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Requirements

for carbohydrate analysis

using the ALEXYS® Carbohydrates Analyzer (180.0055W, 180.0057W)

180.7068, Edition 8, 2024

ISO 9001 certified

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Warning Symbol

The warning sign denotes a warning. It calls attention to a procedure or practice which, if not adhered to, could result in costs, damage or destruction of parts or all of the equipment. Do not proceed beyond a warning sign until the indicated conditions are fully understood and met.

For research purposes only. **The ALEXYS system is not tested by the manufacturer to comply with the In Vitro Diagnostics Directive.**

Observe safety

Operation of an electrochemical detector can involve the use of hazardous materials including corrosive fluids and flammable liquids. The instrument should only be operated by users with the following expertise:

- Completed degree as chemical laboratory technician or comparable vocational training
- Fundamental knowledge of liquid chromatography
- Knowledge and experience in the safe handling of toxic and corrosive chemicals and knowledge of the application safety measures prescribed for laboratories.
- Participation in an end-user training (daily use of system and chromatography software) performed by the manufacturer or a company authorized by the manufacturer.

Unskilled, improper, or careless use of the instrument and the related chemicals can create fire hazards, or other hazards which can cause death, serious injury to personnel, or severe damage to equipment and property.

Observe all relevant safety practices at all times.

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C H A P T E R 1

Introduction

Thank you for ordering an ALEXYS LC-ECD system. For a successful on-site installation of the **Carbohydrates analysis** on the ALEXYS system, please arrange the following requirements at your location in advance:

- a computer (see document 195.7000 for the PC requirements)
- general laboratory conditions and facilities, consumables and chemicals for use with the ALEXYS system (see document 180.7070C)
- application specific chemicals and consumables (see this document)

Arrange these requirements well in advance before the installation to prevent (costly) delays.

This document lists the application specific chemicals for the analysis of various carbohydrates using the following hardware:

ALEXYS® Carbohydrates Analyzer

(180.0055W isocratic, 180.0057W gradient)

For applications requiring post-column addition an additional pump, pulse damper, tubing and gradient mixer might be necessary. A dedicated kit containing all these parts is available under part number 180.0605 - Post Column Kit Carbohydrates.

For LC-ECD applications, only chemicals of sufficient quality should be used to be able to have an optimal system with good performance. The appendix shows detailed descriptions of some of the chemicals that have been used in the Antec R&D laboratory, as an example of what works.

CHAPTER 2

Requirements

There are a number of things to keep in mind when analyzing carbohydrates using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). Most things are related to the anion exchange column and the mobile phase which is a high concentration of hydroxide at high pH (typically in the range of pH 12 – 13.5).

Mobile phase

1. Degassing, (how to) keep mobile phase carbonate free
2. Tubing, inlet filters must be NaOH resistant
3. Bottles: glass vs plastic (PE or PP), and pressure resistant
4. Preparing carbonate-free solvents (avoid dissolving CO_2) and keeping them under an inert gas atmosphere.

Column

1. When to use a borate trap
2. Column temperature stabilization (Elite oven or CT 2.1 column thermostat for separation)
3. Column selection: 2, 3, 4 mm, pre-column, suppliers
4. How to restore column performance

HPLC system

1. Nitric acid passivation, only for metal-containing LC system, once prior to installation & start up
2. Bioinert or metal-free LC system – do not passivate with nitric acid, only prime with 2 M NaOH
3. LPG with 4 channels, or HP binary gradient (low vs high flow rates)
4. Cell current: what is a normal background current
5. Cell potential: pulse type (3 or 4 step)
6. Flow cell selection and REF type
7. What to do when switching off HPLC for more than a few days
8. Some troubleshooting

Do not passivate bioinert or metal-free LC systems with nitric acid, only prime with 2 M NaOH prior to use.

These requirements will be discussed below in more details.

Mobile phase

For carbohydrate analysis, keeping the mobile phase **carbonate free** is one of the key factors towards reproducible retention times. The reason is that carbon dioxide gas present in air will dissolve as CO_3^{2-} ions in the strong

alkaline eluent. The dissolved carbonate ions will increase the ionic strength of the mobile phase, resulting in a shortening of the retention times of the carbohydrates. To prevent this, blanketing of the mobile phase with an inert gas is required. An ET 210 eluent tray (Figure 1) is part of the ALEXYS carbonate analyzer to prepare and keep mobile phases carbonate-free during analysis. See the 192.0010 'ET 210 user manual' for details about how to operate the device and its requirements for installation.

