



# **Analysis of milk allergens by triple – quadrupole mass spectrometry**

**AOAC Europe,  
Paris, November 2009**

- ❖ **composition:** → 3 – 3.5 % protein
  - 2 major protein fractions: caseins (80 %)
  - whey (20%)
  
- ❖ **relevancy:** → 8 % children under the age of 3 affected
  
- ❖ **most important allergens:**
  - caseins ( $\alpha$  - S1,  $\alpha$  - S2,  $\beta$ )
  - $\beta$  - lactoglobulin
  - $\alpha$  - lactalbumin
  
- ❖ **threshold varies with individual**
  - methods for detection: lower mg/kg range

# allergen detection techniques



	+	-
<b>ELISA</b>	<ul style="list-style-type: none"><li>❖ direct analysis of protein</li><li>❖ quick</li><li>❖ easy handling</li><li>❖ semi-quantitative</li></ul>	<ul style="list-style-type: none"><li>❖ single target</li><li>❖ antibody cross-reactivity</li><li>❖ high variability</li></ul>
<b>PCR</b>	<ul style="list-style-type: none"><li>❖ quick</li><li>❖ multi-screening potential</li></ul>	<ul style="list-style-type: none"><li>❖ indirect measurement (DNA)</li></ul>
<b>MS</b>	<ul style="list-style-type: none"><li>❖ direct analysis of protein</li><li>❖ high-throughput</li><li>❖ multi-screening potential</li><li>❖ specific and sensitive</li><li>❖ quantitation possible</li></ul>	<ul style="list-style-type: none"><li>❖ not ready for routine analysis</li><li>❖ technical complexity</li></ul>

# allergen detection techniques



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- ❖ approaches:
  - “top down”
  - “bottom up”

## TOP DOWN:

- MS of intact proteins
- identification via molecular weight
  
- fast, little sample preparation, sensitive
- high molecular masses (10 – 100 kDa)  
high resolution mass spectrometers

- ❖ approaches:
  - “top down”
  - “bottom up”

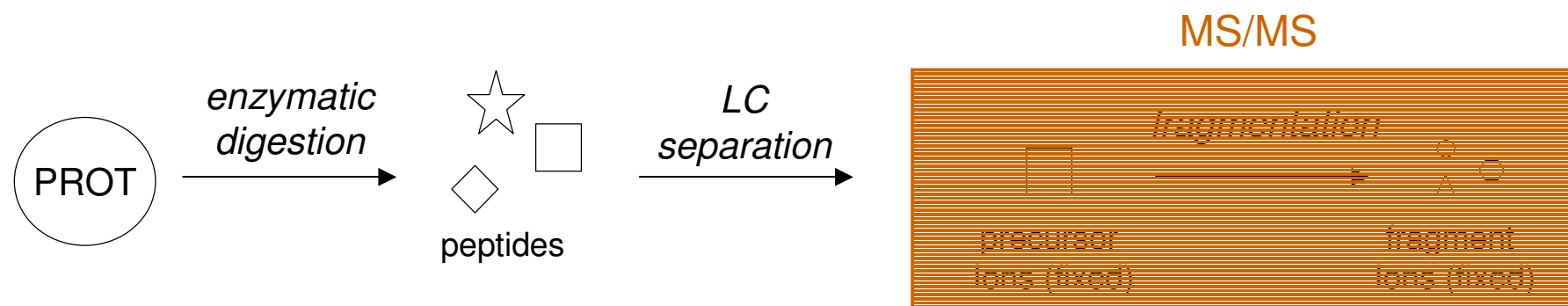
## **TOP DOWN:**

- enzymatic digestion (trypsin)
- MALDI-ESI-MS followed by peptide mass fingerprint for protein ID
- MALDI-ESI-MS sample preparation, database search for ID
  - high molecular masses (10 – 100 kDa)
- m/z = 4000, sensitive, selective
- sample preparation time, no direct molecular weight of protein

## TASK

Method for detection of casein with QqQ MS based on

- Bottom up approach
- MRM mode





## TASK

Method for detection of casein with QqQ MS based on

- Bottom up approach
- MRM mode

### ❖ Why MRM?

- selectivity
- sensitivity
- quantification possibilities
- high linear range

## Requirements for peptides:

1. reproducible occurrence after enzymatic digestion
2. unambiguous for caseins (AA sequence)
3. give “useful” MRM transitions

## @ Eurofins:

- ❖ **HPLC: Agilent 1200**
- ❖ **column: XBridge (Waters) C18 3.5  $\mu$ m x 150 mm**
- ❖ **water / ACN / FA**
  
- ❖ **ESI – QTrap 4000 (Applied Biosystems)**

# Selection of peptides



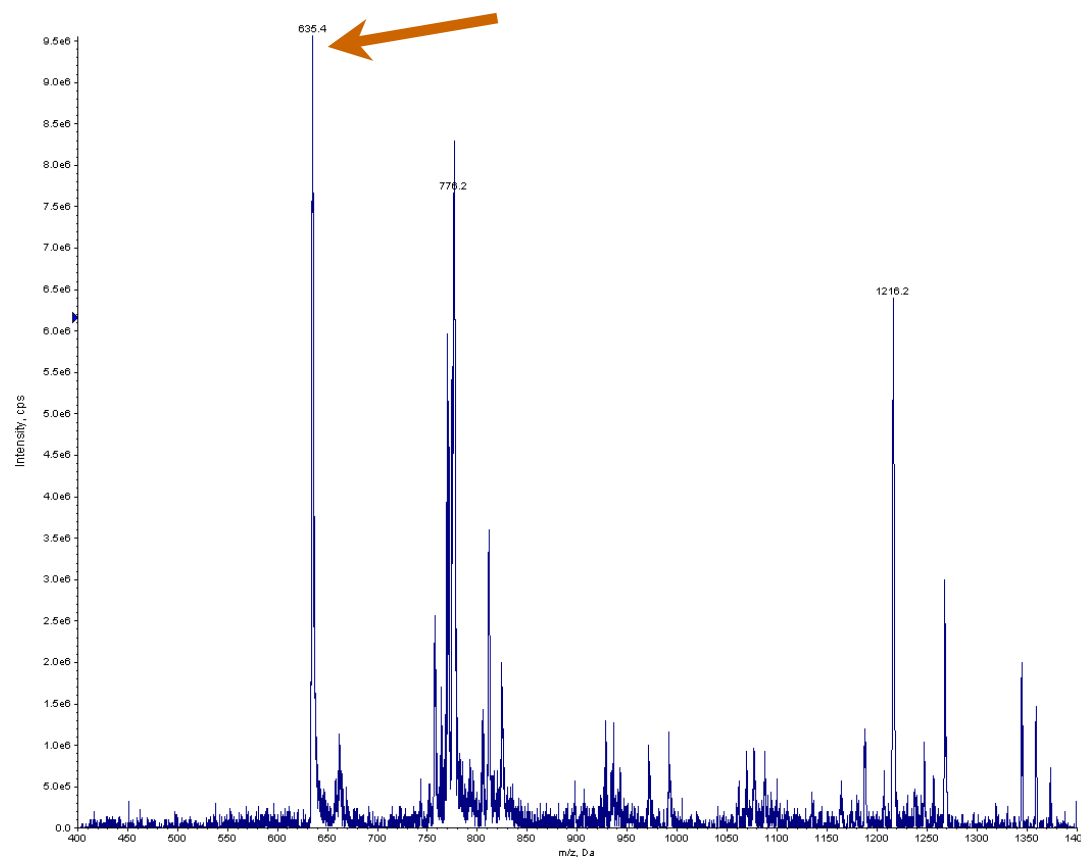
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## 1. Reproducible occurring peptides:

