# Eiwitten in voedsel: kwantitatieve analyse van hydrolyse en modificatie

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voeding & milieu
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# LC-MS in food chemistry (protein/peptides)

- Quantification of indicators of heat treatment and Maillard reactions (LAL/LAN/CML/furosine)
- Quantification and identification of Maillard reaction on intact proteins
- Complete annotation and quantification of peptides in protein hydrolysates

# Heat treatment and Maillard reaction

- **Analysis:** MS using selected reaction monitoring (**SRM**)
- **Samples** : commercial pet foods, cat urine, fish diets, fish faeces, soy protein hydrolysates, ... *(some after acid hydrolysis)*
- **Analytes**: furosine, carboxymethyllysine (CML), lysinoalanine (LAL), and lanthionine (LAN)
- **Internal standard**: C13 labelled Lysine

*In pet foods:*
CML ~ 10-40 mg/ g DM
LAL ~ 5 – 12 mg/ g DM

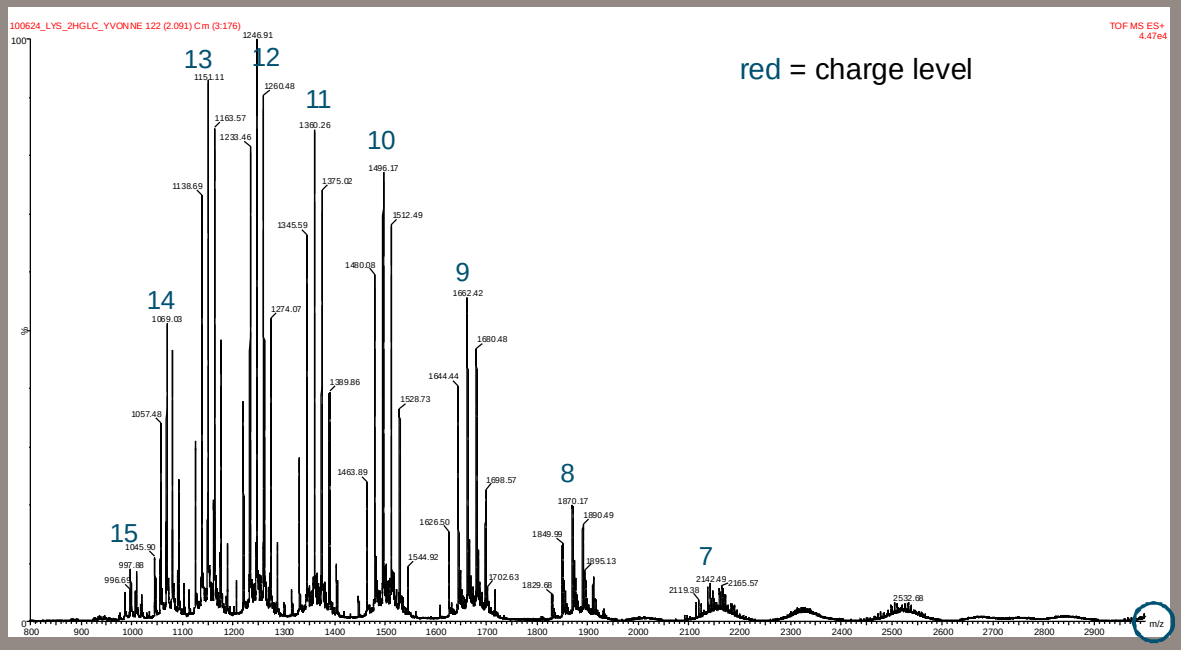

Van Rooijen, C., [...] Wierenga, P.A., Hendriks, W.H., Quantitation of Maillard reaction products in commercially available pet foods, 2014, Journal of Agricultural and Food Chemistry, 62 (35), pp. 8883-8891

Van Rooijen, C., [...] Wierenga, P.A., Hendriks, W.H., Urinary excretion of dietary Maillard reaction products in healthy adult female cats, *submitted*

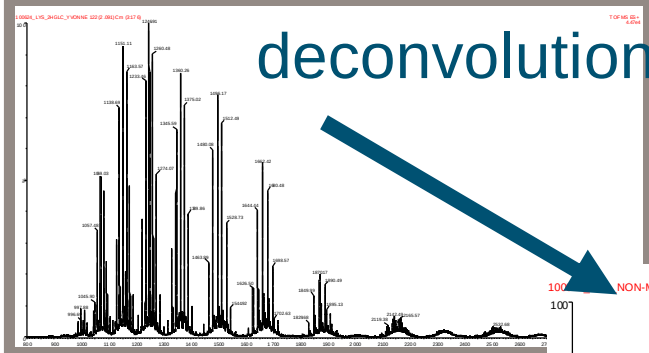
# Maillard reaction in proteins

## Challenge:

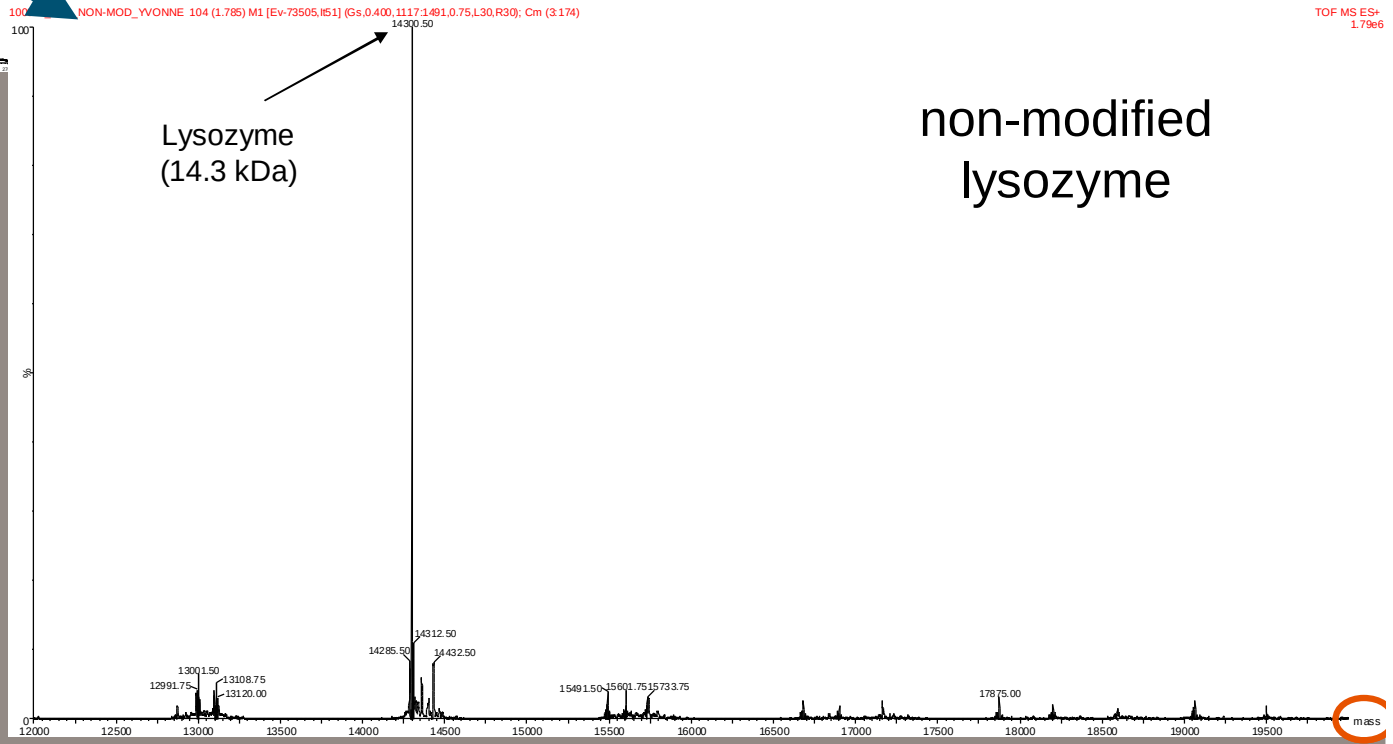
To identify small (162 Da) modifications on large intact proteins (14313 Da)



