Application Note

Clinical & Diagnostics

The most selective LC-EC applications for Clinical & Diagnostics analysis

Catecholamines
Serotonin
Metanephrines
VMA
HVA
5-HIAA

PET imaging tracer
Fluorodeoxyglucose (FDG)
FDG impurities

Sulfides
Homocysteine
Glutathione
Disulfides

Vitamins, minerals
A, C, D, E and K
Iodide
Q10, ubiquinols

Iodide in Urine

- **Sensitive and selective LC-ECD analysis of iodide**
- **Reproducible and linear**
- **Analysis time of only 12 minutes**

Summary

The analysis of iodide was evaluated on an Antec ALEXYS® Iodide Analyzer, using ion-pairing separation on a sub-2 micron C18 column, followed by electrochemical detection on a silver electrode. The method was shown to give repeatable results with an RSD <2% for peak area, the response was shown to be linear with a correlation coefficient >0.999 in the range of 0 25 µM iodide, and the detection sensitivity was about 3 nM (0.4 µg/L) iodide. With a urine sample it was shown that this isocratic analysis is very selective for iodide, resulting in a clear baseline after 14 minutes.

Introduction

Iodide is an essential nutrient, and iodide deficiency is the number one cause of preventable intellectual disability in the world. To asses the iodide deficiency levels of populations and the table salt iodization programs, iodide levels in urine are used as a marker for iodide intake [1].

Urine is a relatively complex matrix of ions and various other polar components that potentially complicates the HPLC analysis. Separation can be achieved on a C_{18} column in combination with a mobile phase containing ion-pairing agent. The subsequent detection of iodide can be done with an electrochemical detector (ECD) on various electrode materials, but the use of a silver electrode in particular makes the method highly selective for iodide. A very low working potential is applied, which makes the method very selective and particularly useful for analysis of urine samples.

The ALEXYS system is a versatile LC-ECD platform that is optimized for sensitive analyses using electrochemical detection. This note shows the measurement conditions and performance for the analysis of Iodide using the ALEXYS system.

Figure 1. ALEXYS Iodide Analyzer

Method

ALEXYS LC-ECD conditions

The ALEXYS Iodide Analyzer (Fig. 1) consists of a P6.1L pump with integrated degasser, DECADE Elite electrochemical detector, AS 110 autosampler and Clarity data acquisition software. Ion-pairing separation on a C_{18} column was followed by electrochemical detection on a silver electrode (Table 1).

Separation

To reduce the amount of chemical waste, a 1 mm ID column was chosen, which runs with a 20 times lower flow rate compared to a regular 4.6 mm ID column. Sub-2 µm column particles show better separation compared to the use of the more traditional 3 µm particles and this feature also helps to minimize run times and solvent consumption. The measurement conditions result in a backpressure below 300 bar: PEEK tubing (and metal connectors) were applied.

When running mobile phase with ion-pairing agent, stabilization times are a bit longer compared to regular HPLC separation. After purging the system up to the injector with mobile phase, a new column needs to stabilize with mobile phase for at least 16 hours (effluent redirected to waste) before connecting the flow cell. A column previously exposed to the mobile phase (and flushed with storage solution, e.g. 50 % acetonitrile) needs to condition for at least 4 hours with mobile phase before retention times have stabilized again.

The choice for a silver electrode makes the analysis very selective for iodide in comparison with other electrodes that need higher working potentials. Chromatograms from urine samples recorded with silver will only show a few additional peaks even though many components elute from the column and the benefit of selectivity in such case is clear (Fig. 2)

