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Improved estimation of *in vitro* protein digestibility of different foods using size exclusion chromatography

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ABSTRACT

While the harmonized INFOGEST model provides a physiologically relevant platform for simulated digestion, it needs to be combined with adequate analytical methods to enable quantification and comparison of protein digestibility in different food matrices. We have shown that size exclusion chromatography (*SEC*) can be used to estimate the proportion of small peptides potentially available for uptake. Combined with determination of total dissolved protein, the % of small peptides per total protein was calculated as a physiologically relevant estimate of protein digestibility (D_{SEC}). Values for D_{SEC} differed for casein (87.6%), chicken mince (72.6%), heated pea protein concentrate (67.8%), bread (63%), beef entrecote (57.7%) and pea protein concentrate (57.8%). In contrast to existing methods (TCA soluble protein, free NH_2-groups), the proposed *SEC* based method gives separate insight into the two fundamental processes during protein digestion (solubilization and break-down), while maintaining the ability to rank digestibility of very different food proteins.

1. Introduction

Dietary protein quality comprises two aspects, i.e., amino acid composition and availability. Availability is defined as "the proportion of the dietary amino acids that are digested and absorbed in a form suitable for body protein synthesis" (Rutherfurd & Moughan, 2012). Therefore, digestibility constitutes one of the key parameters defining quality of a given dietary protein. Protein digestion starts in the stomach through the action of pepsin at low pH and is continued in the small intestine by the combined action of different proteases secreted with pancreatic juice (i.e. trypsin, chymotrypsin and carboxypeptidases). Membrane bound peptidases in the brush border of intestinal epithelial cells further degrade the generated oligopeptide mixture into tri-, di-peptides and amino acids, which are taken up into the epithelial cells by active transport (Freeman, Kim & Sleisenger, 1979).

In vivo methods for protein digestibility often combine digestibility evaluation with assessment of amino acid composition and its suitability for human metabolic needs. Protein efficiency ratio (PER), protein digestibility corrected amino acid score (PDCAAS) and digestible indispensable amino acid score (DIAAS) are among the most common *in vivo* methods. While PER is based on a rat growth assay, PDCAAS and DIAAS use amino acid scoring patterns in combination with protein digestibility based on true fecal nitrogen digestibility (PDCAAS) or true ileal digestibility of individual amino acids (DIAAS). The latter is recommended by FAO and regarded as the gold standard but needs to be determined in pigs or humans using invasive procedures. Recently, a dual-tracer method enabling non-invasive protein digestibility measurements at the ileal level has been developed but has its limitations as it requires proteins labeled with non-radioactive stable isotopes (^2H, ^{13}C) (Devi, Varkey, Sheshshayee, Preston, & Kurpad, 2018). Since *in vivo* methods are expensive, time-consuming and entail ethical problems, they are not suited for multiple samples required to understand ingredient interactions or the effect of processing, which are therefore often assessed using *in vitro* methods.

Different *in vitro* digestion procedures have been harmonized in the international static INFOGEST model (Brodkorb et al., 2019; Minekus et al., 2014), which considerably improved comparability of results between different laboratories (Egger et al., 2016) and gave good estimates of *in vivo* milk protein digestion in humans (Sanchon et al., 2018) and pigs (Egger et al., 2017). But while the harmonized INFOGEST model provides an excellent platform to perform digestion in a relatively higher throughput than the *in vivo* alternatives, it needs to be combined with adequate analytical methods to enable quantification and comparison of protein digestibility in different food matrices.

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Degradation of specific proteins can be monitored by gel electrophoresis, but peptides generated during digestion are usually too small to be separated. Released peptides can be identified using mass spectrometry (MS), which is especially interesting in the search for potentially allergenic or bioactive peptides (Egger et al., 2016; Ribeiro et al., 2017). MALDI-TOF-MS has also been applied to quantify the total number of peptides after simulated digestion (Luo, Taylor, Nebl, Ng, & Bennett, 2018). While giving valuable insight into protein degradation of specific foods with a limited number of different proteins, none of the above-mentioned methods is suitable for comparing complex foods with a multitude of different proteins. For this purpose, many studies have used simpler techniques for quantitative assessment of protein digestibility.

During protein hydrolysis peptide bonds are hydrolyzed and the increasingly smaller protein fragments become more and more soluble. The appearance of free amino acids has been used as a measure of protein digestibility, and this approach can be useful for comparing different samples. However, as free amino acids are only one part of the oligopeptide mixture generated in the intestinal lumen, this method is not suitable for quantification and expectedly underestimates true digestibility. In other studies, the increase in soluble protein released from a solid food matrix during digestion has been used to estimate digestibility (Nordlund, Katina, Aura, & Poutanen, 2013). Precipitation agents such as TCA or sulfosalicylic acid can be used to remove intact but soluble proteins and large peptides from solution. However, TCA precipitation is not only based on size and has been shown to correlate best with hydrophobicity of peptides (Yvon, Chabanet, & Pelissier, 1989). In all these cases, digestibility is defined as the proportion of soluble protein (measured as nitrogen or sum of amino acids) compared to total protein, with no or very little consideration to the significant amount oligopeptides in the digests. The generation of free amino groups through cleavage of peptide bonds is also used to estimate protein digestibility by employing either TNBS (trinitrobenzensulfonic acid) or OPA (o-phthaldialdehyde) as reactive reagents or utilizing the pH drop which accompanies peptide bond cleavage (Hsu, Vavak, Satterlee, & Miller, 1977). Digestibility is thus given either as the number of free NH_2 groups per g protein or as the degree of hydrolysis, which is the number of hydrolyzed peptide bonds divided by the total number of peptide bonds in the sample (h_{tot}). All these quantitative methods are relatively simple to perform and can be used to compare complex foods, but the measured digestibility (in %) does not necessarily reflect the protein portion available for absorption.

One key factor that determines whether a given protein fragment released during digestion could be absorbed or not is its molecular weight or amino acid chain length (Roberts, Burney, Black, & Zaloga, 1999). Hence, there is a need for a method that can account for molecular weight distribution of the protein digest in estimation of food protein digestibility. Size exclusion chromatography (*SEC*) with UV-detection is a well-recognized analytical tool for measuring molecular weight distributions of protein digests (Wubshet et al., 2017) and has been applied to estimate the proportion of peptides with specific size ranges generated during simulated digestion by partial area integration (Le Roux et al., 2020). In addition to that, *SEC* can provide an overall qualitative fingerprint of a given digest in the form of chromatograms. In this study we have further explored the use of *SEC* for characterization and quantification of peptides released during *in vitro* digestion combined with determination of soluble nitrogen. Different animal and plant-based foods ranging from simple (casein) to complex (bread) were digested using the INFOGEST static digestion model. Results were compared with TCA precipitation and the TNBS approach with the aim to evaluate and select a simple, quantitative method which enables the direct comparison of protein digestibility in different food products.