Figure 1. ET 210 with connected PPCO mobile phase bottles.

All tubing and parts must be **high pH resistant.** Ceramic or metal inlet frits for sparging are not allowed. Use PEEK frits or no frit at all. All tubing, inlet filters, connector etc. must be resistant to high pH (12 -14) solutions. Suitable tubing materials are PEEK and FEP,PTFE for the low pressure inlet/suction lines between bottle and pump head. The **HPLC bottles** for solvent must be of thick-walled polypropylene (PP or PPCO) or polyethylene (PE).Glass will slowly release silicate and borate ions and contaminate the HPLC system, and is therefore not allowed. In order to minimize inert gas consumption, the bottles are closed and kept under a small inert gas pressure (typically 0.35 - 0.40 bar/ 5 - 6 psi) . Therefore the plastic bottles must be pressure resistant.

Only use the manufacturer-supplied plastic PPCO bottles (pn 184.0205) in combination with the ET 210 eluent tray. These bottle assemblies are specifically intended for the purpose of inert gas pressurization of the head space above the mobile phase. Before use always check if the bottles are undamaged. Never apply inert gas pressure on damaged bottles. The bottles may never be operated at pressures higher than 0.7 bar / 10 psi. Higher pressures can cause the bottle to explode.

When **preparing mobile phase**, don't just pour out the solutions as this will mix with CO_2 in air and bubbles, which leads to the formation of carbonate ions. The solvents need to be carbonate free. Even when pipetting from the

50% stock solution, the advice is to take the NaOH from the middle, as the CO_2 level is the lowest!

Note: The ET 210 eluent tray and PPCO bottles contain all tubing assemblies for inert gas sparging (mobile phase preparation) and blanketing (analysis) the mobile phase with inert gas.

A high purity inert gas (grade 5.0, ≥ 99.999% purity) supply regulated to a pressure of 2 – 3 bar is required for operation. Read the manual of the ET 210 (pn 192.0010) carefully for more details about the requirements etc.. Please make sure that an inert gas supply is available in your lab prior to installation.

Column

Columns for carbohydrate analysis with HPAEC-PAD are based on Anion Exchange Chromatography (HPAEC). They are quite expensive and analytical columns are always used together with a **guard column** to increase the column lifetime.

In a number of applications a **Borate trap** is used. This is a small column of about 4 x 50 mm which must be installed in the tubing line between pump and autosampler. It removes borate contaminants from the solvent. Borate complexes with certain carbohydrates which might results in tailing peaks (f.e. mannose, fructose, and sugar alcohols).

In some carbohydrates applications an **Amino trap** column is used when analyzing samples which may contain amino acids or small peptides. Amino acids are also EC active and interfere with the carbohydrate peaks. Although the detection potential waveform is optimized for carbohydrates, the amino acids and peptides are still detected. The amines are therefore trapped on a small column located just before the analytical column.

Column temperature is important for reproducibility and retention behavior. Some application require a separation temperature of 20 - 30°C. In such cases the Antec ECD oven is not suitable and an optional CT 2.1 column thermostat (186.ATC00) may be required for separation.

Column diameter is relevant as the consumption of mobile phase is proportional to the column diameter squared. Anion exchange columns with 2 mm ID are available on the market, and are used in a number of our applications. The solvent consumption is 4 times less than the standard 4 mm ID columns.

Always follow the specific advice of the column manufacturer with respect to **storage or regeneration of an anion exchange column**. Therefore consult the user documentation supplied with your column or download it from the manufacturer website. Usually the storage solvent is a low concentration of NaOH (i.e. 10 mmol/L). A column washing (regeneration) procedure is usually based on washing with MSA (methanesulfonic acid) or sulfuric acid followed by a high concentration of NaOH. Between different solvents a

washing step with water, to avoid reactions or precipitation of strong solvents.

HPLC system

Nitric acid **passivation** is necessary for metal-containing LC systems, it covers metal parts with an oxide layer. It helps to minimize unwanted side reactions with Fe^{2+} that would result in noise. Of course, **bioinert systems** that do not contain any metal parts should not be treated with nitric acid.

