Sample preparation for investigating the impurities, extractables and chemical stability of Q Sepharose Big Beads

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1 Introduction

This analytical report describes the sample preparation for investigating the impurities, extractables and chemical stability of Q Sepharose Big Beads.

To investigate residual impurities and extractables, pre-use washed Q Sepharose BB was stored under atmospheric pressure (static extraction) in milli-Q water for 160-170 h at different temperatures. After storage, the storage solution was separated from the beads by vacuum suction and collected into a sample vial. Analytical results from the extracts for total carbon, nitrogen and bromine are shown in the Analytical report 2.

Pre-use washed Q Sepharose BB was also extracted using Pressurised Fluid Extraction (PFE). This was performed in an Accelerated Solvent Extractor (ASE™) where the particles were exposed to high pressure and temperature under static conditions to accelerate the extraction of the organic impurities and extractables from the porous particles into a sample vial. Analytical results for elements and specific compounds are shown in the Analytical reports 2 and 3.

The chemical stability of Q Sepharose BB was evaluated by incubating the medium at different conditions for 160-170 h. Acidic and basic solutions and different temperatures were used. After storage, the amount of carbon, nitrogen and bromine present in the solutions were analysed. Results are shown in Analytical report 4.

2 Determination of the weight of vacuum suctioned and dried Q Sepharose BB

The purpose of these experiments is to make it possible to relate the analytical results to a well defined volume wet sedimented Q Sepharose BB or a well defined weight dried Q Sepharose BB. The vacuum suction procedure is used for simple and accurate determination of a fixed weight of Q Sepharose BB for further treatment. The weight of vacuum suctioned Q Sepharose BB can then easily be re-calculated to volume wet sedimented gel and weight dried gel.

2.1 Materials and methods

2.1.1 Determination of the weight of vacuum suctioned Q Sepharose BB

Equipment

Table 1 lists the equipment used for the determination of the weight of dry suctioned Q Sepharose BB.

Table 1. Equipment for determination of the dry weight of dry suctioned Q Sepharose BB.

Item	Type	Source
Columns for gel volume determination	PD-10, A=1.66 cm^2	GE Healthcare
Ruler for gel-height determination	Precision of ±0.25 mm	
50 mL glass filters	G3 pore size	Scott Duran
Balance	Id 1116272324	Mettler Toledo

Chemicals

Table 2 lists the chemicals used for the determination of the weight of dry suctioned Q Sepharose BB.

Table 2. Chemicals for determination of the dry weight of dry suctioned Q Sepharose BB.

Chemical	Type	Source
Ultra pure water	Milli- Q PLUS water	Millipore corp
Q Sepharose BB	Art no 17-0989-00 Batches: T-301065, manufacturing date Dec 7, 2003 T-301462, manufacturing date Dec 15, 2003 T-302192, manufacturing date Feb 19, 2004	GE Healthcare

Procedure

App. 20 mL of each batch Q Sepharose BB were washed on a 50 mL G3 glass filter with vacuum suction with 5 x 20 mL milli-Q PLUS water. After the last wash, the particles were vacuum suctioned for one minute. App. 5 g of the Q Sepharose BB particles was weighed into a glass beaker exactly with two decimals. Three weighings were made on each batch Q Sepharose BB. ~5 mL milli-Q PLUS water was added into each beaker and a particle concentration of ~50 % was obtained. The gel mixtures were transferred into three PD-10 columns and the gel particles were allowed to settle. The gel height of each PD-10 column was measured using a ruler.

2.1.2 Determination of the dry-weight of Q Sepharose BB dried at 105°C

Equipment

Table 3 lists the equipment used for the determination of the weight of SP Sepharose BB dried at 105°C.

Table 3. Equipment for determination of the weight of Q Sepharose BB dried at 105°C.

Item	Type	Source
Columns for gel volume determination	PD-10, A=1.66 cm^2	GE Healthcare
Ruler for gel-height determination	Precision of ±0.25 mm	
50 mL glass filters	G3 pore size	Scott Duran
Balance	Id 1116272324	Mettler Toledo
Oven 105°C	Id 234	Termaks

Chemicals

Table 4 lists the chemicals used for the determination of the weight of Q Sepharose BB dried at 105°C.

Table 4. Chemicals for determination of the dry weight of Q Sepharose BB dried at 105°C.

Chemical	Type	Source
Ultra pure water	Milli- Q PLUS water	Millipore corp
Acetone	pro analysi art no 1.00014.2511 lot 30332114208	Merck KGaA
Q Sepharose BB	Art no 17-0989-00 Batches: T-301065, manufacturing date Dec 7, 2003 T-301462, manufacturing date Dec 15, 2003 T-302192, manufacturing date Feb 19, 2004	GE Healthcare

Procedure

~7 mL sedimented gel of each batch Q Sepharose BB was added into a PD-10 column and the gel height of the settled gels were measured using a ruler. The gel particles were transferred into pre-weighed G3 glass filters (the glass filters were dried at 105°C for 1 hour and placed into a dessicator before weighing). The gel particles were first dried with 5 x 10 mL acetone and then placed in the 105°C oven over night (19 hours). The dried particles were cooled to room temperature in a dessicator for one hour. Each glass filter with the dried gel was weighed on an analytical balance. Triplicates were made for all investigated batches.

2.2 Results

2.2.1 Results, one minute vacuum suctioned Q Sepharose BB

Table 5 shows the results from the determination of the weight experiment where the gel has been vacuum suctioned for one minute.

Table 5. Results of the weight of vacuum suctioned Q-Sepharose BB.

Batch Q Sepharose BB	Weight, vacuum suctioned gel (g)	Gel height in PD-10 column (cm)	Column volume (mL)	Dry weight (g/mL sedimented gel)
T-301065:1	5.564	4.60	7.64	0.729
T-301065:2	5.854	4.90	8.13	0.720
T-301065:3	5.989	4.975	8.26	0.725
			Average	**0.725 g/mL**
			SD	**0.005 g/mL**
			RSD	**0.62%**
T-301462:1	5.368	4.375	7.26	0.739
T-301462:2	5.250	4.30	7.14	0.736
T-301462:3	5.259	4.25	7.06	0.745
			Average	**0.740 g/mL**
			SD	**0.005 g/mL**
			RSD	**0.68%**
T-302192:1	6.641	5.40	8.96	0.740
T-302192:2	5.560	4.60	7.64	0.728
T-302192:3	5.135	4.325	7.18	0.715
			Average	**0.728 g/mL**
			SD	**0.013 g/mL**
			RSD	**1.76%**

2.2.2 Results, 105^{o}C dried Q Sepharose BB

Table 6 shows the result from the determination where the gel was washed with acetone and then dried in a 105^{o}C oven.

Table 6. Results of the weight of Q Sepharose BB dried at 105°C.

Batch Q Sepharose BB	A Gel height in PD-10 column (cm)	B Column volume, A x1,66 (mL)	C Weight, empty glass filter (g)	D Weight, glass filter + dried gel (g)	Dry weight ((D-C)/B) (g/mL sedimented gel)
T-301065:1	4.60	7.64	50.4698	51.4415	0.1272
T-301065:2	4.90	8.13	50.5037	51.5397	0.1274
T-301065:3	4.975	8.26	50.3164	51.3775	0.1285
				Average	**0,1277 g/mL**
				SD	**0.00068 g/mL**
				RSD	**0.53%**
T-301462:1	4.375	7.26	48.3758	49.2676	0.1228
T-301462:2	4.30	7.14	50.4857	51.3507	0.1212
T-301462:3	4.25	7.06	50.9693	51.8352	0.1227
				Average	**0.1222 g/mL**
				SD	**0.00091 g/mL**
				RSD	**0.75%**
T-302192:1	5.40	8.96	51,6399	52.7793	0.1271
T-302192:2	4.60	7.64	51.7182	52.6872	0.1269
T-302192:3	4.325	7.18	50.5286	51.4245	0.1248
				Average	**0.1263 g/mL**
				SD	**0.00128 g/mL**
				RSD	**1.01%**

2.2.3 Determination of amount moisture in vacuum suctioned Q Sepharose BB

Table 7 shows the calculated amount of moisture in vacuum suctioned Q Sepharose BB.

Table 7. Moisture content in vacuum suctioned gel.

Batch Q Sepharose BB	Dry weight, vacuum suctioned gel (g/mL sed gel)	Dry weight, gel dried at 105°C (g/mL sed gel)	Moisture content in vacuum suctioned gel (g/mL sed gel)
T-301065	0.725	0.128	0.597
T-301462	0.740	0.122	0.618
T-302192	0.728	0.126	0.602

3 Sample preparation for investigating the impurities, extractables and chemical stability of Q Sepharose BB

3.1 Materials

Table 8 shows the equipment required for the sample preparations.

Table 8. Equipment for the sample preparations.

Item	Type	Source
1000 mL glass filters	Pore size G3	Scott Duran
75 mL glass filters	Pore size G3	Scott Duran
250 mL suction flasks		Scott Duran
Pipette 100-1000 μL	Research, id 553	Eppendorff
Balance	Id 1116272324	Mettler Toledo
Vacuum suction equipment		
Oven for 40°C storage	EN 500 SC id 534	Nüve
Oven for 60°C storage	Model 8000 id 5054	Termaks
Sample tubes	8 and 4 mL	VWR international
Storage flasks	100 mL	Scott Duran

3.2 Chemicals and solutions

Table 9 shows the chemicals required for the sample preparations.

Table 9. Chemicals for the sample preparations.

Chemical	Type	Source
Ultra pure water	Milli- Q PLUS water	Millipore corp
NaCl	pa, art no 1.06404.1000 lot K32104204324	Merck KGaA
NaOH	pa, art no 1.06469.1000 lot B948769301	Merck KGaA
HCl	Conc 37%, pa, art no 1.00317 lot K33557117430	Merck KGaA
NaBr	pa, art no 13257-250 lot 98133	Prolabo
Q Sepharose BB	Art no 17-0989-00 Batches: T-301065, manufacturing date Dec 7, 2003 T-301462, manufacturing date Dec 15, 2003 T-302192, manufacturing date Feb 19, 2004	Amersham Biosciences
Allyl Sepharose BB	Art no 30-9861-00 Batches: T-300861, manufacturing date Nov 11, 2003 T-301232, manufacturing date Dec 1, 2003 T-302183, manufacturing date Feb 2, 2004	Amersham Biosciences

3.2.1 Preparation of 1 M NaCl

292 g NaCl was weighed and dissolved to 5000 mL with milli-Q PLUS water. This solution was prepared daily.

3.2.2 Preparation of 0.5 M NaOH

40.0 g NaOH was weighed and dissolved to 2000 mL milli-Q PLUS water. This solution was prepared daily.

3.2.3 Preparation of 0.2 M NaOH

16.0 g NaOH was weighed and dissolved to 2000 mL milli-Q PLUS water. This solution was prepared daily.

3.2.4 Preparation of 0.01 M NaOH

0.8 g NaOH was weighed and dissolved to 2000 mL milli-Q PLUS water. This solution was prepared daily.

Preparation of 0.1 mM HCl

833 μL concentrated HCl was pipetted into a 100 mL volumetric flask and the flask was filled to the mark with milli-Q PLUS water. 2000 μL of this solution was pipetted into a 2000 mL volumetric flask. The 2000 mL flask was filled to the mark with milli-Q PLUS water. This solution was prepared daily.

3.2.5 Preparation of 1 mM HCl

166.6 μL concentrated HCl was pipetted into a 2000 mL volumetric flask. The 2000 mL flask was filled to the mark with milli-Q PLUS water. This solution was prepared daily.

3.3 Procedure

3.3.1 Sampling scheme

Figure 1 shows the structure for preparing all samples for further analysis.

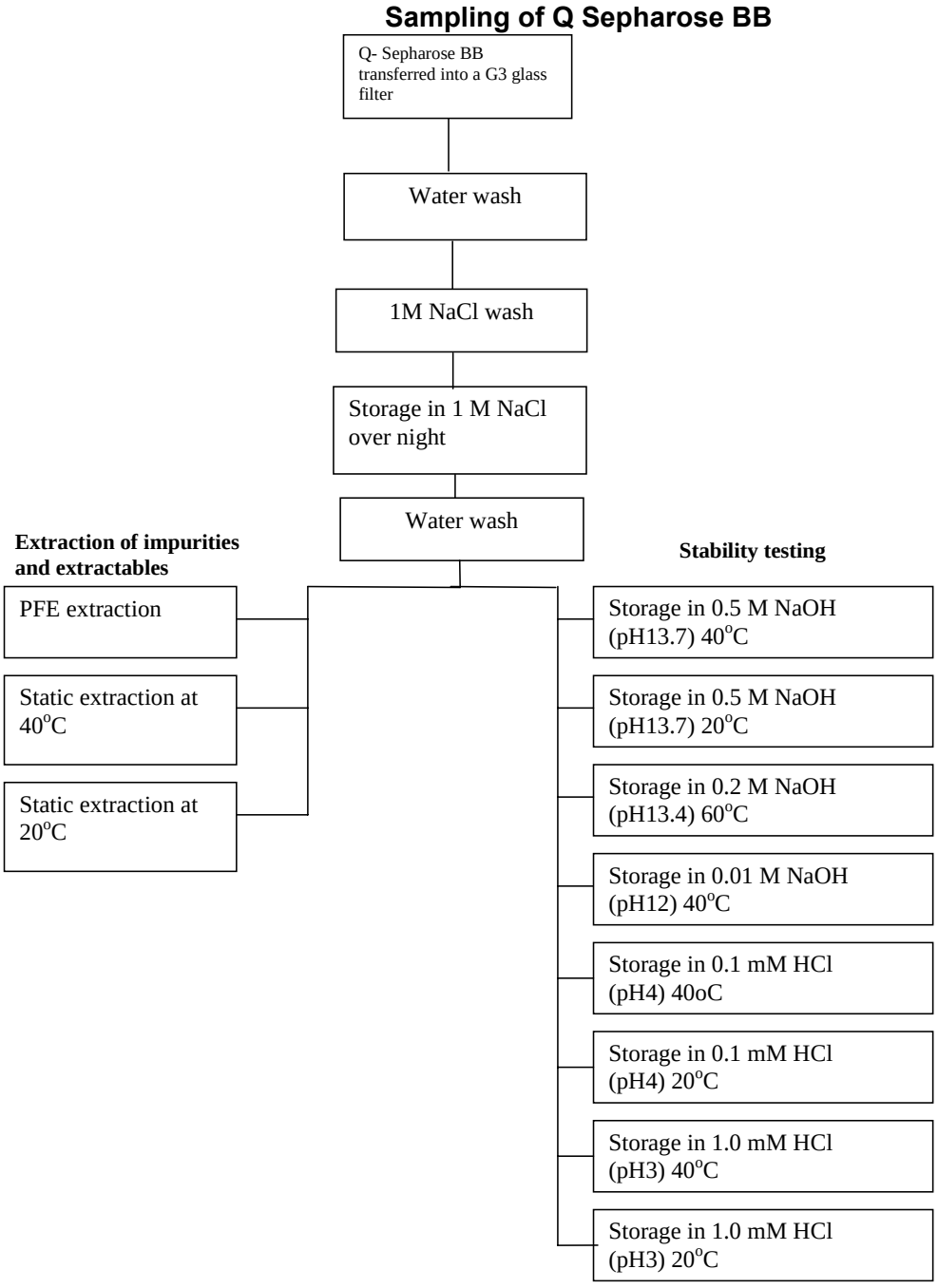

Figure 1. Schematic presentation of the different types of sample preparation

3.3.2 Procedure for washing and sampling

The scheme shown in Figure 1 was followed. The shipping solution, i.e the solution the product is stored in after production, 20% ethanol, was separated from Q Sepharose BB in a G3 glass filter by vacuum suction for one minute. The obtained amount of Q Sepharose BB in the glass filter was weighed and noted. The weight of the gel should be sufficient for all analyses according to the sampling scheme.

Pre-use washing procedure for Q Sepharose BB

First, the gel was washed with 5 x V (V= volume sedimented gel) mL milli-Q water. Between each addition of milli-Q water the solution was drained until the gel was settled and and no more solution emerged through the filter. After the last wash, the gel was vacuum suctioned for one minute and the V mL flow through solution was collected in the suction flask.

Secondly, the gel was washed with 4 x V mL 1 M NaCl. At the 4^{th} wash the gel was vacuum suctioned for one minute. V mL 1 M NaCl was added to the gel and the gel was mixed with the solution and transferred into a 1000 mL storage flask. The gel mixture was stored overnight at room temperature. After storage the gel mixture was transferred back to the glass filter.

Finally, the gel was washed with 5 x V mL milli-Q water and the procedure for the first milli-Q water wash was followed.

Preparation of the washed gel for PFE (Pressurized Fluid Extraction), static extraction and stability testing

For PFE the vacuum suctioned gel was weighed into a 50 mL sample tube. For static extraction and stability testing, the gel was weighed into 75 mL glass filters.

To obtain 40 mL sedimented gel in the sample tube for PFE and in the 75 mL glass filters for static extraction and stability testing, the weight of vacuum suctioned gel that corresponds to 40 mL sedimented gel was calculated and weighed (see section 2).

Procedure for washing and sampling the static extraction and stability testing samples

10 glassfilters, each containing 40 mL sedimented gel, were prepared. Two glass filters were washed with 4 x 40 mL 0.1 mM HCl pH 4, two glass filters were washed with 4 x 40 mL 1 mM HCl, two glass filters were washed with 4 x 40 mL milli-Q water, two glass filters were washed with 4 x 40 mL 0.5 M NaOH and one glass filter was washed with 4 x 40 mL 0.2 M NaOH. These pre-washings are required to equilibrate the gel to the storage conditions. After the 4^{th} wash the gel was vacuum suctioned for one minute. 40 mL of corresponding solution was transferred to the glass filter and mixed with the gel. The gel-mixtures were transferred into 100 mL storage flasks. The storage flasks were stored at different temperatures, according to the sample protocol for each batch Q Sepharose BB on pages 11-13. The incubators were calibrated with thermometer DMT Spezial 15-65 ± 0.01^{o}C (calibrated 2004-04-16 report no 04-161 Swedac ISQ/IEC 17025). Both incubators (40^{o}C and 60^{o}C) differed less than 0.5^{o}C from the adjusted temperatures. All washing solutions were also stored at the same storage conditions as the samples.

After 7 days (160-170 hours, the exact time was noted) the gel-mixtures were transferred from the storage flasks into 75 mL glass filters. The glass filters were vacuum suctioned for one minute and the solutions in the suction flasks were collected into 4-8 mL sample vials. All solutions were stored in a 4^{o}C refrigerator (id 872) before analysis.

The static extraction samples are referred to as the milli-Q water stored samples in the following.

Figure 2 shows a sketch of the equipment used in the washing procedure of Q Sepharose BB.

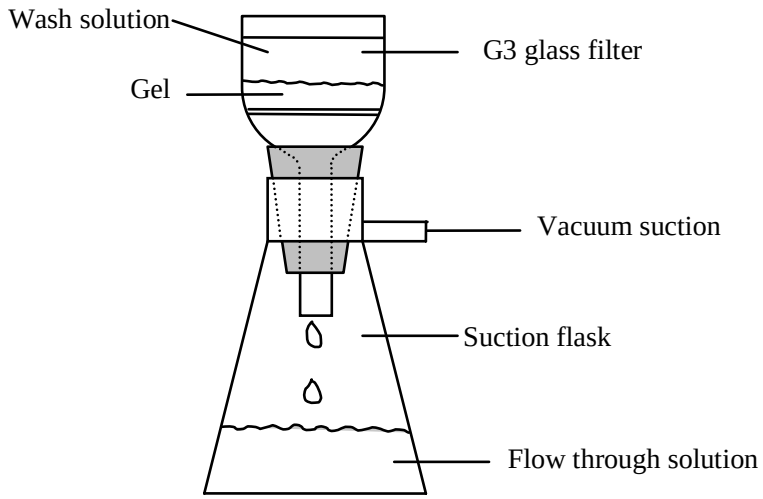

Figure 2. Set-up for washing Q Sepharose BB.

3.3.3 Sampling of Q Sepharose BB T-301065

The scheme (Figure 1) was followed. The density of one-minute vacuum-suctioned gel is 0.725 g/mL sedimented gel (see section 2 Table 5). To obtain more than 500 mL gel, >362 g of the vacuum suctioned Q Sepharose BB was weighed into another 1000 mL G3 glass filter.

Protocol 1 shows the sampling for Q Sepharose BB T-301065 in detail. The gel was washed according to the pre-use washing procedure before distribution of Q Sepharose BB into glass filters for further washing and sampling. 29.0 g (40 mL wet sedimented gel) of vacuum suctioned pre-use washed gel was weighed into the glass filters.

Protocol 1. Sampling of Q Sepharose BB T-301065.

Protocol, sampling of Q-Sepharose BB

Date/Sign: 2005-01-10 start 17.40

Lot nr:	301065
Weight of one minute vaacuum suctioned gel:	390 g
Balance id: 1116272324	
Weight, vacuum suctioned gel for samples:	29.0 g

Start incubation: 2005-01-10 kl 17.40
Stop incubation: 2005-01-17 kl 14.10
Incubation time: 164.5 h

Sample nr	Content	Info
10	Pre-washed vacuum suctioned gel	29.0 g vacuum suctioned gel for PFE extraction
11	Storage solution, 0.1 mM HCl 40°C	Last cv 0.1 mM HCl stored with 40 mL gel for 164.5 h at 40°C
12	Blank, 0.1 mM HCl 40°C	0.1 mM HCl used in sample 11, stored at the same conditions
13	Storage solution, 0.1 mM HCl 20°C	Last cv 0.1 mM HCl stored with 40 mL gel for 164.5 h at 20°C
14	Blank, 0.1 mM HCl 20°C	0.1 mM HCl used in sample 13, stored at the same conditions
15	Storage solution, 1 mM HCl 40°C	Last cv 1 mM HCl stored with 40 mL gel for 164.5 h at 40°C
16	Blank, 1 mM HCl 40°C	1 mM HCl used in sample 15, stored at the same conditions
17	Storage solution 1 mM HCl 20°C	Last cv 1 mM HCl stored with 40 mL gel for 164.5 h at 20°C
18	Blank, 1 mM HCl 20°C	1 mM HCl used in sample 17, stored at the same conditions
19	Storage solutio, 0.2 M NaOH 60°C	Last cv 0.2 M NaOH stored with 40 mL gel for 164.5 h at 60°C
20	Blank, 0.2 M NaOH 60°C	0.2 M NaOH used in sample 19, stored at the same conditions
21	Storage solution, 0.2 M NaOH 20°C	Last cv 0.2 M NaOH stored with 40 mL gel for 164.5 h at 20°C
22	Blank, 0.2 M NaOH 20°C	0.2 M NaOH used in sample 21, stored at the same conditions
23	Storage solution, 0.5 M NaOH 40°C	Last cv 0.5 M NaOH stored with 40 mL gel for 164.5 h at 40°C
24	Blank, 0.5 M NaOH 40°C	0.5 M NaOH used in sample 23, stored at the same conditions
25	Storage solution, 0.5 M NaOH 20°C	Last cv 0.5 M NaOH stored with 40 mL gel for 164.5 h at 20°C
26	Blank 0.5 M NaOH 20°C	0.5 M NaOH used in sample 25, stored at the same conditions
27	Storage solution H_2O 40°C	Last cv H_2O stored with 40 mL gel for 164.5 h at 40°C
28	Blank, H_2O 40°C	H_2O used in sample 27, stored at the same conditions
29	Storage solution, H_2O 20°C	Last cv H_2O stored with 40 mL gel for 164.5 h at 20°C
30	Blank, H_2O 20°C	H_2O used in sample 29, stored at the same conditions

3.3.4 Sampling of Q Sepharose BB T-301462

The scheme (Figure 1) was followed. The density of one-minute vacuum-suctioned gel is 0.740 g/mL sedimented gel (see section 2 Table 5). To obtain more than 500 mL sedimented gel, >370 g of the vacuum suctioned Q Sepharose BB was weighed into another 1000 mL G3 glass filter.