2. Material and methods

2.1. Test foods

Beef entrecote (beef) was cooked in a sealed vacuum bag in a water bath at 70 °C for 30 min, while chicken mince was purchased from a local supermarket and digested raw. White wheat flour bread was prepared as previously described (Rieder, Knutsen, Fernandez, & Ballance, 2019), stored frozen and defrosted overnight at RT. Non-heat-treated pea protein concentrate was provided by AM Nutrition (AM Nutrition, Stavanger, Norway) and used 'as is' or in the form of porridge prepared using a rapid visco analyser as previously described (Mackie et al., 2017), frozen, freeze-dried and ground. The two samples are subsequently designated pea concentrate and heated pea concentrate, respectively. Casein powder was obtained from VWR International (Poole, England). Moisture content was determined in all samples by weight loss during freeze-drying and subsequent oven drying (105 °C, over-night) or only oven-drying (bread crumbs), while protein content was estimated by combustion as described in Section 2.5 The test foods were chosen to resemble a range of different protein contents from 8.4 (bread) to 93.4 (casein) g/100 g and differed in moisture content, which was highest in chicken mince (72.3%) and lowest for pea concentrate, heated pea concentrate and casein (6.1 to 8.4%). Bread and beef samples were ground in a food processor to mimic chewing before simulated gastric and small intestinal digestion.

2.2. In vitro digestion, sample preparation and TCA precipitation

For *in vitro* digestion, the standardized, international consensus model INFOGEST (Minekus et al., 2014) was used as previously described (Rieder et al., 2019). Sample amounts were standardized for protein content, except for beef, which had a higher protein content than expected. For chicken mince 1 g of raw sample containing 170 mg protein was used. For pea protein concentrate and pea protein porridge 350 mg powder containing 170 mg protein was mixed with 650 μL water. For casein 170 mg powder was mixed with 830 μL water. For beef 1 g ground samples was used. However, unlike the chicken, which was raw, the beef contained a higher amount of protein (259 mg protein per g). For some samples different enzyme to substrate ratios were investigated by increasing the amount of protein per test tube e.g. from 84 to 168 mg for bread (corresponding to 1 and 2 g bread crumbs), and 85, 170 and 340 mg for casein while keeping the addition of enzyme solutions constant (corresponding to 1 g sample in INFOGEST model). Enzymes were inactivated after the gastric phase by raising the pH to 7 using 4 mL SIF (without enzymes) and NaOH and at different time points of the intestinal phase (10, 20, 30, 40, 60, 80 and 120 min) by heat inactivation in a boiling water bath for 5 min. Two parallel tubes were prepared for each time point. After inactivation, samples were centrifuged at 4000 rpm for 10 min and separated into supernatant and pellet. Heat inactivation before and after centrifugation were compared but gave similar results (data not shown). Pellets were washed once with 20 mL ice cold water, frozen and freeze-dried. The weight of the freeze-dried pellet was recorded for all tubes. Supernatants were either used directly (mostly for *SEC* and combustion) or stored at – 20 °C before further analysis. Aliquots of 1 mL supernatant were mixed with 200 μL 50% TCA (final concentration of TCA in samples was 8.3%, corresponding to pH 0.5), incubated on ice for 1 h and centrifuged in a bench top centrifuge at 13 000 rpm for 10 min. TCA soluble nitrogen was measured by combustion analysis as described in 2.5.

2.3. Quantification of free NH_2 groups with TNBS reagent and calculation of degree of hydrolysis (DH%)

The degree of protein hydrolysis (DH%), defined as the proportion of cleaved peptide bonds, was measured using a TNBS based method as previously described (Kristoffersen et al., 2020). Supernatants from *in*

vitro digestion were diluted 1:20 or 1:200 with 1% sodium dodecyl sulfate (SDS). Leucine solutions of 0, 0.075, 0.15, 0.3, 0.6, 0.9 1.2 and 1.5 mM in 1% SDS were used for calibration. The assay was performed in 96 well plates and 15 μL of sample or standard solutions were added to each well (in triplicates) followed by 45 μL 0.21 M sodium phosphate buffer (pH 8.2) and 45 μL TNBS solution (0.05% w/v in water). The sealed plate was wrapped in aluminum foil and incubated at 50 °C for 1 h. The reaction was stopped by the addition of 90 μL 0.1 M HCl and absorbance at 340 nm was read in a plate reader (Spectrostar nano, BMG labtec). Absorbance readings were converted into NH_2 concentrations using a linear regression based on the leucine calibration standards. To calculate DH%, estimates for h_{tot} were taken from the literature. For casein 8.2 mmol/g protein was used (El, Karakaya, Simsek, Dupont, Menfaatli, & Eker, 2015), while the approximate value of 8 mmol/g for common food proteins was used for all other proteins (Adler-Nissen, 1977).

2.4. SEC

The peptide size distributions in supernatants after different time points of simulated digestion were determined using size exclusion chromatography (*SEC*) as previously described (Wubshet et al., 2017). Peptide size standards ranged from proteins such as albumin from chicken egg white (385 amino acid residues, 44 000 Da) via peptide hormones of varying size (e.g. Insulin Chain B (30 amino acid residues, 3496 Da) and angiotensin II (8 amino acid residues, 1046 Da)) to tripeptides (ValTyrVal, 379 Da) and free amino acids (tryptophan, 204 Da). A complete list of the standards and retention times can be found in the supplementary (Table S1). Injection solutions of peptide standards were prepared in water at a concentration of 2 mg/mL. Bovine serum albumin (BSA) (Sigma), whey protein hydrolysate (described in a previously published study (Kristoffersen et al., 2020); prepared using 60 min hydrolysis time and alcalase as a proteolytic enzyme) and BSA solution subjected to simulated digestion (17% protein solution, 1 mL per tube) were used as control samples in different dilutions (dilutions prepared with ultrapure water). All samples (standards, digested samples and control samples) were filtered through 0.45 μm syringe filters (Millipore, hydrophilic PVDF). An injection volume of 10 μL was used for standards (in triplicate) and samples (once per tube = twice per time point). The HPLC system consisting of one pump (Dionex UltiMate 3000), an auto injector (Dionex UltiMate 3000), a chromatographic column (BioSep-*SEC*-s2000, Phenomenex, 300–7.8 mm) kept at room temperature and a UV detector at 214 nm. A mixture of acetonitrile (30% v/v) and ultrapure water (70% v/v) containing 0.05% trifluoracetic acid (TFA) was used as eluent with a flow rate of 0.9 mL/min. Isocratic separation was carried out for 17 min. Between 17.0 and 20 min, 100 mM NaH_2PO_4 was used as mobile phase for column cleaning. After 20 min, the mobile phase was switched back to 30% acetonitrile and the column was equilibrated for another 30 min. Chromatographic runs were controlled using Chromeleon software. A proprietary third order polynomial regression (PSS poly 3) was fitted to the retention time of the protein standards plotted against peak molecular weight using a logarithmic scale in PSS WinGPC Unichrome software and had an R^2 of 0.975 (PSS Polymer Standard Service, Mainz, Germany) (Fig. S1). Chromatographic data were processed using PSS WinGPC Unichrome with multiarea slice settings dividing each chromatogram in two parts from 5 to 9.4 min and from 9.4 to 15 min.

