



The harmonized INFOGEST *in vitro* digestion method: From knowledge to action



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ABSTRACT

Within the active field of *in vitro* digestion in food research, the COST Action INFOGEST aimed to harmonize *in vitro* protocols simulating human digestion on the basis of physiologically inferred conditions. A harmonized static *in vitro* digestion (IVD) method was recently published as a primary output from this network. To validate this protocol, inter-laboratory trials were conducted within the INFOGEST network. A first study was performed using skim milk powder (SMP) as a model food and served to compare the different in-house digestion protocols used among the INFOGEST members. In a second inter-laboratory study applying the harmonized protocol, the

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degree of consistency in protein hydrolysis was investigated. Analysis of the hydrolyzed proteins, after the gastric and intestinal phases, showed that caseins were mainly hydrolyzed during the gastric phase, whereas β -lactoglobulin was, as previously shown, resistant to pepsin. Moreover, generation of free amino acids occurred mainly during the intestinal phase.

The study also showed that a few critical steps were responsible for the remaining inter-laboratory variability. The largest deviations arose from the determination of pepsin activity. Therefore, this step was further clarified, harmonized, and implemented in a third inter-laboratory study.

The present work gives an overview of all three inter-laboratory studies, showing that the IVD INFOGEST method has led to an increased consistency that enables a better comparability of *in vitro* digestion studies in the future.

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1. Introduction

One major aim of the COST Action INFOGEST FA1005 INFOGEST (<http://www.cost-INFOGEST.eu/>) was the elaboration and harmonization of *in vitro* protocols simulating human digestion. A basic static model was developed and published in 2014 (Minekus et al., 2014). Many different models have previously been described (Hur, Lim, Decker, & McClements, 2011; Kopf-Bolan et al., 2012; McClements & Li, 2010; Picariello et al., 2010; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005; Wickham, Faulks, & Mills, 2009). In this context, specific aims of the INFOGEST network were i) to find harmonized digestion conditions, ii) to develop a static model that was easy to set up and apply for a large research community, and iii) to be able to compare study results. The parameters proposed by this method were discussed among experts in the field and were chosen based on previously published *in vivo* values. The harmonized method comprises three steps, namely an oral, gastric, and intestinal phase, similar to earlier published models. However, in the past, a major source of discrepancy of results among laboratories, even when applying the same method, was the differences in digestive enzyme activities used for IVD. In most cases, digestive enzymes were added based on weight or non-standardized activity units. To overcome these problems, the new harmonized method proposes a standardized assay for activity determination for each added enzyme and therefore is expected to improve the comparability of experimental results between laboratories.

In this study, the increase in harmonization of IVD results between laboratories of the INFOGEST community was investigated. For that purpose, three rounds of inter-laboratory studies were performed applying IVD to skim milk powder (SMP). SMP was chosen because it is an easy to ship, stable, and complex food matrix. For the first study, the participants digested the SMP with their previously used in-house IVD protocols, and the two following studies were performed with the harmonized method. All three studies were analyzed with the same methods, focusing on protein hydrolysis. Whole proteins were analyzed by SDS-PAGE, peptides by mass spectrometry, and free amino acids by HPLC. In the course of the inter-laboratory trials, pepsin activity was identified as the critical factor in the gastric phase, causing variabilities in protein hydrolysis during this step. Pepsin activity is highly susceptible to pH fluctuations (Piper & Fenton, 1965). The determination of pepsin activity with a classic enzymatic assay adapted from Anson (Anson, 1938; Anson & Mirsky, 1932) was therefore investigated to reduce the variability in the gastric hydrolysis.

2. Materials and methods

2.1. Chemicals and reagents

SMP (Fonterra, NZ, low-heat organic, protein 42.34%, fat 0.89%, lactose 49.8% (w/w)) was shipped to the INFOGEST members. All chemicals and enzymes were from Sigma Aldrich. Three different pepsins were used: P1, Sigma P6887; P2, Sigma P7012; and P3, Sigma P7000. Enzymes and bile extracts used for IVD were of porcine origin.

2.2. Inter-laboratory trials

In total, three inter-laboratory IVD trials were performed, each with SMP as food matrix. The participating laboratories were different between the inter-laboratory trials, therefore the numbering refers only to the corresponding trial (Table 1). The powder from one single batch was shipped to the participating laboratories at ambient temperature. For the first inter-laboratory trial, the participants digested the SMP according to their in-house IVD method using enzymes from different sources. For the second and third trial, the harmonized protocol was followed. Between the second and third trials, the protocols for the enzyme activity measurements and the IVD itself were described in more detail, and the participants in the third study attended a special training school during which they learned to apply the enzymatic activity methods and the harmonized IVD protocol. The same enzyme batches of pepsin P2 and pancreatin were sent to the participants to reduce batch-dependent variability.