Protocol 2 shows the sampling for Q Sepharose BB T-301462 in detail. The gel was washed according to the pre-use washing procedure before distrubution of Q Sepharose BB into glass

filters for further washing. 29.1 g (40 mL wet sedimented gel) of vacuum suctioned pre-use washed gel was weighed into the glass filters.

Protocol 2. Sampling of Q Sepharose BB T-301462

Protocol, sampling of Q-Sepharose BB

Date/Sign: 2005-01-11 start 13.50

Lot nr:	301462
Weight of one minute vaacuum suctioned gel:	390 g
Balance id: 1116272324	
Weight, vacuum suctioned gel for samples:	29.1 g

Start incubation: 2005-01-11 kl 13.50
Stop incubation: 2005-01-18 kl 12.20
Incubation time: 166.5 h

Sample nr	Content	Info
110	Pre-washed vacuum suctioned gel	29.1 g vacuum suctioned gel for PFE extraction
111	Storage solution, 0.1 mM HCl 40°C	Last cv 0.1 mM HCl stored with 40 mL gel for 166.5 h at 40°C
112	Blank, 0.1 mM HCl 40°C	0.1 mM HCl used in sample 111, stored at the same conditions
113	Storage solution, 0.1 mM HCl 20°C	Last cv 0.1 mM HCl stored with 40 mL gel for 166.5 h at 20°C
114	Blank, 0.1 mM HCl 20°C	0.1 mM HCl used in sample 113, stored at the same conditions
115	Storage solution, 1 mM HCl 40°C	Last cv 1 mM HCl stored with 40 mL gel for 166.5 h at 40°C
116	Blank, 1 mM HCl 40°C	1 mM HCl used in sample 115, stored at the same conditions
117	Storage solution 1 mM HCl 20°C	Last cv 1 mM HCl stored with 40 mL gel for 166.5 h at 20°C
118	Blank, 1 mM HCl 20°C	1 mM HCl used in sample 117, stored at the same conditions
119	Storage solutio, 0.2 M NaOH 60°C	Last cv 0.2 M NaOH stored with 40 mL gel for 166.5 h at 60°C
120	Blank, 0.2 M NaOH 60°C	0.2 M NaOH used in sample 119, stored at the same conditions
121	Storage solution, 0.2 M NaOH 20°C	Last cv 0.2 M NaOH stored with 40 mL gel for 166.5 h at 20°C
122	Blank, 0.2 M NaOH 20°C	0.2 M NaOH used in sample 121, stored at the same conditions
123	Storage solution, 0.5 M NaOH 40°C	Last cv 0.5 M NaOH stored with 40 mL gel for 166.5 h at 40°C
124	Blank, 0.5 M NaOH 40°C	0.5 M NaOH used in sample 123, stored at the same conditions
125	Storage solution, 0.5 M NaOH 20°C	Last cv 0.5 M NaOH stored with 40 mL gel for 166.5 h at 20°C
126	Blank 0.5 M NaOH 20°C	0.5 M NaOH used in sample 125, stored at the same conditions
127	Storage solution H_2O 40°C	Last cv H_2O stored with 40 mL gel for 166.5 h at 40°C
128	Blank, H_2O 40°C	H_2O used in sample 127, stored at the same conditions
129	Storage solution, H_2O 20°C	Last cv H_2O stored with 40 mL gel for 166.5 h at 20°C
130	Blank, H_2O 20°C	H_2O used in sample 129, stored at the same conditions

3.3.5 Sampling of Q Sepharose BB T-302192

The scheme (Figure 1) was followed. The density of one-minute vacuum-suctioned gel is 0.728 g/mL sedimented gel (see section 2 Table 5). To obtain more than 500 mL sedimented gel, >364 g of the vacuum suctioned Q Sepharose BB was weighed into another 1000 mL G3 glass filter.

Protocol 3 show the sampling for Q Sepharose BB T-302192 in detail. The gel was washed according to the pre-use washing procedure before distrubution of Q Sepharose BB into glass filters for further washing. 29.1 g (40 mL wet sedimented gel) of vacuum suctioned pre-use washed gel was weighed into the glass filters.

Protocol 3. Sampling of Q Sepharose BB T-302192.

Protocol, sampling of Q-Sepharose BB | Date/Sign: 2005-01-12 start 13.45

Lot nr:	302192
Weight of one minute vaacuum suctioned gel:	390 g
Balance id: 1116272324	
Weight, vacuum suctioned gel for samples:	29.1 g

Start incubation: 2005-01-12 kl 13.45
Stop incubation: 2005-01-19 kl 12.45
Incubation time: 167.0 h

Sample nr	Content	Info
210	Pre-washed vacuum suctioned gel	29.1 g vacuum suctioned gel for PFE extraction
211	Storage solution, 0.1 mM HCl 40°C	Last cv 0.1 mM HCl stored with 40 mL gel for 167.0 h at 40°C
212	Blank, 0.1 mM HCl 40°C	0.1 mM HCl used in sample 211, stored at the same conditions
213	Storage solution, 0.1 mM HCl 20°C	Last cv 0.1 mM HCl stored with 40 mL gel for 167.0 h at 20°C
214	Blank, 0.1 mM HCl 20°C	0.1 mM HCl used in sample 213, stored at the same conditions
215	Storage solution, 1 mM HCl 40°C	Last cv 1 mM HCl stored with 40 mL gel for 167.0 h at 40°C
216	Blank, 1 mM HCl 40°C	1 mM HCl used in sample 215, stored at the same conditions
217	Storage solution 1 mM HCl 20°C	Last cv 1 mM HCl stored with 40 mL gel for 167.0 h at 20°C
218	Blank, 1 mM HCl 20°C	1 mM HCl used in sample 217, stored at the same conditions
219	Storage solutio, 0.2 M NaOH 60°C	Last cv 0.2 M NaOH stored with 40 mL gel for 167.0 h at 60°C
220	Blank, 0.2 M NaOH 60°C	0.2 M NaOH used in sample 219, stored at the same conditions
221	Storage solution, 0.2 M NaOH 20°C	Last cv 0.2 M NaOH stored with 40 mL gel for 167.0 h at 20°C
222	Blank, 0.2 M NaOH 20°C	0.2 M NaOH used in sample 221, stored at the same conditions
223	Storage solution, 0.5 M NaOH 40°C	Last cv 0.5 M NaOH stored with 40 mL gel for 167.0 h at 40°C
224	Blank, 0.5 M NaOH 40°C	0.5 M NaOH used in sample 223, stored at the same conditions
225	Storage solution, 0.5 M NaOH 20°C	Last cv 0.5 M NaOH stored with 40 mL gel for 167.0 h at 20°C
226	Blank 0.5 M NaOH 20°C	0.5 M NaOH used in sample 225, stored at the same conditions
227	Storage solution H_2O 40°C	Last cv H_2O stored with 40 mL gel for 167.0 h at 40°C
228	Blank, H_2O 40°C	H_2O used in sample 227, stored at the same conditions
229	Storage solution, H_2O 20°C	Last cv H_2O stored with 40 mL gel for 167.0 h at 20°C
230	Blank, H_2O 20°C	H_2O used in sample 229, stored at the same conditions

3.3.6 Additional samples, procedure

Two glassfilters, containing 40 mL wet sedimented pre-use washed gel, were prepared. One glass filter were washed with 2 cv 0.5 M NaOH followed with 4 cv 0.01 M NaOH. The other glass filter was washed with 2 cv 0.5 M NaOH followed with 4 cv 1 M NaCl pH12. After the 4th wash the gels in the glass filter were vacuum suctioned for one minute. The gels were transferred into individually storage flasks and 40 mL of corresponding solution was added. The gel-mixtures were stored according to the sample protocol 4 below.

Protocol 4. Additional samples of Q-Sepharose BB T-301065, T-301462 and T-302192, storage with 0.01 M NaOH.

Protocol, sampling of Q-Sepharose BB Date/Sign: 2005-03-09 start 15.10

Lot nr:	301065, 301462, 302192
Weight of one minute vaacuum suctioned gel:	390 g
Balance id: 1116272324	
Weight, vacuum suctioned gel for samples:	29.0 g

Start incubation:	2005-03-09 kl 15.10
Stop incubation:	2005-03-16 kl 14.10
Incubation time:	167 h

Sample nr	Content	Info
311	Storage solution, 0.01 M NaOH T-301065	Last cv 0.01 M NaOH stored with 40 mL gel for 167 h at 40°C
312	Storage solution, 0.01 M NaOH T-301462	Last cv 0.01 M NaOH stored with 40 mL gel for 167 h at 40°C
313	Storage solution, 0.01 M NaOH T-302192	Last cv 0.01 M NaOH stored with 40 mL gel for 167 h at 40°C

3.3.7 Preparation of NaBr standards

NaBr standards were prepared according to Table 10 from a 1000 ppm stock solution. The 1000 ppm stock solution was prepared by weighing 1.285 g NaBr into a 1000 mL volumetric flask. The flask was diluted to the mark with milli-Q PLUS water and mixed. Also a 20 000 ppm Br stock solution was prepared by weighing 2.575 g NaBr into a 10 mL volumetric flask. The flask was diluted to the mark with milli-Q PLUS water and mixed. The purpose of sending NaBr standards to Analytica AB was to investigate if any systematic differences are present between the bromine and bromide analysis and to estimate the performances for both methods. Also NaBr standards in 0.5 M NaOH were sent to Analytica to investigate if the oxidizing environment at high pH leads to any differences between the bromine and bromide analysis.

Table 10. NaBr standards sent to Analytica AB

Sample	ppm Br	Stock solution ppm	Volume of stock solution (μL)	Final volume (mL)
1	10	1000	200	20
2	10	1000	200	20
3	10	1000	200	20
4	50	1000	1000	20
5	50	1000	1000	20
6	50	1000	1000	20
7	100	1000	2000	20
8	100	1000	2000	20
9	100	1000	2000	20
10	500	1000	10000	20
11	500	1000	10000	20
12	500	1000	10000	20
13	1000	1000	20000	20
14	1000	1000	20000	20
15	1000	1000	20000	20
1500 ppm in 0.5 M NaOH	1500	20000	1500	20
1500 ppm in 0.5 M NaOH	1500	20000	1500	20
1500 ppm in 0.5 M NaOH	1500	20000	1500	20
50 ppm in 0.5 M NaOH	50	20000	50	20
50 ppm in 0.5 M NaOH	50	20000	50	20
50 ppm in 0.5 M NaOH	50	20000	50	20

3.3.8 Sampling volumes for different analyses

40 ±1 mL for all samples was obtained for further analysis.

Static extraction samples and PFE extracted samples; 6 mL of the sample to TOC/TN, 6 mL for bromine analysis and 6 mL for bromide analysis. Results are shown in Analytical reports 2 and 3.

Stability testing; 6 mL of the sample to TOC/TN, 6 mL for bromine analysis and 6 mL for bromide analysis. Results are shown in Analytical reports 2 and 4.

4 Pressurised Fluid Extraction (PFE) of pre-use washed Q Sepharose BB

4.1 Materials and methods

4.1.1 Equipment

Table 11 shows the equipment for PFE.

Table 11. Equipment for PFE

Item	Type	Source
Accelerated solvent extractor	ASE™ 200	Dionex corp.
Steel extraction cell	11 mL	Dionex corp.
Cellulose filter	Grade D28, d= 1.983 cm	Whatman
Sample vials	P/N 49465	Dionex corp.
Balance	0.001 g accuracy	Mettler Toledo

Table 12 shows the instrumental settings for the PFE equipment.

Table 12. Instrumental settings for the PFE equipment.

Parameter	Settings
Extraction solvent	Milli-Q PLUS water
Temperature	40^{o}C
Pressure	10 MPa
Heat-up time	5 min
Static time	5 min
Purge time	60 s
No of static cycles	1

4.2 Chemicals

Milli-Q PLUS water, pre-use washed Q Sepharose BB T-301065, T-301462 and T-302192 with the sample numbers 10,110 and 210 respectively and Allyl Sepharose BB T-302183, T-301232 and T-300861. Since the samples don't reach for all analysis a second set of samples were prepared called 10B, 110B and 210B. Also a third set were prepared for GC analysis and these samples were called 10C, 110C and 210C.

4.3 Performance

Before the experiments, the equipment was cleaned by running 5 cycles with fresh solvent. The carbon content was investigated before further extraction , to secure that no carbon is present in the system. It was below 1 ppm C. After this initial cleaning, the extraction cell, 11 mL, was filled with vacuum suctioned Q Sepharose BB in the amounts shown in Table 13.

Table 13. Samples for extraction.

Batch Q Sepharose BB	Weight gel into extraction cell	Set
T-301065 (10)	6.584 g	1
T-301462 (110)	6.552 g	1
T-302192 (210)	6.504 g	1
T-301065 (10B)	6.494 g	2
T-301462 (110B)	6.202 g	2
T-302192 (210B)	6.116 g	2
T-301065 (10C)	6.449 g	3
T-301462 (110C)	6.311 g	3
T-302192 (210C)	6.263 g	3

The extraction procedure started with a 5 min thermal equilibration time during which the cell was heated and continuously filled with solvent. The extraction was continued under static conditions. The extraction temperature, pressure and extraction time were kept constant for all experiments at 40°C, 10 MPa and 5 min, respectively. After the static extraction, the sample was rinsed with fresh solvent, 60% (v/v). Finally, the cell was purged with gaseous nitrogen for 60 s. Milli-Q PLUS water was used as extraction solvent in all experiments.

One extraction cell was left empty and used as a blank sample. This blank extraction cell was placed in position 1 in the extraction cell carousel. The extraction cells containing Q Sepharose BB T-301065, T-301462 and T-302192 were placed into positions 2,3 and 4, respectively. Each extraction cell was extracted 2 with cycles. The extract from each cycle was collected individually into a collection vial.

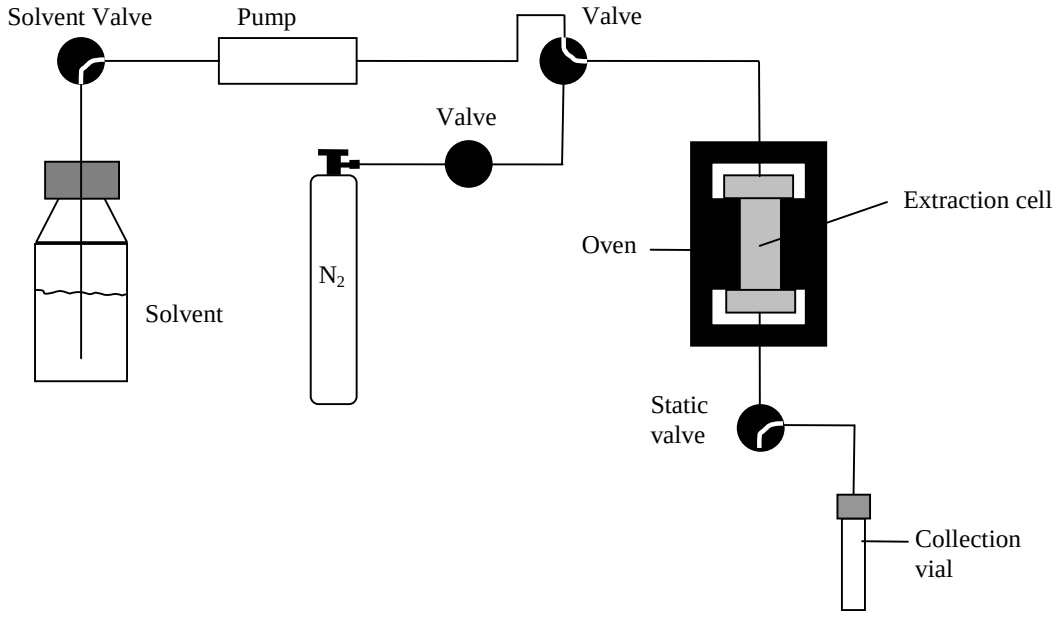

Figure 3. Schematic picture for PFE.

4.3.1 Obtained extraction solutions from the PFE experiment

Table 14 shows the volume of extraction solution that was obtained from each extraction.

Table 14. Volume of extraction solutions.

Sample	Extracion cell	Volume extraction solution (mL)
Blank 1	Blank, cycle 1	23.47
Blank 2	Blank, cycle 2	23.71
10:1	10 (Q Sepharose BB T-301065), cycle 1	17.69
10:2	10 (Q Sepharose BB T-301065), cycle 2	18.95
110:1	110 (Q Sepharose BB T-301462), cycle 1	18.21
110:2	110 (Q Sepharose BB T-301462), cycle 2	18.85
210:1	210 (Q Sepharose BB T-302192), cycle 1	18.09
210:2	210 (Q Sepharose BB T-302192), cycle 2	18.05
Blank 1A	Blank, cycle 1	24.4
Blank 2A	Blank, cycle 2	24.2
10B:1	10B (Q Sepharose BB T-301065), cycle 1	18.8
10B:2	10B (Q Sepharose BB T-301065), cycle 2	18.1
110B:1	110B (Q Sepharose BB T-301462), cycle 1	18.9
110B:2	110B (Q Sepharose BB T-301462), cycle 2	18.6
210B:1	210B (Q Sepharose BB T-302192), cycle 1	18.9
210B:2	210B (Q Sepharose BB T-302192), cycle 2	19.0
Blank 1C	Blank, cycle 1	23.3
Blank 2C	Blank, cycle 2	23.6
10C:1	10C (Q Sepharose BB T-301065), cycle 1	17.6
10C:2	10C (Q Sepharose BB T-301065), cycle 2	18.1
110C:1	110C (Q Sepharose BB T-301462), cycle 1	18.7
110C:2	110C (Q Sepharose BB T-301462), cycle 2	18.2
210C:1	210C (Q Sepharose BB T-302192), cycle 1	18.4
210C:2	210C (Q Sepharose BB T-302192), cycle 2	18.0

Samples 10,110 and 210 were analysed by TOC/TN and samples 10B, 110B, 210B, 10C, 110C and 210C with GC to investigate impurities and extractables obtained from the PFE extractions. Also bromine and bromide were analysed on samples10, 110 and 210. See Analytical reports 2 and 3.

4.3.2 PFE of Allyl-Sepharose BB

The Allyl-Sepharose BB batches, T-302183, T-301232 and T-300861, were transferred into three 11 mL extraction cells, respectively. The cells were filled until the whole cells were filled. The cells were extracted with PFE as described in section 4.3. 16 g extraction solution was obtained for each batch Allyl-Sepharose BB.

Elemental analysis for investigating the impurities, extractables and chemical stability of Q Sepharose BB

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1 Introduction

This analytical report describes the procedure for elemental analysis of the samples that were prepared in Analytical report 1, "Sample preparation for investigating the impurities, extractables and chemical stability of Q Sepharose BB".

Total Organic Carbon (TOC) and Total Nitrogen (TN) were analyzed in-house by a TOC/TN analysator. This analysis has been validated to demonstrate the performance of the equipment. TOC is a method that has been used successfully for monitoring water quality, including the quality of water for injections in pharmaceuticals. TOC analysis offers a number of distinct practical advantages over other commonly used testing methods for residuals because of its high sample throughput, lack of interfering substances and high inherent sensitivity. The TOC method was validated using potassium biphthalate whereas TN was validated using sodium nitrate.

Bromine, phosphorus, heavy metals and other elements have been analysed externally by an accredited test laboratory, Analytica AB. They employed the techniques Inductive Coupled Plasma-Quadropole Mass Spectrometry (ICP-QMS), Inductive Coupled Plasma-Section Field Mass Spectrometry (ICP-SFMS) and Inductive Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) for quantitation of the different elements. For bromide content they employed Ion chromatography (IC).

Total carbon, hydrogen, oxygen, nitrogen and sulphur in the solid dried Q Sepharose BB were analysed by the accredited test laboratory Mikrokemi AB. Elemental analysis of C, H, S and N was performed with combustion using hot wire detection.

2 Estimation of the performance of the TOC/TN analysis

2.1 Introduction

The purpose of this study was to investigate the performance of the TOC/TN analysis in the ranges 0-50 ppm carbon and 0-5 ppm nitrogen.

Total Organic Carbon (TOC) is the organic carbon that is converted into carbon dioxide after oxidation. Direct TOC measurement is done after acidification of the sample. TOC in water samples should ideally include carbon in volatile substances, although many laboratories report TOC samples where the volatiles have already been removed before analysis. The results are still generally accepted as TOC.

The samples are injected by means of the integrated Auto sampler into the high temperature reactor. In the reactor, at the high temperature of 950 ^{o}C (Cobalt oxide catalyst) all organic and inorganic carbon oxidizes into gaseous carbon dioxide (CO_2). The catalyst, present in the reactor, catalyses the oxidation to completion. A flow of oxygen (O_2) transports the carbon dioxide to the Infra Red (IR) detector. The oxygen required for reaction is taken from the airflow. The carbon dioxide is measured at 4.2 μm by IR detection.

The samples described in this analytical report consist of organic carbon and purgable inorganic carbonic acid. Since the carbonic acid is purgeable and removed before analysis it is not necessary to run IC (Inorganic carbon).

