Schweizerische Eidgenossenschaft
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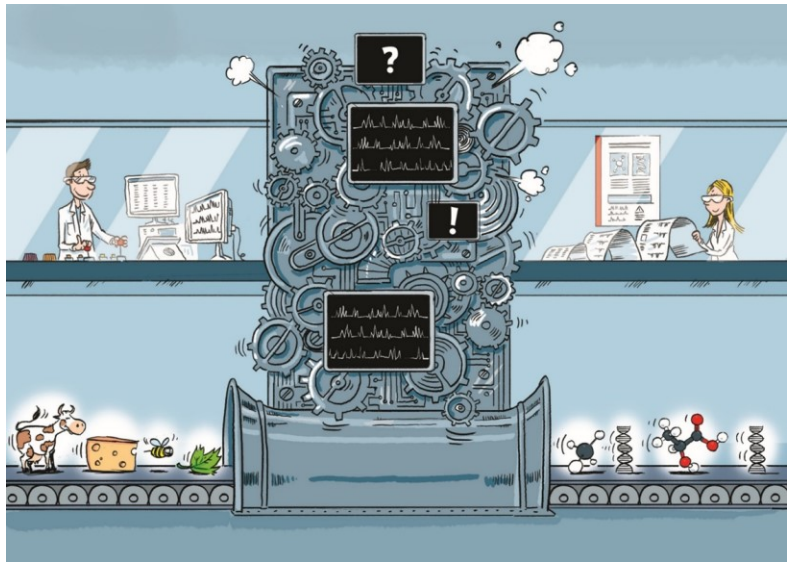
Federal Department of Economic Affairs,
Education and Research EAER

Agroscope

Analytical methods to compare the quality of plant and animal-based protein sources



Lotti Egger, Raquel Sousa and Reto Portmann

Method development and Analytics, **Agroscope, Switzerland**

Starting point: COST Action Infogest 2011-2015

Infogest aims at building an open international network of institutes undertaking multidisciplinary basic research on food digestion gathering scientists from different origins (food scientists, gut physiologists, nutritionists…).

Infogest targets three main scientific goals:

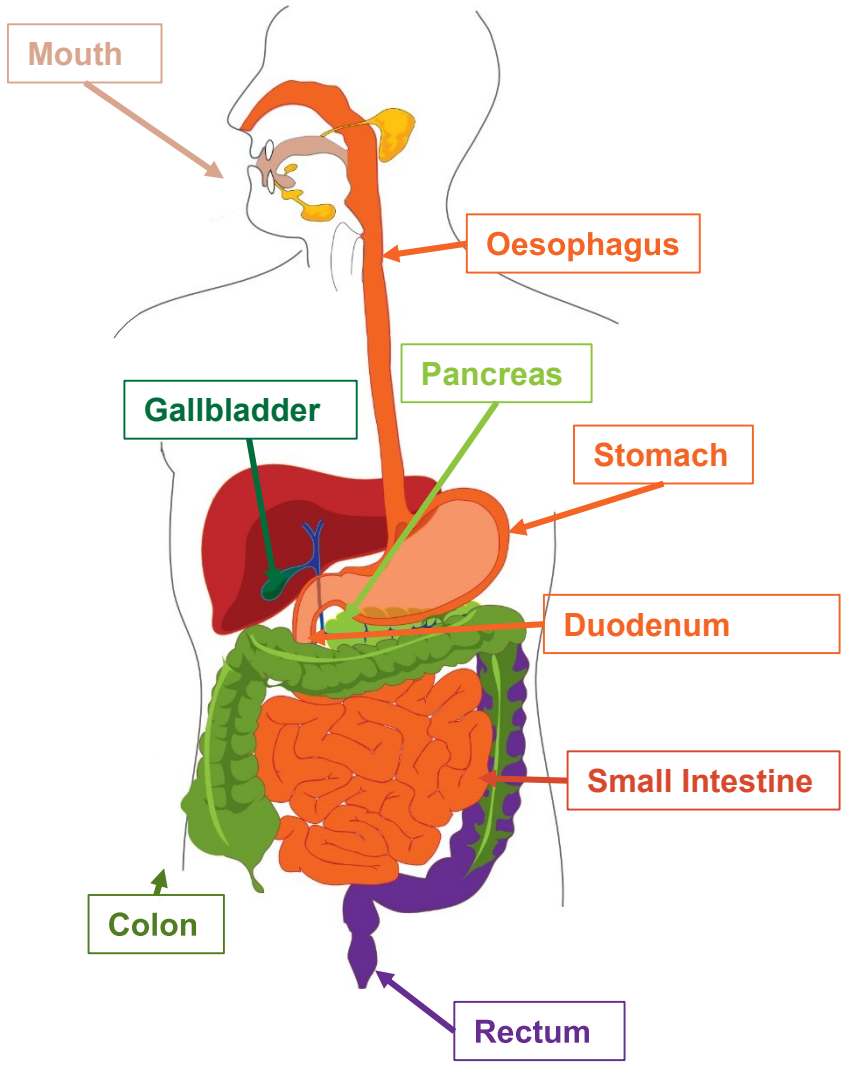
- Identify the beneficial food components released in the gut during digestion
- Support the effect of beneficial food components on human health
- Promote harmonization of currently used digestion models

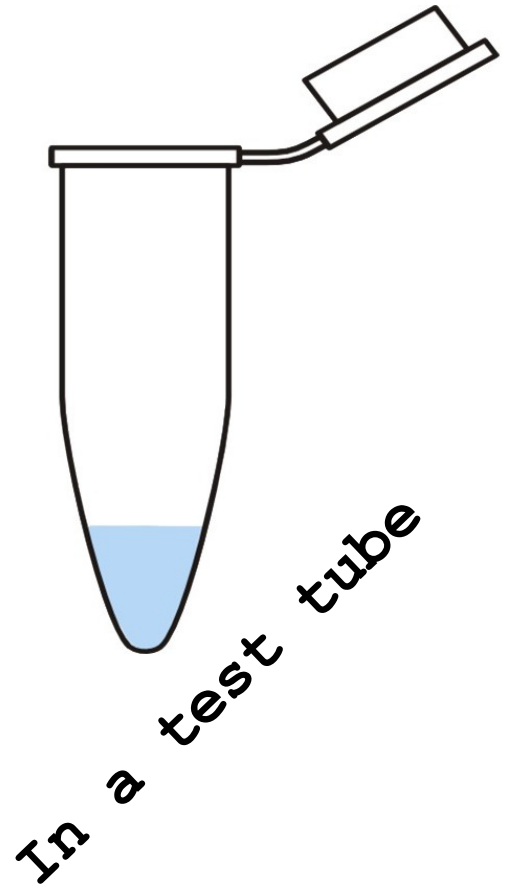
The Human digestion – in a test tube

Oral phase:

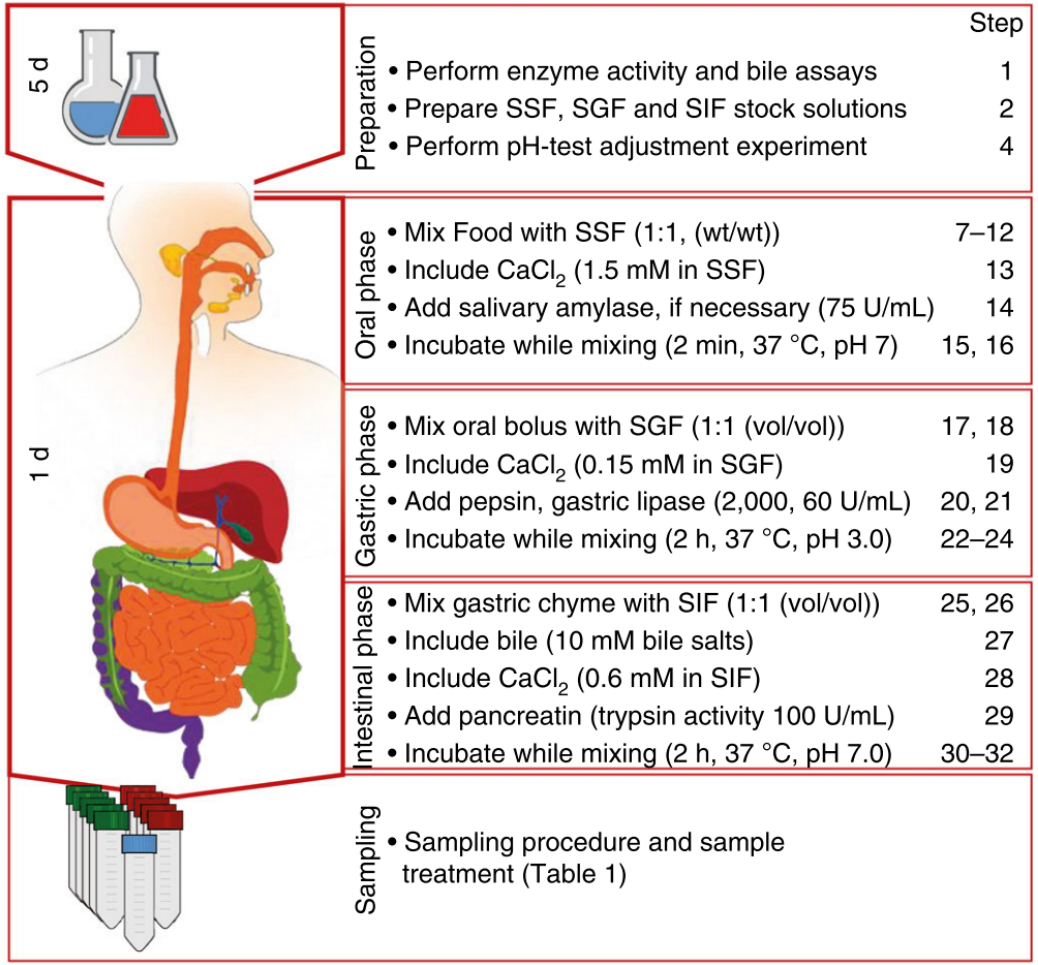
Stomach:

Intestinal Phase:





INFOGEST *in vitro* digestion protocol 2.0



Food & Function

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Cite this: *Food Funct.*, 2014, 5, 1113

A standardised static *in vitro* digestion method suitable for food – an international consensus†