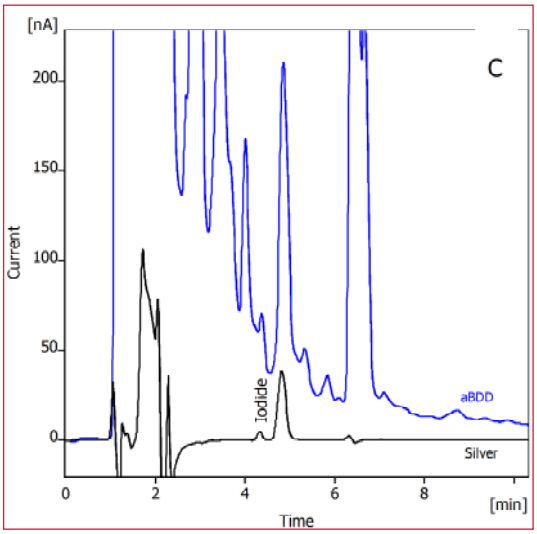

Figure 2. Chromatogram of urine obtained with a silver electrode (black trace) in an overlay with a chromatogram (same sample, injection volume and separation conditions) obtained with an anodized Boron Doped Diamond (BDD) electrode set at 1.8 V (blue trace).

In case of overlap with another peak (poor resolution), the mobile phase composition can easily be adjusted to change the separation. Lowering the ion-pairing agent concentration will result in more retention of iodide, and lowering the acetonitrile concentration will result in more retention (Fig. 2). The other peaks in the chromatogram will change their retention time a bit different compared to iodide and this can help to improve the separation if necessary. Be aware that late eluting peaks become wider thus lower (Fig. 4).

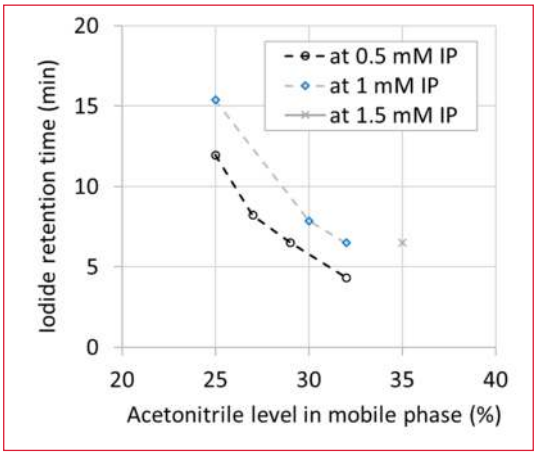

Figure 3. Modification of the mobile phase composition with respect to acetonitrile and ion-pairing (IP) agent changes the retention time of iodide.

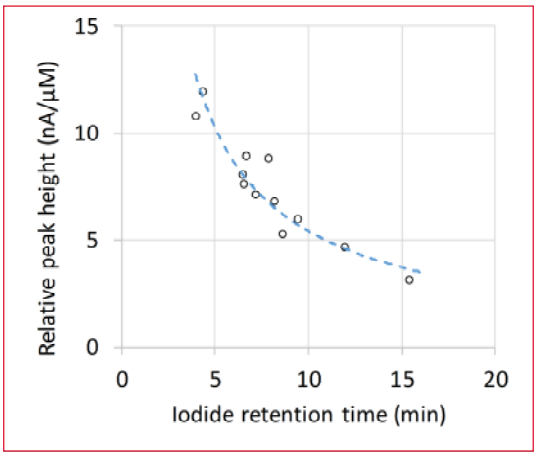

Figure 4. Inverse relationship between peak height and retention time (with peak area about the same for each data point).

Flow cell preparation

In this application note we report the results obtained using a SenCell with silver WE and HyREF. In principle, a FlexCell can be used, but the SenCell model is less prone to internal leakage when running mobile phase with a high organic content. For the specifics when using the FlexCell, some advise and results are given at the end of this application note in a separate additional paragraph.

The SenCell silver working electrode must be polished before use with 1 µm diamond slurry, a polishing pad and/or a cotton rod until shiny. Fill the cell with mobile phase, install it behind the column, and after 10 min a working potential (Ecell) of -100 mV is set. The background current (Icell) must stabilize into the range of -20 nA to +50 nA within 1 minute; otherwise adjust the Ecell up or down accordingly. If the Icell is >500 nA, open, inspect, clean and refill the flow cell and try again, as this can be a sign of a leak.