Total Nitrogen (TN) is all the nitrogen in the sample, this include elementary, organic and inorganic nitrogen. TN is represented as a total mass of nitrogen per amount of sample.

TN analysis is performed simultaneously with the TOC analysis. In the combustion reactor, with a temperature of 950 ^{o}C, the nitrogen is converted to nitric oxide, NO. The flow of oxygen, which is used as a carrier gas and as a source of oxygen, transport the nitric oxide into the chemiluminecent detector where the sample gas is mixed with ozone and formation of excited nitrogen dioxide occurs. Fast decay of the excited nitrogen dioxide produces light, which is measured by a photo multiplier tube, PMT.

2.2 Equipment

Table 1 shows the equipment required for TOC/TN analysis.

Table 1. Equipment required for TOC/TN analysis.

Item	Type	Supplier
TOC analysator	Formacs Combustion TOC analyzer, id 803	Skalar
Total nitrogen detector	ND-10, ser no 00051	Skalar
Oxygene gas, O_2	Alphagas™ O_2 serial no K25163	Air Liquide
Software	TOCWin v 1.4	Skalar
Sample tubes	CA 30, 8 mL	Skalar
Volumetric flasks	10 and 20 mL	Scott Duran
Pipette 10-100 μL	Reference, id 871	Eppendorff
Pipette 100-1000 μL	Research, id 553	Eppendorff
Analytical balance	AT 261 DeltaRange ser no 1117482157	Mettler Toledo
Dessicator		
Incubator 105^{o}C	Id 234	Termaks

Instrumental settings TOC/TN analyser, TOC4Win v1.4

Table 2 shows the instrumental settings of the TOC analyser.

Table 2. Instrumental settings of the TOC/TN analyser.

Parameter	Settings
Default sample time	200 sec
TC temp	950^{o}C
Syringe	250 μL
Flush count	2 counts
Flush volume	200 μL
Rinse time	28 sec
Pick up speed	35
Inject speed	70
Acid to cup	2 x 50 μL
Sparging time	120 sec
Stir time	120 sec
Samples	3 (3 injections/sample)
Extra samples	2 (if the CV% for 3 injections exceed 2%)

2.3 Chemicals and solutions

2.3.1 Chemicals

Table 3 shows the chemicals required for generating TOC and TN calibration curves.

Table 3. Chemicals for TOC calibration.

Chemical	Type	Supplier
Potassium biphthalate	Art no 1.02400.0080 lot 92400E. Quality: Secondary reference material for the alkalimetry traced back to NIST Standard Reference Material (SRM).	Merck KGaA
Sodium nitrate	Art no 1.06537.1000 lot 74137338, pa quality	Merck KGaA
Carbon free water	Milli-Q PLUS water	Millipore corp.
HCl	1.09970 Titrisol, C= 1M	Merck KGaA

2.3.2 Solutions

2 M HCl for automatic acidification

1 ampoule Titrisol 1.09970 (one ampoule gives 1.0 M HCl when diluting to 1000 mL) was added to a 500 mL volumetric flask. The solution was diluted to the mark with milli-Q PLUS water.

1000 ppm organic carbon stock solution

Potassium biphthalate was dried at 105°C over night and stored in a dessicator before weighing.

2.1254 g potassium biphthalate was weighed into a glass beaker (analytical balance id 1117482157). The weighed potassium biphthalate was transferred quantitatively into a 1000 mL volumetric flask with milli-Q PLUS water. The solution was diluted to the mark with milli-Q PLUS water. This solution is stable for one month at 4-8 °C.

1000 ppm nitrogen stock solution

6.0681 g sodium nitrate was weighed into a glass beaker (analytical balance id 1117482157). The weighed sodium nitrate was transferred quantitatively into a 1000 mL volumetric flask with milli-Q PLUS water. The solution was diluted to the mark with milli-Q PLUS water. This solution is stable for one month at 4-8 °C.

2.4 Procedure

2.4.1 Preparation of 100 ppm carbon stock solution

10.0 mL of the 1000 ppm organic stock solution was pipetted into a 100 mL volumetric flask. The solution was diluted to the mark with milli-Q PLUS water and mixed well.

2.4.2 Preparation of carbon calibration standards 0-50 ppm

Calibration standards were prepared from the 100 ppm stock solution according to Table 4. An aliquot of the 100 ppm C solution was pipetted into a 10 mL volumetric flask. Table 4 shows the dilution procedure for all calibration samples.

Table 4. Dilution scheme for the carbon calibration samples 0-50 ppm.

Calibration standard no	Concentration, ppm C	µL from 100 ppm stock solution	Final volume, mL
1	0	0	Only milli-Q PLUS water
2	0.5	50	10
3	1.0	100	10
4	1.5	150	10
5	2.0	200	10
6	2.5	250	10
7	3.0	300	10
8	3.5	350	10
9	10.0	1000	10
10	20.0	2000	10
11	30.0	3000	10
12	40.0	4000	10
13	50.0	5000	10

2.4.3 Preparation of nitrogen standards 0-5 ppm

Calibration standards were prepared from the 1000 ppm stock solution according to Table 5. Table 5 shows the dilution procedure for all calibration samples.

Table 5. Dilution scheme for the nitrogen calibration samples 0-5 ppm.

Calibration standard no	Concentration, ppm N	µL from 1000 ppm stock solution	Final volume, mL
1	0	0	Only milli-Q PLUS water
2	0.5	10	20
3	1.0	10	10
4	2.0	20	10
5	3.0	30	10
6	4.0	40	10
7	5.0	50	10

2.4.4 Procedure for operating the TOC/TN equipment

6 mL milli-Q PLUS water was added into two sample tubes. The first tube was called “Blank” and the second sample tube was called “0 ppm C”. ~6 mL of the calibration samples were transferred into sample tubes, specially designed for the auto sampler, and placed into positions 3-14, respectively.

The nitrogen analysis validation was performed at a different time but the analysis template was equal to the carbon validation procedure.

The oxygen flow was set to 250 mL/min. All other settings were set according to “instrumental settings” in section 2.2. ~100 mL 2M HCl was added into the “Acid to cup” flask.

An analysis template was made containing all sample info. When the baseline of the IR detector was stable and when the oven was at 950^{o}C the analysis was started.

2.5 Validation of the TOC equipment

2.5.1 Demands for the validation of the TOC equipment

- High repeatability and reproducibility for the response, relative standard deviation (cv) of <10% for all samples above 10 ppm carbon, within the range 10-50 ppm C.
- The relative standard deviation (cv) for the random error (Sx_o), for a calculated concentration within the range 10-50 ppm C shall not exceed a cv of 10%.
- High accuracy, linearity of >0.99 for a 0-50 ppm calibration curve.
- Detection limit below or equal to 1 ppm Carbon
- Reproducible standard curve, relative standard deviation (cv) below 5%.

2.5.2 Matrix effects

TOC analysis can not separate different carbon content substances. The result is the total carbon content of all carbon substances present in the sample.

For the TOC technique, matrix effects arise when samples are alkaline and carbon dioxide is produced. This phenomenon is eliminated by acidification and purging the samples with hydrochloric acid and oxygen, respectively, before analysis. It was ensured that all samples were aqueous acidic solutions so that the matrix effect could be ignored. The TOC equipment was validated using potassium biphthalate as calibration standard using carbon free milli-Q water as solvent.

2.5.3 Design of experiment (DOE)

To fulfil the criteria in section 2.5.1 an experimental design was set-up. Table 6 shows the experimental design. For each sample a triplicate analysis was performed if the CV was below 2% for these three samples. If the CV for the triplicate was over 2%, two extra injections were made.

Table 6. DOE for the validation of the TOC equipment between 0-50 ppm carbon.

Sample	Concentration ppm C	Occation	Calibration curve
1	0	1	1
2	0.5	1	1
3	1.0	1	1
4	1.5	1	1
5	2.0	1	1
6	2.5	1	1
7	3.0	1	1
8	3.5	1	1
9	10	1	1
10	20	1	1
11	30	1	1
12	40	1	1
13	50	1	1
14	0	2	2
15	0.5	2	2
16	1.0	2	2
17	1.5	2	2
18	2.0	2	2
19	2.5	2	2
20	3.0	2	2
21	3.5	2	2
22	10	2	2
23	20	2	2
24	30	2	2
25	40	2	2
26	50	2	2
27	0	3	3
28	0.5	3	3
29	1.0	3	3
30	1.5	3	3
31	2.0	3	3
32	2.5	3	3
33	3.0	3	3
34	3.5	3	3
35	10	3	3
36	20	3	3
37	30	3	3
38	40	3	3
39	50	3	3

2.5.4 Results

The total carbon peaks were integrated automatically with the TOCWin software. The response for each sample is the TC (Total Carbon) area obtained from the IR detector. Table 7 shows the response of each calibration sample.

Table 7. The response for each calibration sample.

Sample	Concentration, ppm	Injection 1, TC Area	Injection 2, TC area	Injection 3, TC area	Average, TC area
1	0	0	0	0	**0**
2	0,5	0	0	0	**0**
3	1	128017	136002	142428	**135482**
4	1,5	173123	170254	177004	**173460**
5	2	243287	236285	248844	**242805**
6	2,5	368739	371294	375511	**371848**
7	3	432644	437006	446067	**438572**
8	3,5	513564	518039	529440	**520348**
9	10	1378479	1420104	1374572	**1391052**
10	20	2661108	2615845	2591507	**2622820**
11	30	3714156	3692032	3705597	**3703928**
12	40	4849379	4894601	4889880	**4877953**
13	50	5992919	6057564	5974177	**6008220**
14	0	0	0	0	**0**
15	0,5	0	0	0	**0**
16	1	186046	178682	180706	**181811**
17	1,5	218161	214525	227824	**220170**
18	2	393233	396325	396867	**395475**
19	2,5	351469	362410	357669	**357183**
20	3	447442	441945	444813	**444733**
21	3,5	505350	502943	499327	**502540**
22	10	1337940	1372942	1342382	**1351088**
23	20	2624012	2611401	2618820	**2618078**
24	30	3743623	3772159	3809620	**3775134**
25	40	4966211	5101085	4980101	**5015799**
26	50	6088853	6063835	6065384	**6072691**
27	0	0	0	0	**0**
28	0,5	231765	229904	243086	**234918**
29	1	263433	277217	269330	**269993**
30	1,5	301775	310836	313364	**308658**
31	2	415656	418157	415840	**416551**
32	2,5	553195	563527	472424	**529715**
33	3	505482	505148	485561	**498730**
34	3,5	548102	539254	553891	**547082**
35	10	1334792	1356325	1343474	**1344864**
36	20	2461355	2500310	2482593	**2481419**
37	30	3801385	3684088	3734682	**3740052**
38	40	4829676	4676829	4674542	**4727016**
39	50	5918572	5964759	5898396	**5927242**

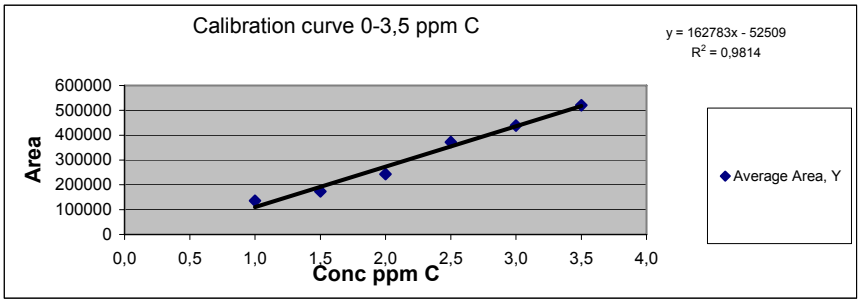
2.5.5 Estimation of the detection limit of the TOC equipment

Part of the data in Table 3 (calibration samples within the range 0- 3,5 ppm C) was used for determination of the detection limit of the TOC equipment as shown in Figures 1-3. The tables in Figures 1-3 shows the statistical data required to calculate the limit of detection (L.o.d). The slope, intercept and regression for each calibration curve were obtained from the Trend function in Microsoft excel. The statistical formulas were obtained from JC Miller and JN Miller, "Statistics and chemometrics for analytical chemistry", 4th edition.

Estimation of the limit of detection, L.o.d., in TOC analysis 0-3,5 ppm

Concentration ppm C, X	X^2	$X-X_a$	$(X-X_a)^2$	Sample 1 Area, Y1	Sample 2 Area, Y2	Sample 3 Area, Y3	Average Area, Y	Nominal Area, Y4	I Y-Y4 I	$(Y-Y4)^2$
0	0	-1,75	3,0625	0	0	0	**0**	N/A	N/A	N/A
0,5	0,25	-1,3	1,5625	0	0	0	**0**	N/A	N/A	N/A
1,0	1	-0,8	0,5625	128017	136002	142428	**135482**	110274	25208	635460069
1,5	2,25	-0,3	0,0625	173123	170254	177004	**173460**	191665,5	-18205	331428093
2,0	4	0,3	0,0625	243287	236285	248844	**242805**	273057	-30252	915163336
2,5	6,25	0,8	0,5625	368739	371294	375511	**371848**	354448,5	17400	302742600
3,0	9	1,3	1,5625	432644	437006	446067	**438572**	435840	2732	7465645
3,5	12,25	1,8	3,0625	513564	518039	529440	**520348**	517231,5	3116	9710495

Concentrations 0 and 0.5 are excluded since they are out of the linear range



k=	162783
m=	-52509
$S_{y/x=}$	23462,58
L.o.d.=	**0,432402 ppm C**

Explanations:
X_a= average of the x-values
k= slope
m= intercept
$S_{Y/X}$= estimation of the random errors in the y-direction
n= number of samples
L.o.d= limit of detection

Formulas:

$$S_{y/x} = \sqrt{\frac{\sum_i (Y - Y4)^2}{n-2}}$$

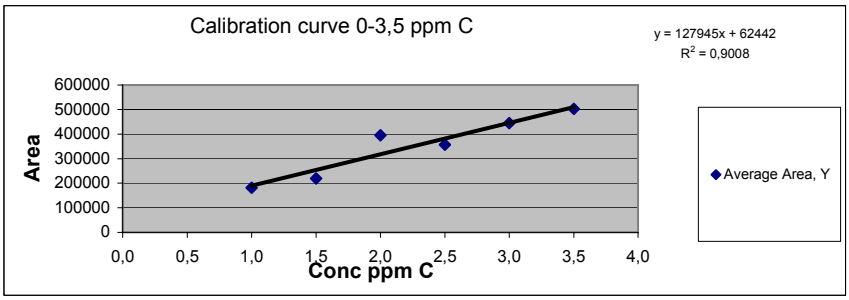
$$L.o.d. = \frac{(m + 3 \times S_{y/x}) - m}{k}$$

Figure 1. Calibration data for occation 1. Calibration curve 1.

Estimation of the limit of detection, L.o.d., in TOC analysis 0-3,5 ppm

Concentration ppm C, X	X^2	$X-X_a$	$(X-X_a)^2$	Sample 1 Area, Y1	Sample 2 Area, Y2	Sample 3 Area, Y3	Average Area, Y	Nominal Area, Y4	I Y-Y4 I	$(Y-Y4)^2$
0	0	-1,75	3,0625	0	0	0	**0**	N/A	N/A	N/A
0,5	0,25	-1,3	1,5625	0	0	0	**0**	N/A	N/A	N/A
1,0	1	-0,8	0,5625	186046	178682	180706	**181811**	190387	-8576	73542059
1,5	2,25	-0,3	0,0625	218161	214525	227824	**220170**	254359,5	-34190	1168921910
2,0	4	0,3	0,0625	393233	396325	396867	**395475**	318332	77143	5951042449
2,5	6,25	0,8	0,5625	351469	362410	357669	**357183**	382304,5	-25122	631106510
3,0	9	1,3	1,5625	447442	441945	444813	**444733**	446277	-1544	2382907
3,5	12,25	1,8	3,0625	505350	502943	499327	**502540**	510249,5	-7710	59436390

Concentrations 0 and 0.5 are excluded since they are out of the linear range



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m=	62442	
$s_{y/x=}$	44402,79	
L.o.d.=	**1,041138**	**ppm C**

Explanations:
X_a= average of the x-values
k= slope
m= intercept
$S_{Y/X}$= estimation of the random errors in the y-direction
n= number of samples
L.o.d= limit of detection

Formulas:

$$S_{y/x} = \sqrt{\frac{\sum_i (Y - Y4)^2}{n-2}}$$

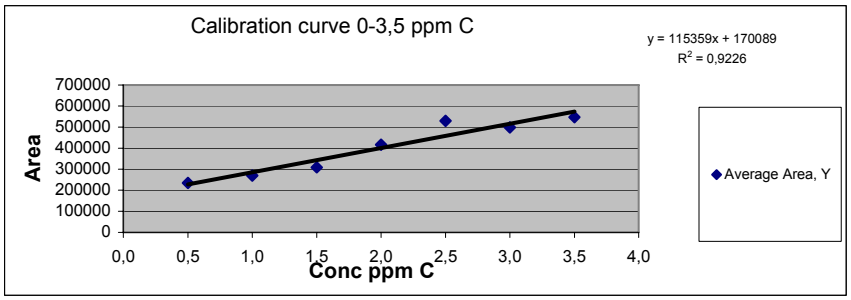
$$L.o.d. = \frac{(m + 3 \times S_{y/x}) - m}{k}$$

Figure 2. Calibration data for occasion 2. Calibration curve 2.

Estimation of the limit of detection, L.o.d., in TOC analysis 0-3,5 ppm

Concentration ppm C, X	X^2	$X-X_a$	$(X-X_a)^2$	Sample 1 Area, Y1	Sample 2 Area, Y2	Sample 3 Area, Y3	Average Area, Y	Nominal Area, Y4	I Y-Y4 I	$(Y-Y4)^2$
0	0	-1,75	3,0625	0	0	0	**0**	N/A	N/A	N/A
0,5	0,25	-1,3	1,5625	231765	229904	243086	**234918**	227768,5	7150	51120117
1,0	1	-0,8	0,5625	263433	277217	269330	**269993**	285448	-15455	238846722
1,5	2,25	-0,3	0,0625	301775	310836	313364	**308658**	343127,5	-34469	1188123451
2,0	4	0,3	0,0625	415656	418157	415840	**416551**	400807	15744	247873536
2,5	6,25	0,8	0,5625	553195	563527	472424	**529715**	458486,5	71229	5073546698
3,0	9	1,3	1,5625	505482	505148	485561	**498730**	516166	-17436	304002472
3,5	12,25	1,8	3,0625	548102	539254	553891	**547082**	573845,5	-26763	716267090

The 0 concentration is exluded since it is out of the linear range



k=	115359
m=	170089
$S_{y/x=}$	39546,88
L.o.d.=	**1,028447 ppm C**

Explanations:
X_a= average of the x-values
k= slope
m= intercept
$S_{Y/X}$= estimation of the random errors in the y-direction
n= number of samples
L.o.d= limit of detection

Formulas:

$$S_{y/x} = \sqrt{\frac{\sum_i (Y - Y4)^2}{n-2}}$$

$$L.o.d. = \frac{(m + 3 \times S_{y/x}) - m}{k}$$

Figure 3. Calibration data for occasion 3. Calibration curve 3.

Estimation of LOD

The average value of LOD from the three calibration curves in the range 0-3.5 ppm is calculated to 0.8 ppm Carbon ((0.43+1.04+1.03)/3). A LOD below or equal to 1 ppm carbon was required. A calibration curve of 2-50 ppm was used for further analysis excluding the 0.5, 1, 1.5, 2.5, 3 and 3.5 ppm samples. In some cases the blank showed a response and it was then used in the calibration curve.

2.5.6 Repeatability and reproducibility of the standard calibration samples

To estimate the errors of the response (TC area) for each calibration sample, as shown in Table 8, the responses were evaluated with the statistical software SAS.JMP v4.0.2. Table 9 shows the results from the evaluation.

Table 8. TC area responses for each calibration sample.

10 ppm Carbon, response TC area

	Injection 1	Injection 2	Injection 3
Sample 1	1378479	1420104	1374572
Sample 2	1337940	1372942	1342382
Sample 3	1334792	1356325	1343474

20 ppm Carbon, response TC area

	Injection 1	Injection 2	Injection 3
Sample 1	2661108	2615845	2591507
Sample 2	2624012	2611401	2618820
Sample 3	2461355	2500310	2482593

30 ppm Carbon, response TC area

	Injection 1	Injection 2	Injection 3
Sample 1	3714156	3692032	3705597
Sample 2	3743623	3772159	3809620
Sample 3	3801385	3684088	3734682

40 ppm Carbon, response TC area

	Injection 1	Injection 2	Injection 3
Sample 1	4849379	4894601	4889880
Sample 2	4966211	5101085	4980101
Sample 3	4829676	4676829	4674542

50 ppm Carbon, response TC area

	Injection 1	Injection 2	Injection 3
Sample 1	5992919	6057564	5974177
Sample 2	6088853	6063835	6065384
Sample 3	5918572	5964759	5898396

Table 9. Evaluation of the TC area responses.

Concentration ppm C	Mean of response, TC area	Within sample Variance, S^2_{within}	Between sample Variance, $S^2_{between}$	S_{total}	CV%	Prob>F[1]
10	1362334	372447150	504045664	25064	1.8	0,0767
20	2574106	556047173	626334e9	80304	3.1	0,0005
30	3739705	1,56042e9	747511304	45539	1.1	0,3091
40	4873589	4,6757e9	1,9305e10	144442	3.0	0.0074
50	6002718	1.08928e9	4.94842e9	72880	1.2	0,0059

[1] If Prob>F is less than 0.05 the between sample variance is significant (for a 95% confidence level)

2.5.7 Evaluation of the slope and intercept of the regression line

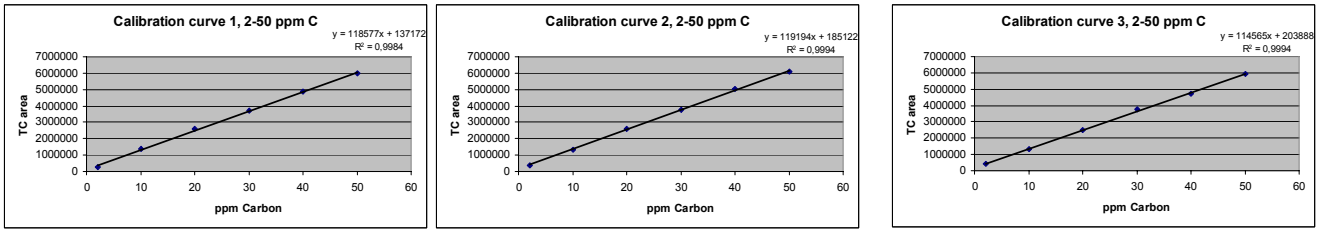
Part of the data from Table 7 was evaluated in Microsoft Excel and three calibration curves were obtained. Table 10 shows the data required for evaluating the slope and intercept of the regression line.

