

PEG, PEG-Steroide und PEG-Peptide aus Plasma – eine wirkliche Herausforderung

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pharm-analyt Labor GesmbH.

Ferdinand-Pichler-Gasse 2

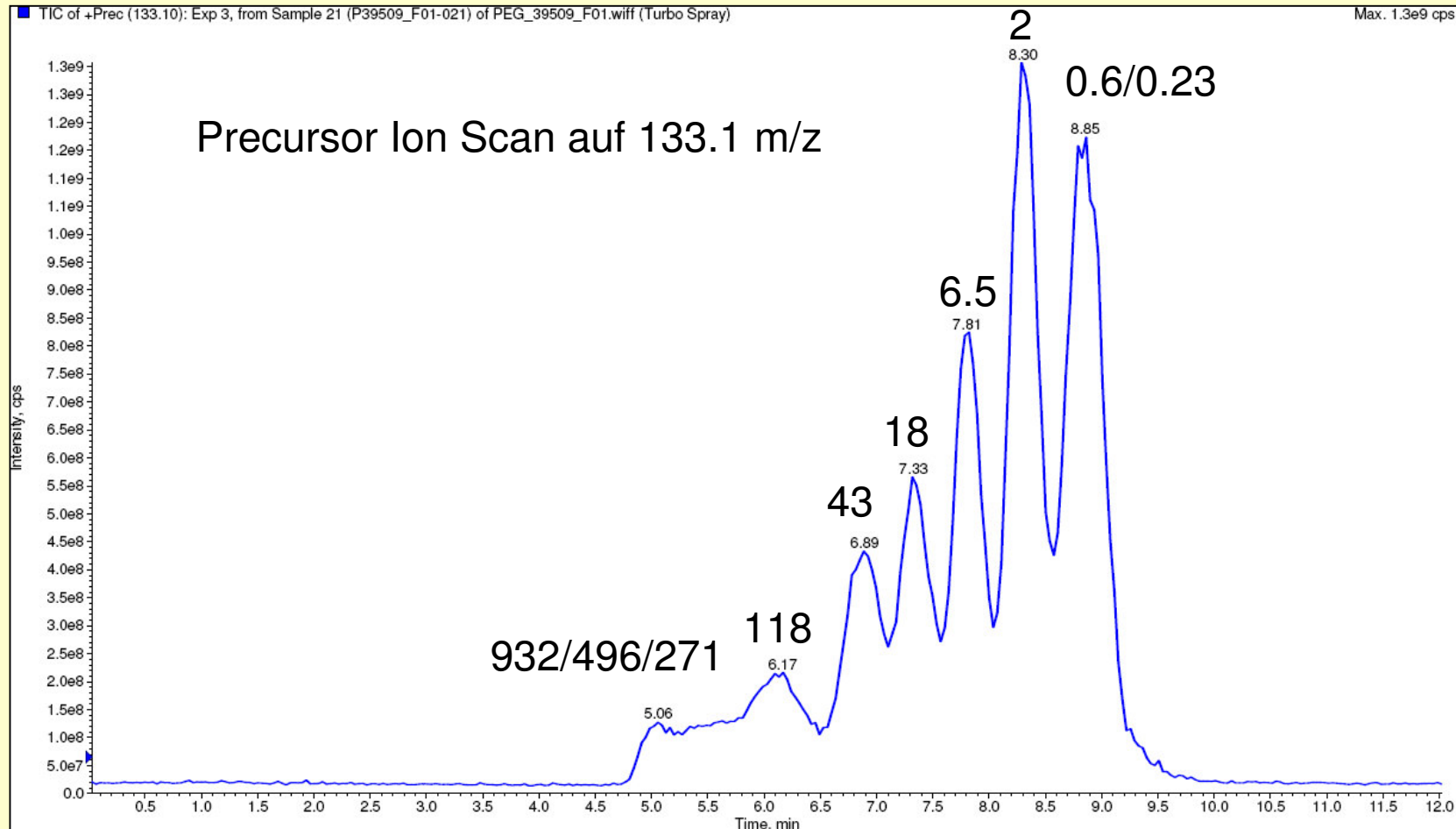
2500 Baden/Österreich

Allgemeine Gedanken zum PEG-Nachweis mit HPLC-MS oder MS/MS

PEG und gekoppeltes PEG

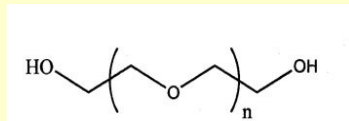
- 1.) Molekülgröße von PEG häufig zwischen 1 – 100 kDa, RP-Chromatographie oder SEC (size exclusion chromatography) ?
- 2.) PEG-Peptid: wenn PEG gewichtsmäßig dominiert, dann kann man vermutlich das MS/MS Signal des Peptids nicht mehr sehen und daher kann dadurch ein selektiver Nachweis PEG-Peptid vs. PEG nur mehr bedingt möglich sein.
- 3.) Plasma: zahlreiche Probeabnahmeröhrchen (von diversen Herstellern) haben verschiedene eher kleinere PEGs (teilweise > 1 kDa) drinnen-ohne Deklaration!
- 4.) Ionisierung von Peptiden mit ESI - na klar!
- 5.) PEGs für Tandem-MS Nachweis, womit ionisieren ? ESI Vielfachladungen vs. APCI Bruchstücke, und welche Fragmente?

PEG Trennung auf einer GFC P4000 Säule, Mischung aus 10 PEGs (in kDa)



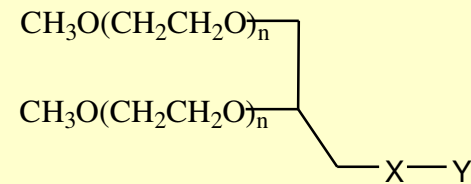
Determination of 60 kDa branched PEG in rat plasma using LC-MS, LLOQ: 1 µg/mL

Linear Polyethylene glycol (PEG)