A number of carbohydrates applications utilize a **gradient HPLC** method. Also for practical reasons, such as method development and to easily change solvents for example, a gradient setup is convenient. The ALEXYS carbohydrates analyzer 180.0057W is equipped with a quaternary Low Pressure Gradient pump (LPG) which enables gradient based on four different eluents.

One frequently asked question is what to do if the **system is not in use** for a few days. We recommend for a short period of time, up to a week, to keep the system running at a low flow rate and 20 mM NaOH solution. The cell is on all the time. For longer period of time (> week) the advice is to flush the column with storage liquid (usually 20 mM NaOH, read manufacturer advise in the column manual) and take out the column. Then flush the system with water and shut it off. Clean the cell and store dry.

The ECD is used in **PULSE mode** because the working electrode must be regenerated every pulse cycle, about twice per second. This pulse cycle 'consumes' a tiny bit of gold and wear of the electrode surface is normal. The flow cell has a gold WE and a HyREF. A salt bridge (sb) REF is also available but not advised. Typical pulse settings are the traditional **3-step pulse** and **4-step pulse**. We advise the use of a 4-step pulse for a DECADE Elite in combination with a SenCell. The 4-step pulse has less gold consumption and a longer lifetime of the electrode. When a DECADE II is used, only the 3-step pulse is available; we advise the use of the FlexCell because the consumption of the gold WE is more severe with 3-step pulse mode. The gold WE in the flexcell is also easier to polish and is replaceable in comparison to SenCell.

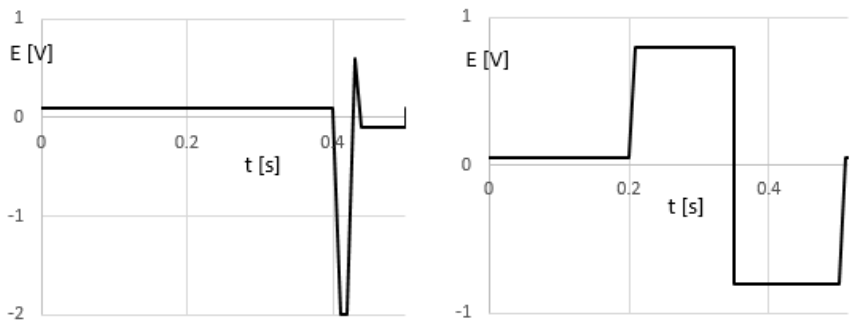

Figure 2. 4-step (left) and 3-step pulse (right).

The **background current** (uncompensated current, I-cell) is an important indicator of the system status, any deviation from the 'normal' background current value might be a sign that there is something wrong. The background current depends on a number of things, such as the mobile phase composition, and the pulse settings used. In most applications the cell current is between 0.1 and 3 µA. In case of significantly larger currents there must be a logical explanation. If not, it indicates the system needs troubleshooting or maintenance.

Troubleshooting

HPLC methods for carbohydrate analysis have certain specific requirements. Sometimes unexpected results are obtained. Often it requires a systematic check to find the root cause. Several root causes & potential remedies are listed below.
See also our Knowledge Base on our website:
https://antecscientific.com/support/knowledge-and-references

In all cases: perform a dummy cell test to rule out any electronic malfunctioning

Problem: Cell current too high

Root cause	Remedy
Contamination	Contamination can be caused by the mobile phase, column bleeding, late eluting peaks or from previous injections, bad piece of tubing or pulse damper. Take out or replace any suspected part. Do not use metal tubing.
Electrode wear	Polish working electrode, remove yellowish layer from AUX electrode, perform maintenance or replace sb REF
Malfunctioning REF	Perform maintenance of REF, check cell cable for a broken wire, replace any suspected part. In case of FlexCell, make sure that the mobile phase is in contact with REF.
Incorrect ECD settings	Pulse t1 is too short in relation with ts. Increase t1 or decrease ts.

Problem: Bad peak height reproducibility

Root cause	Remedy
Contamination of working electrode	Locate the source of contamination and take action.
Incorrect ECD settings	Check literature and application notes, try modified settings.

Problem: Bad retention time reproducibility

Root cause	Remedy
Chromatographic cause	Regenerate the column, check the pre-column, replace if needed. Check temperature stability, as well as pH of the eluent.
Contamination from CO_2.	Apply degassing, sparging, and blanketing to remove the CO_2 from solvents. Check for any possible leaks.
Low hydroxide concentration	Applications with less than 100 mM NaOH often require a 5 min washing step with high concentration between runs.

