Looking into the HPLC Column while Running CIP

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The big application

- By far the biggest RP silica application is insulin purification.
- Insulin is prone to self-aggregation and fibrillation
- The fibrillated goo has to be removed from column, most commonly by NaOH wash

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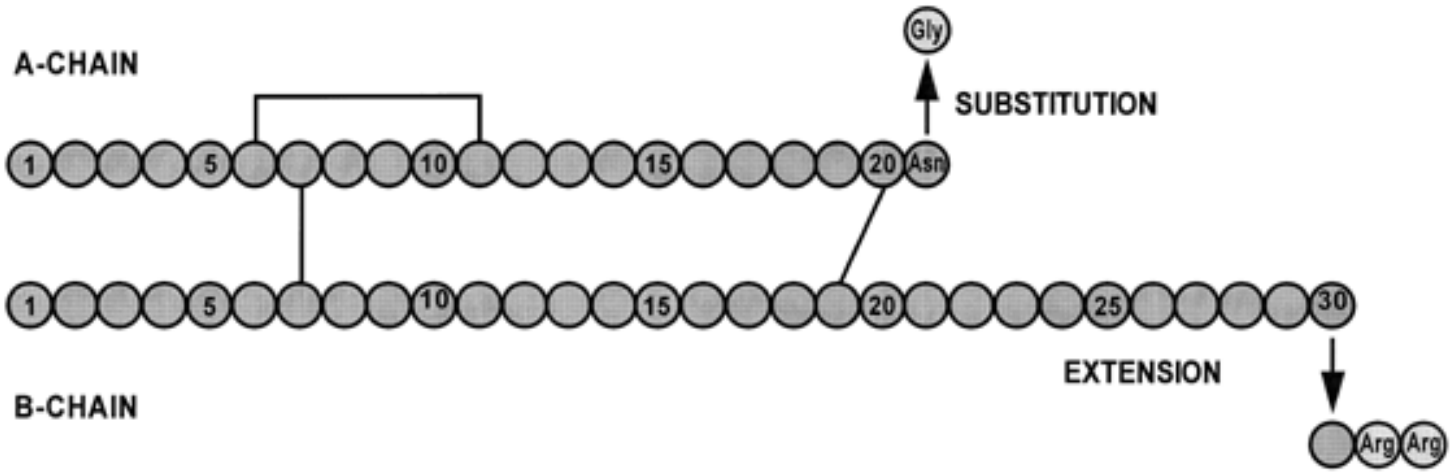
The problem with Insulin



Insulin, insulin analogs and other diabetes treating drugs (GLP-1) are prone to self-aggregation, FIBRILLATION

CIP on silica???

Unprotected (un-bonded) silica melts at pH 13.

Surface modification / bonding makes it last longer.

But still…

NaOH at pH 13 is hydrolyzing siloxane bonds in the silica matrix. First small chunks of silica start falling off (“fines”).

Trouble begins with fragments falling off

The problem with silica

With the chunks of broken off silica bonded ligands are lost.
Problem with LEACHABLES.