Most commonly, an oxidizable component shows a certain working potential threshold value, and the response rises towards a plateau value. Iodide doesn't show such pattern: it shows the same level of response over a wide range of working potentials even well below -300 mV. Research involving cyclic voltammetry even suggested that iodide detection on silver isn't through the straight-forward formation of AgI on the electrode surface [2].

Suitability test

After setting the correct working potential, the baseline is left to stabilize for about 15 min, followed by running 3 analyses of 1 µM potassium iodide in water. The expected peak height for this concentration is around 5-6 nA at a retention time of about 8 min. The expected area response is around 60-70 nA*sec for this concentration. A post-peak dip of up to 10% of the total height is considered normal for detection on silver electrodes.

When the response is far too low, a 2 µL injection of 1000 µM KI in water can help to activate the electrode. Repeat the 1 µM iodide injections again after this activation to check the response and stability again. Also doublecheck for zero carry-over with a blank after having injected such high concentration of iodide. When the response is stabile, the system is ready for use.

Urine sample processing

For each sample, one SPE cartridge (Grace pure™ SPE C-18 fast 1000 mg/ 6 mL) was activated by running 5 mL methanol through (by gravity), followed by 5 mL water and 5 mL sample.

Table 1

LC-ECD conditions	
HPLC	ALEXYS Iodide Analyzer
Column	Acquity UPLC BEH, 1x100 mm 1.8 µm
Mobile phase	25 mM phosphoric acid set to pH 6.8 with NaOH solution, 27% acetonitrile, 0.5 mM hexadecyltrimethylammonium hydroxide
Flow rate	50 µL/min
Backpressure	About 270 bar
Injection	2 µL
Temperature	35°C for separation & detection
Flow cell	Ag SenCell HyREF, AST position 1
E-cell	Between -250 mV and -30 mV vs HyREF
I-cell	-20 nA - +50 nA
ADF	0.05 Hz
Range	500 nA/V; 5 nA/V for near-LOD levels

The first 2 mL of solution running through the cartridge after adding the sample were discarded. The following 1 mL of sample was collected and run through a 0.2 µm PES syringe filter (GVS). The filtrate was collected into a glass autosampler vial and analyzed. Make sure that the SPE cartridge is not run dry, because cracks/channels in the packing will decrease the interaction with the sample. The SPE/filtration recovery of this method was found to be about 98% (comparing the response of an unprocessed and processed iodide standard).
In case that the above SPE/filtration method is not removing enough contaminants from the urine matrix, an additional liquid/liquid extraction could be applied that is described in reference [3].

Samples and standards

For calculation of response linearity, detection limit and repeatability, potassium iodide (KI) standards in the range of 0-25 µM were prepared in water. A set of 0 - 2.5 µM KI standards were prepared in water and in 150 mM NaCl/100 mM KCl (simulating the high concentration of salts in urine) to check for a matrix effect of the salts. To check for a matrix effect of urine, a portion of the SPE/filtration processed urine from a healthy volunteer was spiked with 0 - 2.5 µM KI. An additional set of standards in µg/L concentrations were prepared that corresponded with the different iodide deficiency levels as reported in reference [1]. The iodide level in the urine sample was quantified with the different calibrator sets.

Results

Repeatability

The repeatability of the iodide analysis was investigated based on 6 consecutive injections that were done every 12 minutes (Fig. 2). This was done for two levels of iodide in water and for iodide in a solution of 150 mM NaCl and 100 mM KCl in water, which is close to the concentration of salts in urine.

Table 3 shows the relative standard deviations of the results, which indicates that the analyses were repeatable with RSD below 2% for response and RSD below 0.2 % for retention times.