- ❖ repeated digestion of casein standard

# Selection of peptides

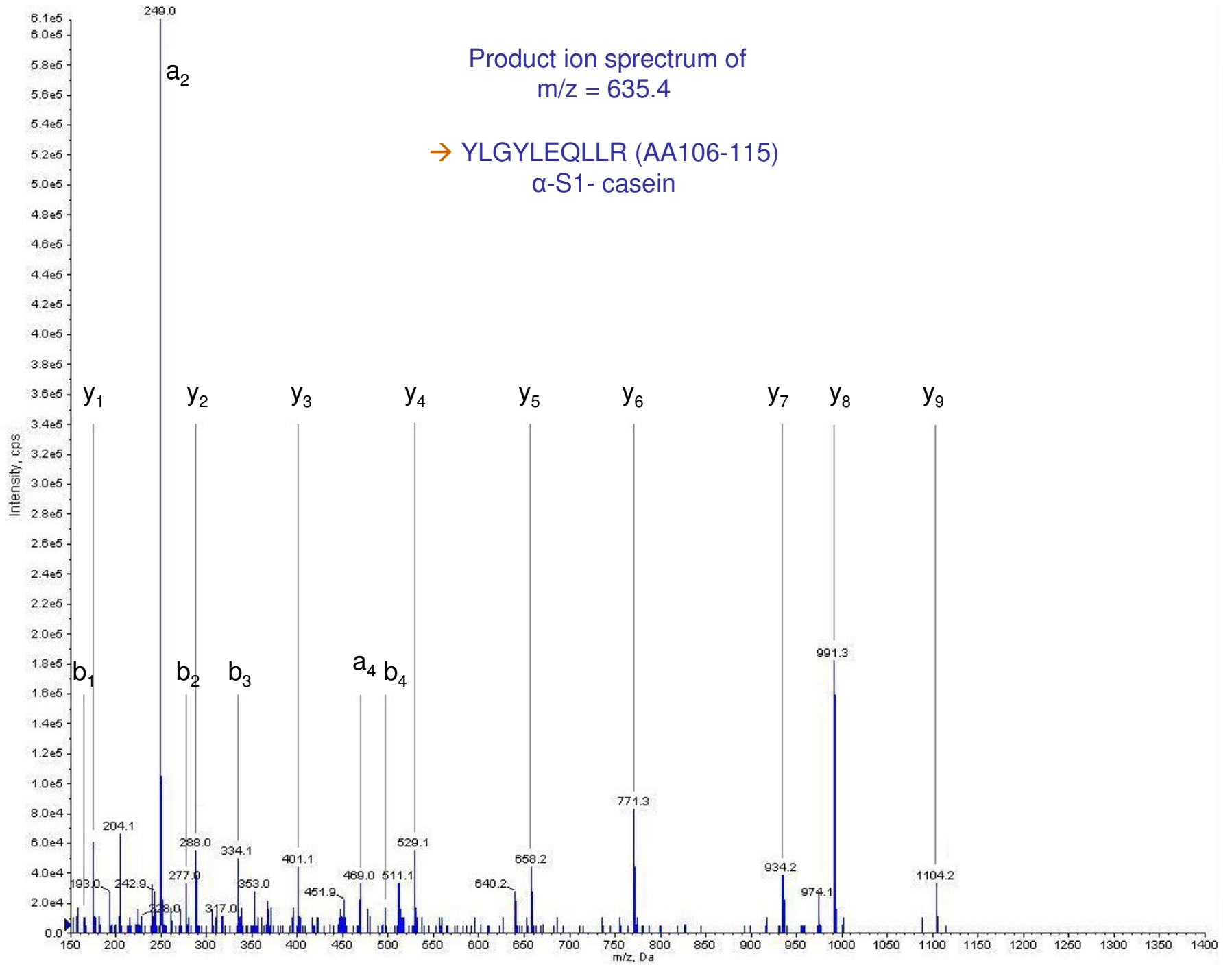
## 1. Reproducible occurring peptides:



MS spectrum, t = 10.05 Min

Product ion spectrum of  
 $m/z = 635.4$

→ YLGYLEQLLR (AA106-115)  
 $\alpha$ -S1- casein



## 2. AA sequence unambiguous for caseins?

→ BLAST search

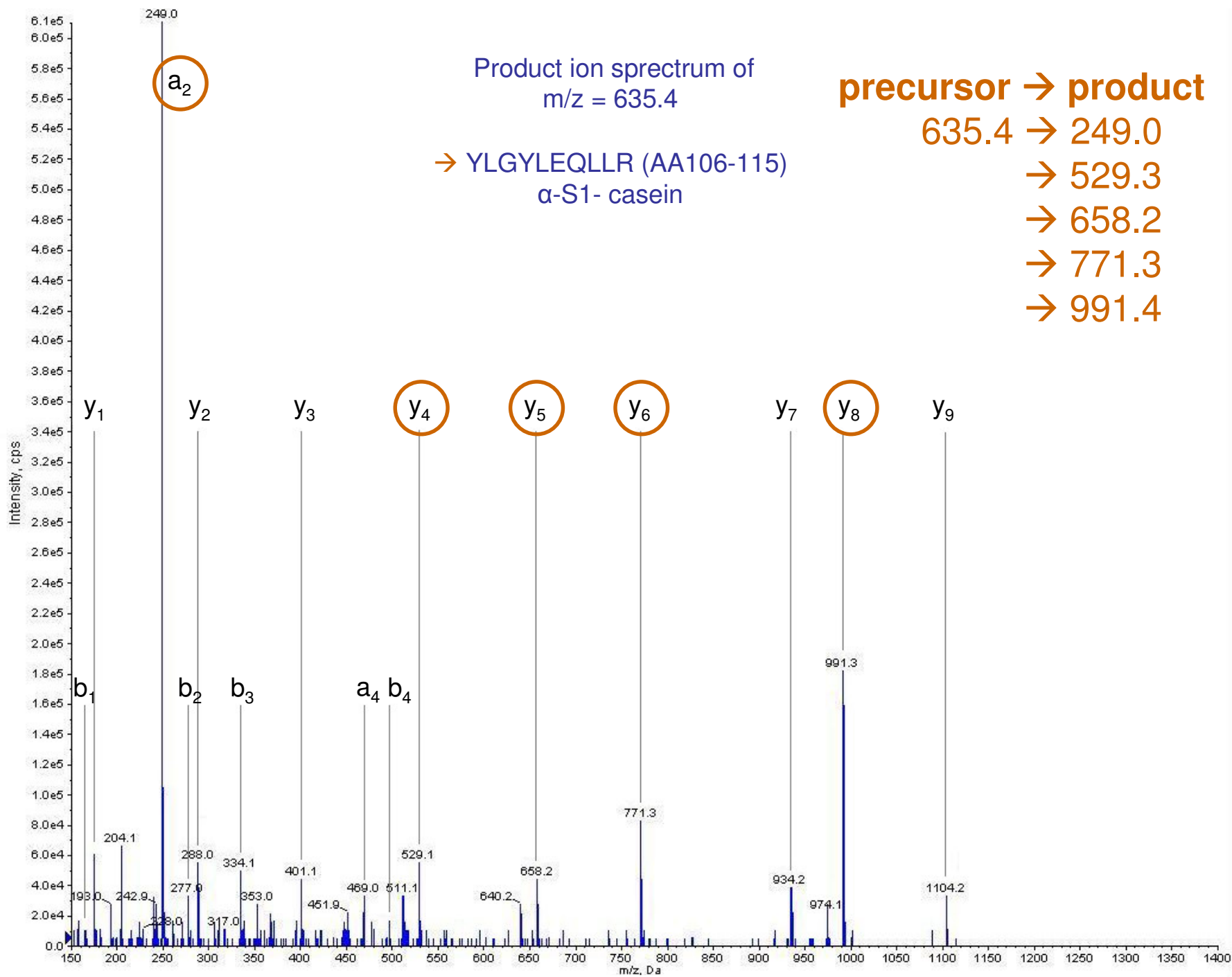
**YES!**

Sequences producing significant alignments:		Score (Bits)	E Value
<a href="#">gb ACJ14317.1 </a>	alpha S1 casein [Bubalus bubalis]	36.3	0.41
<a href="#">gb ACG63494.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98966.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98955.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
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<a href="#">gb ABW98950.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98949.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98948.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98945.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98943.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98942.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98941.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98940.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98939.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98938.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41
<a href="#">gb ABW98937.1 </a>	alpha S1 casein [Bos taurus]	36.3	0.41