2.3. *In vitro* digestion

SMP was digested *in vitro* using in-house methods (Table 1) or according to the harmonized protocol (INFOGEST, 2014; Minekus et al., 2014). The in-house methods were diverse, and the main known parameters are summarized in Table 1. For the harmonized protocol, the SMP (5 mL of a 1/10 dilution of SMP in H₂O, w:v) was mixed with 5 mL of simulated saliva without amylase (due to the absence of starch in SMP), for 2 min and then was mixed with 10 mL of simulated gastric juice containing pepsin (2000 U/mL of digesta, P1, P2 or P3) for 120 min. Subsequently, 20 mL of simulated intestinal juice containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mmol/L in the total digesta) was added and incubated for 120 min. The oral, gastric and intestinal steps were performed at 37 °C under constant gentle mixing. The enzyme activities and the bile concentration were measured according to the assays described in the harmonized protocol (Minekus et al., 2014). The participants were asked to collect samples after the gastric and the intestinal phases. For the first and second trials, the reactions were stopped by the addition of Pefabloc® (Roche, 1 mM) immediately followed by snap freezing (liquid nitrogen) and freeze-drying of the samples. For the third trial, the stopping reactions were improved and consisted of an adjustment of the pH to 7.0 after the gastric phase and by the addition of Pefabloc® (5 mM) at the end of the intestinal phase, prior to the snap freezing and freeze-drying of the samples. The pH shift after the gastric phase is more effective in stopping pepsin activity and at the same time closer to *in vivo* conditions found in the duodenum (Kalantzi et al., 2006). For all three trials, the samples were shipped to one analyzing laboratory (Agroscope, Switzerland) at ambient temperature.

2.4. Sample preparation and gel electrophoresis

The freeze dried samples were reconstituted in ammonium bicarbonate buffer (100 mM) at 1:2 for gastric and 1:1 for intestinal samples

Table 1

	Trial	No. of participants	Laboratory	Gastric Phase			Intestinal Phase			Pancreatin, Enzymes, Human Duodenal Juice	References	
				Short: 30-60 min	Long: 120-180 min	Dynamic	Short: 30-60 min	Long: 120-180 min	Dynamic			
In-house protocols	1	12	1	x			x			P	(Versantvoort et al., 2005)	
			2	x				x		P	(Kopf-Bolanz et al., 2012)	
			3		x				x		P	(Versantvoort et al., 2005)
			4		x				x		P	(Kopf-Bolanz et al., 2012)
			5	x				x			E	(Dupont et al., 2010)
			6		x		x		x	x	P	(Menard et al., 2014)
			7	x					x		J	(Ulleberg et al., 2011)
			8	x				x		x	E	(Shani-Levi, Levi-Tal, & Lesmes, 2013)
			9	x					x		P	(Vreeburg, van Wezel, Ocana-Calahorra, & Mes, 2012)
			10		x					x	E	(Martos, Contreras, Molina, & Lopez-Fandino, 2010)
			11		x					x	P	(Alemay et al., 2013)
			12	x						x	P	(Kenmogne-Domguia, Meynier, Boulanger, & Genot, 2012; Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012)
Harmonized protocol	2. and 3.	10 and 7	1.1-1.10 and 2.1-2.7		x			x	P	(Minekus et al., 2014)		

corresponding to the original volume of SMP input. For gel electrophoresis, equal volumes were mixed with a sample buffer (Tris–HCl 350 mM, pH 6.8, SDS 10%, DTT 100 mM, glycerol 50%) and were separated by SDS–PAGE (15% polyacrylamide). A molecular weight marker (Benchmark™, Invitrogen) and undigested SMP (1:400, mixed with sample buffer) were included on each gel. Thereafter, the gels were stained with colloidal Coomassie Blue (Kang, Gho, Suh, & Kang, 2002).

2.5. LC–MS peptide identification and length analysis

MS analysis was performed as previously described (Kopf-Bolanz et al., 2012). Briefly, samples were filtered through Microcon columns (Ultracel YM-30, Millipore), and the peptides were separated on a Rheos 2200 HPLC (Flux Instruments, Switzerland) equipped with an XTerra MS C18 column (3.5 μ m, 1.0 mm \times 150 mm, Waters). The HPLC was directly coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Switzerland) using an ESI interface. The samples were measured in multiple overlapping narrow mass windows spanning an m/z^{-1} between 100 and 1300, and all raw files were merged for an identification search with Mascot (Matrix Science), using a database containing the major milk proteins of different origin. The search engine parameters were set as follows: instrument configuration ESI-trap, consideration of average masses, peptide charge 1+, 2+, 3+, enzyme: none, variable peptide modifications were Gln \rightarrow pyro-Glu(N-term Q), oxidation (M), phosphor (S, T), peptide and MS/MS tolerance 0.8 Da. Peptides from the five major milk proteins (β -casein, α_{s1} -casein, α_{s2} -casein, κ -casein, β -lactoglobulin) with a minimal length of five amino acids (AA) were considered. The identification results were verified manually. For better visualization of peptide abundance, colored peptide patterns were generated by summing up the number of individual amino acids within the identified peptides from the above mentioned dairy proteins. The colors are ranging from green to red, indicating low and high recurrence of specific amino acids, respectively. Unidentified protein sequences are shown as white stretches. The colors were normalized to the maximal number of identifications within the corresponding protein and digestion phase, e.g. red in α_{s1} -casein can correspond to a different number for red in β -lactoglobulin.

2.6. HPLC analysis

For determination of free amino acids, the freeze dried samples were reconstituted, normalized to SMP input as described for gel electrophoresis and equal volumes were analyzed by HPLC according to the method published by Bidlingmeyer and coworkers (Bidlingmeyer, Cohen, & Tarvin, 1984).

