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Research Article

Quantitative assessment of multi-laboratory reproducibility of SDS-PAGE assays: Digestion pattern of β -casein and β -lactoglobulin under simulated conditions

A digestion protocol was applied in triplicate by ten laboratories, simulating *in vivo* gastric and duodenal conditions. The intra- and inter-laboratory variability in the kinetics of protein degradation was quantified, focussing on the digestion of β -casein under gastric conditions, and of β -lactoglobulin (β -Lg) under duodenal conditions. The addition of surfactants such as phosphatidylcholine (PC) in the digestion mix was also evaluated. Identification and quantification of peptide bands on SDS-PAGE gels formed the basis for analysis. An average intensity loss of 69% (SD = 13.5) at 5 min (89% at 10 min, with SD = 5.5) was observed for β -casein, whereas the β -Lg duodenal digestion showed an 82% loss at 30 min (SD = 14.2). Constant rates of first-order reactions showed that for fast reactions, inaccuracies in the time of first sampling contributed to the variability, which were also affected by image quality, saturation, and the splitting of time courses across gels. Breakdown products for β -casein included ten other polypeptides, with four detected in all and two in most gels, and for β -Lg ten polypeptides, with five detected in most, and two in two-third of the cases. Addition of PC in the gastric phase led to β -Lg intensity loss only a quarter as large as without PC and altered β -Lg proteolysis in the duodenal compartment.

Keywords:

In vitro digestion / Kinetics / Quantitative statistics / Ring trial / SDS-PAGE methodology
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1 Introduction

With the recent growth in proteomic research, there has been increased realisation of the value, as well as the need, to more reproducibly evaluate and quantify the images that are obtained with SDS-PAGE-based work. Methodological developments have been driven by the need to compare results between samples and across gels and accompanied by a substantial number of articles reporting on gel-to-gel variation for the detection and quantitation of protein [1–4]. In particular, several articles have reported on inter-laboratory experiments for characterising the consistency of protein spot positions [5–7], and this remains an issue as shown by more recent studies [8–10].

There has been considerable interest in characterising the reproducibility of 2-D gel patterns and finding ways to surmount the issues that have been identified. In contrast

for 1-D gels, such endeavours have been more restricted, with fewer articles reporting on similar “registration” issues [11]. It is generally recognised that 1-D gels suffer from a number of drawbacks including lack of reproducibility, and yet they remain extremely valuable. This is exemplified in studying the resistance of proteins to proteolysis, such as the pepsin-resistance test used in the risk assessment of genetically modified organisms [12]. There is evidence that resistance to digestion by gastrointestinal proteases such as pepsin is one of the properties that could affect the allergenic potential of a protein [13–15], and 1-D gels provide a relatively simple and low-cost way to examine protein digestion patterns. However, such experiments have sometimes led to inconsistent outcomes. For instance, although in a study of the resistance to pepsin digestion of a selection of major allergenic foodstuff proteins and non-allergenic plant proteins [16], the allergens were resistant to pepsin digestion, other studies showed a less clear distinction between allergens and non-allergenic proteins [17–20].

The lack of a standardised protocol regarding differences in the pH of the assay, enzymes to protein ratios, target protein purity, and method of analysis is likely to be partly responsible for the discrepancies between these digestibility studies [21] and our study previously focussed on this issue [22]. However, even with a given protocol, the

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Abbreviations: β -Lg, β -lactoglobulin; PC, phosphatidylcholine

evidence as to how reproducible results from studies using 1-D gel electrophoresis actually are is usually anecdotal, often consisting of a limited number of trials in a given laboratory, and not well documented. This is particularly true of time-course experiments, which are essential for studying protein digestion patterns. One exception is an inter-laboratory study [21] which systematically analysed the digestion of a variety of proteins by carrying experiments in nine different laboratories, using a common protocol. In this study, the assessment of reproducibility was based on the latest time point in a digestion time course at which a protein could still be observed. There is no extensive report in the literature, though, of studies of the reproducibility of the dynamics occurring during the protein breakdown process. Yet there is no doubt that a robust, more quantitative analysis of such data would enhance their exploitation.

In this study, we have taken a quantitative approach to assess the inter- and intra-laboratory reproducibility of simulated *in vitro* gastric and duodenal digestions across 13 laboratories. A common protocol was used to digest two milk proteins with contrasting properties, being relevant for the study of cow's milk allergy: β -casein and β -lactoglobulin (β -Lg). The kinetics of digestion of the parent proteins and detection of breakdown products were characterised and compared between and within laboratories.

2 Materials and methods

2.1 General

The analysis is based on *in vitro* digestions carried out under conditions mimicking two different physiological conditions: gastric and duodenal. Stage 1 refers to the digestions of β -casein under gastric conditions, and β -Lg under duodenal conditions without any addition of phosphatidylcholine (PC), which were carried out by ten laboratories (assigned a random number 1–10, as described in [22]). Stage 2 refers to the digestion of β -Lg under duodenal conditions, following digestion under gastric conditions with addition of PC, which were carried out by three laboratories (laboratories 1, 9–10). Note that three further laboratories carried out the experiment [22] but the quality of their gel images was not sufficient for quantitative analysis: In two cases, the blurriness of the molecular markers lane did not allow the consistent identification of the polypeptides (for one, this may be due to pre-cast gels not having been used), and for the third the lanes corresponding to different time points from a given digestion were split across many gels and could not be used.

2.2 Experimental protocol

All laboratories performed the digestions by following the protocol described by Mandalari *et al.* [22, 23]. This was carried out in triplicate for each experimental condition. All

laboratories used the same lots of β -casein, β -Lg, pepsin, trypsin, chymotrypsin, and Bowman–Birk trypsin–chymotrypsin inhibitor.

Briefly, aliquots (100 mL) were withdrawn at several time points during digestion and the reactions stopped as described in [22]. For the gastric digestion, aliquots were withdrawn at 0.3, 1, 2, 5, 10, 20, 40, and 60 min and for the duodenal digestion at 0.3, 1, 2, 5, 10, 20, and 30 min. Note that the first sampling was performed as quickly as experimentally possible, at an estimated time of about 15–20 s (~ 0.3 min) from the time of addition of the enzymes; in practice the timing may have varied by ~ 10 s.

The aliquots were then analysed by SDS-PAGE, using 10% polyacrylamide NuPAGE Novex Bis-Tris precast gel (Invitrogen, Carlsbad, CA). The use of pre-cast gels was chosen in order to avoid additional variability due to gel casting by different operators [21]. Laboratories were asked to run molecular weight markers in outer lanes for densitometry analysis (see marker list in [22]).