# Analysis of non-modified proteins



deconvolution

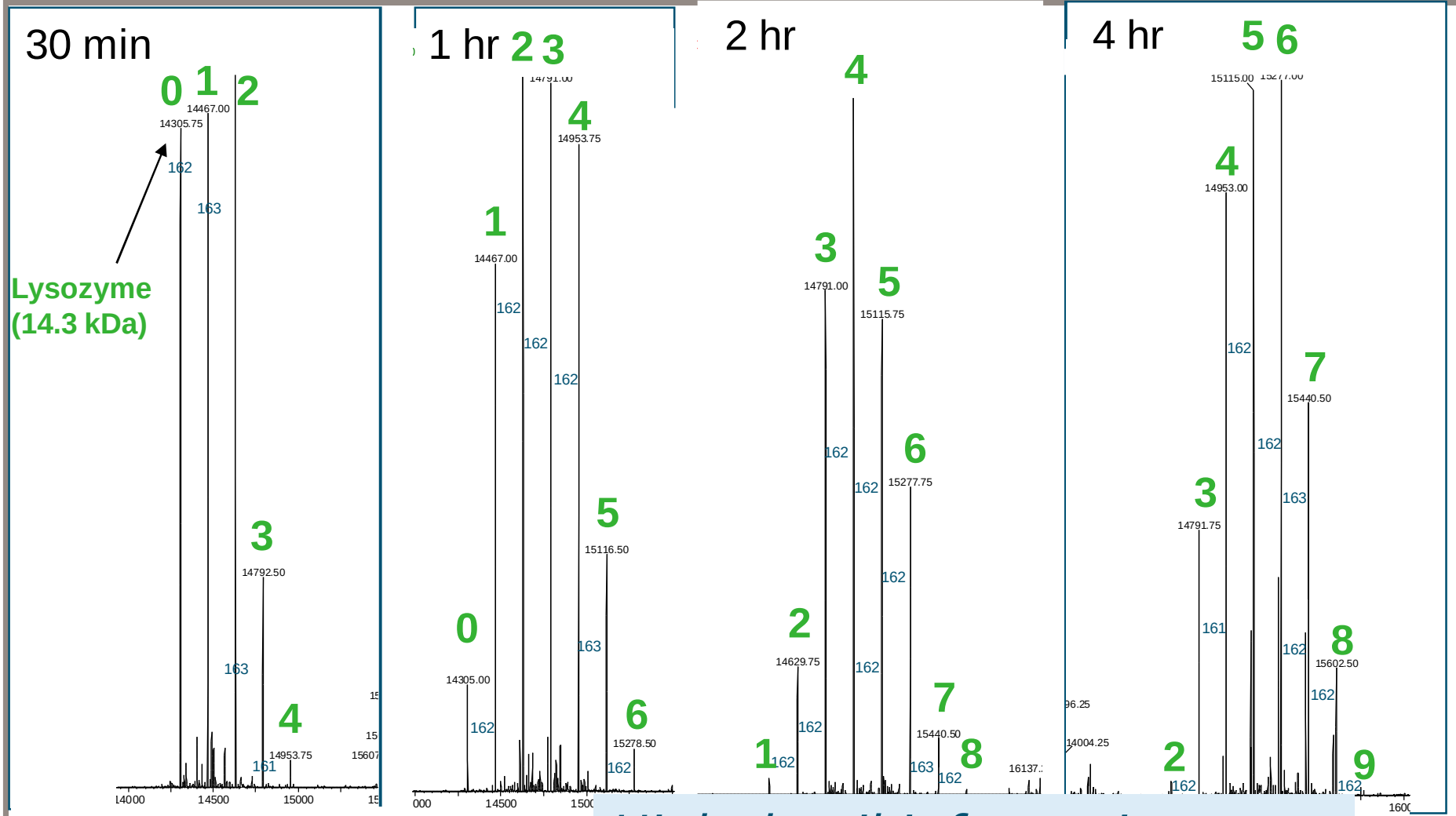



Lysozyme
(14.3 kDa)

non-modified
lysozyme

*Need very high resolution!!*

# Evolution of modification with incubation time

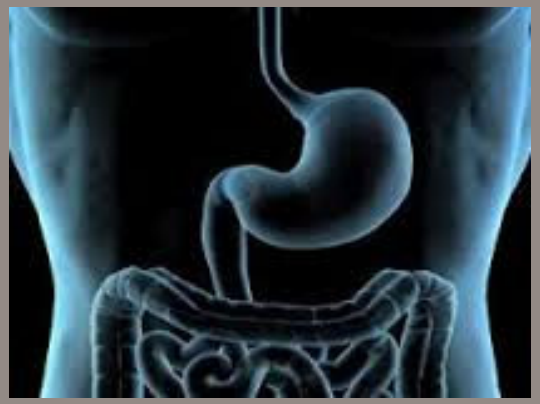



green = numbers of glc attached

*High detail information on distribution of modifications*

# Protein hydrolysis and food science

Digestibility of proteins



Fermented food products

Hydrolysed food ingredients

# Protein hydrolysates in food products

Aims of hydrolysis:

1. Nutritional properties
   a. Easier digestibility
   b. Loss of allergenicity

2. Bio-functional properties
   a. Anti-microbial properties
   b. ACE-inhibition
   c. Anti-oxidant activity
   d. Immunostimulation
   e. Antithrombotic Peptides
   f. Mineral Binding Peptides
   g. Opioid activity