Laboratory equipment

- □ Magnetic stirring plate and stir bars
- □ Inert gas with high purity (grade 5.0, purity > 99.999%, minimum) supply (see previous section).

CHAPTER 3

Chemicals

Have the chemicals and solutions ready at the start of the installation.

For LC-ECD applications, only chemicals of sufficient specific quality should be used to be able to have an optimal system with good performance. The appendix shows detailed descriptions of some of the chemicals that have been used in the Antec R&D laboratory, as an example of what works.

Standard stock solutions

Chemicals

- Standards of the components of interest in high purity grade
- Water with resistivity of 18MOhm.cm and TOC<10ppb
- To minimize degradation and increase the shelf life add 5% Acetonitrile to the stock solution to avoid bacterial/fungi growth.

Preparation

- 10 mL 1 mM of each individual standard in water
  Store at 4 °C until use (max 1 week)

Tip: to extend the storage lifetime of the sugar stock standards in the fridge (4°C) the stock solutions can alternatively be prepared in 95:5 v% Water: Acetonitrile.

Calibrator solutions

Chemicals

- Chemicals for preparation of sample background (e.g. homogenization solution)
- Water with resistivity of 18MOhm.cm and TOC<10ppb

Preparation

- Prepare on the day of use: 0, 2, 4, 6, 8 and 10 µM of the mixed standards for a 5-point linear calibration in a background of water.

Mobile phase

The mobile phase that is applied for the separation of carbohydrates is a solution of NaOH in water, with the addition of sodium acetate in case of a gradient separation.

As different carbohydrates samples require different mobile phase compositions for the separation, we refer to the specific application notes 220.xxx on our website for details about the required mobile phase composition(s).

Do not prepare the mobile phase in glass bottles, as NaOH is a strong etching agent: the glass will release silicates and borates into the mobile phase in such case. Use the dedicated PPCO plastic bottle assemblies delivered with your system (Antec pn. 184.0205).

Chemicals

- 50% w/w NaOH in water, carbonate free (19.1 M), or
- 45% w/w KOH in water, carbonate free (13.5 M)*.
- Water with resistivity of 18 MOhm.cm and TOC<10ppb
- Sodium acetate trihydrate (high purity grade)
  - Modifier, and for gradient separation
  - High purity grade must be used to prevent impurities from causing large baseline shifts when running a gradient.

*** The contents of Hydroxide in commercial hydroxide solutions specified on the bottles are always by approximation. Always use the actual contents of hydroxide as stated in the certificate of analysis to calculate the amount of solution needed to make mobile phases.**

Table 1. Preparation of mobile phase from 50% (19.1M) NaOH

Total volume	Mobile phase constituent	Dissolved in total volume
1 L	10 mM NaOH	0.51 mL 50% NaOH solution
	30 mM	1.53 mL
	60 mM	3.06 mL
	100 mM	5.10 mL
	200 mM	10.2 mL
	1 mM Na-acetate	136 mg Na-acetate.$3H_2O$
	500 mM	68 g
2 L	10 mM NaOH	1.02 mL 50% NaOH solution
	30 mM	3.06 mL
	60 mM	6.1 mL
	100 mM	10.2 mL
	200 mM	20.4 mL
	1 mM Na-acetate	272 mg Na-acetate.$3H_2O$
	500 mM	136 g

Preparation

See the ET 210 manual (pn 192.0010) chapter 4 for details about how to prepare carbonate free mobile phases using the ET 210.

1. Pour 1 or 2 L (minus volume of NaOH solution, see Table 1) of water with high resistivity/low TOC into an empty and clean PPCO mobile phase bottle.
2. Degas the water for 15 min in an ultrasonic bath.
3. Add a clean stir bar and sparge with high-purity inert gas for 15 min under gentle stirring.
4. Pipette the required volume of NaOH solution from the middle part of the commercial 50% NaOH solution and add it to the degassed solution under gentle stirring.
5. Sparge the mobile phase for another 10 min under slow continuous inert gas sparging before use.
6. Install the bottle with headspace pressure in the ET210 as described in the ET210 manual.

Do not store this solution, but prepare when needed.

By any means do <u>not</u> filtrate the mobile phase. Filters can be a source of contamination.

System wash solutions

The piston and needle wash solutions should not contain any alcohols or other organic solvents, as these will be detected when entering the analytical flow path. Therefore, pure water is used as wash solvent for both pistons and autosampler needle.

