Nestlé
Good Food, Good Life

Quantification of milk traces in food matrices using LC/MS

AOAC/ASFILAB workshop, Paris

Petra Lutter
Nestlé Research Center, Lausanne, CH

2009/11/24

Nestlé Research™

Overall motivation

Provide safe food with informative labels for food allergic consumers, and minimize precautionary labeling

Current state-of-the-art

Nestlé
Good Food, Good Life

ELISA technique

- is currently accepted by the authorities and used in Nestlé labs
- selective and sensitive (ppm)
- cross-reactivity possible
- target molecules are not always known
- is fast and easy to use
- little investment costs
- Depended on antibody or kit manufacturers
- is sensitive to protein processing e.g. denaturation and modification
- single-analyte method
- low dynamic range (e.g. 2-20 mg/kg)

LC-MS/MS technique

- is currently evaluated in several labs
- highly selective and sensitive (ppm)
- very specific
- target molecules are known
- is fast, requires MS knowledge
- expensive equipment
- no antibodies required
- not sensitive to protein denaturation, but modification
- Single or multi-analyte formats
- high linear dynamic range (e.g. 2-1000 mg/kg)

Why alternative approaches to ELISA?

Nestlé
Good Food, Good Life

- Need of confirmatory methods
- S/MRM-MS is widely used as the quantification tool of choice for small molecules
- MRM-MS serves as a tool for absolute protein and peptide quantification
- MRM-MS allows detection of multiple unrelated food allergens in a single analysis
- MRM-MS is highly specific
  - allows detection of
    - processed (modified) peptides/proteins
    - peptides/proteins denatured during sample preparation
- Multiplex detection offers control over several peptides/proteins
- Fully transparent
- Offers more flexibility and cost reduction

Outline

Nestlé
Good Food, Good Life

- Analytical strategy
- Selection of target proteins and peptides
- Application in different matrices
  - Sample preparation
  - Determination of milk traces in food
  - Comparison with ELISA/FAPAS
- Conclusion

Protein quantification by MS

Nestlé
Good Food, Good Life

Internal standard by isotope dilution

- **Stable isotope-labelled synthetic peptides**
  - Known from peptidomics and phosphorylation studies
- **QconCAT synthetic proteins**
  - Complex mixtures, protein complexes
- **Full-length isotope-labeled proteins (PSAQ)**
  - Accucate quantification on prefractionated samples

Peptide quantification by isotope dilution – variant I

Nestlé
Good Food, Good Life

Select candidate protein biomarkers

Select suitable quantifiable peptides

K.VLVLDTDYK.K
...

Synthesise ^{13}C-^{15}N-labelled IS heavy peptides

K.VLVLDTDY[K^{13}C^{15}N].K

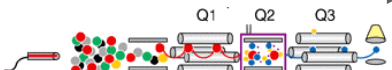
Prepare calibration standards including all heavy and light peptides

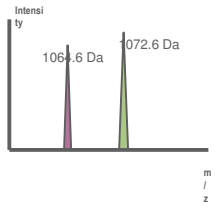
Extract proteins

Digest protein using trypsin

Spike sample with IS heavy peptides

Analyse by MRM LC-MS/MS





Quantify marker proteins using IS heavy peptides

The Quantifiers - selection of peptides

Nestlé
Good Food, Good Life

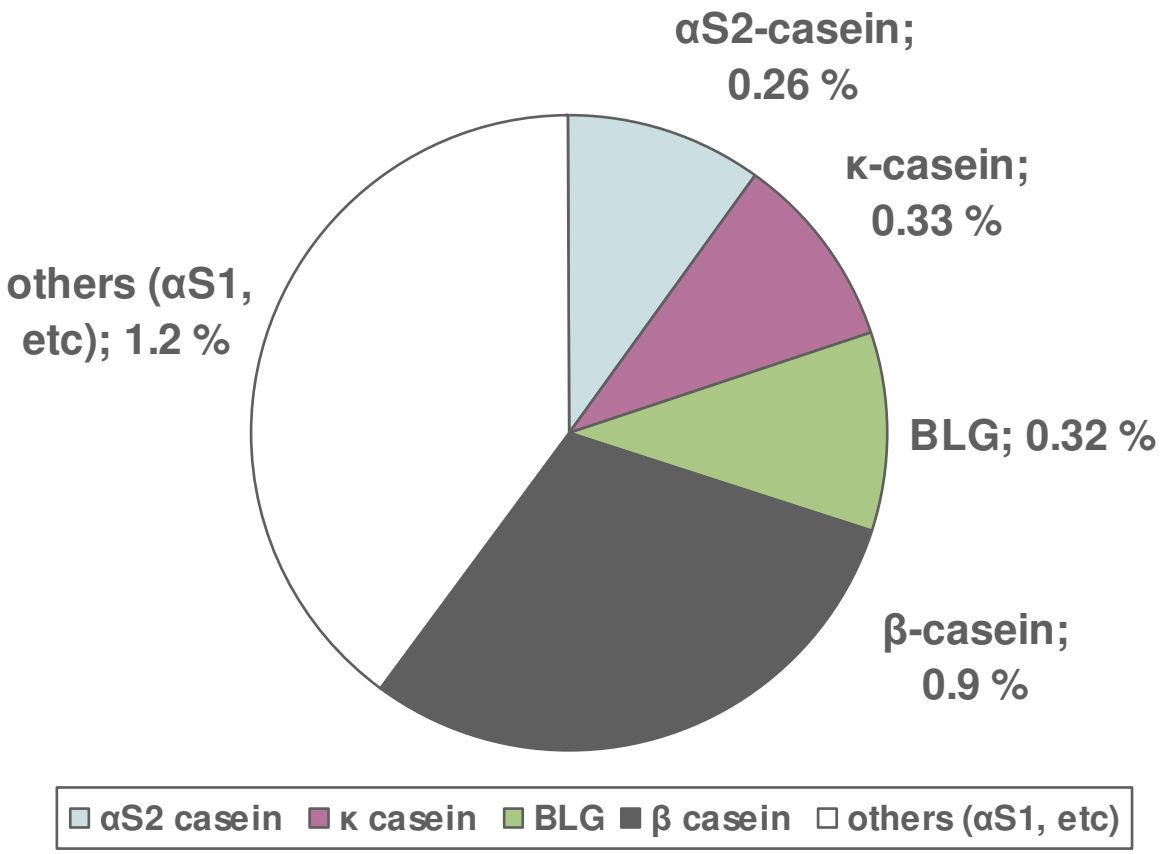
- Selection of reference protein
  - Specific for milk proteins from different species (cow, buffalo) but not specific for other food ingredients (e.g. egg)
  - Ideally 2-3 reference proteins per allergenic compound
  - Good extraction properties and solubility (no membrane proteins)
  - None to few posttranslational modifications, modifications during food processing