3. Techno-functional properties
   a. Solubility
   b. Foam
   c. Emulsion
   d. Taste

# Predicting and controlling hydrolysis

*Ways to characterize the composition of an hydrolysate?*

**Future: Full and complete mass balance**

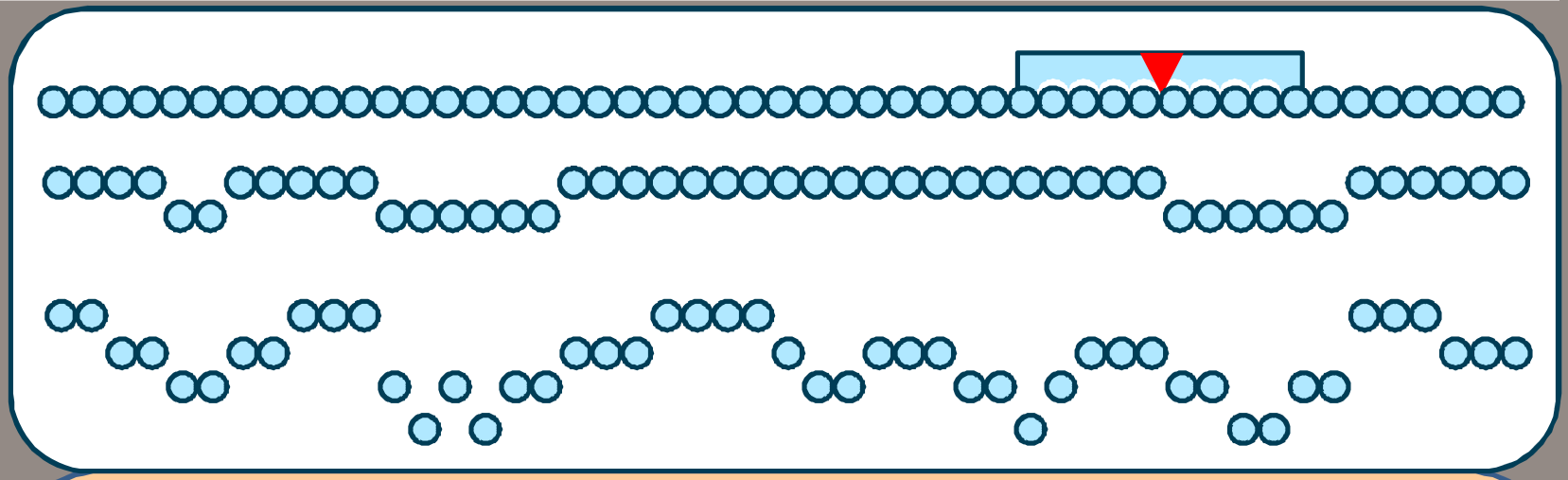
**Complete mass balance**
→ Absolute quantification of all peptides

| Degree of Hydrolysis | Concentration of intact protein | $M_w$ distribution of peptides | Identification of peptides | Quantification of peptides |
|---|---|---|---|---|

*"Proteomics"*
Identify presence of proteins based on peptide patterns

**Current:** Global information,
or: Specific data on few peptides

# Hydrolysates – How big is the challenge?



A protein with N cutting sites
will form ((N+1)*(N+2))/2 different peptides

*for β-lactoglobulin – BLP, 16 cutting sites (E)*
*have been identified ⇒* ***153*** *possible peptides*

# Challenge in peptide quantification

## 1- Annotation

- How to quickly annotate large numbers of peptides
  - That are ranging from 2 to >100AA
  - That may be very similar in structure
- How to test for completeness of annotation

## 2- Quantification

- How to quantify peptides without use of labels
- How to test for completeness of quantification

# Example: a real hydrolysate

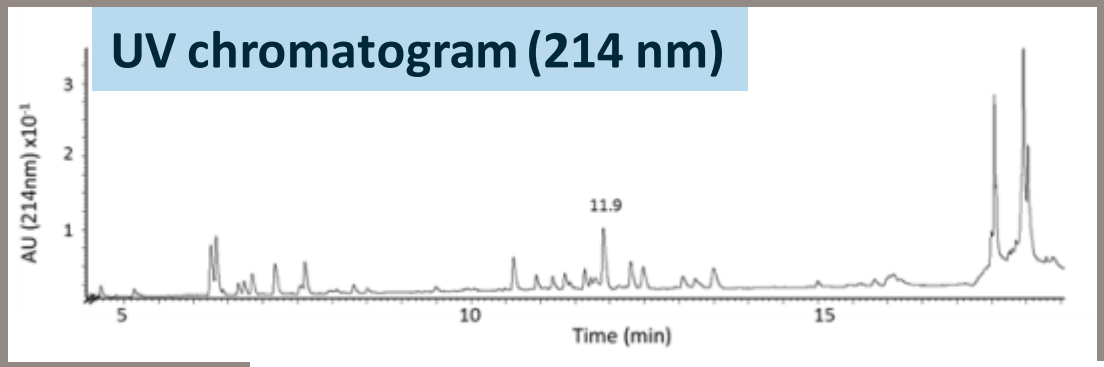
**1 g/L β-lactoglobulin solution (16 Glu per protein)**

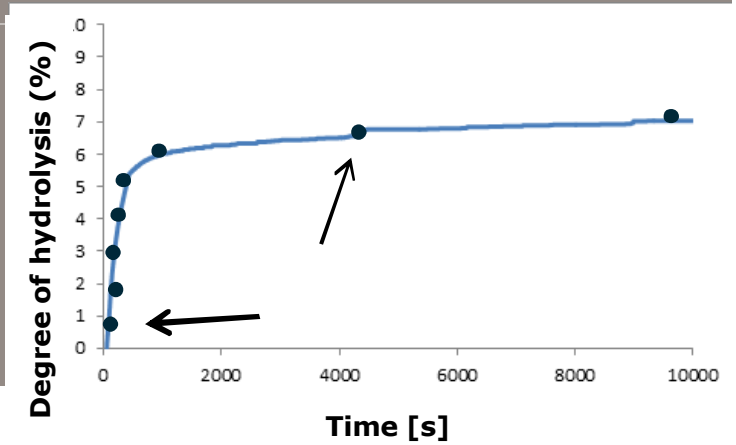
**Hydrolysis by *Bacillus Licheniformis* Protease (Glu specific)**