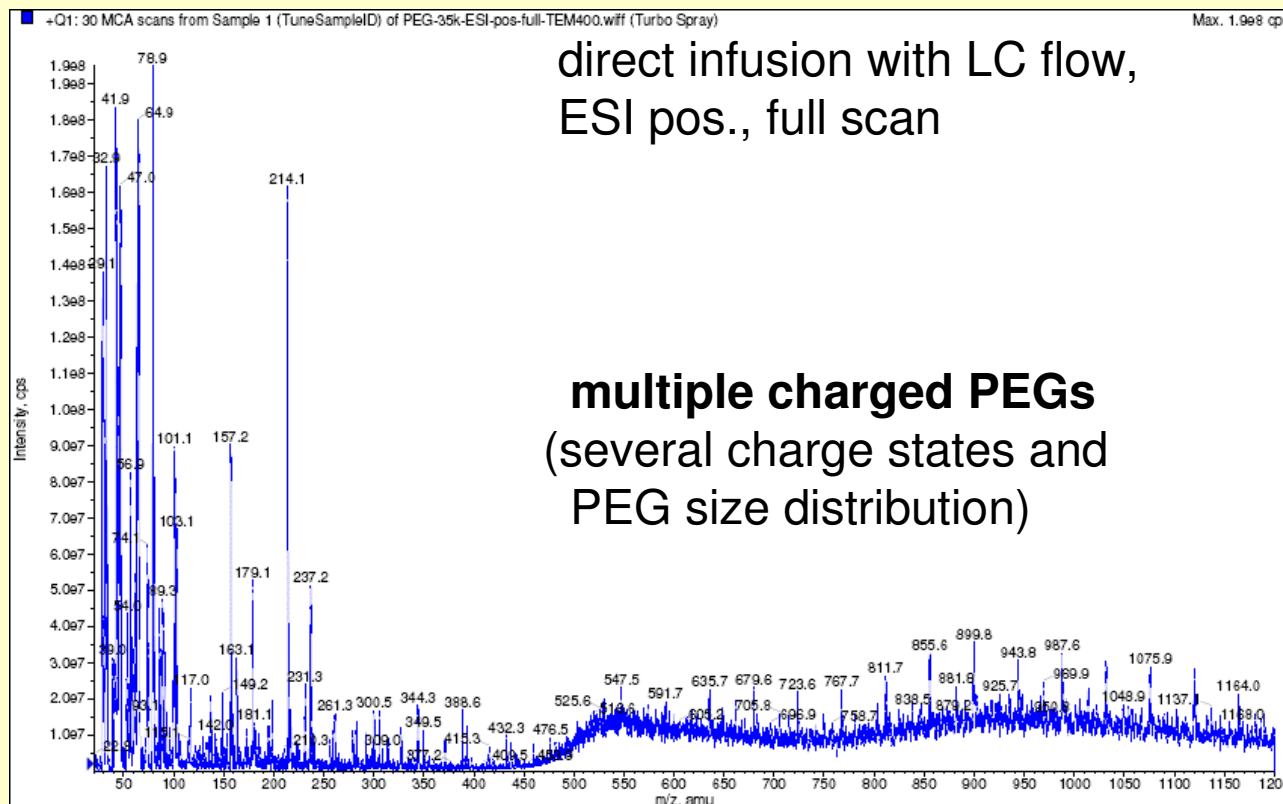
Commonly available:
from 0.3 to 35 kDa

Analyte (branched PEG)



60 kDa

Startversuche mit reinen PEGs ohne Kopplung anderer Moleküle

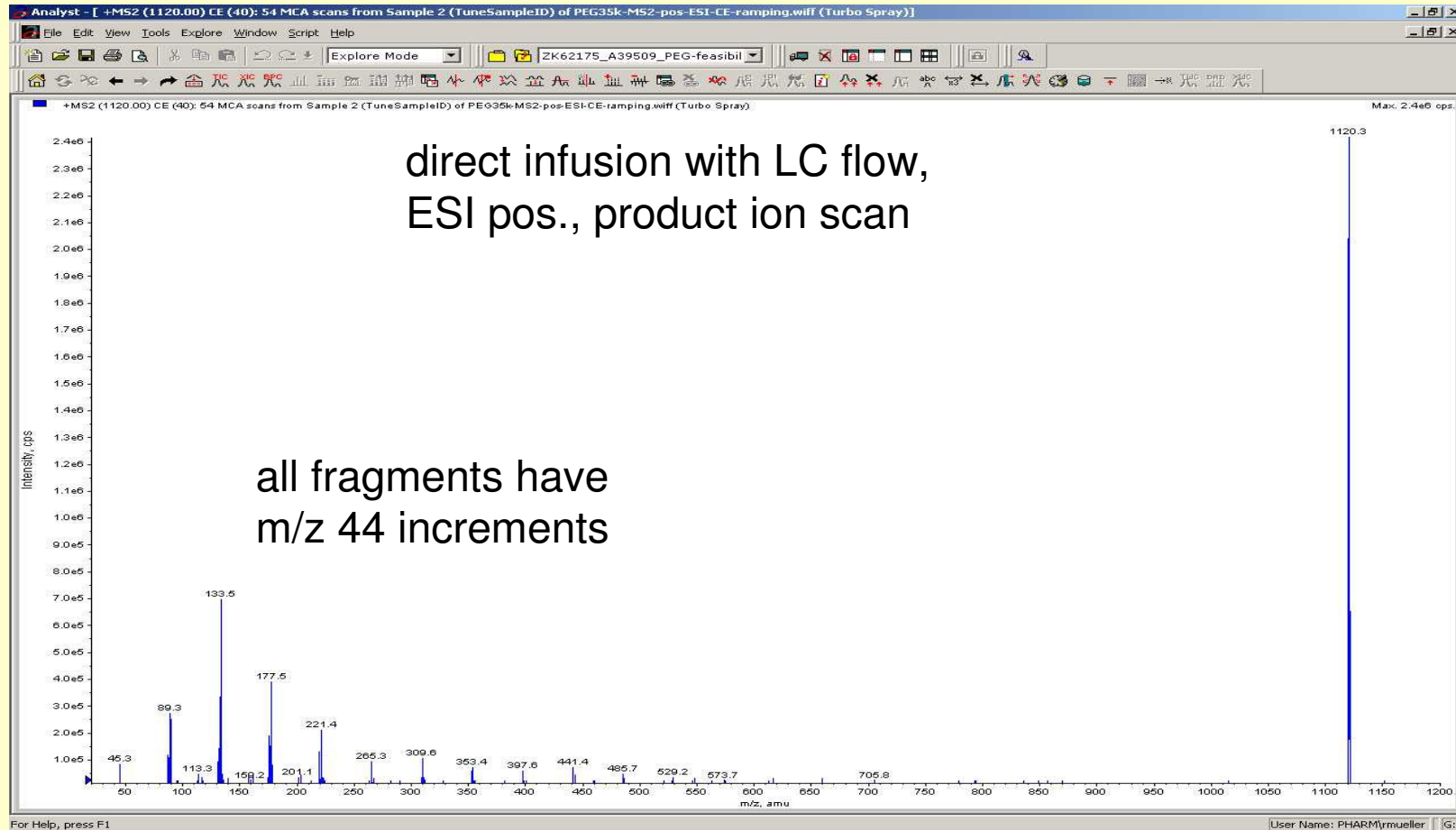


35 kDa PEG

All peaks below m/z 100 are unspecific => MS2 necessary

using APCI: only Na-adducts observed (poor fragmentation of Na-adducts!)