2.7. Determination of pepsin activity

Pepsin activity was measured as described in the Supplemental material of the harmonized protocol (Minekus et al., 2014). Briefly, hemoglobin 2% (w/v) served as substrate, pepsin 1 mg/mL stock solution was dissolved in NaCl 150 mM at pH 6.5 and several dilutions (5–35 μ g/mL) were prepared with HCl 10 mM just before the activity test. For the test, 500 μ L of substrate solution was incubated at 37 °C for 4 min, 100 μ L of enzyme solution was added and after precisely 10 min the reaction was stopped with trichloroacetic acid (TCA, 5%). The samples were centrifuged at 6000 g for 30 min and the absorbance (280 nm) was measured. For each sample, a blank was run in parallel, to which the corresponding pepsin solution was added after the TCA. A ΔA_{280} of 0.001 at pH 2.0 and 37 °C corresponded to 1 U. It was monitored that the activities from each tested pepsin concentration were the same, to ensure that only the linear part of the curve was considered.

2.8. Spearman correlation

Spearman correlations were calculated for the gastric and intestinal phase considering the five investigated proteins (β -casein, α_{s1} -casein, α_{s2} -casein, κ -casein, and β -lactoglobulin) based on the data of the peptide patterns shown in Fig. 2. The number of times an amino acid was found within an identified peptide was summed up, aligned according to the amino acid sequence and compared with each other laboratory within a trial.

2.9. Replication of experiments and statistical analysis

The samples obtained from the participants were analyzed at least three times by gel electrophoresis, analyzed in triplicate by mass spectrometry and in duplicate by HPLC. Representative gels are shown as well as the average values of the results. The Spearman correlation matrix was calculated with SYSTAT 13 software (SYSTAT, Inc. Richmond, CA) using the following settings: one set of variables, rank order data (Spearman).

3. Results and discussion

The COST Action FA1005 INFOGEST (<http://www.cost-INFOGEST.eu/>) aimed at the harmonization of IVD methods. The work of one subgroup (WG 2) was dedicated to the elaboration of a general standardized and practical static IVD protocol allowing a better comparability of experimental data. The methods were established based on available *in vivo* data and published in 2014 (Minekus et al., 2014). Meanwhile, the comparability of IVD experiments was studied in three inter-laboratory trials using SMP and analyzing the hydrolysis of proteins with SDS–PAGE, mass spectrometry and HPLC. The latter analysis was only performed for the harmonized protocol, as it detects total free amino acids and the ratios between SMP substrate and added enzymes were not comparable in the first trial with in-house IVD protocols.

3.1. Protein hydrolysis during *in vitro* digestion

Protein hydrolysis was analyzed with Coomassie-stained SDS PAGE after the gastric and intestinal digestion phases (Fig. 1). To obtain an equal loading of SMP protein in each lane, the samples were reconstituted according to their dilution during *in vitro* digestion. A molecular weight marker and an undigested SMP control were included. For the first inter-laboratory trial, the participants were asked to perform their in-house IVD protocol, and for the second and third inter-laboratory trials, the harmonized IVD method (Minekus et al., 2014) was applied. Bands indicated by arrows are proteins that were previously identified by mass spectrometry (data not shown). In a majority of the samples from all three trials, i) a band of intact β -lactoglobulin was present after the gastric phase as well as ii) a doublet band corresponding to pepsin (Fig. 1a, b and c, arrow), and iii) caseins were mostly hydrolyzed. α -Lactalbumin (α -la, 15–19 kDa) was visible in the undigested sample (SMP) but was not detected after the gastric phase (Fig. 1a, b, c). In contrast to that, the protein pattern of the intestinal phase was more diverse after the first inter-laboratory trial (Fig. 1d), compared to the two trials performed with the harmonized method (Fig. 1e, f). Thus for example, in some samples from the first inter-laboratory trial (5, 6, 11), β -lactoglobulin was still visible after the intestinal phase. One strong band in the intestinal phase, shown with an arrow, was identified by mass spectrometry (data not shown) as α -amylase (Fig. 1d, e, f). The other bands were previously identified as digestive enzymes originating from pancreatin (data not shown).

The improvement in consistency with the harmonized method was clearly visible at the protein level compared to the first inter-laboratory trial (in-house protocols) where discrepancies in the degree of β -lactoglobulin hydrolysis were clearly visible. In some samples, the protein was completely hydrolyzed during the gastric phase, while in