Table 3

Relative Standard Deviation (RSD) for 6 injections

	1 µM iodide in water	*5 µM iodide in water*	*5 µM iodide in 150 mM NaCL/ 100mM KCl*
Retention time	0.18%	0.07%	0.03%
Peak area	0.4%	0.2 %	1.0%
Peak height	0.2%	0.2 %	0.9%

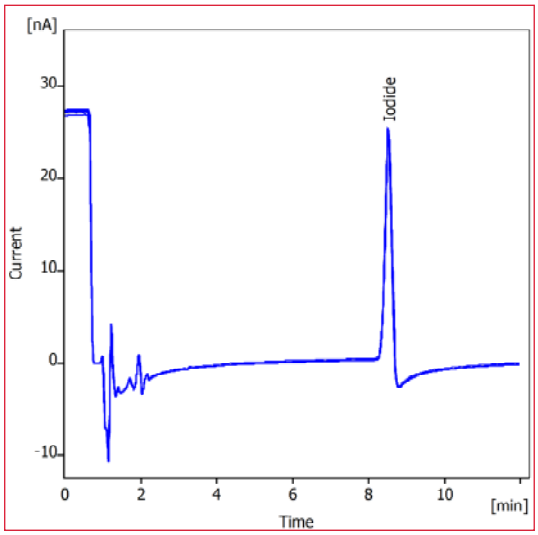

Figure 5. Overlay of 6 chromatograms of 5 µM (0.63 mg/L) potassium iodide in water, recorded with conditions given in Table 1.

Linearity

The response linearity of iodide was measured in the concentration range 0 - 25 µM and resulted in a correlation coefficient >0.999. (Fig. 6).

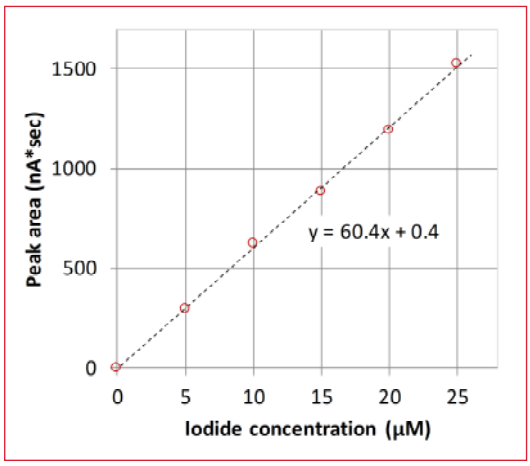

Figure 6: Reponse plot for iodide in water. Data based on analyses with conditions as given in Table 1.

The range of concentrations representing boundary levels for iodide deficiency [1] were all detectable with the same settings (Table 1) and showed a correlation coefficient of >0.999 for both peak area and peak height. A log-log plot of the peak height data is given in Fig. 7.

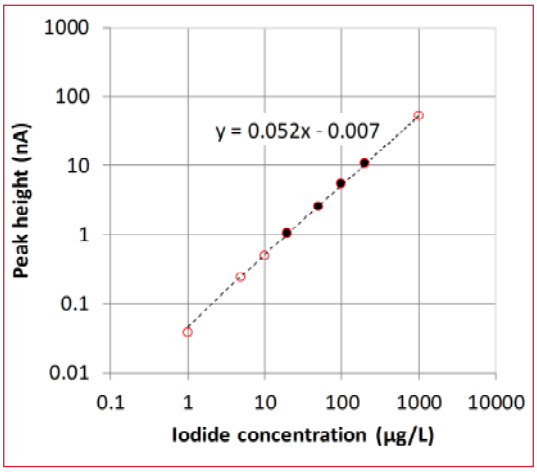

Figure 7: Log-log response plot for iodide peak height, spanning 3 orders of magnitude. The dotted line represents the linear regression line. The filled circles represent concentrations indicating nutritional deficiency boundary levels as given in reference [1]. Data based on analyses with conditions as given in Table 1.