- Selection of marker peptides
  - Sequence specificity
  - No Met, Cys, Trp residues
  - Region with low risk of miscleavages
  - Retention time
  - Ionization capacity
  - 7-15 amino acids/peptide

Selection of proteins

Nestlé
Good Food, Good Life

Distribution of caseins and BLG in total milk protein



The Quantifiers - selection of peptides

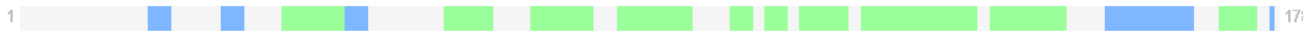
Nestlé
Good Food, Good Life

- Naturally occurring variants and modifications have to be considered
  - All know variants could be excluded when selecting the quantifier peptides for e.g. beta lactoglobulin (source e.g. SWISS PROT database)

Sequence annotation (Features)

	Feature key	Position(s)	Length	Description	Graphical view	Feature identifier
Molecule processing						
☐	Signal peptide	1 – 16	16	Ref.6 Ref.8		
☐	Chain	17 – 178	162	Beta-lactoglobulin		PRO_0000017903
Amino acid modifications						
☐	Disulfide bond	82 ↔ 176		Ref.13 Ref.19		
☐	Disulfide bond	122 ↔ 137		Alternate Ref.13		
☐	Disulfide bond	122 ↔ 135		Ref.13 Ref.19		
Natural variations						
☐	Natural variant	61	1	E → Q in variant D. Ref.10		
☐	Natural variant	72	1	I → L in variant W. Ref.8		
☐	Natural variant	75	1	Q → H in variant C; found only in the Jersey breed. Ref.14		
☐	Natural variant	80	1	G → D in variant A. Ref.6 Ref.1 Ref.9		
☐	Natural variant	134	1	A → V in variant A. Ref.6 Ref.1 Ref.9 Ref.12		
Experimental info						
☐	Sequence conflict	121	1	F → V in CAA32835. Ref.1		
☐	Sequence conflict	136	1	Q → E Ref.12		

Secondary structure



Helix Strand Turn

Sequence homology

Nestlé
Good Food, Good Life

Peptide sequence	Protein	Bovine	Water buffalo	Goat	Sheep	Reindeer	other even-toed ungulate	Human	Bacteria
YIPIQYVLSR	κ-casein	✓	✓	✓	✓	✓	✓	✗	✗
FALPQYLK	α-S2 casein	✓	✗	✗	✗	✗	✗	✗	!!!
ALNEINQFYQK	αS2-casein	✓	✓	✓	✓	✓	n.a.	✗	✗
AVPYPQR	β-casein	✓	✓	✗	✗	✗	✗	✗	1?
VLVLDTDYK	BLG	✓	✓	✓	✓	✓	n.a.	✗	✗
TPEVDDEALEK	BLG	✓	✓	✗	✗	✗	✗	✗	✗

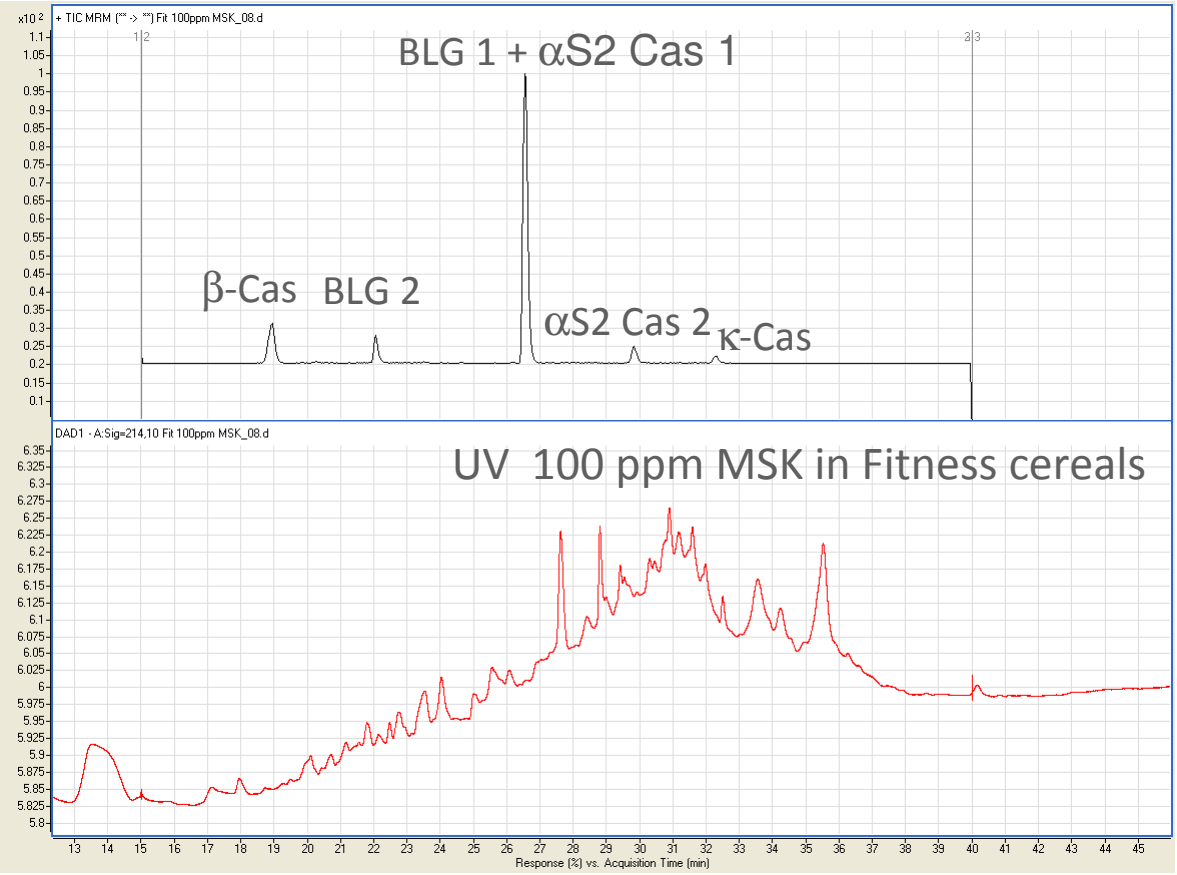
- 5/6 peptides cover both, cow's and buffalo's milk → Asian market !