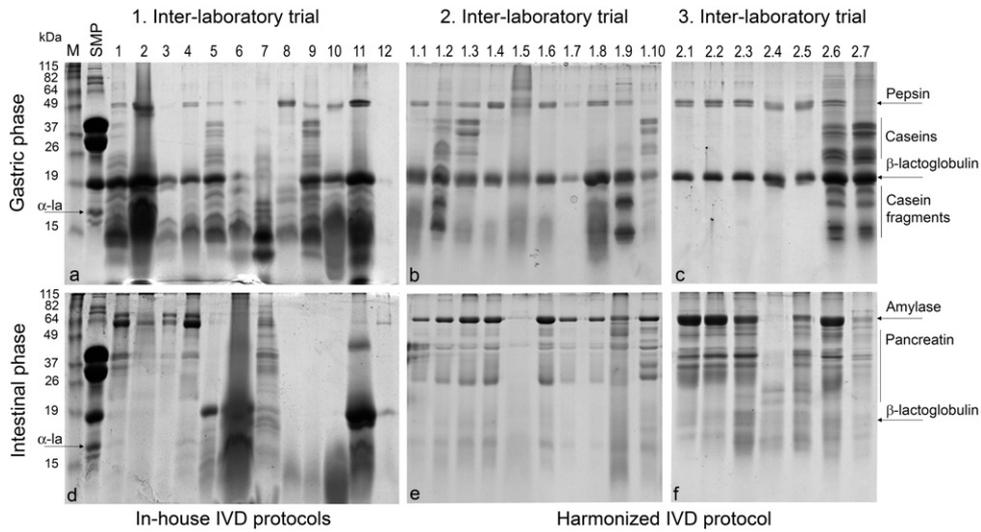


Fig. 1. Protein hydrolysis after gastric and intestinal phases of IVD. Representative Coomassie-stained SDS-PAGE gels of the three inter-laboratory trials; upper gels are samples stopped after the gastric and lower gels after the intestinal phase. The first inter-laboratory trial was performed with in-house IVD protocols, while for the two other trials, the participants applied the harmonized protocol (Minekus et al., 2014). The sample numbering refers only to the corresponding participant in the trial. Bands indicated with arrows have been previously identified as milk protein (β -lactoglobulin, α -lactalbumin (α -la)) or enzymes present in gastric (pepsin) and pancreatic (amylase) juice (Kopf-Bolan et al., 2012). SMP is the undigested skim milk powder; M is a protein marker.

others, it was still present. The harmonized method clearly improved this discrepancy. It was previously shown that β -lactoglobulin was resistant to *in vitro* degradation by pepsin (Mandalari et al., 2009; Reddy, Kella, & Kinsella, 1988), and this observation was confirmed by using the harmonized method. The simulated gastric hydrolysis of caseins was also heterogeneous in the gastric phase in this first trial. Interestingly, this heterogeneity in casein hydrolysis was persistent in some of the samples of the later trials using the harmonized method. These discrepancies were most probably due to variable pepsin activities in the gastric phase. As pepsin is the key enzyme for proteolysis during the gastric step, its activity during IVD and the enzymatic activity test was carefully assessed.

3.2. Pepsin activity as key step for gastric protein degradation

To reduce the observed differences in casein hydrolysis during the gastric phase, the participants in the second trial were asked to indicate the pepsin source and enzymatic activity they had measured before the IVD (Fig. 2). In total, three different products (Fig. 2, P1, P2, P3) were

used and for all three a high variability in activity was reported. In particular the sample digested with P3 (Fig. 2), for which a very low activity was reported, had very few peptides released during the gastric phase, as could be observed by peptide analysis (Fig. 2, sample 1.7). Two possible explanations for the high variability could be advanced. Firstly, to exclude the possibility that the variability originated from the different pepsins preparations, the third inter-laboratory trial was performed using one pepsin from the same batch (Fig. 2, P2), which was shipped between the participants and analyzed for its activity beforehand. However, although the laboratories all applied the same protocol ((Minekus et al., 2014), Supplemental Data) for this determination, the results were again highly variable. The third inter-laboratory trial was performed with the average pepsin activity determined by nine different laboratories, excluding two outliers (2976 ± 591 U/mg). Pepsin activity is known to be strongly dependent on pH and autolysis occurs in the absence of substrate (Cornish-Bowden & Knowles, 1969; Galea, Dalrymple, Kuypers, & Blakeley, 2000). Its optimal activity is between pH 2 and pH 4, it is essentially inactive above pH 6, and it is irreversibly inactivated at higher pH (Piper & Fenton, 1965). Therefore, a second

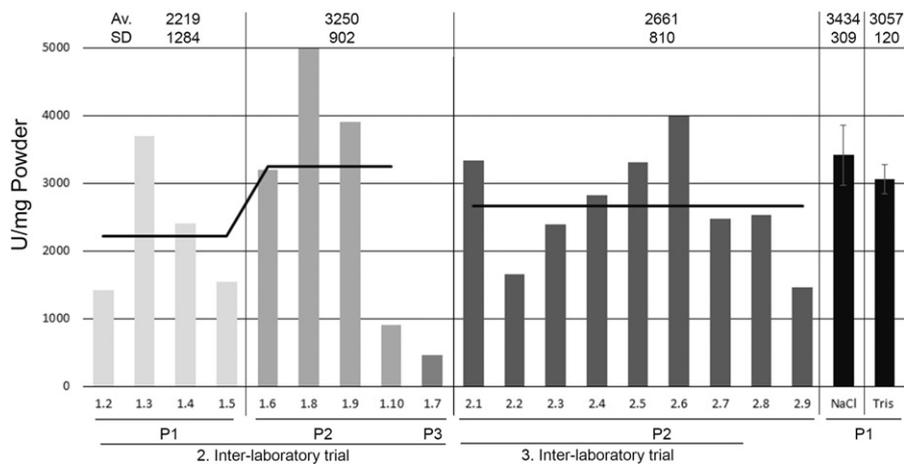


Fig. 2. Determination of pepsin activity according to the harmonized protocol. Pepsin activities determined by the participants in the 2nd and 3rd inter-laboratory trials, both applying the harmonized protocol. Laboratories 2.8 and 2.9 only analyzed pepsin activity but did not participate to the inter-laboratory trial. P1, P2, and P3 stand for different pepsin products (Section 2, Materials and methods), NaCl: pepsin dissolved in NaCl 150 mM, pH 6.5; Tris: pepsin dissolved in Tris 10 mM, NaCl 150 mM, pH 6.5. Average values (Av) with standard deviation (SD) are shown.