## 3. MRM transitions?



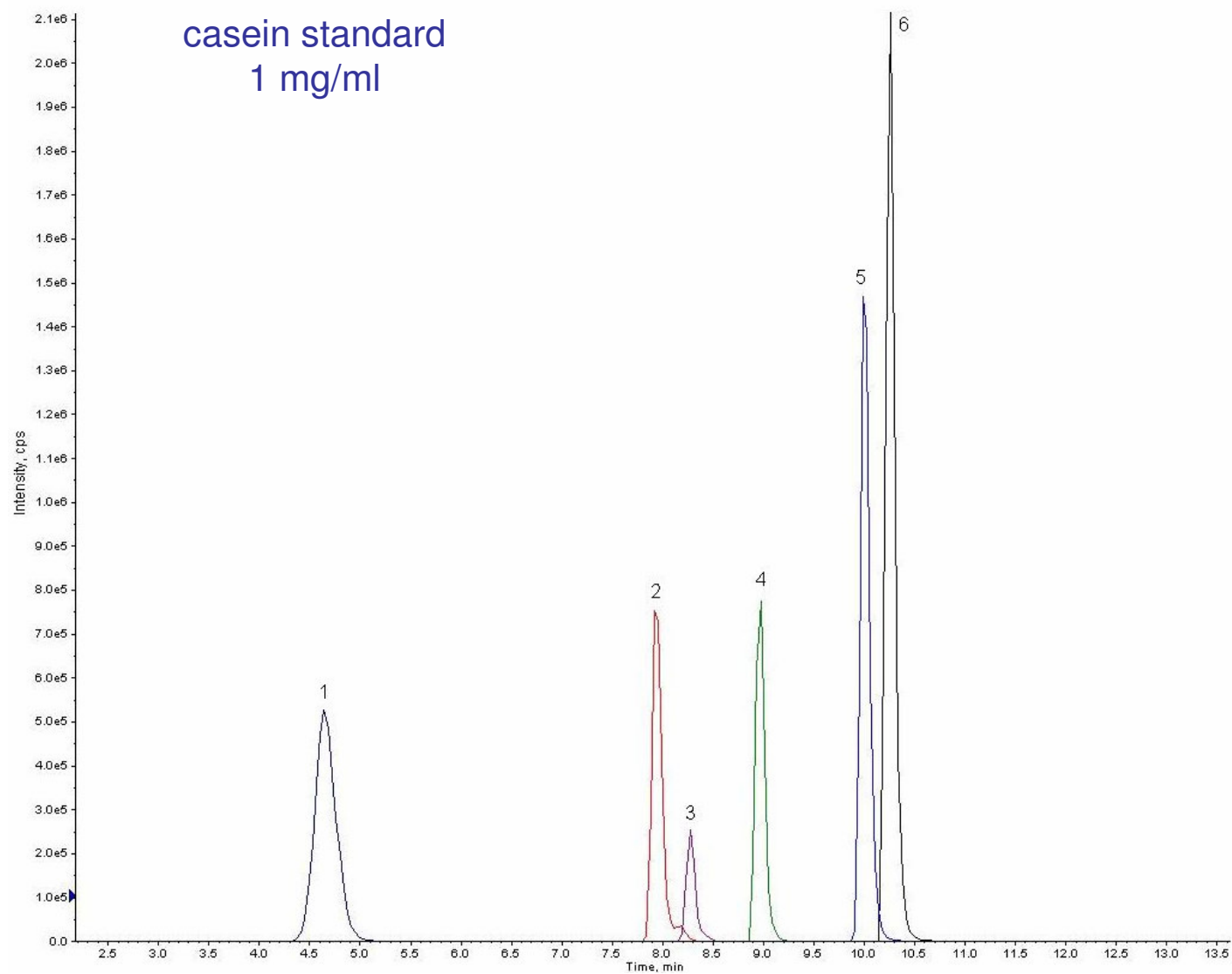


# Selection of peptides

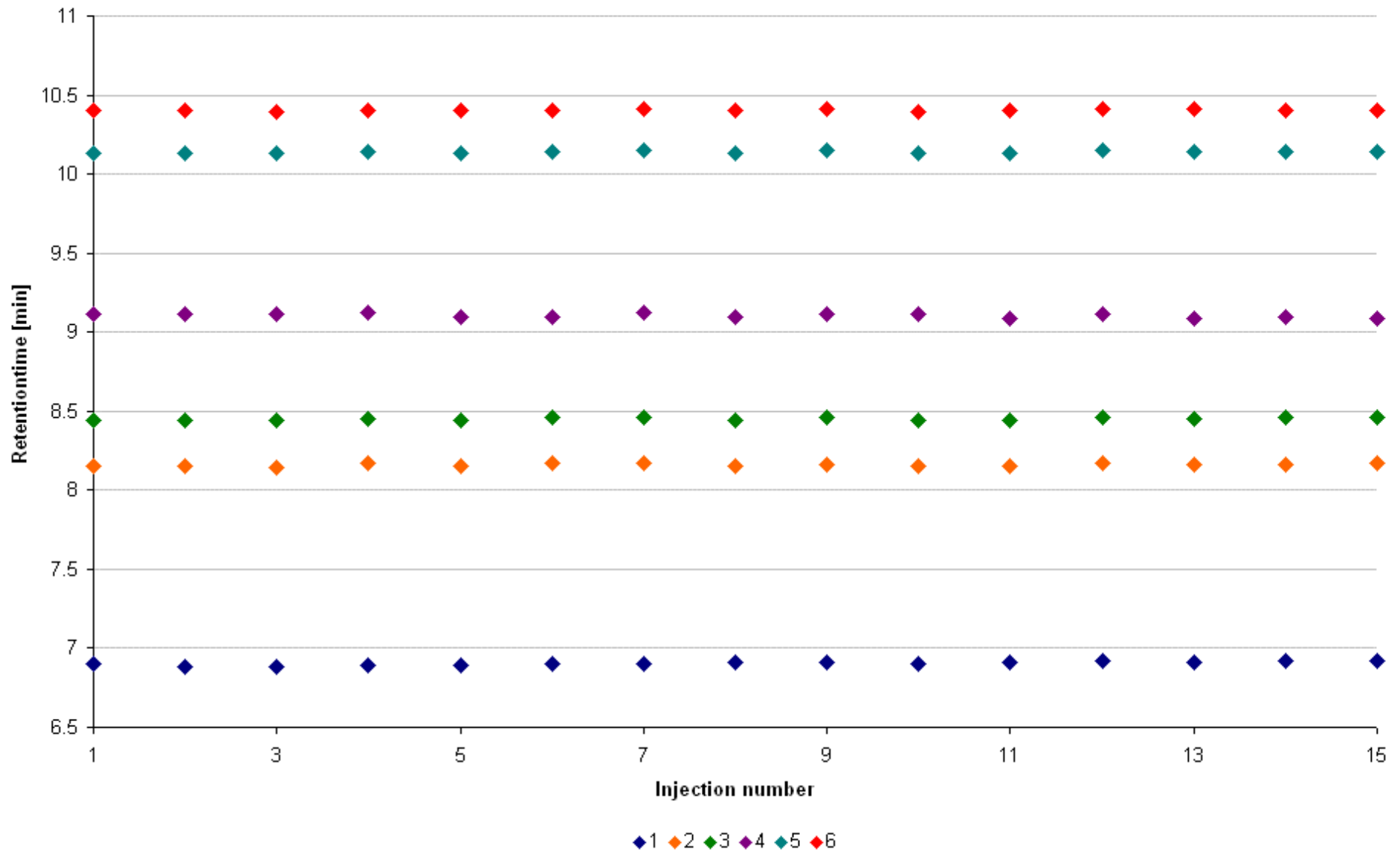


Peptide		Casein
AVPYPQR	1	$\beta$
NAVPITPTLNR	2	$\alpha$ – S2
ALNEINQFYQK	3	$\alpha$ – S2
FALPQYLK	4	$\alpha$ – S2
YLGYLEQLLR	5	$\alpha$ – S1
FFVAPFPEVFGK	6	$\alpha$ – S1