35 kDa PEG (linear, ungekoppelt)



main MS/MS fragments:

m/z 45.0 [C₂H₅O]⁺, m/z 89.1 [C₄H₉O₂]⁺, m/z 133.1 [C₆H₁₃O₃]⁺, m/z 177.1 [C₈H₁₇O₄]⁺

Conditions for HPLC-MS-Detection of different PEGs

Constant parameters:

50 mM formic acid
in water/MeOH

Reversed Phase C-18 and HILIC column:

- PEGs with higher MWs elute later
- Leeching from columns
- Leeching co-elutes with 35 kDa PEG
- less retention on HILIC columns compared to C18

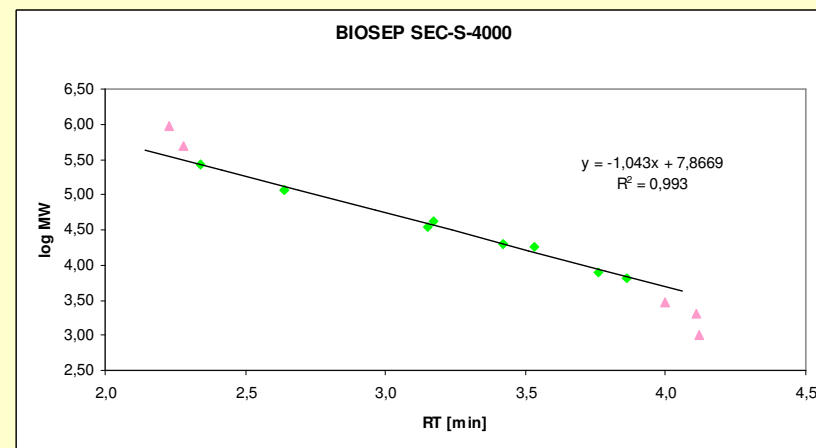
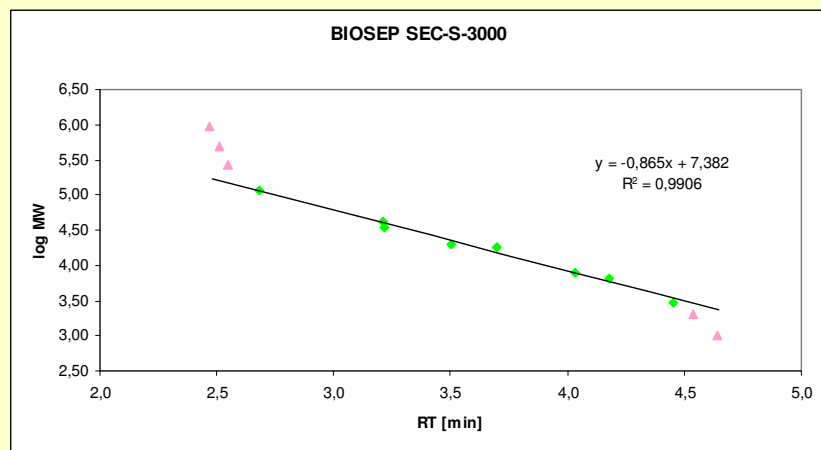
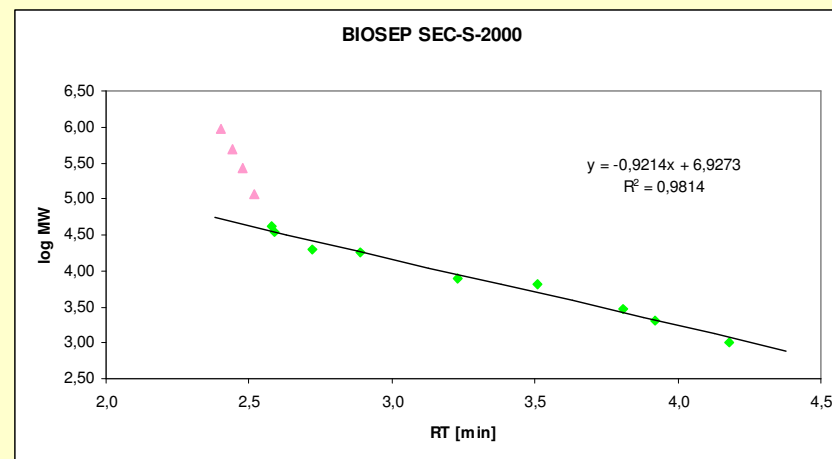
Size exclusion chromatography:

- no benefit when using a gradient => isocratic method
- more than 50 % MeOH necessary to elute PEG from column
- signals are more intensive when using a higher percentage of MeOH

Applied Chromatography:

Biosep SEC,
300x4.6 mm, 5 μ m (Phenomenex)

isocratic, 10 min, 0.8 mL/min
50 mM FA in 90 % MeOH
max. temperature: 50 $^{\circ}$ C
max. back-pressure: 70 bar

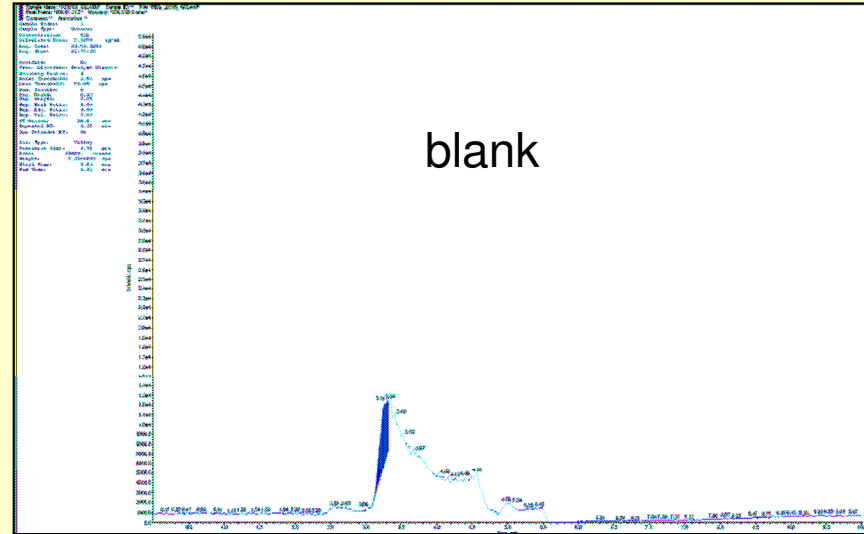
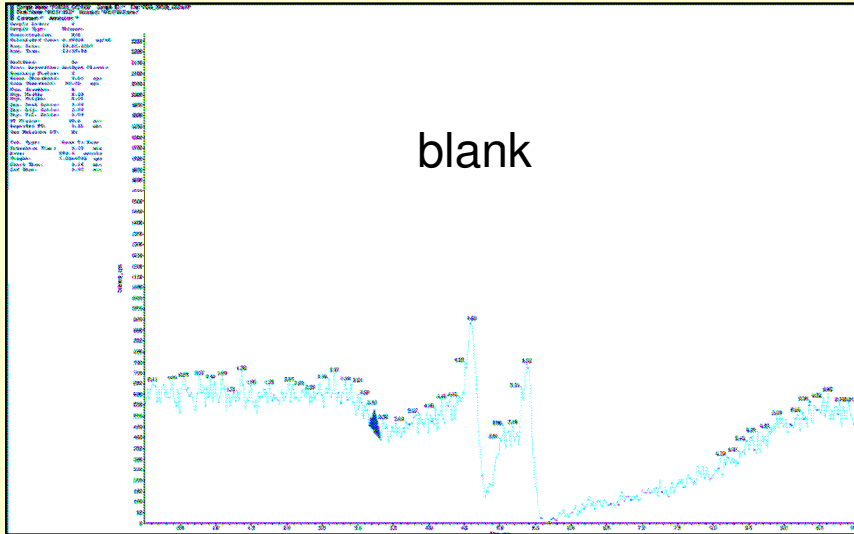