M. Minekus,‡[a] M. Alminger,‡[b] P. Alvito,‡[c] S. Ballance,‡[d] T. Bohn,‡[e] C. Bourlieu,‡[fh] F. Carrière,§[g] R. Boutrou,‡[fh] M. Corredig,‡[i] D. Dupont,§[fh] C. Dufour,‡[j] L. Egger,‡[k] M. Golding,‖[l] S. Karakaya,‡[m] B. Kirkhus,§[n] S. Le Feunteun,‡[o] U. Lesmes,‡[p] A. Macierzanka,‡[q] A. Mackie,‡[r] S. Marze,§[s] D. J. McClements,‖[t] O. Ménard,‡[fh] I. Recio,‡[u] C. N. Santos,‡[vw] R. P. Singh,‖[x] G. E. Vegarud,‡[y] M. S. J. Wickham,‡[z] W. Weitschies‡[aa] and A. Brodkorb‡*[ab]

nature protocols PROTOCOL

https://doi.org/10.1038/s41596-018-0119-1

INFOGEST static in vitro simulation of gastrointestinal food digestion

André Brodkorb[1*], Lotti Egger[2], Marie Alminger[3], Paula Alvito[4], Ricardo Assunção[4], Simon Ballance[5], Torsten Bohn[6], Claire Bourlieu-Lacanal[7], Rachel Boutrou[8], Frédéric Carrière[9], Alfonso Clemente[10], Milena Corredig[11], Didier Dupont[8], Claire Dufour[12], Cathrina Edwards[13], Matt Golding[14], Sibel Karakaya[15], Bente Kirkhus[5], Steven Le Feunteun[8], Uri Lesmes[16], Adam Macierzanka[17], Alan R. Mackie[18], Carla Martins[4], Sébastien Marze[19], David Julian McClements[20], Olivia Ménard[8], Mans Minekus[21], Reto Portmann[2], Cláudia N. Santos[22,23], Isabelle Souchon[24], R. Paul Singh[25], Gerd E. Vegarud[26], Martin S. J. Wickham[27], Werner Weitschies[28] and Isidra Recio[29]

INFOGEST

cost

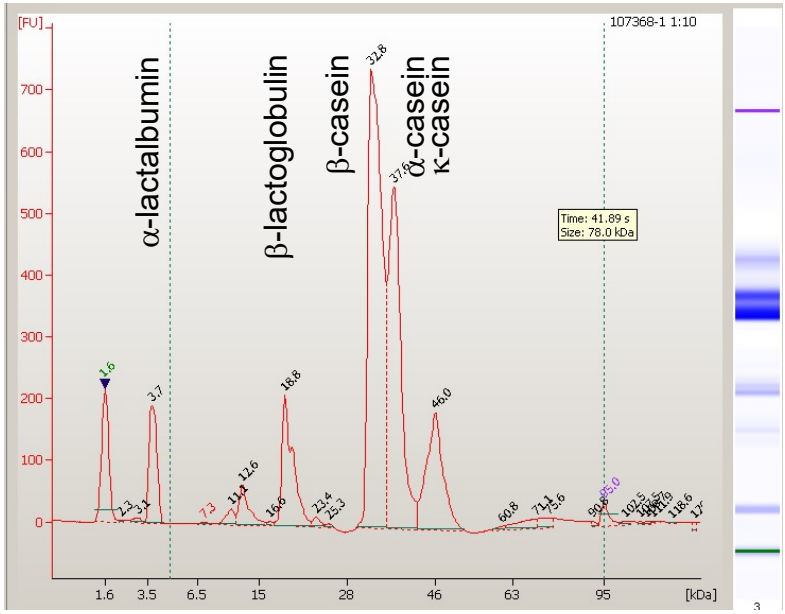
Minekus, M. et al. (2014), A standardised static in vitro digestion method suitable for food – an international consensus, Food Funct.
Brodkorb, Egger, Recio et al. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion, Nature Protocols

Agroscope

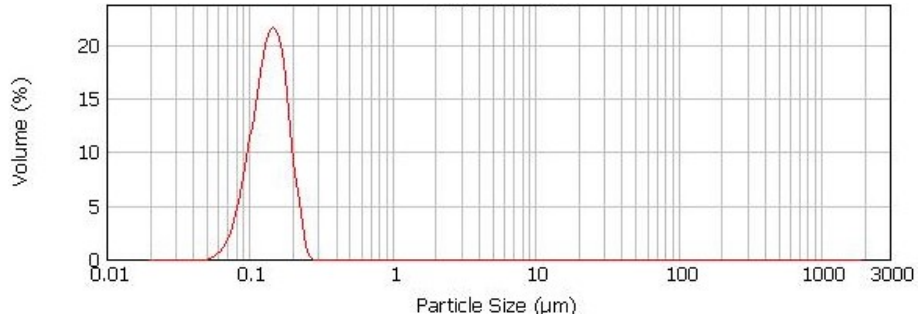
International Comparison using skim milk powder

Per kg Milkpowder (SMP):

- 395 g Protein
  - 3.2% α-lactalbumin
  - 11.3% β-lactoglobulin
  - 28.2% α-casein
  - 45.7% β-casein
  - 10.2% κ-casein
- 8.8 g fat
- 13400 mmol Calcium
- 4980 mmol Lactose
- 9 % denaturation degree



Particle size distribution:



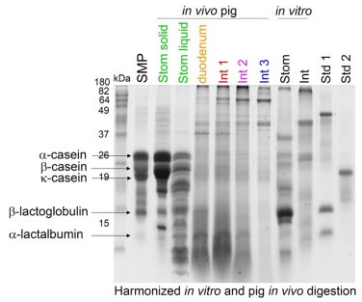
Analytical methods to assess protein hydrolysis

Intact protein

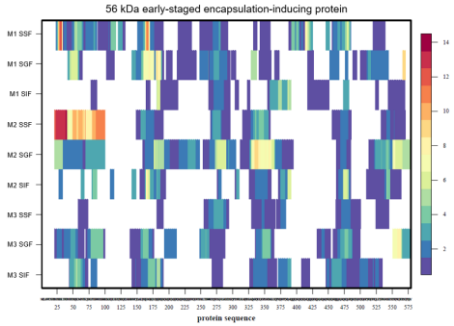
Partially digested protein / peptides

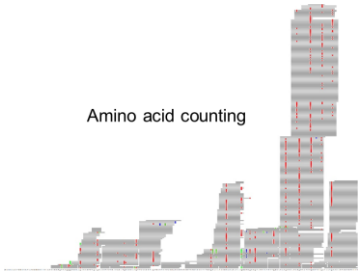
Peptides / free amino acids

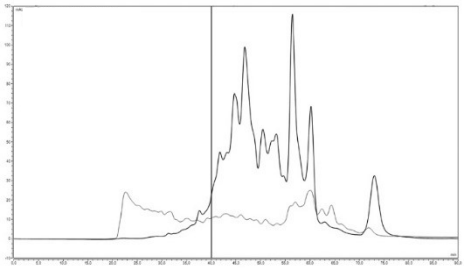
Bioaccessibility

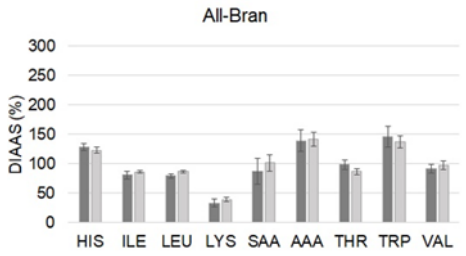


Harmonized *in vitro* and pig *in vivo* digestion





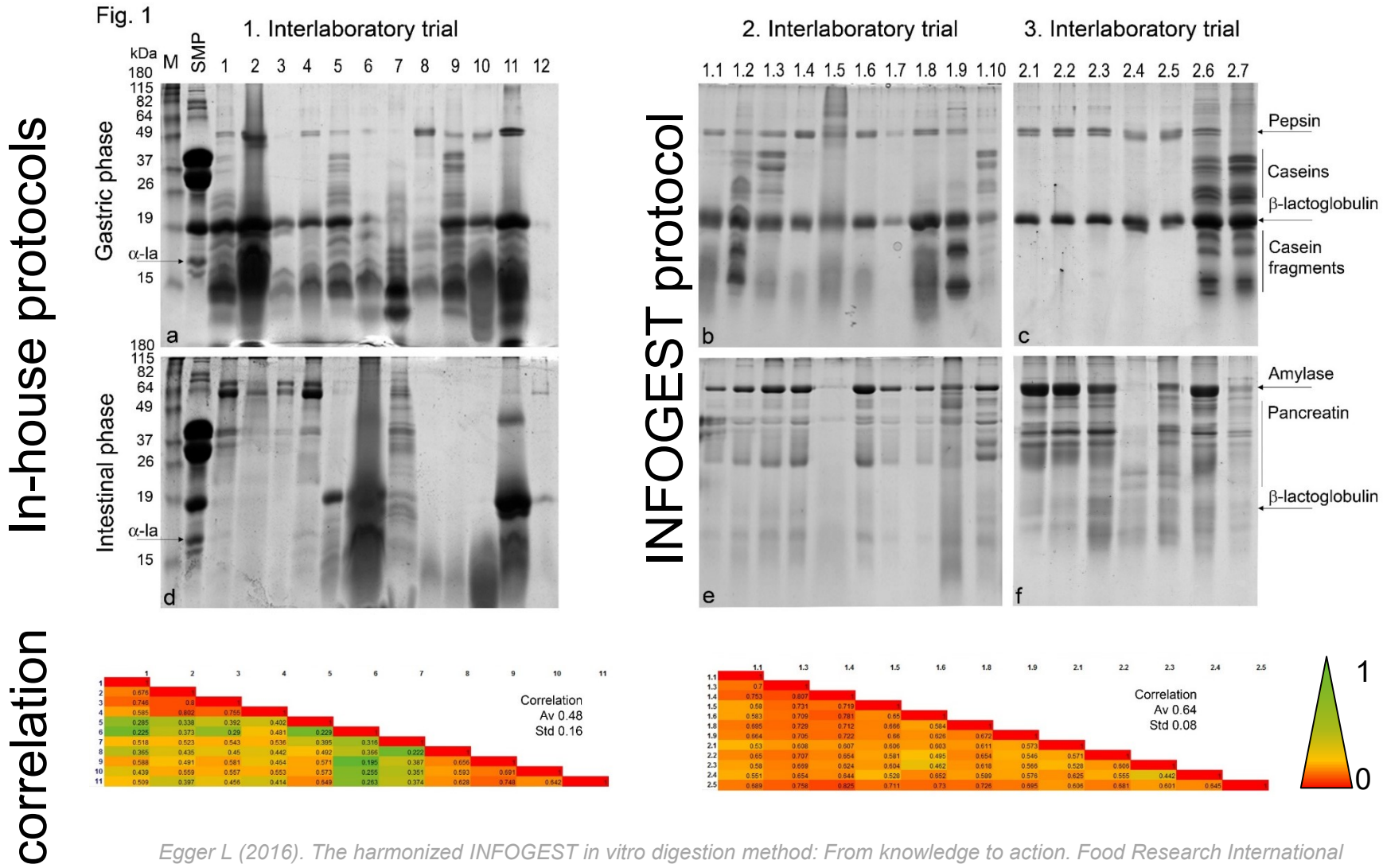




Gel electrophoresis / Mass spectrometry / R-NH_2 / Size exclusion chromatography / HPLC