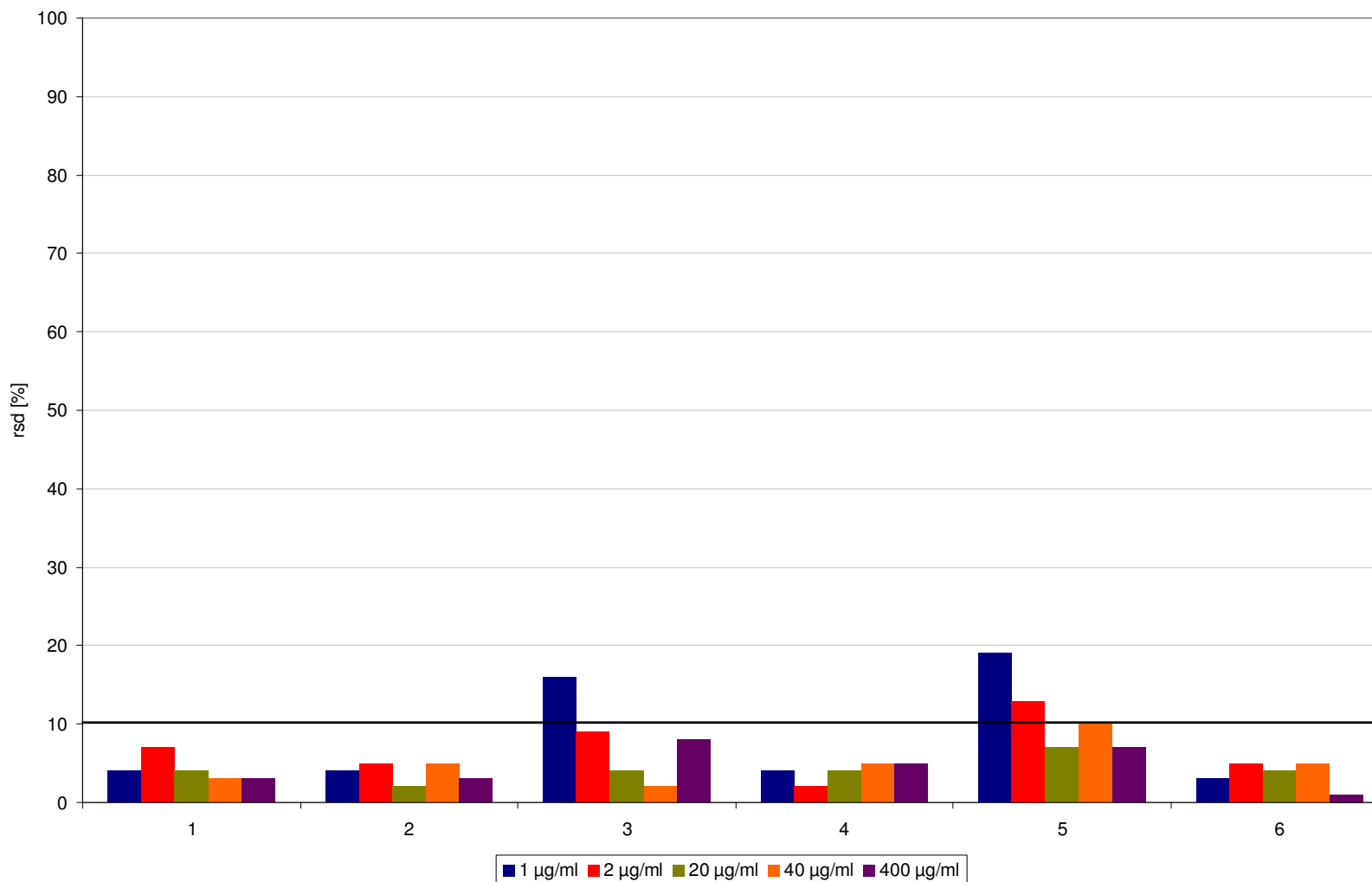
# Selection of peptides



# Selection of peptides



# % rsd (n = 3)



Peptide	Linearity
1	0.9990
2	0.9998
3	0.9999
4	0.9975
5	0.9999
6	0.9974

# Determination of casein in cookie / bread

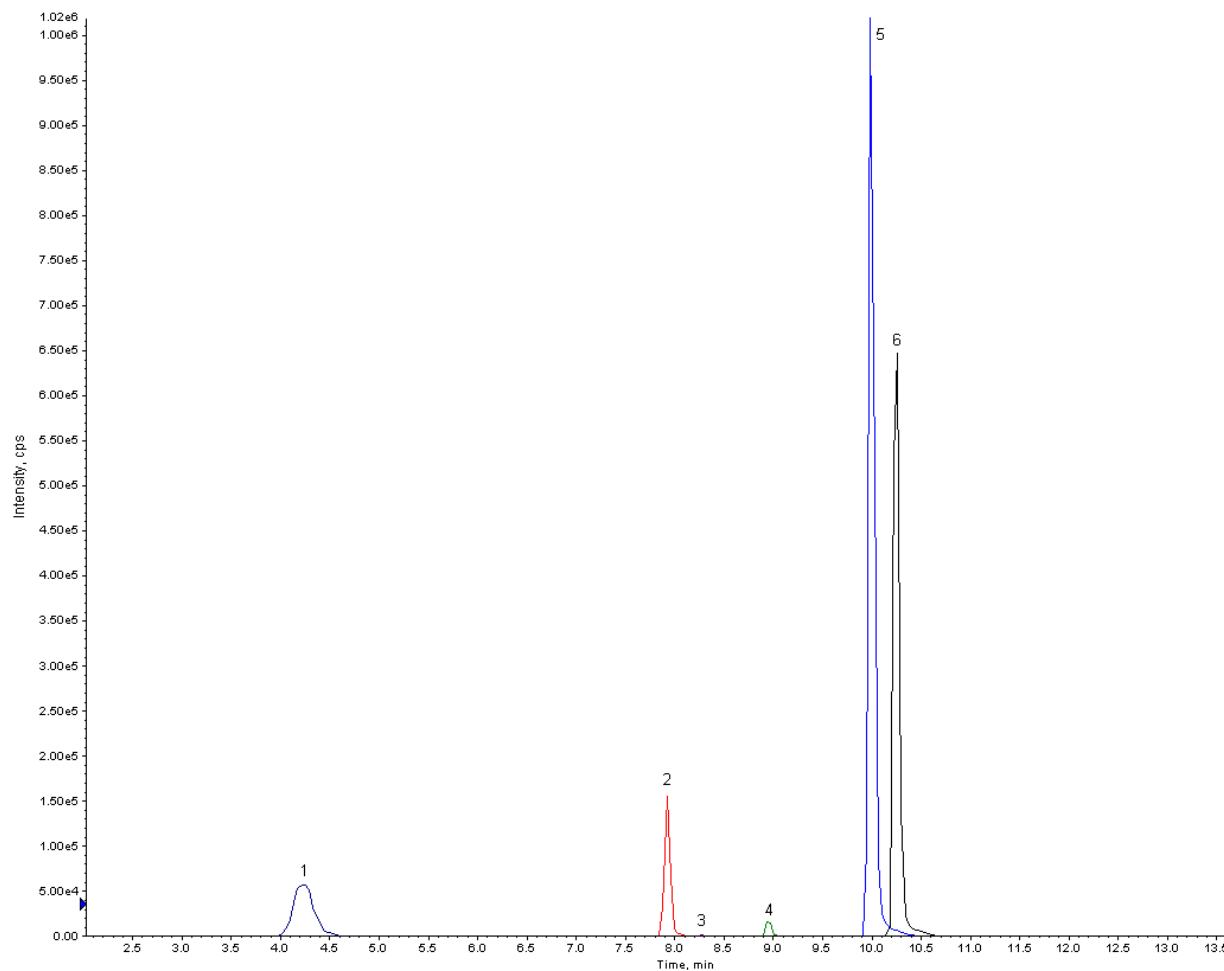
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# Determination of casein in cookie / bread



## ❖ cookie



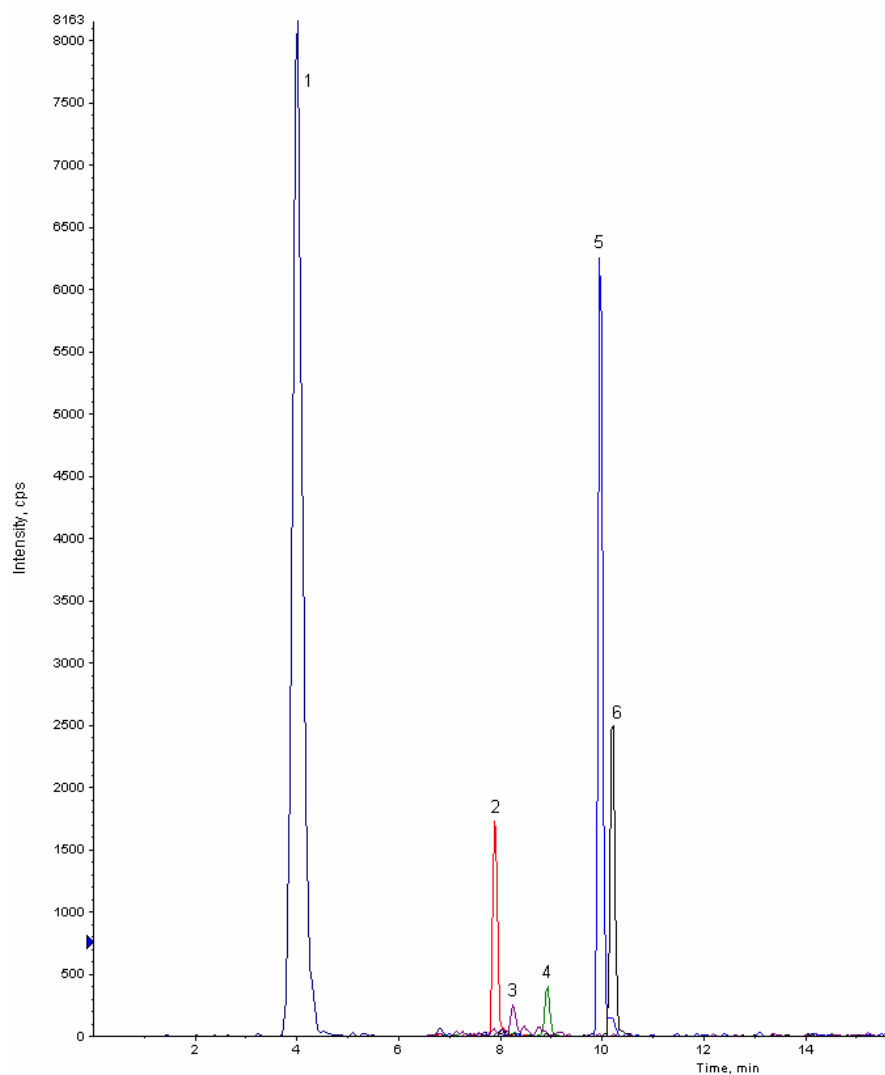


# Determination of casein in cookie / bread



## ❖ bread

100 ppm (ELISA, Tepnel)