Chemicals

- □ Water with resistivity of 18 MOhm.cm and TOC<10ppb

Preparation

- □ 250 mL water, degassed (autosampler needle wash solution)
- □ 125 mL water, degassed (piston wash solution)

Column regeneration solution

When retention times are shorter than expected or have decreased over time, the column may be regenerated by flushing it with about 30 bed-volumes of strong eluting solution. This regeneration solution will wash off adsorbed components from the active sites of an ion-exchange column. See manufacturers column manual for solutions needed for column cleanup.

Chemicals

- □ 50% w/w NaOH in water, carbonate free (19.1 M)
- □ Water with resistivity of 18 MOhm.cm and TOC<10ppb
- □ Sodium acetate trihydrate (high purity grade)

Preparation (the example below is for preparation of 500 mM NaOH + 1 M NaOAc)

1. Dissolve 34 g sodium acetate trihydrate in 243 mL of water with high resistivity/low TOC
2. Pour the solution in a PPCO bottle.
3. Degas the solution for 15 min in an ultra sonic bath.
4. Add a clean stir bar and sparge with high-purity inert gas for 15 min under gentle stirring.
5. Pipette 6.5 mL NaOH solution from the middle part of the commercial 50% NaOH solution and add to the degassed solution under gentle stirring.
6. Sparge the mobile phase for another 10 min under slow continuous inert gas sparging before use.
7. Install the bottle with headspace pressure in the ET210 as described in the ET210 manual.

Do not store this solution, but prepare when needed.

A P P E N D I X

A list of chemicals with purity and purchase details is shown below as a guideline. The listed brands/purities are not necessarily the best chemicals, but these have been giving good results at the Antec R&D laboratory.

If for any reason alternative chemicals need to be purchased, be aware that chemicals that have a specification of high purity may have been tested for UV-active impurities, which can mean that they may still contain electrochemically active impurities. This is one of the reasons why 'HPLC grade' water is not recommended for use with EC detection:

- choose chemicals with the same purity or better
- do not choose ultra-dry grade or anhydrous chemicals
- do not make the NaOH solution from pellets as an alternative, as these contain high concentration of absorbed sodium carbonate on the surface.
- Using sodium acetate with lower purity than specified can cause large baseline shifts during a gradient run.

Table 1. Brands and purities of chemicals used for application development at Antec Scientific.

Component	Purity	Brand	Order no	Mw	kg/L
Sodium hydroxide*					
Sodium hydroxide (NaOH), approx. 50% in water	Pro analyse, carbonate free	Boom	80011912	40.00	D:1.57
Sodium Hydroxide Solution (50% w/w/Certified)	Certified grade	Fisher Scientific	SS254500	40.00	D:1.56
Sodium hydroxide solution, 50-52%	Eluent for IC	Sigma Aldrich	72064	40.00	D:1.53
Potassium hydroxide*					
Potassium hydroxide (KOH), 45% w/v in water (13.5 M)	HPLC grade, carbonate free	Fisher Scientific	15670680	56.105	D:1.45
Sodium Acetate					
Sodium acetate trihydrate (CH3OONa.3H2O)	HPLC grade for EC detection	Fisher Scientific	S/2052/50	136.08	
Sodium acetate trihydrate (CH3OONa.3H2O)	>=99%, BP, Ph.Eur grade	Fisher Scientific	S/2000/60	136.08	
Sodium acetate trihydrate ($CH_3OONa.3H_2O$)	HPLC grade	Baker	0393	136.08	
Acetonitrile	HPLC grade	Acros	268270025	41.05	0.79
Water	TOC <10 ppb and deionised, resistivity >18 MOhm-cm (Barnstead Easypure II)				

* The contents of Hydroxide in commercial hydroxide solutions specified on the bottles are always by approximation. Always use the actual contents of hydroxide as stated in the certificate of analysis to calculate the amount of solution needed to make mobile phases. The certificate of analysis may be delivered with the bottle or can be requested/downloaded from the manufacturer (web site).

Manufacturers/Vendors

JT-Baker	http://www.avantormaterials.com
Sigma-Aldrich	http://www.sigmaaldrich.com
Fluka	http://www.sigmaaldrich.com
Fisher Scientific	http://www.fishersci.com
Barnstead	http://www.thermoscientific.com