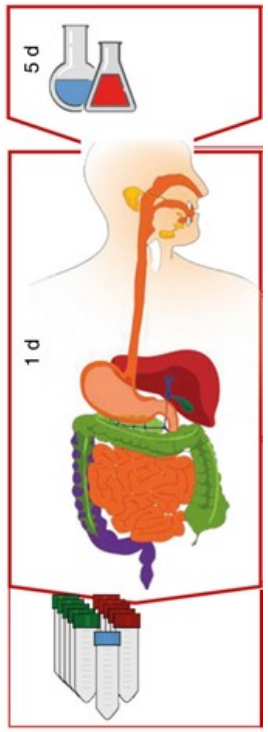
Agroscope

IVD Reproducibility: Inter-laboratory trials



Egger L (2016). The harmonized INFOGEST in vitro digestion method: From knowledge to action. Food Research International

In vitro protocol Validation with *in vivo* data



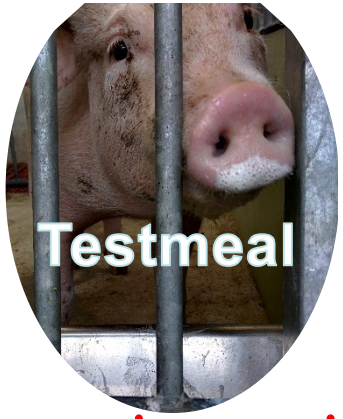
Static *in vitro*
Digestion **(IVD)**
(INFOGEST protocol 2.0)

in vivo
pig system

IVD *in vivo* validation in pigs

Skim milk powder
SMP

Group-housing
during night



-14h

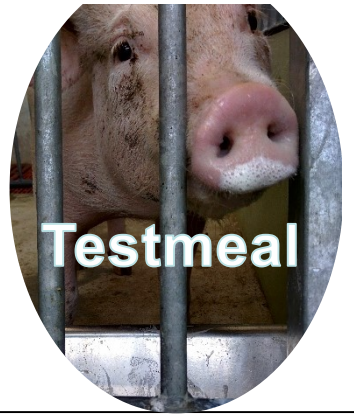
-6h

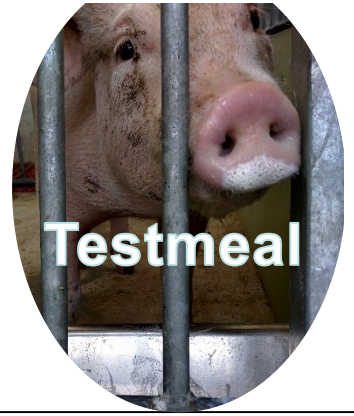
-6.5h

-3h

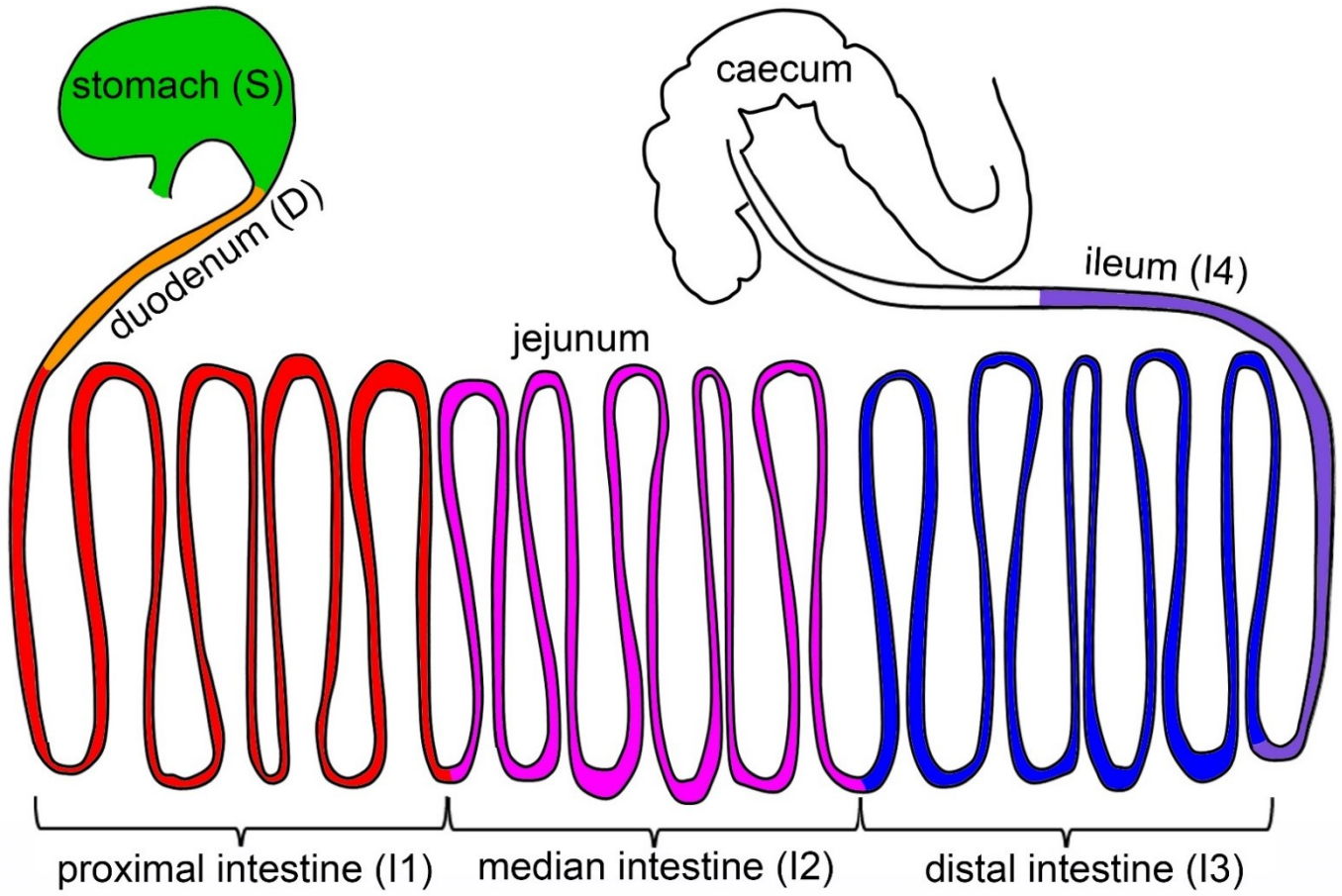
-1.5h

-1h





Sampling along the gastro-intestinal tract of pigs



Egger L (2017). Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. Food Research International