2.3 Quantitative analysis of SDS gel data

The first part of the gel analysis was carried out with Totallab 120 (Nonlinear Dynamics, UK), a software package that can be used to identify and quantify bands in, *e.g.*, 1-D SDS gels. To do this, the gels were scanned and their pictures saved as.tif files by each laboratory, and these were then normalised to 8 bit depth with no compression in Microsoft Photo Editor before being read into Totallab. We describe here the main steps carried out in Totallab, and give an indication of how much manual intervention was necessary in the process. Each lane was first defined (automatic with occasional manual adjustments), ensuring that all lanes on a given gel had the same width, and the average intensity across the width of these lanes was depicted as a function of the distance from the top of the image (thereafter this is referred to as “a profile”). The background was subtracted from each profile using the Rolling Ball method (which sets the background by an algorithm analogous to rolling a ball under the profile of the lane with a radius parameter adjusted on a case-by-case basis), after which the bands were identified (automatic with manual adjustments). Gaussian curves were fitted to the profiles, which allowed overlapping bands to be deconvoluted (automatic with manual adjustments). As there was a degree of subjectivity in all last three steps, all analyses for gels for a given experimental condition were carried out by one individual and attention was paid to ensure consistency throughout the analysis. The lane(s) of standards were then used to reference the position of the bands. The output of this process was a list of bands defined either by their R_f or by their M_r , together with an estimate of the volume under the curve corresponding to each band (volume of fitted Gaussian curve, thereafter referred to as “intensity”).

A list was compiled of all the bands that were detected, at least in some of the lanes, for each of the three experimental conditions. A “detection rate”, which was the

percentage of replicates for which a given band was detected at some point during digestion, was then calculated. Graphs of the intensity *versus* time were drawn for each band for all replicates (data not shown) and from this the typical time span over which each band was present, as well as the typical trend, was determined.

For each replicate, a number of quantities were calculated which characterised the kinetics of digestion for either the β -casein or the β -Lg; first, the percentage intensity loss at a given time point (5 and 10 min for β -casein, and 30 min for β -Lg). Second, models of the kinetics of β -casein and β -Lg were built, based on fitting an exponential curve of the form:

$$y = A \cdot e^{-kt}$$

where y is the intensity of the band, t the time, and A and k the parameters to be calculated. This corresponded to a first-order reaction, y and A being the peptide's concentration, respectively during, and at the start of, digestion. A check on the linearity of the relationship between $\ln(A)$ and t was used to confirm this was appropriate. The models were built in Matlab (The MathWorks, Natick, USA) using a multi-dimensional unconstrained nonlinear optimization (Nelder-Mead) [24], having the advantage of not being hindered by the lack of data at a time of exactly 0 min. For the kinetic parameters (percentage intensity loss, and k), the range, mean, SD, and CV across all repeats were calculated to estimate overall inter-laboratory variation. In addition, the mean, SD, and CV of these quantities were also calculated for each laboratory to estimate the intra-laboratory variation. For β -Lg duodenal digestion, all of the parameters that were estimated (bands present and their overall kinetics, and quantified kinetics for the β -Lg band itself) were compared between Stage 1 (ten laboratories) when digestions were performed in the absence of PC, and Stage 2 (three laboratories) when PC was included.

3 Results

3.1 Visual assessment of *in vitro* gastric and duodenal digestion of β -casein and β -Lg

As reported by others [25], *in vitro* gastric digestion of β -casein led to it being rapidly degraded by pepsin; after 10 min only a faint band of the intact protein could be observed. This was accompanied by the appearance of low-molecular-weight fragments (M_r between 2.5 and 14.4 kDa). On the contrary, β -Lg was pepsin resistant and showed no degradation occurring while subjected to *in vitro* gastric digestion.

After 1 h gastric digestion, the pH was raised to 7.0, irreversibly inactivating pepsin and mimicking the transfer of gastric contents into the duodenal compartment. With the addition of trypsin and chymotrypsin, the low-molecular-weight fragments remaining after pepsinolysis of β -casein were rapidly broken down so that after 5 min duodenal

digestion all traces of β -casein-derived peptides had virtually gone. When transferred into the duodenal compartment, β -Lg was gradually degraded by trypsin and chymotrypsin and after 15 min only a faint band was observed. The low-molecular-weight peptides produced (M_r between 2.5 and 14.4 kDa) ran close to the gel dye front.

SDS-PAGE analysis after *in vitro* gastric digestion of β -casein and β -Lg in the presence of physiological levels of PC showed no differences in the digestion patterns in this digestion phase. The presence of PC did not affect the further breakdown of β -casein into the duodenal compartment either. However, as previously shown [22], including PC at physiological concentrations in the gastric compartment retarded the breakdown of β -Lg in the duodenal digestion phase.

Given these results, the quantitative assessment was focussed on the two following digestion events: (i) digestion of β -casein under gastric conditions and (ii) digestion of β -Lg under duodenal conditions, either with or without the addition of PC. We examined the extent with which the outcome of these experiments was reproducible within- and between-laboratories participating in the inter-laboratory trial.

3.2 Stage 1: Reproducibility of digestion of β -casein under gastric conditions

3.2.1 Kinetics for β -casein polypeptide

First, the percentage intensities loss for the β -casein polypeptide at 5 and 10 min on average across all replicates were 69 and 89% respectively (Table 1), being more consistent across replicates for the 10 min time point (CV of 6.2%) than that of 5 min (CV 19.6%). Thus, although the rate of disappearance may vary somewhat (and hence lead to different stages of advancement early on), the end result was consistent throughout. When fitting an exponential curve to each set of intensity *versus* time data (justified by $R^2 > 0.95$ in regression $\ln(A) = f(t)$ except for four replicates which have specific issues as highlighted below) (Fig. 1), the rate of disappearance of β -casein, k , was on average of 22.2 h^{-1} but varied considerably with a CV of 56.4% (Table 1, Fig. 2A). Analysis of data on an individual basis (Table 1) showed that laboratories 1–4, for which no specific technical issues were noted that may adversely affect the quantitative analysis, showed less intra-laboratory variation. SDS-PAGE image quality was poorer for laboratories 5 and 6 (and $R^2 = 0.92$ in regression $\ln(A) = f(t)$ for Laboratory 5, replicate I), with those from laboratory 5 having high background intensity and those from Laboratory 6 being slightly saturated. For laboratories 7 and 8, the outermost left-h and side lane rather than the second lane along corresponded to the 20 s time point and its distortion may have worsened the quantitation as the bands were not straight (and $R^2 = 0.86$ and 0.88 in regression $\ln(A) = f(t)$ for Laboratory 7, replicates II and III). Similarly laboratory 9