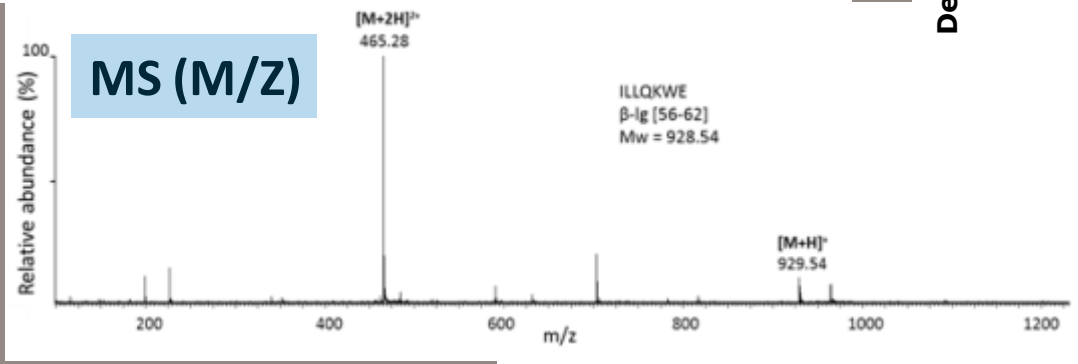
**pH-stat: T = 40 °C; pH 8.0;**

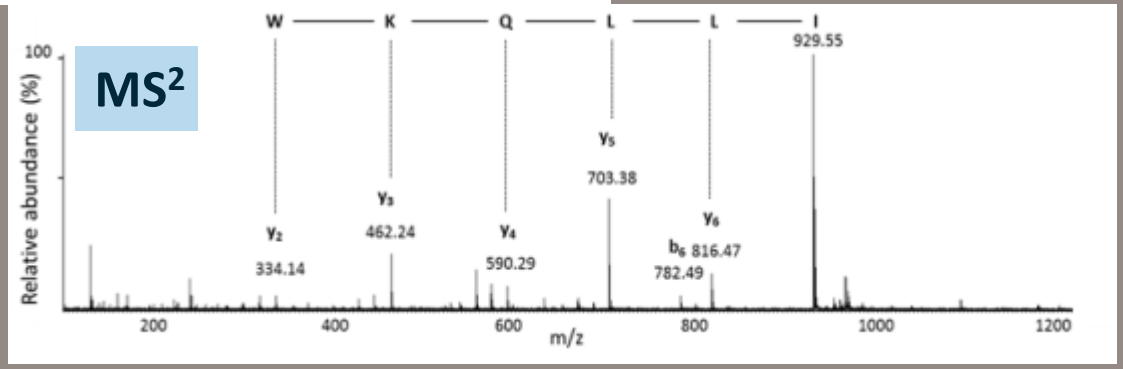
**RP-HPLC – BEH 300 C18 column (1-100 % ACN + 0.1% TFA )**
**Annotation -- MS detection on Synapt High Definition Mass Spectrometer (ESI- Q TOF spectrometer)**
**Quantification -- UV detection at 214 nm**
**Integrate all UV peaks – include from large to small to cover at least 90% of total UV peak area**

# Peptide **annotation**



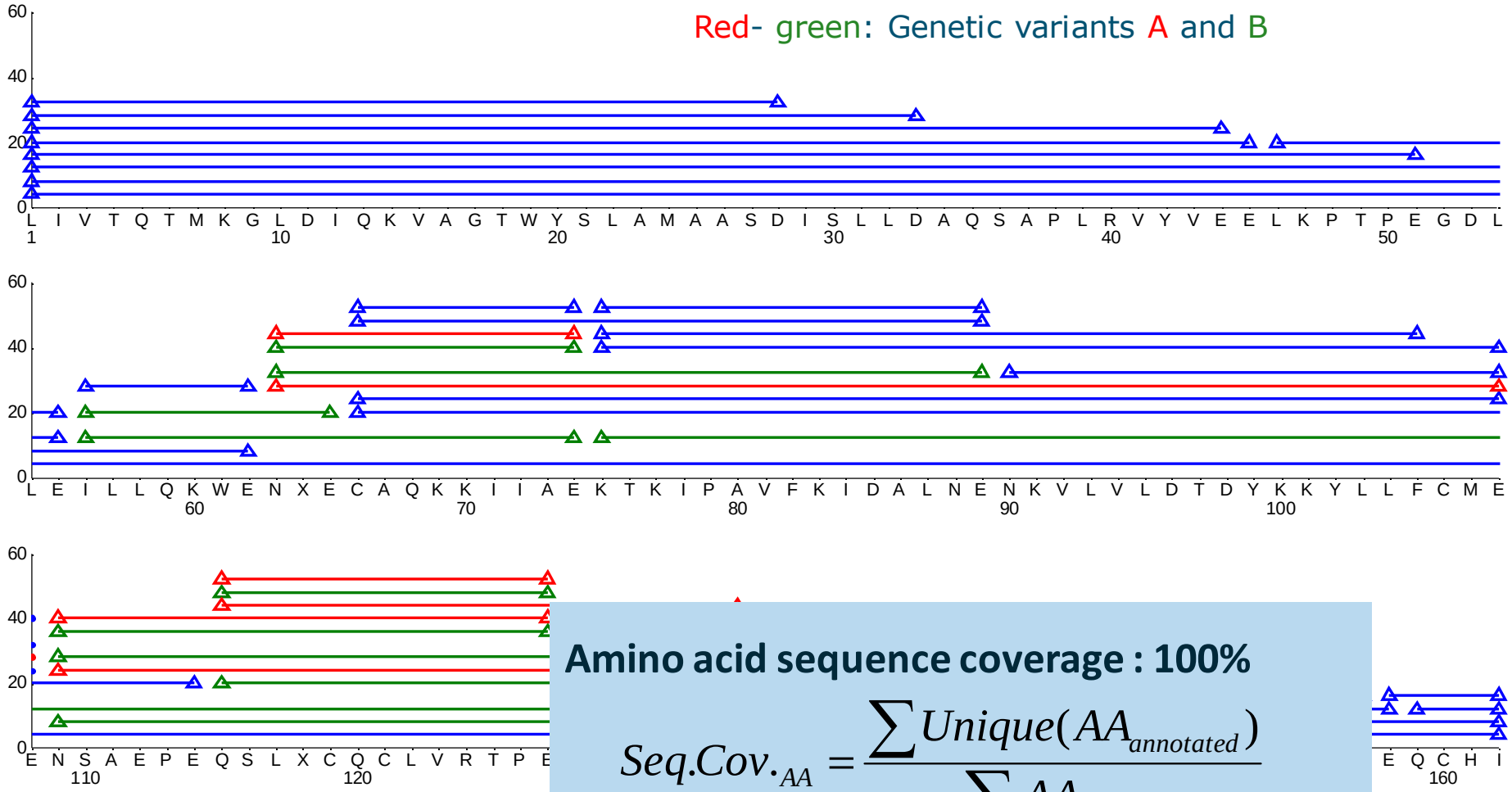




- **44 peptides** per chromatogram
- Automatic, non-targeted
- Only identification on protein sequence and MS/MS fragments