Table 10. Data for evaluation of the slope and intercept of the regression line.

X Concentration (ppm)	X^2	Injection 1 (TC area)	Injection 2 (TC area)	Injection 3 (TC area)	Y, Average (TC area)	Y-X	$(Y-X)^2$	$X-\bar{X}$	$(X-\bar{X})^2$
2	4	243287	236285	248844	**242805**	-131520,7	17297685760	-23,33	544,44
10	100	1378479	1420104	1374572	**1391052**	68109,67	4638926693	-15,33	235,11
20	400	2661108	2615845	2591507	**2622820**	114108	13020635664	-5,33	28,44
30	900	3714156	3692032	3705597	**3703928**	9446,333	89233213,44	4,67	21,78
40	1600	4849379	4894601	4889880	**4877953**	-2298,667	5283868,444	14,67	215,11
50	2500	5992919	6057564	5974177	**6008220**	-57802	3341071204	24,67	608,44
2	4	393233	396325	396867	**395475**	-28035	785961225	-23,33	544,44
10	100	1337940	1372942	1342382	**1351088**	-25974	674648676	-15,33	235,11
20	400	2624012	2611401	2618820	**2618078**	49075,67	2408421059	-5,33	28,44
30	900	3743623	3772159	3809620	**3775134**	14192	201412864	4,67	21,78
40	1600	4966211	5101085	4980101	**5015799**	62917	3958548889	14,67	215,11
50	2500	6088853	6063835	6065384	**6072691**	-72131,33	5202929248	24,67	608,44
2	4	415656	418157	415840	**416551**	-16467	271162089	-23,33	544,44
10	100	1334792	1356325	1343474	**1344864**	-4674,333	21849392,11	-15,33	235,11
20	400	2461355	2500310	2482593	**2481419**	-13768,67	189576181,8	-5,33	28,44
30	900	3801385	3684088	3734682	**3740052**	99213,67	9843351653	4,67	21,78
40	1600	4829676	4676829	4674542	**4727016**	-59472,33	3536958432	14,67	215,11
50	2500	5918572	5964759	5898396	**5927242**	-4895,667	23967552,11	24,67	608,44

The concentration (ppm C), X, was plotted against the response (TC Area),Y, and the slope, k, intercept, m, and regression, R^2, were obtained from the trend function in Microsoft Excel. Figures 4-6 shows the calibration curves for three different occasions. The linear equation (Y=kX+m) of the regression line is also presented in these figures.

Fig 4. Fig 5. Fig 6.



Figures 4-6. 2-50 ppm carbon calibration curves.

Table 11 shows the summary of the calibration curves in Figures 4-6.

Table 11. Summary of calibration curves 1-3.

Calibration curve	Slope, k	Intercept, m	R^2
1	118557	137172	0.9984
2	119194	185122	0.9994
3	114565	203888	0.9994
Average	**117439**	**175411**	**0.9991**

Table 12 shows the errors in the slope and intercept of the averaged regression line. These values were calculated according to JN Miller and JC Miller "Statistics and chemometrics for analytical chemistry", 4th edition. $S_{y/x}$ is the estimated errors in the Y-direction for each regression line and $S_{y/x\ pooled}$ is the pooled errors in the Y-direction for the averaged regression line with the equation Y=117439X+175411. S_k is the standard deviation of the slope and $S_{k\ pooled}$ is the pooled standard deviation of the average value of the slope, 117439. S_m is the standard deviation of the intercept and $S_{m\ pooled}$ is the pooled standard deviation of the average value of the intercept, 175411.

Table 12. Errors in the slope and intercept of the regression line.

Calibration curve	$S_{y/x}$	S_k	S_m
1	97970	2409	72975
2	57515	1414	42841
3	58921	1449	73889
Average calibration curve	$S_{y/x\ pooled}$= 73887	$S_{k\ pooled}$= 1817	$S_{m\ pooled}$= 55036

The formulas used for calculation of the errors in the slope and intercept of the regression line are shown in Figure 7. The formulas are taken from: JN Miller and JC Miller, Statistics and Chemometrics for analytical chemistry, 4th edition.

$$S_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}} \qquad S_k = \frac{S_{y/x}}{\sqrt{\sum_i (x_i - \bar{x})^2}}$$

$$S_m = S_{y/x}\sqrt{\frac{\sum_i x_i^2}{n\sum_i (x_i - \bar{x})^2}} \qquad S_{y/x_{Pooled}} = \sqrt{\frac{(n_1-2)S^2_{y/x_1} + (n_2-2)S^2_{y/x_2} + (n_3-2)S^2_{y/x_3}}{(n_1+n_2+n_3-6)}}$$

$$S_{k\,Pooled} = \frac{S_{y/x\,Pooled}}{\sqrt{\sum_i (x_i - \bar{x})^2}} \qquad S_{m\,Pooled} = S_{y/x\,Pooled}\sqrt{\frac{\sum_i x_i^2}{n\sum_i (x_i - \bar{x})^2}}$$

Figure 7. Formulas for calculation of the errors in the slope and intercept of the regression line.

The relative standard deviation (cv_k) of the slope, k, is:

cv_k= 1817/117439 x 100=1.55%

The relative standard deviation (cv_m) of the intercept, m is:

cv_m=55036/175411 x100=31.4%

The t-value for n-2=4 degrees of freedom and the 95% confidence level is 2.78 (table A.2 in "JC Miller and JN Miller, Statistics and chemometrics for analytical chemistry, 4th edition"). The 95% confidence limit for the slope, k, and intercept, m, are thus:

K= 117439± (2.78 x 1817)= 117439 ± 5051

m= 175411± (2.78 x 55036)= 175411 ± 153000

The regression for all calibration curves is >0.99 which means that all calibration samples fit the regression line properly.

2.5.8 Calculation of a concentration and its random error

The error of a single standard concentration is the standard deviation that is obtained from the formula (obtained from JN Miller and JC Miller, "Statistics and chemometrics for analytical chemistry", 4th edition):

$$S_{x0} = \frac{S_{y/x\,Pooled}}{b}\sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2\sum_i (x_i - \bar{x})^2}}$$

Here *m* is the number of readings to obtain the value of y_0. In this case the number of m=3 since there are 3 calibration points obtained in the averaged calibration curve.

Table 13 shows each calibration concentration and its random error. These errors are calculated considering of the statistical data of the averaged calibration curve.

Table 13. Random errors of each calibration concentration.

Concentration ppm Carbon	Sx_0	CV%
2	0,58	28,9
10	0,50	5,0
20	0,45	2,3
30	0,45	1,5
40	0,50	1,2
50	0,58	1,2

2.6 Validation of the TN equipment

2.6.1 Demands for the validation of the TN equipment

- High repeatability and reproducibility for the response, relative standard deviation of <10% for all samples above 1 ppm, within the range 10-50 ppm Nitrogen.
- The relative standard deviation (cv) for the random error (Sx_0), for a calculated concentration within the range 1-5 ppm N shall not exceed a cv of 10%.
- High accuracy, linearity of >0.99 for a 1-5 ppm calibration curve
- Detection limit equal or below 1 ppm N
- Reproducible standard curve, relative standard deviation (cv) below 5%.

2.6.2 Matrix effects

TN can not separate different nitrogen content substances. The result is the total nitrogen content of all nitrogen substances present in the sample. The nitrogen equipment was validated using sodium nitrate as calibration standard using carbon free milli-Q water as solvent.

2.6.3 Design of experiment

To fulfil the criteria in section 2.6.1 an experimental design was set-up. Table 14 shows the experimental design. For each sample a triplicate analysis was performed if the cv was below 2% for these three samples. If the cv for the triplicate was over 2%, two extra injections were made.

Table 14. DOE for the validation of the equipment between 0-5 ppm nitrogen

Sample no	Concentration (ppm N)	Occasion	Calibration curve no
1	0	1	1
2	0,5	1	1
3	1	1	1
4	2	1	1
5	3	1	1
6	4	1	1
7	5	1	1
8	0	2	2
9	0,5	2	2
10	1	2	2
11	2	2	2
12	3	2	2
13	4	2	2
14	5	2	2

2.6.4 Results

The total nitrogen peaks were integrated automatically with the TOCWin software. The response for each sample is the TN (Total Nitrogen) area obtained from the IR Detector. Table 15 shows the response for each calibration sample.

Table 15. The response for each calibration sample

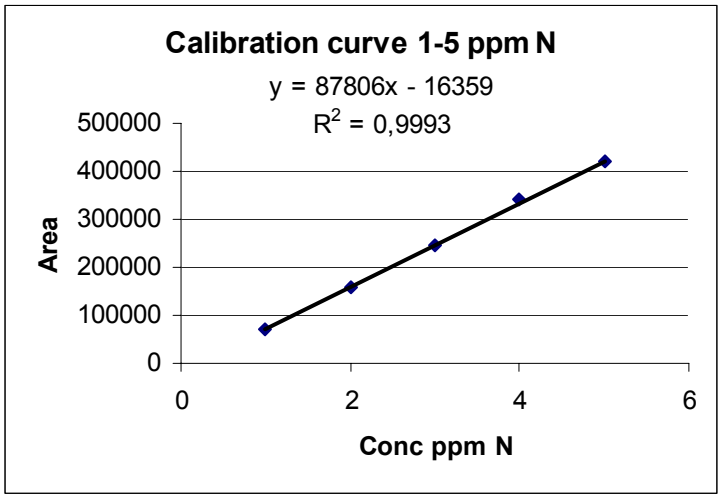
Sample no	Concentration (ppm N)	Response Inj 1	Inj 2	Inj 3	Average
1	0	0	0	0	0
2	0,5	0	0	0	0
3	1	70023	70779	72173	70991,67
4	2	159964	155316	159682	158320,7
5	3	248763	244734	245643	246380
6	4	336355	346120	340029	340834,7
7	5	419231	419234	417826	418763,7
8	0	0	0	0	0
9	0,5	0	0	0	0
10	1	75914	75698	73048	74886,67
11	2	166148	165505	167930	166527,7
12	3	252226	250220	254246	252230,7
13	4	340916	329207	332140	334087,7
14	5	410057	405920	418645	411540,7

2.6.5 Estimation of the detection limit of the TN equipment

Part of the data in Table 15 was used for determination of the detection limit of the TN equipment as shown in Figures 8-9. The tables in Figures 8-9 shows the statistical data required to calculate the limit of detection (L.o.d). The slope, intercept and regression for each calibration curve were obtained from the Trend function in Microsoft excel. The statistical formulas were obtained from JC Miller and JN Miller, "Statistics and chemometrics for analytical chemistry", 4th edition.

Estimation of the limit of detection, L.o.d, in TN analysis 1-5 ppm

Concentration, X (ppm N)	X^2	$X-X_a$	$(X-X_a)^2$	Average Area, Y	Nominal area, Y4	I Y-Y4 I	$(Y-Y4)^2$
1	1	-2	4	70992	71447	-455,3333	207328,4
2	4	-1	1	158321	159253	-932,3333	869245,4
3	9	0	0	246380	247059	-679	461041
4	16	1	1	340835	334865	5969,667	35636920
5	25	2	4	418764	422671	-3907,333	15267254



k= 87806
m= -16359
$S_{y/x}$= 3621
L.o.d= 0,12 ppm N

Explanations:
X_a= average of the x-values
k= slope
m= intercept
$S_{Y/X}$= estimation of the random errors in the y-direction
n= number of samples
L.o.d= limit of detection

Formulas:

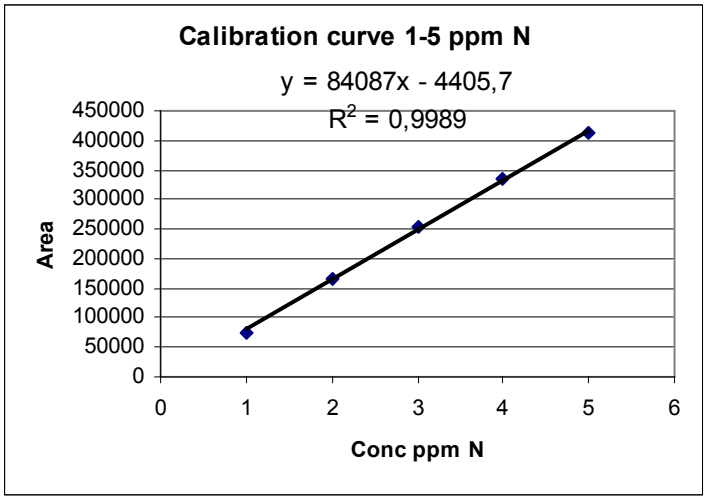
$$S_{y/x} = \sqrt{\frac{\sum_i (Y - Y4)^2}{n-2}}$$

$$L.o.d. = \frac{(m + 3 \times S_{y/x}) - m}{k}$$

Figure 8. Calibration data for occasion 1. Calibration curve 1.

Estimation of the limit of detection, L.o.d, in TN analysis 1-5 ppm

Concentration, X (ppm N)	X^2	$X-X_a$	$(X-X_a)^2$	Average Area, Y	Nominal area, Y4	I Y-Y4 I	$(Y-Y4)^2$
1	1	-2	4	74887	79681	-4794,333	22985632
2	4	-1	1	166528	163768	2759,667	7615760
3	9	0	0	252231	247855	4375,667	19146459
4	16	1	1	334088	331942	2145,667	4603885
5	25	2	4	411541	416029	-4488,333	20145136



k=	84087	
m=	-4406	
$S_{y/x}$=	4316	
L.o.d=	**0,15**	**ppm N**

Explanations:
X_a= average of the x-values
k= slope
m= intercept
$S_{Y/X}$= estimation of the random errors in the y-direction
n= number of samples
L.o.d= limit of detection

Formulas:

$$S_{y/x} = \sqrt{\frac{\sum_i (Y - Y4)^2}{n-2}}$$

$$L.o.d. = \frac{(m + 3 \times S_{y/x}) - m}{k}$$

Figure 9. Calibration data for occasion 2. Calibration curve 2.

Estimation of LOD

The averaged value of LOD from the calibration curves in the range 0-5 ppm N is calculated to 0.14 ppm N.

2.6.6 Repeatability and reproducibility of the standard calibration samples

To estimate the errors of the response (TN area) for each calibration sample , as shown in Table 16, the responses were evaluated with the statistical software SAS.JMP v4.0.2. Table 17 shows the results from the evaluation.

Table 16. TN area responses for each calibration sample.

1 ppm nitrogen, response TN area

	Injection 1	Injection 2	Injection 3
Sample 1	70023	70779	72173
Sample2	75914	75698	73048

2 ppm nitrogen, response TN area

	Injection 1	Injection 2	Injection 3
Sample 1	159964	155316	159682
Sample2	166148	165505	167930

3 ppm nitrogen, response TN area

	Injection 1	Injection 2	Injection 3
Sample 1	248763	244734	245643
Sample2	252226	250220	254246

4 ppm nitrogen, response TN area

	Injection 1	Injection 2	Injection 3
Sample 1	336355	346120	340029
Sample2	340916	329207	332140

5 ppm nitrogen, response TN area

	Injection 1	Injection 2	Injection 3
Sample 1	419231	419234	417826
Sample2	410057	405920	418645

Table 17. Evaluation of the TN area responses.

Concentration ppm N	Mean of response, TC area	Within sample Variance, S^2_{within}	Between sample Variance, $S^2_{between}$	S_{total}	CV%	Prob>F[1]
1	72939	6962724	1868365	2047	2,8	0,0287
2	162424	4184581	3,23E+07	5803	3,6	0,0086
3	249305	4,26E+06	15695521	4137	1,7	0,0292
4	337461	3,07E+07	1,25E+07	4771	1,4	0,3309
5	415152	21395888	18953891	5107	1,2	0,1784

[1] IfProb>F is less than 0.05 the between sample variance is significant (for a 95% confidence interval).

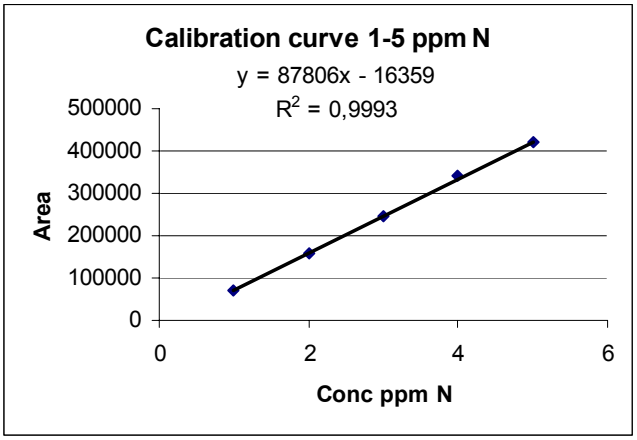
2.6.7 Evaluation of the slope and intercept of the regression line

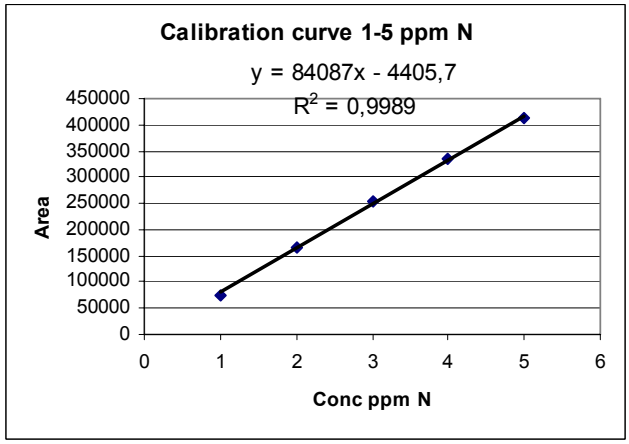
Part of the data from Table 15 was evaluated in Microsoft Excel and two calibration curves were obtained. Table 18 shows the data required for evaluating the slope and intercept of the regression line.

Table 18. Data for evaluation of the slope and intercept of the regression line.

X Concentration (ppm)	X^2	Y, average response (TN area)	$Y-\hat{Y}$	$(Y-\hat{Y})^2$	$X-X_a$	$(X-X_a)^2$
1	1	70992	-176066,5	30999400684	-2	4
2	4	158321	-88737,47	7874337990	-1	1
3	9	246380	-678,1333	459864,8178	0	0
4	16	340835	93776,533	8794038204	1	1
5	25	418764	171705,53	29482790177	2	4
1	1	74887	-172968	29917929024	-2	4
2	4	166528	-81327	6614080929	-1	1
3	9	252231	4376	19149376	0	0
4	16	334088	86233	7436130289	1	1
5	25	411541	163686	26793106596	2	4

The concentration (ppm N), X, was plotted against the response (TN area), Y, and the slope, k, intercept, m, and regression, R2, were obtained from the trend function in Microsoft Excel. Figures 5 and 6 shows the calibration curves for two different occasions. The linear equation (Y=kX+m) of the regression line is also presented in these figures.





Figures 10 and 11. 1-5 ppm nitrogen calibration curves.

Table 19. Summary of calibration curves 1-2.

Calibration curve	Slope, k	Intercept, m	R^2
1	87806	-16359	0.9993
2	84087	-4406	0.9989
Average	**85946**	**-10382**	**0.9991**

Table 20 shows the errors in the slope and intercept of the averaged regression line. These values were calculated according to JN Miller and JC Millller "Statistics and chemometrics for analytical chemistry", 4th edition. $S_{y/x}$ is the estimated errors in the Y-direction for each regression line and $S_{y/x\ pooled}$ is the pooled errors in the Y-direction for the averaged regression line with the equation Y=85946X-10382. S_k is the standard deviation of the slope and $S_{k\ pooled}$ is the pooled standard deviation of the averaged value of the slope, 85946. S_m is the standard deviation of the intercept and $S_{m\ pooled}$ is the pooled standard deviation of the averaged value of the intercept, -10382.

Table 20. Errors in the slope and intercept of the regression line.

Calibration curve	$S_{y/x}$	S_k	S_m
1	3621	1145	3798
2	4316	1365	4526
Average calibration curve	$S_{y/x\ pooled}$= 3983	$S_{k\ pooled}$= 1260	$S_{m\ pooled}$= 4178

The formulas used for calculation of the errors in the slope and intercept of the regression line are shown in Figure 12. The formulas are taken from: JN Miller and JC miller "Statistics and chemometrics for analytical chemistry, 4th edition.

$$S_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}} \qquad S_k = \frac{S_{y/x}}{\sqrt{\sum_i (x_i - \bar{x})^2}}$$

$$S_m = S_{y/x} \sqrt{\frac{\sum_i x_i^2}{n \sum_i (x_i - \bar{x})^2}} \qquad S_{y/x\,\text{Pooled}} = \sqrt{\frac{(n_1 - 2)S^2_{y/x_1} + (n_2 - 2)S^2_{y/x_2}}{(n_1 + n_2 - 4)}}$$

$$S_{k\,Pooled} = \frac{S_{y/x\,Pooled}}{\sqrt{\sum_i (x_i - \bar{x})^2}} \qquad S_{m\,Pooled} = S_{y/x\,Pooled} \sqrt{\frac{\sum_i x_i^2}{n \sum_i (x_i - \bar{x})^2}}$$

Figure 12. Formulas for calculation of the errors in the slope and intercept of the regression line.

The relative standard deviation (cv_k) of the slope, k, is:

cv_k= 1259/85946 x 100= 1.46%

The relative standard deviation (cv_m) of the intercept, m, is:

cv_m= 4178/10382 x 100= 40.2%

The regression for all calibration curves is <0.99 which means that all calibration samples fit the regression line properly.

2.6.8 Calculation of a concentration and its random error

The error of a single standard concentration is the standard deviation that is obtained from the formula (obtained from JN Miller and JC Miller " Statistics and chemometrics for analytical chemistry", 4th edition):

$$S_{x0} = \frac{S_{y/x\,Pooled}}{b} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}}$$

Here *m* is the number of readings to obtain the value of y_0. In this case the number of m=2 since there are 2 calibration points obtained in the averaged calibration curve.

Table 21 shows each calibration concentration and its random error. These errors are calculated considering of the statistical data of the averaged calibration curve.

Table 21. Random errors of each calibration concentration.