1.) PEG Nachweis aus Plasma

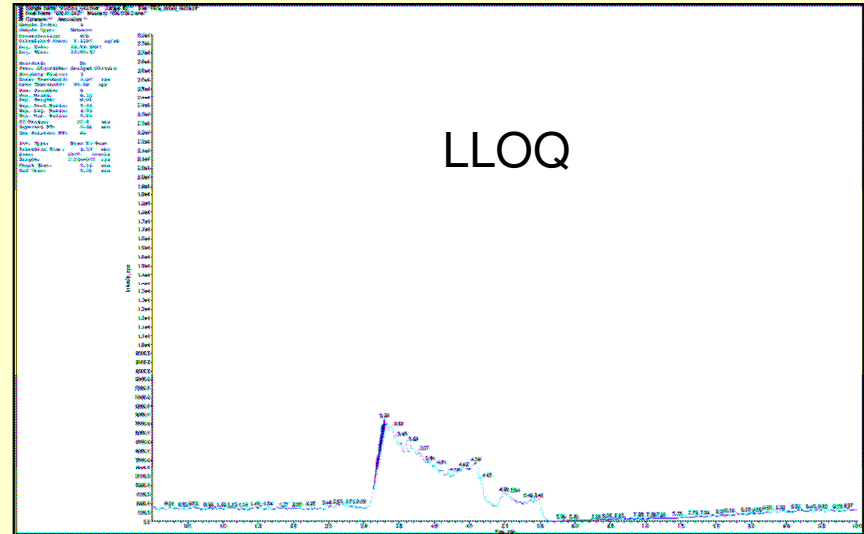
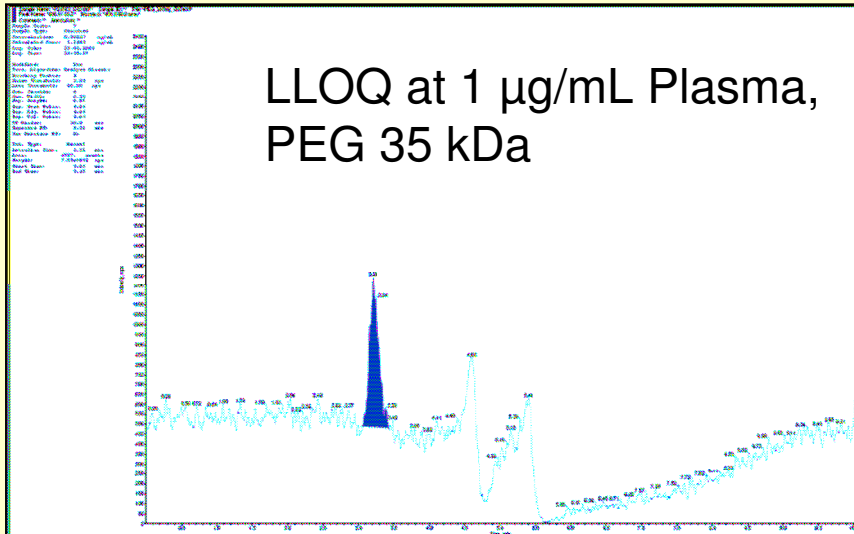
- PEG (linear) mit etwa 35 kDa
- PEG (verzweigt) mit etwa 60 kDa

protein precipitation with MeOH (4+1)

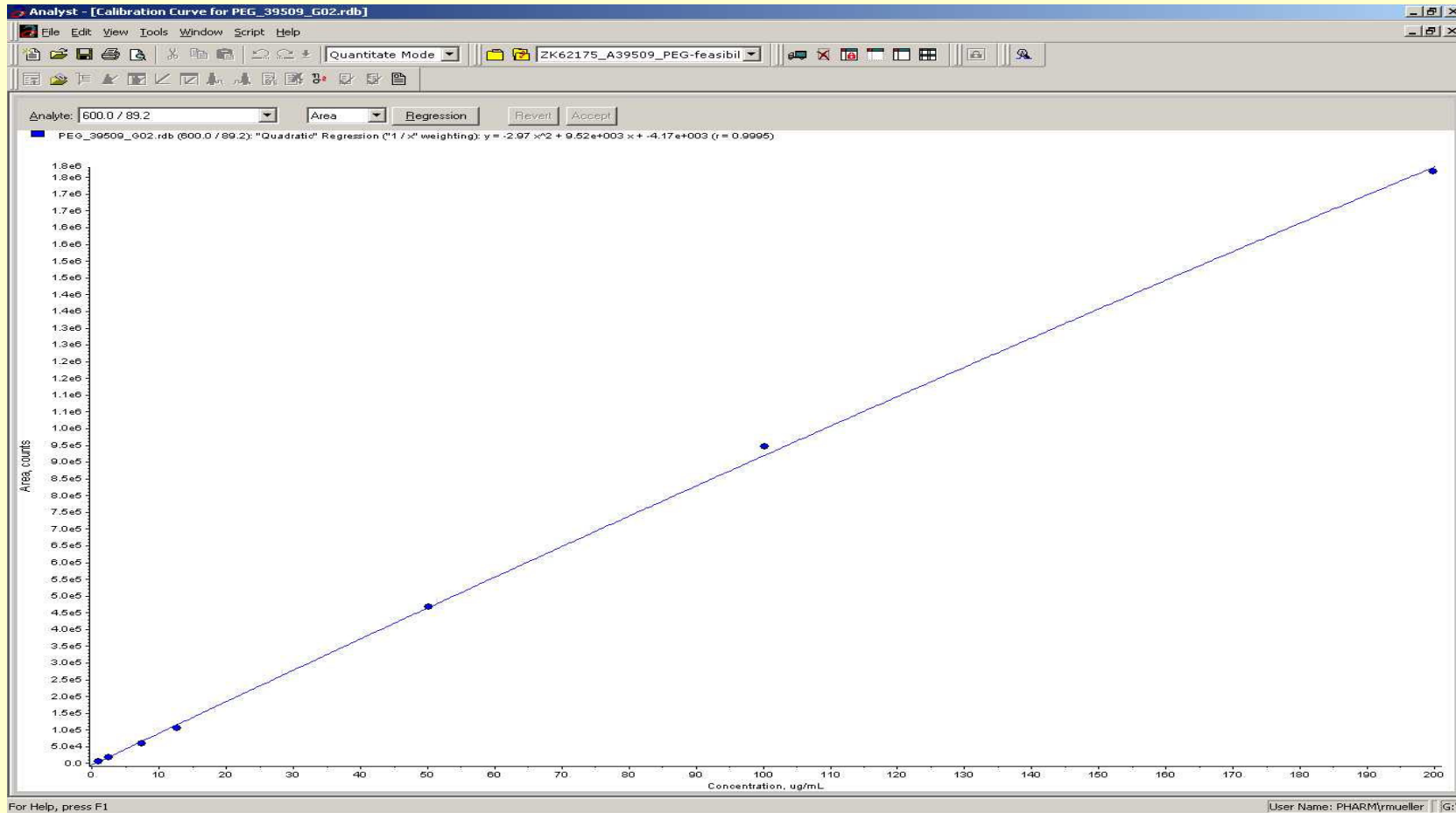
protein precipitation with ACN (4+1)