Typical TIC of selected peptides from milk proteins

Nestlé
Good Food, Good Life

- Total ion chromatogram intensities of selected tryptic peptides in MSK spiked Nestlé Fitness cereals

TIC 100 ppm MSK in Fitness cereals



Selection of transitions - CASEIN

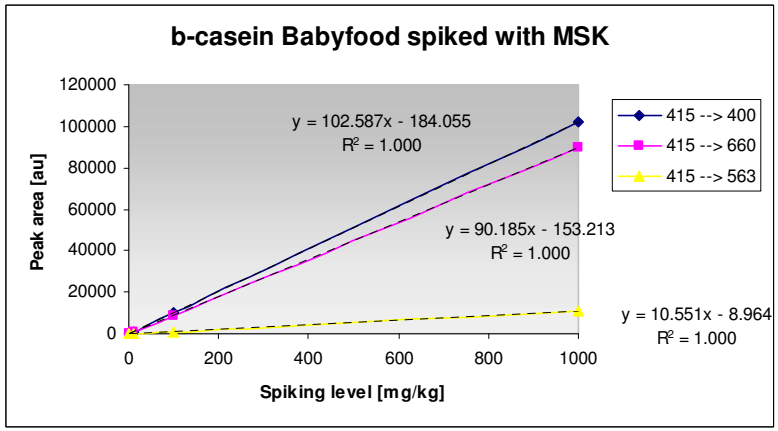
Nestlé
Good Food, Good Life

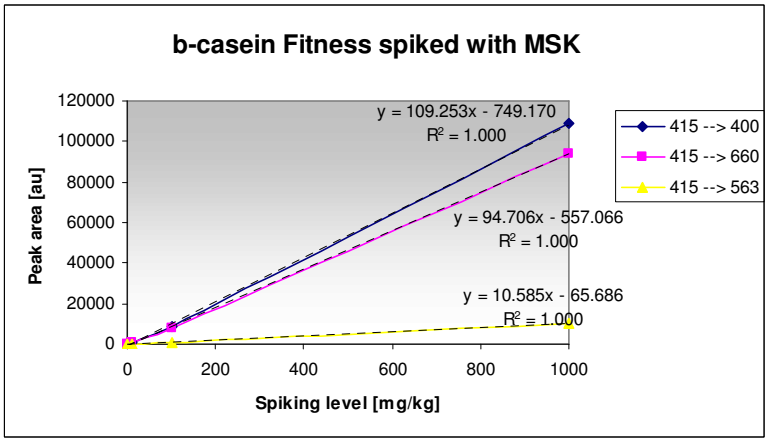
- Linearity and intensity of transitions was analysed in blank baby food and cereals and spiked with MSK at 10, 100, and 1000 mg/kg

Baby food

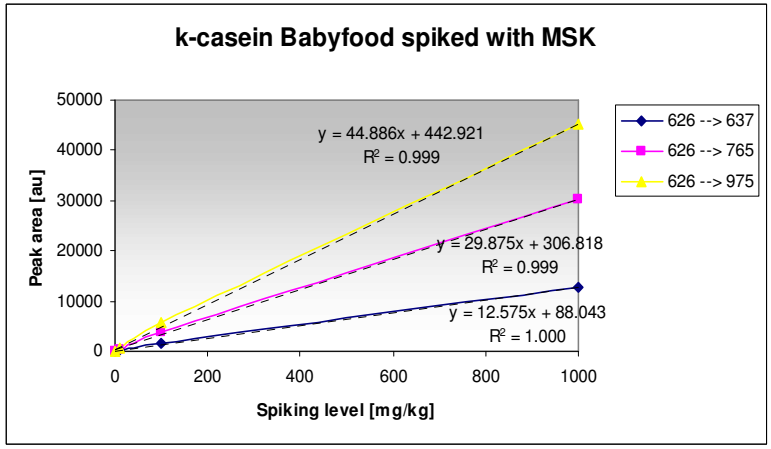
Fitness Cereals

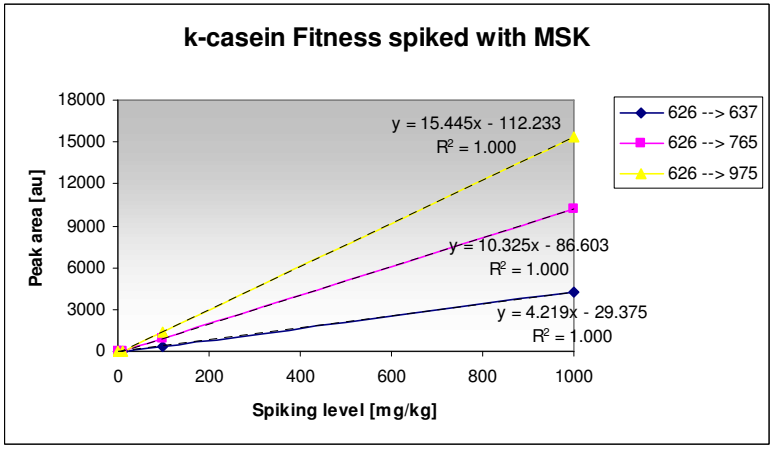
β-casein





κ-casein





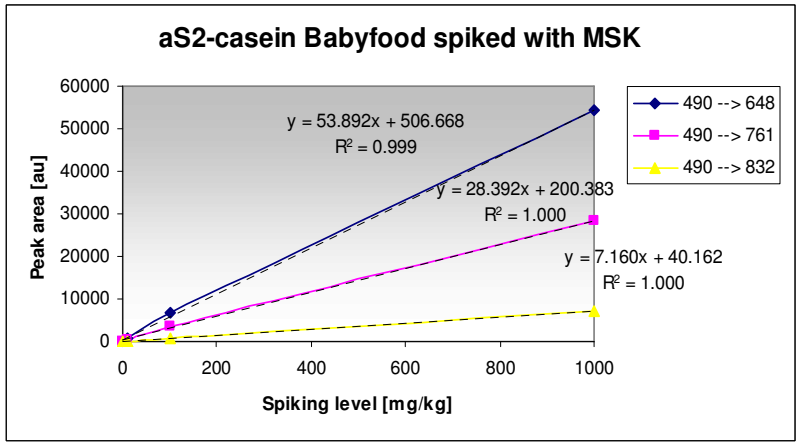
Linearity for different transitions - CASEIN