Matrix effect

The consistency of response and linearity in urine was investigated by comparing it with results from standards in water and a mix of 150 mM NaCl/100mM KCl (Fig. 8). The presence or absence of the salts did not result in a difference in iodide response (Table 4). However, for urine spiked with various concentrations of iodide, a quadratic fit better matched the data. This indicates that there is an additional factor other than the NaCl and KCl in urine affecting the iodide response. This is probably the reason that the linearity value in Fig. 6 is lower compared to the values in Table 4: data of Fig. 6 was analyzed after a set of urine samples.

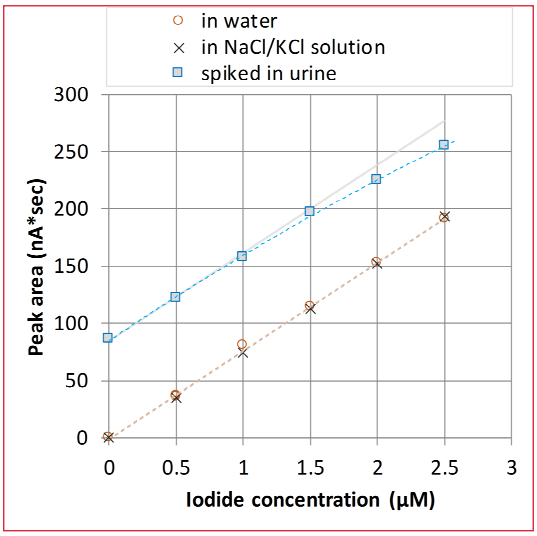

Figure 8: Response plot for iodide in water, in 150 mM NaCl/100 mM KCl, and spiked in urine up to 2.5 µM (317 µg/L). The dotted lines represent the regression lines with equations given in Table 4. The light grey line has the same slope as the non-urine data to help see the curvature. Data based on analyses with conditions as given in Table 1.

After about 8 urine samples, the response to standard was found to be stabile, but about 15% lower. We therefore recommend to recalibrate the response regularly within a sample sequence and clean/polish the electrode after running a set of real samples before running the next set.

Table 4

Regression equations and correlation coefficient (r) for data shown in Fig.8

Matrix	*Equation*	*r*
Water	y = 77.0x - 0.56	0.9996
NaCL/KCl solution	y = 77.4x - 2.4	0.9997
Urine	$y = -4.3x^2 + 78.8x + 85$	0.9996

Detection limit

The detection limit was calculated on the basis of all chromatograms from standards and the peak-to-peak noise evaluation of clear parts of the baselines. With a noise level of about 12 pA, the calculated detection limit (SN-ratio of 3) was about 7 nM (0.9 µg/mL) for iodide. The iodide peak in a chromatogram from a standard near the detection limit concentration is clearly visible (Fig. 9)

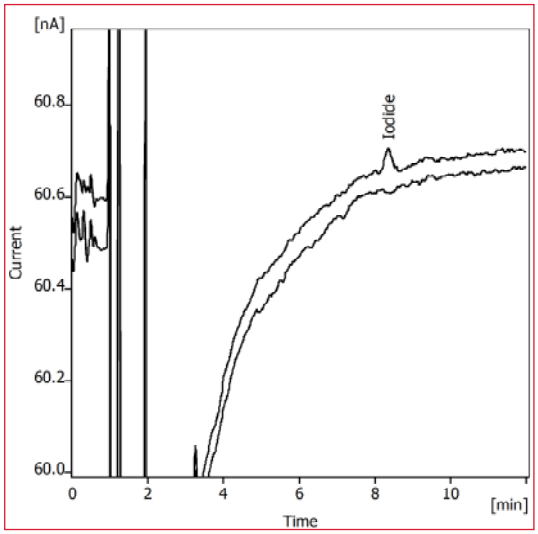

Figure 9: Chromatogram overlay of a blank and 10 nM (1.3 µg/L) iodide in water, recorded with conditions given in Table 1.