Injection volume: 15 μ L



Determination of 35 kDa linear PEG in rat plasma



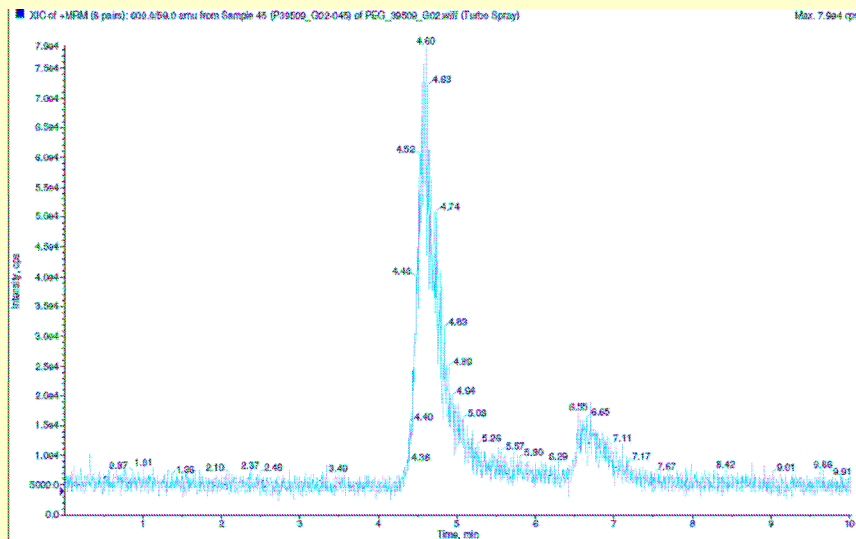
calibration range: 1 - 200 µg/mL
without internal standard

Specific requirements for an internal standard:

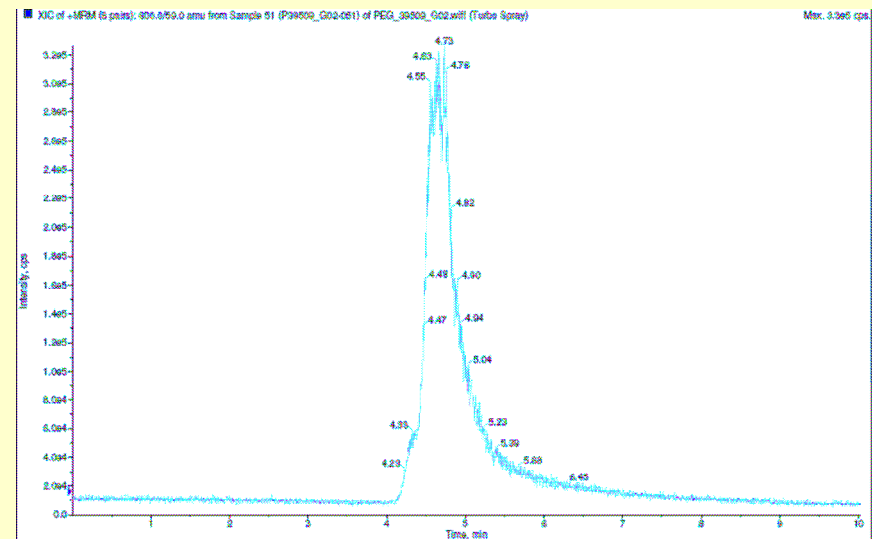
- High molecular weight (about >3 kDa)
- Synthetic, well homologous polymer
- No ethylene glycol building blocks
- Soluble in MeOH (IS-WS solution)

Example: PolyPropyleneGlycol: MRM 600/59.0

PPG 3000 from Applied Biosystems
Calibration Kit (d.f. 100)



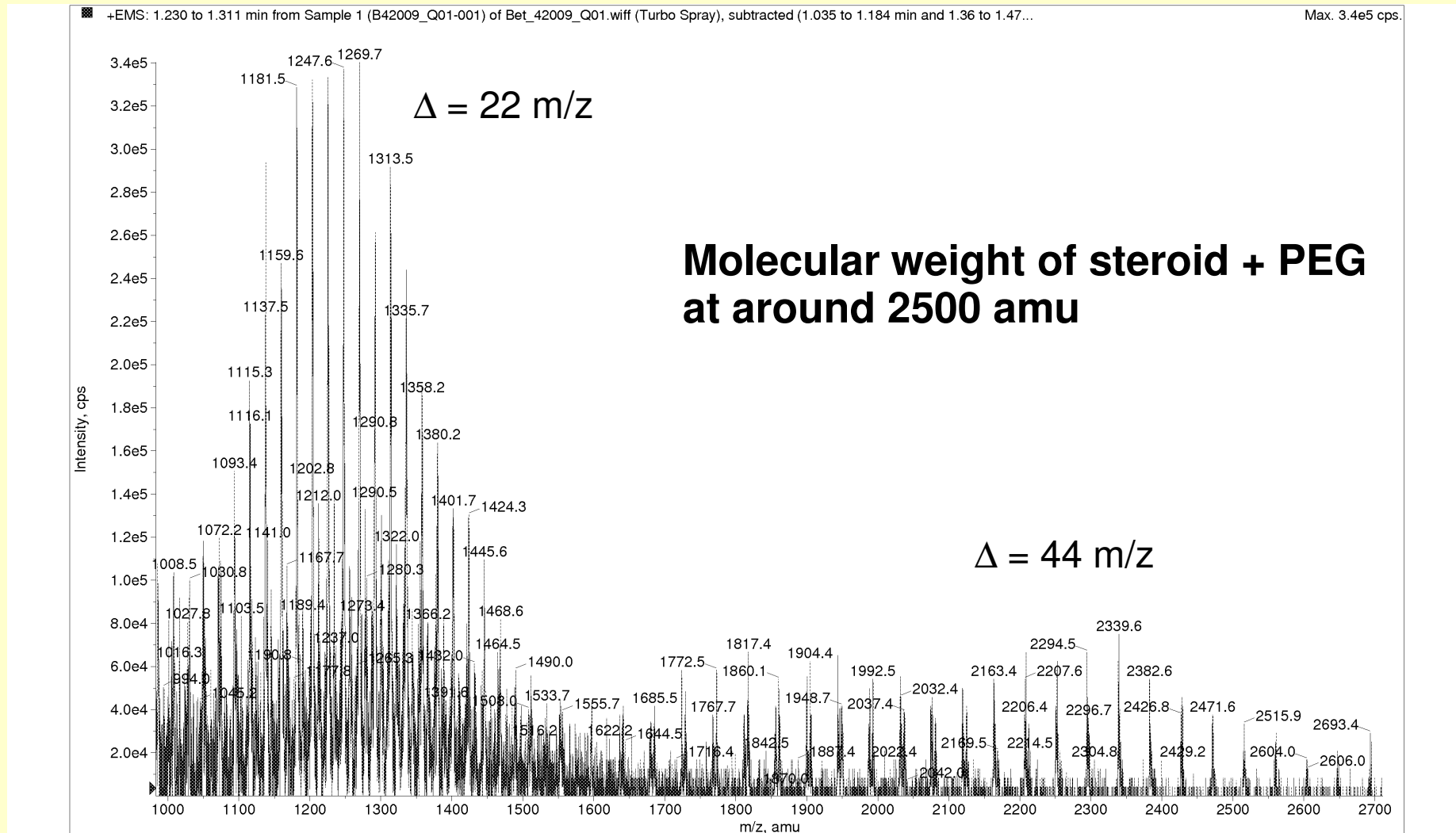
PPG 3.4 kDa from Sigma-Aldrich
(50 µg/mL)



