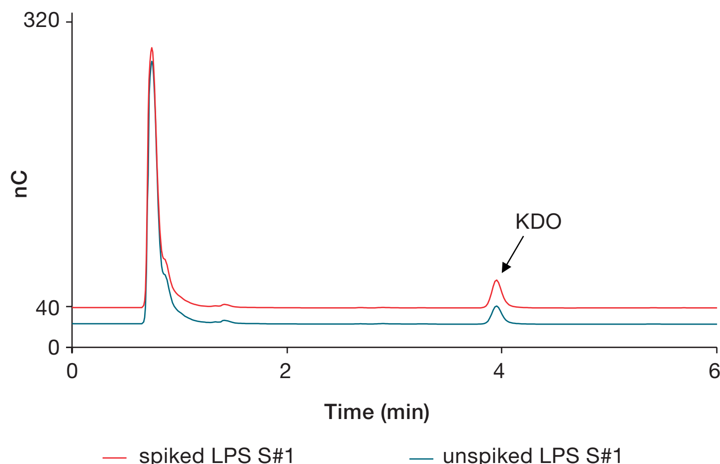


**Figure 7. Chromatograms of LPS sample 1 and sample 1 spiked with 4 µM KDO**

**Table 2. KDO recovery study (n=3)**

| | KDO concentration (µM) | | | |
|---|---|---|---|---|
| LPS standard | $c_{Unspiked}$ | Added | $c_{Spiked}$ | Recovery (%) |
| **S#1** | 6.15 | 4 | 9.55 | 84.9 |
| **S#2** | 0.960 | 1 | 1.76 | 80.4 |
| **S#3** | 0.780 | 1 | 1.61 | 83.4 |
| **S#4** | 0.670 | 1 | 1.56 | 88.5 |

While this method is good for determining the KDO content of an LPS, in separate experiments (not shown) we found that this method does not have enough sensitivity to replace the USP Endotoxins Test. That test requires a calibration curve of 0.05 to 10 EU/mL created with the USP Endotoxins reference standard. The lowest calibration standard 0.05 EU/mL is 0.002 pmol/mL KDO = $2 \times 10^{-6}$ µM, much less than the $1 \times 10^{-2}$ µM LOD. We could not increase sensitivity by increasing injection volume or concentrating sample as that caused column overload.

## Conclusion

Using the Dionex CarboPac PA20-Fast-4µm column, an HPAE-PAD method was successfully developed and validated for KDO determination in four LPS standards. This column allows the separation of KDO from other peaks in less than 5 min with an overall cycle time of 20 min. PAD is sensitive and thus allows the determination of a low concentration of KDO in LPS standards. The method showed good precision and accuracy with a recovery range of 80–90% in the samples tested.

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