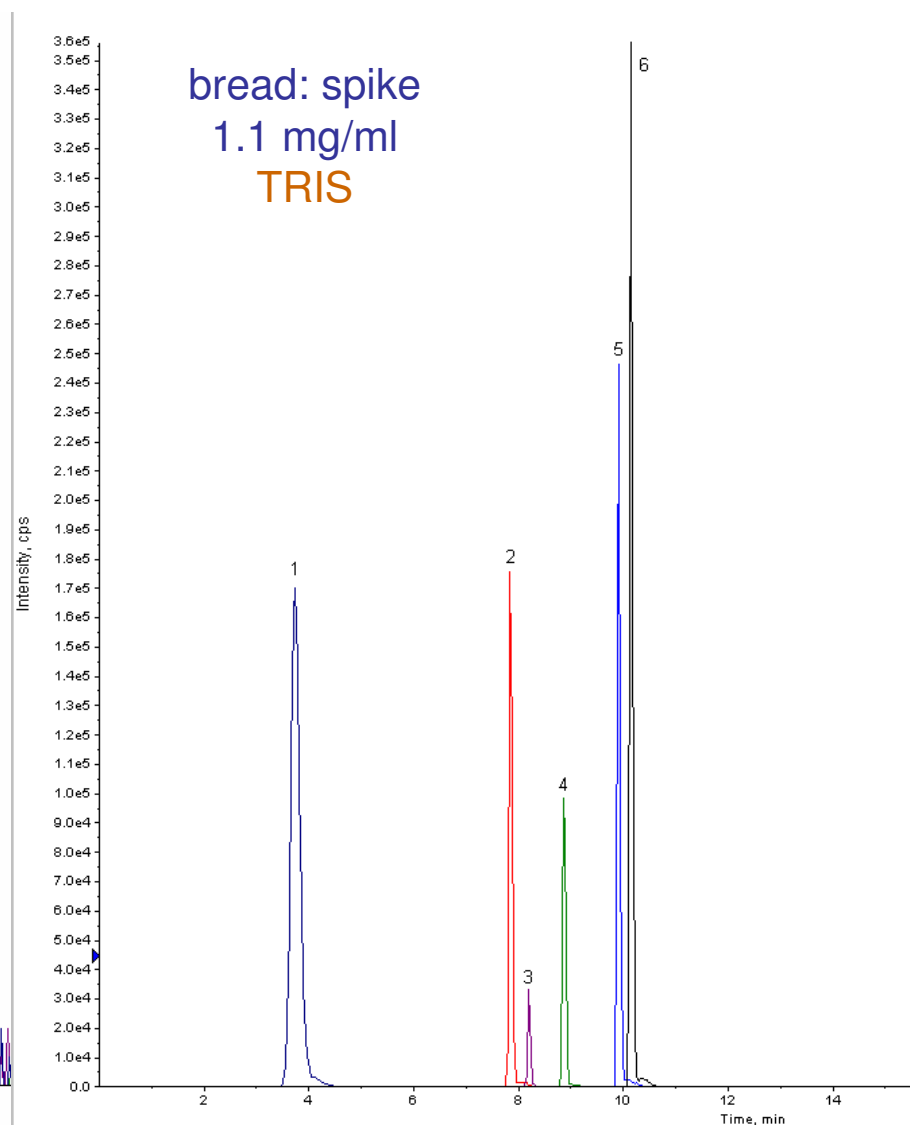
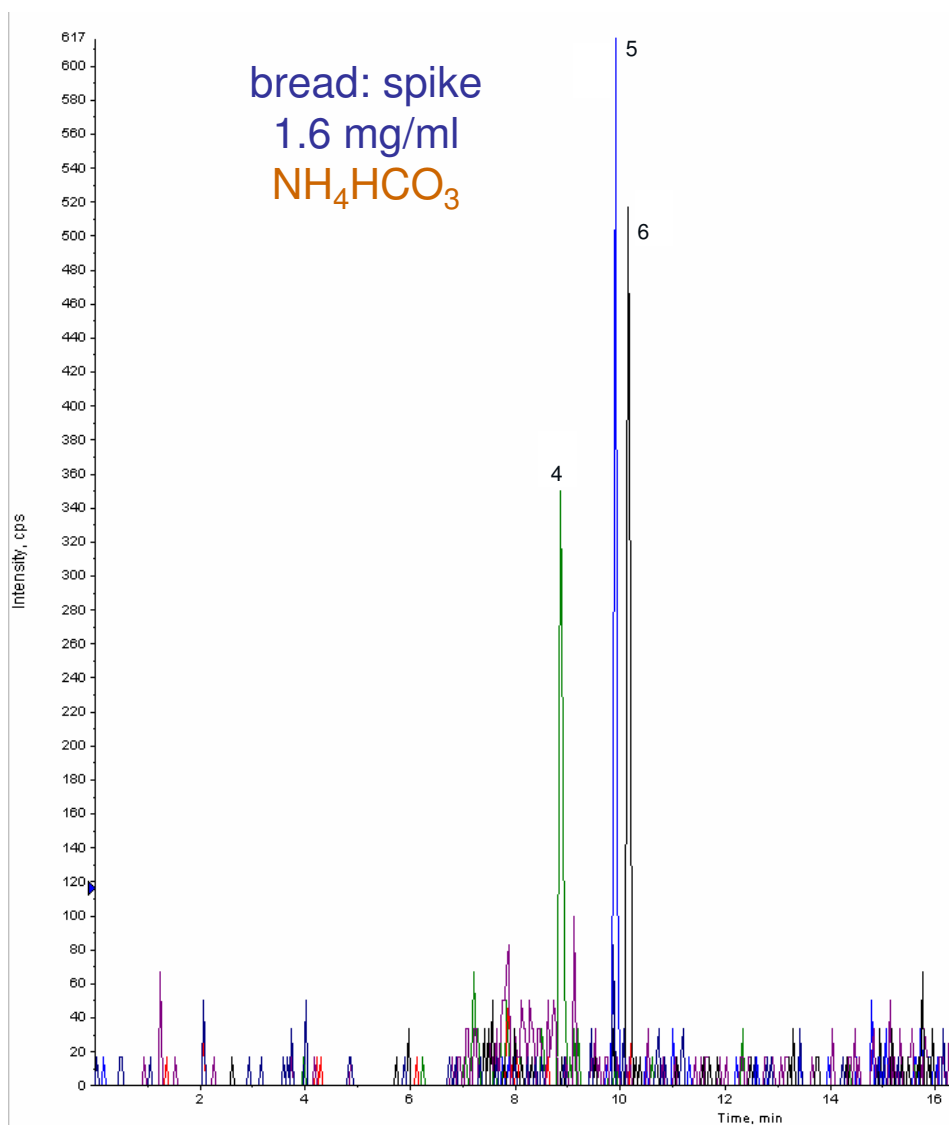
## sample preparation:

- ❖ 2 g sample / 10 ml extraction buffer (60 °C, 3h)
- ❖ filtration (Nylon)
- ❖ ultrafiltration (Amicon 15 kDa cutoff)
- ❖ dilution, reduction, alkylation, digestion