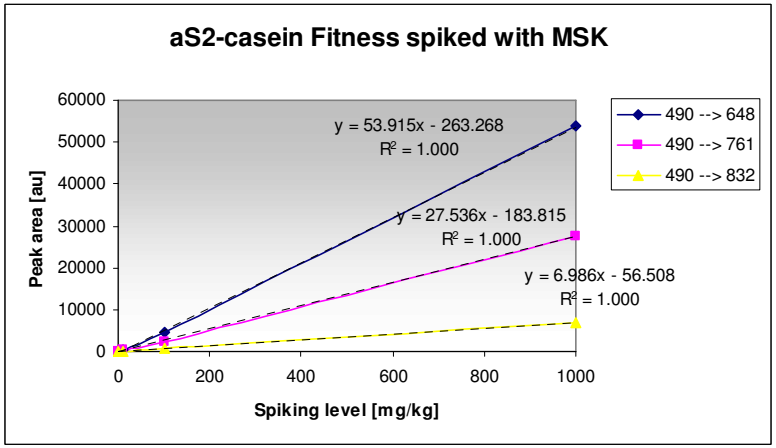
Nestlé
Good Food, Good Life

Baby food

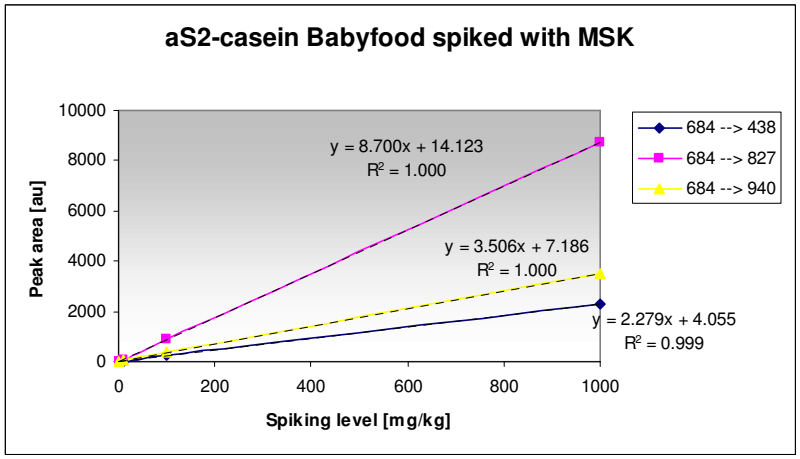
Fitness Cereals

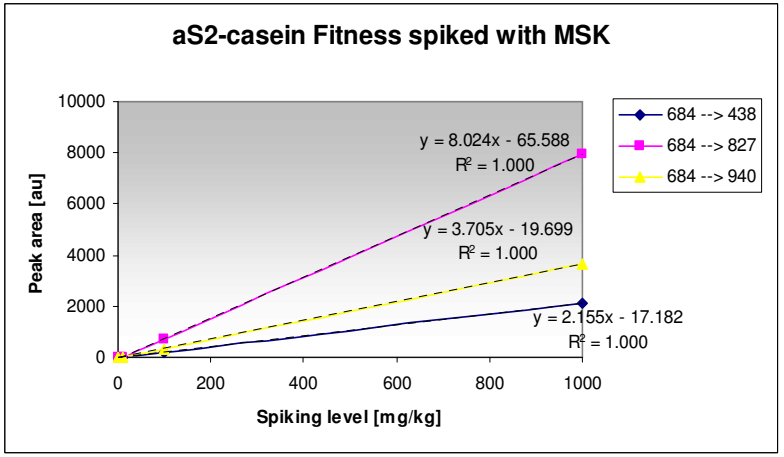
αS2-casein





αS2-casein

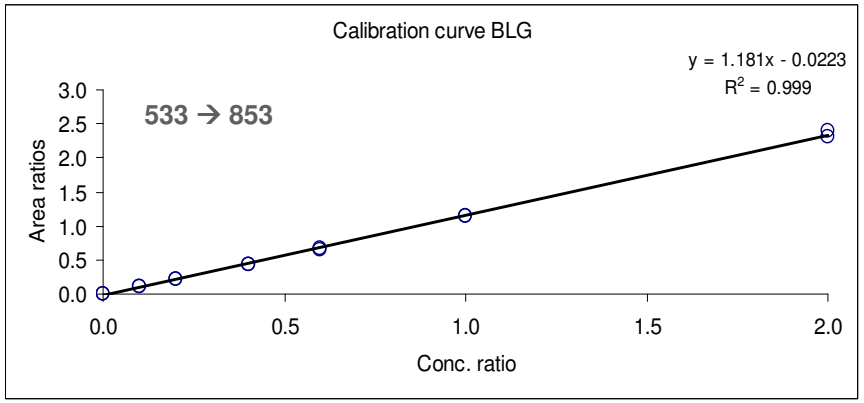


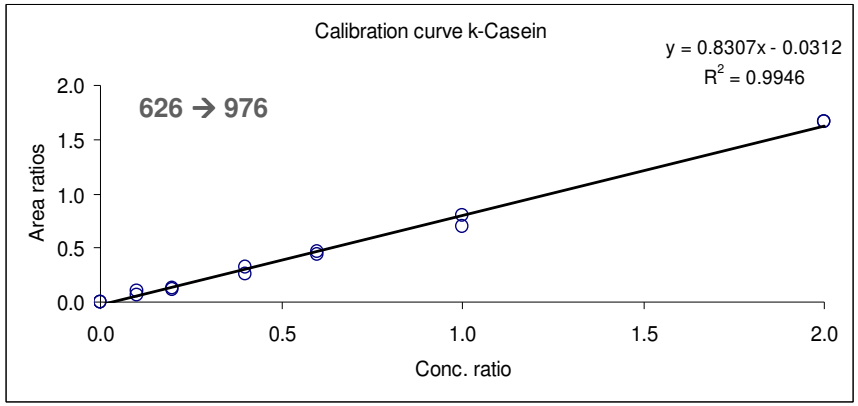


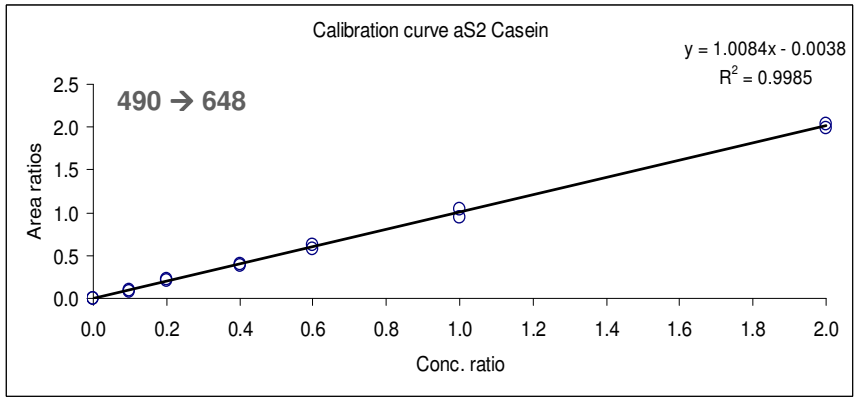
Calibration Mix contains all IS peptides