# Amino Acid sequence coverage:

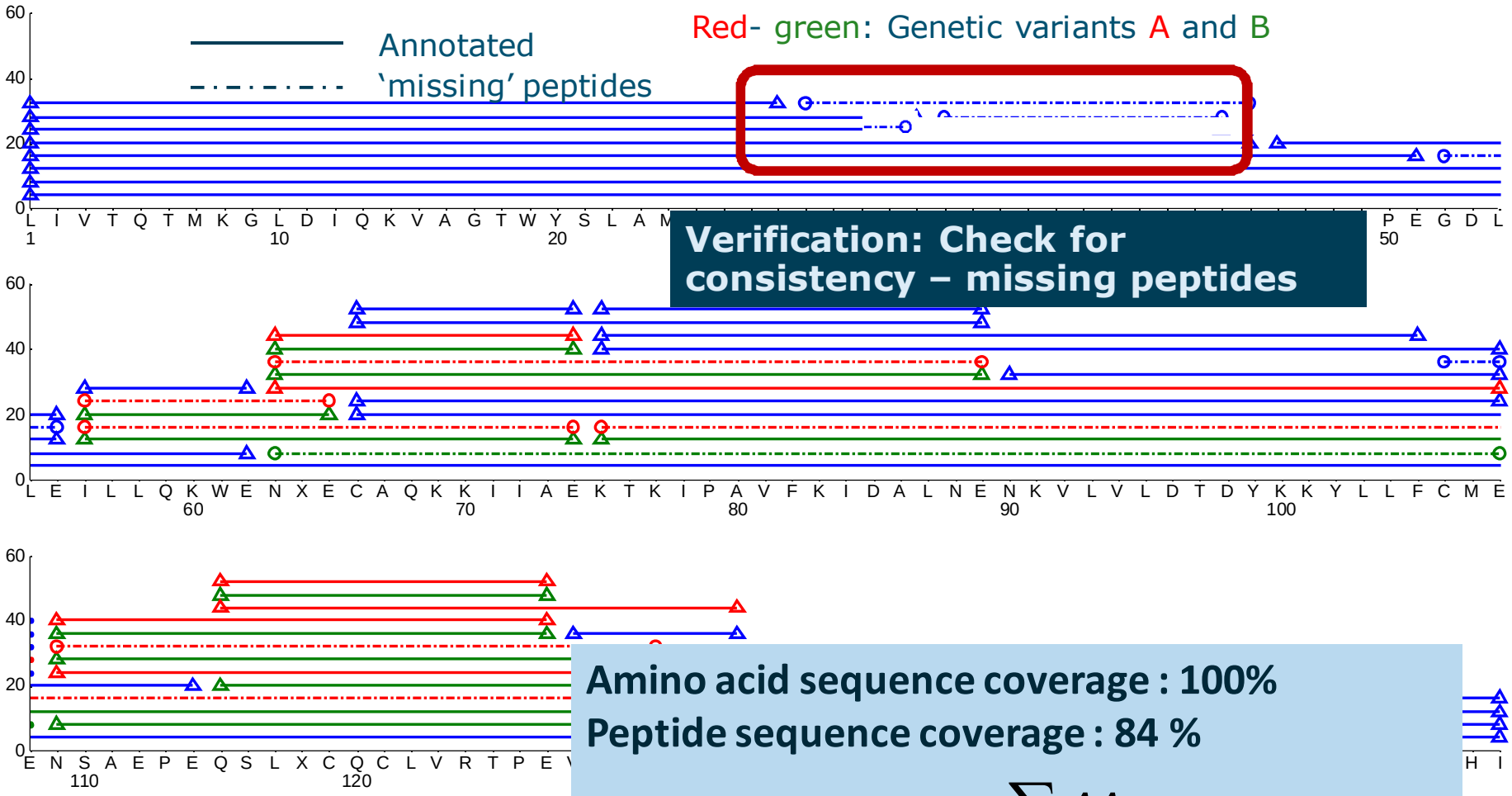



**Amino acid sequence coverage : 100%**

$$Seq.Cov._{AA} = \frac{\sum Unique(AA_{annotated})}{\sum AA_{protein}}$$

**… but is it 'complete'?**

# Sequence coverage: Test consistency

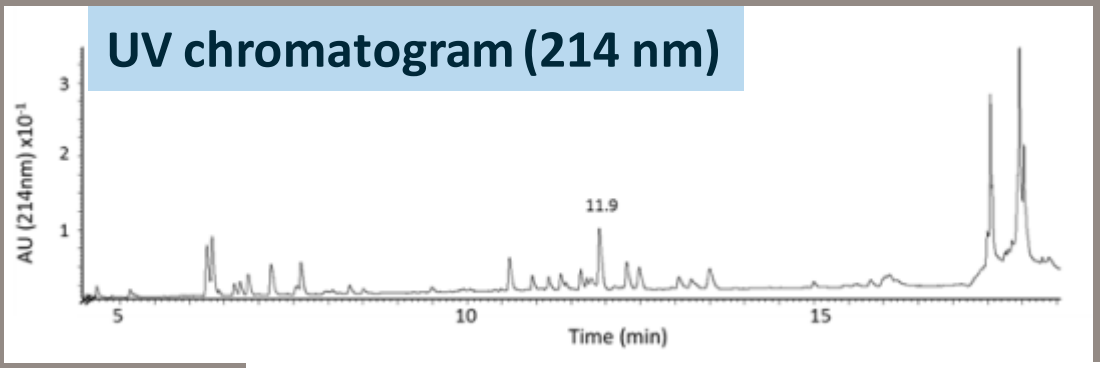


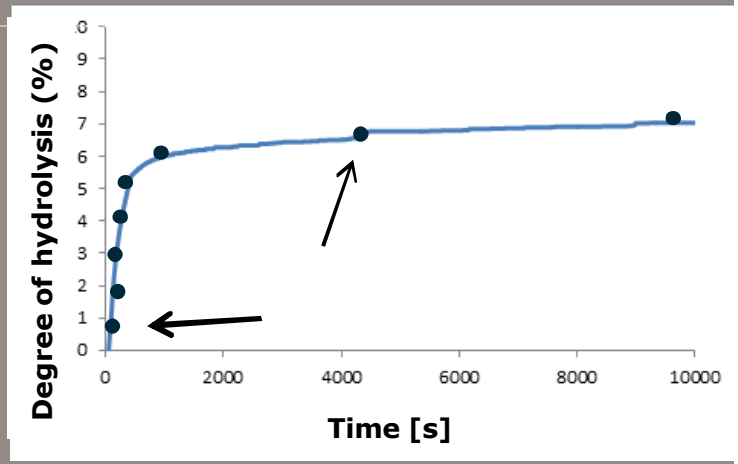

**Amino acid sequence coverage : 100%**

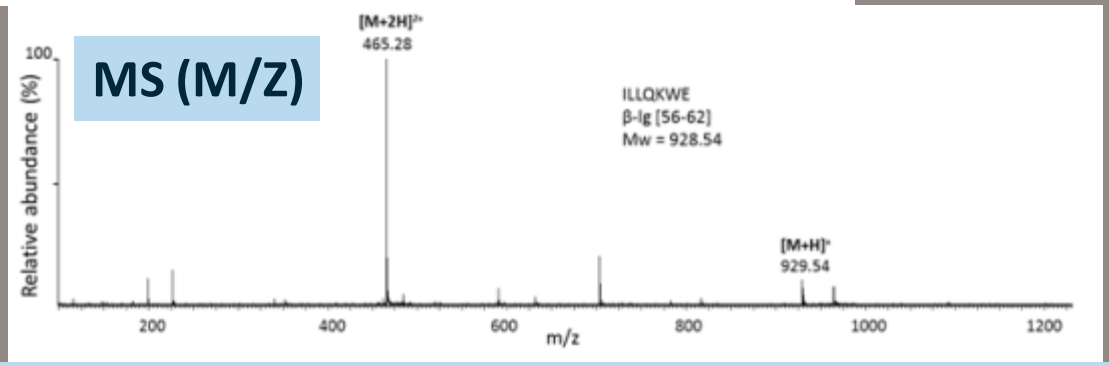
**Peptide sequence coverage : 84 %**

$$Seq.Cov._{peptide} = \frac{\sum AA_{annotated}}{\sum (AA_{annotated} + AA_{missing})}$$

