Evaluation of stationary phases for 2-dimensional HPLC of Proteins
1. Validation of commercial RP-columns

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Abstract:

RP-separation with TFA-based water / acetonitrile eluents is widely used for peptides and small proteins but is well known difficult for large or membrane proteins. Especially in proteomics or other complex biological matrices reliable elution patterns are difficult to achieve. New commercial stationary phases are validated regarding long term stability, protein recovery, carry over, symmetry and selectivity using 10 different proteins with different molar weights, isoelectric points and glycosylation. It could be demonstrated that some stationary phases had poor protein elution performances. They did not elute a protein at all or with minor recovery, peak symmetries. Sometimes bad and formidable carry over effects for peak areas in the following run were observed. Selectivity in separation of different isomers or glycosylated proteins is also different.

The results suggest that neither surface area nor pore diameter play an important role in the application of reversed phases for HPLC of proteins. The investigation leads one to suppose that the bonding chemistry seems to be an important aspect. Most critical fact is that some RP-phases did not elute a protein at all others only 20 % of the injected protein mass, which makes the objective of an RP-chromatogram highly questionable.

1. Introduction:

Protein characterisation in proteomics or quantification in complex biological samples (e.g. serum) is one of the most demanding tasks in modern analytical chemistry.

Separation of many thousand proteins in a biological sample or detection of low abundant factors will be the analytical challenge in the next decade. In the last years two dimensional electrophoresis has often been used for this purpose. As limitations of the conventional 2D-gelelectrophoresis become more obvious, the use of HPLC separation gets more attractive. Especially 2D-HPLC is discussed for this purpose. Two dimensional peptide-separations are presented e.g. by combination of size-exclusion [1] or ionexchange [2,3] as first dimension followed by a reversed-phase-column (RP-column) in the second separation dimension.

Also 2D-separation of complex protein mixtures have been published either combining Isoelectric-Focussing/Reversed-Phase- [4,5,6], Chromatofocussing/Reversed-Phase- [7,8,9] or Ionexchange/Reversed-Phase-separations [10,11]. Especially online coupling 2D-HPLC and electrospray-MS techniques [4 - 9] present a powerful method for differentiation of complex protein mixtures e.g. in biological samples.

Major drawback are differentiation of minor protein components together with high abundant proteins as serum albumine or immunglobulines, separation of relevant protein parameters besides many thousand other proteins and the detection limit for low abundant proteins as relevant physiological factors e.g. tumour markers in human serum.

2D-gelelectrophoresis as highly sophisticated, non automated, multiple stage technique will not be the preferable technique to solve these problems in future tasks. Modern electrospray LC-MS equipment shows sufficient protein detection limit in the low femtomole range and provides a further dimension for improving selectivity of LC-separation.

Major problem in 2D-LC-MS of proteins is the separation power (peak capacity) to differentiate many thousand proteins in biological samples, e.g. for protein characterisation in proteomics or protein quantification in e.g. human serum of diagnostical or pharmaceutical relevant proteins.

Highly stable and efficient separation columns both in the ionexchange- and reversed-phase mode are necessary for routine use. New RP-columns for protein separations have been presented regarding embedded polar phases [12], monolithic [13], non porous phases [14] or different pore diameters [15]. In 1990 we compared different RP-columns for peptide analysis [16]. Ricker et al evaluated commercial RP-Columns for protein separations in 1995 [17] and Bidlingmeyer et.al. evaluated the effect of wide-pore columns for protein separations [18].

Evaluation of the most relevant factors for a good protein column (for instance surface area, pore diameter or chain-length) by examination of different

phases e.g. in a two level factorial experiment would be the correct chemometrical approach. Combining phases with defined high or low surface area, C5- or C18-chains, small or large pores or with and without endcapping should reveal the most important factors to get a column e.g. with best protein recovery or peak symmetry. Finding commercial phases for such a controlled factorial design is very difficult (or sometimes impossible) and synthesis of special phases is difficult. It´s more feasible to use commercial available phases even if they don´t fit the perfect chemometrical design. Besides exploring which parameters are more or less important for a good performing reversed-phase material, this evaluation will also show the actual state of frequently used commercial columns in protein separation.

We will therefore examine some of the most recent developed and popular RP-Phases regarding stability, protein recovery, carry over, symmetry and selectivity using 10 different proteins with different molar weights, isoelectric points and glycosylation.

At present some of the most popular ionexchange columns are examined and the results will be presented in the second publication of this series. At last we will present our effort to develop a new RP-phase especially designed for protein elution.

2. Experimental:
2.1. Reagents:
Acetonitrile, methanol gradient grade, trifluoroacetic acid sequencing grade (TFA), HCl pro analysis, thiourea, aniline, phenol, dimethylaniline, 2-, 3-, 4-ethylaniline, toluene, aceticacidethylester, ethylbenzene were delivered by Merck (Darmstadt, Germany).

Proteins fetuin, bovine serum albumine, ß-lactoglobuline, alkohole-dehydrogenase, glucose-6-phosphate-dehydrogenase, creatinkinase, hexokinase, glycerinkinase, ribonuclease B were supplied by Merck. The mouse monoclonal igG-type antibody was a gift of Boehringer-Mannheim/Roche Diagnostics, Penzberg, Germany.