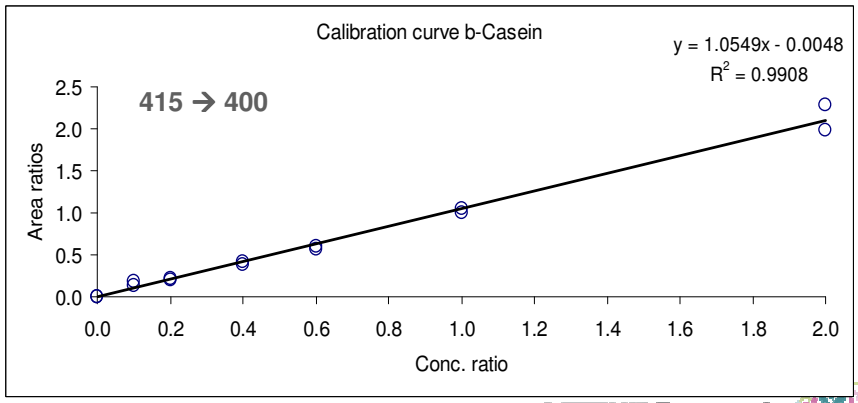
Nestlé
Good Food, Good Life

- The Calibration Mix contains all IS peptides (heavy and light) of the four milk proteins
- IS_{heavy} const. 50 fmol; pure $standards_{light}$ from 1-100 fmol (~30-3000 ng/mL MSK)
- Calibration curves follow linear relationship









Sample preparation + instrumentation

Nestlé
Good Food, Good Life

- Extract 1g of sample with 10 ml 50 mM NH_4HCO_3 + 1 M urea (60°C).
- Shake for 15 min at RT, and centrifuge 10 min (5'000 x g and 4°C).
- Digest an aliquot of supernatant using trypsin at 37°C overnight.
- If needed, dilute with 0.1% Formic Acid.
- Mix with Internal Standard (a mixture of 6 labelled peptides).
- Filter if needed.
- Injection of 20 µl, corresponds to 50 fmol of each IS (41 to 68 pg, according to the MW of peptide).

Instrumentation:

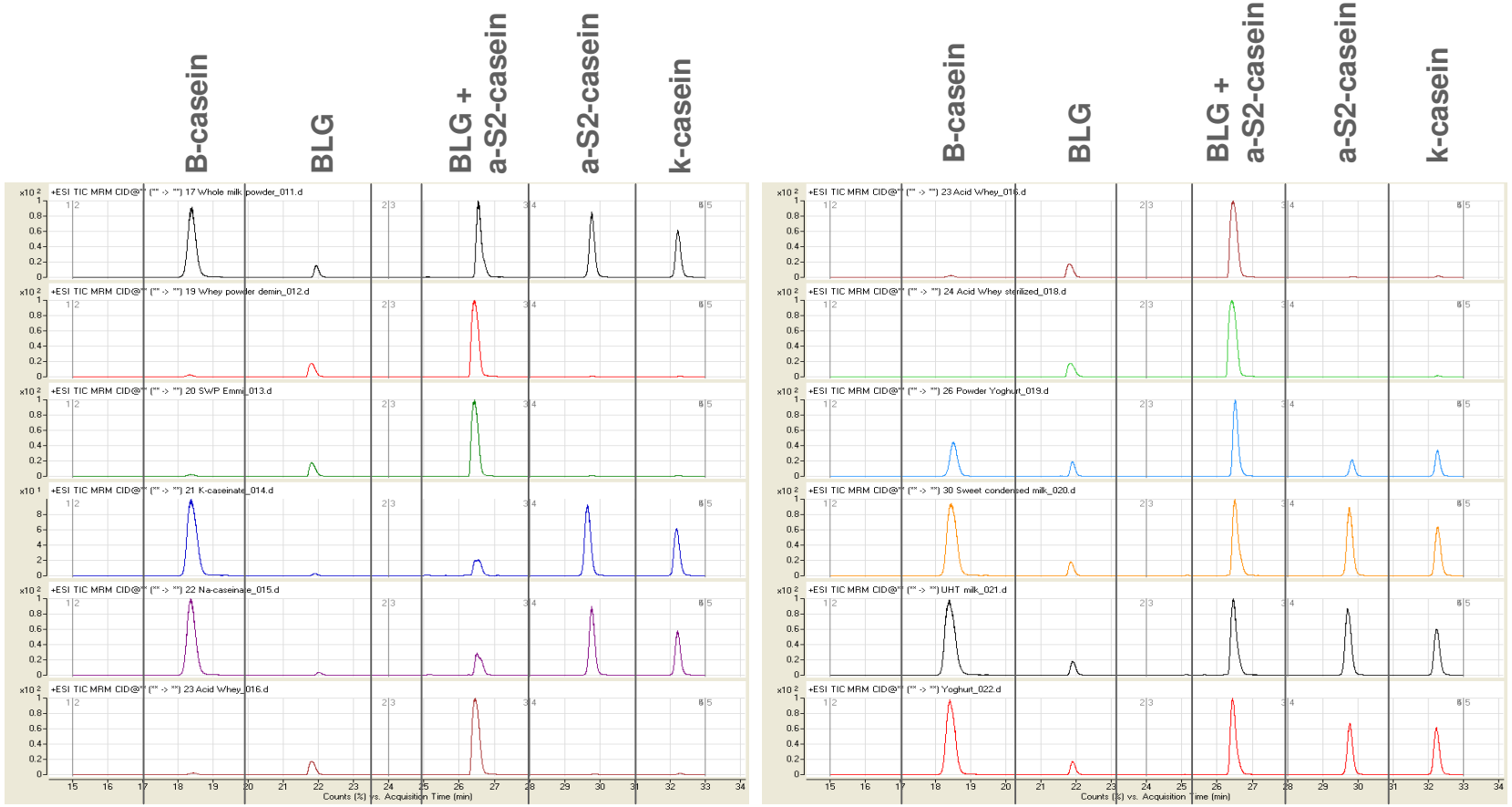
1. **Agilent HPLC 1200 series coupled to Agilent 6460 mass spectrometer**
2. **Agilent HPLC 1100 series coupled to Applied Biosystems 4000 Q TRAP® mass spectrometer**

On both, Waters Symmetry300™ C18 3.5 µm, 2.1 x 150 mm, 1 hour gradient, 0.2 ml/min