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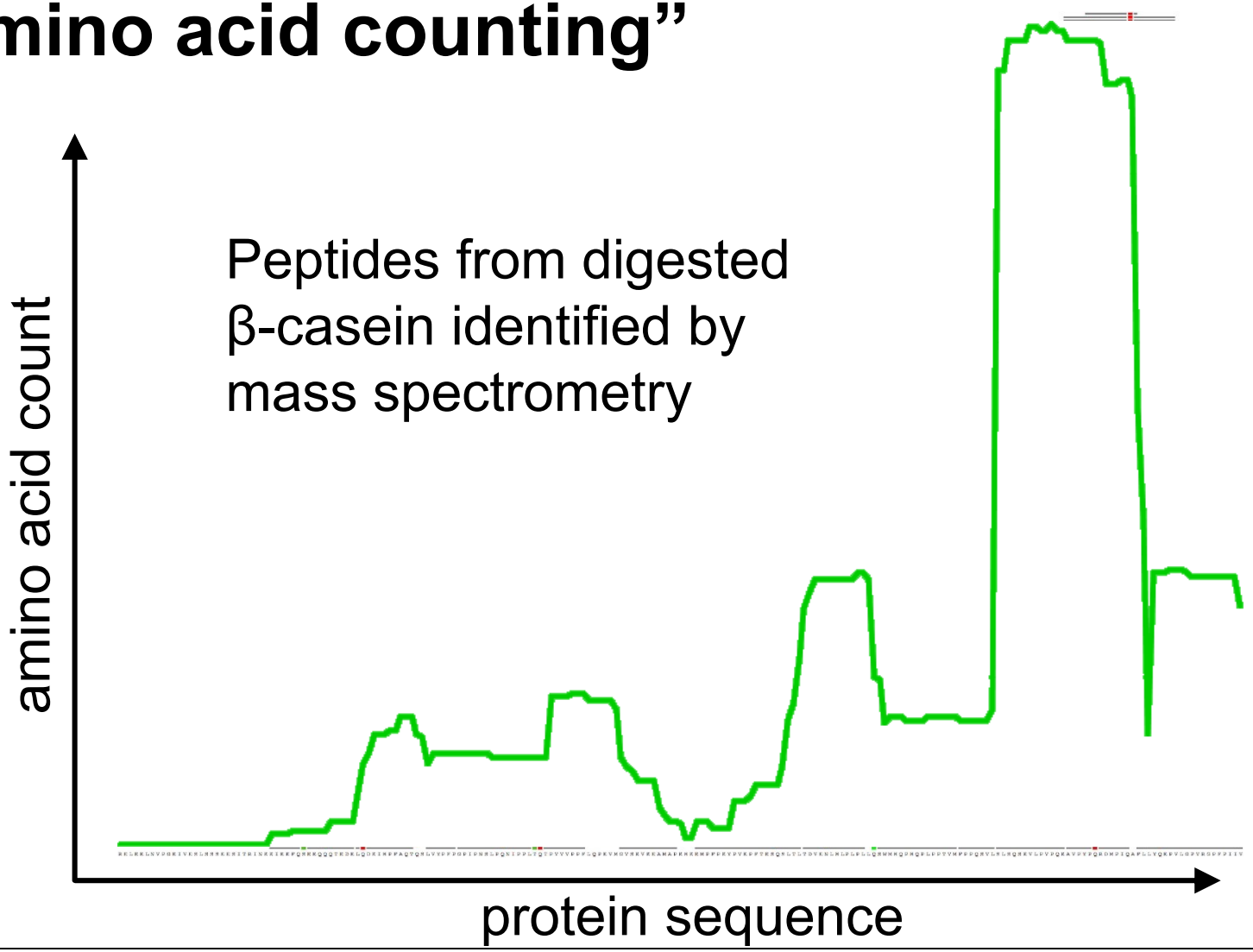
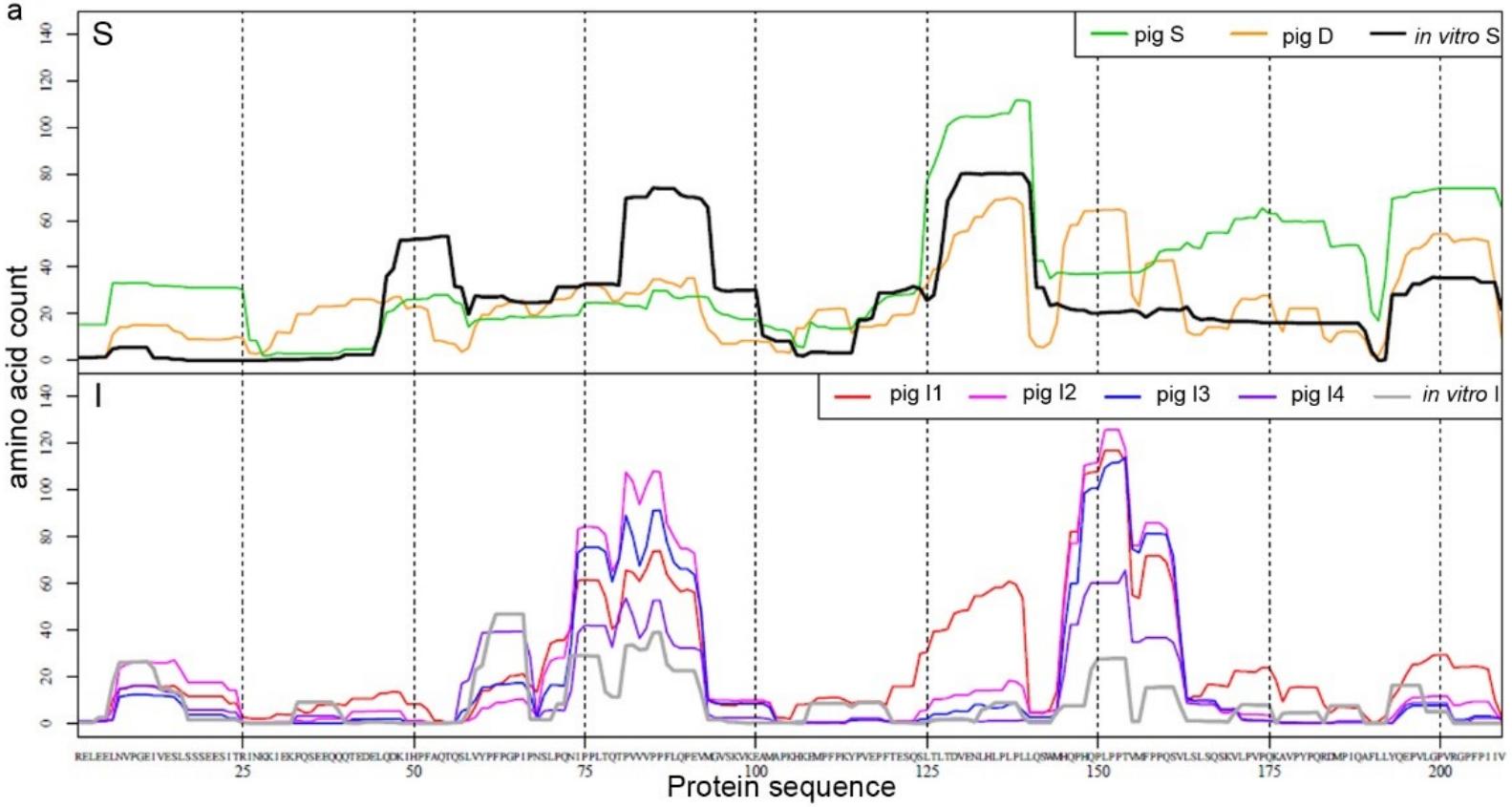
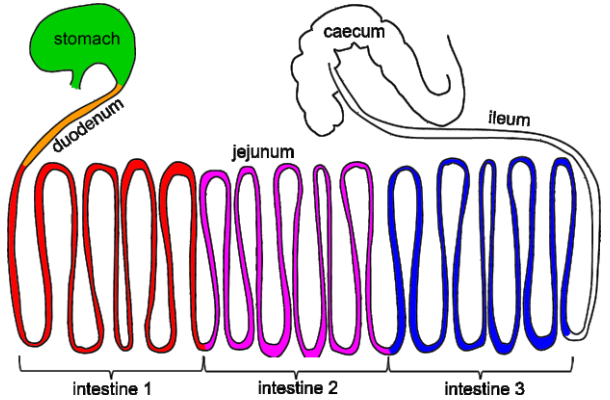
Method peptide quantification: “amino acid counting”



Comparison *in vivo* / *in vitro*: β-Casein peptides

Fig. 5
a

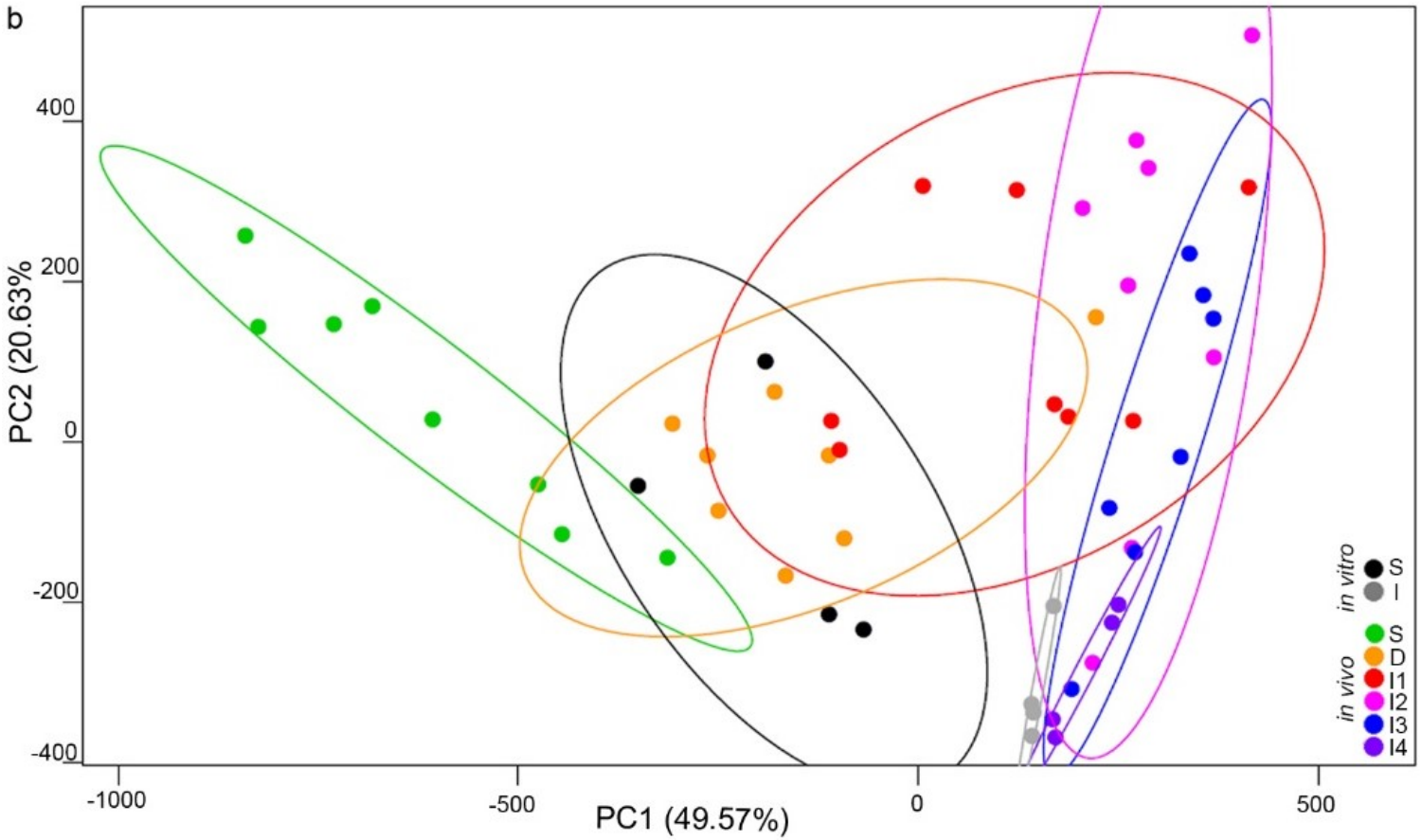


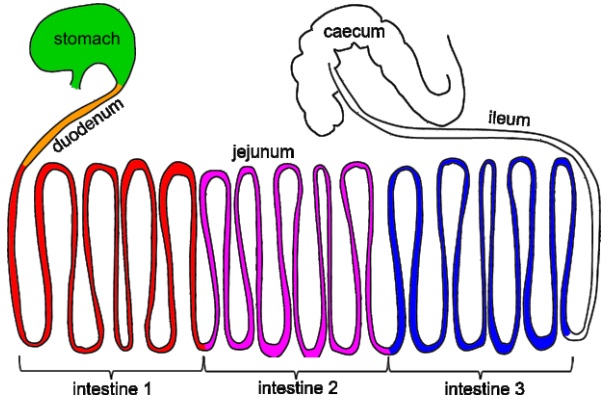


→ Highly similar peptide patterns IVD versus *in vivo*

Agroscope

PC analysis over all peptides: IVD and *in vivo*





→ *in vitro* gastric ≙ *in vivo* gastric-duodenum

→ *in vitro* intestinal ≙ *in vivo* *int.3*- illeum

Validation of *in vitro* results with *in vivo* data

milk protein hydroslysis in pigs

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Physiological comparability of the harmonized INFOGEST *in vitro* digestion method to *in vivo* pig digestion