2.) PEG-Steroid Nachweis aus Plasma

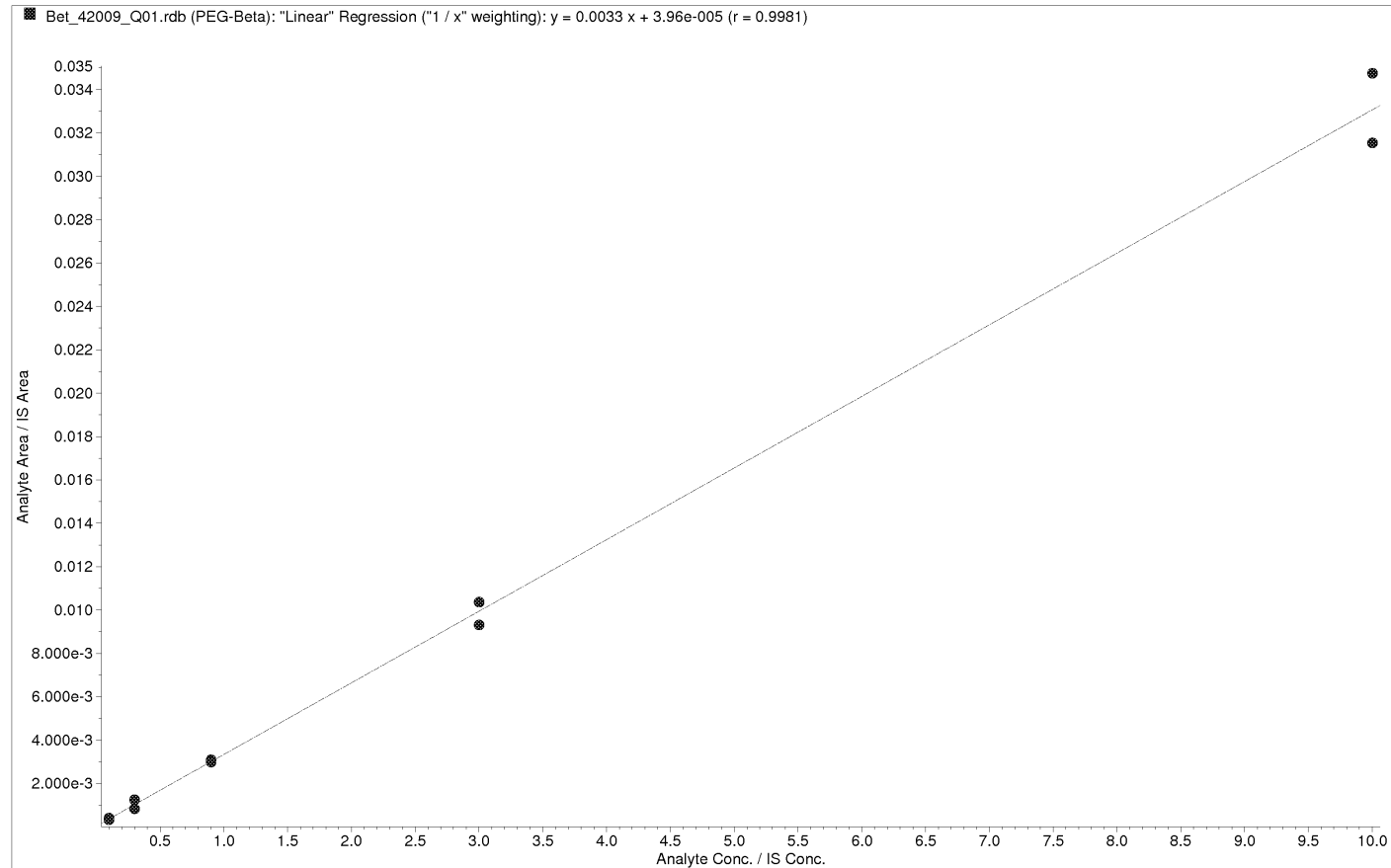
- PEG mit etwa 2000 Da
- Gekoppelt an Steroid mit etwa 400 Da
- API 4000 verwendet, da Scanbereich bis 3000 m/z möglich (API 5000 nur bis 1250 m/z möglich und reduzierte Sensitivität im obersten Bereich)

ESI positive infusion of a pegylated steroid, single and double charged ions clearly visible of the PEG distributions



Calibration Curve for the pegylated Steroid in Human Plasma (from 0.1 to 10 ng/mL)

Method Name: Bet_42009_Q01.qmf Operator: dmascher Analyst Version: 1.4.2
Results Name: Bet_42009_Q01.rdb Workstation: QTRAP



Printing Date: 29-05-2009

Printing Time: 03:46:05 PM

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Chromatograms of pegylated Steroid in Human Plasma at about 0.9 ng/mL and its Internal Standard

Method Name: Bet_42009_Q01.qmf

Operator: dmascher

Analyst Version: 1.4.2

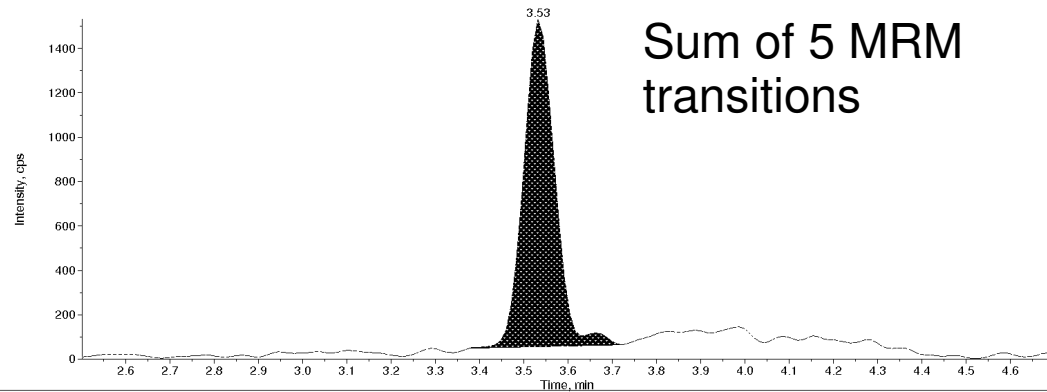
Results Name: Bet_42009_Q01.rdb

Workstation: QTRAP

Sample Name: "B42009_Q01-172" Sample ID: "" File: "Bet_42009_Q01.wiff"
Peak Name: "PEG-Beta" Mass(es): "1247.1/1060.4 amu,1225.1/1038.4 amu,1203.1/1016.4 amu,1269.1/1082.4 amu,1291.1/1104.4 amu"
Comment: "" Annotation: ""
Sample Index: 173
Sample Type: Standard
Concentration: 0.90027 ng/mL
Calculated Conc: 0.89417 ng/mL
Acq. Date: 05/29/2009
Acq. Time: 01:26:11 PM