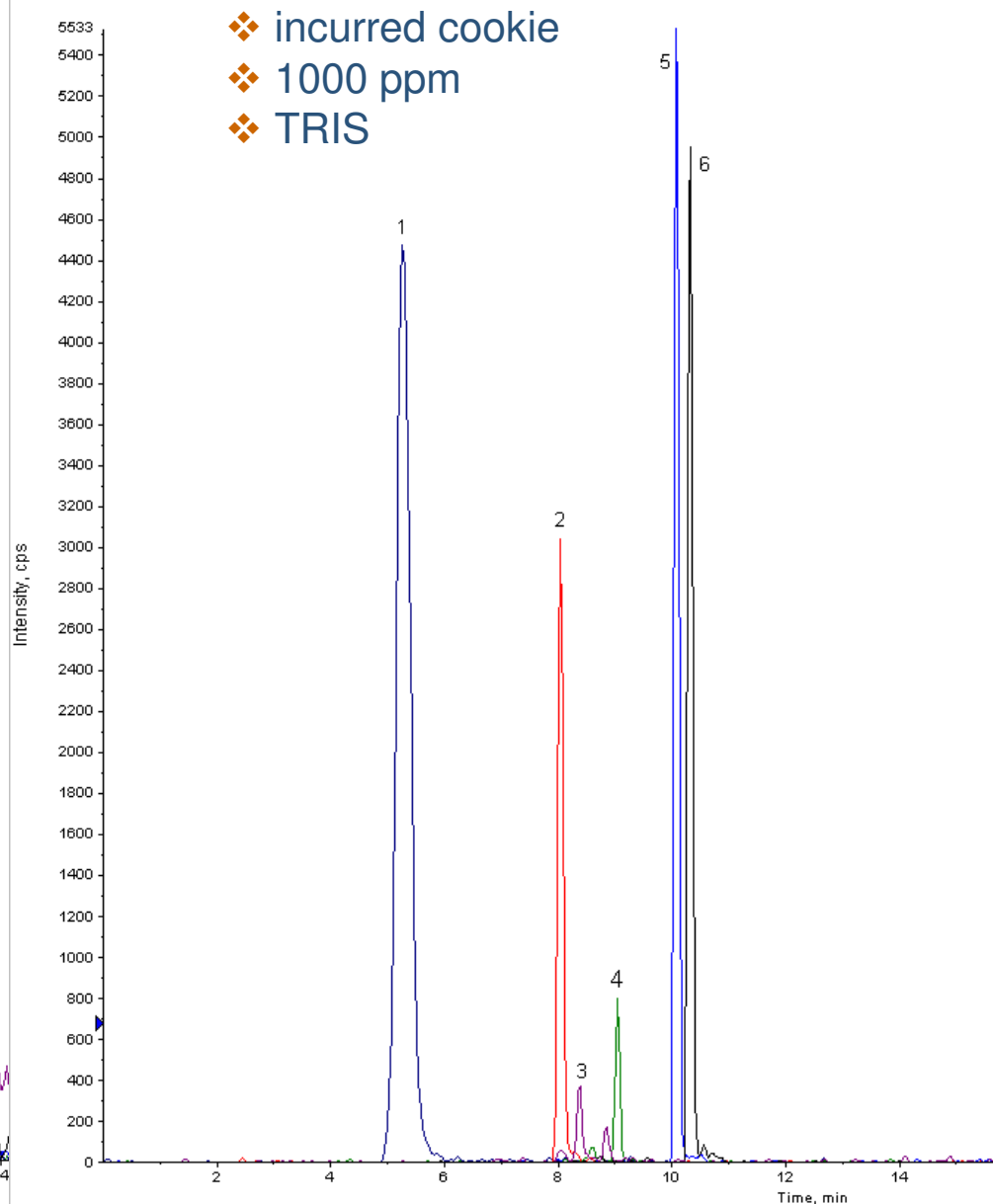
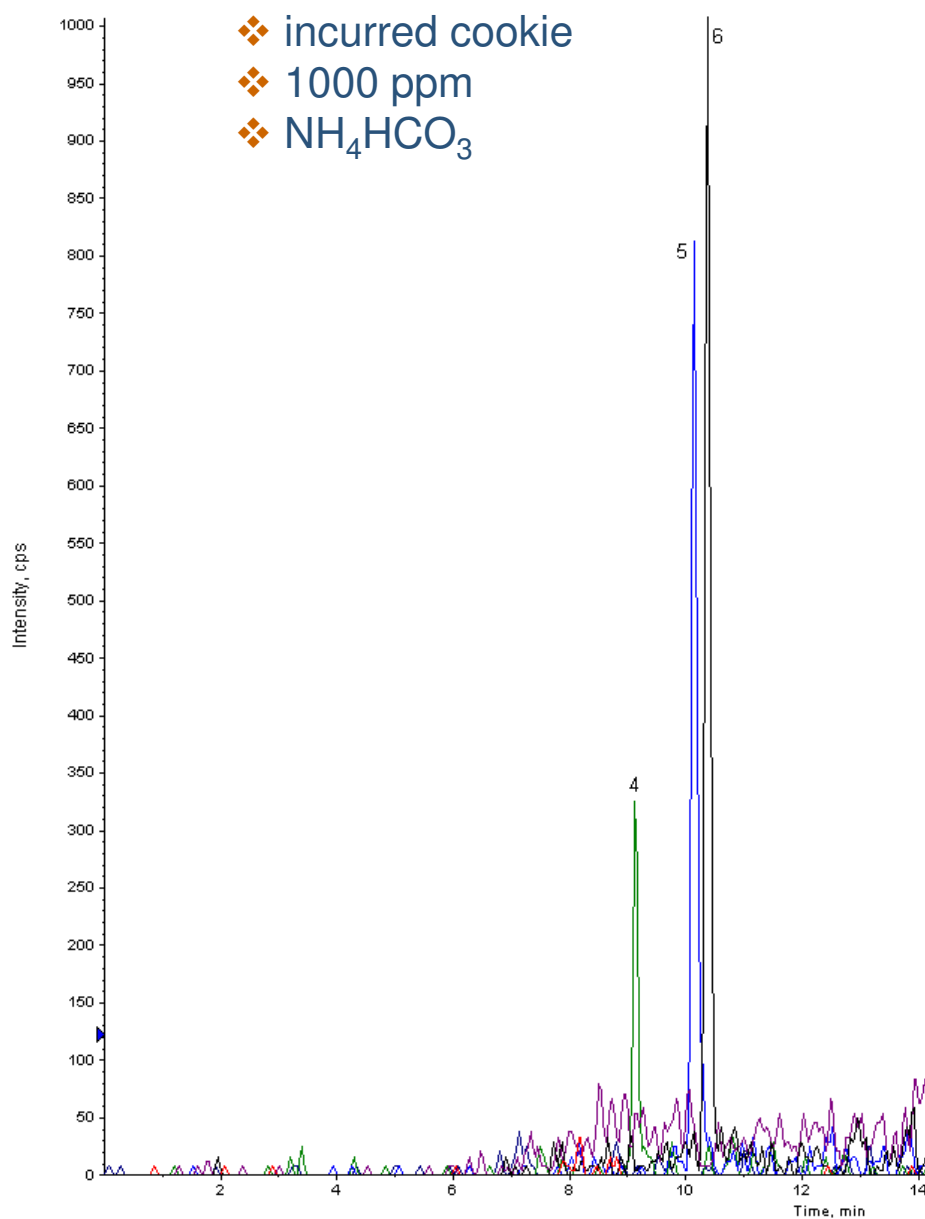
## comparison 2 different extraction buffers:

- $\text{NH}_4\text{HCO}_3$
- Tris(hydroxymethyl)aminomethane (TRIS)  
pH 8.2

# Comparison of different extraction buffer



# Comparison of different extraction buffer



# Matrix suppression 1

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## ion suppression bread and cookie matrix

- ❖ bread and cookie
- ❖ extract with  $\text{NH}_4\text{HCO}_3$
- ❖ spike with digested casein standard

→ recovery rates

## Matrix suppression 1

Peptide	Recovery rates	Recovery rates
	[%] bread, n = 3	[%] cookie, n = 3
1	84 ± 7	103 ± 16
2	50 ± 6	77 ± 0.3
3	77 ± 4	97 ± 7
4	55 ± 2	47 ± 4
5	67 ± 2	90 ± 1
6	56 ± 2	45 ± 5