Lotti Egger[a,*], Patrick Schlegel[b], Christian Baumann[a], Helena Stoffers[a], Dominik Guggisberg[a], Cédric Brügger[a], Desirée Dürr[a], Peter Stoll[b], Guy Vergères[a], Reto Portmann[a]

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ARTICLE INFO

Keywords:
In vitro - in vivo digestion
Dairy proteins
Peptides
Mass spectrometry
Harmonized IVD protocol

ABSTRACT

Recently, a static *in vitro* digestion (IVD) protocol was published by Minekus and coworkers (Minekus et al., 2014) within the COST INFOGEST network. The protocol, concentrating on physiological enzyme activities had the main goal to improve the comparability of experimental data between labs. The protocol was validated in several inter-laboratory studies using skim milk powder (SMP) and indeed demonstrated improved harmonization compared with previous experiments with individual IVD protocols (Egger et al., 2016). Although the enzyme activities and salt concentrations of the harmonized protocol are based on available human *in vivo* data, confirmation of the protocol's physiological relevance has been lacking until now. The main goal of the study was therefore to compare the harmonized IVD protocol with data from *in vivo* digestion. Towards this aim, an *in vivo* pig experiment with the same SMP as used for the validation of the IVD protocol was performed followed by a comparison of protein hydrolysis between *in vivo* and *in vitro* results. Protein hydrolysis at different levels was analyzed with gel electrophoresis, mass spectrometry, high performance liquid chromatography, and spectrophotometric o-phthaldialdehyde determination of free amino acids. Principle component analysis was used for graphical data comparison.

Milk proteins detected after gastric IVD corresponded to gastric and duodenal *in vivo* samples and intestinal IVD samples corresponded to distal jejunal *in vivo* samples. Peptides identified after the gastric phase of IVD, correlated with *in vivo* gastric samples ($r = 0.8$) and intestinal IVD peptides correlated best with in vivo samples collected from the median jejunum ($r = 0.57$). Free amino acids were in both systems mainly released during the intestinal phase of digestion. Protein hydrolysis in the harmonized IVD was similar to *in vivo* protein hydrolysis in pigs at the gastric and intestinal endpoints. Therefore, the harmonized static *in vitro* protocol is suited to study protein hydrolysis at these endpoints.

collected from the median jejunum ($r = 0.57$). Free amino acids were in both systems mainly released during the intestinal phase of digestion. Protein hydrolysis in the harmonized IVD was similar to *in vivo* protein hydrolysis in pigs at the gastric and intestinal endpoints. Therefore, the harmonized static *in vitro* protocol is suited to study protein hydrolysis at these endpoints.

milk protein hydroslysis in human jejunal effluents

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Protein degradation and peptide release from milk proteins in human jejunum. Comparison with *in vitro* gastrointestinal simulation

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ABSTRACT

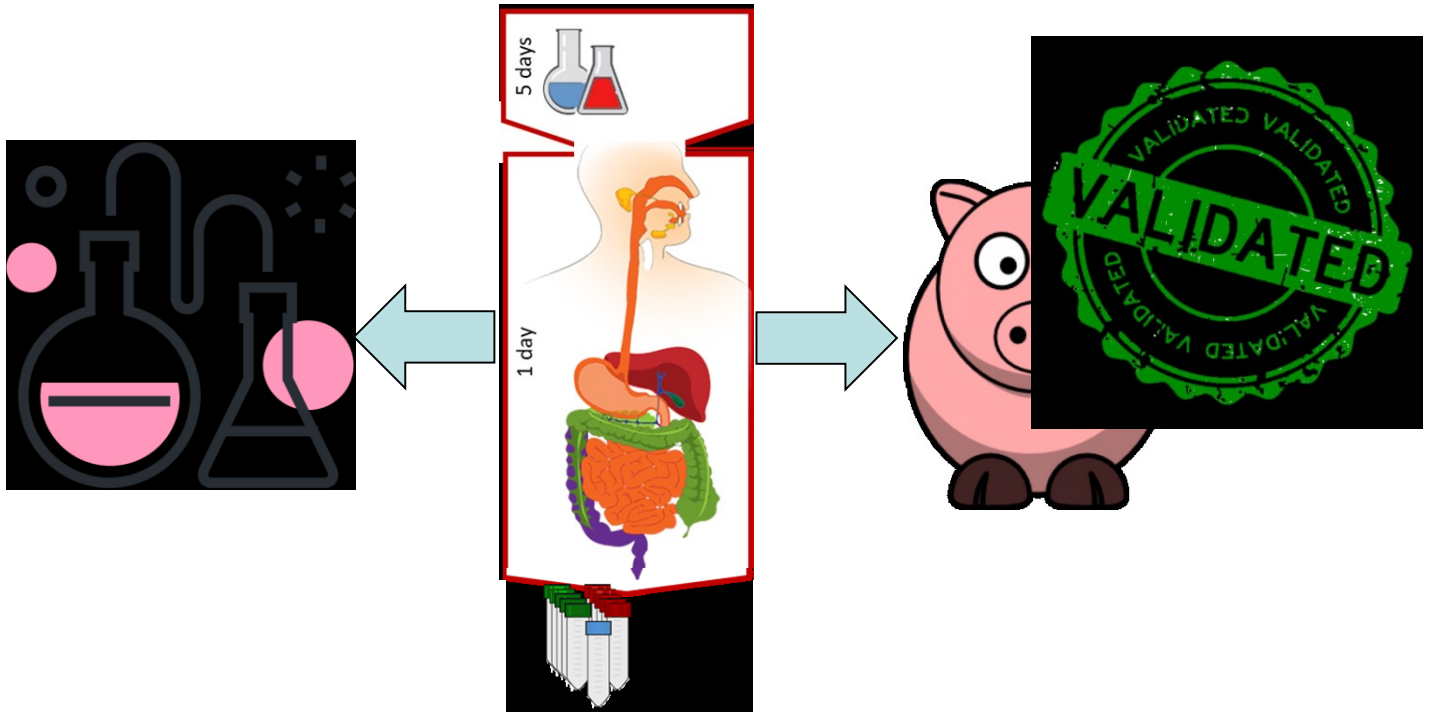
Human jejunal digests after oral ingestion of casein and whey protein were collected by a nasogastric tube and protein degradation and peptide release was compared with that found in the digests of the same substrates using a standardised protocol. No intact casein was detected in the jejunal nor in the *in vitro* samples taken during the intestinal phase, while β-lactoglobulin was found in one hour-jejunal samples in agreement with the *in vitro* digestion. *In vivo* and *in vitro* digests showed comparable peptide profiles and high number of common sequences. A selective precipitation step was used to strengthen the identification of phosphorylated peptides. Most of the sequences found in jejunum, some of them not previously described, were also identified in the simulated digests. Common resistant regions to digestion were identified, revealing that the *in vitro* protocol constitutes a good approximation to the physiological gastrointestinal digestion of milk proteins.

previously described, were also identified in the simulated digests. Common resistant regions to digestion were identified, revealing that the *in vitro* protocol constitutes a good approximation to the physiological gastrointestinal digestion of milk proteins.

→ *in vitro* protein hydrolysis is a good approximation to the *in vivo* situation

Agroscope

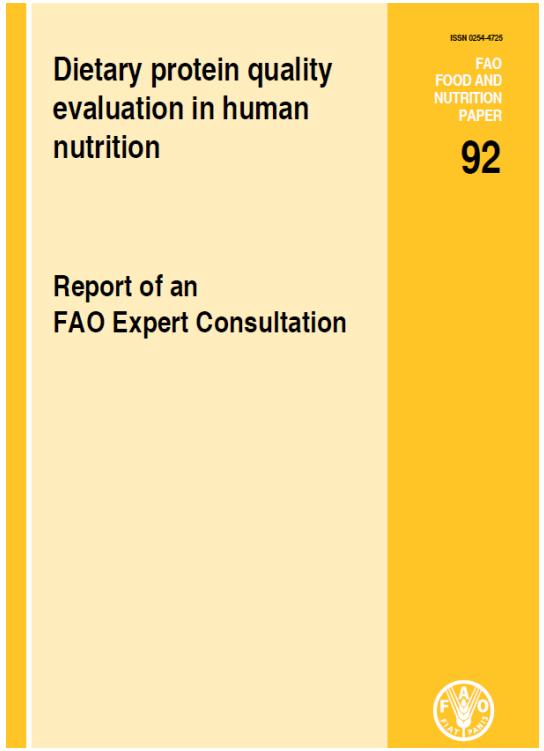
Validation of *in vitro* results with *in vivo* data



Interlaboratory study | Static *in vitro* digestion (INFOGEST protocol) | Pig *in vivo* trial

→ *in vitro* protein hydrolysis is a good approximation to the *in vivo* situation