2.5. Combustion and calculations of total, insoluble, soluble and TCA soluble protein

Combustion of samples (Dumas method) was carried out with a Vario EL cube (Elementar, Langenselbold, Germany) operated in CNS mode and sulfanilamid was used as correction standard. Protein contents were calculated from total nitrogen by different nitrogen to protein conversion factors from Mariotti et al., 2008 (Mariotti, Tome, & Mirand, 2008) depending on the raw material (i.e. 6.15 for casein, 5.49 for wheat, 5.48 for beef, 5.53 for chicken, 5.36 for pea and 6.25 for BSA and all other samples). The protein content of all test foods and pellets after *in vitro* digestion was measured by weighing 5 mg of dry sample (freeze or oven dried see 2.1) into tin foils. Protein concentrations in supernatants (before and after TCA precipitation) were determined by pipetting 50 μL aliquots of each sample into double tin foils. Each series of liquid samples contained at least two blank samples of 50 μL distilled water. Liquid samples were evaporated overnight (for TCA samples at 50 °C, others at room temperature) prior to combustion.

Due to the low protein concentration in digestion blank samples (containing enzymes and bile but 1 mL of water instead of 1 g food sample), the protein concentration of these samples was not only measured with Dumas, but also with a colorimetric assay based on the Biuret reaction (Cu^{2+} reduced to Cu^{+}) using the BCA assay kit from Bio-Rad (Bio-Rad Norway AS, Oslo). The assay was used according to the manufacturer's instructions with bovine serum albumin as standard. Protein concentrations (blank samples) in the gastric phase were 1.11 mg/mL and 0.98 mg/mL using BCA assay and combustion, respectively. While protein concentrations measured in the intestinal phase were 1.59 and 2.31 mg/mL, respectively. The values from combustion analysis were chosen for all calculations due to the comparability of the method. The total protein contribution of enzymes in the gastric and intestinal phase was 7.84 mg and 18.48 mg per tube, respectively (total volume 8 mL). The total dissolved protein of each sample was calculated by multiplying protein concentration with the total liquid volume of each sample (sample moisture content, addition of simulated fluids and pH adjustment) before subtracting the protein contribution of the enzyme blank (for gastric or intestinal samples).

The amount of insoluble protein for each sample and time point was calculated from the weight and protein content of the respective pellets. Soluble and insoluble protein contents were related to the protein content of the starting sample and expressed as % soluble and % insoluble protein. To check the performance of sample preparation and the protein determination method, the sum of soluble and insoluble protein was related to the total protein in the starting material and expressed as % protein recovery e.g. the amount of protein accounted for by the assay procedure.

2.6. Data analysis

Principal Component Analysis (PCA) was used to estimate a dynamic range of intensity increase and intensity decrease in the chromatograms. PCA was performed on chromatograms of time series for all six test foods separately. All chromatograms were normalized against the total area between 5 and 15 min prior to PCA. Subsequently, the loadings of the first principal component for each PCA model were used to estimate the dynamic range. PCA was performed using The Unscrambler X version 10.3 (CAMO Software AS, Oslo, Norway).

The correlation of different methods for protein digestibility determination (% small peptides, % TCA soluble protein, DH%) was evaluated by calculating Pearson correlation coefficients in Minitab (Version 18) on average data for each time point (n = 24) or all end points (n = 6) at a confidence level of 95%. P-values for all correlations are reported in the text.

3. Results and discussion

3.1. Protein solubilization and analytical protein recovery during in vitro digestion.

The test foods covered a range of complexities, from pure protein (i.e. casein) and predominantly protein foods (i.e. chicken and beef) to pea concentrate (containing 49.4% protein, high levels of starch and dietary fiber) and bread (rich in starch and only moderate protein contents, i.e. 8.4% protein). The different complexity of the test foods is reflected in

the differences in protein solubilization during *in vitro* digestion. While casein was highly soluble at all time points of digestion, all other test samples showed an increase of soluble protein and decrease of insoluble protein with digestion time (Table 1). The solid and fibrous beef and chicken samples had the least soluble protein also at the end of the intestinal phase (82 and 83%), followed by bread (87%) as the only other solid food sample and the two pea protein concentrate samples (92 and 94%). The analytical protein recovery values for the sum of soluble and insoluble protein ranged from 98 to 100.7% (SD 1.5–5.9). This is somewhat surprising taking into account the complexity of some of the food matrices, the extensive sample preparation, the correction for protein from digestive enzymes and the conversion of measured protein concentrations to total soluble protein by multiplication with each sample's individual liquid content (based on sample weight and moisture, liquid for pH adjustment and addition of digestive juices) and inspires trust in the used methodology.

3.2. Peptide profiles and quantification of protein digestibility based on SEC

3.2.1. Peptide profiles

Despite their difference in composition, the peptide size distribution profiles of the 6 digested foods showed several similarities, as shown in Fig. 1. All samples showed an expected clear trend with decreasing signals in the low elution volume range (5–9 min) and increasing signals in the high elution volume range (9–13 min) as a function of digestion time. This corresponds well with increasing amounts of small peptides and decreasing amounts of proteins and large peptides. The difference between the 120 min gastric and the 10 min intestinal time point was the largest difference seen in all chromatograms, which can be attributed to the introduction of new proteases in the form of pancreatin at the beginning of the intestinal phase. Except for the bread sample, all samples showed a range of broad but relatively distinct peaks. The first one at 8.7–9 min corresponds to a size range of 1700 – 2100 Da or 15–18 amino acid residues (calculated using a weighted average molecular weight of 113) and decreases with digestion time. The second one at 9.6 to 9.8 min corresponds to a size range of 970 to 1100 Da or 8–10 amino acid residues and clearly increases from the gastric to the intestinal phase. Subsequently, this peak remains relatively constant as the intestinal phase progresses (except for pea concentrate, which shows a time dependent increase, and casein, which shows a time dependent decrease after 10 min intestinal phase). All chromatograms from the intestinal phase (including bread) showed distinct peaks at 11 min (440 Da or 3–4 amino acid residues) and 11.6 min (280 Da or 2–3 amino acid residues) with a smaller but equally consistent broad peak at 12.3 min (170 Da or 1 amino acid), which increased with prolonged digestion time.