When in need of even lower detection limits, the application of the more sensitive range setting of 5 nA reduces the noise level to about 5 pA and the sensitivity to 3 nM (0.4 µg/L) iodide. Note that the more sensitive range setting of 5 nA will limit the measurable concentration of iodide in sample to about 0.7 µM (about 85 µg/L, see also Fig. 7) above which peaks will go off scale.

Sample analysis

A urine sample was processed and analyzed to generate an example chromatogram (Fig. 10). Based on the response of the 0.5 µM spike, the concentration of iodide was quantified as 1.2 µM (151 µg/L). The use of the calibrator set in a background of water resulted in a 5% lower value.

As the method is based on isocratic separation, it is important to check for the presence of later eluting peaks that could interfere with the subsequent chromatogram. As can be seen in Fig. 10, there is a large peak behind iodide, but there is also a small disturbance at about 15 minutes. With a run time of only 12 minutes, this disturbance will elute in the front-peak of the next chromatogram and doesn't need to be waited out. There were no further late eluting signals in a urine chromatogram when applying the conditions as given in Table 1.

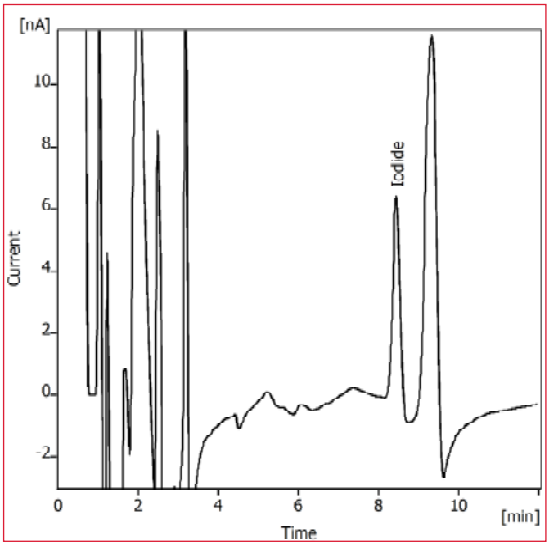

Figure 10: A: Chromatogram of urine with 1.2 µM (151 µg/L) iodide, recorded with conditions given in Table 1.

Using the alternative FlexCell

Activation of the electrode

The FlexCell silver working electrode must be polished before use with 1 µm diamond slurry and a polishing pad until shiny. A very fine but more abrasive polishing paper may be additionally helpful in flattening/polishing this type of electrode.

Right after having polished, rinsed and dried the electrode, apply a droplet of 1 mM KI on the surface and let it sit for 5 minutes. Then wipe it dry and assemble the cell. Fill the reference chamber with mobile phase using a disposable pipette, install the reference electrode (make sure not to trap an air bubble) and connect the cell behind the column. Set a working potential (Ecell) of -50 mV and evaluate the background current (Icell): it must smoothly stabilize into the range of -20 nA to +50 nA within 1 minute; otherwise adjust the Ecell up or down accordingly. If the Icell is >500 nA or spikes are visible in the baseline, open, inspect, clean and refill the flow cell and try again, as this can be a sign of a leak.

If the cell was installed without the KI treatment, it can also be activated with an injection of 1 mM KI solution. Without any treatment the response will start out relatively low and take a few hours to increase and stabilize (Fig. 11).