Following HPLC-columns were used:
Zorbax 300 SBC18, Agilent (Waldbronn, Germany);
Poroshell 300 SBC18, Agilent,
Gemini, Phenomenex (Aschaffenburg, Germany);
Jupiter 300, Phenomenex,
BioWidepore C5, Supelco (Taufkirchen, Germany);
Micra ODS1, Bischoff (Leonberg, Germany);
218TP5205 Grace-Vydac (Hesperia, USA);
238Everest5205 Grace-Vydac; Chromolith Performance C8, Merck (Darmstadt, Germany)

2.2. Apparatus and methods:
LC 1100 HPLC with sampler, degasser, VWD-detector, ChemStation, Agilent.
Aminoacid analysis was performed with an analyser equipped with LC1100 pump, sampler, PU980 reagent pump, Jasco (Groß-Umstadt, Germany), F1000 fluorescence detector, Merck, AA411-column Interaction Chromatography (San Jose, USA), teflon knitted-tube reactor (homemade).

Concentration Evaporator RC 10-22 Jouan (Unterhaching, Germany), microwave oven, MDS 2000 CEM (Kamp-Lintfort, Germany)

2.2.1. Column stability test:
Characterisation of a new column was done with thiourea to ethylbenzene standards (5 µg/Inj., 1 µl Inj. Vol.) with 49 % CH_3OH in H_2O, isocratic elution at 25 °C.
Columns then had been stressed by purging with 50 % CH_3CN + 0.1 % TFA for two weeks (without sample injection). Then the columns had been characterised again.
Flow rate was 0.25 ml/min for all columns but Micra and Chromolith which were operated at 1.0 ml/min.

2.2.2. Protein recovery test:
The peak area of the eluted proteins of all columns was measured using UV-absorbance at 210 nm and calculated as sum of all protein peak-areas.
Absolute mass recovery was gained by injecting each protein with the HPLC-sampler and fractionating after chromatography into a 2 ml LC-Vial. A following injection with HPLC-sampler and fractionating without column was done to get 100 % reference.
After vacuum drying of the fractions the proteins were hydrolysed within the fraction-vials with 6 M HCl for 40 min at 5 bar (150 °C). Aminoacid analysis was done using ionexchange-chromatography with sodium-citrate gradient and o-phthaldialdehyd-post-column derivatisation. Measuring Phe and Ile in the fractions with and without column resulted in the mass recovery in percent. Fractionation and amino acid analysis of the BioWidePore-column elutions served as reference for all other columns.

2.2.3. Protein elution:
Protein concentrations: 100 µg/ml, but fetuin at 200 µg/ml and antibody at 500 µg/ml H_2O. Injection volume: 10 µl; UV-detection: 210 nm (5 mm path length).
Eluent A: 0.1 % TFA in H_2O; Eluent B: 0.1 % TFA in CH_3CN
Gradient: 0 % eluent B after 0 min, 70 % eluent B after 25 min.
Flow rate: 0.25 ml/min, but Micra, Chromolith, 238Everest which were operated at 1.0 ml/min.

2.3. Definitions:

Resolution R (well resolved peaks): $$R = \frac{t_R^2 - t_R^1}{\frac{w_1 + w_2}{2}} \quad (1)$$

with t_R^1, t_R^2: retention times peak 1 and 2, w_1, w_2 peak base width of peak 1 and 2

Resolution δ (severe overlapping peaks): $$\delta = \frac{\overline{AB}}{\overline{AC}} \quad (2)$$

with $\overline{AB}$, $\overline{AC}$ distance points A and B e.g. points A and C (see figure 1a)
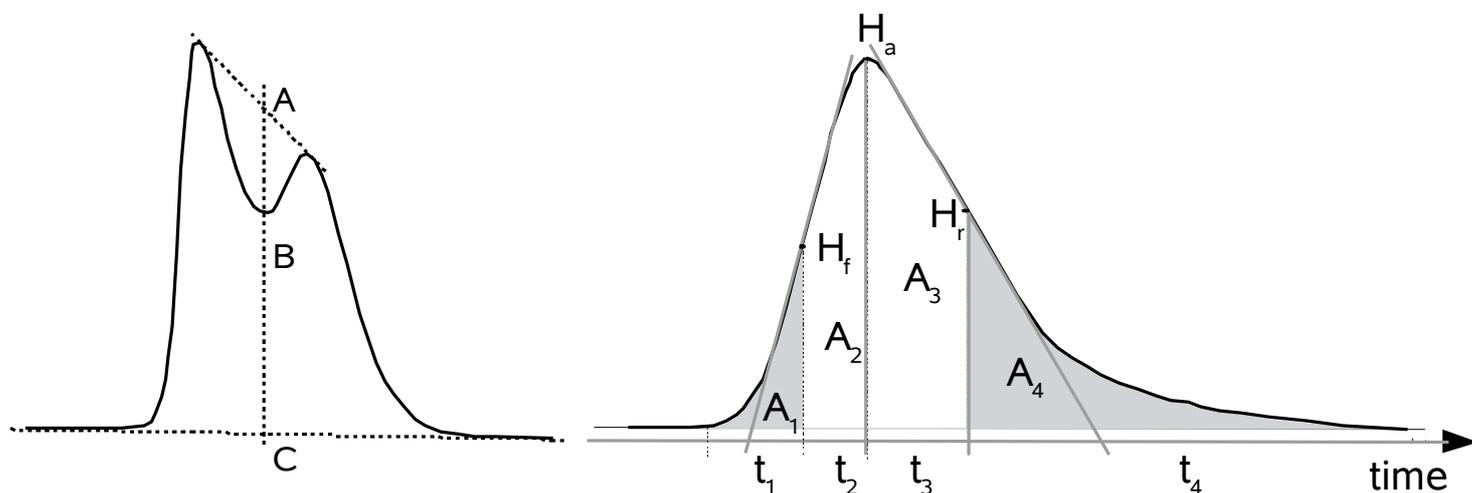
Figure 1: Calculation of a) resolution δ and b) symmetry S