Concentration (ppm N)	Sx_o	cv (%)
1	0,049	4,9
2	0,041	2,1
3	0,039	1,3
4	0,042	1,0
5	0,048	1,0

2.7 Summary, validation of TOC and TN equipment

Table 22 and 23 shows the summary tables for the method validation of the TOC/TN equipment. Parameters that fulfil the demands for the validation are approved.

Table 22. Summary of the method validation of TOC.

Parameter	Demand	Result	Approval
Repeatability and reproducibility of the response	cv of <10%	10 ppm C sample, cv= 1.8%	OK
		20 ppm C sample, cv= 3.1%	OK
		30 ppm C sample, cv= 1.1%	OK
		40 ppm C sample, cv= 3.0%	OK
		50 ppm C sample, cv= 1.2%	OK
Random error of a calculated concentration (calculated in consideration of the statistical data of the averaged calibration curve).	cv of <10%	10 ppm C sample, cv= 4.1%	OK
		20 ppm C sample, cv= 1.9%	OK
		30 ppm C sample, cv= 1.3%	OK
		40 ppm C sample, cv= 1.0%	OK
		50 ppm C sample, cv= 1.0%	OK
Accuracy (how the calibration points fits to the regression line)	R^2>0.99	>0.99	OK
Detection limit	≤ 1 ppm C	0.8 ppm C	OK
Reproducibility of the calibration curve	Relative standard deviation for the slope, cv_k of <5%	cv_k =1.55%	OK

Table 23. Summary of the method validation of TN

Parameter	Demand	Result	Approval
Repeatability and reproducibility of the response	cv of <10%	1 ppm N sample, cv= 2.8%	OK
		2 ppm N sample, cv= 3.6%	OK
		3 ppm N sample, cv= 1.7%	OK
		4 ppm N sample, cv= 1.4%	OK
		5 ppm N sample, cv= 1.2%	OK
Random error of a calculated concentration (calculated in consideration of the statistical data of the averaged calibration curve).	cv of <10%	1 ppm N sample, cv= 4.9%	OK
		2 ppm N sample, cv= 2.1%	OK
		3 ppm N sample, cv= 1.3%	OK
		4 ppm N sample, cv= 1.0%	OK
		5 ppm N sample, cv= 1.0%	OK
Accuracy (how the calibration points fits to the regression line)	R^2>0.99	>0.99	OK
Detection limit	≤ 1 ppm N	0.14 ppm N	OK
Reproducibility of the calibration curve	Relative standard deviation for the slope, cv_k of <5%	cv_k =1.46%	OK

All demands were fulfilled and the combined TOC/TN equipment is satisfactory for its purpose.

3 TOC, TN and elementary analysis

3.1 Preparation of samples for TOC/TN analysis

See Analytical report 1 “Sample preparation for investigating the impurities, extractables and chemical stability of Q Sepharose BB” for sample number and sample preparation procedure of the samples that were analyzed with the TOC/TN equipment. A 2-50 ppm carbon and 1-5 ppm nitrogen calibration curve was prepared on every analysis occasion, see section 2.4. 6 calibration points are necessary for carbon; 2,10, 20,30,40 and 50 ppm carbon. 5 calibration points are necessary for nitrogen; 1,2,3,4 and 5 ppm nitrogen. ~6 mL of the samples were added into sample tubes, specially designed for the TOC/TN analysator. When analyzing samples containing 0.2-0.5 M NaOH, 4.0 mL of the samples were added to the sample tubes containing 2.0 mL 2 M HCl.

TOC/TN analysis data

Figure 13 shows the TOC/TN analysis data for samples 11-30.

TOC and TN analysis

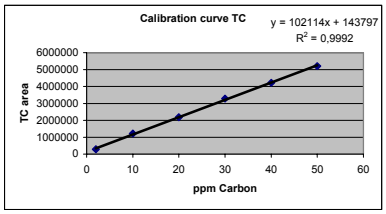
Analysis of samples: Sampling of Q Sepharose BB T-301065, Protocol 1 see Analytical report 1 page 10
Analysis date: 2005-01-27

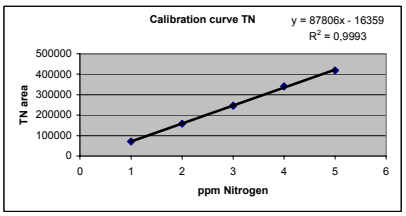
Calibration curve 2-50 ppm Carbon

ppm C	TC area 1	TC area 2	TC area 3	TC area average
2	279702	286992	282081	282925
10	1216153	1215511	1200345	1210670
20	2192184	2186400	2169825	2182803
30	3298219	3223481	3320299	3280666
40	4221654	4232731	4208041	4220809
50	5141312	5151484	5325872	5206223

Calibration curve Nitrogen 1-5 ppm

ppm N	TN area 1	TN area 2	TN area 3	TN area average
1	70023	70779	72173	70992
2	159964	155316	159682	158321
3	248763	244734	245643	246380
4	336355	346120	340029	340835
5	419231	419234	417826	418764





Sample	TC area 1	TC area 2	TC area 3	TC area average	ppm Carbon	Comment
11	248008	230822	241298	240043	0,94	
12	115392	122775	129247	122471	0,00	Below LOD
13	117480	123037	118833	119783	0,00	Below LOD
14	102931	106131	102844	103969	0,00	Below LOD
15	160378	161473	163608	161820	0,18	Below LOD
16	127180	127265	124298	126248	0,00	Below LOD
17	109608	107958	109211	108926	0,00	Below LOD
18	93794	83647	88031	88491	0,00	Below LOD
19	6142502	6238895	6276357	6219251	88,80	Over quantitation limit
20	268786	260880	273995	267887	1,81	
21	1473936	1494976	1505211	1491374	19,70	
22	298742	297440	282289	292824	2,18	
23	2470096	2441161	2466668	2459308	33,84	
24	366387	356504	359535	360809	3,17	
25	1419472	1461957	1422132	1434520	18,87	
26	261802	259978	252898	258226	1,67	
27	300978	287164	274746	287629	1,41	
28	196764	275012	232260	234679	0,89	
29	164821	146784	163745	158450	0,14	Below LOD
30	199502	192634	244626	212254	0,67	Below LOD

Sample	TN area 1	TN area 2	TN area 3	TN area average	ppm Nitrogen	Comment
11	0	0	0	0	0,00	Below LOD
12	0	0	0	0	0,00	Below LOD
13	0	0	0	0	0,00	Below LOD
14	0	0	0	0	0,00	Below LOD
15	0	0	0	0	0,00	Below LOD
16	0	0	0	0	0,00	Below LOD
17	0	0	0	0	0,00	Below LOD
18	0	0	0	0	0,00	Below LOD
19	654306	652919	666262	657829	11,46	Over quantitation limit
20	0	0	0	0	0,00	Below LOD
21	126060	128327	130728	128372	2,46	
22	36192	36003	36097	36097	0,89	
23	214446	206468	209479	210131	3,85	
24	0	0	0	0	0,00	Below LOD
25	105977	103933	106943	105618	2,07	
26	0	0	0	0	0,00	Below LOD
27	0	0	0	0	0,00	Below LOD
28	0	0	0	0	0,00	Below LOD
29	0	0	0	0	0,00	Below LOD
30	0	0	0	0	0,00	Below LOD

Figure 13.TOC/TN analysis data for samples 11-30.

Figure 14 shows the TOC/TN analysis data of samples 111-130.

TOC and TN analysis

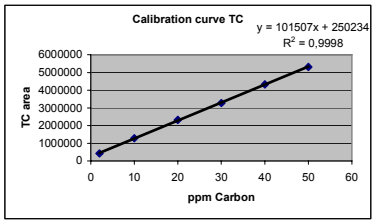
Analysis of samples: Sampling of Q Sepharose BB T-301462, Protocol 2 see Analytical report 1 page 11
Analysis date: 2005-01-28

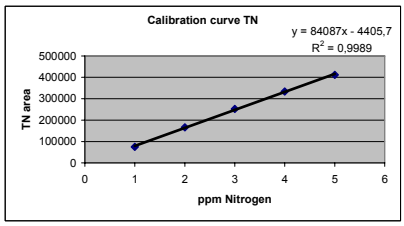
Calibration curve 2-50 ppm Carbon

ppm C	TC area 1	TC area 2	TC area 3	TC area average
2	421432	423272	430065	424923
10	1275778	1285003	1283633	1281471
20	2352517	2286162	2310070	2316250
30	3274534	3217287	3323842	3271888
40	4386978	4329843	4246763	4321195
50	5309378	5264423	5370218	5314673

Calibration curve Nitrogen 1-5 ppm

ppm N	TN area 1	TN area 2	TN area 3	TN area average
1	75914	75698	73048	74887
2	166148	165505	167930	166528
3	252226	250220	254246	252231
4	340916	329207	332140	334088
5	410057	405920	418645	411541





Sample	TC area 1	TC area 2	TC area 3	TC area av	ppm Carbon	Comment
111	206132	197719	207459	203770	0,59	Below LOD
112	158403	158910	148324	155212	0,00	Below LOD
113	141051	145731	138873	141885	0,00	Below LOD
114	130150	141848	130083	134027	0,00	Below LOD
115	178370	167513	184169	176684	0,32	Below LOD
116	129361	135992	130384	131912	0,00	Below LOD
117	239871	253691	250426	247996	0,00	Below LOD
118	147032	148738	133031	142934	0,00	Below LOD
119	7189099	7238032	7254354	7227162	103,53	Over quantitation limit
120	288604	297209	276077	287297	2,10	
121	2080105	2084455	2128266	2097609	28,56	
122	234154	256566	256364	249028	1,54	
123	2522072	2472096	2505090	2499753	34,44	
124	335521	310147	325266	323645	2,63	
125	1620502	1621496	1620451	1620816	21,59	
126	292706	285179	294780	290888	2,15	
127	255124	253941	248442	252502	1,06	
128	219611	245701	219323	228212	0,83	
129	408149	390134	398727	399003	2,50	
130	223782	221672	224143	223199	0,78	Below LOD

Sample	TN area 1	TN area 2	TN area 3	TN area average	ppm Nitrogen	Comment
111	0	0	0	0	0,00	Below LOD
112	0	0	0	0	0,00	Below LOD
113	0	0	0	0	0,00	Below LOD
114	0	0	0	0	0,00	Below LOD
115	0	0	0	0	0,00	Below LOD
116	0	0	0	0	0,00	Below LOD
117	0	0	0	0	0,00	Below LOD
118	0	0	0	0	0,00	Below LOD
119	750855	774801	757685	761114	13,22	Over quantitation limit
120	0	0	0	0	0,00	Below LOD
121	155649	161860	160691	159400	2,99	
122	0	0	0	0	0,00	Below LOD
123	207408	203329	207877	206205	3,78	
124	0	0	0	0	0,00	Below LOD
125	131757	130685	127266	129903	2,49	
126	0	0	0	0	0,00	Below LOD
127	0	0	0	0	0,00	Below LOD
128	0	0	0	0	0,00	Below LOD
129	0	0	0	0	0,00	Below LOD
130	0	0	0	0	0,00	Below LOD

Figure 14. TOC/TN analysis on samples 111-130.

Figure 15 shows the TOC/TN analysis data of samples 211-230.

TOC and TN analysis

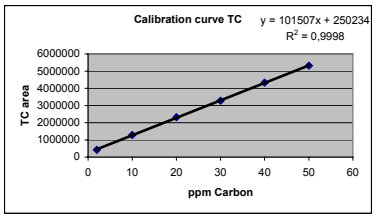
Analysis of samples: Sampling of Q Sepharose BB T-302192, Protocol 3 see Analytical report 1 page 12
Date for analysis: 2005-01-28

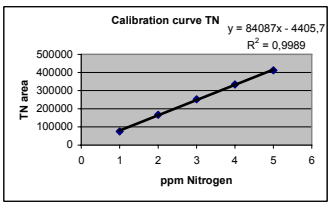
Calibration curve 2-50 ppm Carbon

ppm C	TC area 1	TC area 2	TC area 3	TC area average
2	421432	423272	430065	424923
10	1275778	1285003	1283633	1281471
20	2352517	2286162	2310070	2316250
30	3274534	3217287	3323842	3271888
40	4386978	4329843	4246763	4321195
50	5309378	5264423	5370218	5314673

Calibration curve Nitrogen 1-5 ppm

ppm N	TN area 1	TN area 2	TN area 3	TN area average
1	75914	75698	73048	74887
2	166148	165505	167930	166528
3	252226	250220	254246	252231
4	340916	329207	332140	334088
5	410057	405920	418645	411541





Sample	TC area 1	TC area 2	TC area 3	TC area average	ppm Carbon	Comment
211	235618	229005	231770	232131	0,00	Below LOD
212	423327	408863	426548	419579	1,67	
213	147824	133968	137620	139804	0,00	Below LOD
214	195420	196799	211570	201263	0,00	Below LOD
215	177883	178102	177911	177965	0,00	Below LOD
216	274869	250877	275952	267233	0,17	Below LOD
217	217202	230949	216588	221580	0,00	Below LOD
218	275673	280604	271790	276022	0,25	Below LOD
219	6127176	6234027	6295010	6218738	87,76	Over quantitation limit
220	227038	224476	224799	225438	0,00	Below LOD
221	1860483	1800850	1847696	1836343	15,63	
222	266656	257436	256476	260189	0,10	Below LOD
223	3042299	3118255	3073602	3078052	27,86	
224	346380	376627	372059	365022	1,13	
225	1627649	1677477	1629127	1644751	13,74	
226	249902	2255150	239318	914790	6,55	
227	236368	234810	247825	239668	0,00	Below LOD
228	361196	307828	321858	330294	0,79	Below LOD
229	154155	150008	149993	151385	0,00	Below LOD
230	222074	222921	238265	227753	0,00	Below LOD

Sample	TN area 1	TN area 2	TN area 3	TN area average	ppm Nitrogen	Comment
211	0	0	0	0	0,00	Below LOD
212	0	0	0	0	0,00	Below LOD
213	0	0	0	0	0,00	Below LOD
214	0	0	0	0	0,00	Below LOD
215	0	0	0	0	0,00	Below LOD
216	0	0	0	0	0,00	Below LOD
217	0	0	0	0	0,00	Below LOD
218	0	0	0	0	0,00	Below LOD
219	667121	675899	662703	668574	11,64	Over quantitation limit
220	0	0	0	0	0,00	Below LOD
221	153845	151041	150073	151653	2,86	
222	0	0	0	0	0,00	Below LOD
223	1412667	1461005	1350733	1408135	24,21	Over quantitation limit
224	0	0	0	0	0,00	Below LOD
225	129894	129897	133573	131121	2,51	
226	0	0	0	0	0,00	Below LOD
227	0	0	0	0	0,00	Below LOD
228	0	0	0	0	0,00	Below LOD
229	0	0	0	0	0,00	Below LOD
230	0	0	0	0	0,00	Below LOD

Figue 15. TOC/TN analysis on samples 211-230.

Figure 16 shows the TOC/TN analysis of the extracts from the PFE of Q Sepharose BB.

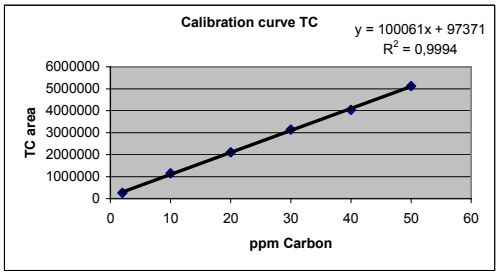
Analysis of samples: PFE samples extracted from samples 10 (T-301065), 110 (T-301462) and 210 (T-302192)

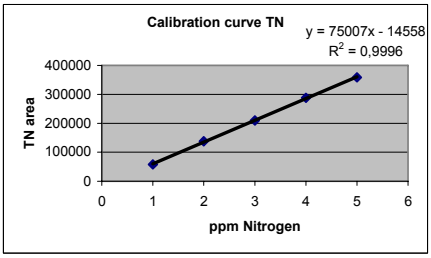
Calibration curve 2-50 ppm Carbon

ppm C	TC area 1	TC area 2	TC area 3	TC area average
2	254628	252999	253714	253780
10	1152362	1162116	1119461	1144646
20	2093459	2067199	2142254	2100971
30	3145097	3165682	3099515	3136765
40	4028003	3973094	4120927	4040675
50	5051749	5170350	5127819	5116639

Calibration curve Nitrogen 1-5 ppm

ppm N	TN area 1	TN area 2	TN area 3	TN area average
1	58972	57209	58985	58389
2	137374	136901	139130	137802
3	211066	208705	209541	209771
4	282492	289219	292505	288072
5	350124	363395	361353	358291





Sample	TC area 1	TC area 2	TC area 3	TC area average	ppm Carbon	Comment
Blank 1	173639	167567	159086	166764	0,69	
Blank 2	96123	91015	92037	93058	0,00	Below LOD
Blank 3	160322	144509	218608	174480	0,77	Below LOD
10:1	270969	280048	279424	276814	1,79	
10:2	87811	87523	83495	86276	0,00	Below LOD
110:1	156715	158820	159047	158194	0,61	Below LOD
110:2	69394	68843	71897	70045	0,00	Below LOD
210:1	362121	381101	392005	378409	2,81	
210:2	64091	73452	78014	71852	0,00	Below LOD

Sample	TN area 1	TN area 2	TN area 3	TN area average	ppm Nitrogen	Comment
Blank 1	0	0	0	0	0,00	
Blank 2	0	0	0	0	0,00	
Blank 3	0	0	0	0	0,00	
10:1	0	0	0	0	0,00	
10:2	0	0	0	0	0,00	
110:1	0	0	0	0	0,00	
110:2	0	0	0	0	0,00	
210:1	0	0	0	0	0,00	
210:2	0	0	0	0	0,00	

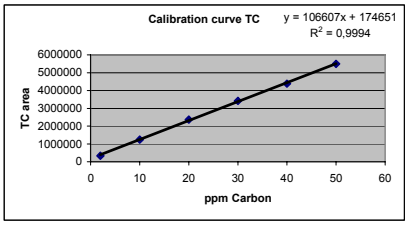
Figure 16. TOC/TN analysis of the extracts from the PFE of Q Sepharose BB.

Figure 17 shows the TOC/TN analysis data for samples 311-313.

TOC/TN analysis

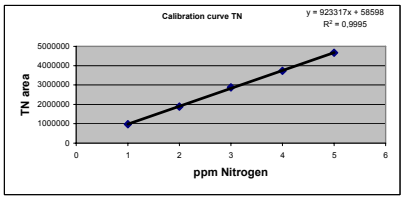
Analysis of samples: 311-313, 4 mL of sample+ 2 mL 1M HCl for TOC no dilution for TN
Data file: 2005-03-21_2.adb and 2005-05-13.adb
Calibration curve 2-50 ppm Carbon

ppm C	TC area 1	TC area 2	TC area 3	TC area average
2	332111	347011		339561
10	1240600	1253239		1246920
20	2383382	2340314		2361848
30	3432507	3407303		3419905
40	4384373	4389047		4386710
50	5485516	5508902		5497209



Calibration curve Nitrogen 1-5 ppm

ppm N	TN area 1	TN area 2	TN area 3	TN area average
1	971268	968080	993755	977701
2	1870015	1852707	1918680	1880467
3	2890798	2873865	2882101	2882255
4	3778444	3725135	3703219	3735599
5	4657509	4693857	4648791	4666719



Sample	TC area 1	TC area 2	TC area 3	TC area average	ppm Carbon	Comment
Blank	112358	115628		113993	0,00	
311	2247922	2245046		2246484	29,01	
312	2123953	2117770		2120862	27,25	
313	2163059	2186546		2174803	28,00	

Sample	TN area 1	TN area 2	TN area 3	TN area average	ppm Nitrogen	Comment
311	3329231	3397211	3443475	3389972	3,61	
312	2892968	2897539	2922805	2904437	3,08	
313	2821103	2895271	2846598	2854324	3,03	

Figure 17. TOC analysis of samples 311-313.

3.2 Bromine data

Table 24 shows the bromine analysis results from Analytica AB. Report id L0501493, L0503424 and L0503976.

Table 24. Bromine analysis results

Sample no	ppm bromine	ppm bromid
10	<0,1	<1
11	0,175	<1
12	<0,01	<1
13	<0,1	<1
15	0,44	<1
16	<0,01	<1
17	0,168	<1
19	1700	1640
20	<0,01	<1
21	810	762
23	1800	1840
24	0,035	<1
25	1100	1180
27	0,16	<1
28	<0,01	<1
29	<0,1	<1
110	<0,1	<1
111	0,14	<1
112	<0,01	<1
113	<0,1	<1
115	0,38	<1
116	<0,01	<1
117	<0,1	<1
119	1600	1580
120	<0,01	<1
121	770	711
123	2100	2000
124	0,066	<1
125	1200	1130
127	0,12	<1
128	0,03	<1
129	<0,1	<1
210	<0,1	<1
211	<0,1	<1
212	0,011	<1
213	0,15	<1
215	0,49	<1
216	<0,01	<1
217	0,11	<1
219	1400	1340
220	<0,01	<1
221	860	828
223	2000	1930
224	0,101	<1
225	1300	1220
227	0,23	<1
228	0,042	<1
229	<0,1	<1

Sample	Bromine mg/l	Bromide mg/l
311	188	189
312	190	193
313	163	170
320	34,9	36,7
321	20,7	21,7
322	26,9	26,9

3.3 Sulphur data

Table 25 shows the sulphur data analysed at Analytica AB, report id 052825.

Table 25. Sulphur analysis data

Sample	Sulphur content (ppm)
27	1.88
29	1.23
127	1.54
129	1.34
227	0.80
229	0.725

3.4 Heavy metals and elements in dried Q Sepharose BB

The dried Q Sepharose BB batches described in Analytical report 1, section 2.2 were sent to an accredited laboratory, Analytica AB, for heavy metals and elemental analysis. Carbon was analysed at another accredited laboratory, Mikrokemi AB. Table 26 shows the results from the heavy metals and elementary analyses of Q Sepharose BB. Table 27 shows the elemental analysis results of C, H, N, O and S.

Table 26. Analysis of heavy metals and elements of dried Q Sepharose BB report no L051492.