# Peptide **Quantification**




- **MS – typically requires internal standards due to variability of ionisation efficiency**
- **UV – more consistent; but can it be calculated from peptide sequence?**

# Quantification based on UV

## Molar absorption coefficients (214 nm)

| building block | $\epsilon$ ($M^{-1}$ $cm^{-1}$) | building block | $\epsilon$ ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|
| group I: $\epsilon_{aminoacid} > \epsilon_{peptidebond}$ | | | |
| proline (not at N terminus) (P) | 2675 | tyrosine (Y) | 5375 |
| histidine (H) | 5125 | tryptophan (W) | 29050 |
| phenylalanine (F) | 5200 | | |
| group II: $\epsilon_{aminoacid} \sim \epsilon_{peptidebond}$ | | | |
| peptide bond | 923 | methionine (M) | 980 |
| group III: 10% < $\epsilon_{aminoacid}$ < 100% $\epsilon_{peptidebond}$ | | | |
| arginine (R) | 102 | glutamine (Q) | 142 |
| asparagine (N) | 136 | cysteine (C) | 225 |
| group IV: $\epsilon_{aminoacid}$ < 10% $\epsilon_{peptidebond}$ | | | |
| glycine (G) | 21 | valine (V) | 43 |
| proline (at N terminus) (P) | 30 | isoleucine (I) | 45 |
| alanine (A) | 32 | leucine (L) | 45 |
| serine (S) | 34 | aspartic acid (D) | 58 |
| lysine (K) | 41 | glutamic acid (E) | 78 |
| threonine (T) | 41 | | |

*These values are used in GPMAW*

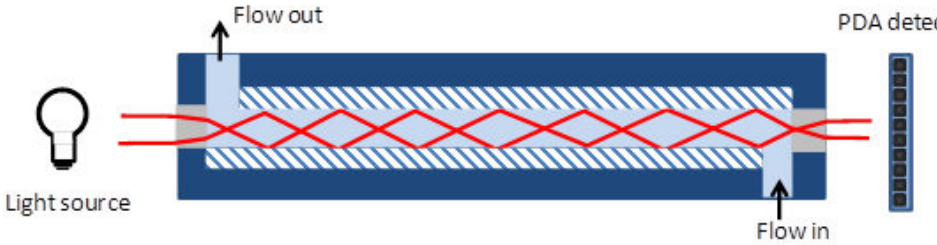
$$\varepsilon_{peptide}\,(M^{-1}cm^{-1}) = \varepsilon_{peptidebond}\ x\ n_{peptidebonds} + \sum_{i=1}^{20} \varepsilon_{amino\ acid(i)}\ x\ n_{amino\ acid(i)}$$

*Kuipers and Gruppen, J. Agric. Food Chem. 2007, 55, 5445-5451*

# Quantification of peptides in LC based on UV

## Determination of cell constant ($k_{cell}$)

$$C_{peptide} = 1 * 10^6 \left( \frac{A_{214}}{\varepsilon_{214} l V_{inj} k_{cell}} \right) Q$$




**Injection of different peptides:**

| | Peptide | Mw [Da] | $\varepsilon_{214}^{*}$ [$M^{-1}$ $cm^{-1}$] |
|---|---|---|---|
| P1 | β-Lg f135-158 | 2827.34 | 44165 |
| P2 | Angiotensin I | 1282.45 | 30332 |
| P3 | Angiotensin II | 1046.18 | 25162 |
| P4 | Bradikinin | 1060.21 | 31600 |
| P5 | Gly-Pro-Arg-Pro | 425.49 | 8980 |
| P6 | Gly-Tyr | 238.2 | 5396 |
| P7 | Val-Tyr-Val | 379.5 | 5461 |
| P8 | met-Enkephalin | 573.7 | 15289 |
| P9 | Angiotensin II | 1046.2 | 25162 |
| P10 | leu-Enkephalin | 555.6 | 14354 |

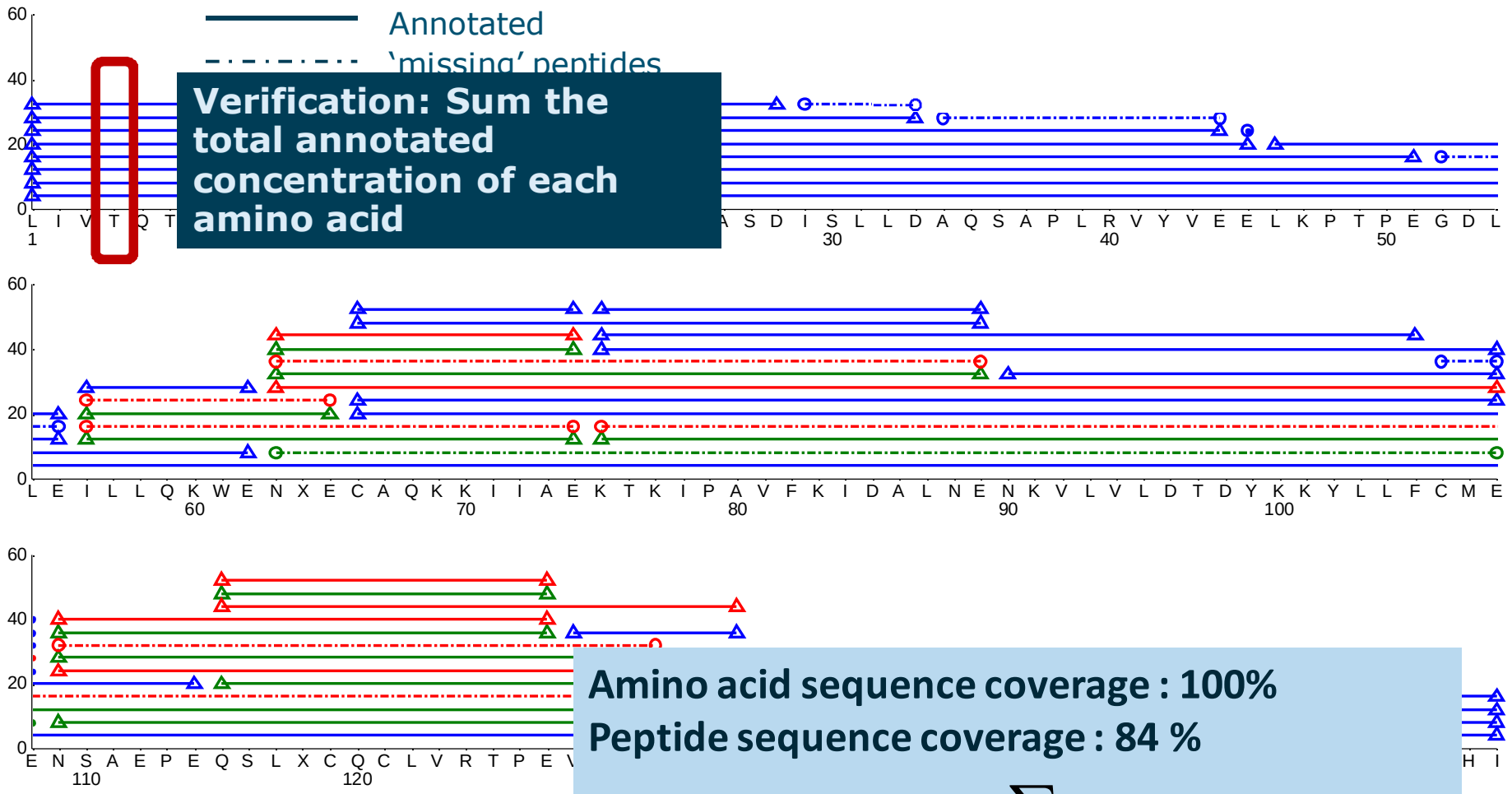
**Under different conditions:**

**Sample volume : 2, 4 µL**
**Injection speed : 75, 750 µL**
**Spectral resolution: 1.2, 6.0 nm**
**Time resolution : 40, 80 ms**