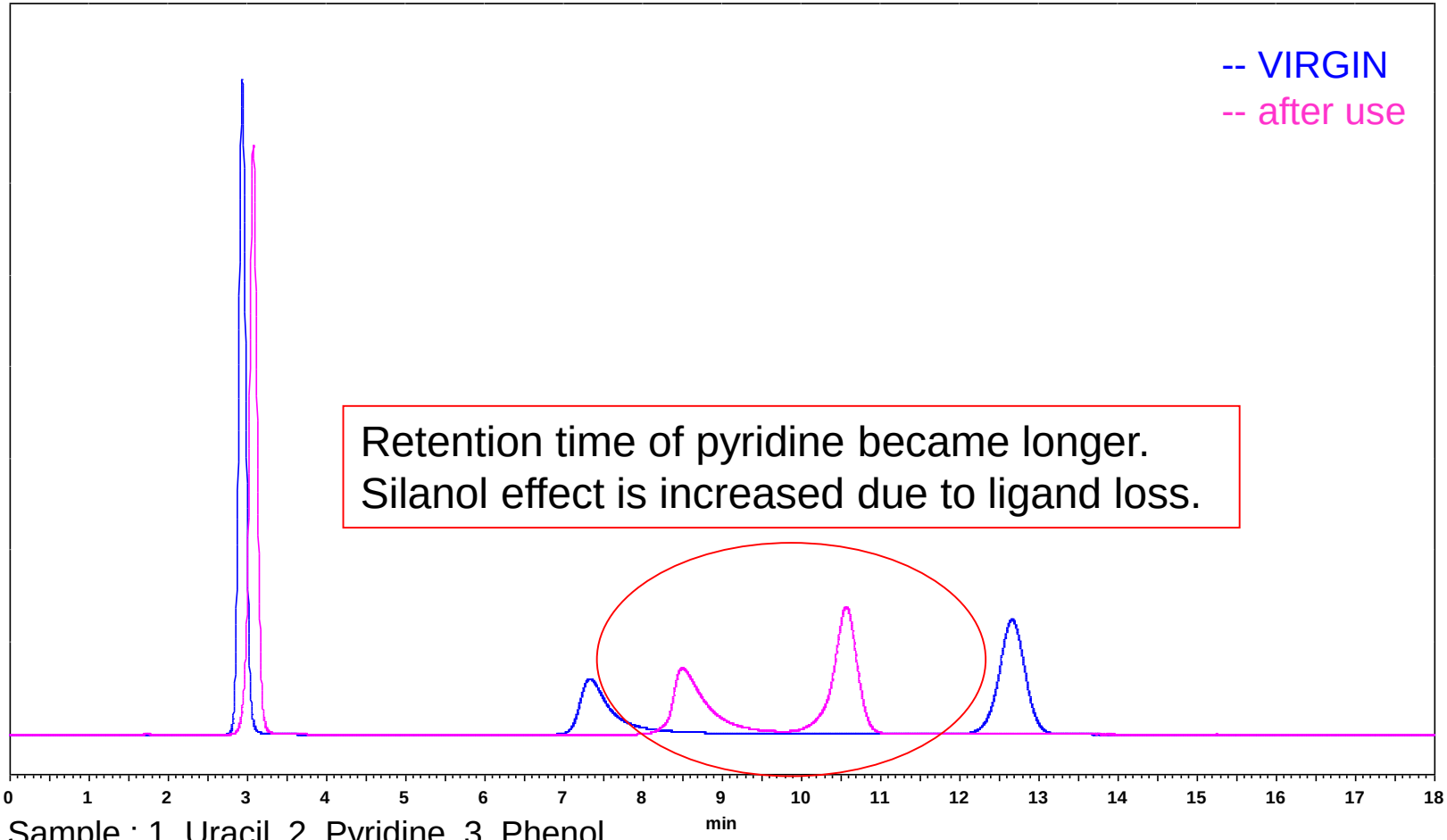
Silanol groups get exposed.
Negatively charged silanol groups decreasing selectivity.

Frequent NaOH wash kills the silica.

What we could not say so far was whether we cleaned the silica to death (over clean) or we did not clean it enough! We had no scientific tool to monitor CIP effect on time.

Pyridine-phenol test

(A model for basic impurities)



Sample : 1. Uracil, 2. Pyridine, 3. Phenol

The problem with degregation

Possible CIP agents:

- Acetic acid or Formic acid

- SDS (with special washing), EDTA if metal ions are sorbed

- Urea, Ammoniumhydroxide, TRIS
  (to suppress hydrophobic interactions on high pH)

- NaOH in combination with organic solvent
  (to fragment aggregates and to suppress hydrophobic interactions)

Evaluating current CIP protocols

Simple protocol from literature:

X CV 0.1 n NaOH *aq*./organic solvent 30/70 (pH 13) is pumped through the column followed by pH adjustment with acid.