Symmetry S according to Agilent ChemStation

software: $S = \sqrt{\dfrac{m_1 + m_2}{m_3 + m_4}}$ (3) with

$$m_1 = A_1 \left(t_2 + \frac{A_1}{1.5} H_f \right) \quad m_2 = \frac{A_2^2}{0.5 H_f + 1.5 H_a}$$

$$m_3 = \frac{A_3^2}{0.5 H_f + 1.5 H_a} \quad m_4 = A_4 \left(t_3 + \frac{A_4}{1.5} H_f \right) \quad \text{(4a-d)}$$

with A_i: area of slice i, t_i: time of slice i, H_f, H_r, H_a: height at front, rear inflection point, apex
(The symmetry parameter is 1.0 of a peak without tailing, leading)

3. Results and Discussion:
3.1. Column characteristics:
In order to check a wide range of RP-columns, stationary phases with different pore diameters, surface areas, alkyl chain length and bonding were evaluated.

Table 1 collects the most relevant parameters of the used phases.
Some aspects have to be emphasised, e.g. the small surface of the nonporous Micra,
the porous-layer-beads of the Poroshell, the monolithic Chromolith with its nanopores,
the special surface organic-coating of the Gemini or the special alkyl-linkage of the Zorbax or Poroshell stablebond phases.

3.2. Stability:
Hydrolysis of the bonded alkyl-groups with acidic eluent (e.g. 0.1 % TFA) is well known and has been a severe drawback in former RP-column generations. This influences the stability of the retention times and the robustness of the method. It has been shown that retention times decreased in peptide maps from run to run [16].
Engelhardt et al. [19] proposed a method to characterise RP-columns with aromatic compounds using thioruea to determine dead volume, amines to check for silanol groups and toluene, ethylbenzene to evaluate the methyl-selectivity and plate height.
According to this method, thiourea, aniline, phenol, dimethylaniline, 2-, 3-, 4-ethylaniline, toluene, acetic acid ethyl ester, ethylbenzene were eluted from a new column to verify its initial state. The column was then flushed with 50 % CH_3CN + 0.1 % TFA for two weeks without injection. The final state was checked the same way as at the beginning.
Degrading of the C-content is indicated with a reduction of the retention times and an enlarged tailing of the amines according to the increasing number of silanol groups.
Table 2 shows the results of this stability test for the used phases.
It can be seen that some phases have good stability even over weeks of operation, which means smaller 10% change of the retention time or plate height (Micra, Poroshell, Zorbax, 218TP), others are less robust (BioWidePore, 238Everest, Jupiter).

Tab. 1: Column data

column	dimension [mm]	particle diameter [μm]	pore diameter [nm]	surface area [m²/g]	Carbon content [%]	alkyl chain	post silanisation
Micra	14 x 4.6	1.5	-	3	ca. 0.8	C18	no
Poroshell	75 x 2.1	5	30	4.5	n.a.	C18	no
Zorbax	75 x 2.1	5	30	45	2.8	C18	no
Chromolith	100 x 4.6	-	-	300	11	C8	yes
Gemini	150 x 2.0	5	11	375	14	C18	yes
Jupiter	50 x 2.0	5	30	170	14	C18	yes
218TP	50 x 2.1	5	30	60-110	8	C18	yes
238Everest	50 x 2.1	5	30	70-110	6	C18	yes
BioWidepore	100 x 2.1	3	30	100	3.5	C5	yes

Tab. 2: Stability test (column change after flushing
with 0.1 % TFA for 2 weeks)

change of [%]	retention ethylbenzene	plate height # toluene	symmetry # dimethylaniline
Zorbax	3	6	-6
BioWidepore	-16	24	17
218TP	-2	2	-8
238Everest	3	-119	16
Chromolith	1	7	27
Jupiter	-4	-10	22
Micra	-9	-4	10
Poroshell	1	2	-6

positive numbers mean improvement of the parameter

Tab. 4. Eluted proteins and detected peak-area
(sum of the area over all detected peaks)

column	protein-peaks detected	peak-area [mV*s]
Zorbax	10	20563
Gemini	7	5854
BioWidepore	10	22953
218TP	9	13596
238Everest	8	14246
Chromolith	10	11161
Jupiter	10	21882
Micra	9	19238
Poroshell	10	22283

3.3. Recovery:
RP-columns are widely used for peptide maps for many years showing good resolution and separation efficiency. Protein elution from RP-columns often shows broad and asymmetric peaks, indicating enhanced denaturing during the sorption on the surface. Sometimes this binding is irreversible and the proteins will not elute at all.
Table 3 shows the used proteins and their properties.