Table 1. Inter-laboratory trial Stage 1: kinetics (% intensity loss and rate constant k (h^{-1}) estimated from the fitting of an exponential curve) of the disappearance of β -casein under gastric conditions, (a) for each digestion, (b) averages (μ), SDs (σ), and CVs for each laboratory

Laboratory (LAB)	(a) Individual digestions					(b) Intra-laboratory variation					
	Replicate	M_r (kDa)	% Intensity loss		K	% Intensity loss			K		
			5 min	10 min		5 min μ (σ) CV	10 min μ (σ) CV	μ (σ) CV			
LAB 1	I	26.77	62.8	81.7	12.9	60.4 (4.4) 7.2%	83.3 (7.8) 9.3%	12.6 (2.4) 18.8%			
	II	27.62	63.1	91.8	14.7						
	III	26.85	55.4	76.5	10.2						
LAB 2	I	27.99	81.8	94	21.4	72.0 (12.6) 17.5%	90.3 (6.4) 7.1%	18.1 (5.9) 32.9%			
	II	27.38	76.5	94	21.7						
	III	26.71	57.8	82.9	11.2						
LAB 3	I	28.63	62.2	89.3	12.7	66.9 (4.5) 6.7%	85.9 (3.6) 4.2%	13.9 (1.1) 8.2%			
	II	28.84	67.4	86.5	14.2						
	III	28.97	71.2	82.1	14.9						
LAB 4	I	27.49	57.3	87.9	11.4	55.1 (2.0) 3.6%	87.7 (0.4) 0.5%	11.2 (0.2) 2.1%			
	II	26.72	54.7	87.3	11.0						
	III	26.84	53.4	88.1	11.1						
LAB 5 ^{a)}	I	28.44	79	91.8	33.9	75.8 (4.4) 5.9%	90.4 (1.9) 2.1%	26.7 (10.2) 38.2%			
	II	29.6	72.7	89.1	19.4						
LAB 6	I	28.34	91.6	ND	32.4	78.1 (13.5) 17.3%	95.7 (—)	22.2 (9.2) 41.2%			
	II	30.47	64.5	95.7	14.6						
	III	29.29	78.2	ND	19.8						
LAB 7	I	28.35	b)	81.3	22.7	b)	86.9 (5.8) 6.7%	39.4 (16.4) 41.6%			
	II	27.42	b)	86.4	40.1						
	III	27.59	b)	92.9	55.5						
LAB 8	I	26.78	66.8	89.9	15.0	79.4 (11.1) 13.9%	93.8 (5.5) 5.9%	25.7 (9.6) 37.2%			
	II	27.68	87.5	ND	33.5						
	III	26.84	84.1	97.7	28.5						
LAB 9	I	28.67	42.4	95.4	10.3	67.9 (—)	95.4 (—)	31.8 (18.9) 59.4%			
	II	28.6/28.07 ^{c)}	c)	c)	39.3 ^{d)}						
	III	26.7/28.9 ^{e)}	93.5	e)	45.7 ^{f)}						
LAB 10 ^{g)}	I	27.9	h)	h)	h)	h)	h)	h)			
	II	28.1	h)	h)	h)						
Range		[26.7–30.47]	[42.4–93.5]	[76.5–97.7] ⁱ⁾	[10.1–55.5]						
Mean		27.9	69.3	88.7 ⁱ⁾	22.2						
SD		0.99	13.54	5.54 ⁱ⁾	12.54						
CV		3.5	19.6	6.2 ⁱ⁾	56.4						

a) The image quality of the gels was satisfactory only for two digestions for Laboratory 5.

b) Aliquots were not withdrawn at time = 5 min.

c) The first time-point aliquot was deposited on a different gel from the remainder aliquots.

d) Curve fitted to time points 1, 2, and 5 min only (as data for time = 20 s were on a different gel).

e) The aliquots were deposited on one gel for times up to 5 min, and another gel for times 10 min onwards.

f) Curve fitted to time points up to 5 min only (as data for time beyond 5 min were on a different gel).

g) It was only possible to reference the β -casein M_r for two of the replicates for Laboratory 10.

h) The β -casein intensity was saturated.

i) Calculation based only on cases where the band was detected.

ND indicates the band is not detected anymore.

Insufficient (–) data to calculate SD and CV.

split digestion experiment samples across gels, a factor likely to impair quantitation and hence intra-laboratory variation (and $R^2 = 0.91$ in regression $\ln(A) = f(t)$ for Laboratory 9, replicate I).

One possible source of error in the estimation of k is that the exact time point at which the sample was taken (aimed to be immediately after adding the enzymes) may

have deviated from the estimated average of 0.3 min. The potential error induced by this was evaluated by fitting exponential curves with the time of the first sampling set to between 6 and 30 s (0.1–0.5 min in 0.01 min steps). As expected, the error (Fig. 2A) is larger for faster digestions, being below 4% for $k < 20 \text{ h}^{-1}$ and up to ~12% for higher rate constants. The laboratories for which this error was