	Q Sepharose BB T-301065			**Q Sepharose BB T-301462**			**Q Sepharose BB T-302192**		
Element	Sample 1 (mg/kg)	Sample 2 (mg/kg)	**Average (mg/kg)**	Sample 1 (mg/kg)	Sample 2 (mg/kg)	**Average (mg/kg)**	Sample 1 (mg/kg)	Sample 2 (mg/kg)	**Average (mg/kg)**
As	0,159	0,167	**0,163**	<0,1	<0,1	**<0,1**	<0,1	<0,1	**<0,1**
B	30,8	30,8	**30,8**	29,8	30,5	**30,15**	42,4	42,9	**42,65**
Cd	<0.006	0,0069	**0,0065**	<0,006	<0,006	**<0,006**	<0,006	<0,006	**<0,006**
Co	0,0091	0,0096	**0,00935**	0,0138	0,0065	**0,01015**	0,0086	<0,006	**0,007**
Cr	0,0888	0,0688	**0,0788**	0,0581	0,0517	**0,0549**	0,0796	0,0693	**0,07445**
Cu	<0,1	<0,1	**<0,1**	0,153	<0.1	**0,13**	0,173	0,158	**0,1655**
Hg	0,016	<0,01	**0,013**	<0,01	<0,01	**<0,01**	<0,01	<0,01	**<0,01**
Mn	<0,05	<0,05	**<0,05**	<0,05	<0,05	**<0,05**	<0,05	<0,05	**<0,05**
Ni	0,0661	0,0808	**0,07345**	0,096	0,0858	**0,0909**	0,106	0,158	**0,132**
P	3,17	<2	**2,6**	<2	<2	**<2**	<2	<3	**<2,5**
Pb	<0,05	<0,05	**<0,05**	<0,05	<0,05	**<0,05**	<0,05	<0,05	**<0,05**
Zn	<0,3	<0,2	**<0,25**	<0,2	<0,2	**<0,2**	<0,2	1,01	**0,6**

Table 27. Elemental analysis on dried Q Sepharose BB, report id 2004-12-21 nr 0403980-0403982 and report id 2005-04-28 nr 0500682-0500686.

Batch Q Sepharose BB	C 1 (%w/w)	C 2 (%w/w)	**C Average (%w/w)**	H 1 (%w/w)	H 2 (%w/w)	**H Average (%w/w)**	N 1 (%w/w)	N 2 (%w/w)	**N Average (%w/w)**	O 1 (%w/w)	O 2 (%w/w)	**O Average (%w/w)**	S 1 (%w/w)	S 2 (%w/w)	**S Average (%w/w)**
T-301065	44.7	44.8	**44.8**	7.1	7.1	**7.1**	2.1	2.1	**2.1**	36.0	35.9	**36.95**	2.2	2.1	**2.15**
T-301462	45.4	45.4	**45.4**	7.2	7.2	**7.2**	2.0	2.0	**2.0**	36.6	36.3	**36.45**	2.0	2.0	**2.0**
T-301086	45.9	46.0	**46.0**	7.3	7.3	**7.3**	2.0	2.0	**2.0**	35.4	35.8	**35.6**	1.83	1.87	**1.85**
T-243424	45.3	45.0	**45.15**	7.5	7.5	**7.5**	2.4	2.3	**2.35**	35.9	36.2	**36.05**	2.4	2.3	**2.35**
T-279882	44.9	44.8	**44.85**	7.3	7.3	**7.3**	2.3	2.2	**2.25**	36.6	36.1	**36.35**	2.2	2.2	**2.2**
T-280981	44.8	44.5	**44.65**	7.3	7.2	**7.25**	2.2	2.2	**2.2**	36.2	36.8	**36.5**	1.9	2.0	**1.95**
T-282557	44.8	44.8	**44.8**	7.5	7.3	**7.4**	2.3	2.3	**2.3**	36.2	36.3	**36.25**	2.1	2.2	**2.15**
T-293719	45.1	44.8	**44.95**	7.4	7.5	**7.45**	2.3	2.3	**2.3**	37.2	36.9	**37.05**	2.2	2.2	**2.2**

4 Evaluation of the PFE extracted samples

4.1 Results TOC analysis

Pressurised Fluid Extraction (PFE) was performed on three batches of Q Sepharose BB T-301065, T-301462 and T-302192. See Analytical report 1 section 4 for detailed sample preparation of the extraction samples.

Table 28 shows the TOC results for the PFE extracted Q Sepharose BB batches. Data was obtained from Figure 16.

Table 28. TOC results for the PFE extracted Q Sepharose BB batches.

Sample	Weight gel into extraction cell (g)	Volume wet sedimented gel (mL)	Volume extraction solution Extraction 1 (mL)	Volume extraction solution Extraction 2 (mL)	Carbon content Extraction 1 (ppm)	Carbon content Extraction 2 (ppm)
Empty extraction cell, Blank	-	-	23	24	0	0
Q Sepharose BB T-301065	6.584	6.584 / 0.725[1] = **9.08**	17.69	18.95	1.8	0
Q Sepharose BB T-301462	6.552	6.552/ 0.740[1] = **8.85**	18.21	18.85	0.6	0
Q Sepharose BB T-302192	6.504	6.504 / 0.728[1] = **8.93**	18.09	18.05	2.8	0

[1] Dry weight, g/mL, obtained from Analytical report 1.

4.2 Discussion

The PFE system was washed with distilled water before extraction, in the attempt to remove residual solvents, until the response of the TOC equipment was zero. The results from the PFE extracted samples show TOC levels similar to those found in the static extraction described in the following.

5 Evaluation of the static extracted samples

5.1 Results TOC/TN and bromine

The result in Table 29 shows the total amount carbon, nitrogen and bromine extracted from Q Sepharose BB after 160-170 hours static extraction time and different extraction temperatures with milli-Q water. Carbon and nitrogen values were obtained from Figures 13-15 in this analytical report. Bromine values were obtained from Table 24 in this analytical report. Sample preparation for the static extracted samples is described in Analytical report 1.

Table 29. Carbon, bromine and nitrogen results of Q Sepharose BB.

Batch Q Sepharose BB	ppm Carbon (sample -blank)	Total carbon extracted from 40 mL gel[1] (μg)	**Carbon content[2]** (μg / mL wet sed gel)	Ppm Bromine (sample-blank)	Total bromine extracted from 40 mL gel[1] (μg)	**Bromine content[2]** (μg / mL wet sed gel)	ppm Nitrogen (sample-blank)	Total nitrogen 40 mL gel[1] (μg)	**Nitrogen content[2]** (μg / mL wet sed gel)	ppm sulphu (sample-blank)	Total sulphur extracted from 40 mL gel[1] (μg)	**Sulphur content[2]** (μg / mL wet sed gel)
T-301065 static extraction at 40°C	1.41-0.89= <0.8	0.8 x 63.9 = <51	**<1.3**	0.16	0.16 x 63.9 = 10.2	**0.26**	<1	1x 63.9= <64	**<1.6**	1.88	1.88x 63.9= 120	**3.0**
T-301065 static extraction at 20°C	<0.8	0.8 x 63.9 = <51	**<1.3**	<0.1	0.1 x 63.9 = <6.4	**0.16**	<1	1x 63.9 = <64	**<1.6**	1.23	1.23x 63.9= 79	**2.0**
T-301462 static extraction at 40°C	1.06-0.83= <0.8	0.8 x 64.7= <52	**<1.3**	0.12	0.12 x 64.7= 7.8	**0.19**	<1	1x 64.7= <65	**<1.6**	1.54	1.54x 64.7= 100	**2.5**
T-301462 static extraction at 20°C	2.50-0.78= 1.7	1.7 x 64.7= 110	**2.75**	<0.1	0.1 x 64.7= <6.5	**0.16**	<1	1x 64.7= 65	**<1.6**	1.34	1.34x 64.7= 87	**2.2**
T-302192 static extraction at 40°C	<0.8	0.8 x 64.1= <51	**<1.3**	0.23	0.23 x 64.1= 14.7	**0.37**	<1	1 x 64.1= 64	**<1.6**	0.80	0.80x 64.1= 51	**1.3**
T-302192 static extraction at 20°C	<0.8	0.8 x 64.1= <51	**<1.3**	0.042	0.042 x 64.1= 2.7	**0.07**	<1	1 x 64.1= 64	**<1.6**	0.725	0.725x 64.1= 46	**1.15**

[1] Total amount of element is calculated by adding the moisture content in vacuum suctioned gel that corresponds to 40 mL sedimented wet sedimented gel (see analytical report 1) plus 40 mL (added solution).

[2] Element content is calculated by dividing the total amount of element with 40 (total volume wet sedimented gel).

5.2 Calculation of potential impurities in Q Sepharose BB

To calculate the maximal content of potential impurities in the gel, the analysis data from the static extraction experiment were used. Table 30 shows the potential impurities in Q Sepharose BB. Since there are three batches of Q Sepharose BB investigated, the batch of Q Sepharose BB that showed the highest amount of the analysed element in the extract (see Table 29) was used for calculation.

Table 30. Calculation of potential impurities in the extraction samples.

Calculation of potential impurities from the extraction experiments

Substance	Formula	Molecular weight (g/mol)	Element for analysis	Weight, element of interest (g/mol)	% (w/w) of element in substance	Amount of element in extract (µg/mL)	Maximal amount of extracted element (µg / mL wet sedimented gel)	Maximal amount of extracted substance (µg / mL wet sedimented gel)
Agarose fragments	$C_{14}H_{18}O_{10\ (subunit)}$	-	C	-	44.8[a]	1.7	2.75	7.1
Betaine	$C_5H_{11}NO_2$	117	C	60	51.3	1.7	2.75	5.8
Bromine	Br_2	159.8	Br	159.8	100	0.23	0.4	0.4
Bromoacetic acid	$BrC_2H_3O_2$	138.95	Br	79.9	57.5	0.23	0.4	0.7
Ethanol	C_2H_6O	46.1	C	24	52.1	1.7	2.75	5.8
Ethyl cellulose	$C_{12}H_{23}O_{6\ (subunit)}$	263	C	144	54.7	1.7	2.75	5.5
Glycerol	$C_3H_8O_3$	92	C	36	39.1	1.7	2.75	7.7
Sodium acetate	$C_2H_3NaO_2$	82	C	24	29.3	1.7	2.75	10.2
Sodium bicarbonate	$CHNaO_3$	84	C	12	14.3	1.7	2.75	21.0
Sodium bromate	$NaBrO_3$	150.9	Br	79.9	62.5	0.23	0.4	0.64
Sodium bromide	NaBr	102.9	Br	79.9	52.9	0.23	0.4	0.76
Sodium chloride	NaCl	58.4	-	-	-	-	-	-
Sodium formate	$CHNaO_2$	68	C	12	17.6	1.7	2.75	17.0
Sodium glycollate	$C_2H_3O_3Na$	98.04	C	24	24.5	1.7	2.75	12.2
Sodium sulphate	Na_2SO_4	142	S	32	22.5	1.9	3.0	14.7
Trimethylamine	C_3H_6N	104	C	36	34.6	1.7	2.75	8.7
Toluene	C_7H_8	92.1	C	84	91.2	1.7	2.75	3.3

[a] Obtained from carbon analysis of dried Q Sepharose BB T-301065, see Table 27.

The maximum levels of potential impurities sodium borate and poly (oxyethylene) nonylphenyl phosphate ester sodium salt were estimated from elemental concentration data on dried Q Sepharose BB. Data was taken for the batch that contained of the highest amount boron and phosphorus (see Table 26). Table 31 shows the maximal amount of sodium borate and Poly (oxyethylene) nonylphenyl phosphate ester sodium salt in the gel.

Table 31. Calculation of sodium borate and Poly (oxyethylene) nonylphenyl phosphate ester sodium salt in dried Q Sepharose BB.

Substance	Formula	Molecular weight (g/mol)	Element for analysis	Weight, element of interest (g/mol)	% (w/w) of element in substance	Amount of element in dried gel (µg/g)	Maximal amount of element (µg / mL wet sedimented gel)	Maximal amount of substance (µg / mL wet sedimented gel)
Sodium borate	$Na_2B_4O_7$	201	B	43	21.39	42.65	5.4	25.2
Poly (oxyethylene) nonylphenyl phospahte ester sodium salt	$C_{54}H_{113}O_{16}P$	1048	P	31	3.0	2.6	0.33	10.9

Analytical report 3, determination of specific compounds

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1 Introduction

This analytical report describes the determination of five specific compounds; epichlorohydrin, glycidol, 3-chloro-1,2-propandiol, allyl glycidyl ether and allyl glycerol ether. It also describes the validation of the methods used.

The compounds are all volatile and consequently suited for gas chromatographic analysis. A concentration step was required to decrease detection limits. The first choice was solid phase micro extraction (SPME). In this technique, a fiber coated with an adsorbing material is held in or above the sample in a closed vial for a specified time to allow the compounds to adsorb onto the fiber. Then the fiber is withdrawn from the sample vial and placed in the injector of a GC. There, the adsorbed compounds are thermally desorbed from the fiber and transported to the GC column for separation and quantification.

However, the SPME technique was successful only for epichlorohydrin and allyl glycerol ether. Evaporation under mild conditions could be used for 3-chloro-1,2-propandiol and allyl glycerol ether. Those compounds have high enough boiling points not to disappear during the evaporation. Glycidol, which had a boiling point of 160 °C disappeared, though. Suitable methods for determination of water solutions of glycidol at low concentrations were not found in the literature. In this case, gas chromatography with injection of a larger volume than normal was done to decrease detection limit.

The tested samples were pressurized fluid extractions (PFE) of Q Sepharose Big Beads and Allyl Sepharose Big Beads according to Analytical Report 1. Samples from experiments testing chemical stability were not analyzed, since these five compounds are not degradation products from the Sepharose resin.

2 Determination of epichlorohydrin and allyl glycidyl ether

2.1 Introduction

This part describes the determination of the compounds epichlorohydrin and allyl glycidyl ether. These compounds are suitable for gas chromatographic analysis (GC) since they are volatile. The concentration technique chosen was head space solid phase micro extraction (HS SPME). In this technique, a fiber coated with an adsorbing material is held above the sample in a closed vial for a specified time to allow compounds to adsorb onto the fiber. Then the fiber is withdrawn from the solution and placed in the injector of a GC, where the adsorbed compounds are thermally desorbed from the fiber and transported to the GC column for separation and quantification.

2.2 Experimental

2.2.1 Chemicals

Epichlorohydrin, 30-0150-00, from Amersham Biosciences

Allyl glycidyl ether, 30-9806-00, from Amersham Biosciences

Ethanol, 99.5 % from Kemetyl, Sweden

Distilled water

Anhydrous sodium sulphate, from Fluka, Switzerland.

2.2.2 Tested samples

Pressurized fluid extractions (PFE) according to Analytical report 1 of the batches of the intermediate Allyl Sepharose Big Beads;

T-300862, T- 301232 and T-302183

and the final Q Sepharose Big Beads:

T-301065, manufacturing date, Dec. 7,2003.

T-301462, manufacturing date, Dec. 15, 2003

T-302192, manufacturing date, Feb. 19, 2004.

2.2.3 Apparatus

A "black" SPME fiber, 75 μm Carboxen/polydimethylsiloxane, from Supelco (Belafonte, PA, USA) was used for sample concentration. The fiber was mounted in a CombiPAL autosampler from CTC Analytic, Switzerland. Samples were injected onto Optic 3 High Performance Injector from ATAS International, The Netherlands, and further to CP-3380 gas chromatograph from Varian, Inc. Lake Forest, CA, USA. An injector liner with 2 mm I.D. was used. The detector was a Saturn 2000 GC/MS/MS ion trap, also from Varian, Inc.

The separation column was a HP-5MSI from Agilent with dimensions, 30 m x 0.25 mm I.D. and film thickness 0.25 μm.

2.2.4 Settings

Adsorption was done at 55 °C for 30 minutes. Desorption was done at an initial injector temperature of 60 °C. After 5 s the temperature was ramped by 16 °C/s to 260 °C. The total desorption time was 2 minutes. The carrier gas was helium and the column flow 1 ml/min. Split less injections were made. After 2 minutes a split flow of 50 ml/min was set. The oven temperature was 30 °C at the start. After 1 minute it was raised by 15 °C/ min to 180 °C. The total GC time was 9 minutes. For detection, EI ionization at 70 eV was done. Ions with 40-85 amu were collected between 1 and 9 minutes. The scan rate was 2 scan/s.

In the case of measuring Allyl Sepharose, the initial time at 30 C was 2 minutes, the collected ions 35 to 65 amu and the scan rate 4 scan/s.

Ion 57 was used for quantification.

2.2.5 Sample preparation.

85 ± 3 mg sodium sulphate and 500 μl of samples were added to 2 ml glass vials with septa and crimp tops. After sealing, the vials were placed in the auto sampler for analysis.

Calibration solutions of approximately 2000 ppm (w/v) of epichlorohydrin and allyl glycidyl ether were prepared in distilled water and further diluted to about 1 or 0.1 ppm. From those solutions, small amounts were added to vials with sodium sulphate and distilled water.

Measurements and calibration were done two days. The first day, a two week old stock calibration solution was used and the solvent in the vials was distilled water. The second day, a

fresh stock solution was prepared, the solvent in the vials was the PFE extract of batch 301065 and a fresh fiber was used. One 50 ppb sample was prepared in water.

2.3 Results

2.3.1 Validation.

Specificity

Spectra of epichlorohydrin and allyl glycidyl ether are shown in Figure 2.1. The retention times of epichlorohydrin and allyl glycidyl ether is 3.5 and 5.4 minutes, respectively. The chromatograms in Figure 2.1 are from the type of runs where the initial oven time was 1 minute. In case of initial oven time of 2 minutes, the retention times are increased to 3.8 and 6.0 minutes, respectively.

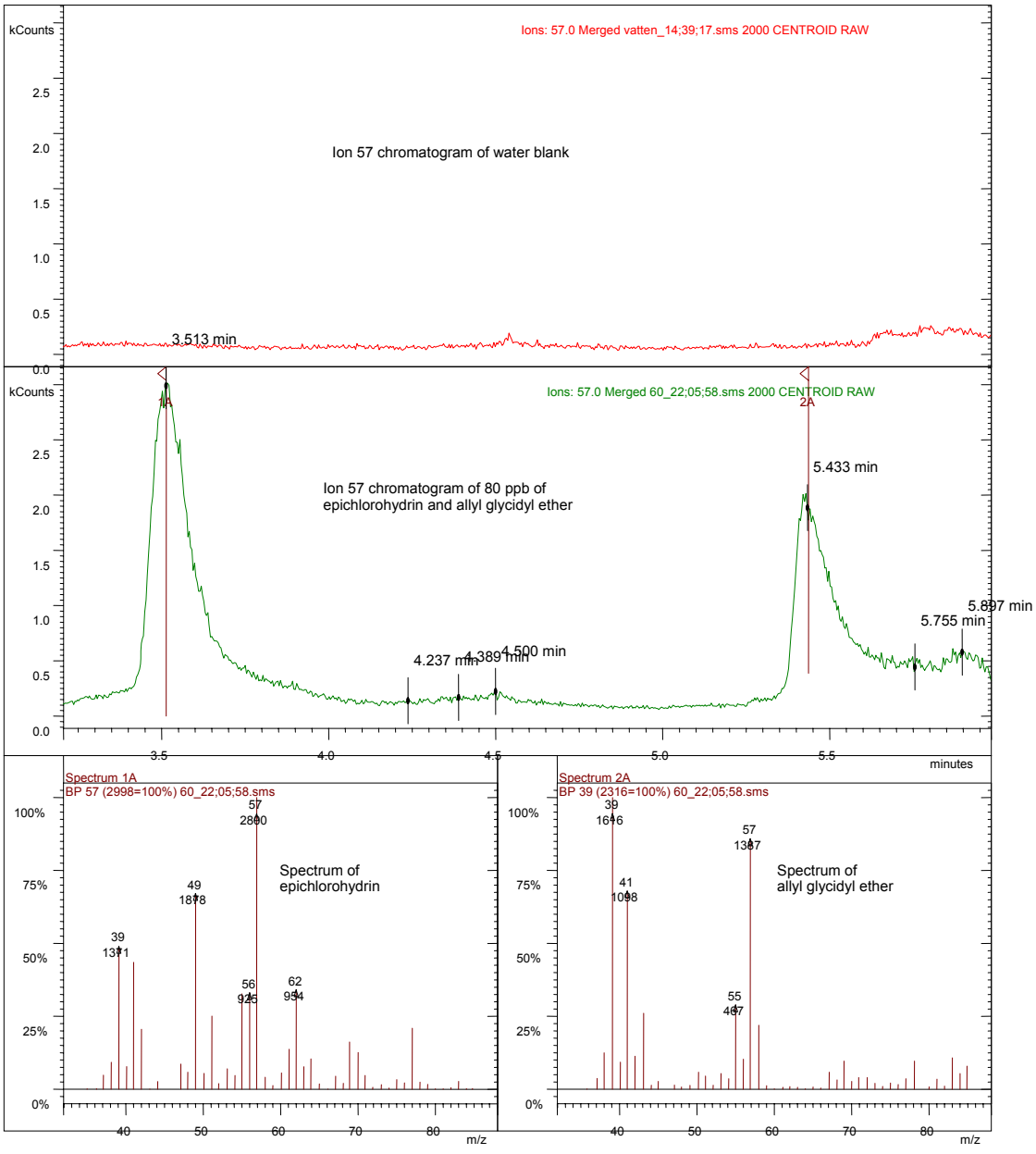

Figure 2.1. Upper window: Ion 57 chromatogram of water. Middle window: Ion 57 chromatogram of an 80 ppb solution of epichlorohydrin and allyl glycidyl ether. Lower windows: spectra of epichlorohydrin (left) and allyl glycidyl ether (right).

Matrix effects and detection limit

Head space methods are sensitive to the matrix. In this case the samples from PFE extraction were very close to water according to carbon analysis in Analytical report 2 and consequently,

matrix effects were not expected. The results of two calibrations, the first prepared in water and the second in a solution from a PFE extraction, are shown in figure 2.2, where the areas of ion 57 are plotted against concentration. The slopes of the graphs indicate that there are no matrix effects of importance. The slopes of the epichlorohydrin graphs differ between the occasions, but this difference has probably other reasons than matrix effects. Day two was run using a new fiber and a freshly prepared stock calibration solution and these things could also influence the result. The result stresses the importance of always running calibration solution together with samples.

Detection limit

The lowest standard, 20 ppb, was easily integrated both days of calibration. Blanks gave no response (Figure 2.1 upper window). Thus, 20 ppb can be set as detection limit.