The proteins were injected in the order listed in table 3, each protein was injected twice.
The injected mass was 1 µg per protein, except for Fetuin 2 µg and MAK 5 µg were used because of the broad, diffuse peaks.

3.3.1. Eluted Peaks and Relative Recovery:
In order to judge protein recovery, the peak area of the UV-detection is registrated for all peaks on all columns. Table 4 depicts, how many proteins could be eluted at all and the measured sum of the peak areas over all protein elutions (areas have been corrected according to the different flow rate).
With Micra ADHGenase is missed, with Gemini ADHGenase, HKinase and MAK are missed,
with 218TP Fetuin and with 238Everest ADHGenase, HKinase are not eluted.
It is an important aspect for validation of a chromatographic protein pattern, that some columns do not elute a protein at all, while others do.

There is also a big difference in the sum of the peak-areas over all eluted proteins.

It is remarkable that the Gemini-column showed only 25 % area, the Chromolith nearly 50 % of the protein peak area compared to the "best" reference column (BioWidePore).
The 218TP- and 238Everest-columns are also not very good candidates in respect of relative recovery. This relative recovery does not represent the absolute mass recovery, meaning how many percent of the injected protein is eluted.

3.3.2. Mass recovery:
Absolute recovery had been measured analysing the protein concentration by aminoacid analysis after fractionating the eluted peak with and without column. In both cases injection of the sample was done with the LC-sampler and manually fractionating the total peak.
Aminoacid analysis of the fractions was done with microwave driven liquid-phase hydrolysis with 6 M HCl within the fractionation vials. Phe and Ile were used for quantification.
This procedure was exemplarily used for the protein elutions of the BioWidePore-column.
Mass recoveries of the different proteins are listed in table 5.

Most of the proteins are eluted with acceptable recovery (> 90%) but it seems to be difficult to elute glycosylated proteins. The mean mass recovery of the best performing BioWidePore-column is 77 %. Related to the measured peak areas (Tab. 4), the mean protein mass recovery of the other columns are shown in table 6.

Tab. 3: Protein-Data

	abbreviation	molweight [kD]	isoelectric point	glycosylation
Bovine serum albumin	BSA	66	4.9	-
ß-Lactoglobulin	ß-LGlobulin	36.8	5.2	-
Alcoholdehydrogenase	ADHGenase	146	5.4	-
Glucose-6-Phosphate-Dehydrogenase	DHGenase	128	5.7	-
Kreatinkinase	KKinase	81	6.3	-
Hexokinase	HKinase	102	4.7	-
Glycerinkinase	GKinase	251	4.2	-
Ribonuclease B	RNase B	12.6	9.2 - 9.6	single glycosylation
Fetuin	Fetuin	48	4 - 8	triple glycosylation
MAK<anti h-AFP>	MAK	156	4.5 - 5.2	multiple glycosylation

Tab. 5: Mass recovery of different proteins from the BioWidePore-column

BSA	ß-LGlobulin	ADHGenase	DHGenase	KKinase	HKinase	GKinase	RNase B	Fetuin	MAK
89%	106%	63%	99%	97%	98%	100%	70%	27%	25%

Tab. 6: Mean mass-recovery of the different stationary phases with the particle parameters

column	mass recovery (mean) [%]	surface area [m²/g]	pore diameter [nm]	alkyl chain	post silanisation
Zorbax	69	45	30	C18	no
Gemini	20	375	11	C18	yes
BioWidepore	77	*100*	*30*	*C5*	*yes*
218TP	46	85	30	C18	yes
238Everest	48	90	30	C18	yes
Chromolith	37	*300*	*0*	*C8*	*yes*
Jupiter	73	170	30	C18	yes
Micra	65	3	0	C18	no

At a first glance the large surface area of the Gemini-column seems to have a fatal effect on mass recovery and the small surface of a porous-layer Poroshell-column is positive. A closer look reveals, that large surface areas of the Jupiter- or Zorbax-column present better mass recoveries than e.g. the minimal surface of Micra-column. All these columns have C18 chain length (other bonding chain length is marked italic in the table).

The Zorbax- and Poroshell-phase, which are declared to have the same bonding chemistry, show a small improvement of the mass recovery with the reduction of the surface area.

Generally there seems to be no correlation between surface area and recovery.

Comparing the results for 218TP-, 238Everest-, Jupiter-, Zorbax-, Poroshell-column (all 30 nm, C18-phases), pore diameter seems not to play the dominant role in protein recovery. Phases without pores are also not compelling for good mass recovery.