This step is implemented after every five number of purification cycles.

- Is the NaOH concentration too low or too high?
- Is the frequency adequate?
- Does the silica get cleaned enough?
- Do we over-clean the silica?

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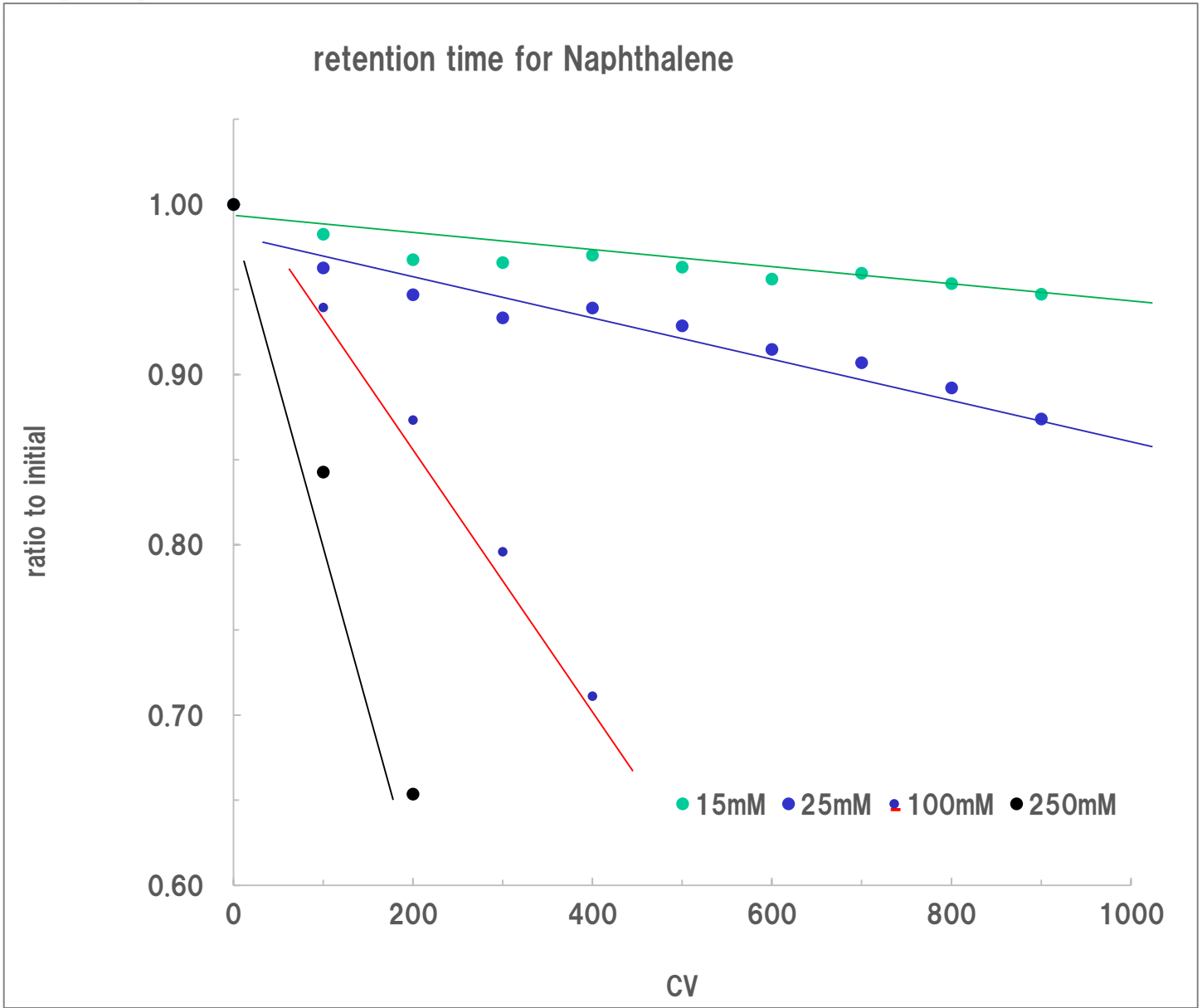
Effect of different NaOH concentration