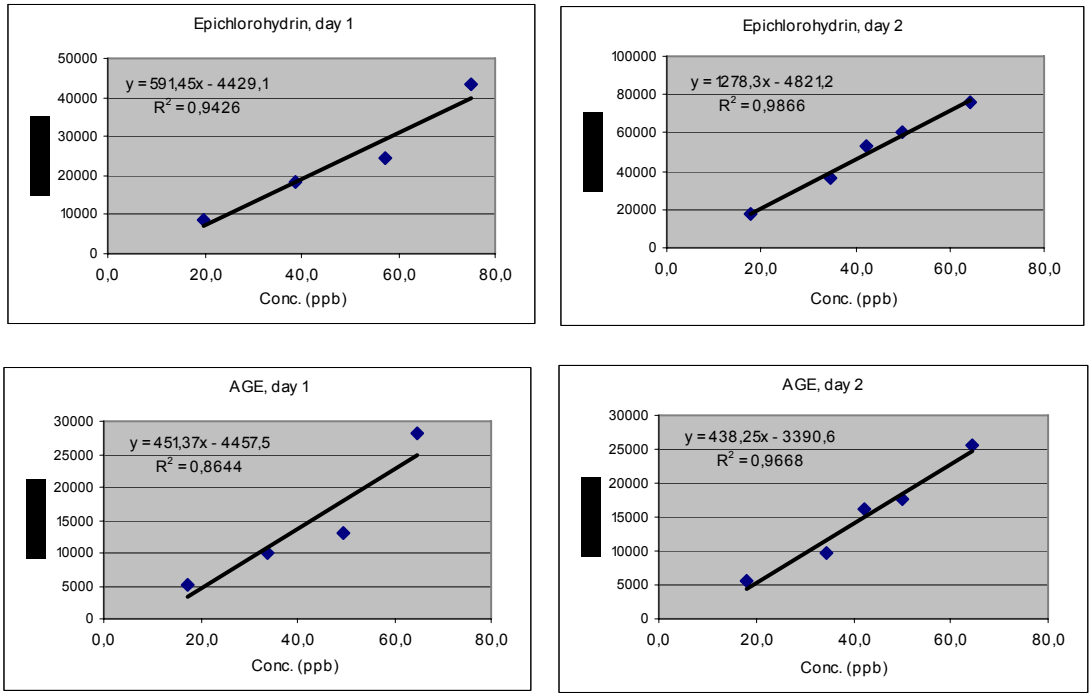

Figure 2.2. Calibration graphs of epichlorohydrin (upper two windows) and allyl glycidyl ether (lower two windows). Calibration at two different occasions are shown (day 1 and day 2), which differ in calibration solution, solvent and fiber.

2.3.2 Sample measurements

Examples of analysis of samples from PFE extractions are shown in Figure 2.3 and 2.4.

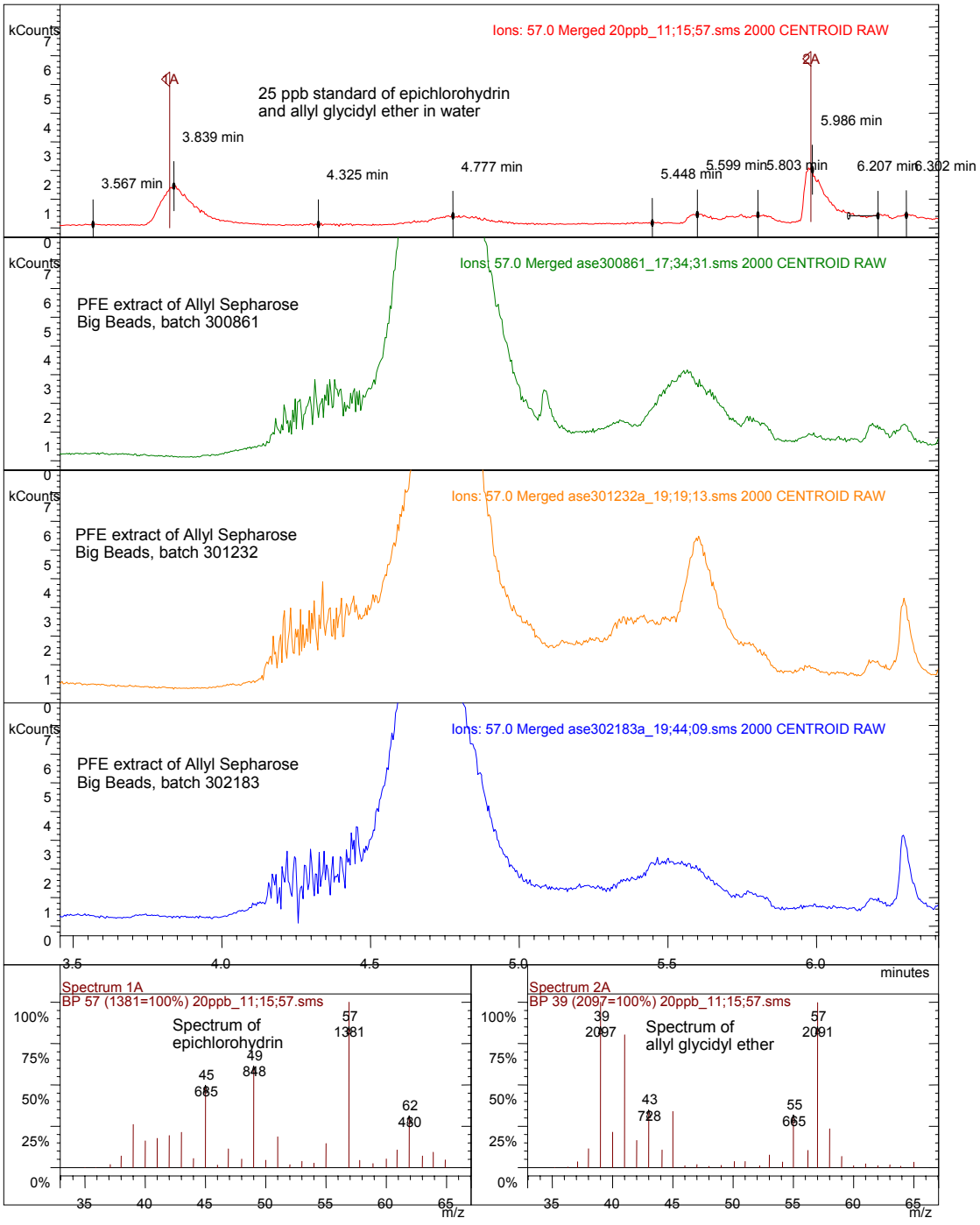

Figure 2.3. Ion 57 chromatograms of PFE extracts of three batches of Allyl Sepharose Big Beads and a standard prepared in water.

Chromatogram Plots

Plot 1: e:\13aprilkal2\qbb1_12;04;53.sms Ions: 57.0 Merged
Plot 2: e:\13aprilkal2\qbb2_12;41;06.sms Ions: 57.0 Merged
Plot 3: e:\13aprilkal2\qbb3_13;17;19.sms Ions: 57.0 Merged
Plot 4: e:\13aprilkal2\20_14;30;46.sms Ions: 57.0 Merged

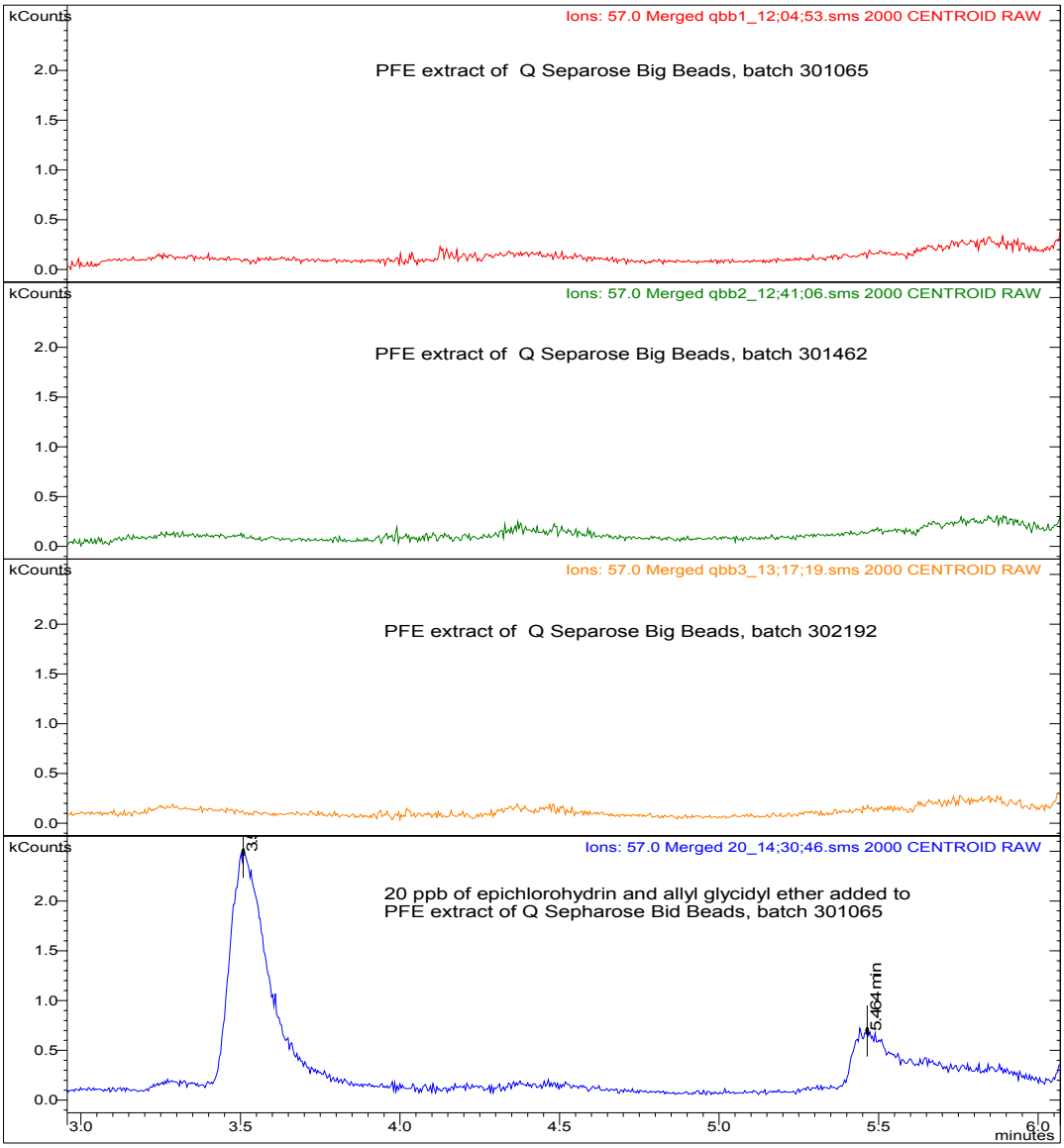

Figure 2.4. Ion 57 chromatograms of PFE extracts of three batches of Q Sepharose Big Beads and the lowest standard of epichlorohydrin and allyl glycidyl ether prepared in the PFE extract of batch 301065.

The most important information in the two figures is that neither epichlorohydrin nor allyl glycidyl ether can be detected in any of the batches. Consequently, the concentration is below 20 ppb.

The retention times of the standard compounds are not exactly the same in the two figures due to the slight difference in GC program used. As can be seen, there are peaks of unidentified

compounds in the chromatograms of the intermediate (Figure 2.3). However, these substances are expected to be washed out during the following process steps. This is evidenced by the fact that the chromatograms of the final Q Sepharose Big Beads batches (Figure 2.4) are not only free from epichlorohydrin and allyl glycidyl ether. They are also free from the large peak between epichlorohydrin and allyl glycerol ether.

Table 3.1. Sample measurement results.

Sample	Area of epichlorohydrin	Area of allyl glycidyl ether
Batch 301065 (10C:1)*	not detected	not detected
Batch 301462 (110C:1)*	not detected	not detected
Batch 302192 (210C:1)*	not detected	not detected

* = notation according to Analytical report 1

2.4 Calculation of the maximum amount of epichlorohydrin and allyl glycidyl ether

The maximum amount of epichlorohydrin and allyl glycidyl ether in the resin, C_m, was calculated from the LOD in the extracts according to the following expression:

C_m = *LOD (in μg/ml solution) x* V_t / V_{resin}

where V_{resin} is the volume of sedimented resin and V_t is the volume of added water + the water present in the resin. The maximum amounts of glycidol in the different samples are given in Table 4.2.

To calculate the overall maximum concentration, the largest value, 0.044 has been taken and been rounded up to the nearest value with one significant figure; 0.05.

Thus, the maximum concentration of epichlorohydrin and allyl glycidyl ether in washed Q Sepharose Big Beads resins is 0.05 μg/ml of sedimented resin.

Table 2.1. Calculation of maximum amounts of analysed compounds.

Sample	V_{resin} (ml)	V_t (ml)	C_m (μg/ml of sedimented resin)
Batch 301065	8.89	17.6	0.040
Batch 301462	8.53	18.7	0.044
Batch 302192	8.60	18.4	0.043

3 Determination of 3-chloro-1,2-propanediol and allyl glycerol ether

3.1 Introduction

This part describes the determination of 3-chloro-1,2-propanediol and allyl glycerol ether. For concentration, evaporation to dryness was done. The boiling points of these compounds were high enough to prevent them from disappearing during evaporation at mild conditions. The compounds were then silylated to improve peak shapes and decrease boiling points.

3.2 Experimental

3.2.1 Chemicals

3-Chloro-1,2-propanediol, 98 %, cat. 10,727-1, from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. This compound is often referred to as chloropropanediol in the text.

Allyl glycerol ether, 99%, Cat. 25,173-9, also from Sigma-Aldrich

Bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane, cat. 270123, from REGIS Technologies, Inc., Morton Grove, Illinois, USA. This chemical is referred to as Regisil in the text.

Distilled water.

3.2.2 Tested samples

Pressurized fluid extractions (PFE) according to Analytical report 1 of the following batches of Q Sepharose Big Beads:

T-301065, manufacturing date Dec. 7, 2003

T-301462, manufacturing date Dec. 15, 2003

T-302192, manufacturing date Feb.19, 2004

3.2.3 Apparatus

GCMS equipment

CombiPAL autosampler from CTC Analytic, Switzerland, was used to inject samples onto CP-3380 gas chromatograph from Varian, Inc. Lake Forest, CA USA. A special injector, Optic 3 High Performance Injector from ATAS International, The Netherlands, was used. The detector was a Saturn 2000 GC/MS/MS ion trap from Varian, Inc.

The separation column was a FactorFour VF-5ms from Varian Inc. with dimensions, 30 m x 0.25 mm I.D. and film thickness 0.1 μm.

Settings

The injector temperature was 60 °C at the start. After 5 s it was raised by 15°/s to 250 °C.The carrier gas flow (helium) was 1 ml/min. The split flow rate was 2 ml/min at the start and after 2 minutes it was raised to 20 ml/min. The oven temperature was 70 °C at the start. After 6 minutes it was raised by 15 °C/min to 185 °C which was held for 1 minute. The total GC time was12 minutes.

The temperature of the transfer line was 250 °C. EI ionization at 70 eV was used and ions between 100 and 299 m/z were collected from 5 to 9 minutes. The scan time was 0.5 s. For quantification, ion 239 was used for chloropropanediol and 117 for allyl glycerol ether.

3.2.4 Method

1.0 ml samples were pipetted into 1.5 ml high recovery glass vials and evaporated to dryness at room temperature under N_2-flux. 60 µl of Regisil were then added and the silylation reaction was allowed to proceed at 60 °C for 45 to 60 minutes. The reacted samples were then moved to the auto sampler for injection onto the GC. 4 µl samples were injected

3.2.5 Validation.

Preparation of calibration samples (standards).

Stock solutions of ca 1500 ppm of 3-chloro-1,2-propanediol and allyl glycerol ether in water were prepared. These solutions were further diluted in water 100 times to about 15 ppm and then further 100 times to 0.15 ppm (w/v). Between 125 and 1000 µl of these solutions were added to 1.5 ml glass vials. The standards were then evaporated to dryness and treated according to the method described in 3.2.4. On day 2, the volumes were added up to 1.0 ml with distilled water before evaporation.

Blank solutions were prepared by treating 1.0 ml of distilled water as a sample.

Standards were run every time samples were analyzed.

3.3 Results

3.3.1 Validation

The two main issues in this validation were specificity and detection limit. Matrix effects have not been tested, since it was known from carbon measurements that the matrix was very close to water (Analytical report 2).

Specificity

The retention times of the silylated chloropropanediol and allyl glycerol ether were 8.9 and 9.6 minutes, respectively. Their spectra are shown in Figure 3.1. Ions 239 (chloropropanediol) and 117 (allyl glycerol ether) were chosen for quantification of the compounds. In blank solutions, those ions are not present at the positions of sought-after compounds as shown in Figure 3.1.

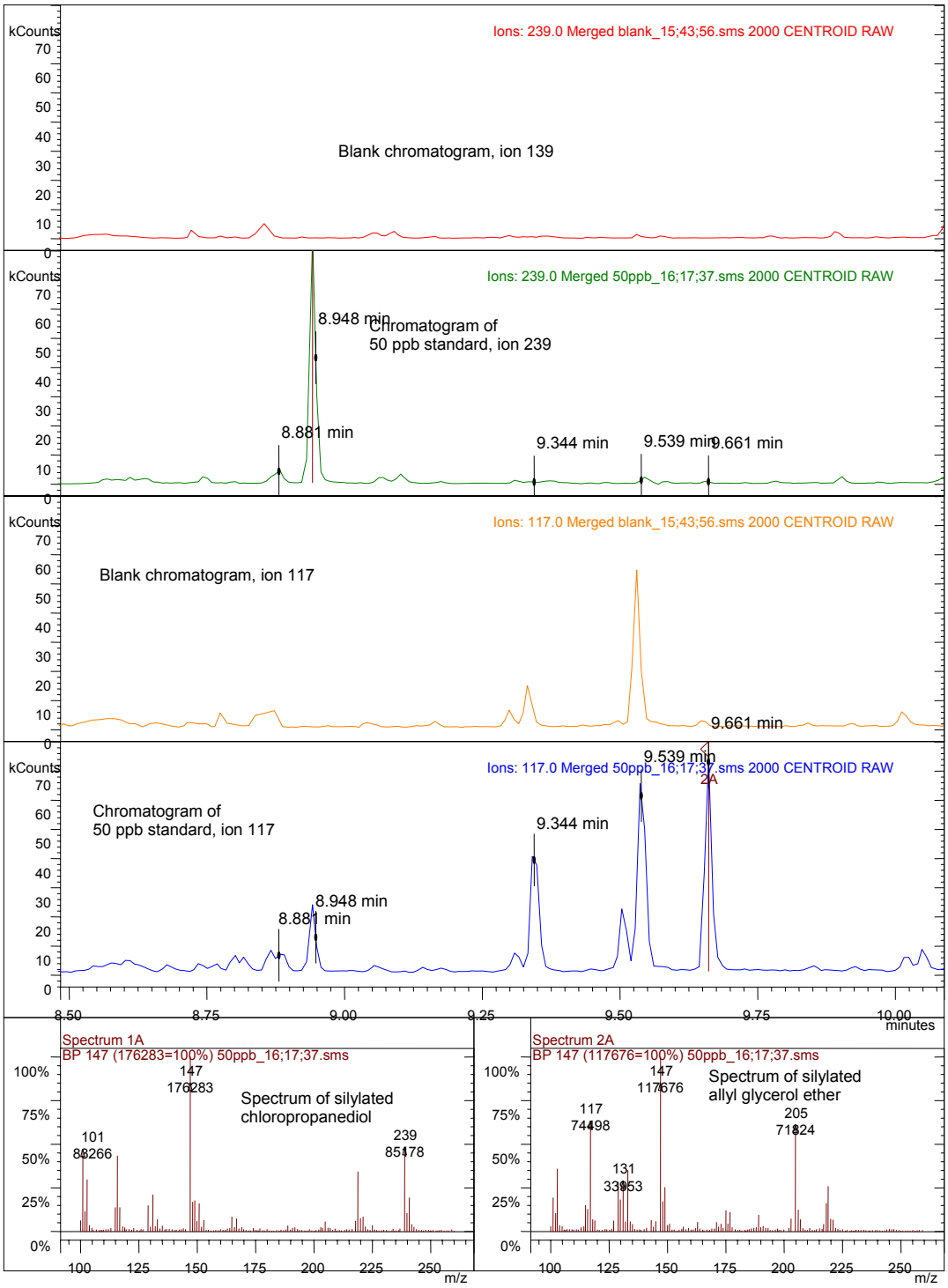

Figure 3.1 The four upper windows show ion chromatograms of blanks and silylated chloropropanediol and allyl glycerol ether. The two bottom windows show spectra of the silylated compounds.

Detection limits

Calibration graphs prepared different days and diluted from different stock solutions are shown in Figure 3. 2.

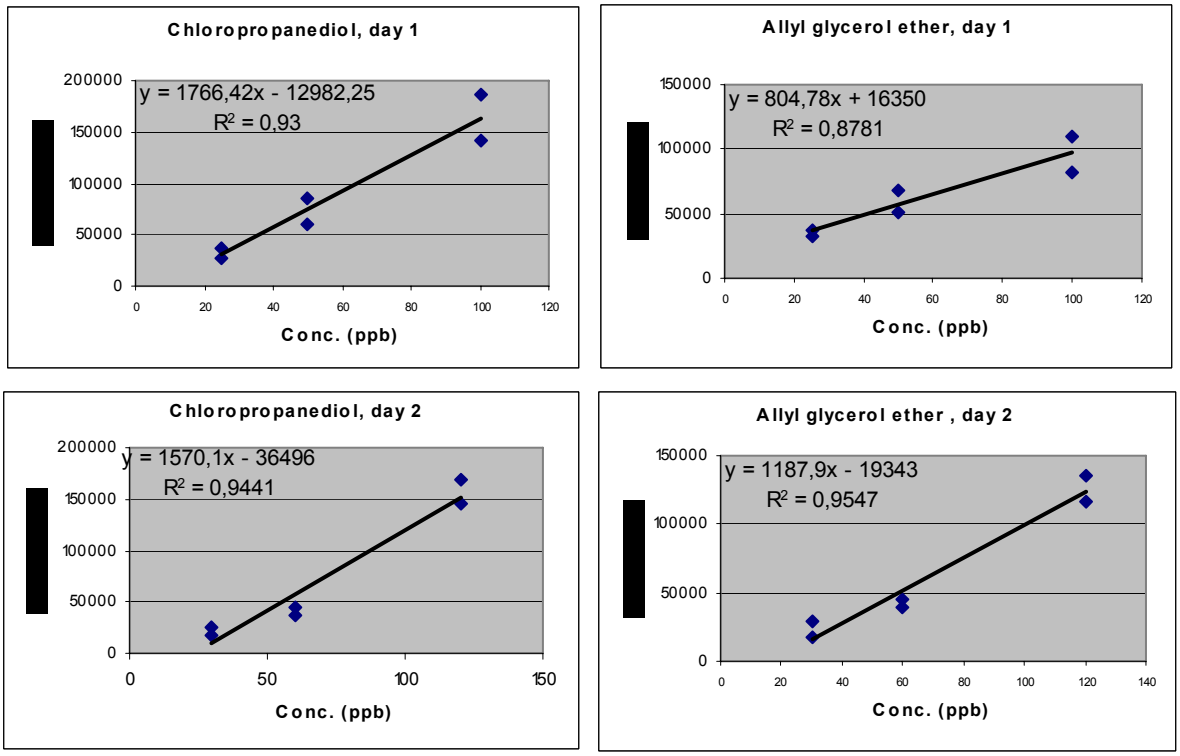

Figure 3.2. Calibration graphs of chloropropanediol and allyl glycerol ether

The lowest standard, which is ca 30 ppb corresponds to an area of about 30000 for chloropropanediol and 35000 for allyl glycerol ether. In blank solutions it was not possible to detect the ions (as illustrated in Figure 3.1). As can be seen it looks as if concentrations far below that would be possible to detect. However, since the lowest standard was about 30 ppb, the detection limit is also set to this value.