Dietary protein quality evaluation by FAO



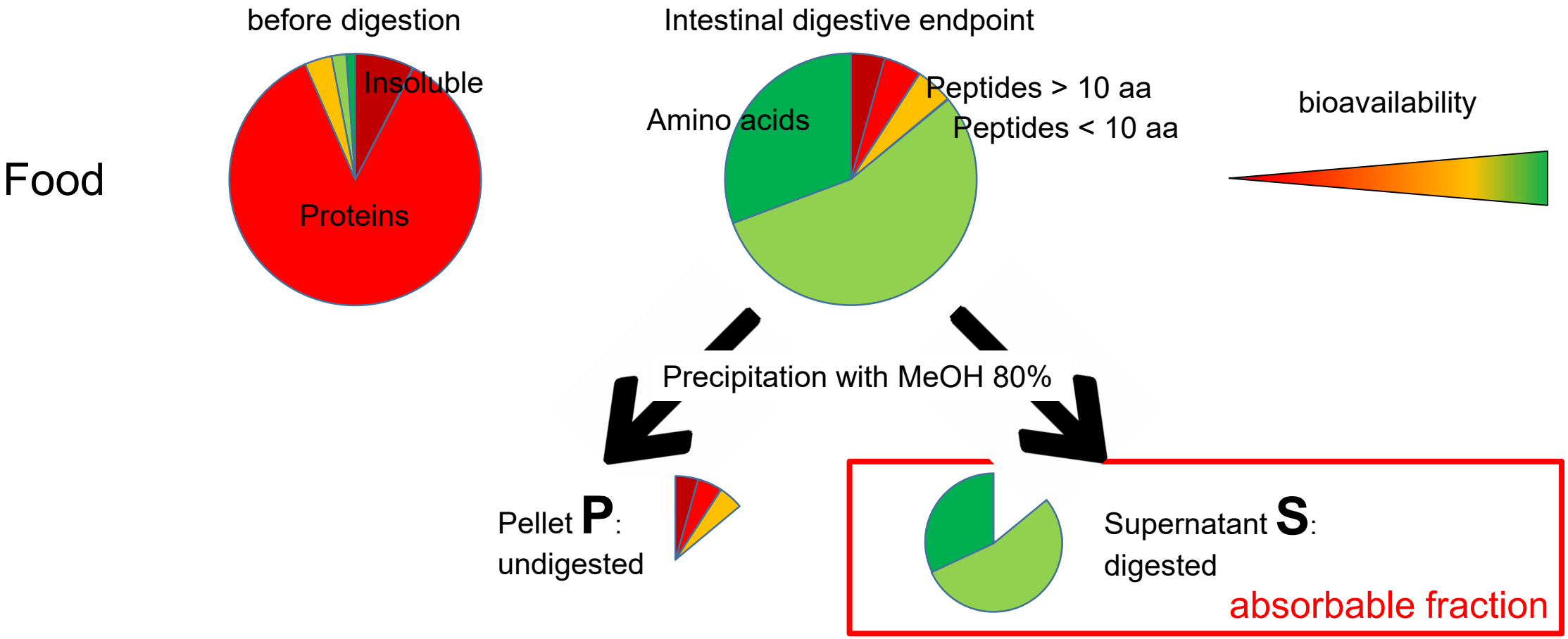
Ileal digestibility

1. Further determine true ileal digestibility of protein and amino acids in a wider range of foods and determine the ileal digestible tryptophan content of human milk.
2. Develop non-invasive accurate methods to determine or predict true ileal dietary protein and amino acid digestibility in humans based on identified biomarkers.
3. Validate the use of animal model data (including providing more robust inter-species prediction equations for true ileal amino acid digestibility) to quantify ileal digestibility in humans, including relating digestibility to functional outcomes.
4. Determine more fully the role of the small intestinal and colonic microflora on ileal amino acid digestibility values.
5. Develop new bioavailability assays such as the reactive lysine assay, for other amino acids.
6. Develop and validate *in vitro* methods for predicting amino acid digestibility and bioavailability in humans.

DIAAS % = 100 x *lowest value* [*"Digestible IAA reference ratio"* for a given amino acid scoring pattern].

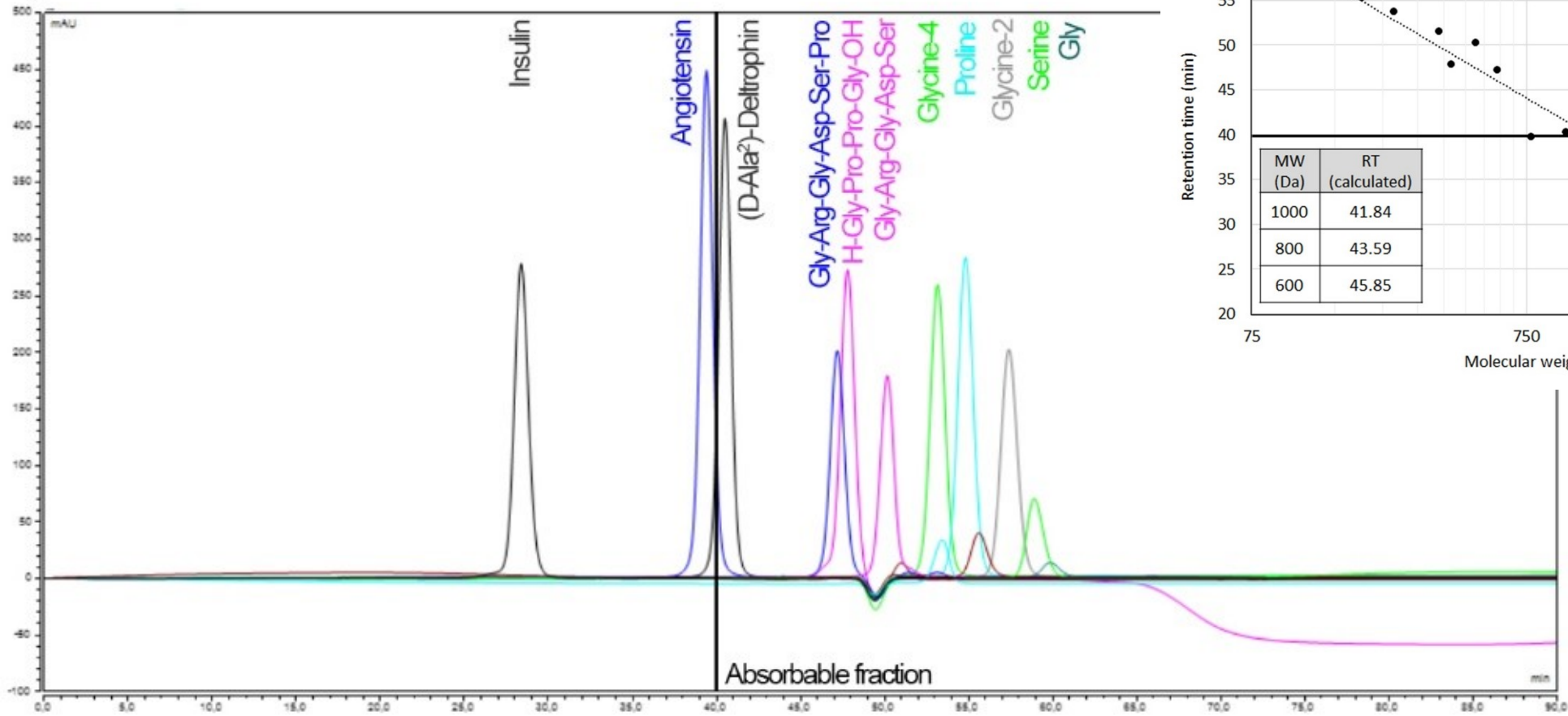
Note that the main difference between DIAAS and PDCAAS is that true ileal amino acid digestibility for the dietary indispensable amino acids is used rather than a single faecal crude protein digestibility value.

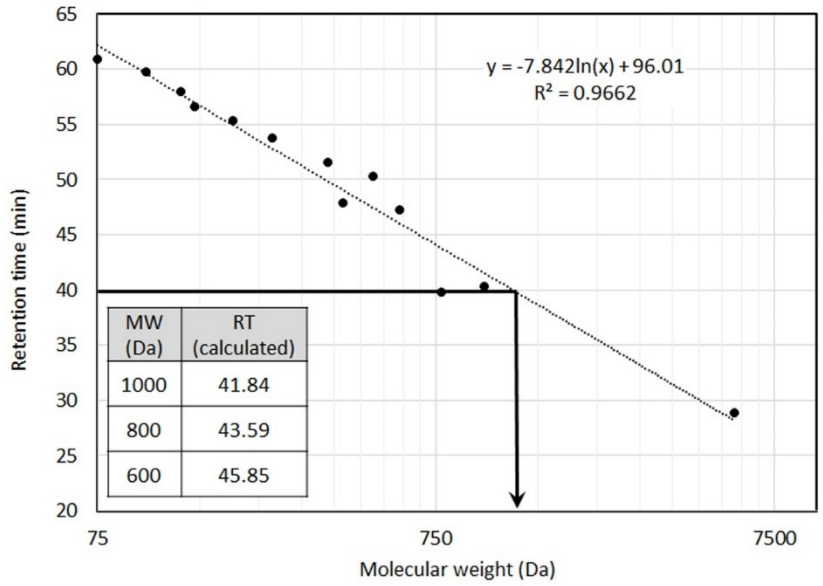
Determination of *in vitro* digestibility



Size exclusion chromatography (SEC)