Effect of processing on detectability of target peptides in typical dairy ingredients

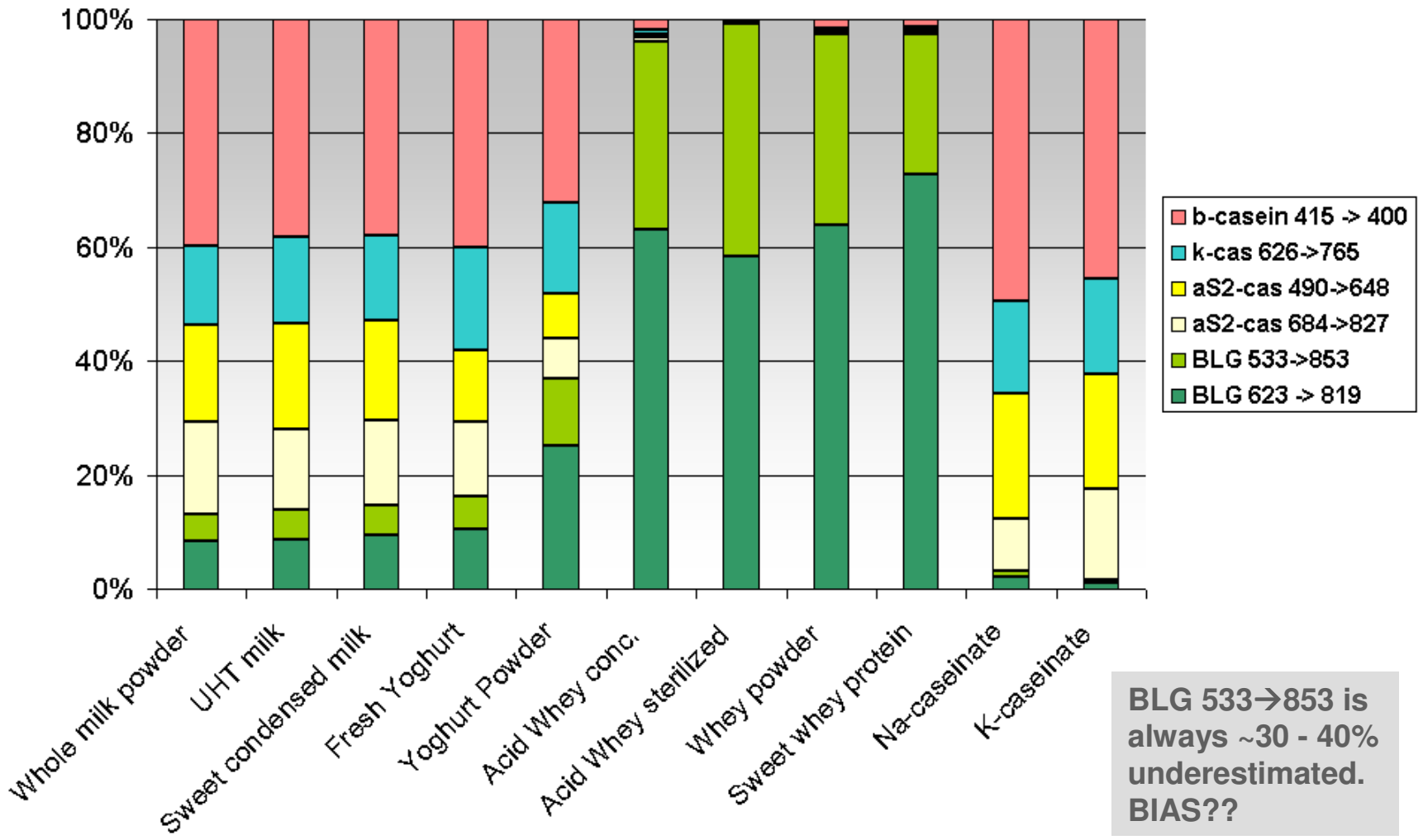
Nestlé
Good Food, Good Life

- Selected processed dairy ingredients MRM TICs
  - spray dried, UHT, concentrated, precipitated, defatted, fermented



Ratios of detected milk protein dairy ingredients

Nestlé
Good Food, Good Life



BLG 533→853 is always ~30 - 40% underestimated. BIAS??

Matrix effects

Nestlé
Good Food, Good Life

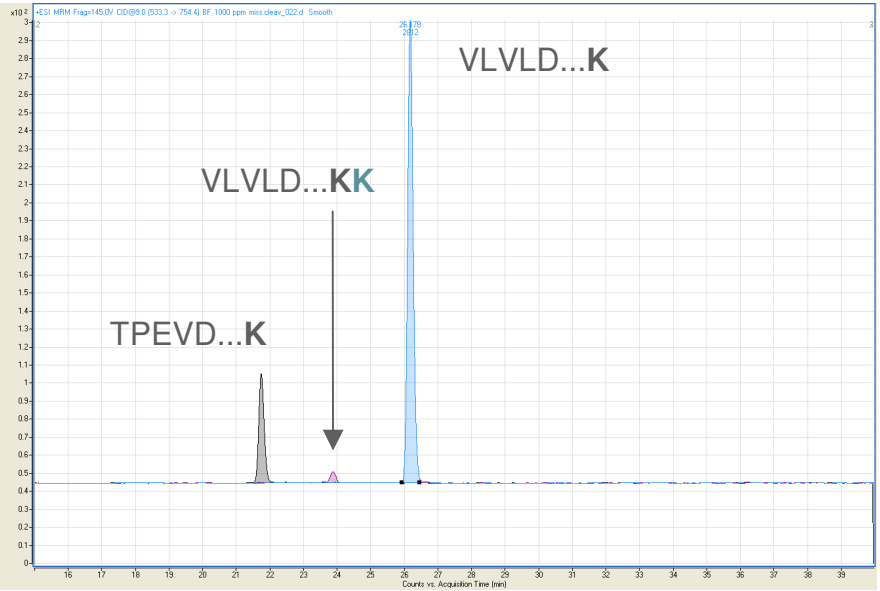
- Matrix components may induce interferences
  - Digestion efficiency
    - pH
    - other proteins
    - protease inhibitors
    - salts, …
    - modifications: polyphenols, blocked lysins, glycation of amino acids
  - Extraction efficiency
    - lipids
    - protein-protein interaction
  - Peptide mass
    - modifications: oxidation, polyphenols, blocked lysins, glycation of amino acids, phosphorylation, glycosylation
    - digestion efficiency (missed cleavages)
    - adducts (salt ions)