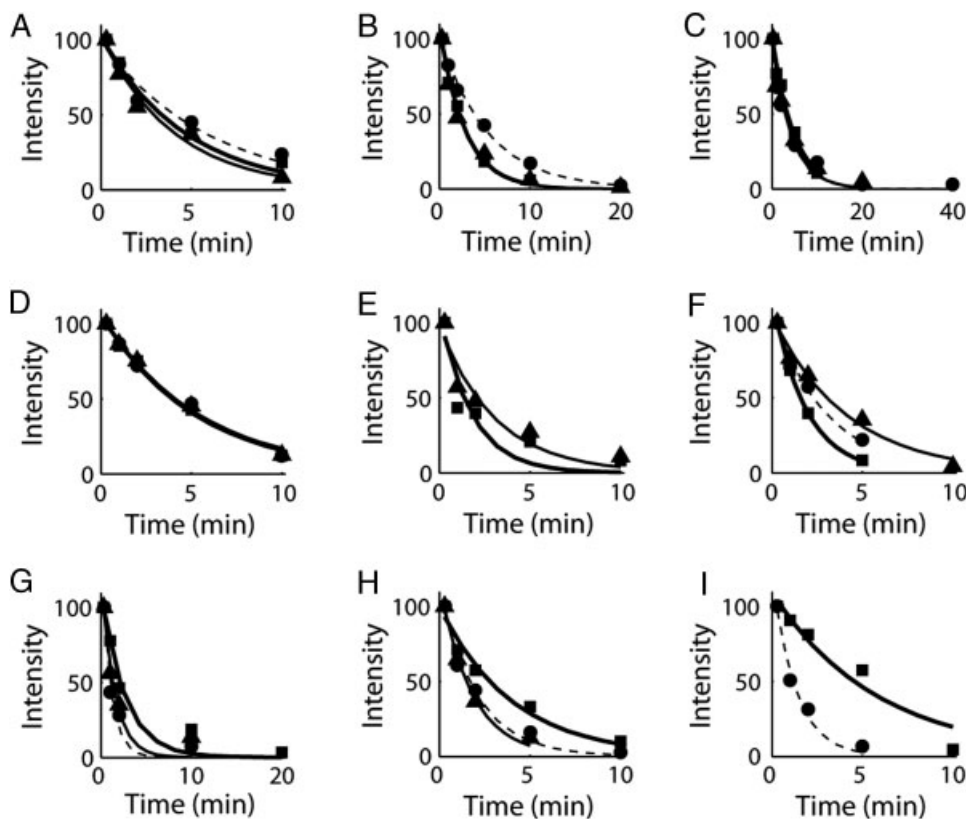


Figure 1. Fitting of exponential curve (—, —, - -) to data points (■, ▲, ●) obtained from quantitative analysis, for digestion of β -casein (band B) during gastric digestion for replicates I, II, and III respectively. Stage 1 of inter-laboratory comparisons for laboratories 1–9 (A–I). Intensities shown as percentage of intensity at first time point.

>4% for at least one replicate digestion were also those with highest intra-laboratory variability. This suggests that variation in sampling at the first time point made a major contribution to the overall intra-laboratory variability, although other sources of error (as discussed above) may also have been involved.

3.2.2 Polypeptides other than β -casein

Table 2 provides a summary of the polypeptides that appear during the course of gastric digestion of β -casein. They were identified as being the same across different gels on the basis of their relative molecular mass. It was found that for an easily recognisable polypeptide, such as β -casein, the molecular weight referenced using the full molecular marker scale from 200 to 2.5 kDa varied by only 3.5% (CV), as compared with 8.2% when working with Rfs based on the same scale. Five polypeptides were detected in all gels (A, B, C, J, and K), two in most gels (D and E), and a further five detected in some of the gels only. The general trend regarding the kinetics of appearance and disappearance of the first group of five polypeptides was also fairly consistent between gels (Table 1), as typified by one gel in Fig. 3A. Band A corresponds to pepsin and as anticipated, only oscillated slightly. Band B, corresponding to β -casein, consistently showed an exponential decrease as digestion proceeded, becoming undetectable after 10–20 min. Three polypeptides, F, G, and H can be grouped together since

both bands F and H ($M_r = 13.4$ and 10.9 kDa, respectively) were almost always observed together, or if this was not the case, band G was identified at the intermediate position of 12.3 kDa. It is therefore highly likely that band G corresponds to at least two fragments sometimes shown as F and H, but that these are not resolved in about 60% of the gels.

3.3 Stage 1: Reproducibility of digestion of β -Lg under duodenal conditions

As expected the β -Lg ran as a $M_r = \sim 18.8$ kDa band which was always detected (Table 3, Fig. 3B). Five bands were also detected in the majority of cases (P–T), and a further two (N–O) in two-third of the cases, whereas three more (U–W) were detected only by a few laboratories and showed no consistent trend, most likely because they run close to the gel dye front. The β -Lg band decreased in intensity as digestion proceeded and could in all cases still be detected after 30 min (see details of kinetics below). Two of the bands, P and S of M_r 11.5 and 5.7 kDa, respectively, were consistently detected, and usually decreased in intensity during digestion. On the contrary, two polypeptides, R and T of M_r 6.9 and 4.7 kDa, respectively, initially increased in intensity and then decreased, as did polypeptides N and O ($M_r = 15.6$ and 13.4 kDa) when they were detected. Band Q, while detected in 93% of cases, did not show a clear,

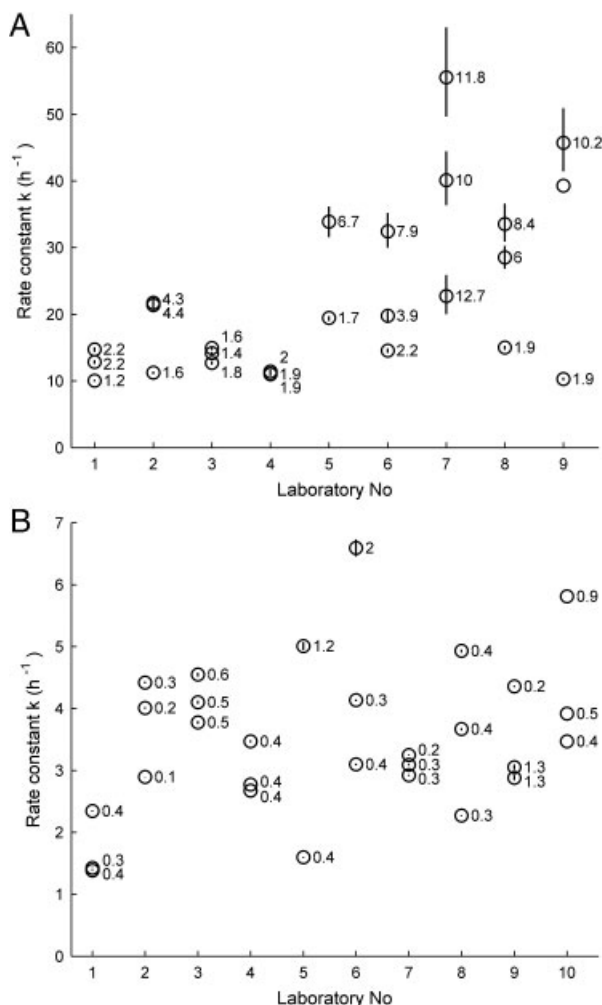


Figure 2. Rate constant k (h⁻¹) for (A) β-casein (band B) during gastric digestion and (B) β-Lg (band M) during duodenal digestion, in Stage 1 of the inter-laboratory comparison. Briefly, ○ represents each replicate digestion with first time point at 0.3 min. — describes mean ± 1SD and the number CV for simulation with first time point between 0.1 and 0.5 min.

consistent trend, indicating that it may arise as a result of co-running of several fragments. This was also observed for several bands (e.g. S, U–W) and may account for the greater variation in the digestion kinetics for β-Lg compared with β-casein.