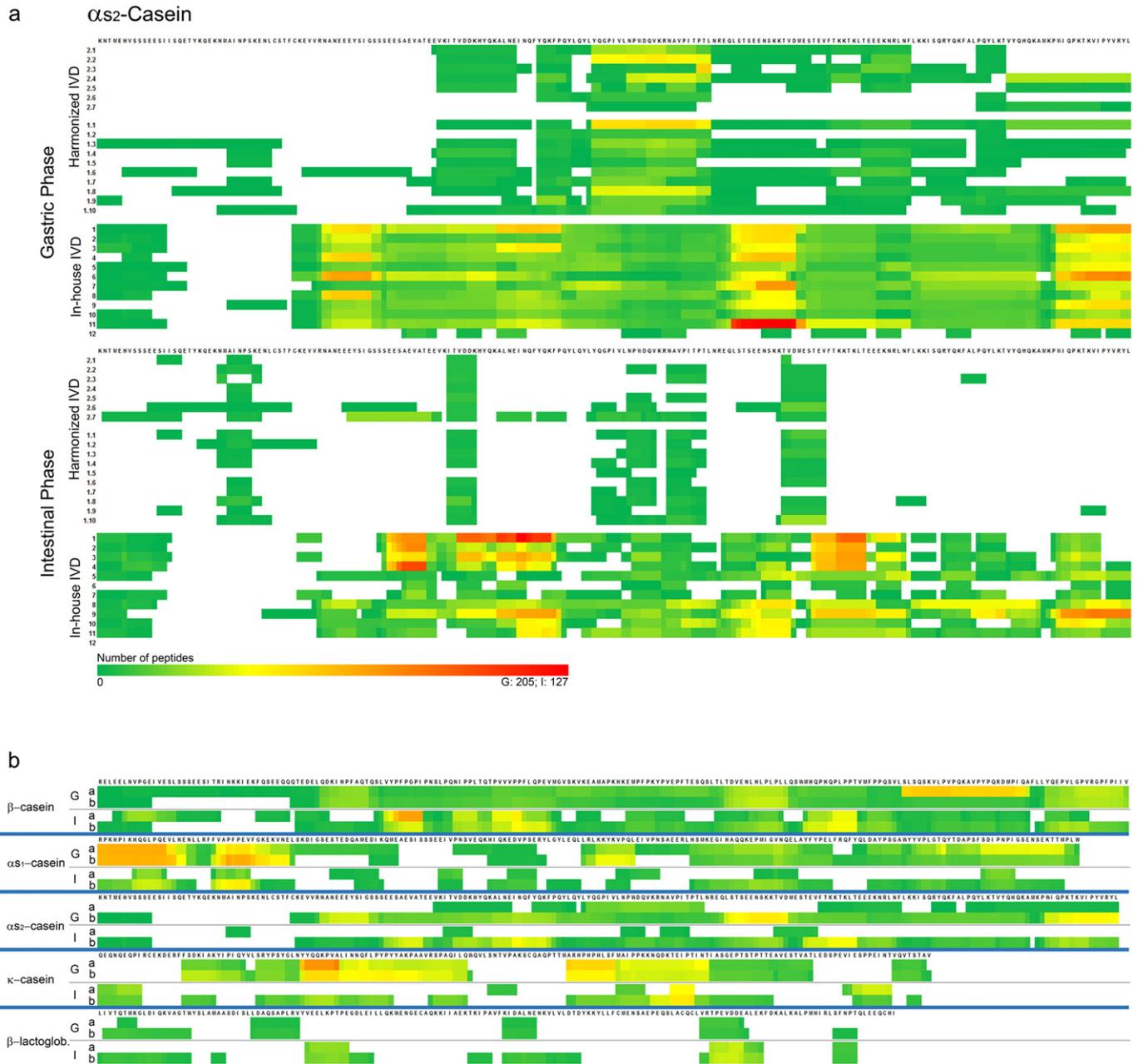


Fig. 3. Peptide patterns after gastric and intestinal phases of IVD. (a) Peptide pattern of α ₂-casein of all three inter-laboratory trials; upper block, two trials applying the harmonized protocol (Harmonized), participants 1.1–7 and 2.1–10, and lower block, with in-house protocols, participating laboratories 1–12. (b) Average peptide patterns of the five most abundant milk proteins, β -casein, α ₁-casein, α ₂-casein, κ -casein, and β -lactoglobulin after the gastric (G) and intestinal (I) phases. Averages were calculated for two groups: harmonized protocol (a) and in-house protocols for IVD (b). The color code is green for low numbers and red for high numbers of detected amino acids. Red color was attributed depending on protein and digestion phase (gastric: G/intestinal: I) and was normalized to the maximal number of detected amino acids within a identified peptide of the corresponding protein: β -casein (G: 312/I: 127), α ₁-casein (G: 92/I: 79), α ₂-casein (G: 205/I: 127), κ -casein (G: 47/I: 30), and β -lactoglobulin (G: 29/I: 54).