Modified: No
Proc. Algorithm: Analyst Classic
Bunching Factor: 1
Noise Threshold: 8.11 cps
Area Threshold: 40.56 cps
Num. Smooths: 4
Sep. Width: 0.20
Sep. Height: 0.01
Exp. Peak Ratio: 5.00
Exp. Adj. Ratio: 4.00
Exp. Val. Ratio: 3.00
RT Window: 30.0 sec
Expected RT: 3.54 min
Use Relative RT: No

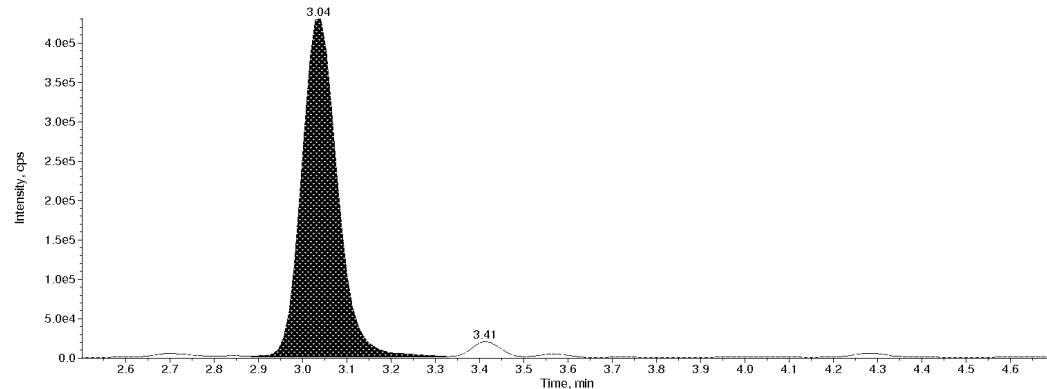
Int. Type: Base To Base
Retention Time: 3.53 min
Area: 7216.9 counts
Height: 1480. cps
Start Time: 3.38 min
End Time: 3.72 min



Sample Name: "B42009_Q01-172" Sample ID: "" File: "Bet_42009_Q01.wiff"
Peak Name: "IS2(IS)" Mass(es): "1335.1/1335.1 amu"
Comment: "" Annotation: ""
Sample Index: 173
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 05/29/2009
Acq. Time: 01:26:11 PM

Modified: No
Proc. Algorithm: Analyst Classic
Bunching Factor: 3
Noise Threshold: 1057.26 cps
Area Threshold: 5286.30 cps
Num. Smooths: 4
Sep. Width: 0.20
Sep. Height: 0.01
Exp. Peak Ratio: 5.00
Exp. Adj. Ratio: 4.00
Exp. Val. Ratio: 3.00
RT Window: 30.0 sec
Expected RT: 3.04 min
Use Relative RT: No

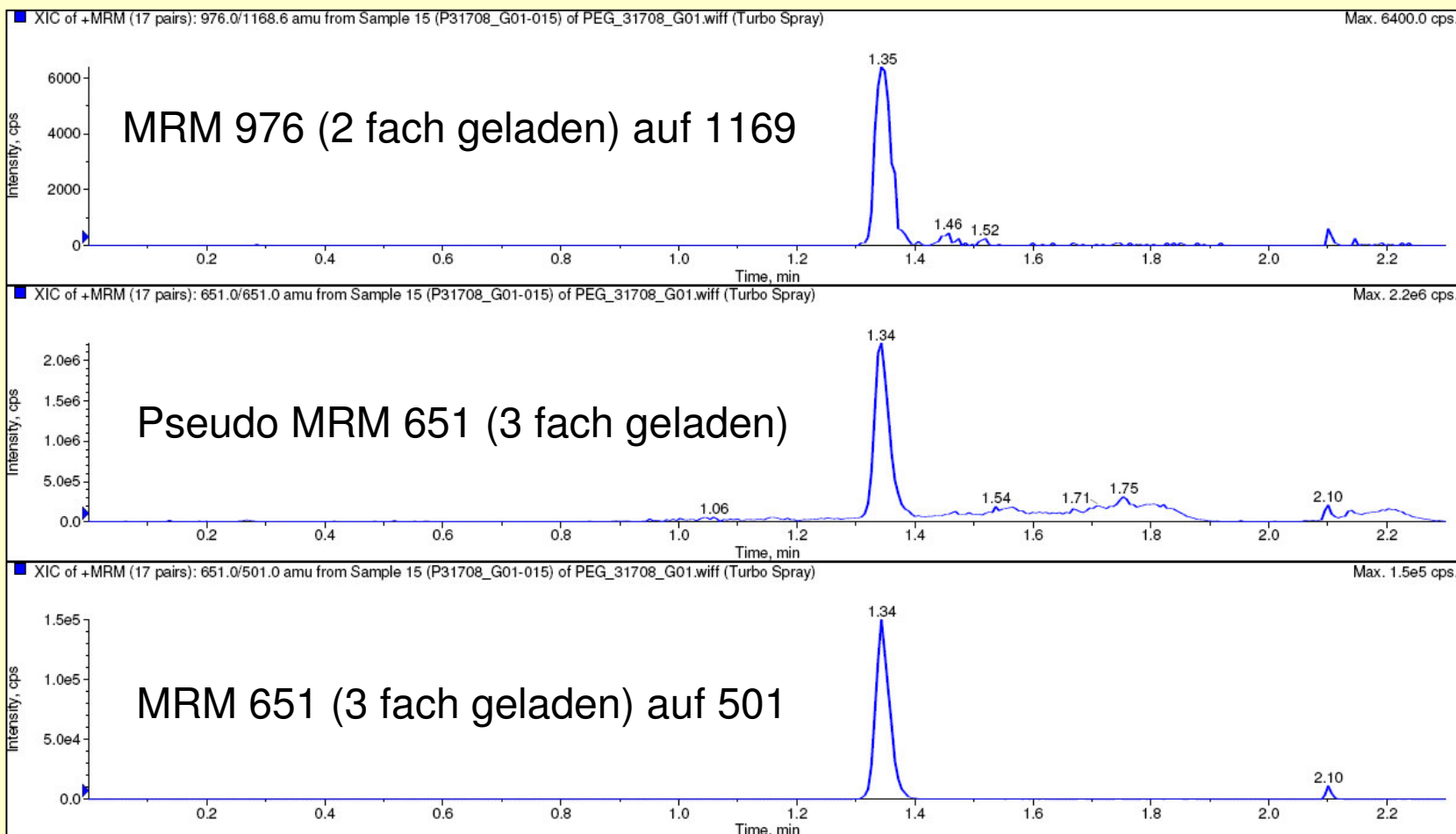
Int. Type: Valley
Retention Time: 3.04 min
Area: 2411500. counts
Height: 434000. cps
Start Time: 2.88 min
End Time: 3.33 min