The persistence of β-Lg for at least 30 min is indicated by an average degradation of 82% (Table 4). The variability of this estimate (CV of 17.2%) is somewhat influenced by five replicates (Laboratory 1, replicates I, II; Laboratory 5, replicates I, II; Laboratory 7, replicate III) with slightly atypical, low, percentage degradation (below 75%). The slower digestive process is also reflected by the smaller rate of disappearance k in the fitted exponential curves (Table 4, Figs. 2B and 4), which are on average of 3.51 h⁻¹ (note that R^2 in the regression $\ln(A) = f(t)$ generally suggests an exponential curve is appropriate, with the exception of a

small number of gels; $R^2 = 0.55$ and 0.67 , respectively, for Laboratory 5, replicate II and Laboratory 7, replicate III). The intra-laboratory variability was fairly consistent in terms of outcome, as the percentage intensity loss at 30 min only varies by about 4–7% except when influenced by the outliers mentioned above, whereas the rate of disappearance is subject to wider variations (Table 4).

The potential error arising from mis-estimating the exact time point of the first sampling was consistently negligible for all digestions (see results from fitting exponential curves with the time of the first sampling set to between 6 and 30 s in Fig. 2B), which is not surprising in view of the lower rate of digestion. Hence, this source of error accounted for little in the overall intra-laboratory variability.

3.4 Stage 2: Reproducibility of duodenal digestion of β-Lg in the presence of PC

The addition of PC in the gastric phase has previously been shown to protect β-Lg from digestion by duodenal proteases [22]. In the inter-laboratory trial, all centres consistently observed the increased stability of the β-Lg band, indicating that PC had inhibited its breakdown (Tables 3 and 4). The percentage intensity loss at 30 min was 18.6% on average, instead of 82.7% without PC, and the R^2 values in the $\ln(A) = f(t)$ regression did not support a first-order decay (being in the range 0.41–0.74) except for two cases; note that for these the rate constant was of an order of magnitude smaller than without PC. Addition of PC has also been found to affect the patterns of proteolysis products (Table 3) and indeed, bands N and O observed in Stage 1 were no longer detected. The other bands did not show any clear trends, as observed in Stage 1, although bands P, Q, S, and T were consistently detected in all cases, band R in two of the three labs, whereas band W, which had been only rarely detected in Stage 1, was consistently detected in all three laboratories.

4 Discussion

The kinetics of protein degradation were evaluated by quantitative statistic methodology and the reproducibility of the assay in different laboratories using a common protocol of digestion was investigated.

Preliminary results from this study (data not shown) [22] demonstrated that the gel electrophoresis system chosen to analyse the digestion fragments could affect the quantitative analysis, and hence we used 10% pre-cast Bis-Tris-reduced gels (Invitrogen). After standardised fixing (30 min), gels were stained with colloidal Coomassie stain (Simply Blue Safe Stain, Invitrogen) which can detect a minimum of 0.1 ng of protein and is more sensitive compared with other dyes [26]. However, variations in fragment detection observed could be due to differences in

Table 2. Inter-laboratory trial Stage 1: summary of polypeptides observed during β -casein gastric digestion

Polypeptide	M_r (kDa)	Detection rate (%) ^{a)}	Time span present	Trend
A (pepsin)	39	100	20 s to 60 min	Oscillating
B (β -casein)	27.9	100	20 s to 10–20 min	Exponential decrease
C	23.2	100	20 s to 10–20 min	Brief increase followed by decrease
D	19.6	72	20 s or 1 min to 10–20 min	Increase up to 2 min, then decrease
E	15.7	88	Usually 1–20 min	Most often, increase up to 5 or 10 min, then decrease. Some variation
F ^{b)}	13.4	60	20 s to 10–20 min	Global increase up to $\sim$ 2 min, then decrease
G ^{b)}	12.3	68	20 s to 10–20 min	Global increase up to $\sim$ 2 min, then decrease
H ^{b)}	10.9	50	20 s to 5–10 min	Global increase up to $\sim$ 2 min, then decrease
I	8.3	32	10–60 min	Variable
J	6.3	100	20 s to 10–20 min	Increase up to 5–10 min, then decrease
K ^{b)}	4.6	100	20 s to up to 60 min	Global increase up to 20–40 min, then decrease
L ^{b)}	3.6	22	Occasional	

a) % Replicates, across all participating laboratories, for which the band is detected at some point during the digestion.

b) Fuzzy band position; identification unsure.

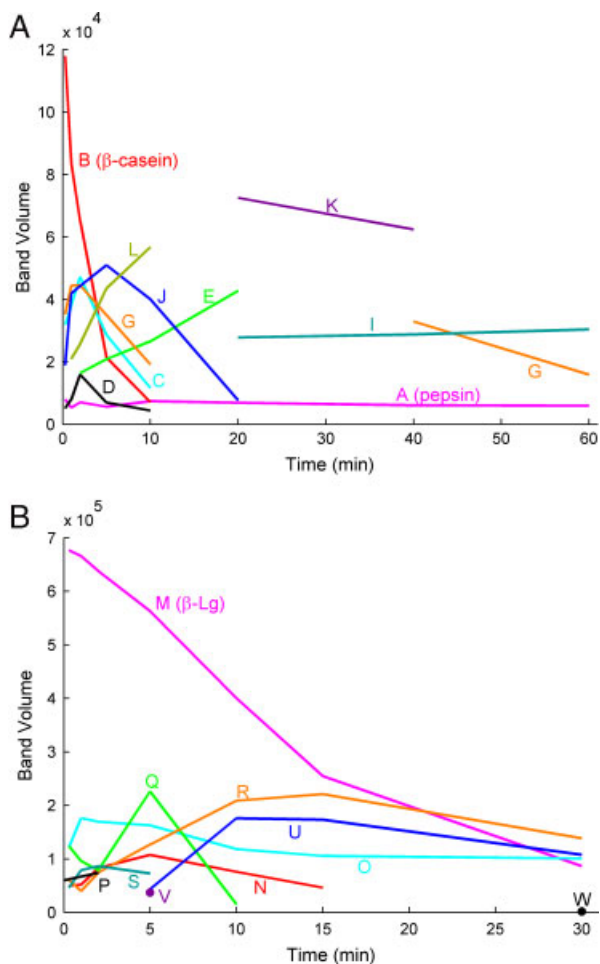


Figure 3. Changes in band volumes ($\sim$ intensity) for: (A) β -casein gastric digestion (Stage 1). Bands are as defined in Table 2 and are designated by the following lines: A (pepsin) —, B (β -casein) —, C —, D —, E —, G —, I —, J —, K —, and L —; (B) β -Lg duodenal digestion (Stage 1). Bands are as defined in Table 3 and are designated by the following lines: M (β -Lg) —, N —, O —, P —, Q —, R —, S —, U —, V — (detected at 5 min only), and W — (detected at 30 min only).

time of destaining used by individual laboratories, together with image capture and processing. Although the colloidal Coomassie stain does not provide a wide dynamic range with a linear response regarding signal intensity, this presented only one problem (Laboratory 9, Stage 1 β -casein study). However, this drawback was considered small compared with the overall benefit on cost of this stain; other stains such as Sypro, while yielding a linear response over a greater range, are considerably more expensive. In practice, there was little evidence that quantitation was skewed, or that fragment detection varied between different laboratories as a consequence of using this stain.