possibility for the variability in pepsin activity, namely the pH stability during pepsin activity determination was further investigated. The pH of the solution used for pepsin dilution (NaCl, 150 mM, pH 6.5) was measured several times during one day of analysis, and it changed from 6.02 to 6.46 (data not shown). To improve pH stability, Tris was added due to its buffering capacity (Tris-(hydroxymethyl)-aminomethane 10 mM, NaCl 150 mM, pH 6.5). Pepsin was dissolved in both solutions and the activity was measured in three independent experiments (Fig. 2, P1). The activity of pepsin in NaCl solution was slightly higher with a greater variability (Fig. 2, NaCl: 3434 ± 309 U/mg powder) than in the Tris buffer, which indeed reduced the variability of the measured activity (Fig. 2, Tris: 3057 ± 214 U/mg

powder). These additional tests were performed in one laboratory and need to be confirmed by a comparison within several laboratories. Note that the average activity of P2 was higher in the third inter-laboratory trial, which may possibly be due to batch variabilities.

3.3. Peptide generation during *in vitro* digestion

Peptide generation during IVD was analyzed by mass spectrometry for all three inter-laboratory trials. To allow a comparison of the results among the different laboratories, a color code was introduced that simultaneously allowed a quantitative evaluation of how many times an amino acid was identified and also visualized regions within a

protein that were more resistant to digestion (Fig. 3a, Supplemental Data). For each sample from all three trials, after the gastric and intestinal digestion phases, a colored peptide pattern was generated according to Section 2.5, for the five most abundant milk proteins (β -casein, α_{s1} -casein, α_{s2} -casein, κ -casein, and β -lactoglobulin). In general, the peptide patterns obtained with the in-house protocols were different between laboratories, comprising a high number of peptides which covered nearly the whole protein sequences (Fig. 3a, in-house 1–12). However, both trials with the harmonized IVD had a similar inter-laboratory pattern with a lower number of peptides which covered only certain regions of the protein (Fig. 3a, harmonized, 1.1–10 and 2.1–7). The number of peptides observed in the harmonized method decreased between the gastric and intestinal phases, indicating the expected progression in the digestion process. Both observations (protein coverage and number of peptides) point toward a higher degree of digestion in the harmonized method.

The differences between the in-house protocols and the harmonized IVD were observed in all investigated proteins in a similar way (Supplemental Data). These differences are summarized in Fig. 3b,

which shows for each of the five proteins an average peptide pattern for (a) the harmonized and (b) the in-house IVD, each time distinguishing the gastric (G) and the intestinal (I) phases. Several peptide sequences persisted at higher frequency after the intestinal phase and were consistent in all three trials, indicating that those sequences were resistant to hydrolysis, possibly because of intrinsic structural traits. Regions that are in yellow or orange in the average peptide pattern (Fig. 3b) indicate such highly resistant regions, and it would be interesting to investigate whether these regions are sources for bioactive peptides or allergenic epitopes. However, the goal of this study was to obtain a measure of the comparability of analytical results between different laboratories.

3.4. Correlation of peptide patterns between in-house and harmonized IVD

The peptide patterns of the different laboratories were aligned, and each peptide pattern was compared to every other pattern using a Spearman correlation between laboratories (Fig. 4). The first two correlations compared the results from the first inter-laboratory trial, for