Reduction the chain length of the C5-BioWidePore-column seems to improve the recovery, but the C8-Chromolith-column shows very poor results compared to C18-Phases of the Jupiter- or Zorbax-column. Chain length seems not to play a critical role for protein recovery, but one has to keep in mind, that the materials in this study with different pore attributes and morphologies make this conclusion difficult.

Opposite to the irreversible binding of amines to the free silanol groups of RP-phases, post silanisation also has no primary effect on the recovery when looking at the good results of the Zorbax- or Poroshell-columns without post silanisation. In these cases one has to keep in mind, that the manufacturer declares a special bonding chemistry with these stable-bond phases.

Perhaps it is the most important aspect to have a more ore less suitable ß bonding chemistry especially for the sorption, denaturation and desorption of proteins on the hydrophobic surface. The effect of a not optimal surface will be reduced, when a lower surface area is used, or will be of minor influence, when higher protein-concentrations are separated e.g. for semipreparative work.

Looking at the practical use of actual commercial protein phases, it has to be mentioned, that 4 of the 9 tested columns presented protein mass recoveries smaller 50 %. In worst case, only 20% of the injected protein is eluted.

Most recent work shows, that using small particle column material (1.4 µm) the resulting high operating pressure in some cases had the added benefit of reduced carryover and improved recovery eluting small proteins [20]. The recovery is not significantly improved e.g. with BSA.

3.4. Carry Over:
Difficulties with protein recovery are often accompanied by a severe carry over of an eluted protein in the following chromatogram. Carry over was measured by injecting the same sample twice. Normally the second peak should show the same peak area as in the first chromatogram.

It had to be seen that in many cases the peak in the second chromatogram was much higher than in the first one. Percentage change of peak area was calculated as carry over related to all eluted proteins. Table 7 shows the results. In most cases BSA is a problematic protein and shows bad carry over values. BSA-carry over gives a valuable hint for recovery problems and can be performed easily in every protein-laboratory.

It has to be mentioned that in some cases peak area of the second chromatogram was significant lower than that of the first elution, which may be explained by an irreversible adsorption of the corresponding protein due to strong interactions with the irreversible retained protein of the first injection.

This holds true for many proteins eluted from the Jupiter-column and some peaks of the BioWidePore column. Almost every time elution of ß-LGlobulin showed a smaller peak area in the second chromatogram, e.g. - 47 % in case of the Chromolith column.

Tab. 7: Carry Over effect

column	carry over (mean)	carry over (max)	worst protein
Zorbax	6	14	ADHGenase
Gemini	9	40	GKinase
BioWidepore	18	54	ADHGenase
218TP	13	37	GKinase
238Everest	9	27	GKinase
Chromolith	14	32	ADHGenase
Jupiter	10	26	MAK
Micra	5	28	BSA
Poroshell	11	29	BSA

Tab. 8: Symmetry of the protein peaks

column	symmetry (mean)	symmetry (BSA)
Zorbax	0.92	0.61
Gemini	0.37	0.19
BioWidepore	0.96	0.59
218TP	0.51	0.20
238Everest	0.44	0.42
Chromolith	0.96	0.40
Jupiter	0.67	0.44
Micra	0.33	0.21
Poroshell	0.37	0.19

In order to calculate the mean carry over, absolute values have been used.

Most of the time a positive carry over effect results from an improved protein recovery from run to run. It can be assumed that in these cases the active centers at the silica surface have to be saturated with protein, to get acceptable results.Repetitive injection of the same BSA-sample showed this effect up to the tenth run. Increasing BSA peak-area and decreasing retention time in following injections are shown with figures 2a and 2b. Even after ten BSA-injections onto a new Micra-column retention conditions seem not to be stable. It can be assumed that this effect can be measured also with better suited phases to a minor extend. Peak symmetry continually declined within the same series from 0.312 at the 1st to 0.097 at the 10th run.

3.5. Peak Symmetry:

Peak shape of protein peaks is most often much worse than with low molecular weight analytes because of the much smaller diffusion coefficients and severe interaction with the stationary phase. While e.g. a peptide separation can be largely improved using smaller particle sizes (1,5 to 2 µm), the theoretical plate height often has no such an effect on protein separations. Protein peaks are often unusual broad and asymmetric (e.g. Fig. 5), which is not an effect of a poor plate height of the packing. Asymmetric protein peaks often indicate intensive interaction with active sites of the stationary phase e.g. remaining silanol groups on the RP-surface. This is often accompanied by a massive protein denaturation on the surface.

This can be a very severe limitation of the separation efficiency in complex protein samples.

Table 8 presents the results of the different columns.

Mean peak symmetry has only been calculated for the proteins with one singular peak (BSA, ADHGenase, DHGenase, HKinase, RNase B).

It is interesting that phases with minimal surface area (Micra-, Poroshell-column) show the worst symmetry, and the Zorbax- and Poroshell-column from the same manufacture with declared same bonding chemistry exhibit very different symmetries.