The quantitative approach that was chosen proved to be a good method to evaluate disappearance of parent protein and appearance of digestion products. It differs from most multi-laboratory studies in that these usually compare either protocols [27, 28], or laboratories [29–31] in terms of a single measurement or at most a few independent measurements. Williams *et al.* [32], who did study a time course of digestion, plotted the average measurement together with the standard error for each time point, which corresponded to nine measurements. Here, for the main proteins, we carried out a quantitative evaluation by calculating the percentage loss intensity of the band at different time points, modelling, and comparing their time course of digestion by curve fitting and examining the consistency of these findings both within and between laboratories. A precise picture of the reproducibility of the appearance of bands was drawn and the kinetics of β -casein and β -Lg decay precisely characterised. In agreement with what had been observed by Thomas *et al.* [21], the observations were more consistent for the protein itself than for the proteolytic fragments. The inter-laboratory CV calculated for the percentage intensity loss of β -casein after 5 min gastric digestion was 19.6%, which decreased to 6.2% by 10 min digestion (Table 1). However, a CV of 17.2% was obtained with β -Lg after 30 min duodenal digestion (Table 4), even though the mean intensity loss of 82.7% is comparable to that of 88.7% observed for β -casein after 10 min digestion.

Table 3. Inter-laboratory trial: summary of polypeptides observed during β -Lg duodenal digestion^{a)}

Polypeptide	M_r (kDa)	Stage 1			Stage 2		
		Detection rate (%) ^{b)}	Time span present	Trend	Detection rate (%) ^{b)}	Time span present	Trend
M (β -Lg)	18.8	100	20 s to 30 min	Exponential decrease	100	20 s to 30 min	Stable
N	15.6	66	20 s to up to 30 min (often less)	Increase up to 5–10 min, then decrease	ND		
O	13.4	62	Usually 20 s to 30 min	Most often, increase up to 5 or 10 min, then decrease. Some variation	ND		
P	11.5	83	20 s to up to 30 min	Overall decrease but inconsistent	100	20 s to 30 min	Oscillating. Possibly slight decrease
Q	8.6	93	20 s to 30 min	Oscillating pattern. Some variation	100	20 s to 30 min	Oscillating, then possibly slight decrease
R	6.9	96	20 s to 30 min	Most often, increase up to 5 or 10 min, then decrease. Some variation	66	20 s to 30 min	Oscillating, then possibly slight decrease
S	5.7	96	20 s to up to 30 min	Overall decrease, sometimes following initial increase	100	20 s to 30 min	Stable/slight decrease
T	4.7	89	20 s to 30 min	Most often, increase up to 10 min, then decrease	100	20 s to up to 30 min	Oscillating
U	4	41	Varied	No consistent trend	ND		
V	3.1	21	Varied	No consistent trend	ND		
W	2.2	7	20 s to 10 min	No consistent trend	100	20 s to 30 min	Stable

a) The observations are based on ten laboratories in Stage 1 and three laboratories in Stage 2.

b) % Replicates, across all participating laboratories, for which the band is detected at some point during the digestion. ND not detected.

Table 4. Inter-laboratory trial: Kinetics (% intensity loss and rate constant k (h^{-1}) estimated from the fitting of an exponential curve) of the disappearance of β -Lg under duodenal conditions, (a) for each digestion, (b) averages (μ), SDs (σ), and CVs for each laboratory

Laboratory (LAB)	(a) Individual digestions					(b) Intra-laboratory variation				
	Replicate	Stage 1			Stage 2	Stage 1				Stage 2
		M_r (kDa)	% Intensity loss 30 min	K		M_r (kDa)	% Intensity loss 30 min	μ (σ) CV	k	% Intensity loss 30 min
LAB 1	I	18.3	48.1	1.38		18.07	17.0	62.0 (21.0) 33.9%	1.72 (0.54) 31.6%	16.9 (2.65) 15.7%
	II	17.85	51.6	1.43		18.49	19.5			
	III	18.32	86.1	2.34		18.06	14.2			
LAB 2 ^{a)}	I	18.75	89	4.42				84.9 (4.0) 4.7%	3.77 (0.79) 20.9%	
	II	18.77	84.7	4.01						
	III	18.86	81	2.89						
LAB 3 ^{a)}	I	19.27	97	4.10				91.8 (4.9) 5.4%	4.14 (0.39) 9.4%	
	II	18.88	91.1	4.55						
	III	18.41	87.2	3.77						
LAB 4 ^{a)}	I	19.68	85.3	3.47				81.6 (3.2) 4.0%	2.97 (0.43) 14.6%	
	II	19.43	80.1	2.77						
	III	19.23	79.3	2.67						
LAB 5 ^{a),b)}	I	19.91	42.5	1.60				57.0 (20.5) 36%	3.30 (2.41) 73.1%	
	II	17.67	71.4	5.01						
	III	20.36	96.6	6.59				93.2 (3.4) 3.7%	4.61 (1.79) 39.0%	
LAB 6 ^{a)}	I	20.09	93.2	4.13						
	II	21.7	89.8	3.10						
	III	18.24	92.4	3.09				79.1 (12.9) 16.4%	3.09 (0.16) 5.3%	
LAB 7 ^{a)}	I	19.05	78.5	2.92						
	II	16.28	66.5	3.25						
	III	17.56	81.7	2.27				89.9 (7.1) 7.9%	3.62 (1.33) 36.7%	
LAB 8 ^{a)}	I	18.18	94.3	3.67						
	II	17.92	93.8	4.93						
	III	19.09	84.8	2.88		18.17	24.5	89.8 (5.3) 5.9%	3.43 (0.81) 23.5%	22.0 (3.6) 16.4%
LAB 9	I	19.11	89.2	3.05		17.94	23.5			
	II	18.85	95.3	4.36		17.77	17.9			
	III	18.46	94.5	5.81		18.79	14.9	89.2 (4.8) 5.3%	4.40 (1.24) 28.3%	16.9 (2.4) 14.2%
LAB 10	I	19.68	85.3	3.47		18.59	19.6			
	II	18.36	87.7	3.92		18.41	16.4			
	III	[16.2–1.7]	[42.4–97.0]	[1.37–6.59]		[17.8–18.8]	[14.2–24.5]			
Range		18.8	82.7	3.51		18.3	18.6			
Mean		1.01	14.2	1.22		0.33	3.56			
SD		5.4	17.2	34.7		1.8	19.1			
CV										

a) Empty areas: Laboratories did not participate in Stage 2.

b) The image quality of the gels was satisfactory only for two digestions for Laboratory 5.