Interestingly, the *SEC* profile of bread deviated from the other samples as it had less distinct peaks and a much broader distribution indicating the generation of a large variety of medium sized peptides during digestion. The signal before the first distinct peak at 8.7 to 9 min decreased for all samples with digestion time but the pattern and intensity of the signal in this area differed a lot between samples. While digested casein showed almost no signal before 8 min for intestinal phase samples (Fig. 1 F), digested pea concentrate contained a much higher proportion of large peptides and proteins eluting before 8 min even after 120 min of simulated intestinal digestion. For the pea concentrate that has been subjected to a heat treatment, this proportion decreased significantly (Fig. 1 C and D). Peptide profiles measured with *SEC* at different time points of digestion clearly enable a qualitative evaluation of differences in protein digestion of different food samples.

3.2.2. Quantification

To use the *SEC* profiles for quantification of protein digestibility, several aspects must be considered. One of them is to decide which part of the chromatogram corresponds to digested protein available for uptake in the body. In a study by Le Roux et al., 2020 using *SEC in vitro* protein digestibility was defined as the fraction of soluble peptides smaller than 10 kDa. This choice was based on previous *in vitro* studies using dialysis or filtration membranes with different cut offs, were 10 or 12 kDa cut off gave the best correlations with *in vivo* data in pigs. However, peptides of 10 kDa length corresponding to 80–90 amino acid residues are not likely to be taken up in the small intestine. Peptides available for uptake clearly include tri-, dipeptides and free amino acids, which are actively transported into intestinal epithelial cells. But small intestinal digestion of proteins results in a mixture of oligopeptides dominated by peptides consisting of 2–6 amino acid residues (Freeman et al., 1979). These oligopeptides are further hydrolysed by brush border peptidase catalysis. The *in vitro* digestion INFOGEST model used does not include brush border peptidases, and we can therefore not expect the protein source to be completely broken down into free amino acids, tri- and di-peptides. Another aspect is that the fraction of oligopeptides should continue to increase with digestion time. One way to reach an operational definition of "digested protein" would therefore be to use the dynamics of peptide molecular weight distributions during digestion. PCA analysis of the chromatograms was used to estimate a dynamic range of intensity increase (small, digested peptides) and intensity decrease (intact proteins and large peptides) (Fig. S2). For most samples this shift between intensity decrease and increase was found around 9.2 min elution volume (for meat and heated pea protein, this shift was found at around 8.9 min elution volume), which would include the peak at 9.6 to 9.8 min (corresponding to 970 to 1100 Da or 8–10 amino acid residues) into the range of small peptides. For subsequent calculations, a cut-off at 9.4 min was used, since there is a trough found in all chromatograms at this time of elution.

Based on the dynamic range estimations, the chromatograms were

Table 1
Insoluble protein and soluble protein at different time points of *in vitro* digestion. Values are averages of two parallel samples. Average protein recovery and standard deviation for all time points (n = 8).

Sample		G 120	I 10	I 20	I 30	I 40	I 60	I 80	I 120	Protein recovery	
										Average	SD
Casein	insoluble protein [%]	2.6	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	100.7	1.5
	soluble protein [%]	95.7	103.0	101.1	101.7	101.5	99.4	100.5	99.9		
Bread	insoluble protein [%]	35.2	20.1	19.5	21.1	18.7	15.7	16.1	12.0	98.9	3.5
	soluble protein [%]	66.8	79.5	76.1	80.2	79.8	81.2	82.2	87.0		
Beef	insoluble protein [%]	47.7	21.0	20.9	22.4	22.6	19.9	37.7	12.8	98.0	5.9
	soluble protein [%]	50.6	74.5	74.4	76.5	76.2	78.5	66.2	82.2		
Chicken	insoluble protein [%]	27.7	21.6	25.6	23.1	23.7	15.5	17.9	9.4	98.7	5.5
	soluble protein [%]	61.8	80.8	76.6	77.1	77.2	85.0	83.6	83.3		
Pea concentrate	insoluble protein [%]	12.1	10.0	8.9	8.4	8.7	9.3	11.1	9.2	98.0	3.9
	soluble protein [%]	78.0	87.6	90.9	87.9	88.6	90.3	90.8	92.3		
Heated pea concentrate	insoluble protein [%]	31.6	14.0	13.9	12.6	12.1	8.6	9.3	8.4	100.3	2.7
	soluble protein [%]	63.8	85.8	87.8	88.9	89.4	90.1	92.3	94.0		