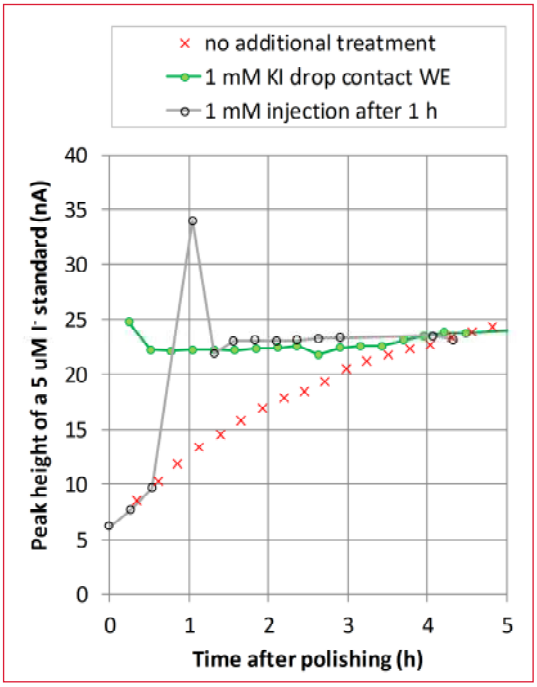

Figure 11: Response of iodide at a FlexCell equipped with a silver working electrode after different treatments. Background current was set to about 25 nA (Ecell -100 mV). Separation conditions as in Table 1.

Suitability test

After setting the correct working potential, the baseline is left to stabilize for about 15 min, followed by running 3 analyses of 5 µM potassium iodide in water. The expected peak height for this concentration is around 20-25 nA at a retention time of about 8 min. When the response is stabile, the system is ready for use.

Performance

The response, repeatability and the noise level of a FlexCell with a silver working electrode was found to be similar to those of a SenCell model. The chromatograms from a urine sample that was processed and recorded with both cells on the same day are also comparable (Fig. 12).

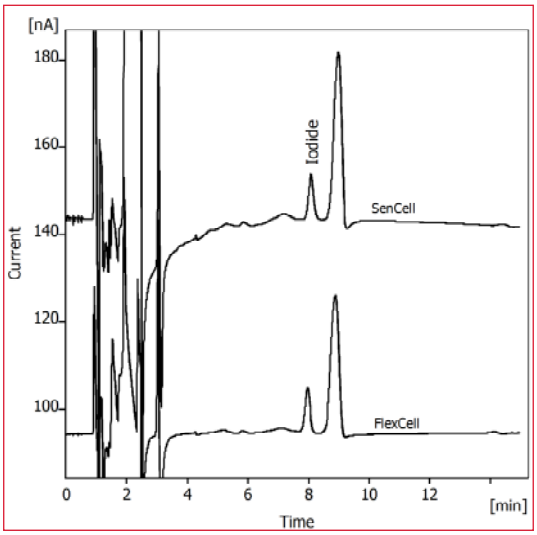

Figure 12: Chromatogram overlay of a sample recorded with two different flow cell types with conditions given in Table 1.

References

1. WHO. Urinary iodine concentrations for determining iodine status deficiency in populations. Vitamin and Mineral Nutrition Information System. Geneva: World Health Organization; 2013 (http://www.who.int/nutrition/vmnis/indicators/urinaryiodine, accessed 31/Jul/2019).
2. H. Below and H. Kahlert (2001). Determination of iodide in urine by ion-pair chromatography with electrochemical detection. Fresenius J Anal Chem 371: 431-436
3. V.T.P. Nguyen, V. Piersoel, T. El Mahi (2012). Urine iodide determination by ion-pair reversed-phase high performance liquid chromatography and pulsed amperometric detection. Talanta 99: 532-537

Conclusion

The ALEXYS Iodide Analyzer is a suitable LC-ECD platform to analyse iodide with a reproducible, linear and sensitive method.

Ordering information

180.0091W	ALEXYS base platform, 1 channel including the DECADE Elite SCC electrochemical detector, Clarity software, tubing set, pulse damper and P6.1L pump with in-line degasser
191.0055UL	AS 110 autosampler UHPLC cool 6p
116.4323	SenCell 2 mm Ag HyREF
250.1160	Acquity UPLC BEH C18, 1.7µm,1 x 100mm (186002346*)
250.1165	Acquity UHPLC in-line filter kit + 6 frits (205000343*)

*) Reordering part numbers at Waters Corporation (Milford, USA).

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