Digestion efficiency in spiked baby food and cereals

Nestlé
Good Food, Good Life

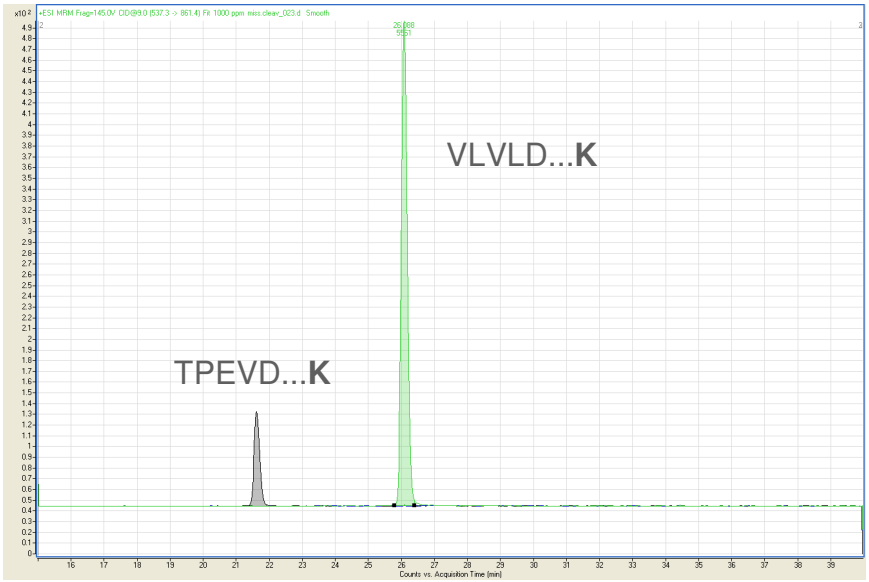
Baby food (1000 ppm MSK)

- Loss dues to **VLVLDTDYKK** is negligible (~ 1.5 area%)
- **TPEVDDEALEKFDK** not present



Cereals (1000 ppm MSK)

- Miscleaved peptides **TPEVDDEALEKFDK** and **VLVLDTDYKK** are not detectable in spiked cereals matrix.



Determination of milk in baby food

Nestlé
Good Food, Good Life

Determination of MSK spiked into baby food sample shows good correlation between the three selected casein proteins.

MSK spiking level in baby food	αS2-Casein		κ-Casein		β-Casein	
	MSK µg/g	% recovery	MSK µg/g	% recovery	MSK µg/g	% recovery
0 ppm	n.d.	---	n.d.	---	n.d.	---
10 ppm	7.5	75	n.d.	---	13.9	139
100 ppm filtered	94	94	n.a.	---	79	79
100 ppm	96	96	78	78	78	78
1000 ppm	793	79	824	82	859	86

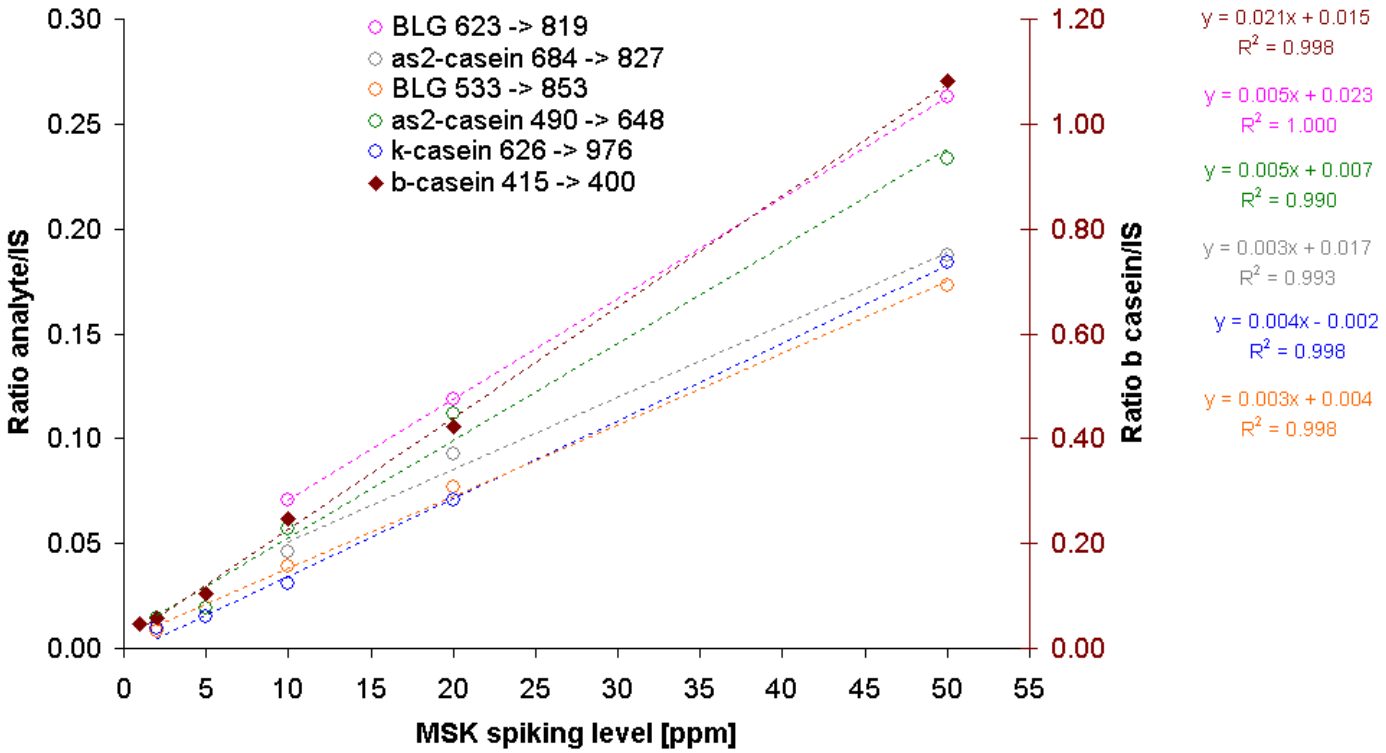
- Filtration of samples does not effect recovery rates (BF and Fit)
- Filtration of standard solutions reduces amounts to ~60 %

Linearity and LOD/LOQ in infant cereals

Nestlé
Good Food, Good Life

Sinlac (milk-free infant cereal, 15.5% total protein) was spiked with 1-50 ppm MSK

- 10 ppm can be quantified based on all reference peptides (based on S/N ratio)
- 2 ppm can be quantified based on β-casein and one BLG peptide (based on S/N ratio)