This might be related to an overloading effect due to the much lower surface area (factor 100) compared to the other packings.

One also should keep in mind that only Micra-, Poroshell- and Zorbax-column have no endcapping and consequently more residual silanol groups available. Especially BSA as a basic analyte will undergo strong interactions with acidic silanol groups. These silanol groups will be overloaded which results in a strong tailing. BSA seems to be a valuable protein to check peak symmetry of RP-columns.

3.6. Selectivity:

Small differences in protein structure will result in very difficult chromatography.

The stationary phase should present optimal selectivity for this task. To examine separation efficiency of a RP-phase the resolution of two ß-LGlobulin-Isomers (equation 2) and an impurity of KKinase and GKinase (equation 1) were examined.
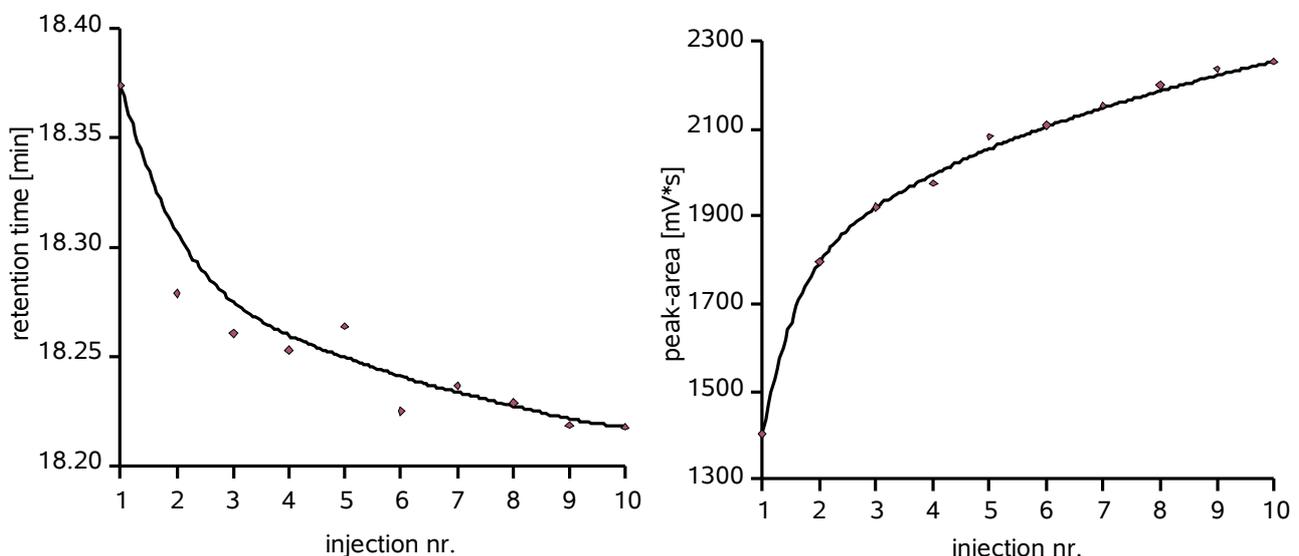

Fig. 2: Peak area and retention time with repetitive injection of 1µg BSA onto a Micra-column.

Tab. 9 Separation efficiency

column	resolution #	resolved peaks with fetuin	resolved peaks with MAK
Zorbax	1.42	6	6 (small peaks)
Gemini	0.00	0	-
BioWidepore	1.63	3	6 (small peaks)
218TP	0.72	1	0
238Everest	0.08	0	0
Chromolith	0.00	0	0
Jupiter	0.92	4	9
Micra	0.55	2	0
Poroshell	0.38	4	4 (small peaks)

One of the most demanding tasks of protein is the separation of proteins that differ only in their glycosylation. RNase B has one glycosylation site, in fetuin 3 glycosylation sites with 17 different glycosylation structures are known, IgG-antibodies have many glycosylation structures, which are impossible to resolve even with very efficient separation techniques such as isoelectric focusssing in gel-electrophoresis. Table 9 summarises the results.

3.6.1. Resolution:
Figure 3, 4, 5 show the chromatograms of ß-LGlobulin-Isomers, KKinase and GKinase with the BioWidePore-, Zorbax- and Poroshell-column. It can be seen that the resolution decreases in the cited order.