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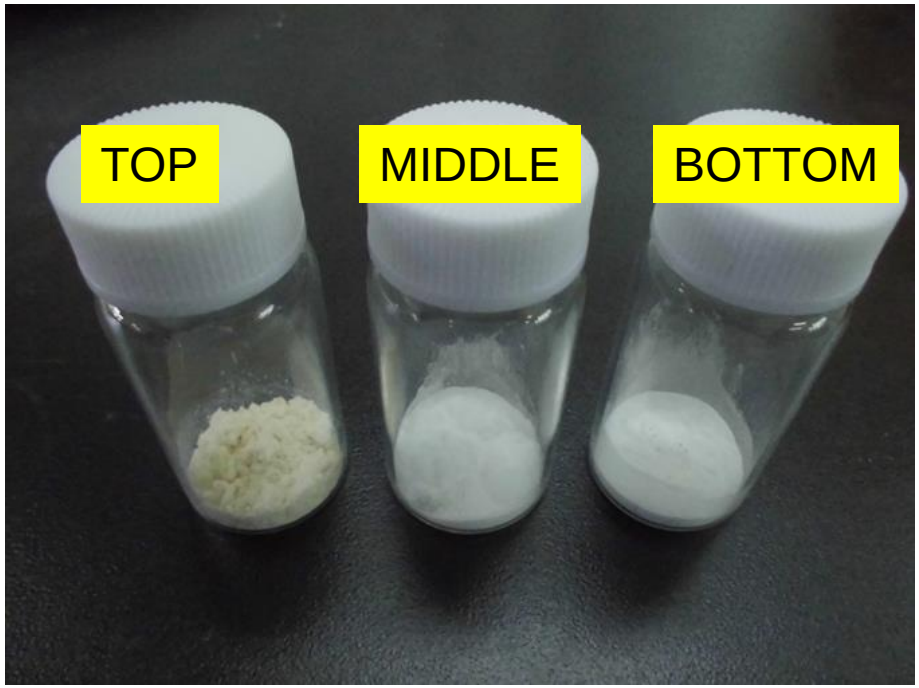
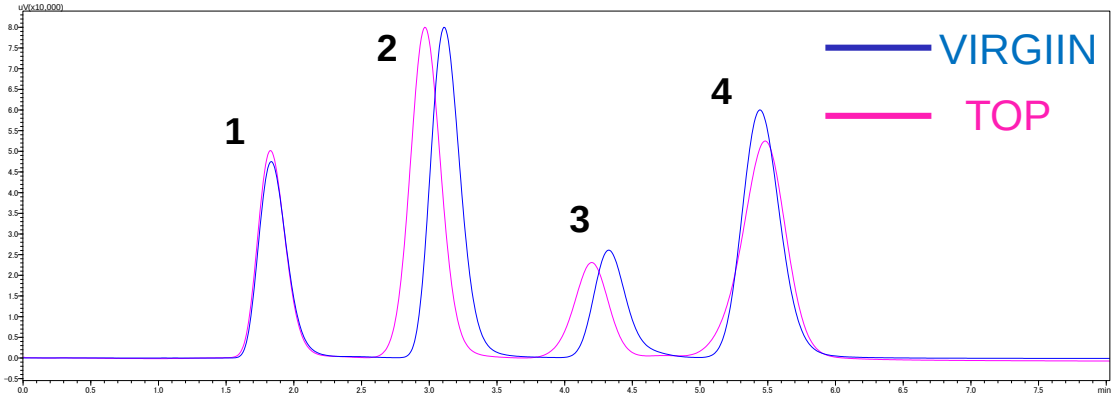
Real, used silica sample analysis: Elemental analysis