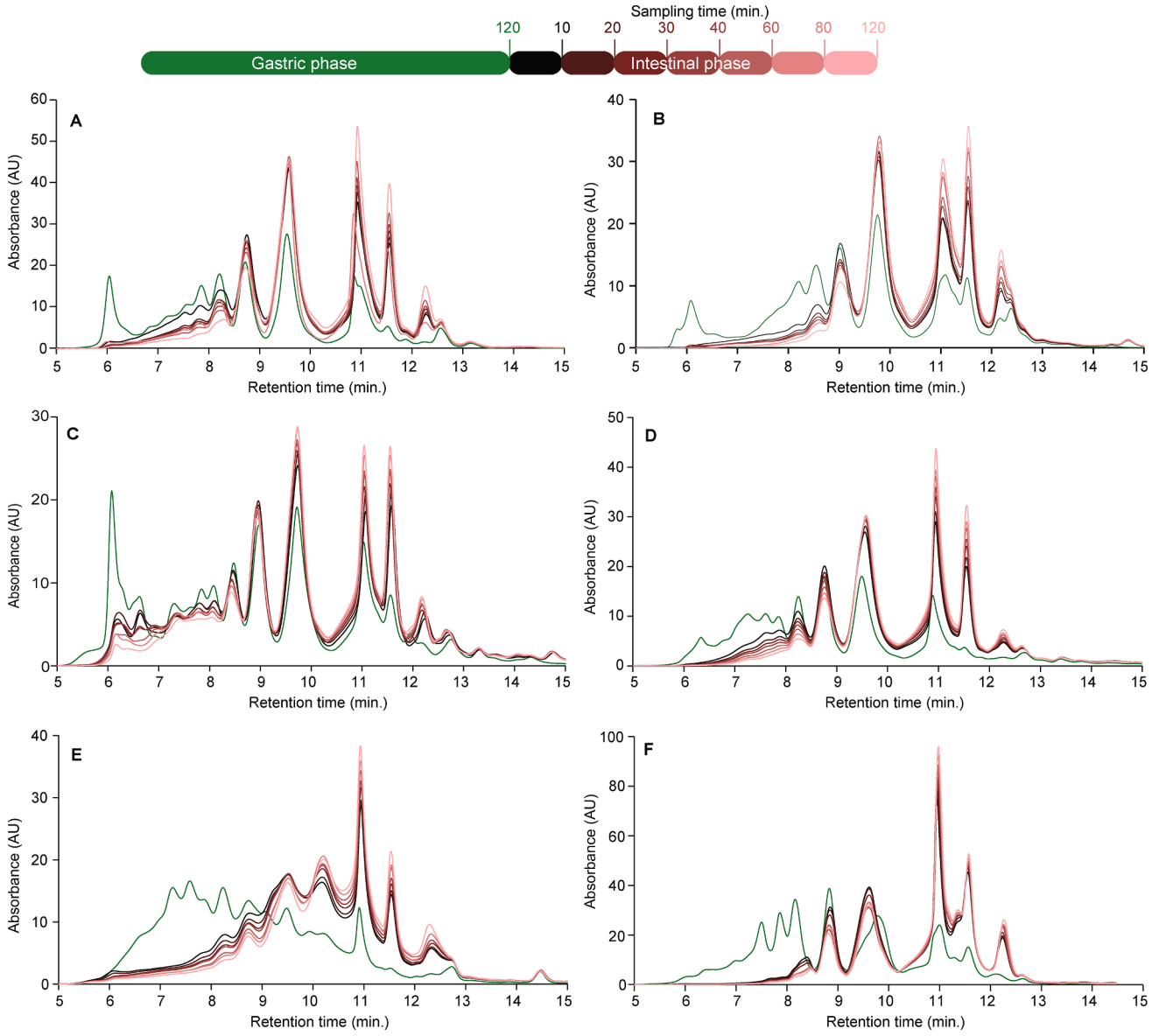

Fig. 1. *SEC* peptide profiles of beef (A), chicken mince (B), pea protein fraction (C), heated pea protein fraction (D) bread (E) and casein (F) after different times of digestion.

divided into an area of large peptides and proteins (a) (from 5 to 9.4 min, subsequently denoted high area) and the area of small peptides (b) (from 9.4 to 15 min, subsequently denoted low area). The % of small peptides in the sample can then be estimated by the proportion of the area under the chromatographic curve from 9.4 to 15 min (b) compared to the total area (a + b) (from 5 to 15 min). This calculation assumes a linear relationship between area (UV signal at 214 nm) and analyte (proteins and peptides) concentration. This approximation can be a potential source of error since both peptide bonds and amino acid side chains contribute to the UV absorption at 214 nm. For pure peptides the different extinction coefficients of the amino acids in the peptide have to be taken into account for accurate quantification based on UV absorption (Bodin, Framboisier, Alonso, Marc, & Kapel, 2015). *SEC* separation depends on hydrodynamic volume and its resolution is too low to separate individual peptides. Each point of the chromatogram therefore represents the signal of a mixture of peptides with similar molecular weight (Bodin et al., 2015). Bodin et al. (2015) showed that local errors due to differences in amino acids composition in peptides with low or high (aromatic amino acids and histidine) extinction coefficients are compensated when the overall signal is integrated. *SEC* analysis and protein contents of BSA, digested BSA and whey protein hydrolysate confirm this finding (Fig. 2 A). Even though the amino acid composition, and importantly the proportion of tryptophan, tyrosine, phenylalanine and histidine, differs between whey and BSA and all three samples have different weight average molecular weights (ranging from 27 000 (BSA) over 4800 (WPH) to 1400 (digested BSA), based on *SEC* analysis), total area from the *SEC* chromatograms was shown to highly correlate with the corresponding protein concentrations (Fig. 2A) ($R^2 = 0.999$).

A plot of total area against protein concentration for each time point and food sample used in the current study (i.e. beef, chicken, bread, pea concentrate, heated pea concentrate and casein) is presented in Fig. 2B. There was a clear relationship between protein concentration and *SEC* total area, and the slope of the linear regression for all samples and time points (3.06) was similar to the slope for BSA (2.79) and whey protein hydrolysate (2.94). However, the fit for the digestion data was much poorer ($R^2 = 0.54$), which is partially due to the limited protein concentration range from 12 to 25 mg/mL. Protein concentration in each supernatant was measured in filtered samples (0.45 μm syringe filters), which is the same filtration as was used for *SEC* measurement. However, variation in protein concentration is also expected to stem from

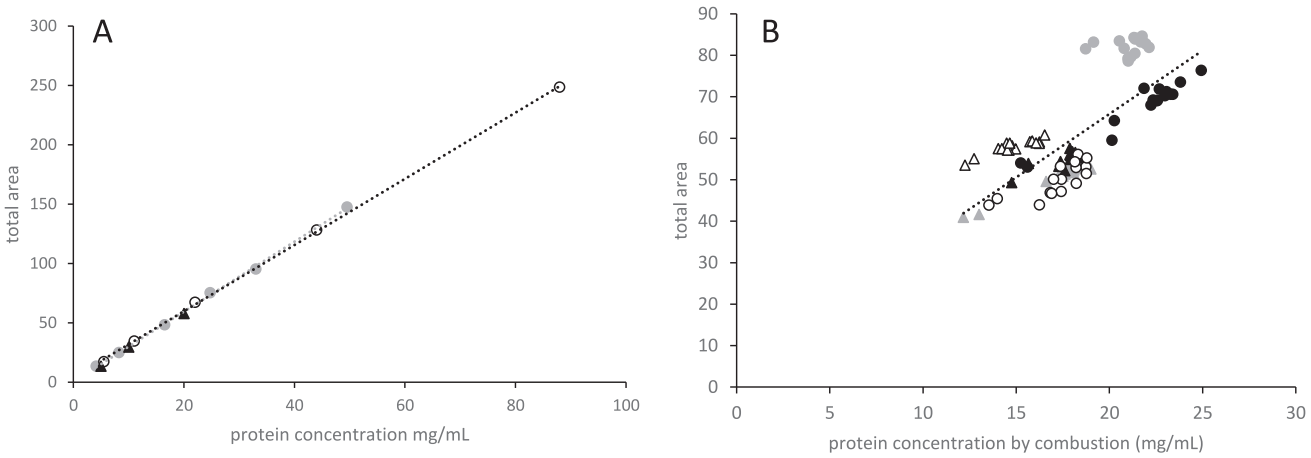

Fig. 2. Relationship between total area measured with *SEC* and protein concentration by combustion: A) different dilutions of BSA (black circles), digested BSA (black triangles) and WPH (grey dots) B) all digested food samples, beef (black dots), chicken (black circles), casein (grey dots), pea concentrate (black triangles), heated pea concentrate (grey triangles) and bread (open triangles), and all time points using individual nitrogen to protein conversion factors (Mariotti et al., 2008).

uncertainties of nitrogen determination (with combustion) and the chosen factor for conversion of nitrogen content to protein content. Individual nitrogen to protein conversion factors based on recommendations from Mariotti et al. (Mariotti et al., 2008) were used, but they are still only an approximation.