LOD/LOQ for MSK in infant cereals

Nestlé
Good Food, Good Life

	BLG 533 → 853		BLG 623 → 819		αs2-casein 684 → 827		αs2-casein 490 → 648		β-casein 415 → 400		κ-casein 626 → 976	
	MSK µg/g	Rec. [%]	MSK µg/g	Rec. [%]	MSK µg/g	Rec. [%]	MSK µg/g	Rec. [%]	MSK µg/g	Rec. [%]	MSK µg/g	Rec. [%]
Sinlac blank	nd	--	nd	--	nd	--	nd	--	nd	--	nd	--
1 ppm MSK	nq	--	nd	--	nd	--	nq	--	2.4	240	nq	--
2 ppm MSK	3.1	157	nq	--	nq	--	nq	--	2.8	140	nq	--
5 ppm MSK	4.2	84	3.4	67	nq	--	3.3	66	4.9	98	6.1	122
10 ppm MSK	6.3	63	5.9	59	2.8	28	8.7	87	11.5	115	8.1	81
20 ppm MSK	10.1	51	11.7	59	9.6	48	16.6	83	19.4	97	13.4	67
50 ppm MSK	19.9	40	29.1	58	23.5	47	34.0	68	49.4	99	28.1	56

- LOQ in Sinlac (milk-free infant cereal, 15.5% total protein) is **5 ppm MSK**
- LOD in Sinlac ~ **2 ppm MSK**
- Best recovery rates were obtained for β-casein

Comparison with ELISA – Recovery rates

Nestlé
Good Food, Good Life

ELISA

RIDASCREEN Fast BLG

Fitness Cereals	Fit 0 ppm	Fit 10 ppm	Fit 100 ppm	Fit 1000 ppm
recovery	nd	63 %	35 %	34 %

LC-MS/MS

Best transitions

Fitness Cereals	Fit 0 ppm	Fit 10 ppm	Fit 100 ppm	Fit 1000 ppm
recovery BLG 533 → 853	nd	51 %	54 %	56 %
BLG 623 → 819	nd	62 %	66 %	68 %

FAPAS – Beta lactoglobulin

Nestlé
Good Food, Good Life

FAPAS 2765	Participants N	BLG in mg/kg Median
ELISA systems	6/9	35.0
R-Biopharm	10/15	148
LC-MS/MS		$128_{(533\rightarrow853)} + 189_{(623\rightarrow819)}$
FAPAS 2751	Participants N	BLG in mg/kg Median
ELISA systems	4/7	18.7
R-Biopharm	17/20	47.6
Tepnel	5/8	20.5
LC-MS/MS		$49_{(533\rightarrow853)} + 71_{(623\rightarrow819)}$

A bias (~0.7) between BLG 533→853 and 623 → 819 was observed analysed ingredients and in both FAPAS samples.

If available, cross contaminating ingredients should be analysed for relative ratios of target peptides.

Conclusion

Nestlé
Good Food, Good Life

- 6 tryptic peptides covering BLG and 3 casein proteins were selected
- Linear calibration curves were obtained for 1-100 fmol (~30-3000 ng/mL MSK)
- LC-MS/MS allows determination of processed milk e.g. spray dried, UHT, concentrated, precipitated, defatted, and fermented
- "Simple" sample preparation w/o any enrichment is sufficient
- Method was evaluated in matrices low, medium and high in protein amount
- LOQ in e.g. protein-rich infant cereal is **5 ppm skim milk powder,** LOD ~ **2 ppm MSK**
  - This corresponds to LOQ of ~ **0.2 ppm for BLG and respective casein proteins**
  - LOQ is comparable with ELISA
  - Comparison with ELISA results show similar or higher values (dep. on ELISA kit)
  - Recovery rates were found similar or higher compared to ELISA
- Allows direct measurement of defined target molecules

The Team

Hans Weymuth

Veronique Parisod

Petra Lutter

Thanks to

Sylvie Benet (NRC), Mathieu Dubois (NRC), Eric Gremaud (NRC), Frank Vanrobaeys (Nestlé R&D Beijing)

Peptide quantification by isotope dilution – variant II

Nestlé
Good Food, Good Life

Select candidate protein biomarkers

Select suitable quantifiable peptides

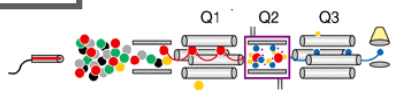
Synthesise ^{13}C-^{15}N-labelled IS heavy peptides

Prepare calibration standards including all heavy and light peptides

Extract proteins

Spike sample with IS heavy peptides

Digest protein using trypsin



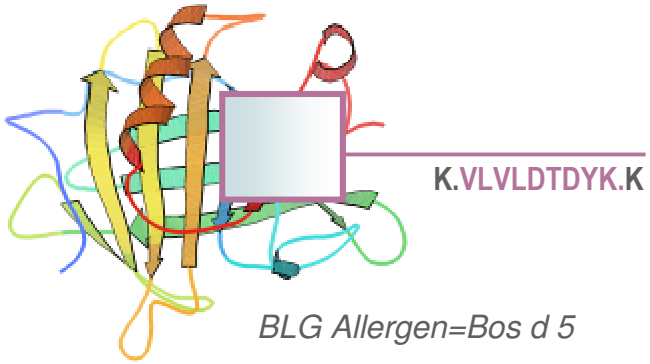
Analyse by MRM LC-MS/MS

Quantify marker proteins using IS heavy peptides

Absolute protein quantification strategy

Nestlé
Good Food, Good Life

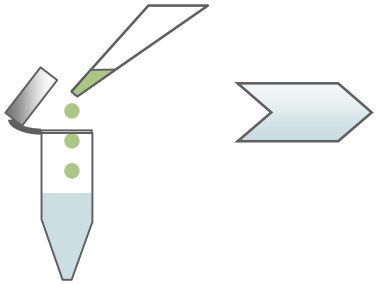
Select allergenic protein



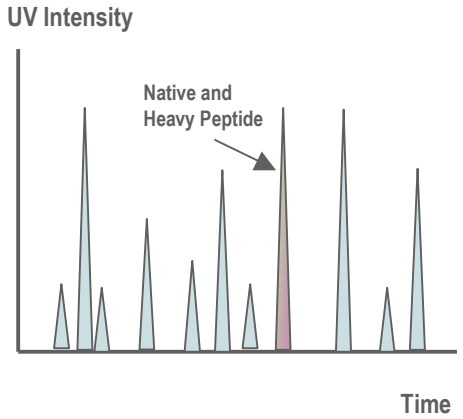
Synthesise heavy peptide analogon (IS)

K.VLVLDTDY[$K^{13}C^{15}N$].K

Spike IS into analyte



Separate by HPLC



Quantify by using MS as detector