**Lowest determined concentration: 2 µM**

$k_{cell} = 0.66 \pm 0.03$

# Testing consistency: Peptide seq. coverage

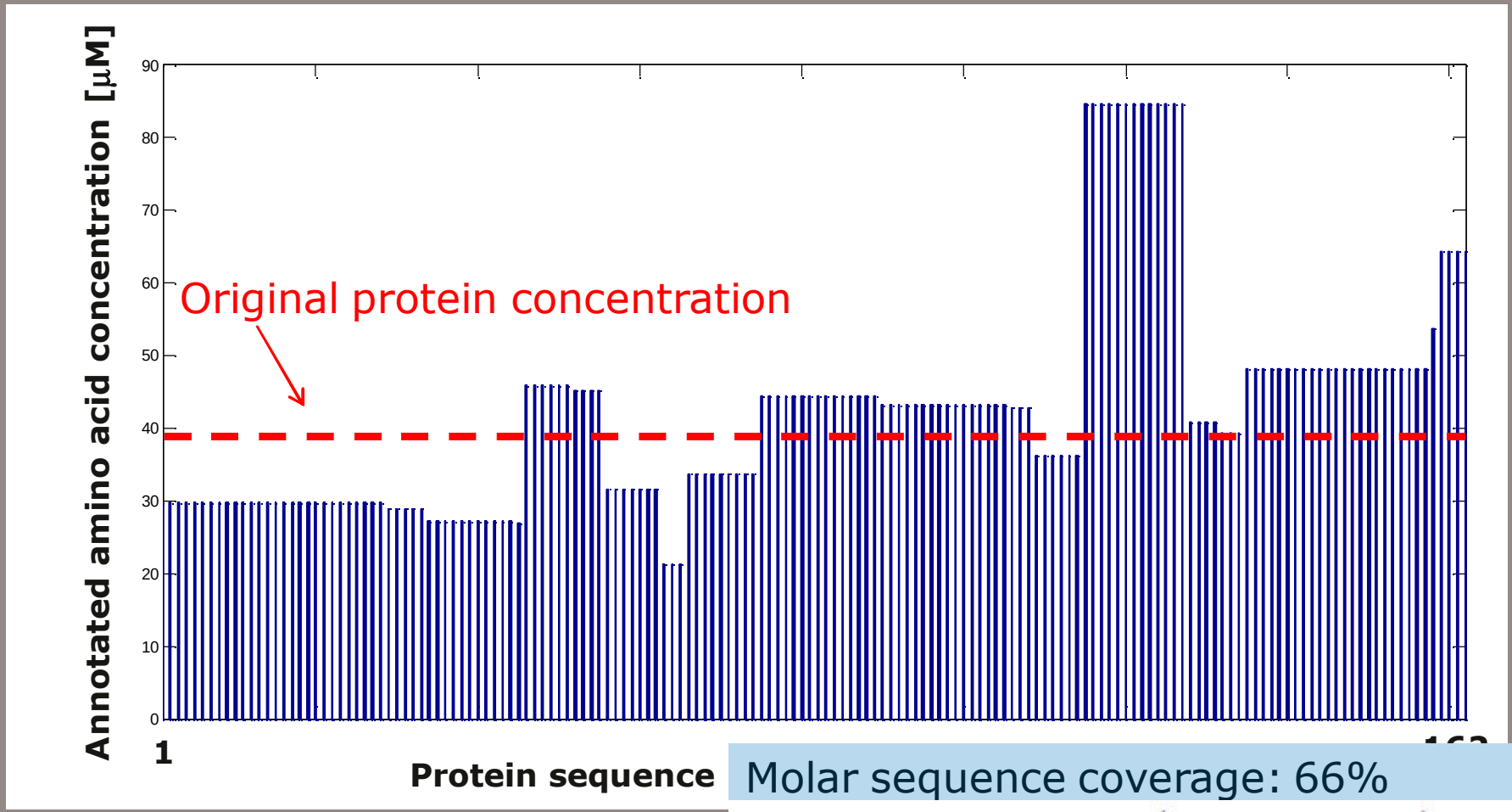



**Amino acid sequence coverage : 100%**

**Peptide sequence coverage : 84 %**

$$Seq.Cov._{peptide} = \frac{\sum AA_{annotated}}{\sum (AA_{annotated} + AA_{missing})}$$

# Quantitative (Molar) sequence coverage




Molar sequence coverage: 66%

$$\text{molar sequence coverage} = \left(1 - \frac{\sqrt{\frac{\sum(C_n - C_0)^2}{(\#\,AA - 1)}}}{C_0}\right) \times 100$$