To estimate the proportion of small peptides in the chromatogram, only a relative (i.e. % of total eluted protein) and not an absolute concentration (i.e. exact peptide concentration) is required. Based on Fig. 2 A and B and the results from Bodin et al. (Bodin et al., 2015) it is reasonable to assume that the % of peptides in a given chromatographic range can be estimated by the corresponding % of area under the chromatographic curve. The % *SEC* area from 9.4 to 15 min (i.e. % low area) can therefore be used as an estimate of the % of small peptides in the sample. However, since this range contains free amino acids, di- and tripeptides, a systematic underestimation of the area % is likely to occur due to the relatively lower proportion of peptide bonds per mass compared to larger peptides and the underestimation of free amino acids with low side chain extinction coefficients. Nevertheless, the % low area can be a useful approximation of the proportion of small peptides generated during simulated digestion of different foods. This can,

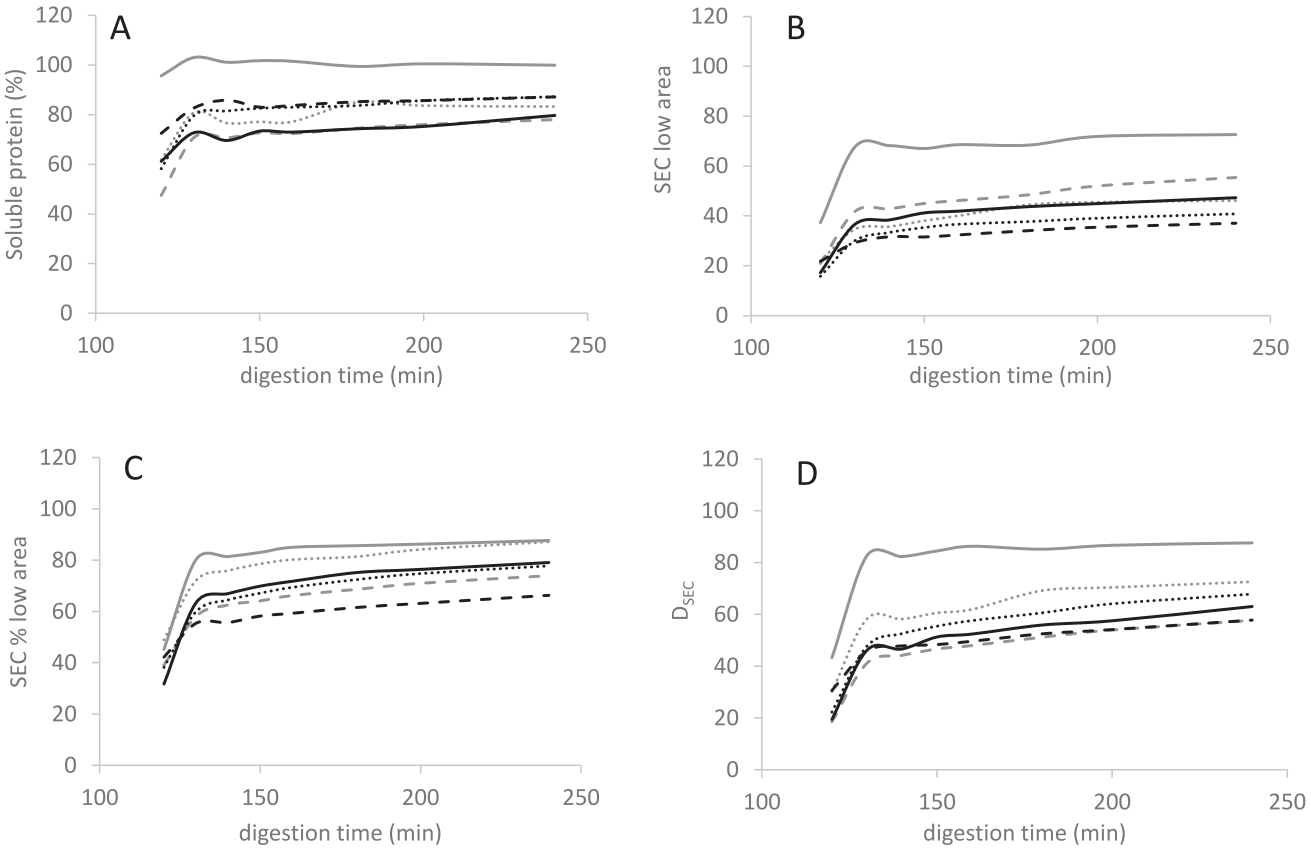

Fig. 3. Protein solubilization (A), *SEC* low area (9.4–15 min) (B), % *SEC* low area of total area (C), and D_{SEC} (D) during *in vitro* digestion. Proteins from animal sources in dark grey: casein (straight line), beef (dashed line), chicken (dotted line). Proteins form plant sources in black: bread (straight line), pea concentrate (dashed line), heated pea concentrate (dotted line).

however, not be used as a measure of protein digestibility directly as it only gives the proportion of small peptides per soluble protein. Therefore, we propose calculation of digestibility as a function of both protein solubility (i.e. soluble protein/total protein) and the extent of protein breakdown (*SEC* area 9.4 to 15 min/*SEC* area 5 to 15 min). Hence, for an improved physiologically relevant approximation, protein digestibility is estimated as the relative proportion of absorbable small peptides from the total protein. This can be calculated from the *SEC* area and the soluble protein as summarized in equation 1:

$$\%Digestibility_{SEC}(D_{SEC}) = \left(\frac{soluble\ protein}{total\ protein} * \frac{SEC\ area\ 9.4\ to\ 15\ min}{SEC\ area\ 5\ to\ 15\ min}\right) * 100$$