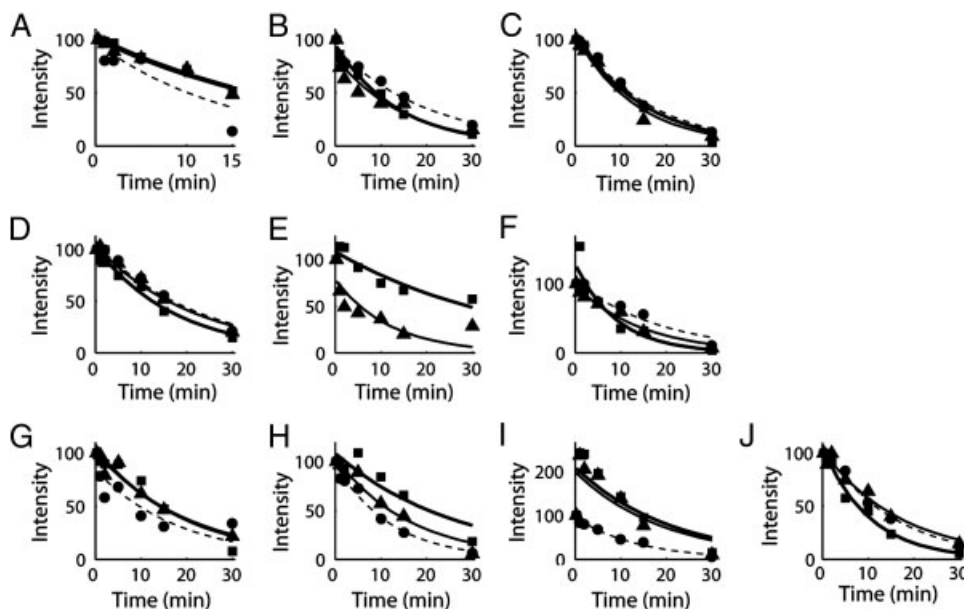


Figure 4. Fitting of exponential curve (—, —, - -) to data points (■, ▲, ●) obtained from quantitative analysis, for digestion of β -Lg (band M) during duodenal digestion for replicates I, II, and III respectively. Stage 1 of inter-laboratory comparisons for laboratories 1–10 (A–J). Intensities shown as percentage of intensity at first time point.

This may be due to three marked outliers (Laboratory 1, replicates I, II; Laboratory 5, replicate I), which showed only a loss of mean intensity of around 47%. The addition of PC to the β -Lg gastric digestion phase led to dramatically lower percentage loss intensity in the duodenal phase (18.6% at 30 min). The CV on this estimate (19.1%) was comparable to Stage 1 and did not guide whether to use biosurfactants on the basis of reproducibility considerations. However, the inclusion of biosurfactants, including PC, can have significant effects on the digestion of some proteins such as α -lactalbumin [33] and β -Lg [23], having complex effects on digestion of protein-stabilised emulsions [25]. Thus, it is our view that biosurfactants should be included in systems attempting to model biochemical aspects of human digestion.

In addition, the study reinforced the importance of following a common protocol rigorously to make cross-comparisons meaningful and highlighted experimental issues which may affect the meaningfulness of densitometry to quantify variation in SDS-PAGE. Poor image quality precluded such analysis when polypeptide bands were blurred and hence poorly referenced in terms of M_r , as well as unsuitable for the curve-fitting step necessary for quantification. We have also found that molecular marker weights have to be sharply defined to reference polypeptides' M_r on a given gel. This is even more the case for inter-laboratory comparisons as they necessitate between-gel band referencing, and we found this to be the step in the analysis which necessitated most expert judgement. Gel distortion, although not a big issue here, can occur in SDS-PAGE experiments [11]. Thus, the use of multiple standard molecular weights lanes is advisable for accurate peptide weights' referencing. Another obstacle to accurate quantification was when lanes from a given digestion were split across gels. Indeed, although results can be described as relative intensity loss or gain within one gel, this is not absolute and differences between gels in terms of background

and lane width can introduce bias. Inaccurate measurement of the exact time point for the first measurement can also affect the parameters of fitted curves, particularly for fast digestions; our estimates are that the rate constant could differ by up to 12% for the fastest of the β -casein gastric digestions (on the basis of the first time point being poorly referenced in the range 6–30 s), though it was negligible for the slowest ones (β -Lg duodenal). Thus, the following recommendations are most essential to optimise a quantitative approach: use the correct gels, use at least two standard lanes on either edges of the gel, avoid splitting experiments across gels, ensure the quality of the picture is such that features are sharp and not saturated, and include a range of molecular weight markers which covers that of the expected fragments. These guidelines are important for any SDS-PAGE work but become compelling for quantitative evaluation. These data also indicate the need for laboratories to work to become proficient in such analysis in order to reduce inter-laboratory variation, as is observed for many other analytical methods. Although challenging from a practical point of view, the referencing of the time at which the first sample is taken contributes greatly to the errors in kinetic parameter estimates for rapidly digested proteins like β -casein. This is likely to be more problematic when using a gross excess of protease, such as the pepsin-resistance test used in risk assessment.