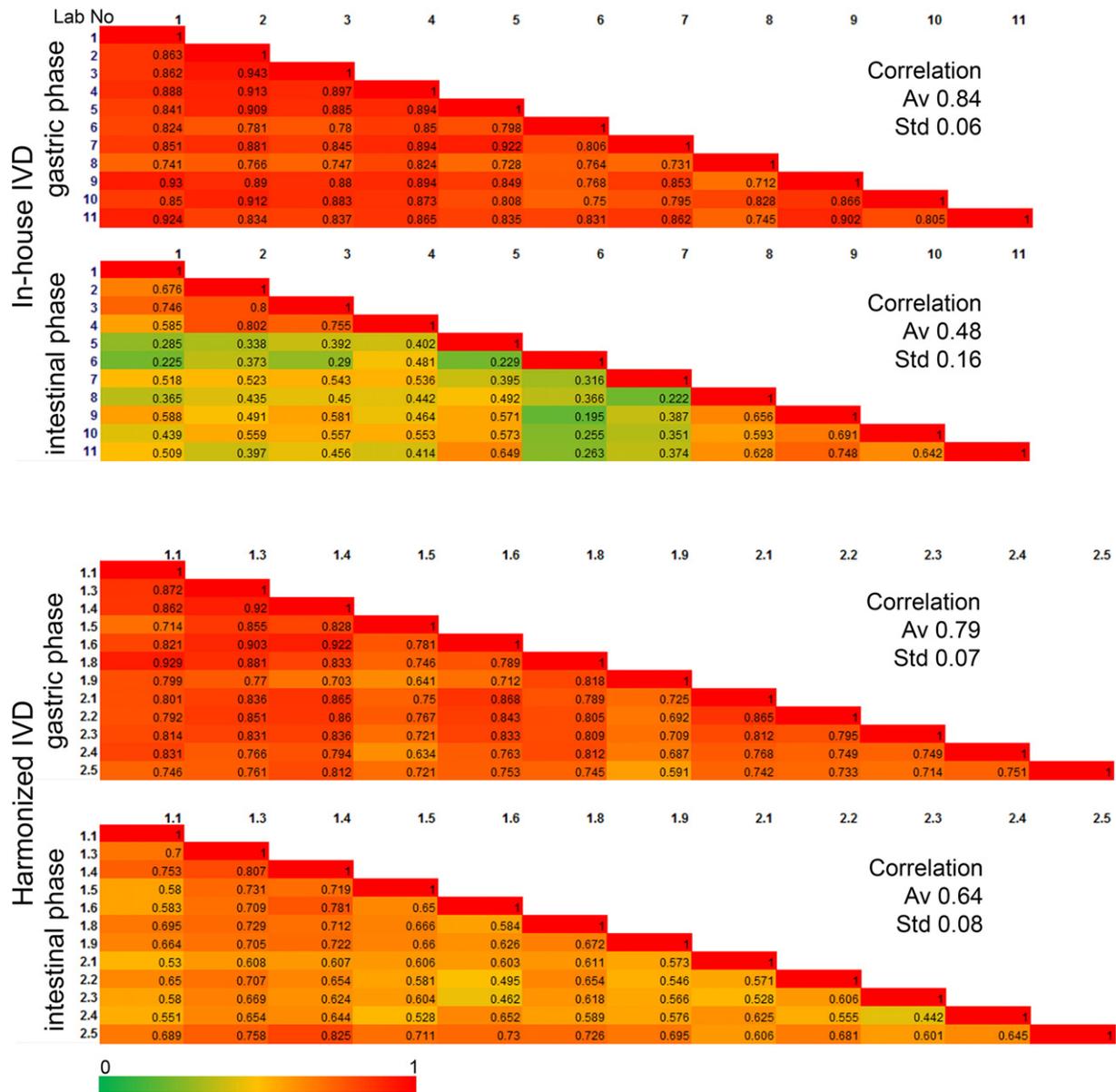


Fig. 4. Spearman correlation matrices showing the degree of harmonization. Spearman correlations were calculated for the in-house trial and the two trials applying the harmonized protocol, separating the gastric from the intestinal phases. Red color indicates good correlation (close to 1), and green indicates low correlation (going to 0). Some laboratories were excluded from the correlation due to various reasons (Section 3.5).

which the participants applied their in-house methods to simulate digestion of SMP, for the gastric and the intestinal phases, respectively (Fig. 4, in-house IVD). Two other correlations (gastric and intestinal phase, respectively) compared the results from the second and third inter-laboratory trials, applying the harmonized IVD method (Fig. 4, harmonized IVD).

For each matrix, the average correlation and the standard deviation were calculated. In addition, the matrix is shown in a color-coded picture, where red indicates high correlation, and green indicates low correlation. Surprisingly, after the gastric phase, both correlations reached similar values, $84 \pm 6\%$ for the in-house protocols and $79 \pm 7\%$ for the harmonized method. This can be explained by a general lower degree of hydrolysis in these samples, as observed on SDS-PAGE (Fig. 1) as well as in lower numbers of peptides identified (Fig. 3, Supplemental Data). However most importantly, after the intestinal phase of digestion, the harmonized method generated a higher correlation of $64 \pm 8\%$ compared to the $48 \pm 16\%$ obtained with in-house IVD. This observation allows the conclusion that the harmonized IVD indeed leads to a clear improvement in result comparability at the end of the intestinal phase.

For this analysis, in total six samples (total 29) were excluded due to inconsistencies with the protocol or high discrepancies of their results compared to the other laboratories. The following were reasons for the exclusion of results. Laboratory 12 (1. trial, in-house IVD) was excluded from the calculation due to the lack of detectable proteins and peptides by gel electrophoresis and mass spectrometry. Laboratory 1.2 (2. trial, harmonized IVD) was excluded because it did not respect the IVD protocol (ratio of SMP compared to digestion juice). Laboratories 1.7 and 1.10 (2. trial, harmonized IVD) were excluded as outliers, due

to very low numbers of peptides after the gastric phase compared to all other laboratories, laboratories 2.6 and 2.7 (3. trial, harmonized IVD) were excluded due to very low casein hydrolysis, as observed by gel electrophoresis (Fig. 1c, lanes 2.6 and 2.7 on the upper right gel).