Fig. 3 gives an overview of different parameters derived from *SEC* and protein quantification analysis of all food samples at different time points of simulated digestion. For all samples, the proportion of soluble protein (Fig. 3 A), *SEC* low area (Fig. 3 B), % *SEC* low area (Fig. 3 C) and D_{SEC} (Fig. 3 D) expectedly increased with digestion time. Protein solubility increased from the gastric to the intestinal phase, but except for bread and meat, the increases during small intestinal digestion were only small. The *SEC* low area is a measure of both protein solubilization and degradation (Fig. 3 B). It was highest for casein, which is highly soluble (100%) and easily degraded. Meat had the second highest *SEC* low area. It is expected that this effect is not so much a function of high solubility (Fig. 3 A) or degradation (Fig. 3 C), but likely related to the higher protein content per tube for the meat samples (see Section 2.2). The % *SEC* low area reflects the degradation of solubilized protein and was highest for casein and chicken (87.7 and 87.2 at 120 min of small intestinal digestion, respectively). The bread sample was also degraded well and had a % *SEC* low area of 79.1 after 120 min of small intestinal digestion, while the beef sample was less well degraded and only had a % *SEC* low area of 73.9 at the end of simulated digestion. There was a big difference in protein degradation between the pea concentrate (% *SEC* low area of 66.3) and heated pea concentrate (% *SEC* low area of 77.8). After accounting for the differences in protein solubilization, casein, with its 100% soluble protein, clearly ranked highest among the samples in terms of digestibility (D_{SEC} 87.6% after 120 min intestinal phase) (Fig. 3 D). Even though chicken had approximately the same % *SEC* low area as casein, the lower protein solubility of chicken compared to casein resulted in a lower proportion of small peptides after digestion (D_{SEC} 72.6% (Fig. 3 D)). For heated pea concentrate and bread, D_{SEC} after digestion was 67.8% and 63.0%, respectively. Non-heated pea concentrate and meat had the lowest proportion of small peptides with D_{SEC} after digestion of 57.8% and 57.7%, respectively. For pea concentrate this was mainly due to a low degradation of solubilized protein (low % *SEC* low area), while for beef proteins the relatively low solubility during digestion was the main reason for the low proportion of small peptides after digestion.

3.3. Comparison of digestibility quantification methods

Protein digestibility estimated as % small peptides based on *SEC* was compared with two other frequently used protein digestibility estimates: 1) % TCA soluble nitrogen; and 2) free NH_2-groups determined as DH% (TNBS). For casein samples, the generated TCA soluble peptides were analyzed with *SEC* (Fig. S3). TCA treatment removed all proteins/peptides eluting before 8 min, left the peaks at 11 and 11.6 min mostly unchanged (slight shift in retention time) and increased and shifted the peak at 12.3 min to 12.8 min (perhaps through acid hydrolysis). Between 8 and 11 min no peaks but very high baselines were observed, indicating incomplete precipitation of some larger peptides, perhaps due to high hydrophilicity (Yvon et al., 1989). All three methods showed a time dependent increase of estimated digestibility in the 4 h simulated digestion (Table S1). Correlation analysis of all time points (average values per time point, $n = 24$) thus expectedly revealed that the three methods are well correlated ($p < 0.01$) with individual correlation coefficients of 0.831 ($D_{DH\%}$ and D_{TCA}), 0.836 (D_{SEC} and D_{TCA}) to 0.861 (D_{SEC} and $D_{DH\%}$). The absolute values and differences between samples, on the other hand, were different (Table S1). For the end point of digestion (120 min intestinal phase), which would be used to rank the digestibility of different foods, correlations were poor ($p > 0.05$) due to the low number of samples ($n = 6$). Correlation coefficients ranged from 0.662 ($D_{DH\%}$ and D_{TCA}) over 0.688 (D_{SEC} and D_{TCA}) to 0.800 (D_{SEC} and $D_{DH\%}$) with p-values from 0.056 to 0.152. Even though absolute values differed, the three methods were all able to pick up time-dependent differences during digestion and give complementary results.

Fig. 4 gives an overview of the results (after 120 min simulated intestinal digestion) obtained with the different methods. $D_{DH\%}$ values ranged from 16% (bread) to 48% (casein) and were considerably lower than D_{TCA}, which ranged from 66% (bread) to 90% (casein). While D_{SEC} ranged in the middle from 58% (beef and pea concentrate) to 87% (casein). All three methods showed good reproducibility. D_{SEC} had the lowest average standard deviation (SD) for duplicate samples at 120 min intestinal digestion (6 samples in parallels, $n = 12$) with 1.6 (range 0.4 to 2.9), followed by D_{TCA} (average 2.1, range 0.3 to 5) and $D_{DH\%}$ (average 2.9, range 0.2 to 4.2). The *SEC* separation alone was very reproducible with SD of % low area from 0.02 to 0.8 (average 0.4).

The ability of an *in vitro* method to reveal differences in protein digestibility due to heating has previously been stated as an important criterion (Tavano, Neves, & Junior, 2016). Grain legumes such as peas contain trypsin inhibitors, which reduce protein digestibility (Gilani, Cockell, & Sepehr, 2005). These can be inactivated by heating to varying degrees (Gilani et al., 2005). Heating also has a positive effect on legume protein hydrolysis due to alterations of their tertiary and quaternary structures (Sousa, Portmann, Dubois, Recio, & Egger, 2020). In our study, food processing (e.g heating) improved the degradation of pea protein concentrate into small peptides during digestion (Fig. 2), which is reflected in the increased % small peptides (58 to 68) and the increased % TCA soluble protein (69 to 88), but not in DH% (30 to 28).

The huge differences in food sample composition, digestion and quantification methods makes it difficult to compare the present results with results from other studies. For casein digested with the INFOGEST model (60 min intestinal sample) approximately 3 μmols free NH_2 groups per mg protein have been reported (Torcello-Gómez et al., 2020), which corresponds well to the 3.9 μmol/mg in the present study (DH% 48 using h_{tot} of 8.2 mmol/g) and is in agreement with results by Tavono et al. (2016) (DH% 47 using TNBS and h_{tot} of 8.85). Bread had the lowest DH% (16) of all samples, which corresponds well with results by Sousa et al., 2020, who reported the lowest amount of free NH_2 groups in digested wheat bran cereal compared to a range of other proteins sources (sorghum, black bean, pigeon pea, peanut, collagen, whey and zein). Some of the values for D_{TCA} soluble protein also corresponded well with literature data. For example, 87% (Tavano et al., 2016) and 90% (present study) for casein, 88% (Menezes, Oliveira, Franca, Souza, & Nogueira, 2018) and 81% (present study) for cooked beef and 85% (Menezes et al., 2018) and 82% (present study) for raw chicken.