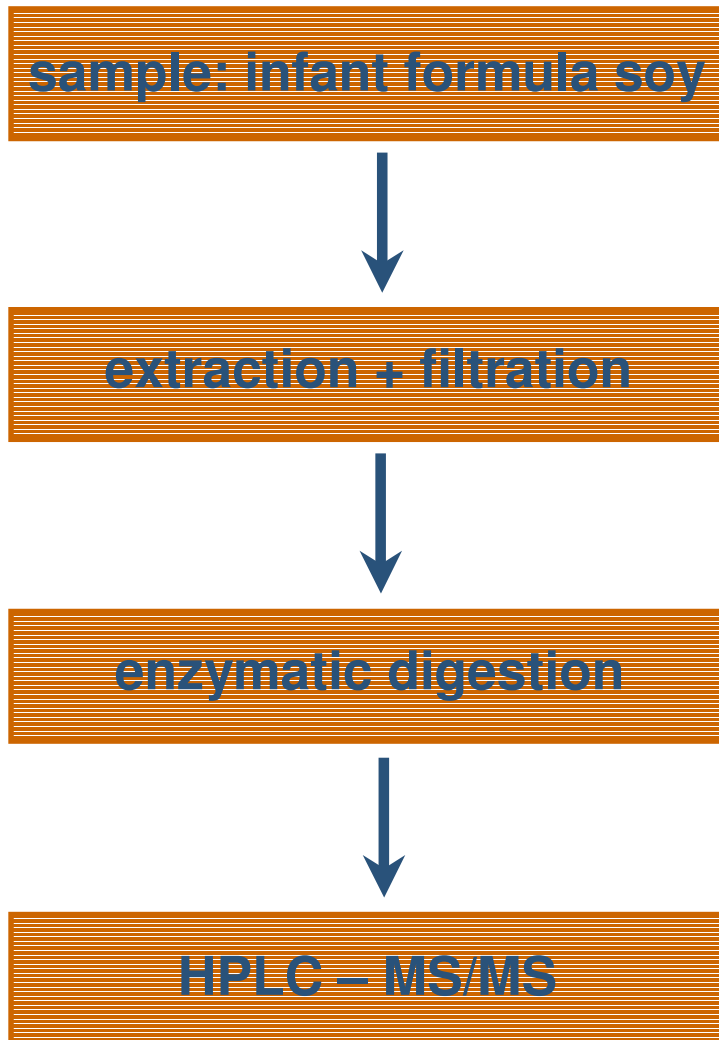
# Matrix suppression 2

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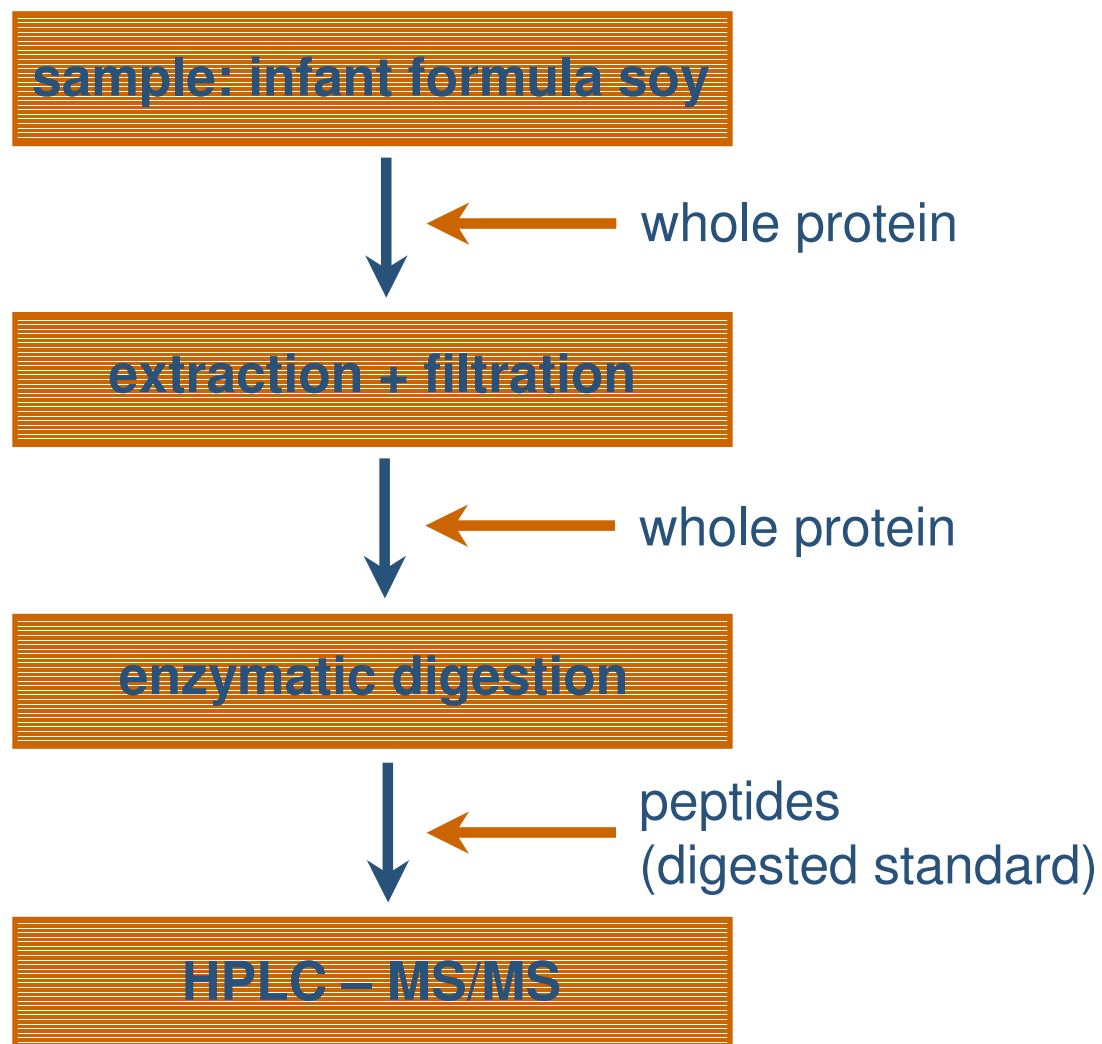


## Matrix suppression 2

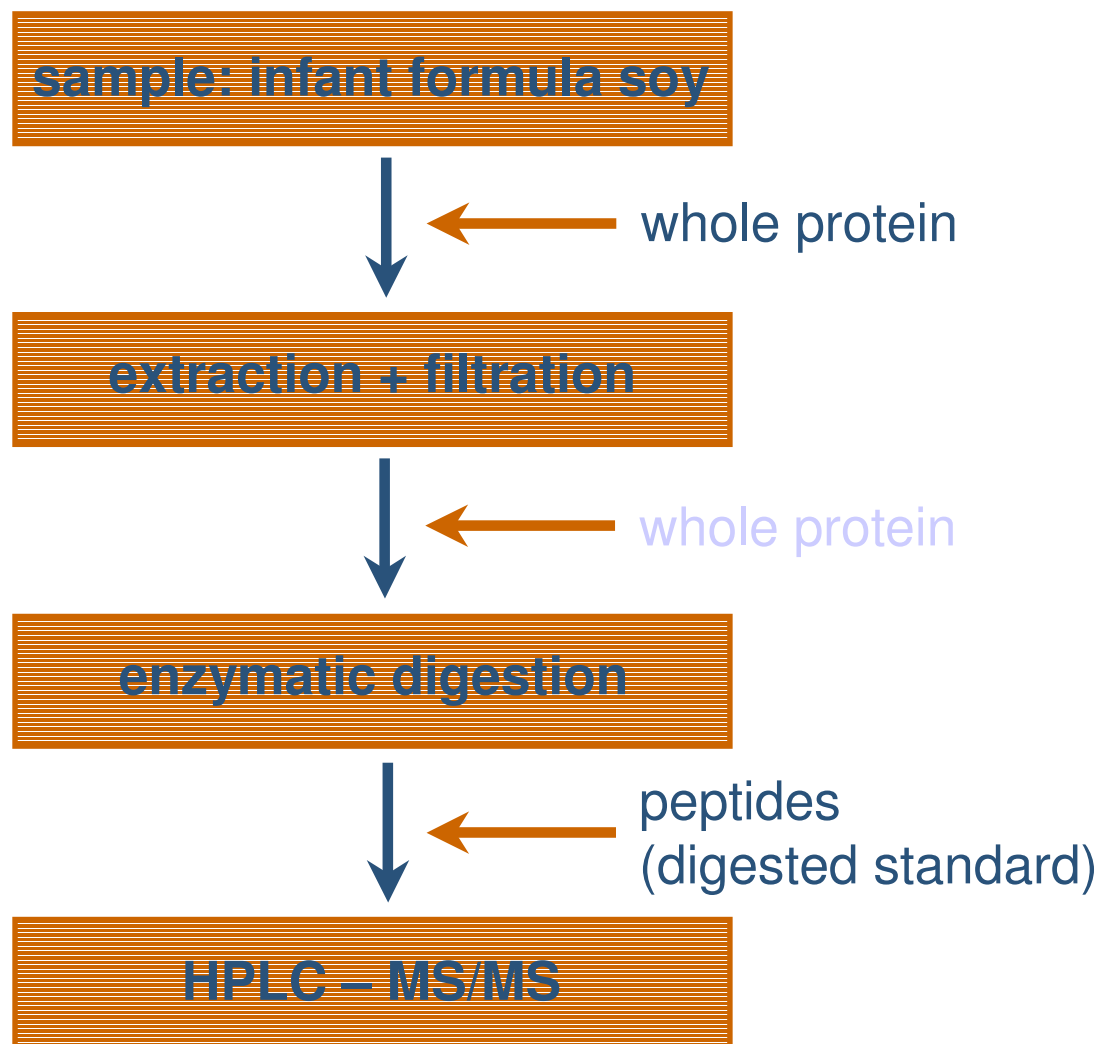


- ❖ sample: blank
- ❖ spiked with whole casein / digested casein at different preparation steps