	C%	H%	N%
VIRGIN	8.6	1.8	0
TOP	12.82	2.27	1.63
MIDDLE	8.43	1.77	0
BOTTOM	8.51	1.77	0



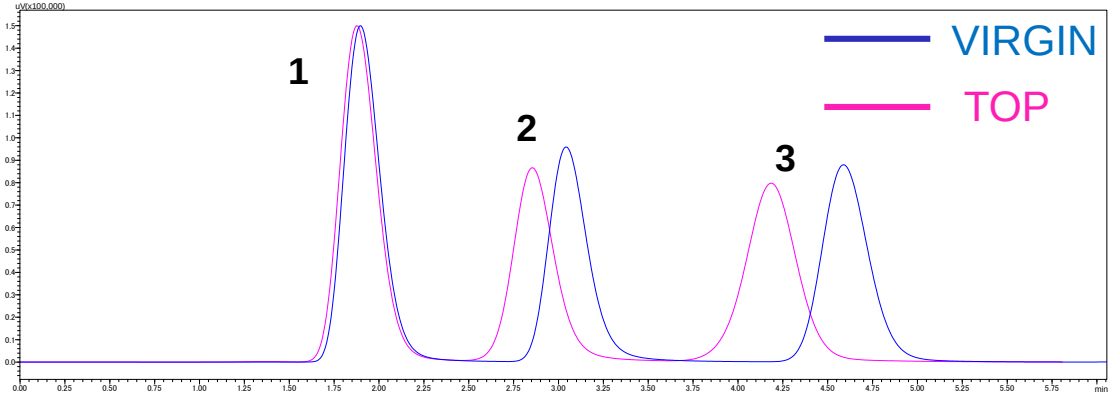
Real, used silica sample analysis: Chromatographic evaluation

Aromatic standard



Mobile Phase : MeOH/H_2O=60/40
Flow rate : 0.2mL/min
Oven temp : 40ºC
Detection : UV, 254 nm
Sample :
1. Uracil
2. Methyl benzoate
3. Toluene
4. Naphthalene