3.3.2 Sample measurements

Figure 3.3 presents examples of measurements of a sample from PFE extraction. The results of the analyzed samples are given in Table 3.1.

Chromatogram Plots

Plot 1: e:\050315\30ppb_14;38;06.sms Ions: 117.0+239.0 Merged
Plot 2: e:\050315\301065_15;25;24.sms Ions: 117.0+239.0 Merged
Plot 3: e:\050315\301462_15;41;10.sms Ions: 117.0+239.0 Merged
Plot 4: e:\050315\302192_15;56;56.sms Ions: 117.0+239.0 Merged

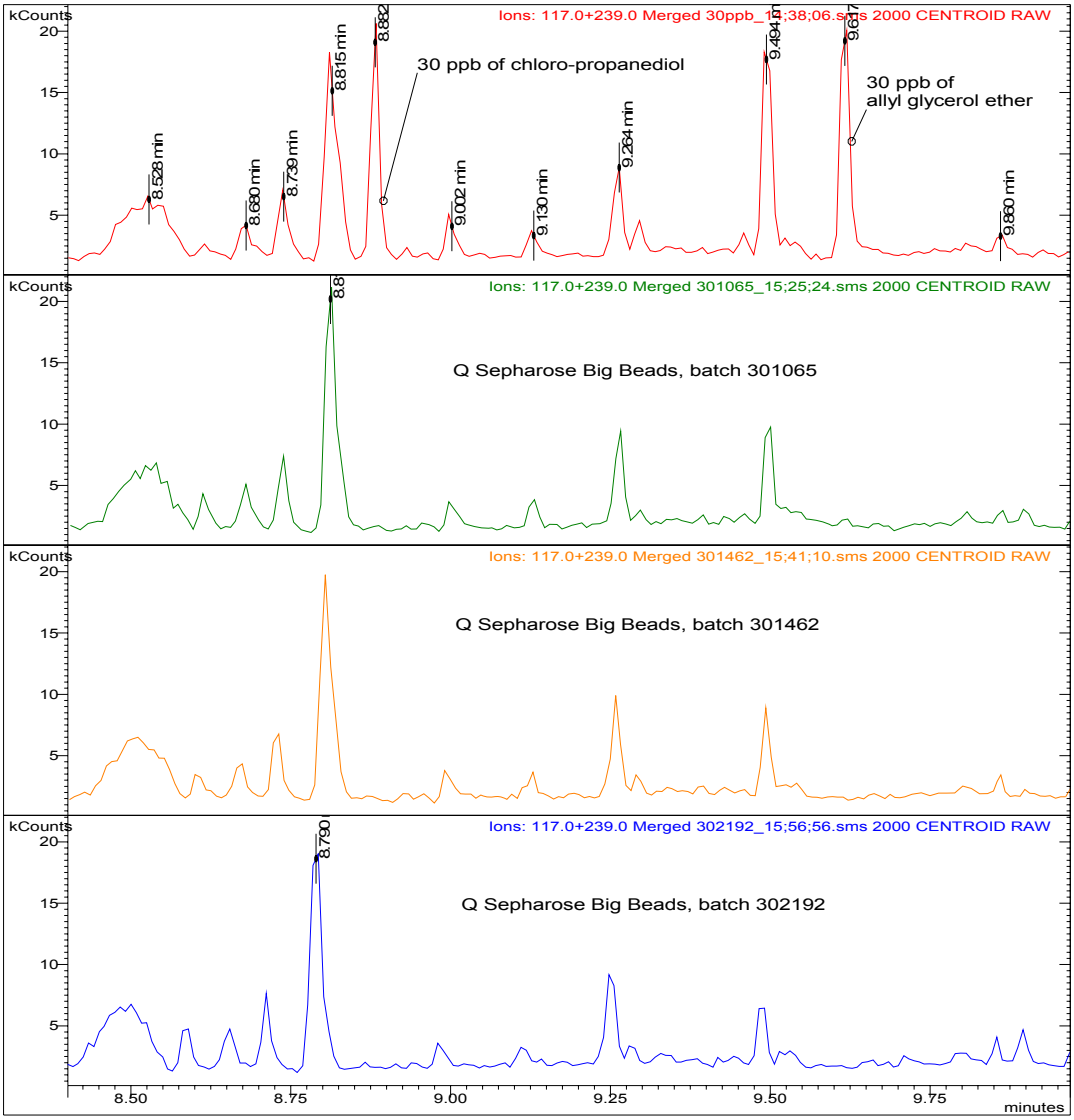

Figure 3.3. Ion 117 + 239 chromatograms of lowest standard (top window) and PFE extracts of batches 301065, 301462 and 302192 of Q Sepharose Big Beads

Table 3.1. Sample measurement results.

Sample	Area of chloropropanediol	Area of allyl glycerol ether
Batch 301065 (10B:1)	not detected	not detected
Batch 301462 (110B:1)	not detected	not detected
Batch 302192 (210B:1)	not detected	not detected

3.4 Calculation of maximum amount of 3-chloro-1,2-propandiol and allyl glycerol ether in washed Q Sepharose Big Beads media

The maximum amount of 3-chloro-1,2-propandiol and allyl glycerol ether in the resin, C_m, was calculated from the LOD in the extracts according to the following expression:

C_m = *LOD* (in μg/ml solution) x V_t / V_{resin}

where V_{resin} is the volume of sedimented resin and V_t is the volume of added water + the water present in the resin. The maximum amounts of 3-chloro-1,2-propandiol and allyl glycerol ether in the different samples are given in Table 3.2.

To calculate the overall maximum concentration, the largest value, 0.068 has been taken and rounded up to 0.07.

Thus, the maximum concentration of 3-chloro-1,2-propandiol and allyl glycerol ether in washed Q Sepharose Big Beads resins is 0.07 μg/ml of wet sedimented resin.

Table 3.2. Calculation of maximum amounts of analysed compounds.

Sample	V_{resin} [ml]	V_t [ml]	C_m [μg/ml of wet sedimented resin]
Batch 301065	8.96	18.8	0.063
Batch 301462	8.38	18.9	0.068
Batch 302192	8.4	18.9	0.068

4 Determination of glycidol

4.1 Introduction

This part describes the determination of glycidol. Glycidol is volatile and consequently suitable for gas chromatographic determination. The ordinary concentration techniques used for the other substances were not possible to use for glycidol. This compound is not well adsorbed by SPME the fiber and it is too volatile for evaporation. Injection onto the GC of a larger volume than usual was the way to decrease detection limit in this case.

4.2 Experimental

4.2.1 Chemicals

Glycidol (2,3-epoxypropan-1-ol), 96 % from Sigma-Aldrich, Cat. G5,80-9.

Distilled water.

4.2.2 Tested samples

Pressurized fluid extractions (PFE) according to Analytical report 1 of the following batches of Q Sepharose Big Beads

T-301065, manufacturing date, Dec. 7,2003.

T-301462, manufacturing date, Dec. 15, 2003

T-302192, manufacturing date, Feb. 19, 2004.

4.2.3 Apparatus

A gas chromatograph HP 5890 from Agilent equipped with injector 7673, electronic pressure controlled injector and an FID detector was used. The separation column was HP-Innowax with dimensions 30 m x 0.32 mm I.D. and film thickness 0.50 μm from Agilent,.

4.2.4 Settings

The carrier gas flow was 3.0 ml/min (helium) and the split flow was 10 ml/min. The injector and detector temperatures were 230 and 240 °C, respectively. The oven temperature was at the start 120 °C. After 1 minute it was raised by 20 °/ min to 160 °C. The total GC time was 4 minutes.

4.2.5 Method

3 µl of the samples were injected onto the GC without preparation.

4.2.6 Validation

Stock solutions of ca 2000 ppm of glycidol in distilled water were prepared. The glycidol amount was weighed. The stock solution was further diluted to ca 1 ppm (w/v). Calibration solutions of ca 0.1, 0.2 and 0.5 ppm were prepared from that solution. Blank solutions were distilled water and blank runs from PFE extraction.

4.2.7 Matrix effects

Matrix effects have not been tested since it was known from carbon measurements that the matrix is very close to water. In addition, direct GC chromatography of neutral substances is not very sensitive to the matrix.

4.3 Results

4.3.1 Validation

Specificity and detection limit were the parameters investigated.

Specificity

The specificity is visualized in Figure 4.1 below. The retention time of glycidol was 2.84 minutes. A peak with a retention time of 2.69 minutes was generally obtained also from distilled water.

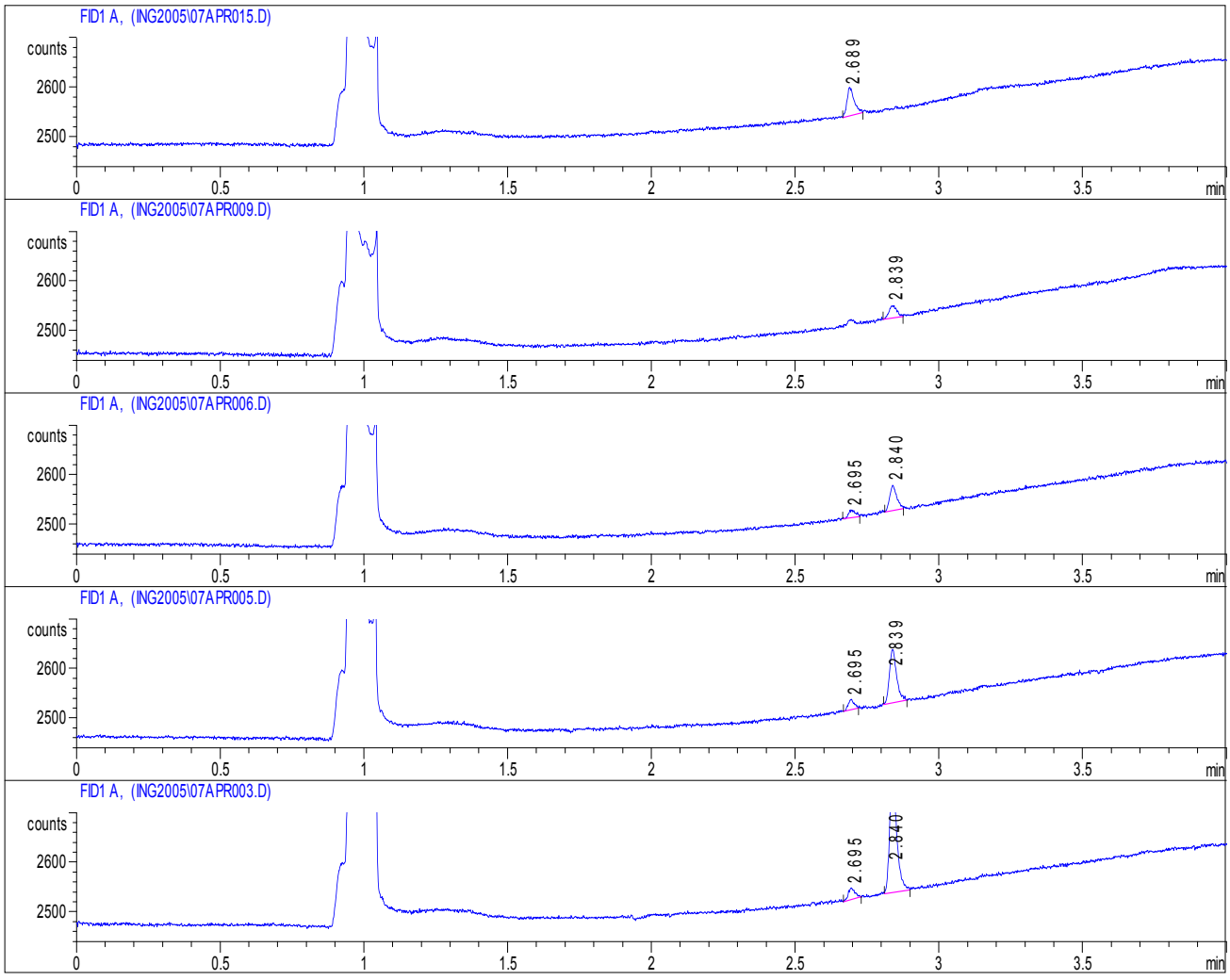

Figure 4.1. Chromatograms of solutions of different concentrations of glycidol in water. From the top the concentrations are 0, 0.1, 0.2, 0.5 and 1.0 ppm.

Detection limit

Two calibration graphs prepared in water from different stock solutions and run on different days are shown below. Those graphs indicate a detection limit of 0.1 ppm, the lowest standard. No signal at the retention time of glycidol is obtained from pure water as shown in Figure 4.1.

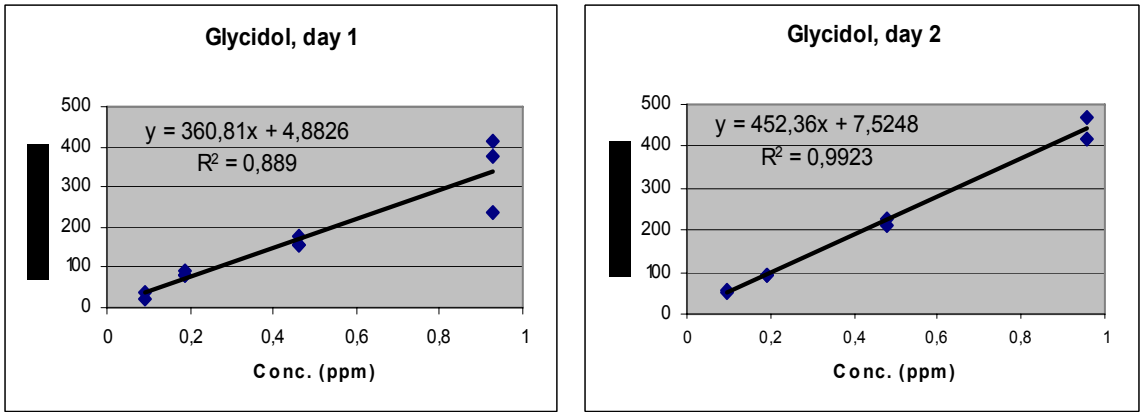

Figure 4.2. Calibration graphs of glycidol in water. The graphs are prepared on two different days from two different stock solutions of glycidol.

4.3.2 Sample measurements

Figure 4.3 below give examples of sample measurements. As is shown, all samples, even PFE blanks, give peaks at the retention time of glycidol. The peaks are broad indicating two compounds eluting very closely. Areas are presented in Table 4.1 below. Those areas include both compounds.

Table 4.1. Areas at the retention time of glycidol from PFE blanks and PFE extracts of three batches of Q Sepharose Big Beads

Sample	Areas
Blank 1	103, 137, 142, 125
Blank 2	118, 95, 96, 130
Sample 301065	139, 129, 57
Sample 301462	90, 124, 96
Sample 302191	158, 122

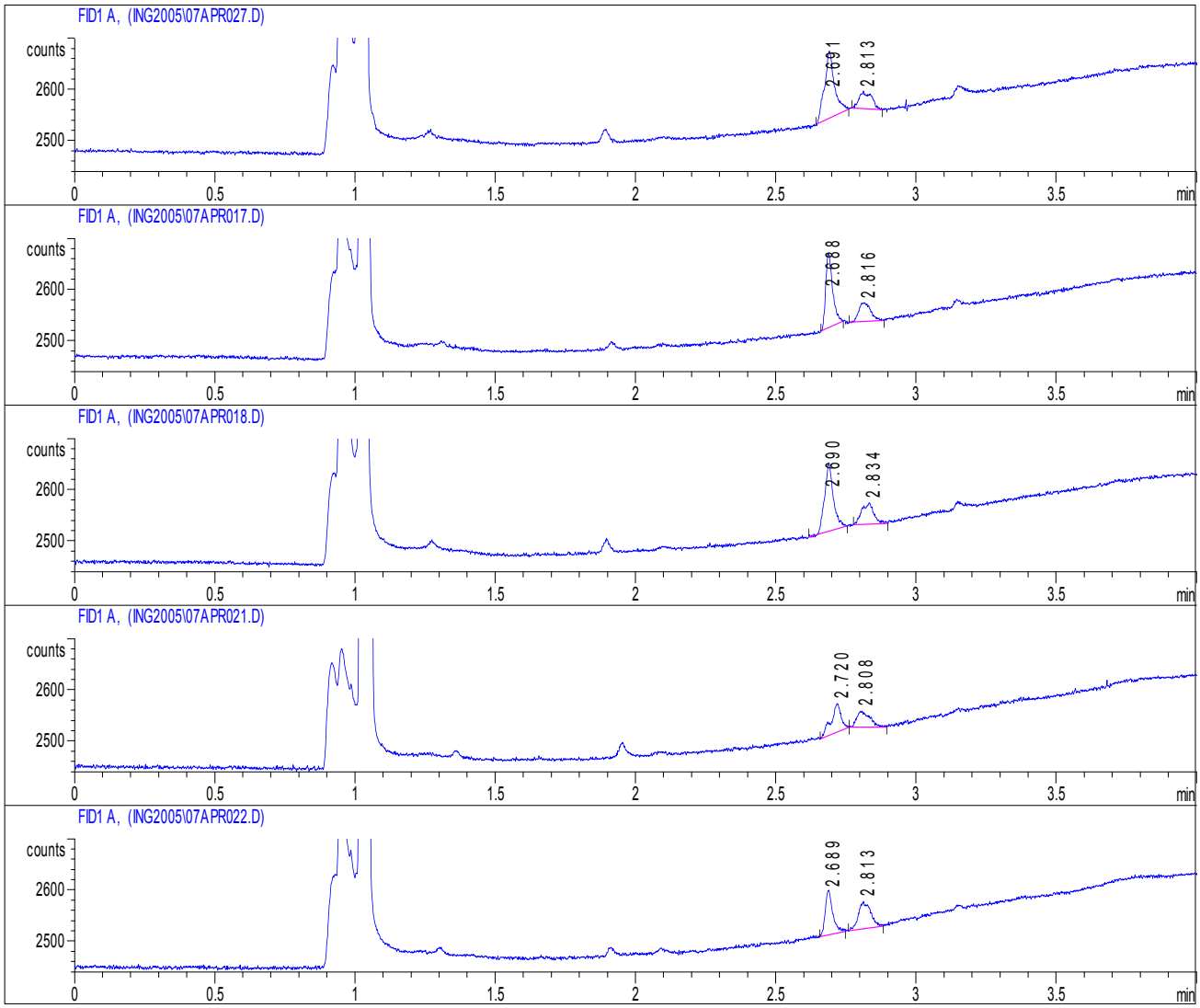

Figure 4.3. Chromatograms of PFE extracts from, counted from the top; batch 301065, batch 301462, batch 302192, blank 1 and at the bottom, blank 2.

Calculations were done to test if the areas from any of the samples were significantly larger than the blank areas. The result is given below.

Pooled standard deviation (s) of measurements	27
Number of degrees of freedom for pooled s	14
Critical t-factor (P = 0.05)	2.14
Mean of blank runs	124
Mean of 301065	108
Mean of 301462	103
Mean of 302192	140

It can be noted that the mean of sample 302192 is larger than that of the blanks. A null hypothesis was adopted that there was no significant difference between the means. A two-sample t-test assuming equal variances was performed. The formula below was used for calculation of the observed t-value:

t = (blank mean – sample mean) /(s * √ (1/N_1+1/ N_2)), where $N_1$and N_2 are the number of measurements for the two samples and s is the pooled standard deviation.

The calculated t-value was (124 – 140) / (27 * √ (1/2+1/10)) = - 0.77.

The observed value of ItI (= 0.77) is less than the critical value 2.14). Thus, the null hypothesis is retained. There is no evidence of the sample mean being larger than the blank mean. In other words, glycidol cannot be detected in the sample.

About detection limit:

A detection limit of 0.1 ppm is indicated in the calibration graph made in water. However, the area counts from 0.1 ppm are at most 50 and could theoretically be hidden in the difference between the blanks and the samples. Instead, 0.2 ppm is set as the practical detection limit.

The overall conclusion is that the amount of glycidol found in PFE extracts of Q Sepharose Big Bead is below 0.2 ppm.

Table 4.2. Sample measurement results.

Sample	Area of glycidol
Batch 301065 (10B:1)	not detected
Batch 301462 (110B:1)	not detected
Batch 302192 (210B:1)	not detected

4.4 Calculation of the maximum amount of glycidol in washed Q Sepharose Big Beads media

The maximum amount of glycidol in the resin, C_m, was calculated from the LOD in the extracts according to the following expression:

C_m = *LOD (in μg/ml solution) x* V_t / V_{resin}

where V_{resin} is the volume of sedimented resin and V_t is the volume of added water + the water present in the resin. The maximum amounts of glycidol in the different samples are given in Table 4.2.

To calculate the overall maximum concentration, the largest value, 0.45 has been taken and been rounded up to the nearest value with one significant figure; 0.5.

Thus, the maximum concentration of glycidol in washed Q Sepharose Big Beads resins is 0.5 μg/ml of sedimented resin.

Table 4.2. Calculation of maximum amounts of glycidol

Sample	V_{resin} (ml)	V_t (ml)	C_m (μg/ml of sedimented resin)
Batch 301065	8.96	18.8	0.42
Batch 301462	8.38	18.9	0.45
Batch 302192	8,40	19	0.45