3.5. Release of free amino acids during harmonized IVD

The degree of free amino acid release after the gastric and the intestinal digestion phase of the harmonized IVD was analyzed by HPLC (Bidlingmeyer et al., 1984) (Fig. 5). Overall, in the gastric phase, a very small amount of free amino acids was released, consisting of 1% of the total amino acids and was consistent between the second and third trial applying the harmonized method. In contrast, during the intestinal phase, most of the proteins were hydrolyzed to amino acids and the results of the first trial applying the harmonized method were more heterogeneous than the final inter-laboratory trial. The high release of free amino acids during the intestinal phase is consistent with the physiological situation in the gastrointestinal tract, where the liberated amino acids are absorbed mainly in the duodenal phase (Barbe et al., 2014; Clemente, 2000). Moreover, the reduction in heterogeneity could possibly be attributed to the better training of the participants during a practical course and a more detailed protocol. Interestingly, after the intestinal phase, the average total release of free amino acids was 88% for the second trial (Fig. 5a) and 60% for the third (Fig. 5b), both trials performed with the harmonized method. A possible explanation for this difference, that was statistically insignificant, could be the change in the procedure for stopping the IVD. For the second trial, a concentration of 1 mM Pefabloc® was recommended, while for the third trial, this was increased to 5 mM due to the observation of incomplete

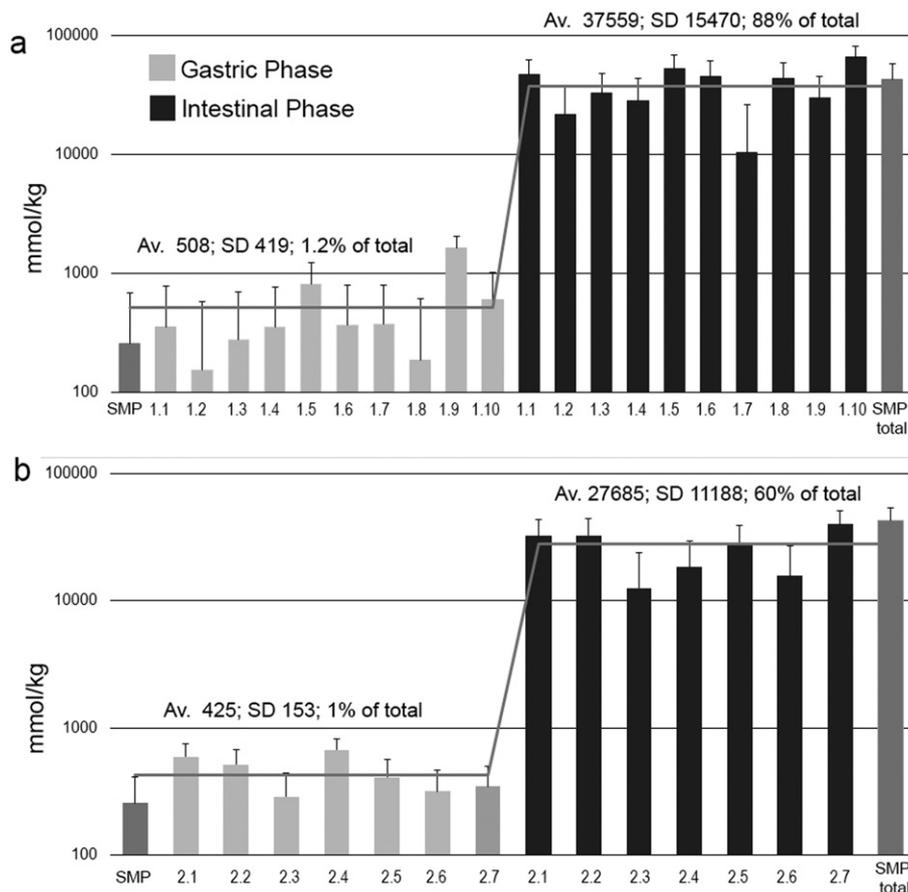


Fig. 5. Release of free amino acids after gastric and intestinal phases of IVD. HPLC analysis of samples from both inter-laboratory trials applying the harmonized protocol, trial 2 (a) and trial 3 (b), after the gastric phase (light gray bars) and after the intestinal phase (dark gray bars). SMP represents the free amino acids of undigested SMP, and SMP total is the theoretical value of amino acids present in the SMP. Average values (Av) with standard deviation (SD) are shown in logarithmic scale in mmol/kg of food, error bars are SD between all samples of the corresponding digestion phase.

inhibition. This highlights the importance of stopping the reaction effectively after sampling. The release of free amino acids, determined at the third trial is in a close agreement (58%) with that recently measured after gastric-duodenal digestion of SMP with a model closely related to the harmonized IVD (Picariello et al., 2015).

3.6. Validation of IVD

The harmonized IVD method published by Minekus and coauthors (Minekus et al., 2014) has been established based on physiologically available and published *in vivo* enzyme and salt concentrations. However, static models are simplified models of a highly complex *in vivo* digestion process. In particular, the status of hydrolysis during the gastric phase is likely to differ from the *in vivo* situation, especially for solid and semi-solid food matrices, keeping in mind that *in vivo*, the chyme is dynamically processed and gradually released to the duodenum during digestion rather than all at once and the pH is not fixed but gradually decreases. Therefore, it is difficult to fully mimic the kinetic behavior of the *in vivo* situation with a static model. The analyses presented in this article confirm that the harmonized method improves the comparability of IVD data and supports the advantages of this methodology as a robust and simple tool to study the digestive fate of oral intake. However, true evaluation of digestive fate remains to be further investigated and corroborated by *in vivo* trials. Therefore, a direct comparison between results from IVD and *in vivo* would be a major step to validate the IVD model.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.foodres.2015.12.006>.

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