Fig. 3: Protein-chromatograms BioWidePore-column
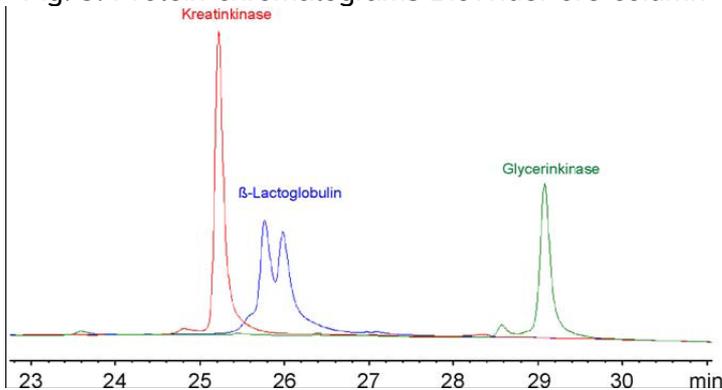
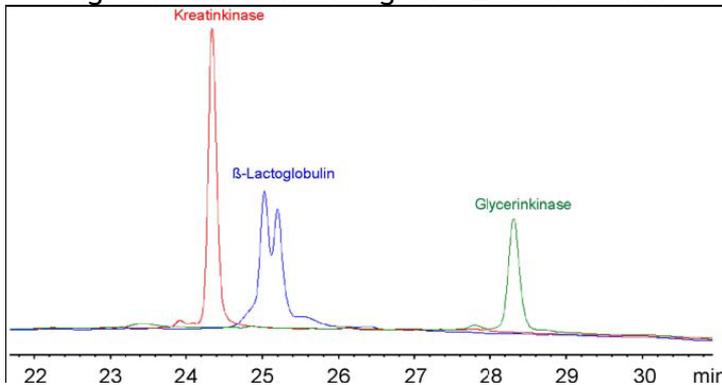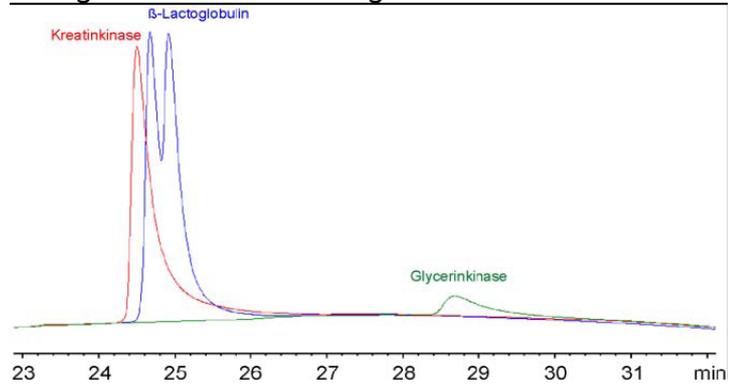Fig. 4: Protein-chromatograms Zorbax-column

Fig. 5: Protein-chromatograms Poroshell-column

3.6.2. Glycosylation differentiation:
It was surprising that with all columns there was only one peak when eluting RNase B.
Elution of the glycosylated isomers of Fetuin or MAK presents very different chromatograms.

Many RP-columns show some differentiated peaks when eluting Fetuin (Fig. 6, 7).

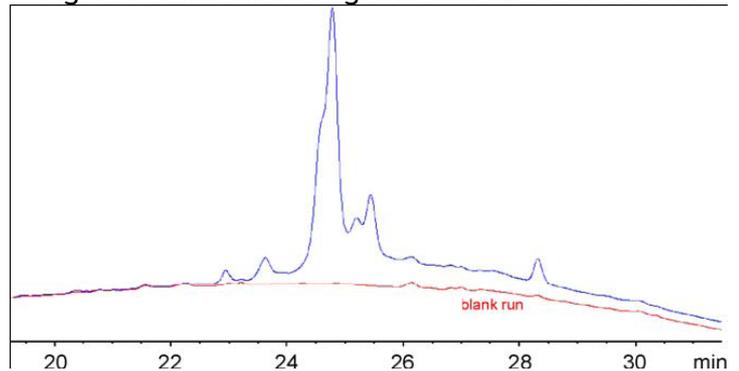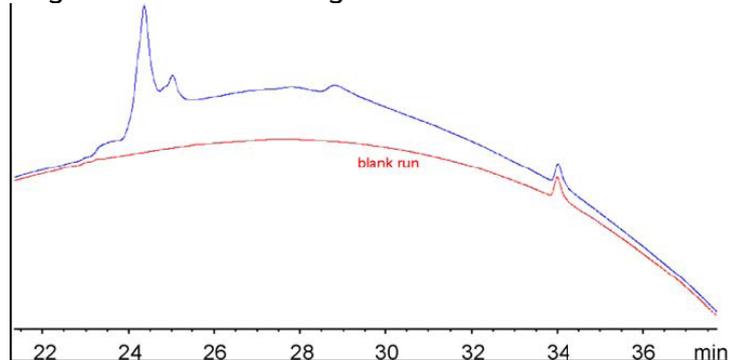Fig. 6: Fetuin-chromatogram of the Zorbax-column

Fig. 7: Fetuin-chromatogram of the Poroshell-column

The Gemini- and 238EV505-column show no Fetuin peaks at all. Only the Jupiter-column presents many well discriminated peaks in the MAK-chromatogram (Fig. 8). The BioWidePore- and Poroshell-column show some minor peaks (Fig. 9, 10), while the other columns present only one broad peak. The Gemini-column does not elute the MAK at all.
It can be stated that the Jupiter-, Poroshell and BioWidePore-column present the "best separation efficiency" with these proteins.