Fig. 2a





MW (Da)	RT (calculated)
1000	41.84
800	43.59
600	45.85

→ SEC shows size distribution in samples

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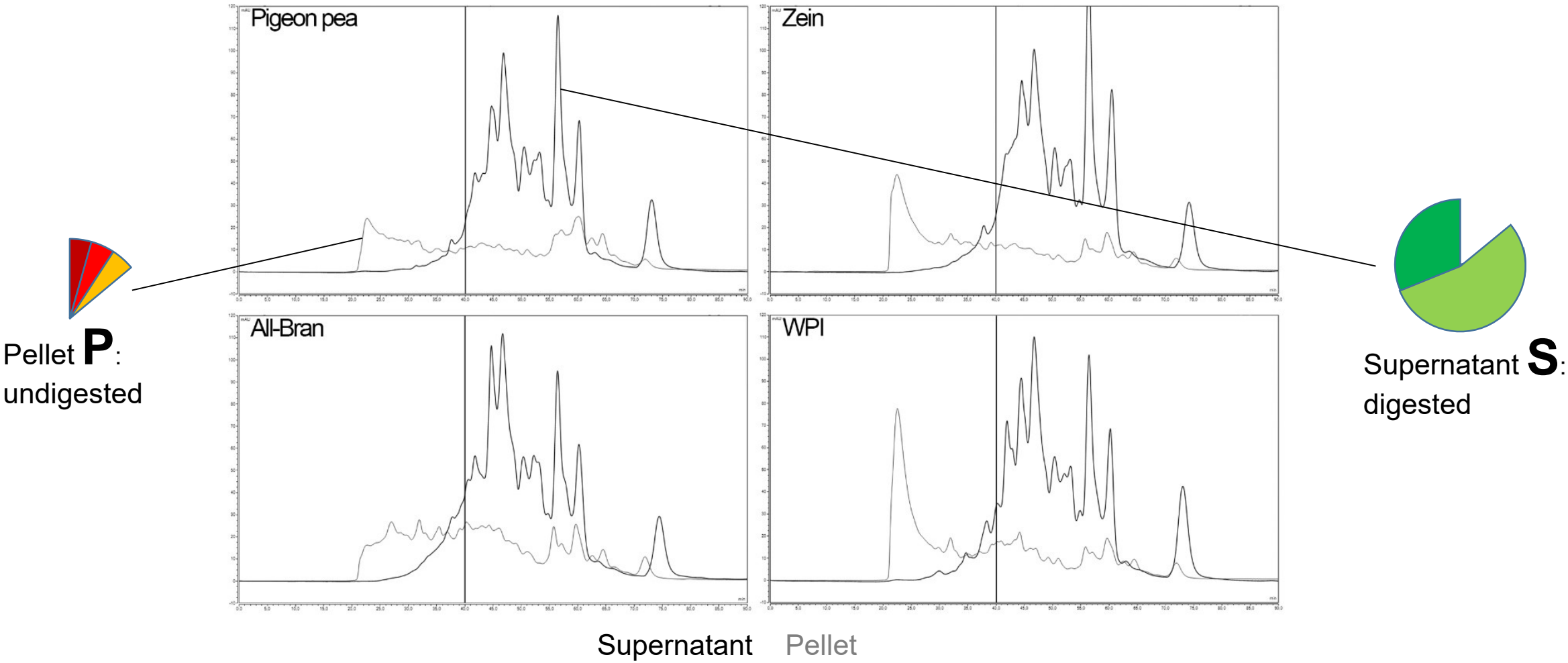
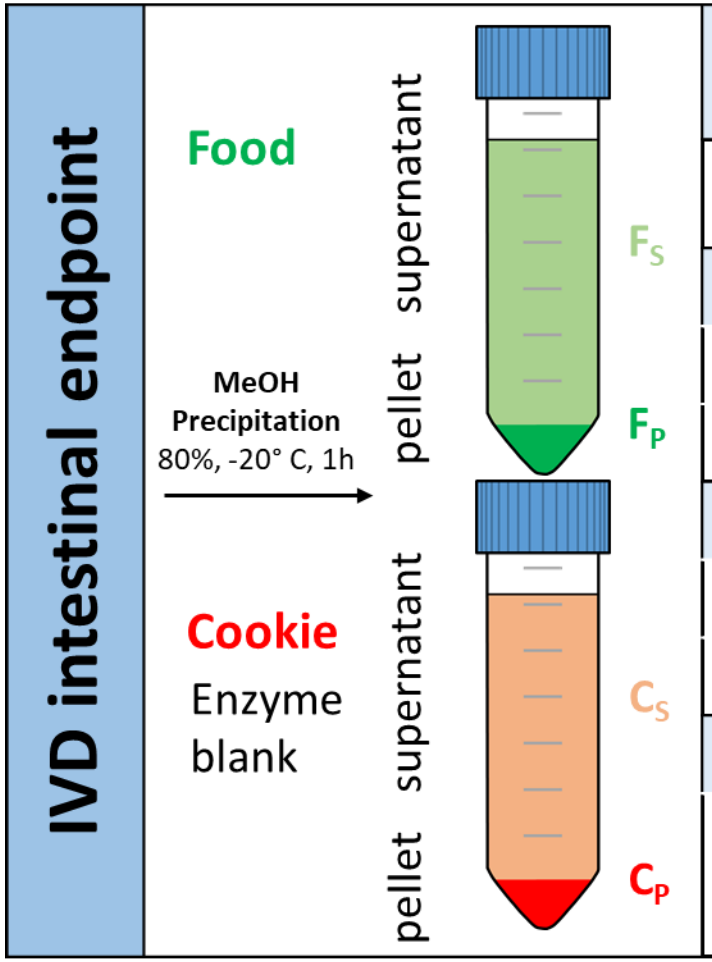
Precipitation with 80 % MeOH



→ precipitation separates bioavailable from non-available components

Lotti Egger, Raquel Sousa, Reto Portmann

The different analytical endpoints



Total digestibility			*In vitro* DIAAS
TN	$R\text{-}NH_2$	TAA	
Supernatant			
	Hydrolysis 6 N HCl, 110°C, 15 h		
Kjeldahl	OPA	HPLC	
Pellet			
	Hydrolysis 6 N HCl, 110°C, 15 h		
Kjeldahl	OPA	HPLC	
Calculation			

$$Digestibility[\%] = \frac{F_S - C_S}{(F_S - C_S) + max(0; F_P - C_P)} \times 100$$

Digestible indispensable amino acid score (DIAAS)

mg amino acid per g food protein

mg indispensable amino acid per g food protein

$\times$ $Digestibility_{Lys}$

mg digested indispensable amino acid per g food protein ($DIAA_{measured}$)

mg amino acid per g reference protein ($DIAA_{reference}$)

Recommended amino acid scoring patterns for infants, children and older children, adolescents and adults

Age Group	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
	scoring pattern mg/g protein requirement								
Infant (birth to 6 months)[1]	21	55	96	69	33	94	44	17	55
Child (6 months to 3 year)[2]	20	32	66	57	27	52	31	8.5	43
Older child, adolescent, adult[3]	16	30	61	48	23	41	25	6.6	40

[1] Infant is based on the gross amino acid content of human milk from Table 4.
[2] Child group is from the 6 month (0.5 y) values from Table 3.
[3] Older child, adolescent, adult group is from the 3-10 y values from Table 3.

FAO: Dietary protein quality evaluation in human nutrition (ISBN 978-92-5-107417-6)

DIAAS: Digestible indispenable amino acid score

$$DIAAS = \frac{DIAA_{measured}}{DIAA_{reference}} \times 100$$

*lowest DIAAS is reported as limiting amino acid

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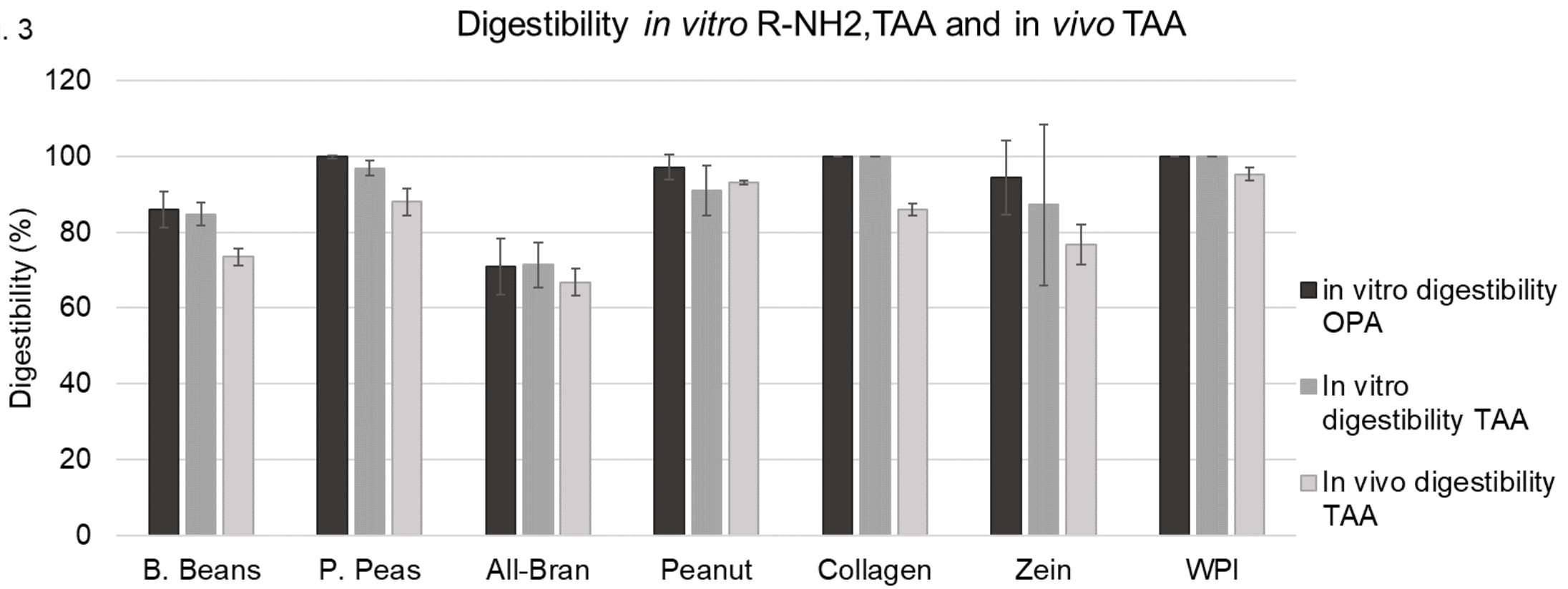
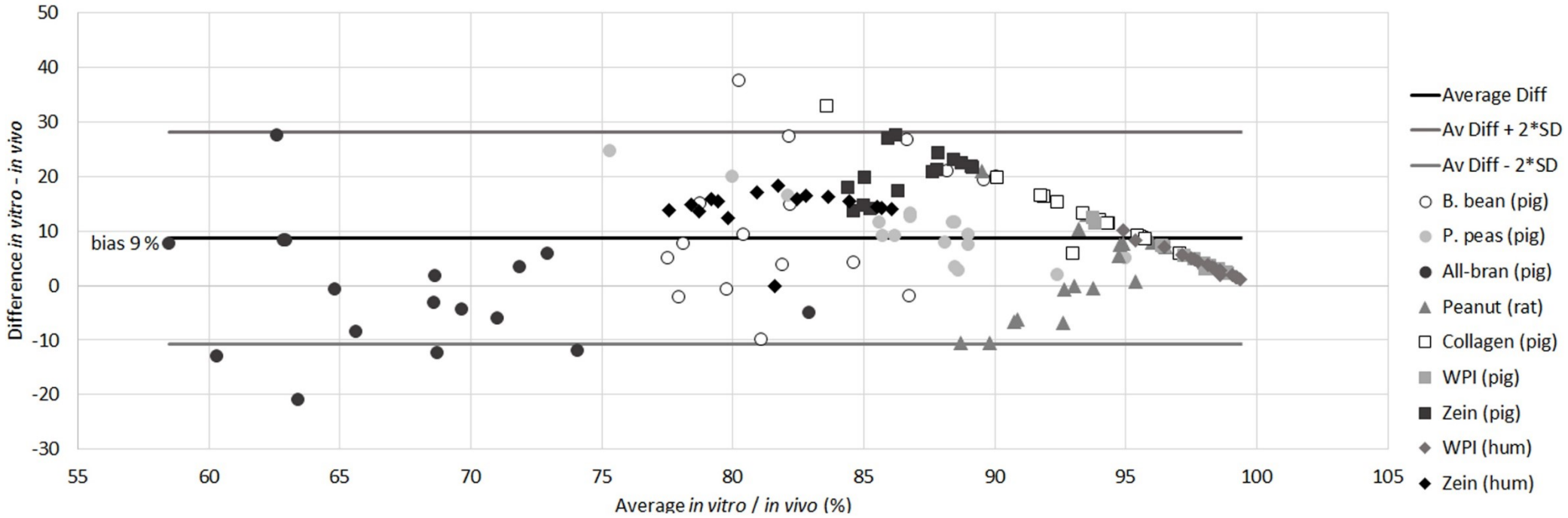
Proteos *in vitro* versus *in vivo* digestibility