In vivo data for true fecal protein digestibility vary between human and rat assays (Bodwell, Satterlee, & Hackler, 1980), between different studies (Bodwell et al., 1980; Gilani et al., 2005; Hsu et al., 1977) between different laboratories (Gilani et al., 2005) and depending on the used correction factor for endogenous nitrogen loss (Bodwell et al., 1980). Nevertheless, true fecal protein digestibility values are generally high for milk, meat and poultry proteins e.g. 90–100% (Gilani et al., 2005). White bread, wheat gluten and soybean protein isolate also range high 93–101% (Bodwell et al., 1980; Gilani et al., 2005), while soybean flour and bean protein range slightly lower with average values of 86 and 84% (Gilani et al., 2005; Hsu et al., 1977). However, the question is not only which of the *in vitro* methods best represents *in vivo* results, but also if protein digestibility determined by the fecal balance method (in rats or humans) truly represents the part of the protein available for absorption in the small intestine. Recent studies using dual tracer methods to determine true ileal protein digestibility in humans have

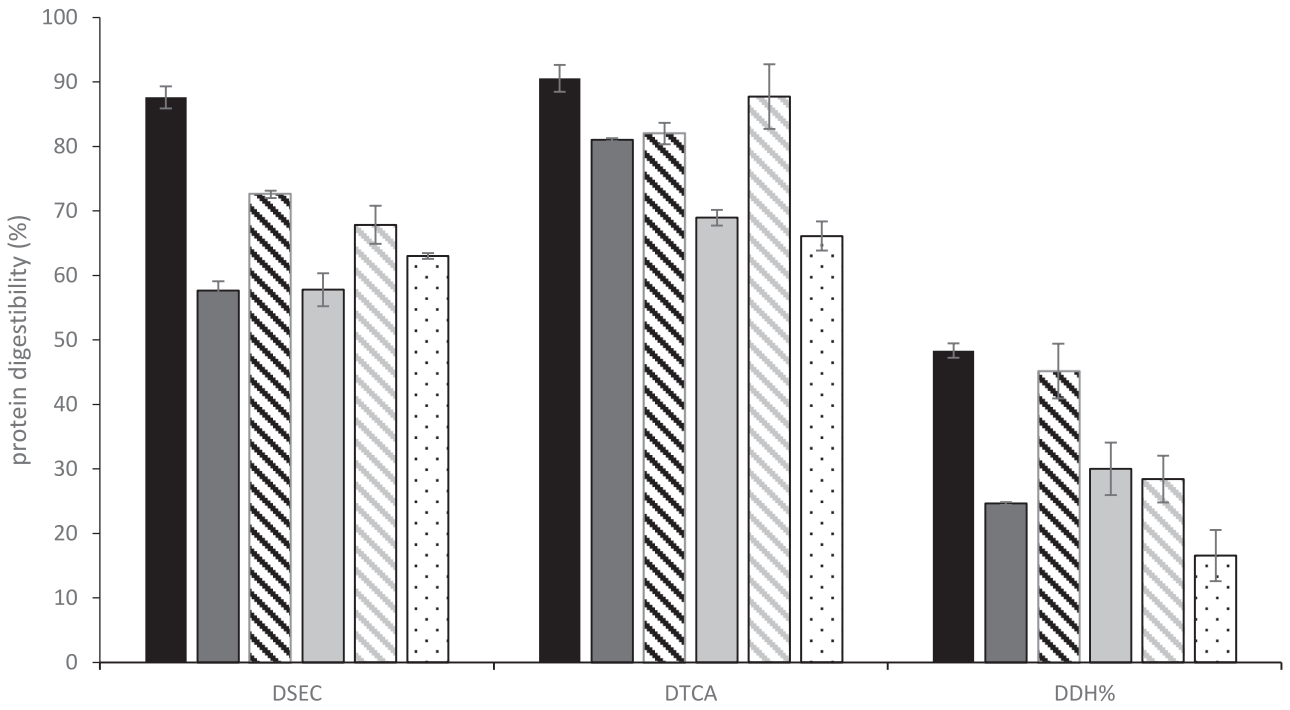

Fig. 4. Protein digestibility of casein (black), beef (dark grey), chicken (dark grey stripes), pea concentrate (light grey), heated pea concentrate (light grey stripes) and bread (dots) after 120 min intestinal phase using different quantification methods.

reported relatively low values for chickpea and mung bean protein (57 and 58%), but only slightly lower values for cooked chicken meat (92%) (Devi et al., 2018; Kashyap et al., 2018).

As true ileal digestibility values of different food proteins in humans are still scarce, it is difficult to correlate *in vitro* data with relevant *in vivo* data. The INFOGEST method has been shown to correlate well with *in vivo* digestion of milk proteins in humans and pigs (Egger et al., 2017; Sanchon et al., 2018) as similar peptide patterns were found. However, from a quantitative perspective a static *in vitro* digestion model will always be in danger of underestimating digestibility due to product inhibition as digestion products are, unlike in the *in vivo* situation, not removed from the reaction mixture. Indeed, we have seen that increasing amounts of casein (with constant enzyme concentrations) resulted in decreased % low area and lower % TCA soluble protein. For 85, 170 and 340 mg casein the % low area was 90.1, 87.7 and 84.9, respectively. While % TCA soluble protein decreased from 93.6 (85 mg) to 90.5 (170 mg) and 82.5 (340 mg). Interestingly, this dose dependence was not found for bread samples were 1 g bread (84 mg protein) and 2 g bread (168 mg protein) resulted in equal % low area of 79.1. One possible explanation for this difference could be the presence of many different proteins in wheat resulting in less product inhibition, while the more homogeneous casein break-down products may have resulted in product inhibition of digestive enzymes.

Clearly, *in vitro* methods are not able to mimic all aspects of human digestion and absorption. There seems to be some consistency in data obtained with the same quantification method (D_{TCA} or $D_{DH\%}$) even though the values cannot be easily compared with each other or with *in vivo* data. However, *in vitro* methods are indispensable to increase our understanding of the effect of processing and formulation (different ingredients) on protein digestibility in foods. This requires both the ability to rank different foods (with different protein sources) and an increased understanding of the underlying molecular processes of protein solubilization from a solid food matrix and protein degradation. While the proposed *SEC* based method (D_{SEC}) was not proven as a method better correlated to the *in vivo* digestibility, it presents an improved alternative to the existing methods by taking into consideration the two fundamental processes during protein digestion, namely solubilization and hydrolysis of peptide bonds. The *SEC* chromatograms give valuable information on size distributions of proteins, large peptides, and smaller protein degradation products. D_{SEC} is thus complementary to other quantification methods such as D_{TCA} and $D_{DH\%}$ Unlike more detailed molecular methods, the *SEC* method can be easily used to rank digestibility of very different food proteins, while giving insight into their digestion process. The method can be used to study *in vitro* protein digestibility in specific groups of the population, as new and emerging *in vitro* digestion models are being developed, simulating the digestive process in older adults and infants, as well as simulating the physiological conditions related to various disorders in the human gastrointestinal tract (Shani-Levi et al., 2017). It may also be well suited to study the effect of food processing on protein digestibility and was able to pick up the difference in pea protein digestibility upon heating. Last, but not least, the *SEC* method has a very good reproducibility.

CRediT authorship contribution statement

Anne Rieder: Conceptualization, Methodology, Validation, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Nils Kristian Afseth:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Ulrike Böcker:** Conceptualization, Methodology, Writing - review & editing. **Svein Halvor Knutsen:** Conceptualization, Methodology, Writing - review & editing. **Bente Kirkhus:** Writing - review & editing. **Hanne K. Mæhre:** Writing - review & editing. **Simon Ballance:** Writing - review & editing. **Sileshi Gizachew Wubshet:** Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129830.

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