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5 References

- [1] Andersen, H. U., Fey, S. J., Larsen, P. M., Nawrocki, A., Hejnaes, K. R., Mandrup-Poulsen, T., Nerup, J., *Electrophoresis* 1997, **18**, 2091–2103.
- [2] Malm, C., Hadrevi, J., Bergstrom, S. A., Pedrosa-Domellof, F., Antti, H., Svensson, M., Frangsmyr, L., *Scand J. Clin. Lab. Invest.* 2008, **68**, 793–800.
- [3] Schroder, S., Zhang, H., Yeung, E. S., Jansch, L., Zabel, C., Watzig, H., *J. Proteome Res.* 2008, **7**, 1226–1234.
- [4] Seferovic, M. D., Krugchkov, V., Pinto, D., Han, V. K. M., Gupta, M. B., *J Chromatogr. B* 2008, **865**, 147–152.
- [5] Corbett, J. M., Dunn, M. J., Posch, A., Gorg, A., *Electrophoresis* 1994, **15**, 1205–1211.
- [6] Blomberg, A., Blomberg, L., Norbeck, J., Fey, S. J., Larsen, P. M., Larsen, M., Roepstorff, P., Degand, H., Boutry, M., Posch, A., Gorg, A., *Electrophoresis* 1995, **16**, 1935–1945.
- [7] Lopez, M. F., Patton, W. F., *Electrophoresis* 1997, **18**, 338–343.
- [8] Zhan, X. Q., Desiderio, D. M., *Electrophoresis* 2003, **24**, 1834–1846.
- [9] Zhan, X. Q., Desiderio, D. M., *Electrophoresis* 2003, **24**, 1818–1833.
- [10] Zhang, H., Li, N., Chen, Y., Huang, L. Y., Wang, Y. C., Fang, G., He, D.-C., Xiao, X.-Y., *Biomed. Environ. Sci.* 2007, **20**, 24–32.
- [11] Olias, R., Maldonado, B., Radreau, P., Le Gall, G., Mulholland, F., Colquhoun, I. J., Kemsley, E. K., *Electrophoresis* 2006, **27**, 1288–1299.
- [12] Astwood, J. D., Leach, J. N., Fuchs, R. L., *Nat. Biotechnol.* 1996, **14**, 1269–1273.
- [13] Metcalfe, D. D., Astwood, J. D., Townsend, R., Sampson, H. S., Taylor, S. L., Fuchs, R. L., *Crit. Rev. Food Sci. Nutr.* 1996, **36**, S165–S186.
- [14] Taylor, S. L., Hefle, S. L., *J. Allergy Clin. Immunol.* 2001, **107**, 765–771.
- [15] Mills, E. N. C., Jenkins, J., Alcocer, M. J. C., Shewry, P. R., *Crit. Rev. Food Sci. Nutr.* 2004, **44**, 379–407.
- [16] US Pharmacopoeia, The National Formulary, 1995. USP XXIII, NF XVIII, US Pharmacopoeia Convention, Inc., Mack Printing Co., Easton, PA, p.2053.
- [17] Asero, R., Mistrello, G., Roncarolo, D., de Vries, S. C., Gautier, M. F., Ciurana, C. L. F., Verbeek, E., Mohammedi, T., Knul-Brettlova, V., Akkerdaas, J. H., Bulder, I., Aalberse, R. C., van Ree, R., *Int. Arch. Allergy Immunol.* 2001, **122**, 20–32.
- [18] Fu, T. J., *Ann. NY Acad. Sci.* 2002, **964**, 99–110.
- [19] Fu, T. J., Abbott, U., Hatzos, C., *J. Agric. Food Chem.* 2002, **50**, 7154–7160.
- [20] Tanaka, K., Matsumoto, K., Akasawa, A., Nakajima, T., Nagasu, T., Iikura, Y., Saito, H., *Int. Arch. Allergy Immunol.* 2002, **129**, 49–56.
- [21] Thomas, K., Aalbers, M., Bannon, G. A., Bartels, M., Dearman, R. J., Esdaile, D. J., Fu, T. J., Glatt, C. M., Hadfield, N., Hatzos, C., Hefle, S. L., Heylings, J. R., Goodman, R. E., Henry, B., Herouet, C., Holsapple, M., Ladics, G. S., Landry, T. D., MacIntosh, S. C., Rice, E. A., Privalle, L. S., Steiner, H. Y., Teshima, R., van Ree, R., Woolhiser, M., Zawodny, J., *Regul. Toxicol. Pharmacol.* 2004, **39**, 87–98.
- [22] Mandalari, G., Adel-Patient, K., Barkholt, V., Baro, C., Bennett, L., Bublin, M., Gaier, S., Graser, G., Ladics, G. S., Mierzejewska, D., Vassilopoulou, E., Vissers, Y. M., Zuidmeer, L., Rigby, N. M., Salt, L., Defernez, M., Mulholland, F., Mackie, A. R., Wickham, M. S. J., Mills, E. N. C., *Regul. Toxicol. Pharm.* 2009, **55**, 372–381.
- [23] Mandalari, G., Mackie, A. M., Rigby, N. M., Wickham, M. S. J., Mills, E. N. C., *Mol. Nutr. Food Res.* 2009, **53**, S131–S139.
- [24] Nelder, J. A., Mead, R., *Comput. J.* 1965, **7**, 308–313.
- [25] Macierzanka, A., Sancho, A. I., Mills, E. N. C., Rigby, N. M., Mackie, A. R., *Soft Matter* 2009, **5**, 538–550.
- [26] Syrový, I., Hodný, Z. J., *Chromatography* 1991, **569**, 175–196.
- [27] Linssen, C. F. M., Jacobs, J. A., Beckers, P., Templeton, K. E., Bakkers, J., Kuijper, E. J., Melchers, W. J. G., Drent, M., Vink, C., *J. Med. Microbiol.* 2006, **55**, 1229–1235.
- [28] VandenBroeke, T., Dumont, L. J., Hunter, S., Nixon, J., Murphy, S., Roger, J., Herschel, L., AuBuchon, J. P., Gulliksson, H., Dengler, T., Hornsey, V., Prowse, C., *Vox Sang.* 2004, **86**, 183–188.
- [29] Chun, D. T. W., Bartlett, K., Gordon, T., Jacobs, R. R., Larsson, B. M., Larsson, L., Lewis, D. M., Liesivuori, J., Michel, O., Milton, D. K., Rylander, R., Thorne, P. S., White, E. M., Brown, M. E., Gunn, V. S., Wurtz, H., *Am. J. Ind. Med.* 2006, **49**, 301–306.
- [30] Ferris, N. P., King, D. P., Reid, S. M., Hutchings, G. H., Shaw, A. E., Paton, D. J., Goris, N., Haas, B., Hoffmann, B., Brocchi, E., Bugnetti, M., Dekker, A., De Clercq, K., *Vet. Microbiol.* 2006, **117**, 130–140.
- [31] Khaparde, V. V., *Environ. Monit. Assess.* 2006, **116**, 217–231.
- [32] Williams, B. J., Cameron, C. J., Workman, R., Broeckling, C. D., Sumner, L. W., Smith, J. T., *Electrophoresis* 2007, **28**, 1371–1379.
- [33] Moreno, F. J., Mackie, A. R., Mills, E. N. C., *J Agric Food Chem.* 2005, **53**, 9810–9816.