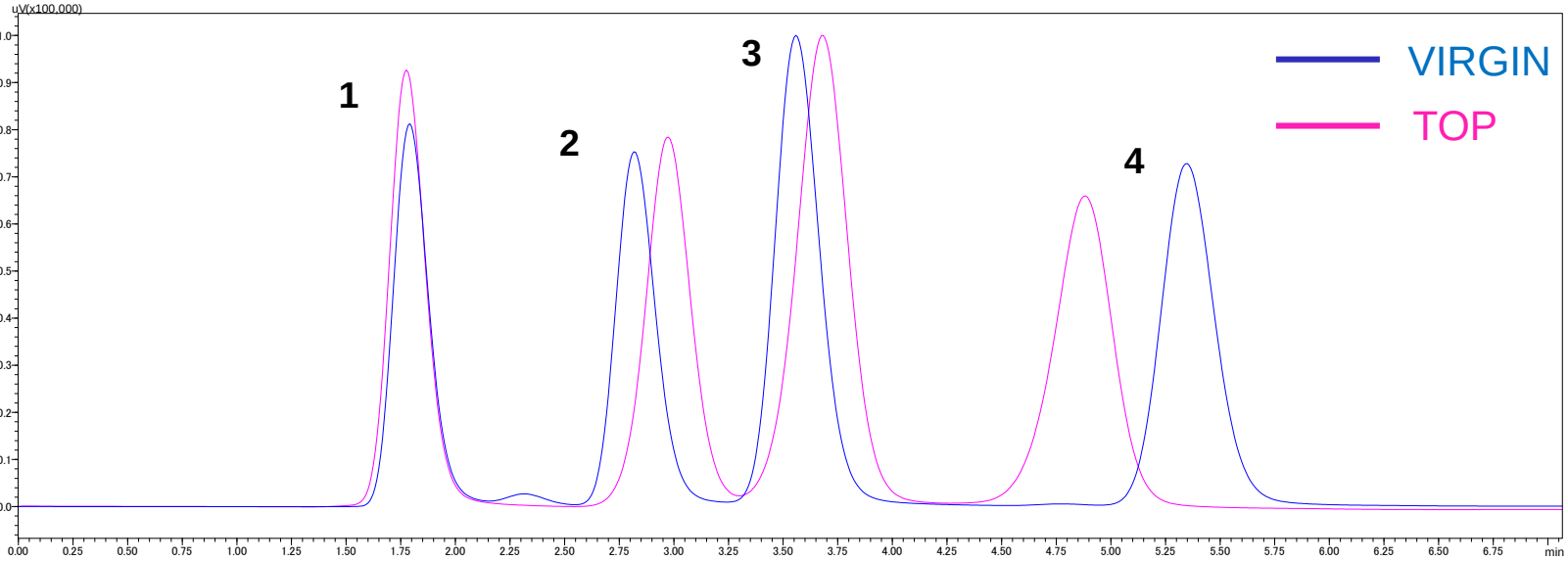
Basic standard



Mobile Phase : MeOH/H_2O=30/70
Flow rate : 0.2mL/min
Oven temp : 40ºC
Detection : UV, 254nm
Sample :
1. Uracil
2. Pyridine
3. Phenol

Real, used silica sample analysis: Chromatographic evaluation

Acidic standard



Mobile Phase : MeCN/20mM Potassium Phosphate Buffer (pH=3.2)=35/65
Flow rate : 0.2mL/min
Oven temp : 40ºC
Detection : UV, 254nm
Sample :

1. Uracil
2. Benzoic acid
3. *p*-Toluic acid
4. Methyl benzoate

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Real, used silica sample analysis: Summary of chromatographic evaluation

	Aromatic standard				Basic standard			Acidic Standard		
	N4	As4	k'4	Pressure (MPa)	As2	As3	k'2/k' 3	As3	As4	k'3/k' 4
VIRGIN	1,655	1.11	1.97	1.0	1.20	1.13	0.43	1.12	1.09	0.50
TOP	1,227	0.92	2.00	1.1	1.12	1.00	0.42	0.99	0.93	**0.61**
MIDDLE	1,183	0.89	1.88	1.0	1.07	0.94	**0.46**	0.96	0.90	0.51
BOTTOM	1,344	1.02	1.87	1.0	1.20	1.08	**0.48**	1.10	1.05	0.51

N : plate number, As ; asymmetry, k' : $(t-t_0)/t_0$

Acidic standard test is good indicator to judge how dirty silica is.

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Elemental analysis after CIP using alternative agents

	C%	H%	N%
VIRGIN	8.6	1.8	0
TOP	12.82	2.27	1.63
0.1M NaOH（15%ACN）10CV	9.87	1.65	0.46
6M Guanidine hydrochloride（15%ACN）10CV	11.08	2.00	0.98
8M Urea（15%ACN）10CV	11.11	2.06	1.05
HCOOH（15%ACN）4CV	8.67	1.75	0.18

Standard chromatographic tests and what they can show us

Aromatic Standard

Deceptive

k’4 : Retention time for Naphtalene

The shorter it goes the more ligand we lose.

Basic Standard

Shows damage of silica

k’2/k’3 : Ratio of retention time for the peaks

The number gets bigger with increasing silanol exposure.

Acidic Standard

Indicator of dirty silica

k’3/k’4 : Ratio of retention time for the peaks

The number gets smaller with more Nitrogen removed.

SUMMARY

Classic chromatography standard tests are used to indicate the state of the silica stationary phase providing “non-invasive” way.

Now we can “see” whether the silica is cleaned enough or not.
We can “see” how much damage has been inflected on the silica.

These most valuable new tools provide way to re-evaluate the CIP step in the biggest RP HPLC applications.

Better CIP provides longer silica life, resulting in better API production on more affordable way.

This way we contribute to the elimination of suffering from this planet.