Fig. 3



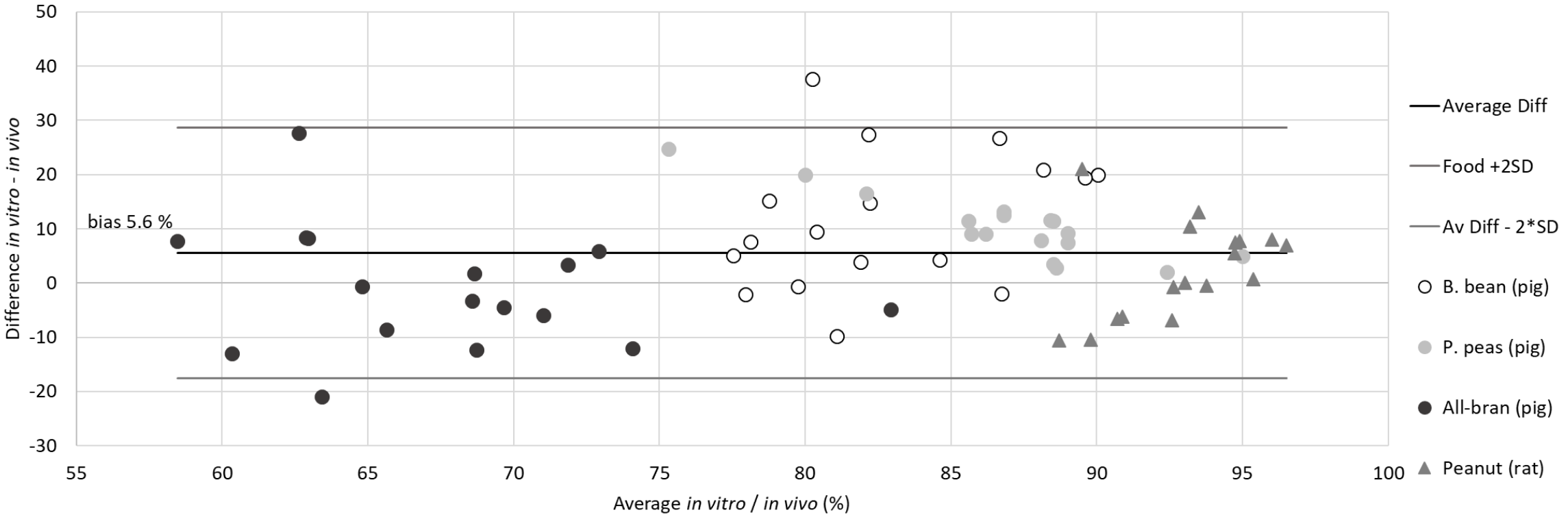
→ *in vitro* digestibility gives a good estimate for *in vivo* digestibility

Proteos *in vitro* versus *in vivo* digestibility all substrates



→ *in vitro* digestibility compared to *in vivo* digestibility represented with Bland-Altman plot

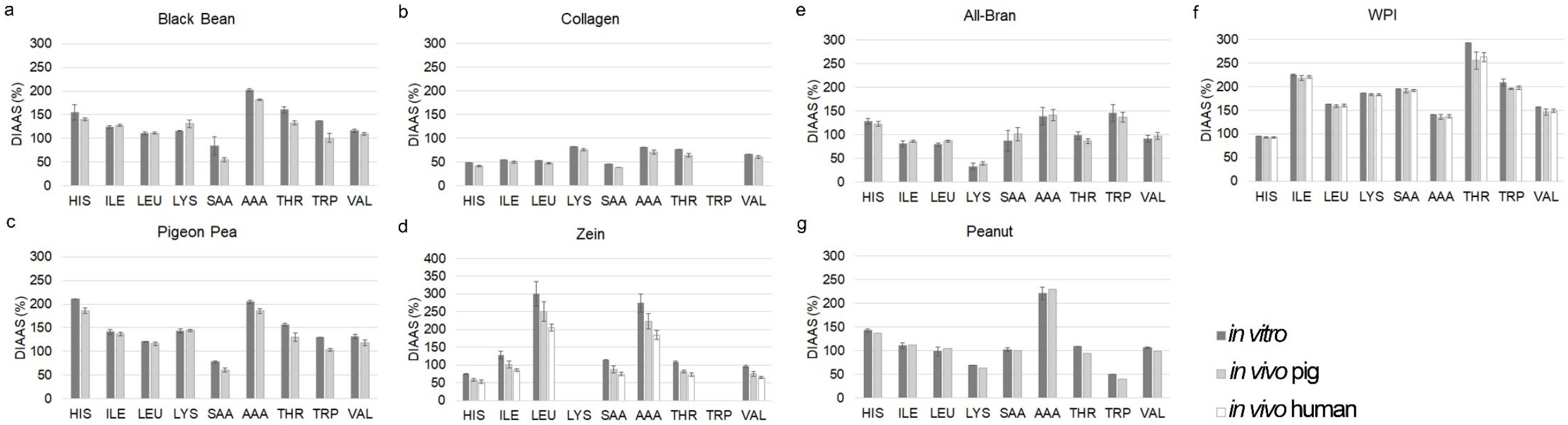
Proteos *in vitro* versus *in vivo* digestibility foods



→ *in vitro/in vivo* digestibility of foods: bias of 5.6 %

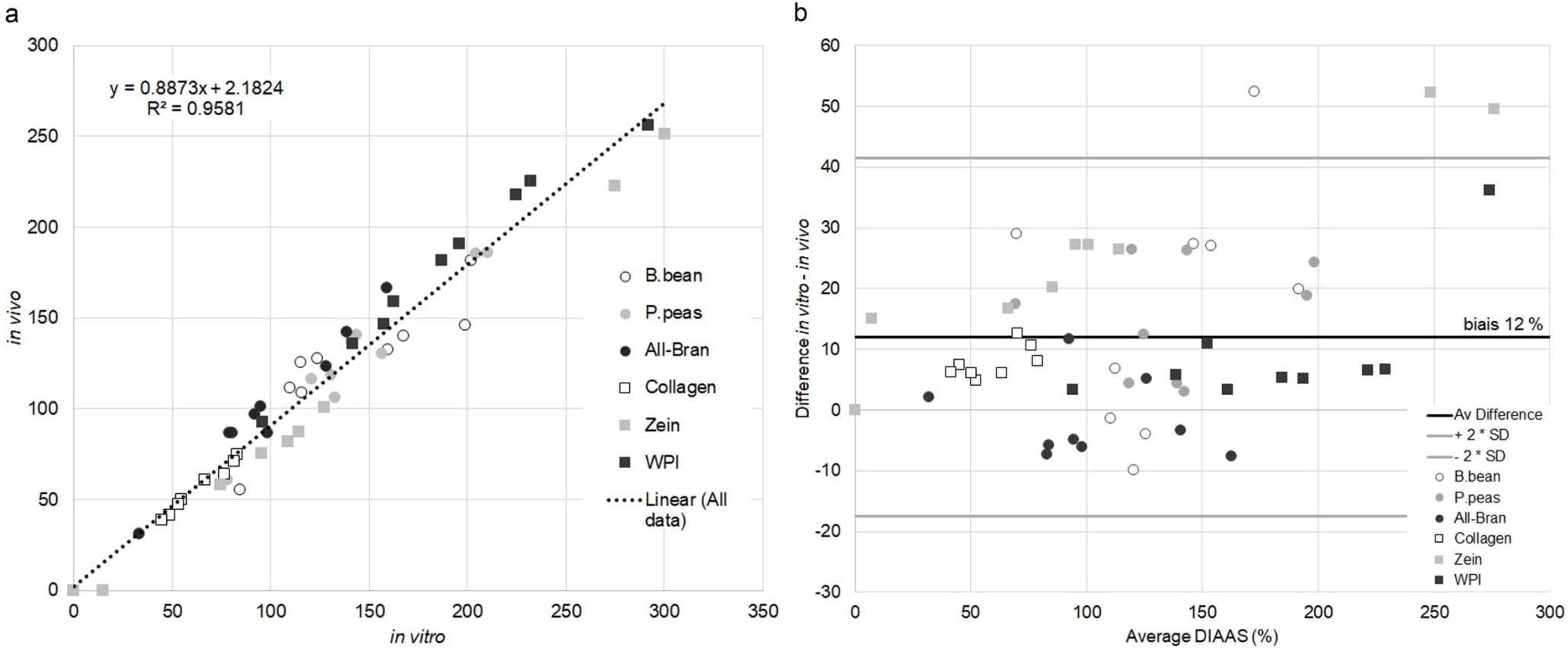
Agroscope

Proteos *in vitro* versus *in vivo* DIAAS



Agroscope

Proteos *in vitro* versus *in vivo* DIAAS



→ *in vitro* DIAAS gives a good estimate for *in vivo* DIAAS














Thank you for your attention

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