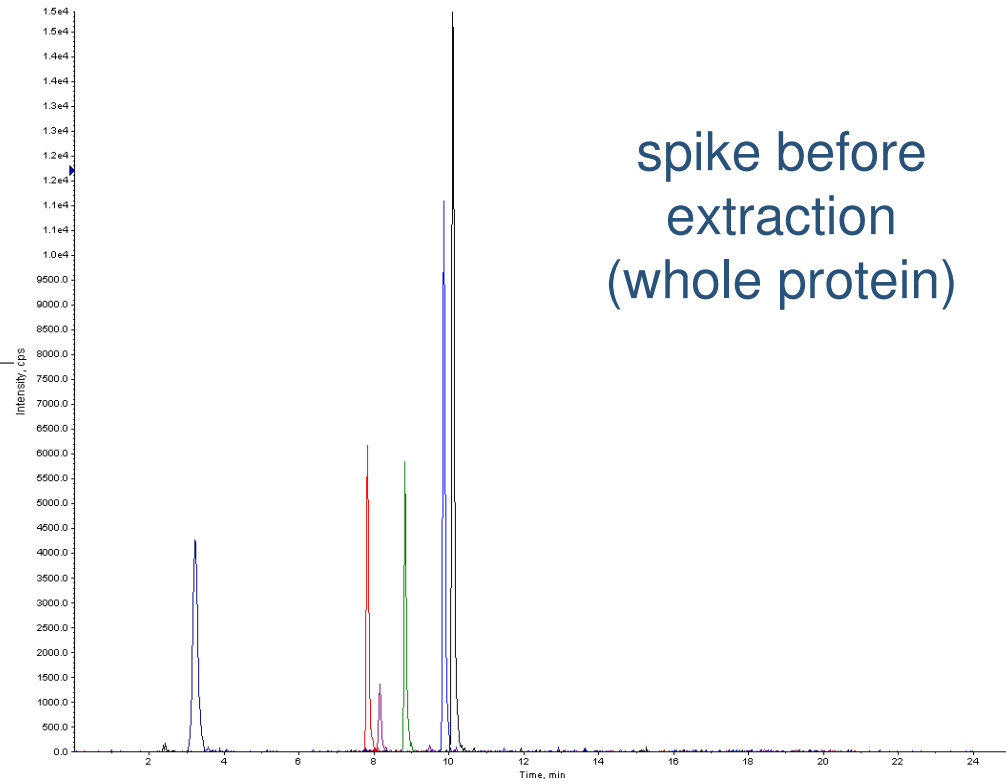
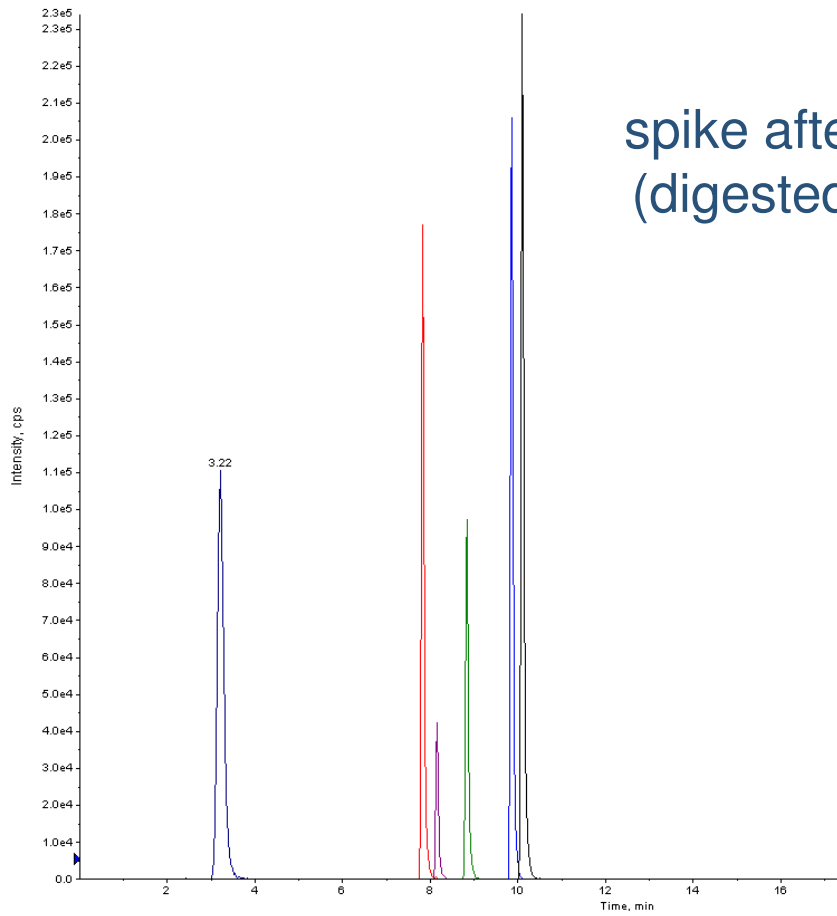
## Matrix suppression 2



## Matrix suppression 2



spike after digestion  
(digested standard)



spike before  
extraction  
(whole protein)

## Matrix suppression 3 (bread, n = 3)

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# Linearity of the method

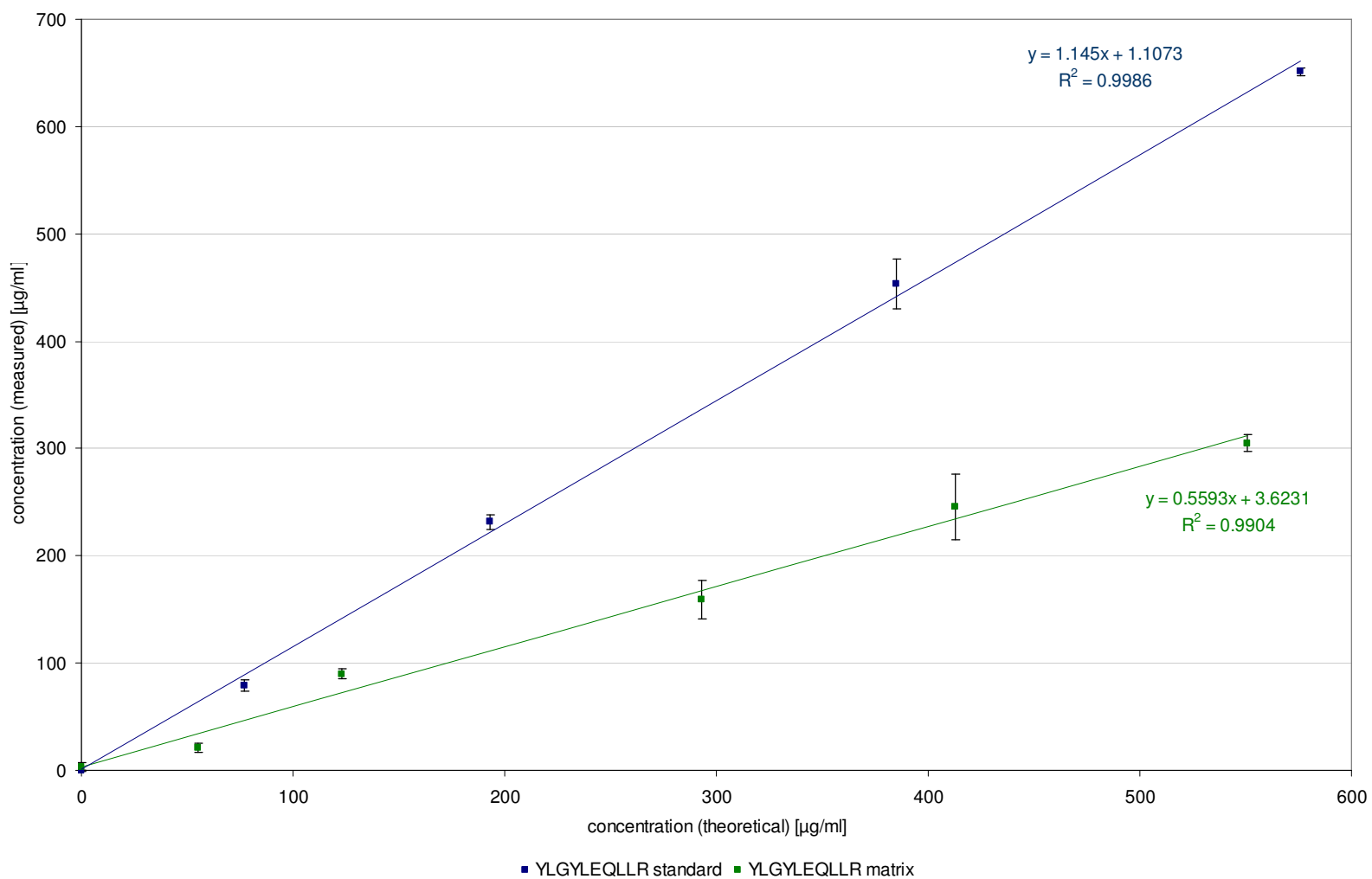
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- ❖ bread
- ❖ spike with standard solution
- ❖ extract
- ❖ digest

→ linearity

# Matrix suppression 3 (bread, n = 3)



**THANK YOU!**



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**Dr. Bert Pöpping, Eurofins**  
**Prof. Dr. Markus Fischer, Universität Hamburg**