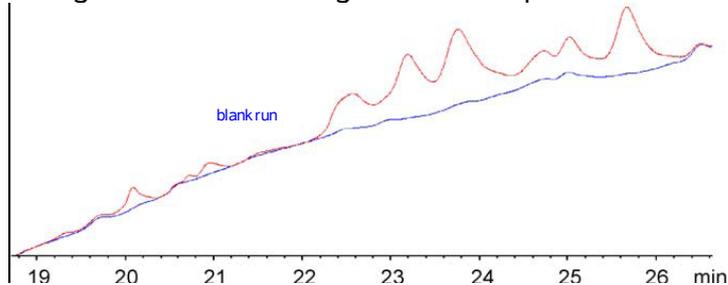Fig. 8: MAK-chromatogram of the Jupiter-column

blank run

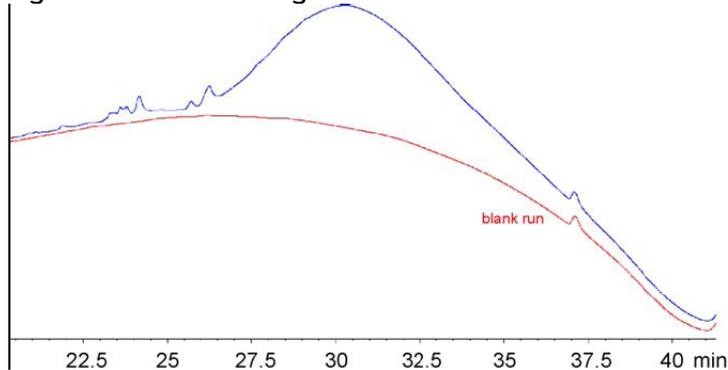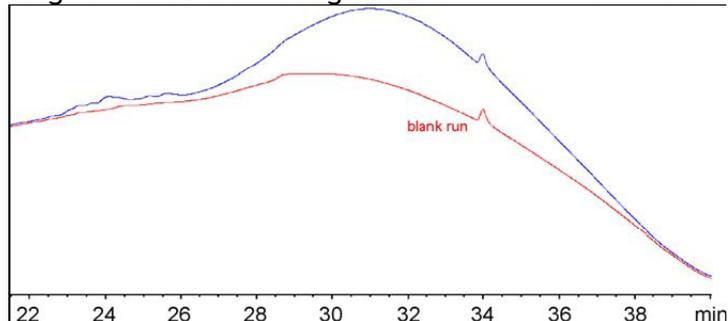Fig. 9: MAK-chromatogram of the BioWidePore-column

blank run

Fig. 10: MAK-chromatogram of the Poroshell-column

blank run

4. Conclusion:
This overview compares some commercial RP-columns for protein separations regarding stability, recovery, carry over and selectivity.
Besides the BioWidePore-, 238Everest-, Jupiter-column all stationary phases show acceptable stability for the use with acidic eluent in the pH-range of 2, using 0.1 % TFA as organic modifier. Even after 2 weeks retention time or peak symmetry were constant. The BioWidePore-column showed severe reduction of retention time due to loss of carbon content by hydrolysis. This may be explained by the good accessibility of the surface due to the shorter chain length of this stationary phase.

Most important aspect is recovery of the proteins which showed severe differences. Worst recovery present Gemini-, 218TP-, 238Everest- and Chromolith-column, where the Gemini- eluted only 7 and the 238Everest-column 8 of the injected 10 proteins.
Some phases showed quite acceptable mass-recoveries of more than 90 % for most of the used proteins, glycosylated proteins seem to have worse recoveries.
Many stationary phases exhibit low mass recovery (< 50 % for Gemini-, Chromolith-, 218TP-, 238Everest-column). Using the wrong column can result in < 20 % recovery of the injected proteins, which is unacceptable for a validated chromatographic information.

Keeping in mind the limited number of different phases examined, there seems to be no correlation of the recovery to alkyl-chain-length, surface area or pore diameter. Regarding the complex interaction and partially denaturation of larger proteins on the hydrophobic surface, the bonding chemistry of the stationary phase may play an important role in protein interaction. It is therefore very important to choose an optimal stationary phase for protein characterisation in complex mixtures.

Carry over of the protein can also be severe problem, showing that the BioWidePore-, Chromolith- and 218TP-column have more than 10 % mean carry over and that up to 40 or 50 % of the injected protein can elute in the following chromatogram. Most often BSA shows this unwanted effect and can also serve as an easy to use marker for recovery and carry over problems. It is well known that these deficiencies of HPLC columns can be improved with the course of repetitive injections or by coating of the stationary phase with a large amount of protein at the beginning.

Limited resolution based on the poor protein peak-width is often made worse by significant peak asymmetry. Zorbax-, BioWidePore- and Chromolith-column present best peak symmetries near to 1, Gemini-, Micra- and Poroshell-column show symmetry parameters below 0.4.

As known from separation of low molecular weight compounds, different phases show different selectivities. This also holds true for protein separations with the examined columns. Zorbax- and BioWidePore-column show best selectivity for the separation of ß-Lactoglobulin, Kreatinkinase or Glycerinkinase peak pairs, while the Jupiter column performed best in the case to separate glycosylation isomers of the monoclonal antibody.

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