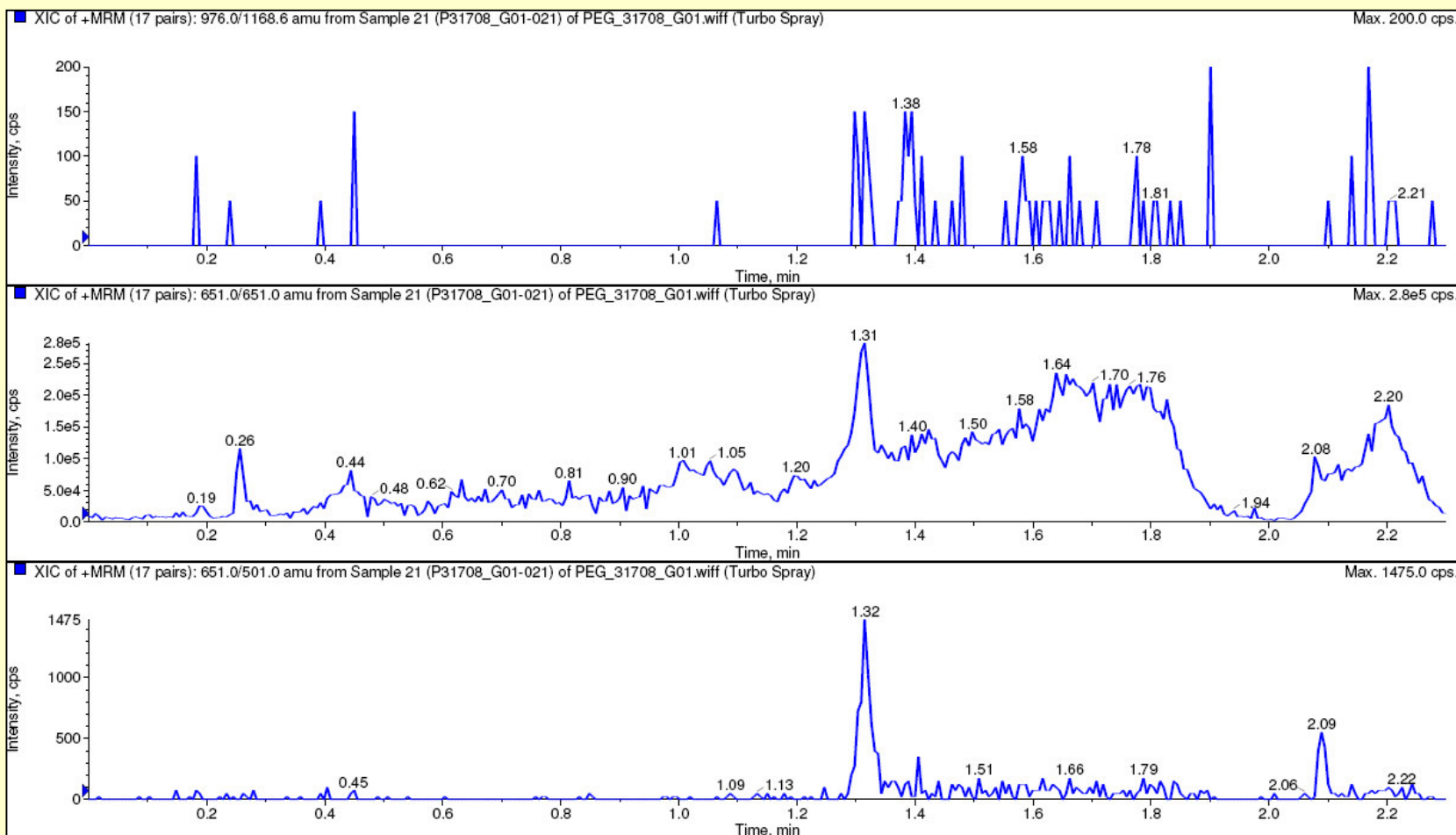
3.) PEG-Peptid Nachweis aus Plasma

- PEG mit etwa 20 kDa
- Gekoppelt daran ein Peptid mit etwa 3 kDa

PEG-Peptid 40 µg/mL Plasma, online tryptisch gespalten nach C18 Trennung



PEG-Peptid Leerplasma, online tryptisch gespalten nach C18 Trennung



Verschleppung > 1-2 % durch Trypsin Säule



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StyrosZyme™ Trypsin: Immobilized TPCK-Trypsin on Simulated Monolith Bed Cartridge.

Operating Instructions

Product description

StyrosZyme™ TPCK-Trypsin is a monolithic, fully uniform hydrophilic polymer based packing with TPCK-Trypsin covalently tethered to the surface of a Simulated Monolith Bed.

It is intended to perform online digestion of protein in a flow through setting.

The base matrix that is fully pervious, is made of highly crosslinked poly(styrene-divinylbenzene). Our proprietary polymerization technique ensures that the beads are not brittle and as a result, free of any leachables. The unique macroporous structure takes full advantage of the inner bead's surface area, making it possible to run high speed, high resolution separations without any mass transfer restrictions.

The polystyrenic matrix is rendered hydrophilic through a covalently bound coating.

The packed cartridges offer extremely high-pressure tolerance, and can be used routinely up to 2,000 psi.

The advantages offered by immobilized enzyme in a flow through setting are numerous:

- Digestion time is reduced to a few minutes as compared to hours.
- The enzyme cartridge can be used as a direct inlet to either a LC or a MS system for the analysis of the resulting peptides,

substantially reducing and simplifying the sample handling process and allowing it to be fully automated.

- Changing the flow rate and the temperature can control the extent of digestion. It can also be made fully reproducible.
- The immobilized enzyme displays high stability towards pH's, organic solvents, high flow rates, temperatures and back pressures.
- The possibility of using fast flow rates allows the cartridge to be reconditioned quickly, further reducing the process time.
- Due to the absence of contact in the immobilized format between enzyme molecules, no autolysis occurs.

It is essential for the media not to leach. Leaching depletes the enzyme from the surface, reduces the enzyme activity and contaminates the products.

StyrosZyme™ TPCK-Trypsin cartridges are offered in a number of formats to provide a wider selection.

Class	Column ID
NB Narrow Bore	2.2.1mm
MB Micro Bore	1 mm
uB Micron Bore	0.75-0.5 mm
nB Nano Bore	0.25 mm

The present instructions provide an example of protein digestion process carried out with StyrosZyme™ TPCK-Trypsin enzyme reactor in a Narrow Bore column.

Hermann Mascher

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