# Comparing Sequence coverage

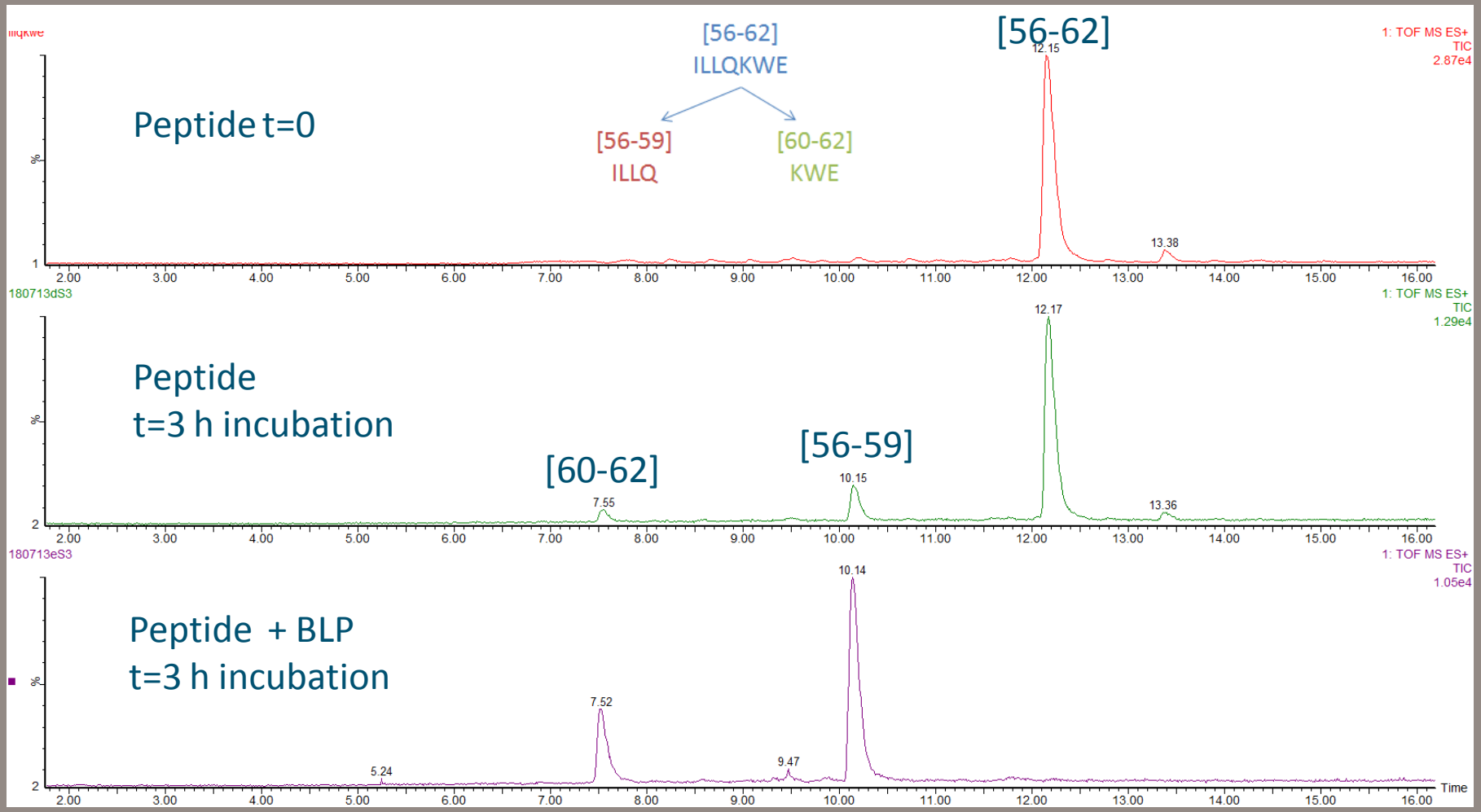
| | Presence | Consistency | Quantification |
|---|---|---|---|
| Amino acid sequence Coverage : | ✓ | ✗ | ✗ |
| Peptide sequence Coverage : | ✓ | ✓ | ✗ |
| Molar sequence Coverage : | ✓ | ✓ | ✓ |

# Comparing Sequence coverage

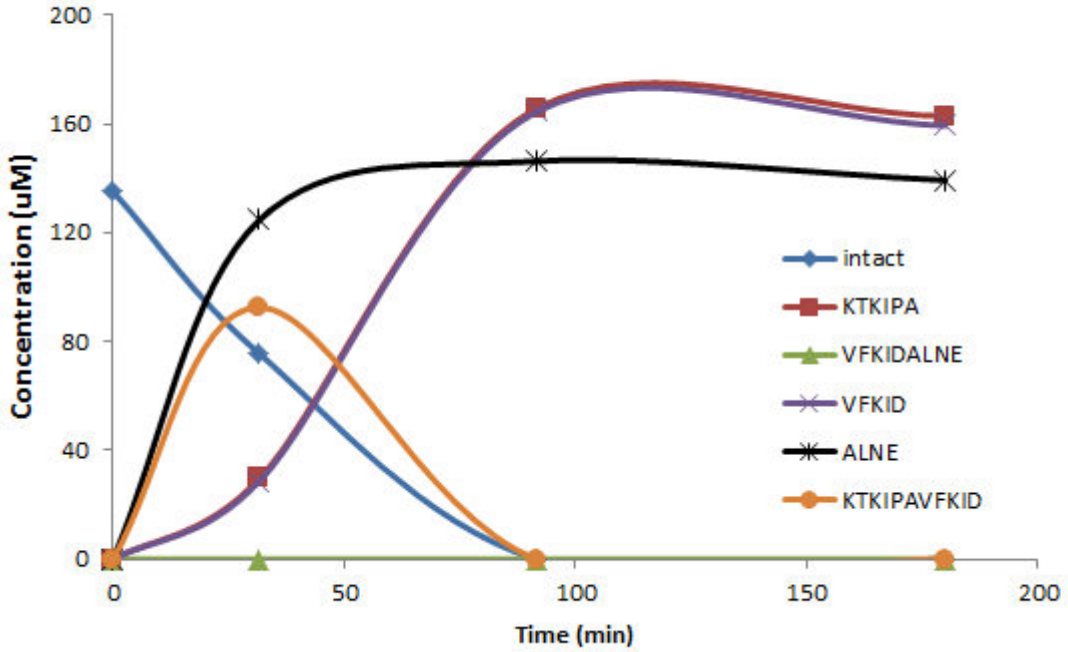
| DH | 1.8% | 7% |
|---|---|---|
| Traditional sequence Coverage : | 100 % | 97 % |
| Corrected sequence Coverage : | 84 % | 89% |
| Average concentration (microM): | 25 | 32 |
| Molar sequence Coverage : | 66 % | 85 % |
| Enzyme specificity : | 98% | 70% |
| Cutting sites : | D E F | Q D E L X A N F Y S K M |
| Cutting site frequency : | 2 45 1 | 6 11 43 4 2 1 1 1 1 2 3 2 |

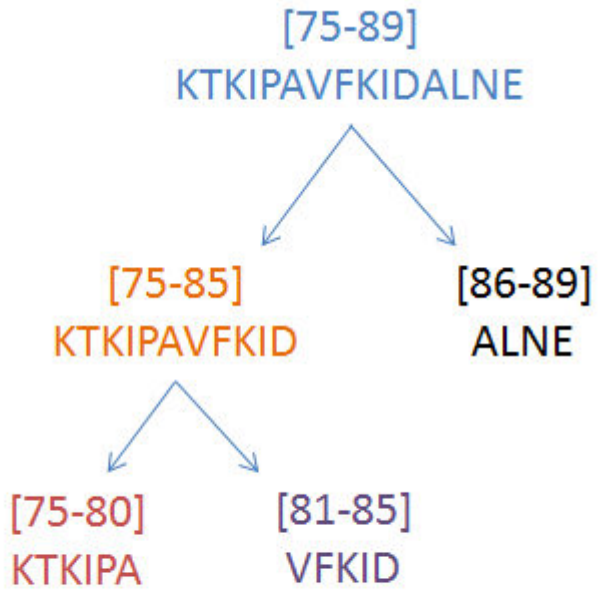
**At high DH a lot of a-specific cleavage:**
**Hypothesis: even 'specific' enzymes can hydrolyse 'a-specific' bonds**

# Confirmation of 'a-specific' cleavage



# Confirmation of ‘a-specific’ cleavage



# Summarizing LC-MS in food chemistry

**Extremely powerful combination:**

**UV quantification of peptides:**

- LABEL-FREE
- Fast, easy, accurate
- Absolute molar concentrations

**MS annotation of peptides**

- Strongly improved by lock-mass

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